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INHIBITION OF *TRICHOMONAS VAGINALIS* ORNITHINE DECARBOXYLASE BY AMINO ACID ANALOGS*

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Abstract—Ornithine decarboxylase (ODC) from *Trichomonas vaginalis* was inhibited irreversibly by several substrate analogs. Of these, DL-α-monofluoromethyldehydroornithine (MFMDO) and DL-α-monofluoromethylornithine (MFMO) were the most potent. The enzyme was unaffected by putrescine analogs suggesting that differences exist between the regulation of the trichomonad enzyme and that in other eukaryotes. In culture the ornithine analogs strongly inhibited putrescine synthesis and increased the generation time after 24 hr of exposure. In a semi-defined growth medium MFMDO methyl and ethyl esters increased the generation time from 4.5 hr to 9.0 and 8.2 hr, respectively. In standard undefined growth medium the trichomonad ODC was fully induced only after 15 hr (late log) and had an extended half-life of greater than 8 hr.

Trichomonas vaginalis is a parasite of the human urogenital tract. It is considered to be the most prevalent non-viral sexually transmitted disease, with an estimated 180 million new cases per year worldwide [1]. As a result of infection, the amino acid composition of the vaginal fluid changes from one rich in arginine to one containing a high concentration of putrescine [2, 3]. Putrescine is also the major polyamine produced by *T. vaginalis* during *in vitro* growth [4–6]. Inhibition of ornithine decarboxylase (ODC‡; EC 4.1.1.7) and subsequent reduction in polyamine biosynthesis lead to a reduction in cell proliferation in a wide variety of cell types [7–12]. The most successful ODC inhibitor, DL-α-difluoromethylornithine (DFMO) is a potent antitrypanosomal agent [13], and reduces intracellular polyamines in several other protozoa [9], including *T. vaginalis* grown in a medium devoid of exogenous putrescine [6]. However, *in vivo* DFMO fails to cure mouse intravaginal model infections, although it does block cytotoxicity of the parasite to mammalian cells in culture, and delays the development of subcutaneous abscesses due to *T. vaginalis* model infections [14]. In addition, 5 mM DFMO arrests cell division of *T. vaginalis* grown in a semi-defined medium lacking exogenous putrescine [6].

The trichomonad enzyme had minor activity with lysine or arginine as substrate, and was not affected by high concentrations of putrescine (Yarlett N, Goldberg B, Moharrami MA and Bacchi CJ, unpublished observations). In this study we further characterize the trichomonad enzyme by comparing several ODC inhibitors that are potential trichostatic agents. The apparent dissociation constants (K_i) of each inhibitor for the trichomonad enzyme were determined. The polyamine inhibitors are discussed with respect to *in vivo* depletion of polyamines and efficacy in a standard drug assay.

MATERIALS AND METHODS

Chemicals. ODC inhibitors were gifts from Drs. P. P. McCann and A. Bitonti, Marion Merrell Dow Research Institute, Cincinnati, OH. HPLC grade acetonitrile was from Fisher Scientific, Springfield, NJ. Unless otherwise stated, all chemicals were of the purest grade available from the Sigma Chemical Co., St. Louis, MO.

Cultures. T. vaginalis C1-NIH (ATCC 30001) was grown in tryptose–yeast extract–maltose (TYM) medium supplemented with 10% horse serum [15]. Cultures were harvested by centrifugation at 2500 *g* for 10 min at 8° and washed in a buffer containing 30 mM sodium phosphate, 0.6 mM $CaCl_2$, 74 mM NaCl and 1.6 mM KCl, pH 6.4 (washing buffer) and resuspended in 0.02 M acetate buffer, pH 6.5, containing 1 mM 2-mercaptoethanol, 0.02% Brij 35 and 60 μM pyridoxal phosphate (buffer A). Cell-free extracts were prepared by homogenization using a Teflon pestle fitted with a motor drive. The homogenate was diluted 10-fold in ice-cold buffer A, and centrifuged at 400 *g* for 30 min to obtain a supernatant fraction which was used to semipurify the ODC.

Partial purification of ornithine decarboxylase. The ODC was partly purified by elution from DEAE Sephacel using a linear gradient from 0 to 1 M KCl in buffer A. The fractions having peak activity (0.4 M KCl) were pooled and concentrated 2-fold by ultrafiltration with a 50 kDa filter (Amicon

* This paper is dedicated to Dr. S. H. Hutner on the occasion of his 80th birthday.

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‡ Abbreviations: ODC, ornithine decarboxylase; DFMO, DL-α-difluoromethylornithine; MFMO, DL-α-monofluoromethylornithine; DFML, DL-α-difluoromethyllysine; MFMDO, DL-α-monofluoromethyldehydroornithine; DFMA, DL-α-difluoromethylarginine; DFMC, DL-α-difluoromethylcadaverine; MFMC, DL-α-monofluoromethylcadaverine; MAP, *R*,*R*′-δ-methyl-α-acetylenic-putrescine; MO, α-methylornithine and TCA, trichloroacetic acid.

Microfiltration Systems, Dublin, CA). The preparation was applied to a 1 × 90 cm agarose column and eluted with 2 column volumes (110 mL) of buffer A. The peak fractions were again pooled and concentrated as previously described. This resulted in a 56-fold purification over the crude extract.

Enzyme assays. ODC was assayed as previously described [16] using 0.2 M acetate buffer, pH 6.5, containing 60 μM pyridoxal phosphate, 1 mM 2-mercaptoethanol, 3 mg/mL bovine serum albumin and 1 μCi of L-[1-^{14}C]ornithine (42.2 mCi/mmol; DuPont, NEN Research Products, Wilmington, DE). About 6 μg of the semipurified protein was added to the reaction vial and the [^{14}C]CO_2 was trapped using hyamine (30 μL) soaked filters in center wells. After 30 min (37°) the reaction was stopped using 1 mL of 40% trichloroacetic acid (TCA) and allowed to incubate for a further 30 min. Radioactivity was quantified using liquid scintillation counting.

ODC Inhibition Kinetics. Time-dependent, irreversible inhibition of ODC was determined as previously described [7]. Trichomonad ODC was incubated at 37° with various concentrations of the inhibitor. At selected times 1 μCi of L-[1-^{14}C]-ornithine was added to the flask and the assay incubated for a further 30 min before stopping with 1 mL of 40% (v/v) TCA. The degree of reversibility was determined by overnight dialysis in buffer A of enzyme preparations previously incubated for 30 min with a 100 μM concentration of the respective inhibitor. The percent remaining activity was compared to controls lacking inhibitor.

In vitro *activity of ODC inhibitors.* Log phase cells were inoculated to a final density of 10^5 cells/mL into TYM medium containing a 5 mM concentration of the polyamine antagonist and transferred daily for 4 days into fresh medium containing the antagonist. Cell counts were made daily and cultures were collected by centrifugation. Cell pellets were resuspended in the washing buffer. The cell-free medium was brought to a final concentration of 6% (v/v) TCA and stored at 4°. The cell pellets were brought to a final volume of 0.4 mL with 6% (v/v) TCA and stored at 4°.

Polyamine analysis. Polyamines were derivatized with *o*-pthalaldehyde (0.8 g/L) dissolved in 3 mL methanol and added to 30.9 g/L boric acid containing 24 g/L KOH, and 1 mL mercaptoethanol, pH 10.4, and quantitated using reverse phase HPLC. Samples and standards were separated using a percosphere 10 μm particle size C-18 column (Perkin Elmer Corp., Norwalk, CT). The polyamine derivatives were separated using a discontinuous gradient starting with 85% buffer A (2.5 g/L lithium citrate, pH 2.65, containing 0.22 g/L octane sulfonic acid), changing in 25 min to 40% buffer B (acetonitrile), and held isocratic at this step for 20 min before changing to 50% buffer in 10 min and finally to 85% buffer B in 15 min. Detection was by fluorescence using an emission wavelength of 455 nm and an excitation wavelength of 320 nm, as described previously [6].

Minimum inhibitory concentration assay. Susceptibility of the organism to polyamine antagonists was determined by the assessment of growth and viability in a multiwell plate assay. An aliquot of the drug (50 μL) was added to an equal volume of growth medium containing 10% horse serum and serially diluted. A 24-hr stock culture was diluted into fresh medium and 66,666 cells were added to each well of a 96-well plate (Linbro/Titertek, Flow Laboratories Inc., McLean, VA). Each drug was tested in triplicate and compared to controls lacking polyamine antagonists, and to wells containing Flagyl® (1-ethoxy-5-nitroimidazole; metronidazole), a standard trichomonacide. Plates were scored for growth and motility after 24 and 48 hr of incubation at 37° [17].

The effect of the amino acid analogs on the generation time of the parasite was determined using Linstead's semi-defined medium [18] with the following modifications: ferrous gluconate was replaced by 72 mg/L ferrous ammonium sulfate in 7.2 mg/L 5-sulfosalicylic acid and 100 mg/L calcium succinate replaced 350 mg/L calcium chloride; the pH was brought to 6.2 with Quadrol [*N*,*N*,*N*′,*N*′-tetrakis(2-hydroxypropyl)ethylenediamine]. The medium contained a 5 mM concentration of the respective amino acid analog and was inoculated with 10^5 cells/mL; aliquots were removed at 4-hr intervals and cells counts performed using a Neuberg counting chamber. The generation time of control and drug-treated cultures was determined from a plot of the log of cell number versus time over 24 hr.

ODC induction and half-life studies. T. vaginalis was grown to stationary phase (6×10^6 cells/mL), diluted to 3×10^5 cells/mL in fresh culture medium, and incubated for a further 15 hr at 37° either in the presence or absence of 50 μg/mL cycloheximide. At 1-hr intervals an aliquot of cells was removed, centrifuged at 5000 *g* at room temperature, and resuspended in the washing buffer at room temperature; then ODC preparations or polyamine extracts were made as described previously. Protein synthesis was determined in control and cycloheximide-treated cells by measuring the ability to incorporate [^{3}H]leucine into TCA-precipitable material. These cells were incubated in TYM medium containing 10% (v/v) horse serum and 1 μCi/mL [^{3}H]leucine. Aliquots were removed at 1-hr intervals, pelleted and washed as described above; the washed cell pellets were dried on filter paper (Whatman GF/C) and treated as described by Bacchi *et al.* [16]. In a second experiment cells were grown to late exponential phase (16 hr) in TYM medium. At 16 hr 50 μg/mL of cycloheximide was added to one flask and at set intervals aliquots were removed and the cells pelleted and washed in acetate buffer. The cell pellets were subjected to three cycles of freeze–thawing and assayed for ODC activity as described previously.

Proteins were estimated by the method of Lowry *et al.* [19].

Statistics. The results are presented as the means ± the sample standard deviation (SD) of the coordinate values in the matrix. The sample variance of the coordinate values in the matrix is also presented.

RESULTS

Kinetics of ODC inhibition. Time-dependent

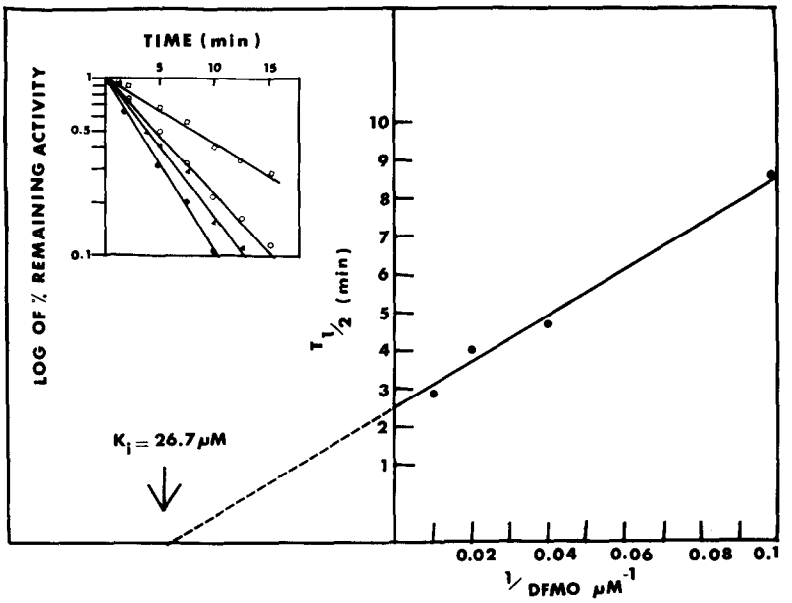

Fig. 1. Time-dependent, irreversible kinetics of *T. vaginalis* ODC inhibition by DFMO. Insert: The log of the percent remaining activity with time of preincubation with DFMO was plotted for 10 μM (□), 25 μM (○), 50 μM (▲) and 100 μM (●) DFMO. Right panel: The reciprocal of each DFMO concentration used is plotted against the time taken to reach 50% of the remaining activity ($T_{1/2}$) from which the K_i is extrapolated from the intercept on the abscissa. Similar plots were obtained in 4 separate experiments with an average determination of 27 μM (± 2.7). The assay method is described in Materials and Methods.

kinetic studies of several ornithine, lysine, arginine and putrescine analogs were determined using a partially purified *T. vaginalis* ODC preparation. The times for 50% inhibition of the trichomonad ODC with various concentrations of the substrate analogs were determined from plots of the log of percent activity remaining at various times (0–15 min) with fixed concentrations of the inhibitor (Fig. 1). From plots of the half-life of the enzyme activity versus the inverse of the inhibitor concentration it was possible to determine an apparent dissociation constant (K_i) for each inhibitor and the half-life ($T_{1/2}$) of the enzyme activity at an infinite concentration of inhibitor (Table 1). Inhibition of *T. vaginalis* ODC activity by all the analogs tested was time dependent and irreversible. Incubation of the semi-purified enzyme for 30 min with 100 μM DFMO, DL-α-monofluoromethylornithine (MFMO) or DL-α-monofluoromethyldehydroornithine (MFMDO) free base resulted in total inhibition of enzyme activity in samples assayed immediately after the incubation period. Overnight dialysis of the ornithine analog inactivated enzyme resulted in an activity that was 5, 3.5 and 2% of the control for DFMO, MFMO and MFMDO, respectively, confirming that inhibition was irreversible. The most potent compounds tested were MFMDO free base and MFMO with K_i values of 2.9 and 3.4 μM, respectively. Using ornithine as substrate, DL-α-difluoromethyllysine (DFML) was a less potent inhibitor of the trichomonad ODC than DFMO. DFML, however, proved to be equally as potent an inhibitor as DFMO using 1-[^{14}C]lysine as substrate (28.6 μM; data not shown). α-Methylornithine (MO), DL-α-difluoromethylarginine (DFMA) and (*R*,*R'*)-δ-methyl-α-acetylenicputrescine (MAP) were non-inhibitory up to the maximum concentration used (0.5, 2.5 and 1.5 mM, respectively).

In vitro *effects of inhibitors on putrescine production*. The effects of several different polyamine antagonists on putrescine production were tested using growing cultures of *T. vaginalis*. With the exception of MAP all of the compounds tested caused a reduction in intracellular putrescine concentration. MFMDO free base and the methyl ester were the most effective in lowering intracellular putrescine levels, with 5 mM causing about 80% reduction (Table 2), whereas 5 mM DFML had only a slight effect on intracellular putrescine (21% reduction). DFMA, although non-inhibitory to the trichomonad ODC at up to 2.5 mM, did cause a marked reduction in polyamine content of intact cells (Table 2).

Effects of polyamine antagonists on cell growth and viability. Using undefined TYM medium supplemented with horse serum, none of the polyamine antagonists were lethal to *T. vaginalis* up to a maximum concentration of 750 μg/mL. Metronidazole had a minimum inhibitory concentration (value which results in no motility or growth) of 3.12 μg/mL (not shown). The generation time of the parasite in the presence of a 5 mM concentration of each of the amino acid analogs was determined in semi-defined medium (Table 3).

Table 1. Activity of ornithine, arginine, lysine and putrescine analogs against semipurified *T. vaginalis* ODC using [1-^{14}C]ornithine as substrate

Drug	K_i ODC (μM)	$T_{1/2}$ (min)	$T_{1/2}$ at 10 μM (min)	N*
DFMO	27 ± 2.7	2.4 ± 0.1	8.2 ± 0.4	4
MFMDO methyl ester	712 ± 27	1.2 ± 0.6	75 ± 4.6	3
MFMDO ethyl ester	213 ± 14	3.1 ± 0.4	59 ± 4.2	3
MFMDO free base	2.9 ± 0.9	0.7 ± 0.1	1.2 ± 0.1	3
MFMO	3.4 ± 0.5	1.1 ± 0.3	2.9 ± 0.2	3
DFML	113 ± 18	7.1 ± 1.7	62 ± 4.9	3
DFMA†	2500	ND‡	ND	2
MAP†	1500	ND	ND	3
MO†	500	ND	ND	2

Abbreviations: DFMO, DL-α-difluoromethylornithine; MFMDO, DL-α-monofluoromethyldehydroornithine; DFML, DL-α-difluoromethyllysine; DFMA, DL-α-difluoromethylarginine; MAP, (*R*,*R'*)-δ-methyl-α-acetylenicputrescine; MFMO, DL-α-monofluoromethylornithine; and MO, α-methylornithine. Values are means ± SD.

* Number of experiments.

† Not inhibitory up to the concentration shown.

‡ Not determined.

Table 2. Inhibition of putrescine synthesis in intact *T. vaginalis* after 24-hr growth in the presence of a 5 mM concentration of the ornithine decarboxylase inhibitors shown

Inhibitor	Putrescine (nmol/10^7 cells)	% Inhibition
None	46.0 ± 2.5	0
DFMO	13.4 ± 0.9	71.5
MFMDO methyl ester	9.8 ± 0.5	79.1
MFMDO ethyl ester	15.9 ± 1.6	66.2
MFMDO free base	9.4 ± 0.9	80.0
DFML	37.0 ± 1.0	21.3
DFMA	25.3 ± 0.8	46.2
MAP	47.0 ± 1.5	0
MFMO	14.0 ± 0.6	70.0

Results are averages ± range of duplicate experiments. See Table 1 for abbreviations.

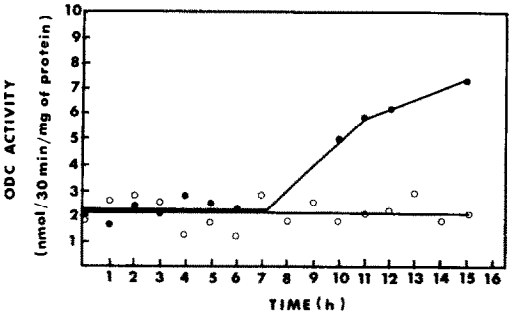

Fig. 2. ODC activity of whole cell homogenates with time. Stationary phase cells were diluted into fresh medium (3×10^5 cells/mL) containing 50 μg/mL cycloheximide (○) and compared to control cultures (●) lacking cycloheximide. Samples were removed at 1-hr intervals and ODC activity was measured as described in Materials and Methods. Each point is an average of triplicate determinations, and the plot was identical in duplicate experiments.

Under these conditions 5 mM MFMDO methyl ester doubled the generation time of *T. vaginalis* from 4.5 to 9.0 hr; MFMDO ethyl ester also caused a significant increase in the mean generation time of the parasite. MFMDO free base, MFMO and DFMO had only a modest effect on the growth of the parasite (Table 3). The other analogs tested did not affect the growth of *T. vaginalis* at a maximum concentration of 5 mM.

Induction and half-life of trichomonad ODC. To determine the induction time and half-life ($T_{1/2}$) of ODC in *T. vaginalis*, stationary phase cells were diluted to 10^5 cells/mL in fresh medium containing 50 μg/mL of cycloheximide. Samples were removed at 1-hr intervals and ODC activity was determined and compared to control cultures lacking cycloheximide. The ODC activity of control cells varied from 1.7 to 2.8 nmol/30 min/mg protein (average 2.26 ± 0.32 SD for 8 determinations) over the first 7 hr (Fig. 2). From 8 hr onwards the ODC activity increased steadily to a maximum of 7.3 nmol/30 min/mg protein (Fig. 2). Cycloheximide-treated cells, however, showed no induction of ODC activity at 8 hr and the activity varied from 1.2 to 2.9 nmol/30 min/mg protein (average 2.12 ± 0.54 SD for 16 determinations) over the 15-hr experiment (Fig. 2). Since no clear decline in activity was observed in this and similar studies, it appears that the half-life of ODC in *T. vaginalis* is greater than 15 hr. Incorporation of [^{3}H]leucine into TCA-precipitable material, in the presence or absence of 50 μg/mL cycloheximide, was monitored at the same time in parallel sets of flasks containing TYM supplemented with 10% (v/v) horse serum and 1 μCi [^{3}H]leucine/mL. The results indicate that protein synthesis was significantly (70%) reduced in cycloheximide-treated

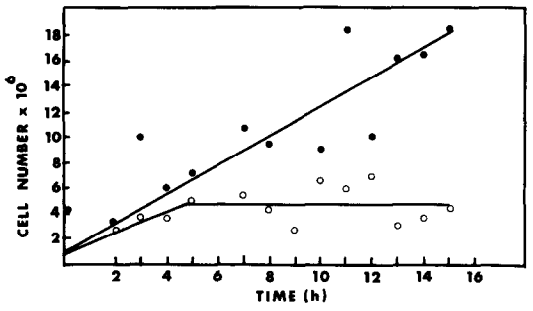

Fig. 3. Growth curves for *T. vaginalis*. Stationary phase cells were diluted into fresh medium (3×10^5 cells/mL) containing 50 μg/mL cycloheximide (○) and compared to controls (●) lacking cycloheximide. Samples were removed at 1-hr intervals and counted using a hemacytometer as described in Materials and Methods. A typical result of duplicate experiments is shown.

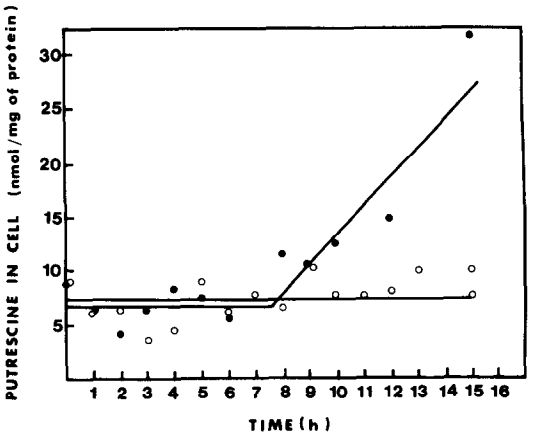

Fig. 4. Putrescine content of whole cells. Stationary phase cells were diluted in fresh medium (3×10^5 cells/mL) containing 50 μg/mL cycloheximide (○) and compared to controls (●) lacking cycloheximide. Samples were removed at 1-hr intervals and polyamine content was determined by HPLC analysis of acid extracts as described in Materials and Methods. A typical plot of duplicate experiments is shown. Each point is an average of triplicate determinations.

Table 3. Effects of polyamine antagonists on the growth of *T. vaginalis* in semi-defined medium

Polyamine antagonist	Generation time (hr)
None	4.5
DFMO	5.5
MFMDO free base	6.4
MFMDO methyl ester	9.0
MFMDO ethyl ester	8.2
DFML	4.5
DFMA	4.5
MAP	4.5
MFMO	5.6
DFMC	4.5
MFMC	4.5
MO	4.5

The amino acid analogs were added to the medium to a final concentration of 5 mM as described in Materials and Methods. Cell counts were determined at 4-hr intervals and the generation time was calculated from a semi-log plot of cell number versus time. DFMC = DL-α-difluoromethylcadaverine; and MFMC = DL-α-monofluoromethylcadaverine; all other abbreviations are defined in Table 1.

cells (not shown), and this was confirmed in cell counts which indicated no increase in cell numbers of cycloheximide-treated cultures after 5 hr (Fig. 3). In a second experiment cells were grown to late exponential phase prior to the addition of cycloheximide, and ODC activity was measured at 30-min intervals for 8 hr. In these cells the ODC activity did not vary significantly for control cells (7.90 ± 1.04 nmol/30 min/mg protein, variance 1.07; SD for 11 values) or for cycloheximide-treated cells (7.10 ± 0.85 nmol/30 min/mg protein, variance 0.73; SD for 12 values: not shown), indicating that the half-life of the trichomonad enzyme when fully induced is greater than 8 hr.

Intracellular polyamines. The putrescine content of control and cycloheximide-treated cells was also determined. In control cells intracellular putrescine increased coincident with the increase in ODC activity (Fig. 4). For the first 7 hr the putrescine levels in control cells had an average value of 6.84 nmol/mg protein ± 1.57 SD for 8 determinations (variance 2.45); from 8 to 16 hr intracellular putrescine increased to a maximum of 33 nmol/mg protein (Fig. 4). Cycloheximide-treated cells showed no increase in intracellular putrescine and had an average intracellular putrescine content of 7.31 nmol/mg protein ± 1.8 SD for 16 determinations (variance 3.25), which is within the statistical range of the control cells up to 7 hr (Fig. 4).

Putrescine excretion. The putrescine content of TYM medium is approximately 36 nmol/mL [6]. The medium from the control cells had an average putrescine concentration of 35.1 nmol/mL ± 2.68 SD for 5 determinations (variance 7.18) for the first 4 hr (Fig. 5). This then steadily increased, as evidenced by the increasing SD and variance from normal. By 15 hr the extracellular putrescine content had almost doubled (Fig. 5). Cycloheximide-treated cultures showed no increase in medium putrescine levels and after 9 hr of exposure an abrupt decline in external putrescine took place as determined by increasing SD and variance from normal (Fig. 5).

DISCUSSION

As with the majority of eukaryotes, putrescine synthesis by *T. vaginalis* occurs solely via ODC [5, 6, 20]. Unlike most other eukaryotes, however, *T. vaginalis* derives ATP from the arginine dihydrolase pathway leading to putrescine biosynthesis [20], and appears to be deficient in the synthesis of spermidine and spermine from putrescine

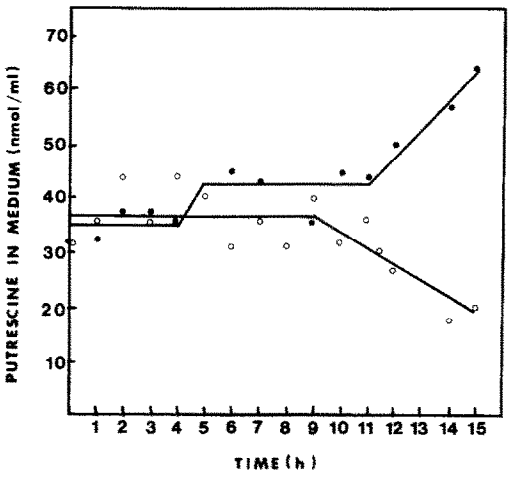

Fig. 5. Putrescine content of the *T. vaginalis* culture medium. Stationary phase cells were diluted into fresh medium (3×10^5 cells/mL) containing 50 μg/mL cycloheximide (○) and compared to controls (●) lacking cycloheximide. Samples were removed at 1-hr intervals and whole cells removed by centrifugation. The polyamine content of the medium was analyzed by HPLC as described in Materials and Methods. A typical plot of duplicate experiments is shown. Each point is an average of triplicate determinations.

[6]. As a consequence, large concentrations of putrescine accumulate and are exported into the environment. The polyamine antagonist DFMO has been shown to reduce intracellular putrescine content in *T. vaginalis*, grown both in non-defined and semi-defined (lacking exogenous putrescine) media [6]. DFMO, however, is neither cytostatic in non-defined medium [6, 21], nor is it chemotherapeutic in mouse intravaginal model infections [14]. The amine analogs used in this study are potent inhibitors of both mammalian and trypanosome ODC activity [22–27], but a complete study of polyamine antagonists has not been reported for *T. vaginalis*. In common with all other ODC activities examined to date, the trichomonad enzyme exhibited time-dependent irreversible inhibition with DFMO. The apparent K_i obtained for *T. vaginalis* ODC with DFMO was 27 μM, which is similar to that determined for rat liver, 39 μM [27], but much lower than the 130 μM determined for *Trypanosoma brucei brucei* [27]. The K_i value determined by time-dependent inhibition kinetics is slightly higher than the value determined by Lineweaver–Burk analysis (12 μM: [5]; 9 μM: Yarlett N, Goldberg B, Moharrami MA and Bacchi CJ, unpublished observations). The trichomonad enzyme was very sensitive to inhibition by MFMDO free base, the most potent ODC inhibitor (K_i approx. 3 μM) and the most efficient at reducing intracellular putrescine levels *in vitro* (80% at 5 mM). However, the methyl and ethyl esters of MFMDO were poor inhibitors of the enzyme and, as stated by Bitonti *et al.* [27], they probably act as prodrugs which are cleaved by esterases upon entering the cell releasing the free acid. The high K_i values determined for these compounds, therefore, reflect the rather slow hydrolysis of these analogs by the semipurified ODC used in this study. The observed reduction of intracellular putrescine pools indicates that there was no difference between the activities of the free base or the methyl ester forms of MFMDO. MFMO was also a very effective inhibitor of the trichomonad ODC and had a K_i 10-fold lower than that demonstrated for the *T. b. brucei* ODC [27]. In contrast to the trypanosome and mammalian ODC the trichomonad enzyme has been found to be insensitive to MAP [27, 28]. *T. b. brucei* ODC has a K_i for MAP of 5 μM [27], making it one of the most potent inhibitors of the trypanosome enzyme. These differences between inhibition of the trichomonad enzyme and the trypanosome and mammalian enzymes strongly suggest that significant differences exist in the structure and regulation of the *T. vaginalis* ODC. With the exception of MAP, all of the analogs tested effectively reduced the intracellular putrescine content of growing cells. MFMDO, MFMO and DFMO acted similarly in reducing the intracellular putrescine content by more than 60% after 24 hr. The reduction of intracellular putrescine content of whole cells by DFMA is an interesting observation. *T. vaginalis* lacks arginine decarboxylase activity and since arginine is able to act as a weak substrate for *T. vaginalis* ODC (with a V_{max} of only 0.03% of ornithine: Yarlett N, Goldberg B, Moharrami MA and Bacchi CJ, unpublished observation), it is possible that *in vivo* DFMA does act as a rather weak substrate, resulting in suicide inhibition of the ODC. The long half-life and slow turnover of the trichomonad enzyme would effectively accentuate even a weak inhibitor over 24 hr. It is, however, more likely that DFMA reduces intracellular putrescine content by an effect not directly attributable to inhibition of ODC, but rather by affecting a preceding enzyme, possibly arginine deiminase (L-arginine iminohydrolase, EC 3.5.3.6). As noted previously, *T. vaginalis* does not possess arginine decarboxylase activity [5] and putrescine is produced solely via the arginine dihydrolase pathway; hence, it is unlikely to be due to an effect on an alternative pathway of putrescine biosynthesis. The argument for an alternative to direct inhibition of ODC by DFMA is bolstered by the observation that DFML is less effective at reducing intracellular putrescine than DFMA (21 and 46% at 5 mM, respectively) despite the fact that DFML is a more potent inhibitor of the trichomonad ODC and lysine is a better substrate than arginine for the enzyme (3.4 and 0.03%, respectively: Yarlett N, Goldberg B, Moharrami MA and Bacchi CJ, unpublished observation).

The sensitive nature of the trichomonad enzyme to the fluorinated amino acid analogs tested is not reflected in their efficacy as cytostatic agents. In this respect *T. vaginalis* behaves like mammalian cells whose ODCs are extremely sensitive to inhibition by amine analogs [22–26]. However, the methyl and ethyl esters of MFMDO had a market effect on the growth rate of *T. vaginalis* in semi-defined medium. It is likely that these esters are taken up more rapidly from the environment and then hydrolyzed to the free acid by esterases as suggested for the significant

activity of MFMDO methyl ester against *T. b. brucei* [27]. One mechanism postulated to be operating in the efficacy of the methyl ester of MFMDO is the potential cytotoxicity of intracellular methanol generated by this hydrolysis. In *T. vaginalis*, the methanol released is not the cytotoxic agent, however, because controls containing methanol at levels equivalent to 100% of the hydrolyzed drug concentration did not differ in generation time from controls lacking both methanol and drug.

The ODC from *T. vaginalis* was found to be very stable with a turnover rate in excess of 8 hr when fully induced. The trypanosome enzyme also has an extended half-life (> 6 hr: [29]) in contrast to the mammalian ODC which is turned over rapidly [29]. The trypanosome ODC differs from the mammalian enzyme primarily in lacking a 36 amino acid C-terminal sequence (PEST sequence) which confers rapid turnover of the protein in mammalian cells [30]. We are presently purifying the trichomonad enzyme in preparation for sequence homology studies with other eukaryotic cells.

The trichomonad ODC appears to be highly unregulated as compared to mammalian cells, a finding which may relate to the function of the anaerobic metabolism typical of this parasite. The ODC from *T. vaginalis* was not regulated by putrescine, spermidine or spermine (Yarlett N, Goldberg B, Moharrami MA and Bacchi CJ, unpublished observation), which is the reverse of the situation found in other eukaryotic cells, in which polyamines act as strong feedback regulators of ODC activity [29, 31–34]. This observation is consistent with our finding that in *T. vaginalis* putrescine was the byproduct of an energy yielding pathway.

The long half-life of the *T. vaginalis* ODC would appear to confer similar susceptibility to growth inhibition by DFMO as found with the trypanosomes; however, this is not supported by culture or mouse model infection studies [5, 6, 14, 21]. This may be due to the presence of exogenous polyamines, but if putrescine functions as an end product of an energy producing pathway, this would not seem to be a likely cause. It is possible that the observed effect of DFMO in earlier studies [6] is due to interference with the arginine dihydrolase pathway caused by transiently increasing ornithine pools, which decrease after 48 hr, coinciding with inhibition of cell division [6]. This may be due to the production of Δ'-pyrroline-5-carboxylate, a toxic product of the transamination of ornithine [35], or to direct interference with ATP production from this pathway. It can be calculated that *T. vaginalis* depletes 1 mM arginine from the medium after 24 hr, corresponding to an increase in cell number of 10^6 cells/mL [18]. This would result in a flow of 5 nmol arginine consumed/min/mg protein. Each mole of arginine can generate a mole of ATP via the arginine dihydrolase pathway. With hexose sugar, about 50 nmol ATP are produced/min/mg protein [36]; hence, the arginine dihydrolase pathway is capable of producing about 10% of the parasite energy requirements. It is likely, therefore, that antagonists aimed at the step preceding carbamate kinase and hence directly affecting ATP synthesis would have greater efficacy than inhibitors of ODC, and this is presently under investigation.

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