PYRIMIDINE SALVAGE IN *GIARDIA LAMBLIA*

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The intestinal protozoan parasite *Giardia lamblia* is the causative agent of a condition known as giardiasis, which is quickly becoming a major worldwide public health concern. In several surveys of apparently healthy populations in the United States, 2–22% of the screened individuals tested positive for *G. lamblia* infections (1–4).

Giardiasis is a debilitating disease characterized by severe diarrhea, abdominal pain, anorexia, and stunted growth in children. Mammals become infected with *G. lamblia* through ingestion of food or water contaminated with *G. lamblia* cysts. Currently there is no satisfactory treatment for giardiasis; the two drugs available, metronidazole (Flagyl) and quinacrine (Atabrine), both produce undesirable side effects.

It has become increasingly apparent in recent years that parasite metabolism can differ significantly from that of the mammalian host, and that these differences can be viewed as attractive targets for the design of antiparasitic agents. As an example, many parasitic protozoa examined to date have been found to lack purine *de novo* synthesis and thus depend exclusively on salvage of preformed purines to supply necessary purine nucleotide needs (5–9). *Trypanosoma cruzi* and *Leishmania donovani* are two examples whose dependence on salvage of purines has been successfully exploited in designing antitrypanosomal and antileishmanial agents effective in controlling the parasite without ill effects to the host (10–12). Recently (13), we have observed that *G. lamblia* also lacks *de novo* purine synthesis and relies on a very simple scheme of purine salvage involving a guanine phosphoribosyltransferase and an adenine phosphoribosyltransferase.

In contrast to the deficiency of purine *de novo* synthesis, most protozoan parasites are fully capable of synthesizing pyrimidine nucleotides *de novo*. However, two anaerobic, flagellated parasitic protozoa, *Tritrichomonas foetus* and *Trichomonas vaginalis* have been found (14, 15) to lack *de novo* synthesis of both purine and pyrimidine nucleotides, and thus rely on salvage of exogenous purines, pyrimidines, and their nucleosides to satisfy metabolic needs. These two organisms were also found (14, 15) to lack dihydrofolate reductase and thymidylate synthetase, and must rely on salvage of exogenous thymidine for DNA synthesis. In addition, Lindmark and Jarroll (16) have suggested that *G. lamblia* lacks *de novo* pyrimidine metabolism, as indicated by its inability to incorporate orotate or aspartate into cold trichloroacetic acid–insoluble fractions and by the absence of *de novo* pyrimidine synthesis enzymes. In previous studies (Wang,

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unpublished results), it was found that *G. lamblia* also lacks dihydrofolate reductase and thymidylate synthetase.

These results indicate that *G. lamblia*, like *T. foetus* and *T. vaginalis*, is incapable of both purine and pyrimidine *de novo* biosynthesis and must rely on the host to provide for its nucleotide needs. This dependence on the host would be of critical importance to the survival of the parasite.

It was our aim in this study to verify this dependence on salvage of pyrimidines by *G. lamblia* and to establish the pyrimidine salvage pathway used by this parasite. This information could then be used to propose rational targets for design of antigiardial agents.

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 (17); 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 2×10^6/ml. These cultures were used to inoculate fresh media at 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 orotate, aspartate, bicarbonate, pyrimidines, and pyrimidine nucleosides were purchased from New England Nuclear, Boston, MA; ICN Pharmaceuticals, Inc., Irvine, CA; or Amersham Corp., Arlington Heights, IL. 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 2×10^7/ml, and incubated at 37°C. Radiolabeled substrates were each added to the cell suspension to a final concentration of 40 μM, and aliquots were taken at various time intervals. Aliquots of 300 μl were taken for uptake studies of [5,6-^{3}H]uracil, [5,6-^{3}H]uridine, [2-^{14}C]cytosine and [5-^{3}H]cytidine. Aliquots of 3 ml, concentrated 10-fold to 300 μl, were taken for uptake studies of [6-^{14}C]orotate, [2,3-^{3}H]aspartate, [^{14}C]bicarbonate, [2-^{14}C]thymine, and [2-^{14}C]thymidine. Each aliquot was treated with perchloric acid–KOH and filtered through glass fiber filters loaded with polyethyleneimine (PEI)[1]-cellulose in 5 mM ammonium acetate, pH 5.0, as previously described (9). The nucleotide-loaded filter was washed with three 5-ml portions of the ammonium acetate buffer, dried, and soaked in Aquasol 2 (New England Nuclear). Levels of radioactivity were determined with a liquid scintillation spectrometer (LS-3133T; Beckman Instruments, Inc., Fullerton, CA).

High Performance Liquid Chromatography (HPLC). Pyrimidine nucleotides were separated, identified, and quantitated in an ion-exchange HPLC system with an Ultrasil AX (10 μm; Beckman Instruments, Inc., Fullerton, CA) 4.6 × 250 mm column. Perchloric acid–KOH extracts of incorporation experiments concentrated fivefold ([5,6-^{3}H]uracil, [5,6-^{3}H]uridine, [2-^{14}C]cytosine, [5-^{3}H]cytidine) or 10-fold ([6-^{14}C]orotate, [2,3-^{3}H]aspartate, [^{14}C]bicarbonate, [2-^{14}C]thymine, [2-^{14}C]thymidine) were injected (100 μl) 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 an

[1] *Abbreviations used in this paper:* CMP, cytidine monophosphate; HPLC, high performance liquid chromatography; PEI, polyethyleneimine; TMP, ribosylthymine monophosphate; UMP, uridine monophosphate.

ultraviolet (UV)-absorbance detector (Beckman Instruments, Inc.), mixed with Aquasol 2 at a 1:3 ratio, and the radioactivity recorded in a radioactive flow detector (Flo-one; Radiomatic Instruments & Chemical Co., Inc., Tampa, FL). Both UV absorbance and radioactivity data were recorded and analyzed by 3390A integrator (Hewlett-Packard Co., Palo Alto, CA) (9).

For analysis of pyrimidine bases and nucleosides, a reverse phase HPLC system with an ODS (5 μm; Beckman Instruments, Inc.) reverse phase column was used, eluted with a programmed gradient, from 7 mM KH_2PO_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 (9).

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 $MgCl_2$, and 1 mM dithiothreitol (TKMD). Cells were disrupted, using a sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, 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 pelletable fractions. Protein concentrations were determined by the method of Lowry et al. (18), with bovine serum albumin as the standard.

Pyrimidine phosphoribosyl transferases were assayed according to a modified procedure by Schmidt et al. (19). The reaction mixture, containing 100 μl of 100 mM Tris-HCl, pH 7.8, 8 mM $MgCl_2$, 1.0 mM 5-phosphoribosyl-1-pyrophosphate (PRPP), 50 μg/ml bovine serum albumin, and 200 μM of [2-^{14}C]cytosine (61 mCi/mmol), or [2-^{14}C]uracil (53 mCi/mmol) was incubated at 37°C. The reaction, initiated by adding enzyme sample, was terminated by adding an equal volume of ice-cold, 5 mM ammonium acetate, pH 5.0. Aliquots of the mixture were filtered through PEI-cellulose and the trapped radioactivities were counted as described previously.

Pyrimidine 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 $MgCl_2$, 30 mM phosphoenolpyruvate, 40 IU/ml pyruvate kinase, and 0.2 mM of [5-^{3}H]cytidine (48 mCi/mmol), [5,6-^{3}H]uridine (52.1 mCi/mmol), or [2-^{14}C]-thymidine (58.4 mCi/mmol), 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.

Pyrimidine nucleoside phosphotransferases were assayed by using *p*-nitrophenylphosphate (10 mM) as the phosphate donor in an assay mixture containing 100 mM Na-acetate, pH 5.4, 0.2 mM of the radiolabeled pyrimidine nucleosides, and the enzyme. Assay procedures were the same as for the kinases.

Pyrimidine nucleoside hydrolase was assayed in TKMD with 1.0 mM of a pyrimidine nucleoside in the absence of added phosphate for varying lengths of time, terminated by perchloric acid, neutralized with KOH, and the reaction products analyzed by reverse phase HPLC to identify and quantitate the pyrimidine bases.

Cytosine/cytidine deaminase was assayed in 50 mM Tris HCl, pH 7.5 and 0.2 mM substrate. Reaction was initiated by addition of enzyme and then incubated at 37°C for varying lengths of time. The reaction was terminated by perchloric acid–KOH treatment. The extract was then analyzed by reverse phase HPLC.

Results

De Novo Pyrimidine Synthesis. Log phase *G. lamblia* cells were incubated at 37°C with [^{14}C]bicarbonate (50 mCi/mmol), [6-^{14}C]orotic acid (61 mCi/mmol), or [2,3-^{3}H]L-aspartic acid (28.7 mCi/mmol) for 60 min. Perchloric acid–KOH extracts of cell suspensions concentrated 10-fold were analyzed by PEI cellulose absorption and HPLC. No radioactivity could be detected in pyrimidine nucleotides of *G. lamblia*.

Incorporation of Pyrimidines and Pyrimidine Nucleosides. When radiolabeled

pyrimidines or pyrimidine nucleosides were tested for incorporation into the *G. lamblia* nucleotide pool, all substrates except thymine were incorporated to a detectable level (Fig. 1). Uridine and uracil were incorporated at the highest initial rate of 2.25 pmol/min per 10^6 cells, followed by cytosine and cytidine at an initial rate of 0.8 pmol/min per 10^6 cells. Thymidine was incorporated at the lowest rate, 0.2 pmol/min per 10^6 cells, and was detected only after time point aliquots were concentrated 10-fold and assayed for PEI absorption. Thymine incorporation was below the minimal level of detection (0.08 pmol/min per 10^6 cells) even after we incubated samples for 60 min and concentrating them 10-fold.

HPLC Analysis of Radiolabeled Nucleotide Pools. When perchloric acid–KOH extracts of cells incubated for 60 min with [^{14}C]HCO_3^-, [6-^{14}C]orotate, [2,3-^{3}H]-aspartate, or [2-^{14}C]thymine were analyzed by HPLC, no radiolabel incorporation into nucleotide pools could be detected.

HPLC profiles of *G. lamblia* nucleotide pools after incubation with either [5,6-^{3}H]uracil, [5,6-^{3}H]uridine, [2-^{14}C]cytosine, or [5-^{3}H]cytidine all showed a similar pattern. Radioactivity was observed in both the uracil nucleotides and cytosine nucleotides after pulse labeling for 45 min (Fig. 2). [5,6-^{3}H]uracil and [5,6-^{3}H]-uridine had similar incorporation profiles, with radioactivity found predominantly as uridine monophosphate (UMP), UDP-hexose, UTP, and, to a smaller extent, UDP. However, substantial radioactivity was found associated with cytidine monophosphate (CMP), CDP, and CTP. Similarly, when either [2-^{14}C]-cytosine or [5-^{3}H]cytidine was used as the radioactive precursor, radioactivity was equally distributed into CMP, CDP, and CTP as well as UMP, UDP, UDP-hexose, and UTP.

These data indicate that there is extensive conversion between pyrimidine nucleoside uridine and cytidine nucleotides. This interconversion could take place at the pyrimidine or pyrimidine nucleoside level or between pyrimidine

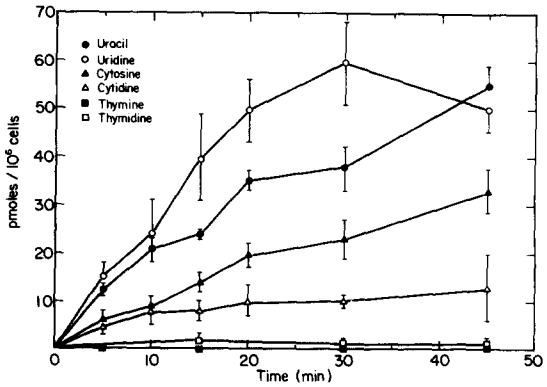

FIGURE 1. Incorporation of radiolabeled pyrimidines and pyrimidine nucleosides into the nucleotide pool of *G. lamblia*. [5,6-^{3}H]uracil (52 mCi/mmol) (●); [5,6-^{3}H]uridine (52.1 mCi/mmol) (○); [2-^{14}C]cytosine (61 mCi/mmol) (▲); [5-^{3}H]cytidine (48 mCi/mmol) (△); [2-^{14}C]-thymine (54 mCi/mmol) (■); [2-^{14}C]thymidine (58.4 mCi/mmol) (□). Each data point was from six independent experiments.

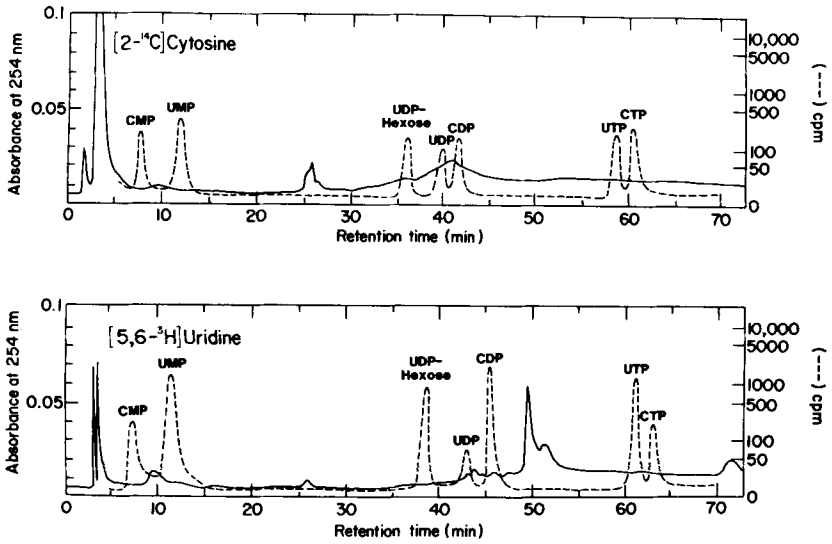

FIGURE 2. HPLC analysis of *G. lamblia* nucleotide pool pulse-labeled by 40 μM of [2-^{14}C]-cytosine (61 mCi/mmol) or [5,6-^{3}H]uridine (52.1 mCi/mmol).

nucleoside triphosphates, using an enzyme similar to that found in other systems: a cytidine triphosphate synthetase (20–23).

HPLC analysis of [2-^{14}C]thymidine incorporation into *G. lamblia* nucleotide pools quantitated radioactivity exclusively in three peaks, corresponding to ribosylthymine monophosphate (TMP), TDP, and TTP (data not shown).

Enzyme Assays. The results of various enzyme assays from *G. lamblia* soluble and pellet fractions are summarized in Table I. Thymidine salvage occurs apparently through a thymidine phosphotransferase that is found mainly in the 10^5 *g* pellet fraction, with only residual activity in the supernatant fraction. Very low amounts of thymidine kinase activities [0.0123–0.0142 nmol/(min·mg)] were detected in the pellet and supernatant fractions.

One of the major enzyme activities in the supernatant fraction of *G. lamblia* crude extracts was uracil phosphoribosyltransferase, which is apparently responsible for salvaging and converting exogenous uracil to UMP. A uridine phosphotransferase was also present in the supernatant fraction when uridine is used as a substrate and *p*-nitrophenylphosphate as the phosphate donor for the formation of UMP. No uridine kinase or cytidine kinase activities could be detected in the pellet or supernatant fractions of the extracts.

Other enzymes exhibiting extremely high levels of activity in *G. lamblia* were thymidine hydrolase and uridine hydrolase. Cytidine hydrolase was present at a much lower level. These enzymes, which convert thymidine, uridine, and cytidine to their corresponding pyrimidine bases, are not dependent upon the presence of orthophosphate for their activities and are thus unlikely to be phosphorylases. The relatively low level of cytidine hydrolase may be due to the extremely high amount of cytidine deaminase activity found in the supernatant fraction. No cytosine deaminase enzyme activities were detected in either the supernatant or pellet fraction. However, cytosine can be salvaged by the cytosine phosphoribo-

TABLE I
Pyrimidine Salvage Enzymes in G. lamblia Trophozoites

Enzyme	Specific activity [nmol/(min · mg) protein]	
	Pellet	Supernatant
Thymidine kinase	0.0142 ± 0.007	0.0123 ± 0.001
Uridine kinase	<0.001	<0.001
Cytidine kinase	<0.001	<0.001
Thymidine phosphotransferase	0.090 ± 0.003	0.01505 ± 0.0045
Uridine phosphotransferase	0.058 ± 0.019	<0.001
Cytidine phosphotransferase	<0.001	<0.001
Uracil phosphoribosyltransferase	0.025 ± 0.004	1.87 ± 0.62
Cytosine phosphoribosyltransferase	<0.001	0.1325 ± 0.04
Thymidine hydrolase	<0.001	155 ± 18
Uridine hydrolase	<0.001	168 ± 14
Cytidine hydrolase	<0.001	7.6 ± 0.4
Cytidine deaminase	<0.001	183 ± 33
Cytosine deaminase	<0.001	<0.001

Each data value was from four independent experiments.

syltransferase activity present at a low level in the supernatant fraction of *G. lamblia*.

Discussion

Our investigation has confirmed the absence of *de novo* pyrimidine nucleotide synthesis in *G. lamblia*, caused by the failure to detect incorporation of orotate, bicarbonate, or aspartate into the *G. lamblia* pyrimidine nucleotides pool. This result agrees with observations published earlier by Lindmark and Jarroll (16), who could not detect the *de novo* pyrimidine synthesis enzymes.

From our results we are able to propose a fairly simple scheme of pyrimidine salvage by *G. lamblia*, which is summarized in Fig. 3. Incorporation of uracil into UMP by uracil phosphoribosyltransferase seems to be the predominant pathway supplying pyrimidine nucleotides. Uridine is mostly converted to uracil first, because of the very high level of uridine hydrolase in *G. lamblia*, before incorporation into UMP via uracil phosphoribosyltransferase. This conclusion is supported by the similar incorporation rates of radiolabeled uracil and uridine, as well as the HPLC profiles of their radioactive nucleotide pools. These HPLC profiles also indicate that labeled uracil or uridine give rise to radioactive peaks corresponding not only to UMP, UDP-hexose, UDP, and UTP, but also to CMP, CDP, and CTP. This indicates an active conversion from uracil nucleotides to cytosine nucleotides, probably by the action of cytidine triphosphate synthetase (20–23).

The HPLC profile of cytidine incorporation is similar to those of uracil and uridine, indicating that it too is shuttled through the uracil salvage pathway. This is confirmed by the substantial amount of cytidine deaminase activity in the

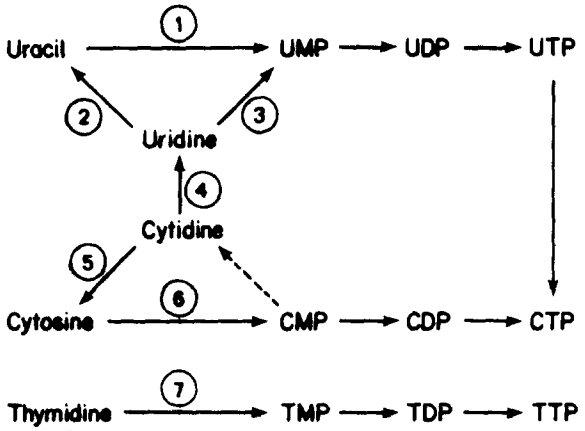

FIGURE 3. The pyrimidine salvage pathway in *G. lamblia*: (*1*) uracil phosphoribosyltransferase, (*2*) uridine hydrolase, (*3*) uridine phosphotransferase, (*4*) cytidine deaminase, (*5*) cytidine hydrolase, (*6*) cytosine phosphoribosyltransferase, (*7*) thymidine phosphotransferase.

crude extract of *G. lamblia*, which converts nearly all the available cytidine to uridine within minutes. The uridine is then rapidly hydrolyzed to uracil before incorporation into the pyrimidine nucleotide pool. A small amount of cytidine might be hydrolyzed to the corresponding base cytosine, which could be incorporated into the nucleotide pool by the action of cytosine phosphoribosyltransferase.

HPLC analysis of the radioactive nucleotide pool after incubation of *G. lamblia* trophozoites with radiolabeled cytosine revealed labeled CMP, CDP, and CTP peaks, as well as UMP, UDP, UDP-hexose, and UTP peaks. The labeling of UMP, UDP-hexose, UDP, and UTP cannot be readily explained, because we did not detect cytosine deaminase activity deaminating cytosine to uracil, or cytosine phosphorylase activity converting cytosine to cytidine, in the soluble or pelletable fractions of *G. lamblia*. Direct conversion of cytosine nucleotides to uracil nucleotides has not yet been demonstrated in *G. lamblia* crude extracts. When we incubated soluble fractions of *G. lamblia* for various time intervals with CMP, and analyzed the enzyme extract by ion exchange and reverse phase HPLC, we detected no UMP formation but significant amounts of uridine, uracil, cytidine, and cytosine (data not shown). We believe that the phosphatase and hydrolase activities of *G. lamblia* may account for the formation of uracil nucleotides from cells incubated with radiolabeled cytosine. CMP could be converted to cytidine by the phosphatase and then to uridine by cytidine deaminase. Uracil generated from uridine could then be recycled through uracil phosphoribosyltransferase and incorporated into UMP, UDP, and UTP. CMP could also be converted to CDP and CTP, presumably by kinase actions. These activities may account for the parallel increase in radioactivity of cytosine nucleotide peaks as compared with uracil nucleotide peaks in HPLC profiles of cytosine-labeled *G. lamblia* nucleotides.

G. lamblia must rely on the salvage of exogeneous thymidine via thymidine phosphotransferase to satisfy its nucleic acid needs. This is supported by the fact that *G. lamblia* lacks both dihydrofolate reductase and thymidylate synthetase.

The estimated rate of thymidine incorporation, 0.2 pmol/min per 10^6 cells (Fig. 1) corresponds to an incorporating activity of 0.025 nmol/(min·mg) protein (there is 8 μg protein in the pelletable fraction of 10^6 *G. lamblia* trophozoites). The specific activity of thymidine phosphotransferase (0.09 nmol/(min·mg) protein, Table I) is more than adequate to supply all the TMP needed by *G. lamblia*. There is also evidence of thymidine kinase activity in *G. lamblia*, but at such a low level (Table I) that it is not likely a primary means of supplying thymidine nucleotides.

These results indicate that salvage of pyrimidines by *G. lamblia* is very similar to that reported (14) for pyrimidine salvage by *T. foetus*, which also is primarily dependent on the activity of uracil phosphoribosyltransferase and thymidine phosphotransferase to supply necessary nucleotides. The parasite *T. vaginalis* has been found (15) to depend on a membrane-bound deoxyribonucleoside phosphotransferase for all deoxynucleotides. It would be interesting to investigate the various substrate specificities of the thymidine phosphotransferase of *G. lamblia* to determine if it is a similar type of enzyme.

Obviously, the uracil phosphoribosyltransferase and thymidine phosphotransferase are particularly attractive targets for antigiardial drug design, since the activity of these two enzymes seems crucial to the survival of the parasite. Future work will concentrate on the purification and characterization of these two enzymes.

Summary

We have found that the anaerobic protozoan parasite *Giardia lamblia* is incapable of *de novo* pyrimidine metabolism, as shown by its inability to incorporate orotate, bicarbonate, and aspartate into the pyrimidine nucleotide pool. Results from high performance liquid chromatography of pyrimidine and pyrimidine nucleoside pulse-labeled nucleotide pools and enzyme assays suggest that the parasite satisfies its pyrimidine nucleotide needs predominantly through salvage of uracil by a cytoplasmic uracil phosphoribosyltransferase. Exogenous uridine and cytidine are primarily converted to uracil by the action of uridine hydrolase and cytidine deaminase before incorporation into nucleotide pools. Direct salvage of cytosine occurs to a relatively limited extent via cytosine phosphoribosyltransferase. *G. lamblia* relies on salvage of exogenous thymidine for ribosylthymine monophosphate (TMP) synthesis, accomplished primarily through the action of a 100,000 *g*–pelletable thymidine phosphotransferase.

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