

## PURINE SALVAGE NETWORKS IN *GIARDIA LAMBLIA*\*

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*Giardia lamblia* is an anaerobic flagellated protozoa and a common inhabitant of human intestine. It is transmitted mainly by ingestion of the cysts in contaminated food or drinking water, and is present in at least 10% of the population in the United States (1). The infection causes diarrhea, abdominal pain, weight loss, and retarded growth in children. The infectious dose of cysts appears to be quite low, whereas a large number of cysts can be shed from infected individuals. Metronidazole (Flagyl) and atabrine are the only two drugs available for treating the infection; both having undesirable side effects (2). No chemoprophylactic drugs are yet available for giardiasis.

Recent success in establishing axenic cultures of *G. lamblia* trophozoites (3) has made it possible to study the metabolisms in this organism. In one recent report (4), *G. lamblia* was found incapable of incorporating orotic and aspartic acid into nucleic acids, and no activities of de novo pyrimidine-synthesis enzymes were detected in the crude extracts of *G. lamblia*. This finding, which suggests the absence of pyrimidine de novo synthesis in *G. lamblia*, agrees with our recent observations on two other anaerobic, flagellated protozoan parasites, *Tritrichomonas foetus* (5) and *Trichomonas vaginalis* (6). These two parasites are unable to synthesize pyrimidines de novo but rely on salvaging uracil (5) or cytidine and uridine (6) for their requirement for pyrimidine ribonucleotides. They have no detectable dihydrofolate reductase or thymidylate synthetase activity; TMP can be only acquired via the action of a unique enzyme, thymidine phosphotransferase, for salvaging exogenous thymidine. Preliminary studies in this laboratory also indicated the absence of dihydrofolate reductase and thymidylate synthetase in *G. lamblia* (Wang, unpublished results). Thymidine salvage by these three parasites thus could be an attractive target for chemotherapeutic control of these three parasites.

On the other hand, *T. foetus* (7) and *T. vaginalis* (8) have also been found incapable of de novo synthesis of purine nucleotides, as would have been expected from the absence of dihydrofolate reductase. It is thus likely that *G. lamblia* may lack this synthetic capability. In fact, all the protozoan parasites investigated to date appear to lack the activity in de novo purine synthesis and depend solely on purine salvage for purine nucleotides (7–9). This common deficiency among parasites has provided ample opportunities for chemotherapeutic attack. Allopurinol is effective against *Trypanosoma cruzi* because the drug is recognized as a

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substrate by the parasite hypoxanthine phosphoribosyl transferase (HPRT)<sup>1</sup> and is eventually incorporated into the parasite RNA (10). Allopurinol riboside (11) and formycin B (12) are effective antileishmanial agents due to the presence of a unique enzyme, purine nucleoside phosphotransferase, in the *Leishmania* spp. that incorporates the compounds into the parasites. *T. foetus* depends mainly on HPRT (7), whereas *T. vaginalis* relies solely on adenosine and guanosine kinase (8) for their needs for purine nucleotides. In recent studies (13), we were able to demonstrate that 8-azaguanine (inhibitor of HPRT), mycophenolic acid (inhibitor of IMP dehydrogenase), and hadacidin (inhibitor of succinyl AMP synthetase) were inhibitors of *T. foetus* growth without affecting *T. vaginalis*. Conversely, tubercidin, toyocamycin, sangivamycin, and araA, all known to inhibit adenosine kinase (13), exert significant inhibition only on the growth of *T. vaginalis*.

It is the purpose of the present investigation to verify whether *G. lamblia* is indeed devoid of purine de novo synthesis and to delineate the entire network of purine salvage in *G. lamblia* as compared with those in *T. foetus* and *T. vaginalis*. Probable targets for chemotherapeutic control of *G. lamblia* and possible common targets in purine salvage among the three parasite species are explored.

### Materials and Methods

***G. lamblia* Cultures.** The *G. lamblia* Portland I strain was a generous gift from Dr. Donald Lindmark of Cornell University. *G. lamblia* trophozoites were cultivated axenically in vitro under anaerobic conditions at 37°C in Diamond's BI-S-33 medium, pH 7.05 (14), with the following modifications: (a) sterilization of the medium was by filtration through a 0.22 µm Millipore filter (Millipore/Continental Water Systems, Bedford, MA); (b) the vitamin-Tween 80 mixture was eliminated from the medium. Cultures reached stationary phase after 96 h of growth and had a final cell density of  $2 \times 10^6$ /ml. These cultures were used to inoculate fresh media in a 2:10 ratio. Mid-log phase growth was achieved after 74 h incubation with a cell density of  $10^6$ /ml. These cells were harvested and used for all studies. Cell countings were routinely performed in a Coulter ZF counter (Coulter Electronics Inc., Hialeah, FL).

**Chemicals.** Radiolabeled glycine, formate, purines, and purine nucleosides were purchased from New England Nuclear, Boston, MA or ICN, Irving, CA. Formycin B was purchased from Sigma Chemical Co., St. Louis, MO. Mycophenolic acid was a gift from Dr. W. Sadée of the School of Pharmacy, University of California, San Francisco. All other chemicals used in the studies were of the highest purities commercially available.

**Precursor Incorporation into Nucleotide Pools.** *G. lamblia* harvested by centrifugation at 3,000 g for 5 min was washed three times and resuspended in phosphate-buffered saline glucose (20 mM), pH 7.0, with 2 mM cysteine, to a final cell density of  $2 \times 10^7$ /ml, and incubated at 37°C. Radiolabeled substrates were each added to the cell suspension to a final concentration of 40 µM, and aliquots of 300 µl were taken at various time intervals. Each aliquot was treated with perchloric acid-KOH and filtered through glass fiber filters loaded with polyethyleneimine (PEI)-cellulose in 5 mM ammonium acetate, pH 5.0, as previously described (7). The nucleotide-loaded filter was washed with three 5-ml portions of the ammonium acetate buffer, dried, and soaked in Aquasol 2. Levels of radioactivity were determined with a Beckman LS-3133T liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, CA).

**High Pressure Liquid Chromatography (HPLC).** Purine nucleotides were separated, iden-

<sup>1</sup> Abbreviations used in this paper: APRT, adenine phosphoribosyl transferase; GPRT, guanine phosphoribosyl transferase; HPLC, high pressure liquid chromatography; HPRT, hypoxanthine phosphoribosyl transferase; PEI, polyethyleneimine; PRPP, 5-phosphoribosyl-1-pyrophosphate; TKMD, 25 mM Tris-HCl, pH 7.2, 20 mM KCl, 6 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol; XPRT, xanthine phosphoribosyl transferase.

tified, and quantitated in an ion-exchange HPLC system with an Ultrasil AX (10  $\mu\text{m}$ ) 4.6  $\times$  250-mm column. Samples concentrated fivefold from the incorporation experiments (100  $\mu\text{l}$ ) were injected and eluted with 7 mM phosphate buffer, pH 3.8, at a flow rate of 1.0 ml/min. A programmed gradient elution from 7 mM phosphate buffer, pH 3.8, to 250 mM phosphate buffer, pH 4.5, plus 500 mM KCl was applied. The effluent was monitored at 254 nm in a Beckman 160 UV-absorbance detector, mixed with Aquasol 2 at a 1:3 ratio, and the radioactivity was recorded in a Flo-one radioactive flow detector (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL). Both UV absorbance and radioactivity data were recorded and analyzed by a Hewlett-Packard 3390A integrator (Hewlett-Packard Co., Palo Alto, CA) (7).

For the analysis of purine bases and nucleosides, a reverse-phase HPLC system with an ODS (5  $\mu\text{m}$ ) reverse-phase column was used and eluted with a programmed gradient from 7 mM  $\text{KH}_2\text{PO}_4$ , pH 6.0, to 50% acetonitrile at a flow rate of 0.75 ml/min. UV monitoring and continuous radioactivity measurement of the effluent were as previously described (7).

**Enzyme Assays.** Mid-log phase *G. lamblia* trophozoites were washed and resuspended in an equal volume of 25 mM Tris-HCl, pH 7.2, 20 mM KCl, 6 mM  $\text{MgCl}_2$ , and 1 mM dithiothreitol (TKMD). Cells were disrupted using an Ultrasonics sonicator (Ultrasonic Instruments International, Westbury, NY) in an ice bath for three 10-s pulses at an output setting of 4 and duty cycle setting of 40. The homogenate was centrifuged at  $10^4 g$  for 30 min to remove cell debris. The crude supernatant fraction was then centrifuged at  $10^5 g$  for 1 h to separate the soluble and pellet fractions. Protein concentrations were determined by the method of Bradford (15), with bovine serum albumin as the standard.

Purine phosphoribosyl transferases were assayed according to a modified procedure by Schmidt et al. (16). The reaction mixture, containing 100  $\mu\text{l}$  of 100 mM Tris-HCl, pH 7.8, 8 mM  $\text{MgCl}_2$ , 1.0 mM 5-phosphoribosyl-1-pyrophosphate (PRPP),  $50 \mu\text{g} \cdot \text{ml}^{-1}$  bovine serum albumin, and 20  $\mu\text{M}$  of [ $8\text{-}^{14}\text{C}$ ]hypoxanthine (51.1 mCi  $\cdot$  mmol $^{-1}$ ), [ $8\text{-}^{14}\text{C}$ ]adenine (49.1 mCi  $\cdot$  mmol $^{-1}$ ), [ $8\text{-}^{14}\text{C}$ ]xanthine (41 mCi  $\cdot$  mmol $^{-1}$ ), or [ $8\text{-}^{14}\text{C}$ ]guanine (56.7 mCi  $\cdot$  mmol $^{-1}$ ) was incubated at 37°C. The reaction, initiated by adding the enzyme sample, was terminated by adding an equal volume of ice-cold, 2 mM unlabeled purine solution. Aliquots of the mixture were filtered through PEI-cellulose and the trapped radioactivities were counted as described previously.

Purine nucleoside kinases were assayed by a procedure similar to that described by Nelson et al. (11). The assay mixture, consisting of 100 mM Tris-HCl, pH 7.5, 20 mM ATP, 20 mM  $\text{MgCl}_2$ , 80 mM phosphoenolpyruvate, 40 IU  $\cdot$  ml $^{-1}$  pyruvate kinase, and 1.5 mM of [ $8\text{-}^{14}\text{C}$ ]inosine (12.0 mCi  $\cdot$  mmol $^{-1}$ ), [ $2,8\text{-}^3\text{H}$ ]adenosine (49.4 mCi  $\cdot$  mmol $^{-1}$ ), or [ $8\text{-}^3\text{H}$ ]guanosine (45.7 mCi  $\cdot$  mmol $^{-1}$ ), was incubated at 37°C for 10 min after the addition of enzyme. The reaction was stopped by a 10-fold dilution of the reaction mixture in cold 5 mM ammonium acetate, pH 5.0. The PEI-adsorbable radioactivity was measured.

Purine nucleoside phosphotransferases were assayed by using *p*-nitrophenylphosphate (10 mM) as the phosphate donor in an assay mixture also consisting of 100 mM Na-acetate, pH 5.4, 1.0 mM of the radiolabeled purine nucleosides, and the enzyme. Assay procedures were the same as for the kinases.

Purine nucleoside hydrolase was assayed in TKMD with 1.0 mM [ $2,8\text{-}^3\text{H}$ ]adenosine (10 mCi  $\cdot$  mmol $^{-1}$ ) or [ $8\text{-}^3\text{H}$ ]guanosine (10 mCi  $\cdot$  mmol $^{-1}$ ). Reactions were run at 37°C for varying lengths of time, terminated by perchloric acid, neutralized with KOH, and the reaction products were analyzed in reverse-phase HPLC to identify and quantitate the purine bases.

## Results

**Precursor Incorporation into Nucleotide Pool.** [ $^{14}\text{C}$ ]Formate (56.0 mCi  $\cdot$  mmol $^{-1}$ ), [ $2\text{-}^3\text{H}$ ]glycine (59.2 mCi  $\cdot$  mmol $^{-1}$ ), [ $\text{G}\text{-}^3\text{H}$ ]hypoxanthine (60.4 mCi  $\cdot$  mmol $^{-1}$ ), [ $8\text{-}^{14}\text{C}$ ]xanthine (41.0 mCi  $\cdot$  mmol $^{-1}$ ), and [ $8\text{-}^{14}\text{C}$ ]inosine (55.0 mCi  $\cdot$  mmol $^{-1}$ ) were tested in the incorporation assay. All the substrates fell below the minimal level of detection for incorporation (0.80 pmol  $\cdot$   $10^6$  cells $^{-1}$ ) after 1 h incubation.

Radiolabeled adenine, adenosine, guanine, and guanosine were all able to enter the nucleotide pool of *G. lamblia*. Data presented in Fig. 1 indicate that adenine was incorporated at the highest initial rate of  $3.5 \text{ pmol/min per } 10^6$  cells. It was followed by adenosine ( $2.0 \text{ pmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ ), guanine ( $1.5 \text{ pmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ ), and guanosine ( $0.3 \text{ pmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ ).

When the incorporation was carried out in the presence of another unlabeled substrate at 10-fold higher concentration, the results presented in Fig. 2 suggested highly specific competition among the four substrates: (a) Adenine incorporation was only inhibited significantly by unlabeled adenine or adenosine (Fig. 2A); (b) adenosine incorporation was only inhibited significantly by unlabeled adenine or adenosine (Fig. 2B); (c) guanine incorporation was only inhibited significantly by unlabeled guanine or guanosine (Fig. 2C); (d) guanosine incorporation was only inhibited significantly by unlabeled guanine or guanosine (Fig. 2D). It is clear that the route of adenine/adenosine incorporation into the nucleotide pool of *G. lamblia* is well separated from that of guanine/guanosine incorporation.

**HPLC Analysis of the Radiolabeled Nucleotide Pool.** Pulse labeling of *G. lamblia* trophozoites with  $[8\text{-}^3\text{H}]$ adenine (Fig. 3A) and  $[2,8\text{-}^3\text{H}]$ adenosine (Fig. 3B) for 60 min resulted primarily in the labeling of AMP, ADP, ADP-hexose, and ATP in the nucleotide pool. There was no radioactivity detected in GMP, GDP, or GTP.

Similar experiments with  $[8\text{-}^{14}\text{C}]$ guanine (Fig. 4A) and  $[8\text{-}^3\text{H}]$ guanosine (Fig.

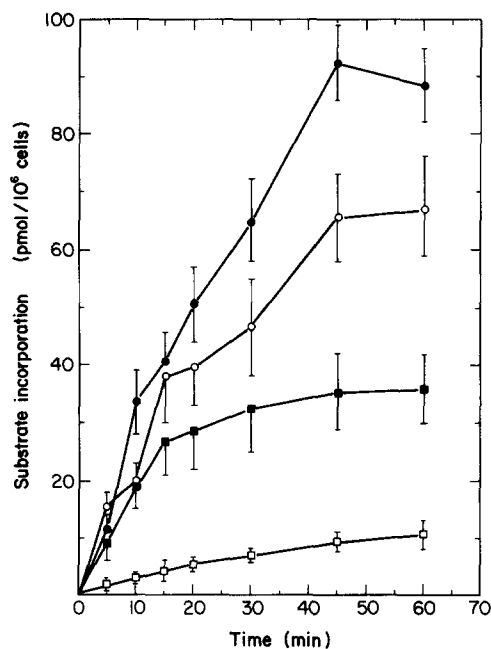


FIGURE 1. Incorporation of radiolabeled substrates into *G. lamblia* nucleotide pool.  $[8\text{-}^3\text{H}]$ Adenine ( $64.4 \text{ mCi} \cdot \text{mmol}^{-1}$ ) (●);  $[2,8\text{-}^3\text{H}]$ Adenosine ( $52.4 \text{ mCi} \cdot \text{mmol}^{-1}$ ) (○);  $[8\text{-}^{14}\text{C}]$ guanine ( $54.67 \text{ mCi} \cdot \text{mmol}^{-1}$ ) (■);  $[8\text{-}^3\text{H}]$ guanosine ( $63.2 \text{ mCi} \cdot \text{mmol}^{-1}$ ) (□). Each data value was from six independent experiments.

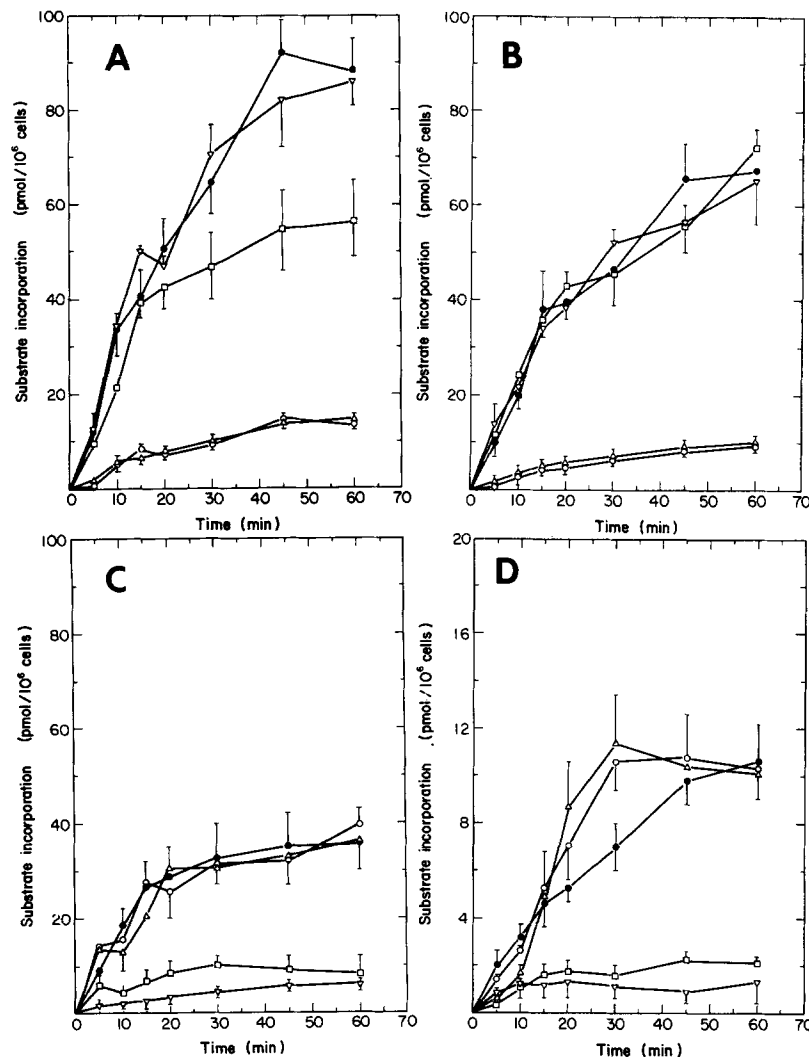


FIGURE 2. Inhibition of incorporation of radiolabeled substrates into *G. lamblia* nucleotide pool by unlabeled substrates. The radiolabeled substrates are as described in Fig. 1. (A) [8-<sup>3</sup>H]adenine, (B) [2,8-<sup>3</sup>H]adenosine, (C) [8-<sup>14</sup>C]guanine, (D) [8-<sup>3</sup>H]guanosine. The unlabeled substrates are each present at a concentration (400  $\mu$ M) 10 times higher than the concentration of radiolabeled substrates. Adenine (O), adenosine ( $\Delta$ ), guanine ( $\square$ ), guanosine ( $\nabla$ ), none ( $\bullet$ ). Each data value was from three independent experiments.

4B) as substrates resulted in heavy labeling of GMP, GDP, and GTP without any radioactivity found in AMP, ADP, or ATP. However, when [5'-<sup>3</sup>H]guanosine was used as the substrate for pulse labeling (see Fig. 4C), only 3% as much radioactivity as that from [8-<sup>3</sup>H]guanosine was incorporated into the nucleotide pool. Of the little label in the pool, none was associated with GDP or GTP, but some was detected in AMP, ADP, ATP, ADP-hexose, and GMP (Fig. 4C).

These data clearly indicate that (a) adenine/adenosine can only be incorporated into adenine nucleotides; (b) guanine/guanosine can only be incorporated

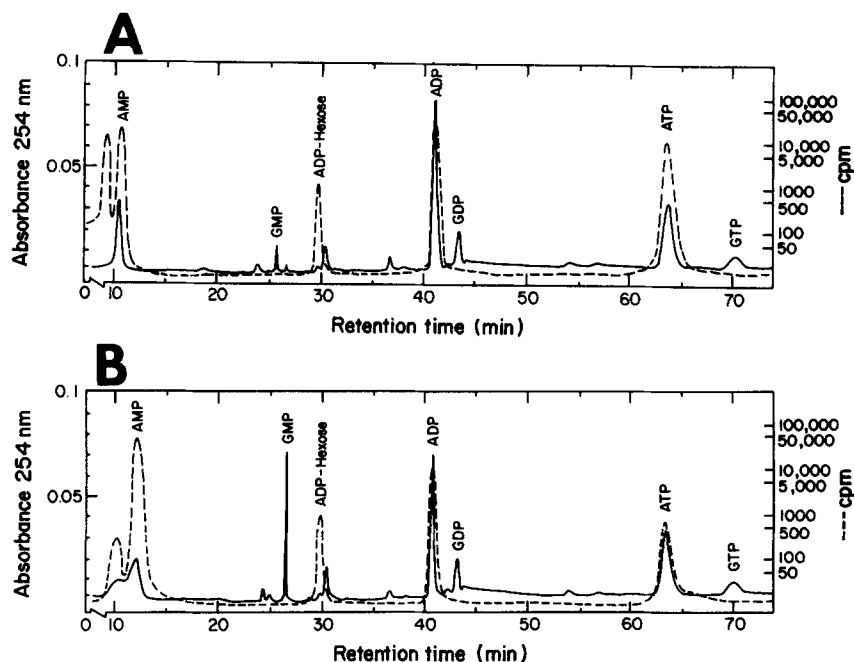


FIGURE 3. HPLC analysis of *G. lamblia* nucleotide pool pulse labeled by (A)  $[8\text{-}^3\text{H}]$ adenine ( $25 \text{ Ci} \cdot \text{mmol}^{-1}$ ) and (B)  $[2,8\text{-}^3\text{H}]$ adenosine ( $48 \text{ Ci} \cdot \text{mmol}^{-1}$ ) for 60 min.

into guanine nucleotides; (c) there is no interconversion between adenine nucleotide and guanine nucleotide; (d) the ribose moiety of guanosine does not incorporate with the guanine moiety, i.e., only guanine can be specifically incorporated into guanine nucleotides. The ribose may have been converted to PRPP and become randomly incorporated into nucleotides.

**Enzyme Activities.** No purine nucleoside kinase or purine nucleoside phosphotransferase activity could be detected above the minimal level of  $1 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  in the supernatant and  $10^5 \text{ g}$  pellet fractions of *G. lamblia* crude extracts. Substantial levels of adenine phosphoribosyl transferase and guanine phosphoribosyl transferase (GPRT) were, however, found in the supernatant fraction (see Table I). But HPRT and xanthine phosphoribosyl transferase (XPRT) were below the level of detection (see Table I).

Among the other enzymic activities assayed, adenosine hydrolase and guanosine hydrolase activities were detected at extraordinarily high levels in the supernatant fraction (Table I). These activities, hydrolyzing adenosine and guanosine to the corresponding purine bases, were not dependent on the presence of orthophosphate. They are thus not phosphorylases.

**Drug Sensitivity Test.** The inhibitor of IMP dehydrogenase, mycophenolic acid, which blocks the conversion of IMP to XMP and GMP (17), was tested and titrated in the *in vitro* culture of *G. lamblia*. The growth of *G. lamblia* was unaffected up to  $100 \mu\text{M}$  of the drug.

Formycin B, a nonhydrolyzable purine nucleoside analog (18) whose 5'-monophosphate derivative is known to block conversion of IMP to AMP (12),

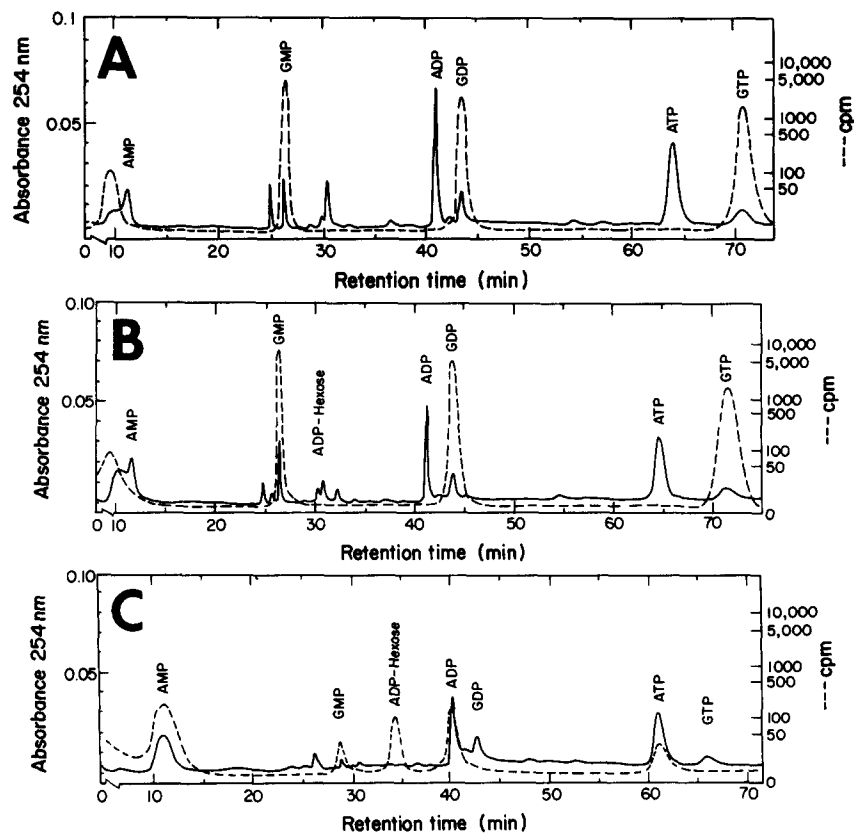


FIGURE 4. HPLC analysis of *G. lamblia* nucleotide pool pulse labeled by (A)  $[8\text{-}^{14}\text{C}]\text{guanine}$  ( $54.67 \text{ mCi} \cdot \text{mmol}^{-1}$ ), (B)  $[8\text{-}^3\text{H}]\text{guanosine}$  ( $11.0 \text{ Ci} \cdot \text{mmol}^{-1}$ ), and (C)  $[5'\text{-}^3\text{H}]\text{guanosine}$  ( $21.3 \text{ Ci} \cdot \text{mmol}^{-1}$ ) for 60 min.

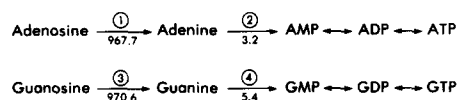


FIGURE 5. The purine salvage pathways in *G. lamblia*. (1) Adenosine hydrolase, (2) adenine phosphoribosyl transferase, (3) guanosine hydrolase, (4) guanine phosphoribosyl transferase. Numbers beneath the arrows represent specific enzyme activities in  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  (see Table I).

was also tested. No growth inhibition was observed up to 1 mM of formycin B in the medium.

### Discussion

Our investigation has confirmed the absence of de novo purine synthesis in *G. lamblia* by showing the failure of glycine and formate to enter the nucleotide pool. We have also revealed a remarkably simple scheme of purine salvage in *G. lamblia* that can be best presented in Fig. 5.

The total lack of incorporation of hypoxanthine, inosine, and xanthine into *G.*

*lamblia* nucleotides suggests the absence of HPRT and XPRT, which has been supported by the data from enzyme assays. It also suggests the absence of IMP and the conversion of IMP to AMP and GMP in the parasite. This suggestion has also been confirmed in the HPLC profiles by the total lack of interconversion between adenine nucleotides and guanine nucleotides. It is further supported by the insensitivity of *G. lamblia* toward mycophenolic acid and formycin B. The competition experiments (Fig. 2) and the HPLC profiles demonstrate that adenine and adenosine are incorporated through the same channel, and that they are converted only into adenine nucleotides. Guanine and guanosine are apparently incorporated by another route and they can only enter the guanine nucleotides. The HPLC profile in Fig. 4C further suggests that guanosine cannot be directly converted to guanine nucleotides; it has to be hydrolyzed first to guanine before the incorporation. Owing to the unavailability from commercial sources of adenosine with radiolabel only in the ribose moiety, we were unable to perform a similar analysis on adenosine. But the enzyme profile has clearly demonstrated that *G. lamblia* has no apparent means of directly salvaging either adenosine or guanosine. The most likely mechanism of purine salvage in the parasite is simply adenine salvage by adenine phosphoribosyl transferase (APRT) and guanine salvage by GPRT with no connection in between (Fig. 5). The presence of extraordinarily high adenosine hydrolase and guanosine hydrolase in *G. lamblia* provides added proof to this simple scheme of purine salvage. Adenosine and guanosine are apparently rapidly hydrolyzed to adenine and guanine before incorporation into AMP and GMP.

This purine salvage network in *G. lamblia* is not only simple but also totally different from those in *T. foetus* (7) and *T. vaginalis* (8). These differences may provide many interesting subjects for future studies. Meanwhile, our own attention for the immediate future will be centered on the GPRT of *G. lamblia*. It is apparently a very interesting enzyme since it does not recognize either hypoxanthine or xanthine as substrate. Since HGPRT activities usually reside in one enzyme in eukaryotes, and since XGPRT activities are associated in a single enzyme in bacteria (19), the GPRT (or, less likely, AGPRT) in *G. lamblia* must have a somewhat different substrate specificity than its mammalian host HGPRT,

TABLE I  
Profile of Purine Salvage Enzymes in *G. lamblia* Trophozoites

Enzymes	Specific activities* (nmol · min <sup>-1</sup> · mg protein <sup>-1</sup> )
HPRT	<0.001
GPRT	5.40 ± 1.61
XPRT	<0.001
APRT	3.16 ± 0.52
Adenosine hydrolase	967.7 ± 32.4
Guanosine hydrolase	970.6 ± 23.5

\* The activities were detected in the 10<sup>5</sup> g supernatant fraction of the crude extract of *G. lamblia* trophozoites. Each data value was from three independent experiments.



to allow specific inhibitor design. We believe that specific inhibition of *G. lamblia* GPRT will lead to effective chemotherapeutic control of the parasite.

### Summary

Purine metabolism in *Giardia lamblia* was investigated by monitoring incorporation of radiolabeled precursors into purine nucleotides in the log-phase trophozoites cultivated in vitro in axenic media and incubated in buffered saline glucose. The lack of incorporation of formate, glycine, hypoxanthine, inosine, and xanthine into the nucleotide pool suggests the absence of de novo purine nucleotide synthesis and the inability to form IMP as the precursor of AMP and GMP in *G. lamblia*. Only adenine, adenosine, guanine, and guanosine were incorporated. Further analysis of the labeled nucleotides by HPLC indicated that adenine and adenosine are converted only to adenine nucleotides whereas guanine and guanosine are only incorporated into guanine nucleotides. There is no competition of incorporation between adenine/adenosine and guanine/guanosine, and there is no interconversion between adenine and guanine nucleotides. Results from analyzing [5'-<sup>3</sup>H]guanosine incorporation indicate that the ribose moiety is not incorporated with the guanine base. Assays of purine salvage enzymic activities in the crude extracts of *G. lamblia* revealed the presence of only four major enzymes; adenosine and guanosine hydrolases and adenine and guanine phosphoribosyl transferases. Apparently, *G. lamblia* has an exceedingly simple purine salvage system; it converts adenosine and guanosine to corresponding purine bases and then forms AMP and GMP by the actions of corresponding purine phosphoribosyl transferases. The guanine phosphoribosyl transferase in *G. lamblia* is interesting because it does not recognize either hypoxanthine or xanthine as substrate. It thus must have a unique substrate specificity and may be regarded as a potential target to attack as a rational approach to chemotherapeutic control of giardiasis.

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