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ADENOSINE ANALOG METABOLISM IN *GIARDIA LAMBLIA*

IMPLICATIONS FOR CHEMOTHERAPY*

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Abstract—Certain adenosine analogs can inhibit the growth of *Giardia lamblia*. This biological action correlates with the ability of the organism to phosphorylate the nucleoside directly to the nucleotide. Four of these, 8-azaadenosine, 1-deazaadenosine, 7-deazaadenosine, and 9-deazaadenosine, were very effective. The respective bases of the first three were ineffective. The base of 9-deazaadenosine was not tested as this C-nucleoside is non-cleavable. Metabolic studies using radioactive 7- and 9-deazaadenosine showed that these compounds were phosphorylated by the organism. Enzymatic assay confirmed the presence of nucleoside phosphotransferase activity; no nucleoside kinase activity was found. Preliminary characterization of this phosphotransferase suggests that it has different substrate and phosphate donor specificities than the mammalian enzyme and, therefore, may be a potential site for chemotherapeutic attack.

Giardia lamblia, the etiologic agent of giardiasis, has a worldwide distribution with a prevalence estimated as high as 30% in parts of the developing world [1]. It is the leading protozoan cause of diarrhea among travelers [2]. In the United States, this parasite has caused water-borne disease epidemics and is endemic in Colorado, Washington, Utah, New Hampshire, Vermont, Oregon, and Montana [3]. Giardiasis is also a serious problem in veterinary medicine [4]. Current drugs such as quinacrine, metronidazole, and furazolidone are efficacious but have undesirable side-effects, and none is completely effective [5]. There is also some suggestion that these drugs may be carcinogenic or mutagenic [5].

An ideal chemotherapeutic compound for giardiasis would not be absorbed or metabolized by the host and would be active against some unique metabolic capability of the parasite. Studies of other flagellated protozoan pathogens, such as *Leishmania* spp. [6], *Trypanosoma* spp. [7, 8], and *Trichomonas* spp. [9, 10], which have some peculiar purine metabolic sequences, suggest that purine metabolism in *Giardia lamblia* might be an area of potential chemotherapeutic interest. Wang and Aldritt [11] investigated purine metabolism in the Portland-1 (P-1‡) strain of *G. lamblia* and found that this parasite, like the above organisms, lacks *de novo* purine synthesis and must rely on exogenous purine sources [11]. Adenine, guanine and their ribonucleosides were salvaged but were used only for the synthesis of their respective nucleotides. There was no salvage of hypoxanthine, xanthine or inosine. The inability to interconvert between adenine and guanine nucleotides suggested that IMP was not a common intermediate as it is in most organisms. This was supported by the inability of the parasite to salvage hypoxanthine and its ribonucleoside. Analysis of purine salvage enzymes by the authors confirmed the above and showed a relatively simple purine salvage network that differed from other parasitic flagellates. Only four purine salvage enzymatic activities were demonstrated: adenosine and guanosine hydrolases and adenine and guanine phosphoribosyltransferases. There was no evidence for the direct phosphorylation of adenosine or guanosine to their respective nucleotides. From these results it was concluded that the ribonucleosides of adenine and guanine were hydrolyzed to their respective bases and that phosphoribosylation was the only route to AMP and GMP for exogenous purines.

Investigations in this and other laboratories [6, 12] have shown that the protozoan parasite *Leishmania donovani* also salvages purine ribonucleosides principally by first cleaving them to their respective bases. However, it also was found that certain purine ribonucleoside analogues, such as allopurinol ribonucleoside [13] and formycin B [14], are not cleaved to their bases but are phosphorylated directly by a nucleoside phosphotransferase. Because the appar-

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‡ Abbreviations: P-1, Portland 1; 9-DINO, 9-deazainosine; 9-DADO, 9-deazaadenosine; Tris–mannitol, 0.23 M mannitol, 0.07 M sucrose, 0.05 M Tris–HCl buffer (pH 7.0) and 0.001 M EDTA; LSC, liquid scintillation counter; HPLC, high performance liquid chromatography; DTT, dithiothreitol; PEI, polyethyleneimine; ADA, adenosine deaminase; dCF, deoxycoformycin; TYI, TYI-S-33 medium; 5-ITU, 5-iodotubercidin; MEM, Eagle's Minimum Essential Medium; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; and PIPES, 1,4-piperazinediethanesulfonic acid.

ent similarities in the salvage of purine ribonucleosides by *G. lamblia* and *L. donovani*, we investigated the biological effects and metabolism of adenosine analogues in *G. lamblia*.

MATERIALS AND METHODS

Materials. Culture media components were obtained from the following sources: casein digest peptone and yeast extract from BBL Microbiological System, Cockeysville, MD; fetal calf serum, MEM components and purine-free medium 199 from KC Biologicals, Lenexa, KS; amino acids and vitamins, from the Sigma Chemical Co., St. Louis, MO; and plasticware from Corning Glass Works, Corning, NY. Tritium-labeled formycin A and B, 7-deazaadenosine, 9-deazaadenosine and 9-deazainosine were purchased from Moravek Biochemicals, Brea, CA, and were [^{3}H(G)]-labeled except for 9-deazainosine which was [2,8-^{3}H]-labeled. All other radiolabeled purines were from RPI, Mount Prospect, IL, and were [8-^{14}C]-labeled. Purine analogues were gifts from Dr. R. L. Miller, Burroughs Wellcome Co., Research Triangle Park, NC, with the following exceptions: formycin A and B, 7-deazaadenosine and 7-deazaadenosine monophosphate were purchased from the Sigma Chemical Co.; 9-deazainosine and 9-deazaadenosine were gifts from Dr. R. S. Klein, Sloan-Kettering Institute, New York, NY; deoxycoformycin was obtained from the National Cancer Institute, Bethesda, MD; and 5-iodotubercidin was a gift from Dr. L. B. Townsend, University of Michigan School of Pharmacy, Ann Arbor, MI. All other chemicals were from commercial sources and were of analytical grade or better.

Culture techniques. The Portland-1 (ATTC no. 30888) and WB (ATTC no. 30957) were obtained from the American Type Culture Collection, Rockville, MD, and were cultured in Diamond's filter-sterilized TYI-S-33 (TY1) medium modified as described by Keister [15]. Stock cultures were maintained in 25 cm^2 culture flasks filled to the top and sealed. Cells for radiolabel incorporation experiments were grown in 75 cm^2 tissue (T-75) culture flasks containing 25–30 ml of medium and incubated in an anaerobic culture system (Forma model 1024, Forma Scientific, Marietta, OH) under microaerobic conditions (0.5% O_2, 10% CO_2, balance N_2 atmosphere). Cells for enzyme assay experiments were grown in "outside-in" roller bottles (Bellco Glass, Vineland, NJ) as described by Farthing *et al.* [16]. All cultures were incubated at 37°; cell numbers were determined by counting in a hemocytometer.

Growth inhibition studies. These were performed in 100 × 13 mm screwcap tubes filled to the top and sealed. Drug was added to TYI medium (0.1 ml or less in 10 ml medium) and filter-sterilized. Tubes (three replicate tubes per drug concentration) were inoculated with 0.1 ml medium containing 1–$2 \cdot 10^5$ cells to give a final cell density of 1–$2 \cdot 10^4$ organisms/ml. Tubes were incubated for 4 days at 37°, at which time control cultures were in stationary phase and had a cell density of 1–$2 \cdot 10^6$ cells/ml. Cells were counted by chilling tubes in ice for 30 min, then reading the optical density at 650 nm in a Gilford 300-N micro-sample spectrophotometer and comparing the absorbance to a standard curve constructed from numbers obtained by counting cells in a hemocytometer. This relationship was linear from $1 \cdot 10^5$ to $2 \cdot 10^6$ cells/ml. Results are expressed as average percent control growth.

High performance liquid chromatograph (HPLC) analysis. The HPLC system consisted of the following Varian instruments (Varian Instrument, Sugarland, TX): a 5060 liquid chromatograph system, a UV-1 ultraviolet (254 nm) detector connected in series with a UV-50 variable wavelength detector set to 295 nm and a Vista CSD-401 data system. Injections were done using a Valco C6U valve (Valco Instruments, Houston, TX) with a 100-μl sample loop. Nucleotides were separated by strong anion exchange (SAX) HPLC using a 4.6 × 250 mm Whatman Partisil 10 SAX column (Whatman, Inc., Clifton, NJ). Samples were eluted at a flow rate of 0.5 ml/min with a gradient of 0.015 to 1 M potassium phosphate buffer, pH 3.5. The gradient was isocratic for 15 min and then linear for 75 min. The eluate was collected (0.5 ml/fraction), and radioactivity was determined with 5 ml of 3a70 B counting fluid (RPI) in a Beckman (Beckman Instruments, Fullerton, CA) LS-7000 liquid scintillation counter (LSC). Bases and nucleosides were separated by reverse phase (RP) HPLC on a Vydac (Separations Group, Hesperia, CA) HS 201 (10 μm) 4.6 × 250 mm column using a linear gradient starting with 1.2% acetonitrile in 4.8 mM potassium acetate buffer (adjusted to pH 5.0 with trifluoroacetic acid) and finishing with 25% acetonitrile in H_2O over 40 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected, and their radioactivities were determined as described above.

The concentrations and identifications of compounds separated by HPLC were determined by comparison of their peak areas and retention times with known concentrations of standards, using the Vista 401 external standard method. Identities of nucleotide analogues were verified by obtaining their 254/290 nm absorbance ratios from an HPLC separation of the authentic nucleotides [17].

Radiolabel incorporation studies. Short-term radiolabeled experiments (less than 6 hr) were performed as follows. A T-75 culture in late log phase was placed in an ice bath for 20–30 min to free adherent cells from the flask, the culture was shaken, the cell density was determined, and the suspension was centrifuged at 4° for 20 min at 1000 *g*. The supernatant fraction was decanted and the pellet was resuspended in cold Tris–mannitol (0.23 M mannitol, 0.07 M sucrose, 0.05 M Tris–HCl buffer, pH 7.0, and 0.001 M EDTA) and centrifuged again. This pellet was washed with Tris–mannitol. The final pellet was resuspended in 20 ml of a purine-defined incubation medium (designated MSS-1, see below) which contained the radiolabeled compound. This was incubated as above and then placed in an ice-bath to release the cells which were counted, harvested, and resuspended in 1 ml of cold 0.8 N perchloric acid. The acid-soluble nucleotides were extracted from the perchloric acid using 0.5 M tri-*N*-octylamine in trichlorotrifluoroethane [18] and analyzed by SAX-HPLC and LSC; the purine or purine analogue base

and nucleoside content of the incubation medium was analyzed by RP-HPLC and LSC [19].

Incubations in the presence and absence of 5-iodotubercidin (5-ITU) were performed as above with the following modifications. The final cell pellet was resuspended in MSS-1 with or without 1 μM 5-ITU and preincubated for 1 hr. Then, tritiated 9-deazaadenosine (9-DADO) was added (8.9 μM; sp. act. 37 μCi/mole), the cells were incubated for an additional 2 hr and harvested, and their nucleotide pools were analyzed by SAX-HPLC and LSC.

The MSS-1 medium was prepared by modification of our purine-defined African trypanosome medium SDM-79 [19]: the MOPS and HEPES buffers were replaced with a phosphate buffer (1 g K_2HPO, and 0.6 g KH_2PO_4 per 850 ml), and ascorbic acid, L-cysteine and bovine albumin were added (0.2, 1.5 and 2.5 g/850 ml respectively); the volume was reduced from 1 liter to 800 ml (to increase osmolarity from 330 to 380 mOsM, approximately that of the TYI medium [20]). The final pH was adjusted to 6.8.

Long-term (24 hr) radiolabel incorporation studies were done by adding sterile radioactive compound directly to TYI medium of a mid-log phase T-75 culture. At the end of the incubation, cells were harvested for HPLC analysis as in the short-term experiments.

Cell-free extracts. A roller bottle culture was harvested and cells were washed as described for a T-75 culture. The final pellet ($3–6 \cdot 10^8$ cells) was resuspended in 1.5 ml of cold 50 mM PIPES buffer, pH 6.8, containing 2 mM DL-dithiothreitol (DTT) and 1 mM $MgCl_2$. This suspension was sonified with a microtip for 1 min on ice at a power setting of 6 with a Systems model W185 cell disruptor (Plainview, NY). This was followed by centrifugation at 40,000 *g* for 1 hr at 4°. The supernatant fraction was desalted by a series of four ultrafiltrations and reconstitutions (50 mM PIPES, 2 mM DTT) using a Centricon-10 microconcentrator (Amicon, Davners, MA). The final retentate, the enzyme source, was reconstituted in 1.5 ml of 50 mM PIPES and 2 mM DTT and was stored at −80°.

Enzyme assay. Assay procedures were a modification of those of Miller and Lindstead [9]. A standard purine nucleoside kinase reaction mixture contained 50 mM PIPES buffer (pH 6.8), 5 mM $MgCl_2$, 50 mM KCl, 0.25 mM sodium phosphoenolpyruvate, 2 I.U./ml pyruvate kinase, 0.1 mM radioactive substrate (sp. act. 340 μCi/μmole), 4 mM ATP and 30 μl of enzyme preparation in a final volume of 75 μl. Reactions were incubated at 37° and initiated by the addition of enzyme. They were terminated by spotting a 10-μl aliquot directly onto a PEI cellulose thin-layer chromatography plate (MC/B Manufacturing, Cincinnati, OH) at 0, 15, 30, 45, 60, 90 and 120 min. Analysis of the reaction mixture at 120 min by SAX-HPLC showed an ATP concentration of 3.8 mM and confirmed the effectiveness of the ATP regenerating system. Plates were developed to 16 cm from the origin in water and then air-dried. This procedure was repeated twice. Radioactivities of the nucleotide monophosphate (at the origin) and the substrate (at the solvent front) were determined by cutting out these regions and quantitating them by LSC.

Nucleotide formation was verified with SAX-HPLC analysis by the coelution of radioactivity with the corresponding nonlabeled authentic nucleotide monophosphate. To confirm the identity of the products, the fractions from SAX-HPLC separation containing the radioactivity were treated with calf intestine alkaline phosphatase (Calbiochem, San Diego, CA). The radioactive nucleoside released by enzymatic hydrolysis was then identified by coelution on RP-HPLC with the original substrate [17].

Nucleoside phosphotransferase activity was determined in the same manner except that AMP replaced ATP and the ATP regenerating system (pyruvate kinase and phosphoenolpyruvate) was deleted.

Nucleotide stability of both the phosphate donor and the reaction product was assessed by replacing the nucleoside in the phosphotransferase reaction mixture with its respective nucleoside monophosphate. Aliquots were withdrawn after 0, 15, 30, 45 and 60 min of incubation, acid-extracted, and analyzed by RP-HPLC.

Nucleoside cleavage was assayed by the spectrophotometric methods of Miller and Lindstead [9]. A standard reaction mixture contained a 50 mM concentration of either PIPES (hydrolytic conditions) or phosphate (phosphorylase conditions), pH 6.8, and a 100 μM concentration of purine nucleoside in a total volume of 1 ml. The inosine reaction mixture contained 0.1 I.U. xanthine oxidase (Sigma Chemical Co.).

Protein concentration was determined using a Bio-Rad Laboratories (Richmond, CA) protein assay kit.

RESULTS

Growth inhibition studies. Seven analogues of adenosine were tested for their abilities to inhibit growth of the P-1 and WB strains of *G. lamblia.* As shown in Table 1, four of these compounds, 8-azaadenosine and 1-, 7-, and 9-deazaadenosine, caused a greater than 50% inhibition of growth. These drugs only appeared, however, to inhibit growth since organisms exposed to a 37 μM concentration of either 7- or 9-deazaadenosine for 48 hr

Table 1. Effects of adenosine analogs on the growth of *G. lamblia*

Analog* (37 μM)	% of Control growth† (strain) P-1	WB
Adenosine	96	102
Aminopyrazolopyrimidine ribonucleoside	83	132
Formycin A	100	95
3-Deazaadenosine	95	95
8-Azaadenosine	32	37
1-Deazaadenosine	4	6
7-Deazaadenosine	38	49
9-Deazaadenosine	11	20

* Addition of 50 μM deoxycoformycin did not affect results.

† Percent of control values represents the average of three replicate cultures. Ranges were $< \pm 6\%$.

Table 2. Incorporation of nucleoside analogs into *G. lamblia* (P-1) cellular nucleotides

Ribonucleoside analog	Resulting nucleotide analog (pmoles/10^6 cells) IMP	AMP	ADP	ATP
7-Deazaadenosine		8.2	11.2	62.8
9-Deazaadenosine		1.8	8.5	29.0
9-Deazainosine	3.2			
Formycin A				
Formycin B				

Approximately 10^7 cells were incubated with radioactive nucleoside analog (37 μM; 5 μCi in 25 ml TYI-S-33 media) for 24 hr. Cellular nucleotides were extracted in perchloric acid and analyzed by anion-exchange HPLC. Limit of detection was <1 pmole/10^6 cells.

underwent normal replication, after a 12- to 14-hr lag, when the drug was removed. Similar studies were done using the respective bases of three of the four compounds (8-azaadenine, 1-deazadenine, and 7-deazaadenine). None of these inhibited growth. The base of 9-deazaadenosine (9-DADO) was not tested as this riboside is a C-nucleoside (i.e. the ribose is bonded to the base by a carbon-to-carbon bond instead of the normal carbon-to-nitrogen bond) and is resistant to hydrolytic cleavage. Growth inhibition by these nucleosides is not a result of cleavage to their respective bases.

Formycin A (FORA) can be deaminated to formycin B (FORB) by adenosine deaminase (ADA) which is present in the serum component of the TYI-media. To ascertain if deamination was responsible for this lack of toxicity, FORA was retested in the presence of 50 μM deoxycoformycin (dCF), an ADA inhibitor [17]. Formycin A remained ineffective. The dCF did not alter the activities of the other compounds nor did it affect the ability or lack of ability of the other compounds to inhibit growth.

Metabolic studies. The above results suggested that these adenosine analogues are phosphorylated directly and that their respective nucleotides may be responsible for their toxicity. To test for direct phosphorylation, both the P-1 and WB strains were incubated for 24 hr in TYI media containing radiolabeled 9-DADO or 7-deazaadenosine (7-DADO). The acid-soluble nucleotides were analyzed by SAX-HPLC. Table 2 shows the results with the P-1 strain. Also shown are incubations with 9-deazainosine (9-DINO), FORB, and FORA plus 50 μM dCF. These were added to determine the specificity of the phosphorylating enzyme. Both of the 9-deaza compounds and 7-DADO were phosphorylated to their respective monophosphates; 7-DADO and 9-DADO were further converted to their respective ADP and ATP analogues. There was no phosphorylation of either FORA or FORB detected. Analysis of the cell-free media by RP-HPLC after these incubations showed no cleavage or deamination of the original nucleosides. Incubation of the WB strain with 7- and 9-DADO also resulted in the formation of their AMP, ADP, and ATP analogues (7-DADO: 10.0, 13.6, and 57.3 pmoles/10^6 cells respectively; 9-DADO: 2.3, 14.3, and 38.0 pmoles/10^6 cells respectively). Similar incubations with 8-azaadenosine and 1-deazaadenosine were not done as radiolabeled compounds were not available.

Table 3. Salvage of purine bases and their respective nucleosides by *G. lamblia* (P-1)

Nucleotide	Labeled precursor ± Unlabeled base [^{14}C]Ade	[^{14}C]Ado	[^{14}C]Ado + Ade	[^{14}C]Gua	[^{14}C]Guo	[^{14}C]Guo + Gua
AMP	12.3* (0.48)÷	10.5 (0.47)	11.1 (0.12)	29.9 (ND)	29.9 (ND)	33.6 (ND)
GMP	7.1 (ND)	6.5 (ND)	5.7 (ND)	10.5 (0.38)	8.6 (0.28)	21.0 (ND)
ADP	106.6 (0.13)	98.0 (0.15)	114.1 (0.04)	79.3 (ND)	84.2 (ND)	90.1 (ND)
GDP	38.0 (ND)	34.4 (ND)	30.4 (ND)	40.7 (0.26)	39.8 (0.11)	51.2 (ND)
ATP	781.3 (0.11)	886.7 (0.10)	995.0 (0.01)	715.7 (ND)	692.0 (ND)	810.2 (ND)
GTP	121.5 (ND)	140.2 (ND)	178.0 (ND)	219.8 (0.24)	221.3 (0.07)	271.6 (0.02)
% of Nucleotide pool derived from radioactive precursor	11.5	10.3	1.7	25.1	8.0	1.3

Approximately 10^7 cells were incubated in 20 ml of MSS-1 medium containing radiolabeled precursor (5 μCi; 50 μM) for either 45 min (adenine series) or 75 min (guanine series); labeled base, if present, was added at a concentration 5-fold that of its respective labeled nucleoside. Cellular nucleotides were extracted and analyzed as in Table 2. Abbreviations: Ade, adenine; Ado, adenosine; Gua, guanine; and Guo, guanosine.

* Nucleotide concentrations are given as pmoles/10^6 cells.

÷ Values in parentheses represent the relative specific activities of the respective nucleotide (pmoles radiolabeled nucleotide/total pmoles of nucleotide). ND = <1 pmole radiolabeled nucleotide per 10^6 cells.

As these results indicated the presence of one or more rather specific nucleoside phosphorylating activities and those of Wang *et al.* did not, we performed our own whole-cell purine incorporation experiments. It was possible that our "P-1 strain" salvaged purines differently from the "P-1 strain" used by Wang and Aldritt since a recent report questioned the identities of the P-1 strains from various sources [21]. There have been no reports of purine metabolic studies on the WB strain. These incubations were done in purine-defined MSS-1. This medium is an enriched version of the modified medium 199 which was shown by Gillin and Reiner [20] to maintain viability (based on attachment and mobility) of *G. lamblia* for up to 24 hr.

Table 3 shows the results of incubation of our P-1 strain with radiolabeled adenine and guanine plus their respective ribonucleosides. Adenine and adenosine labeled only the adenine nucleotides; guanine and its ribonucleoside labeled only the guanine nucleotides. There was no interconversion between the two nucleotide pools. Adenine and adenosine were incorporated at the same rate; guanine was incorporated more efficiently than its ribonucleoside. This suggested cleavage of the ribonucleosides to their bases before nucleotide synthesis with guanosine hydrolysis the rate-limiting step in that sequence. This was confirmed by the addition of nonradiolabeled base at 5-fold the concentration of its radiolabeled nucleoside to determine if excess base would force direct nucleoside phosphorylation. Under these conditions, both [^{14}C]adenosine incorporation were reduced about 84%. This reduction is the expected decrease if all the ribonucleoside were cleaved to the base before incorporation (i.e. radiolabel would be diluted by unlabeled base 1:6). A similar series of incubations was performed with the WB strain. The results of these (data not shown) were similar to those found for the P-1 strain.

All of the above results are consistent with the direct phosphorylation of certain non-hydrolyzable adenosine analogues by either a nucleoside kinase or phosphotransferase. To differentiate between these two, organisms were incubated with radiolabeled 9-DADO in the presence and absence of 5-iodotubercidin (5-ITU), an inhibitor of adenosine kinase [22]. Addition of 5-ITU resulted in an 11% decrease of incorporation of 9-DADO into the nucleotide pools of the parasites. Under similar conditions, 5-ITU caused a greater than 90% inhibition of 9-DADO incorporation by both mouse L cells and *L. donovani* [17]. Both cell types have been shown to possess adenosine kinase [17, 23]. The results with *Giardia* suggest that a phosphotransferase is responsible for the phosphorylation of these adenosine analogues and not a kinase.

Enzyme assays. The above data were confirmed by an *in vitro* assay using 7-DADO and 9-DADO as the substrates. No kinase activity was detected but both compounds were phosphorylated (specific activities: 7-DADO, 0.36, and 9-DADO, 0.27 nmole·min^{-1}·mg^{-1}) by a phosphotransferase with AMP as the phosphate donor. This activity was linear with time for 1 hr and required both enzyme and a phosphate donor (Table 4). Both 7- and 9-DADO as well as 9-DINO were good substrates for the enzyme while formycin A and B were poor substrates. The formation of the monophosphates was confirmed by SAX-HPLC analysis. Analysis of the reaction mixture by RP-HPLC showed that there was no cleavage of the C-nucleosides FORA, FORB, and 9-DADO. There was, however, a low rate of 7-DADO (0.01 nmole·min^{-1}·mg^{-1}) cleavage. This rate of cleavage was about 3% of the rate of phosphorylation. These results are consistent with the growth and metabolic experiments (Tables 1 and 2).

Product stability under conditions in which the nucleoside phosphotransferase was assayed was assessed as described. After 1 hr, 26% of the 7-deazaadenosine monophosphate was converted to its nucleoside while 24% of the AMP was converted to adenosine (2%) and adenine (22%). The monophosphates of FORA, FORB and 9-DADO were not tested but it is expected that they would undergo similar rates of hydrolysis. These data suggest that the above specific activities may be artifactually low by no more than 25% due to simultaneous product formation and breakdown. The amount of phosphate donor (AMP) breakdown, from 4 to 3 mM, should not have affected the reaction rate.

Purine ribonucleoside cleavage activity also was looked for *in vitro* to see if our P-1 strain rate of nucleoside cleavage was different from the value reported by Wang and Aldritt [11]. Specific activities

Table 4. Substrate and phosphate donor specificity of *Giardia lamblia* (P-1) purine nucleoside phosphotransferase

Substrate	PO_4 Donor	% Relative specific activity
9-Deazaadenosine	AMP	100
9-Deazainosine	AMP	127
7-Deazaadenosine	AMP	134
Formycin A	AMP	4.7
Formycin B	AMP	12.6
Adenosine	AMP	0
9-Deazaadenosine	AMP	100
9-Deazaadenosine	GMP	99
9-Deazaadenosine	TMP	8.0
9-Deazaadenosine	*p*-Nitrophenylphosphate	26
9-Deazaadenosine	Phenylphosphate	12.4

in nmoles product · min^{-1} · (mg protein)$^{-1}$ for adenosine, inosine, and guanosine cleavage were 1375, 1087 and 894 respectively. Addition or deletion of phosphate from the reaction mixture had no effect on these activities and suggests that the enzymes are hydrolases and not phosphorylases. The cleavage rates we found for adenosine and guanosine are essentially the same as those reported by the above authors. They did not assay for inosine cleavage.

DISCUSSION

These results confirm those of Wang and Aldritt with respect to purine salvage. We found no difference between these authors' Portland-1 strain and our own, nor with the WB strain. Thus, the presence of the phosphotransferase was activity not due to strain differences. In addition to the reported presence of adenosine and guanosine hydrolysis activity [11], we found that this parasite also hydrolyzed inosine to hypoxanthine. The specific activity for inosine cleavage was intermediate to those of adenosine and guanosine.

The finding that the growth of both the P-1 and WB strains was inhibited by certain adenosine analogues but not by their bases suggested that *G. lamblia* can phosphorylate certain purine ribonucleoside analogues directly to their monophosphates even though the principal route of salvage of normal purine ribonucleosides is by cleavage to the base [11]. Phosphoribosylation of the respective base analogues cannot be ruled out since radiolabeled incorporation studies were not done, although we consider this unlikely since these bases were not toxic.

Both radioactive 7-DADO and 9-DADO were phosphorylated by whole cells to their AMP, ADP and ATP analogues. As 9-DADO is a C-nucleoside and resistant to hydrolytic cleavage and ADA deamination [24], this phosphorylation must also be direct. RP-HPLC analysis of postincubation media from both the 7-DADO and 9-DADO experiments resulted in only one radioactive peak from each. Both coeluted with authentic standard, suggesting that neither compound is hydrolyzed. *Giardia* also can phosphorylate the inosine analogue 9-DINO to its IMP analogue but this monophosphate is not metabolized further. The pyrazolo(4,3-*d*)pyrimidines, FORA and FORB, which are *C*-nucleoside analogues of adenosine and inosine, respectively, were not detectably phosphorylated. The phosphorylation of these purine ribonucleoside analogues seems to be highly selective.

When whole-cell incubations with 9-DADO were repeated in the presence of 5-ITU, an inhibitor of adenosine kinase [17, 22], there was no significant inhibition of phosphorylation. This suggests that a nucleoside phosphotransferase was responsible for the phosphorylation rather than a kinase. This was confirmed when nucleoside phosphotransferase activity was found in a crude cell-free extract. While 7-DADO, 9-DADO, and 9-DINO (and to a much lesser extent FORA and FORB) were phosphorylated, no activity was detected with adenosine as the substrate. If one makes the assumption that adenosine is as good a substrate for the phosphotransferase as 9-DADO, then the rate of adenosine cleavage would be about 5000 times the rate of its phosphorylation [1375 verus 0.27 nmole · min^{-1} · (mg $protein^{-1}$)]. It is not surprising that Wang and Aldritt could not detect this activity using adenosine as the substrate.

The nucleoside phosphotransferase of *G. lamblia* has certain properties that make it a potential target for chemotherapy. It appears to have different substrate and phosphate donor specificities from the enzymes present in other eucaryotic cells. The enzyme from *L. donovani* is equally efficient at phosphorylating both 9-DINO [17] and FORB [14], whereas that of *G. lamblia* is not. The enzyme from various human tissues also phosphorylates a wide range of nucleosides [25]. The phosphate donor specificity is also different. The *L. donovani* [13] and mammalian [25] enzymes use *p*-nitrophenylphosphate and phenylphosphate, respectively, better than either AMP and GMP. The reverse is true for the giardial enzyme. Thymidine monophosphate, while a good donor for the enzymes of the former organisms [13, 25], proved to be ineffective with this enzyme.

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REFERENCES

1. WHO Scientific Working Group, *Bull. Wld Hlth Org.* **58**, 819 (1980).
2. G. F. Craun, in *Giardia and Giardiasis* (Eds. S. L. Erlandsen and E. A. Meyer), pp. 243–61. Plenum Press, New York (1984).
3. M. S. Wolfe, in *Principles and Practice of Infectious Diseases* (Eds. G. L. Mandell, R. G. Douglas and J. E. Bennett), pp. 2143–7. John Wiley, New York (1979).
4. G. Johnson, *Small Animal Practice*, pp. 969–70. W. B. Saunders, Philadelphia (1977).
5. M. S. Wolfe, in *Giardia and Giardiasis* (Eds. S. L. Erlandsen and E. A. Meyer), pp. 147–59. Plenum Press, New York (1984).
6. J. J. Marr, R. L. Berens and D. J. Nelson, *Biochim. biophys. Acta* **544**, 360 (1978).
7. R. L. Berens, J. J. Marr, S. W. LaFon and D. J. Nelson, *Molec. biochem. Parasit.* **3**, 187 (1981).
8. W. R. Fish, D. L. Looker, J. J. Marr and R. L. Berens, *Biochim. biophys. Acta* **719**, 223 (1982).
9. R. L. Miller and D. Lindstead, *Molec. biochem. Parasit.* **7**, 41 (1983).
10. C. C. Wang, R. Verham, A. Rice and S. Tzeng, *Molec. biochem. Parasit.* **8**, 325 (1983).
11. C. C. Wang and S. Aldritt, *J. exp. Med.* **158**, 1703 (1983).
12. G. W. Koszalka and T. A. Krenitsky, *J. biol. Chem.* **254**, 8185 (1979).
13. D. J. Nelson, S. W. LaFon, J. V. Tuttle, W. H. Miller, R. L. Miller, T. A. Krenitsky, G. B. Elion, R. L. Berens and J. J. Marr, *J. biol. Chem.* **254**, 11544 (1979).
14. B. A. Carson and K. P. Chang, *Biochem. biophys. Res. Commun.* **100**, 1377 (1981).
15. D. B. Keister, *Trans. R. Soc. trop. Med. Hyg.* **77**, 487 (1983).

16. M. J. G. Farthing, M. E. A. Pereira and G. T. Keusch, *Parasitology* **54**, 410 (1982).
17. S. W. Lafon, D. J. Nelson, R. L. Berens and J. J. Marr, *J. biol. Chem.* **260**, 966 (1985).
18. S. C. Chen, P. R. Brown and D. M. Rosie, *J. chromat. Sci.* **15**, 218 (1977).
19. W. R. Fish, J. J. Marr and R. L. Berens, *Biochim. biophys. Acta* **714**, 422 (1982).
20. F. D. Gillin and D. S. Reiner, *Molec. cell. Biol.* **2**, 369 (1982).
21. M. A. Bertram, E. A. Myer, J. D. Lile and S. A. Morse, *J. Parasit.* **69**, 793 (1983).
22. T. P. Zimmerman, R. D. Deeprose, G. Wolberg, C. R. Stopford, G. S. Duncan, W. H. Miller, R. L. Miller, M. I. Lim, W. Y. Ren and R. S. Klein, *Biochem. Pharmac.* **32**, 1211 (1983).
23. D. M. Iovannisci and B. Ullman, *Molec. biochem. Parasit.* **12**, 139 (1984).
24. M. Y. Chu, L. B. Zukerman, S. Sato, G. W. Crabtree, A. W. Bogden, M. I. Lim and R. S. Klein, *Biochem. Pharmac.* **33**, 1229 (1984).
25. N. K. Ahned and V. L. Green, *Int. J. Biochem.* **15**, 1469 (1983).