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Biochemistry

Pyrimidine metabolism in *Tritrichomonas foetus*

(uracil phosphoribosyltransferase/thymidine phosphotransferase/5-fluorouracil)

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ABSTRACT **The anaerobic parasitic protozoa *Tritrichomonas foetus* is found incapable of *de novo* pyrimidine biosynthesis by its failure to incorporate bicarbonate, aspartate, or orotate into pyrimidine nucleotides or nucleic acids. Uracil phosphoribosyltransferase in the cytoplasm provides the major pyrimidine salvage for the parasite. Exogenous uridine and cytidine are mostly converted to uracil by uridine phosphorylase and cytidine deaminase in *T. foetus* prior to incorporation. *T. foetus* cannot incorporate labels from exogenous uracil or uridine into DNA; it has no detectable dihydrofolate reductase or thymidylate synthetase and is resistant to methotrexate, pyrimethamine, trimethoprim, and 5-bromovinyldeoxyuridine at millimolar concentrations. It has an enzyme thymidine phosphotransferase in cellular fraction pelleting at 100,000 × g that can convert exogenous thymidine to TMP via a phosphate donor such as *p*-nitrophenyl phosphate or nucleoside 5′-monophosphate. Thymidine salvage in *T. foetus* is thus totally dissociated from other pyrimidine salvage.**

It has become apparent in recent years that parasitic protozoa are generally incapable of *de novo* synthesis of purine nucleotides. *Trypanosoma cruzi* (1), *Leishmania donovani* (2), *Plasmodium lophurae* (3), *Eimeria tenella* (4), and *Trichomonas vaginalis* (5), to name but a few examples, depend on specific networks of salvage pathways to fulfill their purine requirements. Because of this deficiency in metabolic activities, rational approaches to controlling some of the parasites have been possible. Allopurinol exhibits antitrypanosomal and antileishmanial activities because it is recognized by the parasite salvage enzymes as a hypoxanthine analog (6, 7). Allopurinol riboside (8), formycin B (9), and 4-thiopyrazolopyrimidine riboside (10) have antileishmanial activities because of the nucleoside phosphotransferase in leishmania, which incorporates the compounds into parasite's nucleotide pool.

De novo pyrimidine biosynthesis, on the other hand, takes place in most of the parasitic protozoa. Recently, it has been reported that the anaerobic flagellates *Trichomonas vaginalis* and *Giardia lamblia* may not, however, have even the capability of pyrimidine *de novo* synthesis. The former lacks aspartate transcarbamoylase, dihydroorotase, dihydroorotate dehydrogenase, and orotate phosphoribosyltransferase in its crude extract (11), whereas the latter indicates no incorporation of aspartate into the cold trichloroacetic acid-insoluble fraction (12). These results suggest that anaerobic flagellates may differ from other protozoan parasites in lacking both purine and pyrimidine *de novo* synthetic abilities and thus may offer even more opportunities for chemotherapeutic attack.

To verify these possibilities, we studied pyrimidine metabolism in *Tritrichomonas foetus*, a cattle parasite that is closely related to *Trichomonas vaginalis*. Our results show that *T. foetus* cannot perform *de novo* pyrimidine synthesis; a very simple scheme of pyrimidine salvage is providing the needs for the parasite.

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MATERIALS AND METHODS

Cultures. *T. foetus* strain KV_1 was cultivated in Diamond's TYM medium, pH 7.2/10% heat-inactivated horse serum at 37°C (13). Stationary cultures having a cell density of about 2×10^7/ml were used to inoculate fresh media at a 1:10 ratio. Midlogarithmic phase of growth, with a cell density of 10^7/ml, was achieved after 17 hr of incubation. These cells were used for all the studies. Cell number was determined in a Coulter ZF Counter.

Chemicals. Radiolabeled bicarbonate, L-aspartate, pyrimidines, nucleosides, and nucleotides were purchased from New England Nuclear, Amersham, or ICN. Enzyme samples were obtained from Sigma. Other chemicals used were all of the highest purities commercially available.

Precursor Incorporation into the Nucleotide Pool. *T. foetus* cells were washed, suspended in phosphate-buffered saline, pH 7.2 (P_i/NaCl)/20 mM glucose to a final cell density of 10^8/ml, and incubated at 37°C. A radiolabeled substrate was added, and aliquots were taken at different times for perchloric acid–KOH extraction (4). The extract was filtered through polyethyleneimine (PEI)-cellulose in 5 mM ammonium acetate (pH 5.0), and the adsorbed radioactivity was determined with a Beckman LS-3133T liquid scintillation spectrometer. For pulse–chase experiments, the incubated cell suspension was washed with P_i/NaCl/glucose at 37°C to remove the radioactive substrate and resuspended in P_i/NaCl/glucose together with unlabeled substrate, and incubation was continued.

HPLC. Nucleotides in the perchlorate–KOH extract were separated, identified, and quantitated in an ion exchange HPLC system with an Ultrasil AX (10-μm) 4.6 × 250 mm column. Samples (100 μl) 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, pH 4.5/500 mM KCl was used.

The effluent was monitored at 254 nm in a Beckman 160 UV absorbance detector and then mixed with Aquasol-2 (1:3); radioactivity was recorded continuously in a Flo-one radioactive flow detector (Radiomatic, Tampa, FL). UV absorbance and radioactivity were synchronously recorded in a Kipp and Zonen BD41 dual recorder, and the data were analyzed with a Hewlett-Packard 3390A integrator.

Precursor Incorporation into Nucleic Acid. Radiolabeled *T. foetus* cells were washed with P_i/NaCl/glucose and dissolved in 0.25 M NaOH containing calf thymus DNA at 0.5 mg/ml and unlabeled precursor at 0.5 mg/ml, and the solution was incubated at 37°C overnight. DNA and protein were precipitated with cold 5% trichloroacetic acid, washed, and collected on a

Abbreviations: P_i/NaCl, phosphate-buffered saline, pH 7.2; PEI, polyethyleneimine.

0.45-μm Millipore cellulose nitrate filter. Radioactivities were determined as described previously; those in the hot 5% trichloroacetic acid pellets were considered with the proteins.

To measure precursor incorporation into RNA, cells were dissolved in a solution of $NaDodSO_4$ at 10 mg/ml containing *Escherichia coli* ribosomal RNA at 500 μg/ml and unlabeled precursor at 250 μg/ml and immediately treated with cold 5% trichloroacetic acid overnight. The resulting pellet was washed and treated with 0.3 M KOH at 37°C for 16 hr. Perchloric acid was added to 0.3 M; radioactivity in the supernatant fraction was measured by scintillation counting.

Enzyme Assays: *Phosphoribosyltransferase.* Phosphoribosyltransferase activities were assayed in a 0.10 M Tris·HCl, pH 7.8/7 mM $MgCl_2$/1.0 mM 5-phosphoribosyl-1-pyrophosphate/bovine serum albumin (50 μg/ml)/20 μM [2-^{14}C]uracil (55.2 mCi/mmol; 1 Ci = 37 GBq)/20 μM [6-^{14}C]orotic acid (61.0 mCi/mmol)/20 μM [*methyl*-^{14}C]thymine (54.0 mCi/mmol) or 20 μM [2-^{14}C]cytosine (61.0 mCi/mmol) (4). The reaction was carried out at 37°C for 10 min and terminated by addition of 2 mM unlabeled pyrimidine solution, the mixture was filtered through PEI-cellulose, and the trapped radioactivity was determined.

Nucleoside kinase. Kinase activity was assayed according to Nelson *et al.* (8). The mixture, consisting of 0.10 M Tris·HCl, pH 7.5/20 mM ATP/20 mM $MgCl_2$/80 mM phospho*enol*pyruvate/pyruvate kinase (40 units/ml)/1.5 mM [5,6-^{3}H]uridine (55.4 mCi/mmol)/1.5 mM [5-^{3}H]cytidine (50.1 mCi/mmol) or 1.5 mM [6-^{3}H]thymidine (42.4 mCi/mmol), was incubated at 37°C for 10 min and the PEI-cellulose-adsorbable radioactivity was determined.

Nucleoside phosphotransferase. p-Nitrophenyl phosphate (10 mM) was the phosphate donor (8) in the presence of 0.10 M NaOAc (pH 5.4), 1.0 mM radioactive pyrimidine nucleoside, and the enzyme. Assay procedures were the same as for the kinases.

Other enzymes. Nucleoside phosphorylases were assayed in 50 mM Tris·HCl, pH 7.5/1.0 mM radiolabeled pyrimidine nucleoside/10 mM potassium phosphate. Cytidine deaminase was assayed in 50 mM Tris·HCl, pH 7.5/0.2 mM [5-^{3}H]cytidine (50.1 mCi/mmol). Both reactions were run at 37°C for 60 min and terminated by perchloric acid–KOH treatment. The extract was analyzed by HPLC with an octadecylsilyl (5-μm) reversed-phase column, which was eluted with a programmed gradient from 1 mM KH_2PO_4 (pH 6.0) to 100% acetonitrile at a flow rate of 0.75 ml/min. The UV monitoring and radioactivity measurements of the effluent were as described previously. Thymidylate synthetase was assayed by the 5-fluorodeoxy[^{3}H]uridine monophosphate (18 Ci/mmol) filter-binding method, which is sensitive to picomolar quantities (14). Dihydrofolate reductase was assayed spectrophotometrically by the procedure of Hillcoat *et al.* (15).

Preparation of *T. foetus* Extracts. Cells were washed, suspended in 1 vol of 25 mM Tris·HCl, pH 7.2/20 mM KCl/6 mM $MgCl_2$/1 mM dithiothreitol, and homogenized in a Brinkman Polytron for two 15-sec periods. The homogenate was centrifuged at 10,000 × *g* for 30 min to remove cell debris and then at 100,000 × *g* for 1 hr to separate the soluble and pellet fractions. Protein concentrations were determined by the method of Bradford (16), using bovine serum albumin as standard.

RESULTS

***De Novo* Pyrimidine Nucleotide Synthesis.** Radiolabeled precursors of *de novo* pyrimidine nucleotide synthesis—bicarbonate, L-aspartate, and orotic acid (see Table 1)—were incubated with *T. foetus* at 37°C. Samples taken at various times indicated, by HPLC analysis, that none of the precursors had been incorporated into the nucleotide pool (data not shown).

Table 1. Incorporation of radiolabeled substrates into *T. foetus* nucleic acids

Substrate	Conc., mM	Label, mCi/mmol	Incorporation, pmol per 10^6 cells	
			DNA fraction	RNA fraction
$H^{14}CO_3^-$	2.0	58.0	<0.05 (3)	<0.05 (3)
[^{14}C]Aspartate	6.8	14.8	<0.05 (3)	<0.05 (3)
[5-^{14}C]Orotate	2.5	15.1	<0.05 (3)	<0.05 (3)
[*methyl*-^{3}H]-Thymidine	1.6	61.9	0.64 ± 0.11 (6)	<0.05 (3)
[6-^{3}H]Uracil	2.2	40.6	<0.05 (6)	5.77 ± 0.79 (4)
[6-^{3}H]Uridine	3.3	30.1	<0.05 (6)	5.30 ± 1.60 (3)

Incorporation was determined after 2 hr of incubation. Conc., concentration. Numbers in parentheses represent numbers of experiments. Results represent mean or mean ± SEM.

Possible precursor incorporation into *T. foetus* nucleic acids was examined after 2 hr of incubation (Table 1). No radioactivity from bicarbonate or orotic acid was found in DNA, RNA, or protein. L-Aspartate was incorporated only into protein.

In the phosphoribosyltransferase assays, no orotate phosphoribosyltransferase activity could be detected in extracts (Table 2).

Salvage of Pyrimidines and Pyrimidine Nucleosides. As shown in Fig. 1, exogenous uracil, uridine, cytidine, and thymidine can be taken up by *T. foetus* and converted to nucleotides. Uracil incorporation has an initial rate of 1.67 pmol/min per 10^6 cells, uridine, and cytidine are taken up at a rate that is about 10% of this, and thymidine is incorporated at a rate about 1% of that for uracil. Cytosine and thymine are not incorporated at all. Results of competition experiments between radiolabeled substrates and 10-fold higher concentration of unlabeled substrates are presented in Fig. 2. Thymine and cytosine, themselves not incorporated, exert no effect on the incorporation of

Table 2. Pyrimidine salvage enzyme activities in extracts of *T. foetus*

Enzyme type	Substrate	Activity (nmol/min)/mg of protein	
		Supernatant	Pellet
Phosphoribosyltransferase	Uracil	0.516 ± 0.004	<0.001
	Cytosine	<0.001	<0.001
	Thymine	<0.001	<0.001
	Orotate	<0.001	<0.001
Kinase	Uridine	<0.001	<0.001
	Cytidine	<0.001	<0.001
	Thymidine	<0.001	<0.001
Phosphotransferase	Uridine	<0.001	0.254 ± 0.050
	Cytidine	<0.001	0.039 ± 0.003
	Thymidine	<0.001	0.144 ± 0.022
Phosphorylase	Uridine	1.64 ± 0.02	<0.001
	Cytidine	<0.001	<0.001
Deaminase	Cytosine	<0.001	<0.001
	Cytidine	1.84 ± 0.02	<0.001
Dihydrofolate reductase	Dihydrofolate	<0.01	<0.01
Thymidylate synthetase		<0.0001*	<0.0001*

Each enzyme activity value is derived from three independent assays. Results represent mean or mean ± SEM.
* Binding value expressed as nmol of 5-fluorodeoxyuridine monophosphate bound per mg of protein.

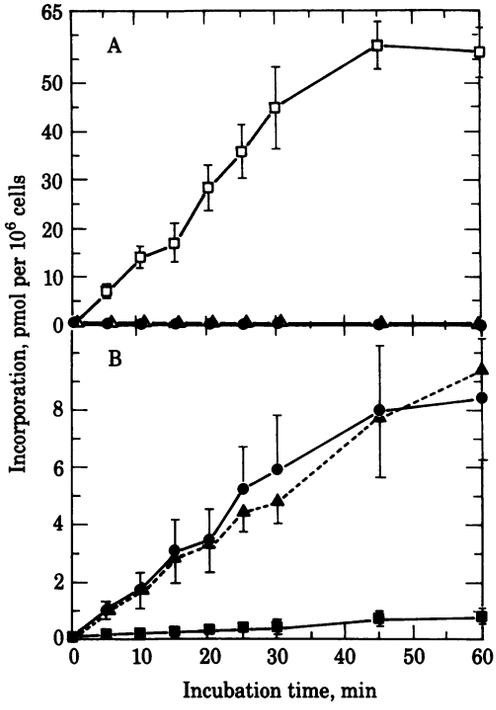

FIG. 1. Substrate incorporation into *T. foetus* nucleotides. Substrates were used at 20 μM. (*A*) □, [2-^{14}C]Uracil (58 mCi/mmol); △, [2-^{14}C]cytosine (61 mCi/mmol); ●, [*methyl*-^{14}C]thymine, (54 mCi/mmol). (*B*) ●, [5,6-^{3}H]Uridine (50 mCi/mmol); ▲, [5-^{3}H]cytidine (40 mCi/mmol); ■, [*methyl*-^{3}H]thymidine (47 mCi/mmol).

other substrates. Uracil and uridine compete with each other (Fig. 2 *A* and *B*) and also with cytidine (Fig. 2*C*). Cytidine, however, has little effect on the incorporation of uracil or uridine (Fig. 2 *A* and *B*). Thymidine incorporation is not affected by any other pyrimidines or pyrimidine nucleosides (Fig. 2*D*).

The incorporation of thymidine, uracil, and uridine into *T. foetus* nucleic acids was examined. The results indicate exclusive incorporation of thymidine into the DNA fraction and of uracil and uridine into the RNA fraction (Table 1).

HPLC Analysis of Nucleotide Pools. Radiolabeled pyrimidines and pyrimidine nucleosides were used to label *T. foetus* and then chased with unlabeled substrates. The distribution of radioactivity in the nucleotide pools was analyzed in HPLC. The data indicate the following. (*i*) Thymidine is converted to TMP, TDP, and TTP. Incorporated radioactivities are rapidly chased off by unlabeled thymidine (Fig. 3). (*ii*) Uracil and uridine have a similar pattern of incorporation; they are mainly converted to UMP, UDP, UTP, and UDP-hexose. Prolonged chasing reduces the amount of radioactive UMP and increases that of radioactive CTP and CDP (Fig. 4). (*iii*) Cytidine is incorporated partly into CMP, CDP, and CTP and partly into UMP, UDP, UTP, and UDP-hexose. A substantial portion of the label can be chased from the CMP, CDP, CTP, and UMP in 30 min (Fig. 5).

Enzyme Profiles. Enzyme activities found in freshly prepared soluble and pellet fractions of *T. foetus* extract are summarized in Table 2. There is no dihydrofolate reductase, thymidylate synthetase, or thymidine kinase detectable in *T. foetus*. A thymidine phosphotransferase activity was found in the fraction pelleting at 100,000 × *g*. The product of this enzyme reaction has been identified as TMP by HPLC analysis (data not shown). *p*-Nitrophenyl phosphate, AMP, GMP, UMP, and CMP are all equally effective phosphate donors when tested at 10 mM whereas sugar phosphates—e.g., fructose 1,6-diphosphate—are inactive. Kinetic studies on the crude enzyme preparation indicated a K_m of 10 mM for thymidine. Uridine and cytidine had no effect on the enzyme activity, but guanosine was a noncompetitive inhibitor with an estimated K_i of 1 mM.

Uracil phosphoribosyltransferase is a major enzyme in the supernatant fraction of *T. foetus* extract. There is also a uridine phosphotransferase in the pellet fraction, but the uridine phosphorylase present may convert most of the uridine to uracil first. The cytidine phosphotransferase detected in *T. foetus* pellets may be responsible for converting some cytidine directly to CMP. But the presence of cytidine deaminase and uridine phosphorylase in *T. foetus* suggests also conversion of cytidine to uridine and then to uracil before incorporation into nucleotide.

Drug Testing. *T. foetus* cells were cultivated *in vitro* in the presence of various concentrations of antifolates and pyrimidine derivatives. Cell densities monitored after 24 hr of in-

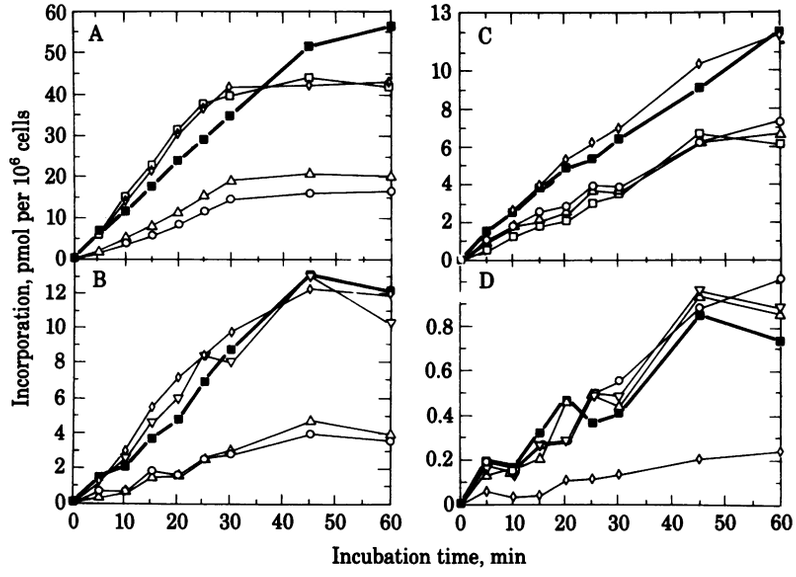

FIG. 2. Incorporation of radiolabeled uracil (*A*), uridine (*B*), cytidine (*C*), and thymidine (*D*) into *T. foetus* nucleotides in the presence of a 10-fold excess of unlabeled substrate. ■, No unlabeled substrate; ○, unlabeled uracil; △, unlabeled uridine; ◇, unlabeled cytidine; □, unlabeled thymidine.

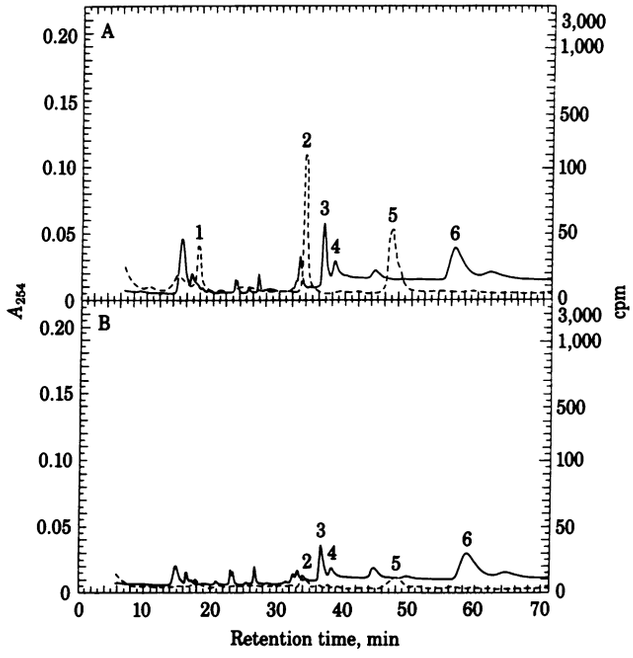

FIG. 3. HPLC analysis of *T. foetus* nucleotides labeled for 30 min with 20 μM [*methyl*-^{3}H]thymidine (500 mCi/mmol) (*A*) and labeled for 60 min and then chased for 60 min with 20 μM unlabeled thymidine (*B*). ——, A_{254}; ---, radioactivity. Peaks: 1, TMP; 2, TDP; 3, ADP; 4, GDP; 5, TTP; 6, ATP.

cubation indicate no appreciable growth inhibition by 0.5 mM methotrexate, 0.25 mM pyrimethamine, 0.5 mM trimethoprim, 0.1 mM azomycin riboside, or 0.1 mM 5-bromovinyldeoxyuridine. 5-Fluorouracil has a moderate inhibitory effect with a IC_{50} of 25 μM. It is also a competitive inhibitor of *T. foetus* uracil phosphoribosyltransferase, having a IC_{50} value of 70 μM

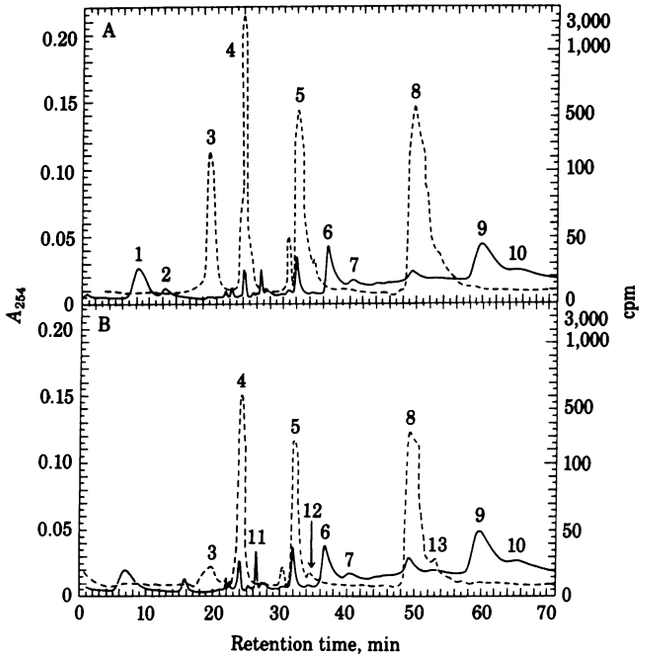

FIG. 4. HPLC analysis of *T. foetus* nucleotides labeled for 30 min with 20 μM [2-^{14}C]uracil (58 mCi/mmol) (*A*) and labeled for 30 min and then chased for 30 min with 20 μM unlabeled uracil. ——, A_{254}; ---, radioactivity. Peaks: 1, NAD; 2, AMP; 3, UMP; 4, UDP-hexose; 5, UDP; 6, ADP; 7, GDP; 8, UTP; 9, ATP; 10, GTP; 11, XMP; 12, CDP; 13, CTP.

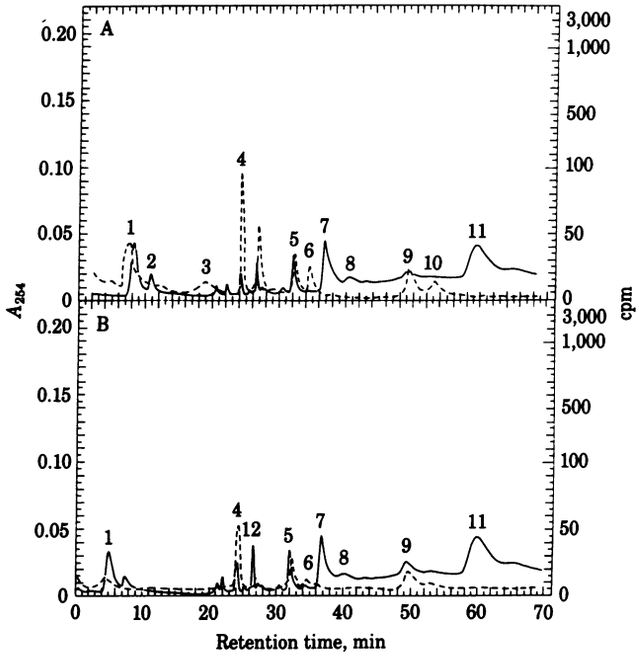

FIG. 5. HPLC analysis of *T. foetus* nucleotides labeled for 30 min with 20 μM [5-^{3}H]cytidine (500 mCi/mmol) (*A*) and labeled for 30 min and then chased for 30 min with 20 μM unlabeled cytidine. ——, A_{254}; ---, radioactivity. Peaks: 1, CMP; 2, AMP; 3, UMP; 4, UMP-hexose; 5, UDP; 6, CDP; 7, ADP; 8, GDP; 9, UTP; 10, CTP; 11, ATP; 12, XMP.

against 20 μM uracil. 5-Fluorouracil (0.5 mM) had no effect on thymidine incorporation into TMP, TDP, and TTP. The incorporation of uracil, uridine, or cytidine into nucleotides was, however, significantly inhibited by the drug. HPLC analysis showed that labeling of all the nucleotides by each of the three substrates was reduced. When 5-fluoro[6-^{3}H]uracil (32.8 mCi/mmol) was used to label cells and the products were analyzed by HPLC, radioactive peaks corresponding to 5-fluorouridine mono-, di-, and triphosphate were detected, suggesting that it may be eventually incorporated into *T. foetus* RNA.

DISCUSSION

Our studies indicate that bicarbonate, aspartate, and orotate cannot be incorporated into the nucleotide pool or the nucleic acids of *T. foetus*. Orotate phosphoribosyltransferase was absent in the extracts of *T. foetus*. Thus, *T. foetus* does not have the capability of *de novo* pyrimidine synthesis.

Incorporation of uracil into UMP by the action of uracil phosphoribosyltransferase is apparently the main source of pyrimidine nucleotides for *T. foetus*. The similar profiles of uracil and uridine incorporation, the competition between the two substrates, and the presence of a uridine phosphorylase suggest a predominant conversion of uridine to uracil before incorporation. The HPLC profile of cytidine incorporation is similar to those of uracil and uridine but differs in also suggesting significant amounts of labeled CMP, CDP, and CTP. Thus, the deamination of cytidine and further conversion to uracil may be one pathway of cytidine incorporation (supported by inhibition of uracil or uridine on cytidine incorporation and the presence of cytidine deaminase), but there must be another pathway in which cytidine is directly converted to CMP by cytidine phosphotransferase. The relative contributions of the two pathways are not known, but Fig. 5 suggests that the first pathway may prevail.

The lack of incorporation of radioactive uracil and uridine

into *T. foetus* DNA suggests that TMP cannot be formed from them. This suggestion is supported by the lack of dihydrofolate reductase and thymidylate synthetase in *T. foetus* extracts and the insensitivity of *T. foetus* toward millimolar concentrations of antifolates and thymidylate synthetase inhibitors. The only apparent pathway for supplying *T. foetus* with TMP is by the action of thymidine phosphotransferase. The enzyme, using *p*-nitrophenyl phosphate as well as nucleoside 5′-monophosphate as a phosphate donor to thymidine, has a specific activity of 0.144 ± 0.022 (nmol/min)/mg of protein, which can more than account for the rate of thymidine incorporation into the *T. foetus* nucleotide pool (estimated at 0.02 pmol/min per 10^6 cells; see Fig. 1); the fraction pelleting at 100,000 × *g* contains ≈2 μg of protein per 10^6 *T. foetus* cells. The same pellet fraction also contains guanosine phosphotransferase (17). In view of the inhibition of thymidine phosphotransferase by guanosine, it is not impossible that GMP may be the natural phosphate donor and that the conversion of thymidine to TMP may be dictated by the *T. foetus* cellular content of GMP. The scheme of pyrimidine salvage networks in *T. foetus* is summarized in Fig. 6.

This simple pyrimidine metabolism in *T. foetus* has made this parasite one of the most unusual living organisms—not only does it lack *de novo* pyrimidine synthesis, it also lacks the ubiquitous enzymes dihydrofolate reductase, thymidylate synthetase, and thymidine kinase. Other wild-type organisms are not known to exhibit these deficiencies. The dependence on thymidine phosphotransferase for thymidine salvage also finds no parallel in other organisms. As mentioned above, there are indications that *de novo* pyrimidine synthesis may also be absent in *Trichomonas vaginalis* (11) and *G. lamblia* (12). We have also observed that extracts of these two parasites lack dihydrofolate reductase and thymidylate synthetase; both parasites grow well in the presence of millimolar concentrations of antifolates such as methotrexate (unpublished data). This family of anaerobic flagellates thus may share the same metabolic deficiencies.

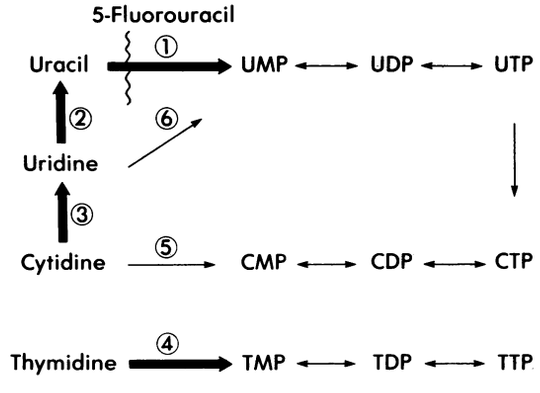

FIG. 6. Pyrimidine salvage pathways in *T. foetus*. 1, Uracil phosphoribosyltransferase; 2, uridine phosphorylase; 3, cytidine deaminase; 4, thymidine phosphotransferase; 5, cytidine phosphotransferase; 6, uridine phosphotransferase.

From a pharmacological point of view, it is easy to see how inhibition of thymidine phosphotransferase or uracil phosphoribosyltransferase may lead to inhibition of *T. foetus* growth (Fig. 6). 5-Fluorouracil is a moderately competitive inhibitor of uracil phosphoribosyltransferase and also a moderate inhibitor of *T. foetus in vitro* growth. Although the drug may eventually be incorporated into *T. foetus* RNA to slow the growth of the organism (because it is converted to 5-fluorouridine triphosphate in *T. foetus*), its inhibition of incorporation of uracil, uridine, and cytidine also emphasizes the importance of a functioning uracil phosphoribosyltransferase for *T. foetus*. This enzyme may have unique properties because it has no orotate phosphoribosyltransferase activity and has not been found in most mammalian tissues (18).

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