



## International Journal of Current Research and Academic Review

ISSN: 2347-3215 Volume 4 Number 4 (April-2016) pp. 185-192

Journal home page: <http://www.ijcrar.com>

doi: <http://dx.doi.org/10.20546/ijcrar.2016.404.023>



### Enzymes of the De novo Pyrimidine Biosynthetic Pathway in *Leishmania tropica*

Husain F. Hassan\*

Department of Biology, College of Science, University of Kirkuk, Iraq

\*Corresponding author

#### KEYWORDS

*Leishmania*,  
de novo,  
pyrimidine,  
enzymes.

#### A B S T R A C T

The pathway of *de novo* pyrimidine biosynthesis in the amastigote and Promastigote forms of *Leishmania tropica* has been investigated. Both parasite forms contained all six enzyme activities. Carbamoyl phosphate synthase (CPSase) utilizes L- glutamine and not ammonia as the amine group donor. Dihydroorotate dehydrogenase (DHO-DHase) was found to be cytoplasmic, where as orotate phosphoribosyltransferase (OPRTase) and orotidylate decarboxylase (ODCase) were found to be particulate. N-(phosphonacetyl)-L-aspartate, dihydro-5azaorotate, 5-azaorotate and 6-aza-UMP were found to be a potent inhibitor of the aspartate transcarbamoylase (ATCase), DHO-DHase, OPRTase, and ODCase, respectively.

#### Introduction

There are two mechanisms by which an organism can produce pyrimidine nucleotides which are primarily required for nucleic acid synthesis. These are, the *de novo* biosynthesis from non-pyrimidine precursors (Fig.1) and salvage synthesis utilizing preformed exogenous pyrimidines. Much of the evidence to suggest the presence of the *de novo* pyrimidine biosynthesis in parasites has come mainly from studies of the composition of minimal defined media for the growth of organism (Kar *et al.*, 1990, Kar, 1997) and the incorporation of radioactive biocarbonate and orotate into nucleic acid pyrimidine rings (3-5). Preliminary investigations have

demonstrated the existence of a number of *de novo* enzymes in *Leishmania mexicana amazonensis*, *L. m. mexicana* and *L. major* (Hassan and Coombs, 1988; Berens *et al.*, 1995; Carter *et al.*, 2003; Hill *et al.*, 1981; Hammond and Gutteridge, 1982; French *et al.*, 2011; Wilson *et al.*, 2012), *vis*, orotate phosphoribosyltransferase and orotidylate decarboxylase. I report here the presence of the activities of the six enzymes of the pyrimidine *de novo* biosynthetic pathway in *L. tropica*. The results indicate that these parasites have the potential for obtaining their pyrimidine requirements by *de novo* synthesis in a manner analogous to the closely related trypanosomal parasite.

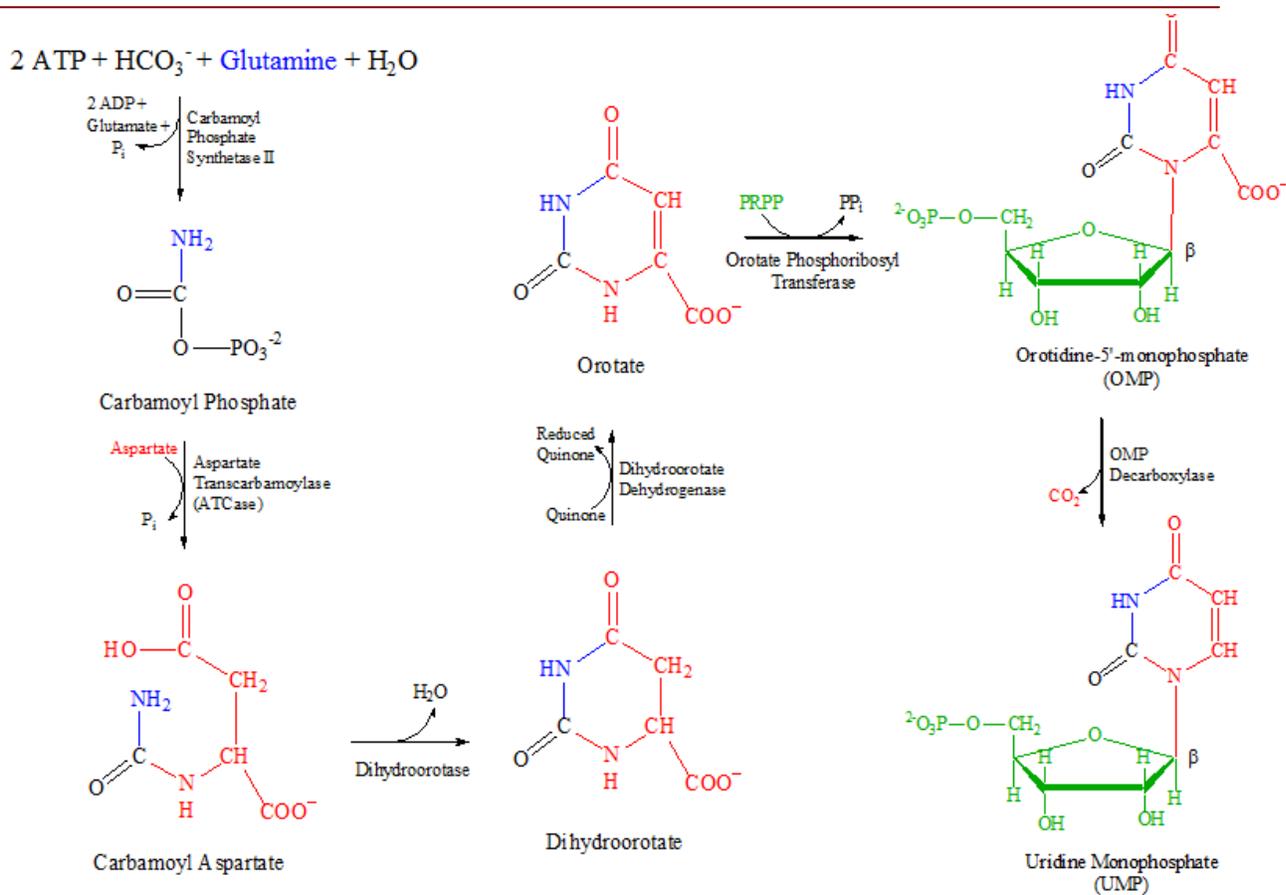


Fig. 1 Denovo pathway of uridine monophosphate

## Material and Methods

### Isolation, cultivation and fractionation of cells

*Leishmania tropica* amastigotes were isolated from lesion of NIH mice by a method involving Saponin Lysis of host cells, anion exchange chromatography and isopycnic centrifugation, which will be detailed in (10). Promastigote of *L. tropica* were grown in vitro in HOMEM medium, with 10 % (v/v) heat inactivated fetal calf serum at 26 C°. Crude homogenates of cells in 0.25 M sucrose were obtained by 3 cycles of freezing (-180 C°) and thawing (30Co) and fractionated by centrifugation at 100000 Xg for 1 hr to yield particulate and supernatant fractions.

### Enzymes assays

Assays for carbamoylphosphate synthase (CPSase, EC 2.7.2.5), aspartate transcarbamoylase (ATCase, EC 2.1.3.2), dihydroorotase (DHOase, EC 3.5.2.3), dihydroorotate dehydrogenase (DHO-DHase, EC 1.3.3.1), orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) and orotate decarboxylase (ODCase, EC 4.1.1.23) were based on published methods. Enzymes activities for these enzymes were measured at 26 C° and expressed as nmol product/min/mg protein. Protein concentrations were measured by the Lowery method (Lowery *et al.*, 1951) using bovine serum albumin as standard. The effect of pH (range 6.0 - 10.0) on reaction

velocity was determined in 20 mM Tris-maleate-glycine buffer.

### Results and Discussion

The activities of the six enzymes of pyrimidine biosynthesis *de novo*, CPSase, ATCase, DHOase, DHO-DHase, OPRTase and ODCase, were demonstrated in both promastigote and amastigote of *L.tropica* homogenates (Table 1). The CPSase activity measured was L-glutamine dependent. The addition of ammonium ions (15 mM) did not stimulate the enzyme activity. Thus it seems that *L.tropica* contains only CPSase (II)-glutamine dependent and totally lacks CPSase (I) ammonia dependent activity. The results indicated that the UTP phosphoribosyl pyrophosphate (PRPP) stimulate the activity by 15 %.

High activities of aspartate transcarbamoylase (ATCase) and dihydro-orotase (DHOase), the second and the third enzymes of the pyrimidine biosynthetic pathway, were detected in both promastigote and amastigote of *L.tropica* (Table 1). This pattern of low CPSase and high ATCase is typical of a number of organisms. Apparent Michaelis – Menten constant (Km) values for both aspartate and carbamoylphosphate were determined for ATCase (Table 2). Substrate inhibition at concentrations above 10 mM was observed for aspartate. This has

been demonstrated for ATCase from other systems (Inagaki and Tatibana, 1970). N-phosphonacetyl-L-aspartate (PALA), was shown to be competitive inhibitor of promastigote and amastigote ATCase with Ki of 6 µM and 9 µM, respectively (Table 2).

On the other hand, the Km value for dihydroorotate (L-DHO) was 0.76 mM and 1.1 mM for promastigote and amastigote DHOase respectively (Table 2). The activity of DHOase of both parasite forms did not inhibited by O-phenanthroline (1 mM) and L-cysteine (1 mM).

The activity of dihydroorotate dehydrogenase (DHO-DHase) of promastigotes was shown to be five times higher than that of amastigotes (Table 1). The Km values for L-DHO were determined as 8.2 µM and 16.1 µM for promastigotes and amastigotes DHO-DHase, respectively (Table 2). The activity of DHO-DHase was inhibited by dihydro-5-azaorotate, an analog of L-DHO, with Ki values of 9.6 µM and 18.1 µM for promastigotes and amastigotes enzyme respectively (Table 2). DHO-DHase activity in the both parasite forms was not inhibited by cyanide (5 µM) antimycin A (0.1 mg/ml) or amytal (1mM) indicating that the reaction was not coupled to the conventional respiratory chain.

**Table.1** Specific activities of the enzymes of the pyrimidine biosynthetic pathway in *Leishmania tropica*

Enzyme	Promastigote		Amastigote	
	pH optimum	Specific activity <sup>a</sup>	pH optimum	Specific activity <sup>a</sup>
CPSase II	7.2	4.6 ± 0.4	7.2	4.1 ± 0.5
ATCase	8.4	112 ± 11	8.0	98 ± 13
DHOase	6.4	27 ± 1	6-6.5	23 ± 2
DHO-DHase	7.0	0.98 ± 0.1	7.0	0.21 ± 0.03
OPRTase	7.5	0.14 ± 0.04	7.5	0.09 ± 0.01
ODCase	8.0	1.96 ± 0.1	8.2	0.82 ± 0.1

a = nmol/min/mg protein; The figures represent mean ± SD of three determinations

**Table.2** Summary of kinetic data and inhibition constants for the *L.tropica* pyrimidine biosynthetic enzymes

Enzyme	Promastigote		Amastigote	
	K <sub>m</sub>	K <sub>i</sub>	K <sub>m</sub>	K <sub>i</sub>
<b>CPSase II</b>				
Glutamine	18 μM	-	24 μM	-
UTP	-	720 μM	-	910 μM
<b>ATCase</b>				
Aspartate	2.1 mM	-	2.8 mM	-
Carbamoylphosphate	11 μM	-	13 μM	-
PALA	-	6 μM	-	9 μM
<b>DHOase</b>				
L-DHO	0.76 mM	-	1.1 mM	-
<b>DHO-DHase</b>				
L-DHO	8.2 μM	-	16.1 μM	-
Dihydro-5-azaorotate	-	9.6 μM	-	18.1 μM
<b>OPRTase</b>				
Orotate	5.4 μM	-	4.2 μM	-
5-Azaorotate	-	6.8 μM	-	5.2 μM
<b>ODCase</b>				
OMP	0.92	-	0.71	-
UMP	-	146 μM	-	124 μM
XMP	-	115 μM	-	91 μM
6-Aza-UMP	-	0.46	-	0.37 μM

Apparent K<sub>m</sub> values and inhibition constants (K<sub>i</sub>) for the most effective inhibitors tested, were determined from weighted regression procedures (19).

**Table.3** Distribution of enzyme activities in *Leishmania tropica* between particulate and soluble fractions of cell extracts<sup>a</sup>

Enzyme	Promastigote		Amastigote	
	P	S	P	S
<b>CPSase II</b>	0.39	2.8	0.26	2.3
<b>ATCase</b>	0.47	4.2	0.41	3.8
<b>DHOase</b>	0.3	4.9	0.07	1.8
<b>DHO-DHase</b>	0.5	4.1	0.15	2.1
<b>OPRTase</b>	1.6	0.05	1.2	0.04
<b>ODCase</b>	1.6	0.05	1.4	0.06

a = Crude homogenates were prepared as described in materials and methods, were centrifuged at 100000 Xg for 60 min at 4 C°. The pellet was resuspended in a volume of preparation buffer equal to the supernatant recovered. The fractions produced were assayed for pyrimidine biosynthetic enzymes. The results are expressed as relative specific activities (% total activity / % total protein) in the particulate (P) and the soluble (S) fractions.

The two enzymes that comprise the latter part of the pyrimidine biosynthetic pathway were found to be present in the crude homogenate of both parasite forms. The activity of orotidylate decarboxylase (ODCase) was significantly higher than the orotate phosphoribosyltransferase (OPRTase). The Km values for orotic acid was determined to be 5.4  $\mu$ M and 4.2  $\mu$ M for promastigotes and amastigotes OPRTase, respectively. The substrate analog, 5-azaorotic acid, was demonstrated to be a competitive inhibitor with respect to orotic acid with Ki of 6.8  $\mu$ M and 5.2  $\mu$ M for promastigotes and amastigotes enzymes, respectively. On the other hand, the Km for orotidylic acid (OMP) was determined to be 0.92  $\mu$ M and 0.71  $\mu$ M for promastigotes and amastigotes ODCase respectively. As detailed in Table 2, the product of the reaction UMP was a weak inhibitor, 6-aza-UMP a good inhibitor, and the purine nucleotide XMP, only a moderate inhibitor of the ODCase reaction in both parasite forms. It is of interest to note that the allopurinol and oxipurinol which inhibit human OPRTase and ODCase, did not have any marked inhibitory effect on the terminal two enzymes in the pathway from *L.tropica*. The distribution of the pyrimidine biosynthetic enzymes in the various cell fractions are shown in (Table 3). CPSase I, ATCase, DHOase and DHO-DHase were found to be predominantly in the supernatant fraction indicating the cytosolic nature of the enzymes. In contrast, the OPRTase and ODCase activity in *L.tropica* promastigotes and amastigotes were shown to be associated with the 100000 xg pellet, indicating the particulate nature of the enzymes.

For the first time all the enzyme activities necessary for the *de novo* biosynthesis of UMP have been detected in crude homogenates of promastigote and

amastigote forms of *L.tropica*, thus providing evidence that both parasite forms depends primarily on the *de novo* pathway as a source of pyrimidine. Notably CPSase II activity was observed. Carbamoyl phosphate synthase of *L. tropica* shares the properties of the synthase II of higher animals (Wahl *et al.*, 1979) in that (a) it utilizes L-glutamine as source of ammonia; (b) it does not require N-acetyl-L-glutamate; (c) it is localized in the cytosol fraction; (d) it is subject to feedback inhibition by pyrimidine nucleotides; (e) it is stimulated by PRPP.

As reported for all other sources, the highest specific activity was observed for ATCase. Its kinetic behavior appears to be similar to the enzyme from other systems (Ali *et al.*, 2013). However, the inhibition by PALA is consistent with the idea that the measured activity was ATCase and not an artifact.

In common with other known pyrimidine biosynthetic pathways, CPSase II, ATCase and DHOase are soluble enzymes. In contrast to other cells, OPRTase and ODCase are particulate enzymes, not only in *L.tropica* (Table 3) but also in *Trypanosoma cruzi* in agreement with previous studies (Shi *et al.*, 1999). Location of DHO-DHase in *L.tropica* is also unusual in that it is cytosolic. A soluble DHO-DHase has also been reported in other kinetoplastida and requires oxygen for activity but not sensitive to inhibition by cyanide or antimycin A. In contrast, in mammalian cells, DHO-DHase has been found to be membrane bound, mitochondrial and intimately connected to the respiratory chain to which it passes electron directly probably at the ubiquinone level (Ritt *et al.*, 2013). The qualitative differences found between the last three enzymes of the pyrimidine *de novo* biosynthetic pathway in *L.tropica* and mammalian cells may make this area of

metabolism a potentially useful target at which to aim in the search for much needed new antileishmanial drugs (Hassan, 2002).

## References

- Ali, J.A., Tagoe, D.N., Munday, J.C., Donachie, A., Morrison, L.J., de Koning, H.P. 2013. Pyrimidine biosynthesis is not an essential function for *Trypanosoma brucei* bloodstream forms. *PLoS One*, 8: e58034.
- Ali, J.A., Tagoe, D.N., Munday, J.C., Donachie, A., Morrison, L.J., *et al.* 2013. Pyrimidine biosynthesis is not an essential function for *Trypanosoma brucei* bloodstream forms. *PLoS One*, 8: e58034.
- Arakaki, T.L., Buckner, F.S., Gillespie, J.R., Malmquist, N.A., Phillips, M.A., Kalyuzhniy, O., Luft, J.R., Detitta, G.T., Verlinde, C.L., Van Voorhis, W.C., Hol, W.G., Merritt, E.A. 2008. Characterization of *Trypanosoma brucei* dihydroorotate dehydrogenase as a possible drug target. Structural, kinetic and RNAi studies. *Mol. Microbiol.*, 68: 37–50.
- Berens, R.L., Krug, E.C., Marr, J.J. 1995. Purine and pyrimidine metabolism. In *Biochemistry of Parasitic Organisms and its Molecular Foundations* (Marr, J.J. and Muller, M.eds) PP. 89-117. Academic Press.
- Carter, N., Rager, N., Ullman, B. 2003. Purine and pyrimidine transport and metabolism, in *Molecular and Medical Parasitology* (Marr, J. J., and R. Komuniecki, eds) pp. 197–223, Academic Press Limited, London.
- Fox, B.A., Bzik, D.J. 2002. De novo pyrimidine biosynthesis is required for virulence of *Toxoplasma gondii*. *Nature*, 415: 926–929.
- French, J.B., Yates, P.A., Soysa, D.R., Boitz, J.M., Carter, N.S., Chang, B., Ullman, B., Ealick, S.E. 2011. The *Leishmania donovani* UMP synthase is essential for promastigote viability and has an unusual tetrameric structure that exhibits substrate-controlled oligomerization. *J. Biol. Chem.*, 286: 20930–20941.
- Hammond, D.J., Gutteridge, W.E. 1982. UMP synthesis in the kinetoplastida. *Biochem. et Biophys. Acta*, 718: 1-10.
- Hashimoto, M., Morales, J., Fukai, Y., Suzuki, S., Takamiya, S., Tsubouchi, A., Inoue, S., Inoue, M., Kita, K., Harada, S., Tanaka, A., Aoki, T., Nara, T. 2012. Critical importance of the de novo pyrimidine biosynthesis pathway for *Trypanosoma cruzi* growth in the mammalian host cell cytoplasm. *Biochem. Biophys. Res. Commun.*, 417: 1002–1006.
- Hassan, H.F. 2002. Effect of some purine analog on growth and metabolism of *Leishmania major* promastigotes. The 2nd. *Int Con. Biol. Sci., Egypt*, PP. 51.
- Hassan, H.F., Coombs, G.H. 1985. *Leishmania mexicana*: Purine metabolizing enzymes of amastigotes and promastigotes. *Exp. Parasitol.*, 59: 139-150.
- Hassan, H.F., Coombs, G.H. 1986. A comparative study of the purine and pyrimidine metabolizing enzymes of a range of trypanosomatid. *Comp. Biochem. Physiol.*, 84 B: 217-223.
- Hassan, H.F., Coombs, G.H. 1988. Purine and pyrimidine metabolism in parasitic protozoa. *FEMS Microb. Rev.*, 54: 47-84.
- Hill, J.K., Rogerson, G.W., McIntosh, R.T., Ginger, C.D. 1981. The enzymes of pyrimidine biosynthesis in a range of parasitic protozoa and helminthes. *Mol. Biochem. Parasit.*, 2: 123-134.

- Inagaki, A., Tatibana, M. 1970. Control of pyrimidine biosynthesis in mammalian tissues, *Biochem. et Biophys. Acta*, 220: 491-502.
- Kar, K. 1997. Folic acid the essential supplement to brain heart infusion broth for cultivation and cloning of *Leishmania donovani* promastigotes. *Parasit.*, 115: 231-235.
- Kar, K., Mukerji, K., Nasker, K., Bhattacharya, A., Ghosh, D. 1990. *Leishmania donovani* : a chemically defined medium for cultivation and cloning of promastigotes and transformation of amastigotes to promastigotes. *J. Protozool.*, 37: 277-279.
- Lowery, O.M., Rosenbrough, N., Farr, A.C., Randall, R.J. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Ritt, J.F., Raymond, F., Leprohon, P., Legare, D., Corbeil, J., Ouellette M. 2013. Gene amplification and point mutations in pyrimidine metabolic genes in 5-fluorouracil resistant *Leishmania infantum*. *PLoS Negl. Trop. Dis.*, 7(11): 1-11.
- Shi, W., Schramm, V.L., Almo, S.C. 1999. Nucleoside hydrolase from *Leishmania major*. Cloning, expression, catalytic properties, transition state inhibitors, and the 2.5-Å crystal structure. *J. Biol. Chem.*, 274: 21114–21120.
- Soysa, R., Wilson, Z.N., Elferich, J., Forquer, I., Shinde, U., Riscoe, M.K., Yates, P.A., Ullman, B. 2013. Substrate inhibition of uracil phosphoribosyltransferase by uracil can account for the uracil growth sensitivity of *Leishmania donovani* pyrimidine auxotrophs. *J. Biol. Chem.*, 288: 29954-29964.
- Wahl, G.M., Padgett, R.A., Stark, G.R. 1979. Gene amplification causes overproduction of the first three enzymes of UMP synthesis in N-(phosphonacetyl)-L-aspartate-resistant hamster cells. *J. Biol. Chem.*, 254: 8679–8689.
- Wilson, Z.N., Gilroy, C.A., Boitz, J.M., Ullman, B., Yates, P.A. 2012. Genetic dissection of pyrimidine biosynthesis and salvage in *Leishmania donovani*. *J. Biol. Chem.*, 287: 12759–12770.

**How to cite this article:**

Husain F. Hassan. 2016. Enzymes of the De novo Pyrimidine Biosynthetic Pathway in *Leishmania tropica*. *Int.J.Curr.Res.Aca.Rev.*4(4): 185-192.  
doi: <http://dx.doi.org/10.20546/ijcrar.2016.404.023>