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DIFFERENTIAL EFFECTS OF INHIBITORS OF PURINE METABOLISM ON TWO TRICHOMONAD SPECIES*

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Abstract—*Tritrichomonas foetus* and *Trichomonas vaginalis* are both incapable of *de novo* purine nucleotide synthesis. Previous studies indicated that *T. foetus* relies mainly on the salvage of hypoxanthine and subsequent conversion of IMP to AMP and GMP, whereas *T. vaginalis* depends on direct conversions of exogenous adenosine to AMP and guanosine to GMP without much interconversion between the two nucleotides. These two different types of purine salvage suggest the possibility of differential sensitivities between the two species of trichomonad flagellates toward different purine antimetabolites. Mycophenolic acid, hadacidin, 8-azaguanine, and formycin B inhibited the growth of *T. foetus* but had no effect on *T. vaginalis*. Mycophenolic acid acted by blocking conversion of IMP to GMP, hadacidin inhibited conversion of IMP to AMP, and 8-azaguanine was incorporated into the *T. foetus* nucleotide pool, likely via hypoxanthine phosphoribosyl transferase. Formycin B was converted to 5′-monophosphate in *T. foetus* and inhibited the conversion of IMP to AMP. Its precise mechanism of action on *T. foetus* remains, however, to be elucidated. Alanosine, whose ribonucleotide derivative is a potent inhibitor of adenylosuccinate synthetase, had no effect on the growth or hypoxanthine incorporation in *T. foetus*, which may be due to the lack of converion of alanosine to the ribonucleotide because of the absence of *de novo* purine nucleotide synthesis in parasites. Four adenosine analogs, adenine arabinoside, tubercidin, sangivamycin, and toyocamycin, were found inhibitory to the growth of *T. vaginalis* but showed little effect on *T. foetus* growth. Further investigations suggested that these four compounds acted on *T. vaginalis* by blocking incorporation of adenosine into the adenine nucleotide pool.

Trichomonad flagellates represent a family of flagellated protozoan parasites inhabiting the gastrointestinal tract or vagina of mammals. *Tritrichomonas foetus*, a cattle parasite, and *Trichomonas vaginalis*, a human parasite, are among the best studied species with the most biochemical information available on them [1]. Our recent investigation indicated that *T. foetus* is incapable of *de novo* synthesis of both purines [2] and pyrimidines [3]. Its survival depends on simple salvage pathways; conversion of exogenous hypoxanthine to IMP provides the main source of purine nucleotides [2], whereas a direct incorporation of uracil into UMP serves as the main channel for pyrimidine ribonucleotides [3]. It possesses no dihydrofolate reductase or thymidylate synthetase activity and thus has a totally isolated and highly specific single pathway for thymidine salvage [3].

These unusual metabolic activities are apparently not unique to *T. foetus*. Similar observations have been made also on *T. vaginalis* [4–7]. One major difference between these two species appears to be in the purine salvage; *T. vaginalis* cannot incorporate hypoxanthine into nucleotides but, instead, converts adenosine and guanosine directly to AMP and GMP through its kinase activities [5, 6]. This difference could be reflected in different susceptibilities of the two species toward various known inhibitors of purine metabolism.

In the present investigation, we have tested two known inhibitors of adenylosuccinate synthetase, alanosine [8] and hadacidin [9], and a specific inhibitor of IMP dehydrogenase, mycophenolic acid (MPA)‡ [10], on the two trichomonad flagellates. The effects of the antineoplastic agents and substrates of adenosine kinase, adenine arabinoside (ara-A), sangivamycin, toyocamycin, and tubercidin, on the two parasites were also examined [11, 12]. Formycin B (FoB), an effective antileishmanial agent [13] converted by *Leishmania* spp. [14, 15] and *Trypanosoma cruzi* [16] to formycin A-5′-triphosphate, was also tested. 8-Azaguanine (8-AG), a substrate for the hypoxanthine phosphoribosyl transferases [17], was examined. The results confirmed the two different schemes of purine salvage postulated in *T. vaginalis* [5] and *T. foetus* [2], and they provided more in-depth understanding on the purine metabolism in trichomonads as a potential target for chemotherapy of trichomoniasis.

MATERIALS AND METHODS

Cultures and assays for growth inhibition. T. foetus KV_1 strain was cultivated in Diamond TYM medium, pH 7.2, plus 10% heat-inactivated horse serum [18]. *T. vaginalis* ATCC 30001 was cultured in a similar TYM medium, pH 6.2, plus 10% heat-inactivated bovine serum [18]. For drug sensitivity studies, the

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‡ Abbreviations: MPA, mycophenolic acid; ara-A, adenine arabinoside; FoB, formycin B; and 8-AG, 8-azaguanine.

cells grown to stationary phase were inoculated at a 1:10 ratio to the fresh media containing the drug under testing. Cultures were then incubated at 37° in a Forma anaerobic incubator model 1024 under N_2 for 24 hr. The number of cells in the incubated culture was monitored in a Coulter counter model ZF. For more detailed analyses of the relationship between drug effects on the growth of *T. foetus* and purine supply in the media, a semi-defined HUT medium containing 147 μM hypoxanthine, 178 μM uracil and 0.8 μM thymidine [19] was used for culturing *T. foetus*. The procedures of inoculation, incubation, and cell counting of the HUT cultures were the same as previously described. Another semi-defined medium, DL-8 [20], was used for cultivating *T. vaginialis*, but it could not sustain *T. vaginalis* growth beyond two serial transfers with an inoculation ratio of 1:10 in our hands.

Precursor incorporation into nucleotide pool. Log-phase parasites suspended in phosphate-buffered saline glucose solution (PBSG) to a final concentration of 10^8 *T. foetus* cells/ml at pH 7.2 [2] or 3×10^6 *T. vaginalis* cells/ml at pH 6.2 [7] were labeled with radioactive substrates at 37° and washed, and the intracellular nucleotide pool was extracted by the perchloric acid-KOH procedure [3]. The extract was analyzed by high pressure liquid chromatography (HPLC) with an ion-exchange column Ultrasil AX according to a previously described procedure [2]. For quantitative estimation of radiolabeled substrate incorporation into nucleotide pool, the extract was passed through glass-fiber filter loaded with PEI-cellulose and washed, and the resin-trapped radioactivity was counted in a Beckman LS-3133T liquid scintillation spectrometer [3].

Chemicals. Radiolabeled purine bases and purine nucleosides were purchased from Amersham (Arlington Heights, IL), New England Nuclear (Boston, MA), and ICN (Irvine, CA). [^{3}H]FoB was a gift from Dr. P. Rainey [15]. FoB, 8-AG, ara-A, and tubercidin were obtained from the Sigma Chemical Co., St. Louis, MO. Alanosine, hadacidin, sangivamycin, and toyocamycin were provided to us by Dr. W. Sadée of the School of Pharmacy, UCSF. All the other chemicals used in the study were of the highest purity commercially available.

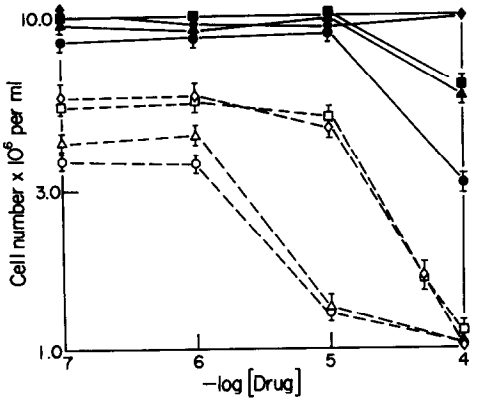

Fig. 2. Effects of ara-A (♦, ◊), tubercidin (■, □), sangivamycin (▲, △) and toyocamycin (●, ○) on the growth of *T. foetus* (♦, ■, ▲, ●) and *T. vaginalis* (◊, □, △, ○). Culturing conditions were as described in the legend of Fig. 1.

RESULTS

Drug effects on in vitro *growth of* T. foetus *and* T. vaginalis. Various drugs were tested on the growth of these two parasites in TYM media over a period of 24 hr. The results indicated no detectable effect of 8-AG, alanosine, or hadacidin on either organism up to a drug concentration of 1 mM. The rest of the drugs would be classified roughly into two categories: (1) Those inhibiting the growth of *T. foetus* but showing little effect on *T. vaginalis*. It consists of MPA and FoB (see Fig. 1). (2) Those blocking *T.*

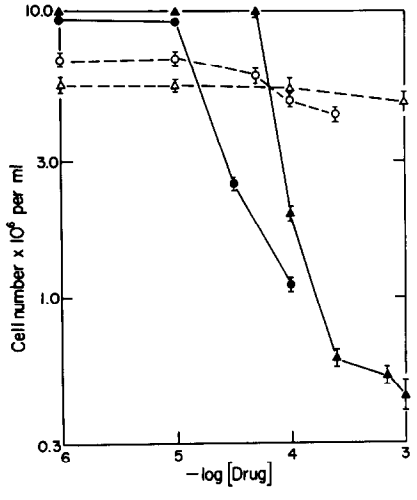

Fig. 1. Effects of MPA (●, ○) and FoB (▲, △) on the growth of *T. foetus* (●, ▲) and *T. vaginalis* (○, △). *T. foetus* was cultivated in TYM medium, pH 7.2, and *T. vaginalis* in TYM medium, pH 6.2. Incubation was at 37° for 24 hr.

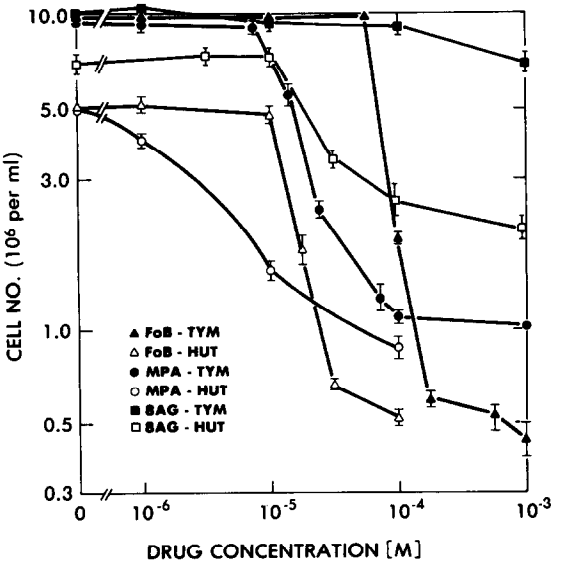

Fig. 3. Effects of drugs on the growth of *T. foetus* in the TYM medium vs growth in the HUT medium.

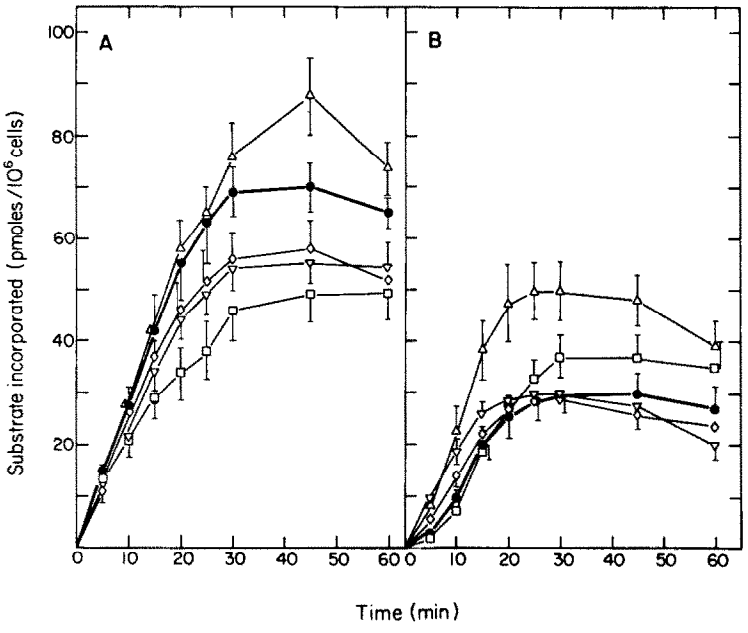

Fig. 4. Drug effects on the incorporation of hypoxanthine and guanine into the *T. foetus* nucleotide pool. [8-^{14}C]Hypoxanthine (51.1 mCi/mmole) (A) and [8-^{14}C]guanine (56.7 mCi/mmole) (B) were the substrates present at 20 μM. The drugs added to the incubation mixture were: 100 μM MPA (△), 1 mM FoB (□), 1 mM hadacidin (▽); and 1 mM 8-AG (◊); closed circles (●) represent no-drug control.

vaginalis growth but demonstrating no appreciable effect on *T. foetus*. The group includes toyocamycin, sangivamycin, tubercidin, and ara-A (see Fig. 2). When MPA or FoB was tested again on *T. foetus* in the semi-defined medium HUT [19], both compounds demonstrated higher potencies in blocking *T. foetus* growth (Fig. 3). 8-AG, which showed no inhibition of *T. foetus* growth in TYM medium, limited the parasite in HUT medium with an estimated 50% inhibitory concentration (IC_{50}) of 30 μM (Fig. 3).

Drug effects on substrate incorporation into nucleotide pools of T. foetus *and* T. vaginalis. Incorporation of radiolabeled hypoxanthine or guanine into the nucleotide pools of *T. foetus* was examined in the presence of various drugs (Fig. 4). Alanosine (1 μM) showed, again, no detectable effect on the incorporation of either substrate, whereas hadacidin (1 mM) caused about 20% decrease in the rate and level of hypoxanthine incorporation (Fig. 4A), but showed no apparent effect on the guanine incorporation (Fig. 4B). FoB at 1 mM acted like hadacidin with some-

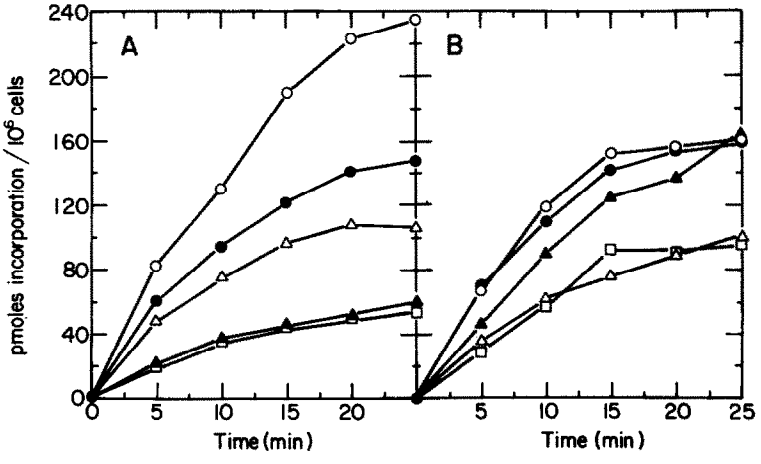

Fig. 5. Drug effects on the incorporation of adenosine and guanosine into the *T. vaginalis* nucleotide pool. [2,8-^{3}H]Adenosine (46.7 mCi/mmole) (A) and [8-^{3}H]guanosine (66.6 mCi/mmole) (B) were the substrates present at 20 μM. The drugs added to the incubation mixture were: 10 μM toyocamycin (▲—▲), 50 μM tubercidin (□—□), 10 μM sangivamycin (△—△), and 50 μM ara-A (●—●); open circles (○—○) represent no-drug control.

what stronger inhibition of hypoxanthine incorporation (Fig. 4A). Similar effects were also observed with 1 mM 8-AG. MPA (100 μM), on the other hand, stimulated the incorporation of both hypoxanthine and guanine. When the *T. foetus* incorporation of [8-^{14}C]adenine (49.1 mCi/mmole) was monitored in the presence of different drugs, the time courses of incorporation were very much similar to those of hypoxanthine incorporation (see Fig. 4A). This observation is in agreement with our previous finding [2], that adenine is exclusively converted to hypoxanthine before entering the nucleotide pool in *T. foetus*. The incorporation of [2,8-^{3}H]adenosine (94.7 mCi/mmole) was not appreciably affected by any of the drugs tested, suggesting direct incorporation of adenosine into AMP through the action of *T. foetus* adenosine kinase which may not be susceptible to the drugs.

Incorporation of radiolabeled adenosine and guanosine into the nucleotide pool of *T. vaginalis* was assayed in the presence of ara-A, tubercidin, toyocamycin, or sangivamycin, each at its IC_{50} value estimated from the data in Fig. 2. The results presented in Fig. 5 indicate that the rate of adenosine incorpoartion was reduced significantly by 10 μM toyocamycin or 50 μM tubercidin to 25% of the control value. Sangivamycin (10 μM) and ara-A (50 μM) inhibited adenosine incorporation by about 50% (Fig. 5A). At the same concentrations, sangivamycin and tubercidin exerted 50% inhibition on the rate of guanosine incorporation. Toyocamycin exerted about 25% inhibition whereas ara-A showed no appreciable effect.

There was no detectable incorporation of radiolabeled hypoxanthine, adenine, guanine, or inosine into the nucleotide pool of *T. vaginalis* in our studies.

HPLC analysis of radiolabeled nucleotide pools. The nucleotide pools in *T. foetus* pulse-labeled with 20 μM [8-^{14}C]hypoxanthine (51.1 mCi/mmole) for 45 min in the presence of different drugs were extracted and analyzed by ion-exchange HPLC. Data presented in Fig. 6 demonstrate that 100 μM MPA completely inhibited incorporation of hypoxanthine into XMP, GMP, GDP, or GTP. Meanwhile, labels in IMP, AMP, ADP, and ATP were enhanced markedly (Fig. 6B). This observation agrees with the knowledge that MPA is a specific inhibitor of IMP dehydrogenases of both bacterial and mammalian origins [10]. When 1 mM hadacidin or 1 mM FoB was present in the pulse-labeling experiments, incorporation into AMP, ADP, and ATP was decreased appreciably, whereas incorporation into IMP, XMP, GMP, GDP, and GTP was enhanced only slightly (Fig. 6, panels C and D). The inhibitory effects of FoB appeared to be stronger than those of hadacidin, which may explain why the latter inhibited somewhat less the overall hypoxanthine incorporation (see Fig. 4). These results also agree with the previous findings that hadacidin is an inhibitor of adenylosuccinate synthetase [9], and FoB-5′-phosphate has an inhibitory effect on the same enzyme from *Leishmania* [13]. 8-AG at 1 mM had a slight inhibitory effect on incorporation of radioactivities in all the purine nucleotides (data not shown).

In *T. vaginalis*, adenosine was incorporated only into AMP, ADP, ATP, NAD and ADP-glucose, and guanosine only into GMP, GDP, GTP and GDP-glucose (Fig. 7). The four adenine nucleoside analogs, tubercidin, sangivamycin, toyocamycin and ara-A, significantly and uniformly reduced adenosine incorporation into all adenine nucleotides, whereas guanosine incorporation was only moderately but also uniformly decreased among all the guanine nucleotides. The profiles in panels A and B of Fig. 7 show the general inhibitory effects of 50 μM tubercidin on adenosine and guanosine incorporation. This across-the-board inhibition suggests that tubercidin may block the conversion of purine nucleosides to the monophosphates in *T. vaginalis*.

Analysis of drug actions. While all the indications suggest that the inhibition of *T. foetus* growth by MPA may be due to blocked conversion of IMP to GMP in the parasite, the theory could be further tested in the HUT medium where hypoxanthine is the only source of purine for *T. foetus*. By adding guanine or guanosine to the medium, one should be able to bypass the block and provide GMP by the actions of guanine phosphoribosyl transferase and guanosine phosphotransferase in *T. foetus* [2]. The

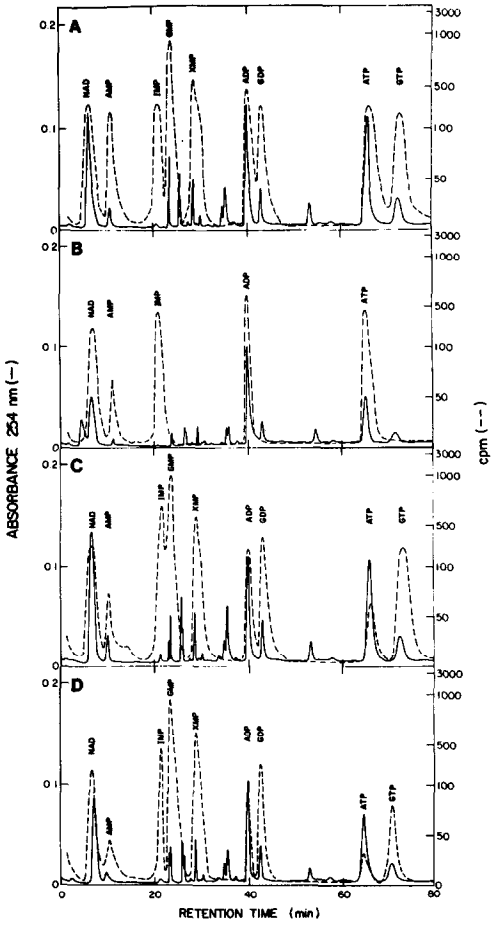

Fig. 6. HPLC profiles of nucleotides in *T. foetus* pulse-labeled with 20 μM [8-^{14}C]hypoxanthine (51.1 mCi/mmole) for 45 min in the presence of (A) no drug control, (B) 100 μM MPA, (C) 1 mM hadacidin, and (D) 1 mM FoB.

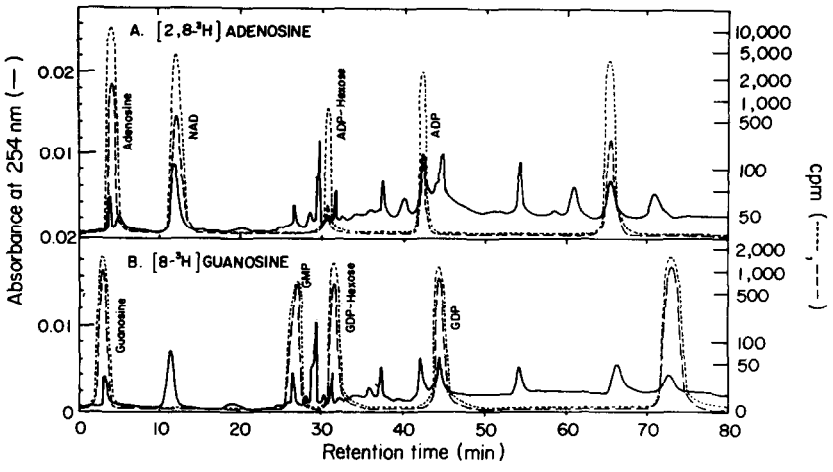

Fig. 7. HPLC profile of nucleotides in *T. vaginalis* pulse-labeled with 20 μM [2,8-^{3}H]adenosine (500 mCi/mmole) (A) or [8-^{3}H]guanosine (500 mCi/mmole) (B) for 30 min, in the no drug control (- - - -) and in the presence of 50 μM tubercidin (— — —).

experimental results presented in Fig. 8 indicate that both guanine and guanosine are indeed capable of reversing the inhibitory effect of MPA on *T. foetus* growth. The postulated mechanism of anti-*T. foetus* activity of MPA has been thus further confirmed.

When *T. foetus* was incubated with [^{3}H]FoB and the nucleotide pool was extracted and analyzed in HPLC, the elution profile (Fig. 9) indicated the presence of only a single radiolabeled band whose retention time corresponded to that of FoB-5′-monophosphate [15]. Should this nucleotide derivative be the active entity blocking growth of *T. foetus*, it could act by inhibition of adenylosuccinate synthetase, as suggested by data in Fig. 4A and 6D and indicated previously in *L. donovani* by Carson and Chang [13]. Cultivation of *T. foetus* in HUT medium with added adenosine would then be expected to reverse the inhibition by FoB since adenosine can be converted directly to AMP by adenosine kinase in *T. foetus*. The actual data presented in Fig. 8, however, indicate that the increasing concentrations of adenosine in the HUT medium have no effect on the inhibition of growth by 100 μM FoB. The mechanism of FoB action on *T. foetus* thus remains unknown.

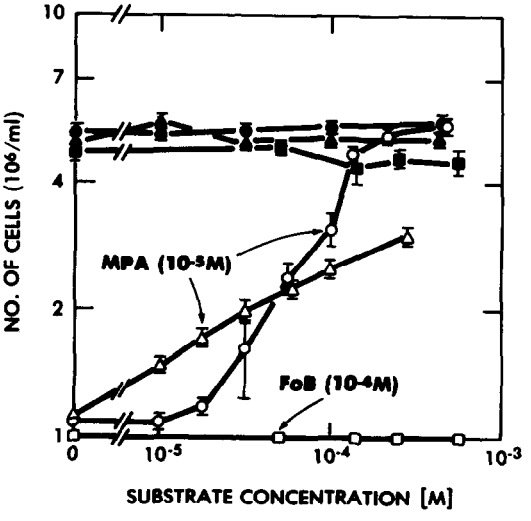

Fig. 8. Reversal of growth inhibition exerted by MPA or FoB on *T. foetus* cultivated in HUT medium. The added substrates were: guanine, (○, ●), guanosine (△, ▲), and adenosine (□, ■).

The inhibitory effects of 8-AG on *T. foetus* growth in HUT medium (Fig. 3) and on hypoxanthine incorporation into *T. foetus* nucleotides (Fig. 4A) fulfilled the expected action of 8-AG as a substrate of hypoxanthine phosphoribosyl transferase [21]. Since radiolabeled 8-AG is not commercially available, we incubated *T. foetus* with 1 mM unlabelled 8-AG and analyzed the u.v.-profile of nucleotide pools in HPLC. The data presented in Fig. 10 indicated the emergence of three u.v.-absorbing bands with retention times corresponding to those of mono-, di-, and triphosphates of 8-AG [21]. It is thus likely that the mode of anti-*T. foetus* action of 8-AG is that of incorporation into the parasite nucleic acids.

DISCUSSION

The obvious differences in purine salvage pathway networks between *T. foetus* [2] and *T. vaginalis* [5] have suggested to us that these two parasites may have different susceptibilities toward various antimetabolites of purines and purine nucleosides. Since *T. foetus* can survive on hypoxanthine alone [19], the hypoxanthine phosphoribosyl transferase, IMP dehydrogenase, and adenylosuccinate synthetase in the parasite must be capable of performing functions essential for its survival. This is demonstrated by the growth inhibition exerted by the IMP dehydrogenase inhibitor MPA [10] (Figs. 1 and 3), its inhibition of hypoxanthine incorporation into XMP, GMP, GDP, and GTP (Fig. 6B), and the reversal of its growth inhibition by guanine or guanosine (Fig. 8). The inhibitor of adenylosuccinate synthetase, hadacidin [9], has only weak activity in reducing the incorporation of hypoxanthine into AMP (Fig. 6D) which may explain why hadacidin at up to 1 mM concentration

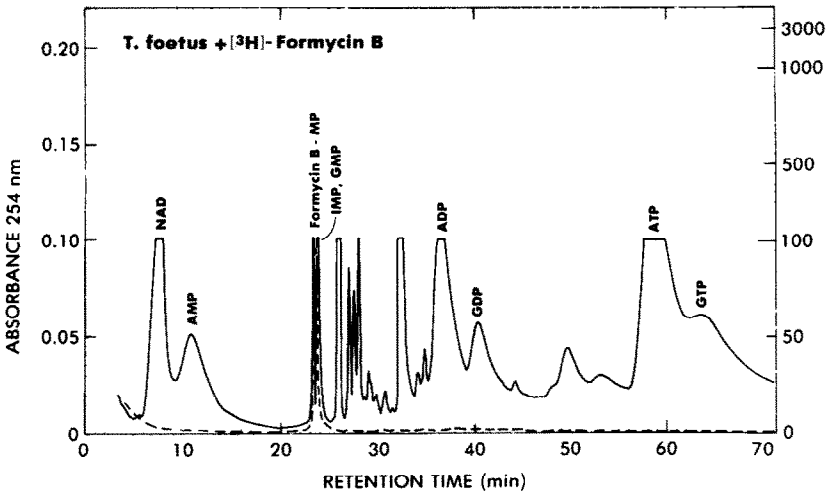

Fig. 9. Incorporation of FoB into the nucleotide pool of *T. foetus*. *T. foetus* was incubated with 3 μM [^{3}H]FoB (450 mCi/mmole) for 2 hr.

shows no apparent inhibition of *T. foetus* growth. This lack of activity in hadacidin, which has a low K_i value of 4.2 μM on *Escherichia coli* adenylosuccinate synthetase [9], could be due to different kinetic properties between the *E. coli* and *T. foetus* enzymes. Or, it could be attributed to a probable reduction of the hadacidin molecule to the inactive amino alcohol derivative by the power of extraordinarily low redox potential in *T. foetus* [1]. The other inhibitor of adenylosuccinate synthetase, alanosine [8], has no detectable effect on either the growth of *T. foetus* or the hypoxanthine incorporation into nucleotides. The reason for this lack of activity is probably because alanosine itself is a poor inhibitor of adenylosuccinate synthetase (K_i = 57.23 mM) [22]. It has to be converted to L-alanosyl-5-amino-4-amidazole carboxylic acid ribonucleotide before turning into a potent inhibitor (K_i = 0.228 μM) [22]. The enzyme catalyzing this conversion, 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide synthetase, is an enzyme involved in *de novo* purine nucleotide synthesis. It is apparently missing from *T. foetus* since the latter is incapable of *de novo* purine nucleotide synthesis [2].

The mechanism of FoB inhibition of *T. foetus* growth is far from being clear. The IC_{50} value of growth inhibition was about 20 μM in HUT medium with the presence of 147 μM hypoxanthine (see Fig. 3), whereas the IC_{50} for inhibiting 20 μM hypoxanthine incorporation into nucleotides was around 1 mM (see Fig. 4A and 6D). Since FoB was converted only to FoB-5′-monophosphate in *T. foetus* (Fig. 9), and since it did inhibit conversion of hypoxanthine to AMP, ADP, and ATP (Fig. 6D), it is likely that one of the actions of FoB in *T. foetus* is inhibition of adenylosuccinate synthetase by its 5′-monophosphate, as was suggested in *Leishmania* by Carson and Chang [13]. But this inhibition might not

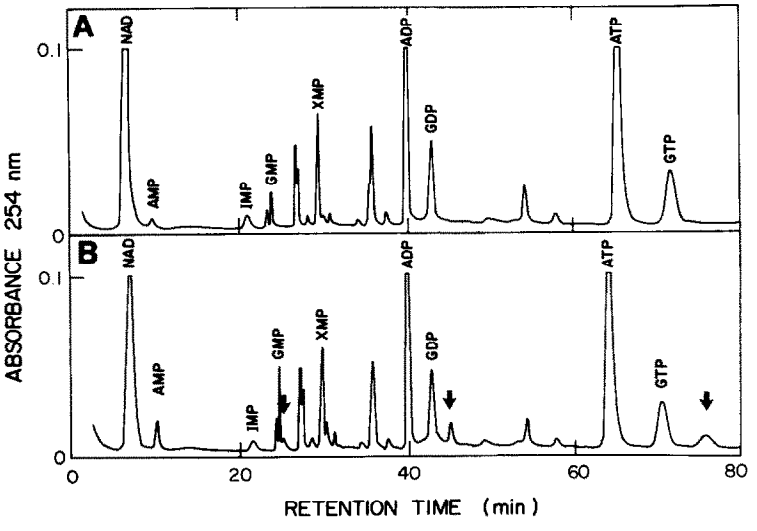

Fig. 10. Incorporation of 8-AG into the nucleotide pool of *T. foetus*. Key: (A) no drug control, and (B) with 1 mM 8-AG for 2 hr.

be the sole mechanism of FoB inhibition of *T. foetus* growth since supply of AMP to the parasite from adenosine cannot reverse the inhibited growth (Fig. 8). Unless the AMP supplied from adenosine is proven to be inadequate, another yet unidentified action(s) of FoB-5′-monophosphate in *T. foetus* may be responsible for the growth inhibition. Whatever the action may be, it cannot be the incorporation of formycin A-5′-triphosphate into RNA as has been suggested for the mode of FoB action on *Leishmania* [14, 15] and *T. cruzi* [16], because there is no conversion of FoB to formycin A-5′-triphosphate in *T. foetus*.

Out data suggest that 8-AG may be converted to the corresponding nucleotide by *T. foetus* hypoxanthine phosphoribosyl transferase and proceed to form di- and triphosphates (see Fig. 10). It is likely that the triphosphate may be incorporated eventually into *T. foetus* RNA as in the bacterial and mammalian systems [21], and that could precipitate the cessation of cell growth witnessed in Fig. 3. The partial inhibition of hypoxanthine incorporation by 8-AG (Fig. 4A) might not be the cause of growth inhibition, since further increase of hypoxanthine level in HUT medium failed to reverse the inhibited growth (data not shown).

Since *T. vaginalis* cannot incorporate hypoxanthine or inosine, shows no purine phosphoribosyl transferase in its extracts, and can only convert adenosine to AMP and guanosine to GMP through kinases [5, 6], it is likely that the interconversion network among IMP, AMP and GMP is completely absent from *T. vaginalis*. This probability has been verified in our laboratory by repeated HPLC analyses of nucleotide pools of *T. vaginalis* which were pulse-labeled and chased with adenosine or guanosine; no interconversion between adenine and guanine nucleotides has ever been observed (unpublished observation). There is also a total absence of IMP dehydrogenase and adenylosuccinate synthetase from the crude extracts of *T. vaginalis* (unpublished observation). It is thus of little surprise to see the total insensitivity of *T. vaginalis* toward MPA, 8-AG, and FoB (Fig. 1).

On the other hand, the four adenosine analogs, ara-A, tubercidin, toyocamycin, and sangivamycin, showing inhibition on *T. vaginalis* growth, have little effect on *T. foetus* (Fig. 2). One possible explanation for this discrepancy may be attributed to the lack of adenosine deaminase in *T. vaginalis* [5] but the abundant presence of the same enzyme in *T. foetus* [2]. The latter may simply deaminate the four adenosine analogs to destroy their biological activities. The *T. vaginalis* adenosine kinase, on the other hand, may behave like other adenosine kinases of bacterial or mammalian sources by recognizing the adenosine analogs as substrates [11, 12]. These drugs could be eventually incorporated into *T. vaginalis* RNA and stop cell growth. The preferential and general inhibition of adenosine incorporation into *T. vaginalis* AMP, ADP, and ATP by these drugs (see Fig. 7) tends to support this suggested mechanism of action. These drugs may have the potential of being developed into chemotherapeutic agents against trichomoniasis in humans.

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