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PURINE AND PYRIMIDINE SALVAGE PATHWAYS IN *LEISHMANIA DONOVANI*

STEPHEN W. LAFON* and DONALD J. NELSON
Wellcome Research Laboratories, Burroughs Wellcome Co.,
Research Triangle Park, NC 27709, U.S.A.
and
RANDOLPH L. BERENS and J. JOSEPH MARR
Department of Medicine and Microbiology, St. Louis University, St. Louis, MO 63104, U.S.A.

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Abstract—*Leishmania donovani*, grown in culture, salvaged radiolabeled purine bases which were distributed into adenine and guanine ribonucleotides and into the RNA of these cells. *De novo* synthesis of purines in *L. donovani* does not occur [J. J. Marr, R. L. Berens and D. J. Nelson, *Biochim. biophys. Acta* **544**, 360 (1978)]. [8-^{14}C]Adenine was rapidly deaminated to hypoxanthine via the action of an adenine aminohydrolase (EC 3.5.4.2). [8-^{14}C]Guanine was also rapidly deaminated by guanase (EC 3.5.4.3) to form xanthine in these cells. Therefore, the formation of nucleotides of hypoxanthine and xanthine are the first committed steps of purine salvage in *L. donovani*. While purines are efficiently conserved by this parasite, the salvage of pyrimidines is not so dramatic. [2-^{14}C]Orotic acid was converted to OMP and then incorporated into the pyrimidine nucleotides and into RNA, indicating the existence of the later steps of *de novo* pyrimidine synthesis. [6-^{14}C]Thymidine was salvaged by *L. donovani*, being incorporated into the thymine deoxyribonucleotides and into DNA. The major pathway of thymidine metabolism in this parasite, however, was cleavage of the deoxyriboside linkage to form thymine, probably via the action of a thymidine phosphorylase (EC 2.4.2.4).

Previous work in our laboratory has demonstrated the lack of *de novo* purine synthesis in *Leishmania donovani* and *L. braziliensis* and the existence of a purine salvage pathway similar to that of other eukaryotic organisms [1]. These studies indicated the presence of an active adenine aminohydrolase (EC 3.5.4.2) as well as a guanine deaminase (EC 3.5.4.3). The adenylosuccinate synthetase (EC 6.3.4.4) of this organism differs from that of man in its ability to convert the inosinic acid analogue, allopurinol ribonucleoside monophosphate (HPP-Rib-5′-P)† to 4-aminopyrazolopyrimidine ribonucleoside monophosphate (APP-Rib-5′-P) [2–4]. Three different purine phosphoribosyltransferases have been isolated in *L. donovani*; these have specificity for hypoxanthine-guanine (EC 2.4.2.8), adenine (EC 2.4.2.7) and xanthine (EC 2.4.2.22) [5].

Little is known about the pathways of pyrimidine metabolism in *L. donovani*. A study of the pyrimidine enzymes in *L. mexicana mexicana* has revealed the existence of both an orotate phosphoribosyltransferase (EC 2.3.2.10) and an orotidine-5′-monophosphate decarboxylase (EC 4.1.1.23) [6].

In this paper, the interconversion of purines and pyrimidines in *L. donovani* has been investigated. The data have been presented in terms of the flux of metabolites through the different metabolic pathways as described by Refs. 7 and 8.

METHODS

Materials. Carbon-14 radioisotopes were purchased from the New England Nuclear Corp., Boston, MA, except xanthine, which was purchased from ICN, Irvine, CA. Purine bases were labeled in the eight position, thymidine in the six position and orotic acid in the two position. Bases, ribonucleosides and ribonucleotides used as standards were purchased from P-L Biochemicals, Milwaukee, WI, and culture media materials from GIBCO, Grand Island, NY.

Purine and pyrimidine metabolism studies. Leishmania donovani (Strain S1) was cultured as previously described by Marr and Berens [9]. Each incubation mixture consisted of 10 ml of medium and a cell density of 50×10^6 cells/ml. The radiolabeled precursor was added to a final concentration of 100 μM (1 μCi/ml, except xanthine with 0.5 μCi/ml) and the cells were incubated for 60 min. At the end of the incubation, the cells were chilled to 4° and the cell free incubation medium (CFM) and the acid soluble (AS) and acid insoluble (AIS) fractions were prepared as previously described [1].

Analysis of extracts from purine metabolism experiments. The AS fractions were analyzed for various purine and pyrimidine nucleotides as previously described using anion-exchange high performance liquid chromatography (h.p.l.c.) followed by

* Author to whom all correspondence should be addressed: Mr. Stephen W. LaFon, Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, NC 27709, U.S.A.

† Abbreviations: HPP-Rib-5′-P, allopurinol ribonucleoside 5′-monophosphate; APP-Rib-5′-P, 4-aminopyrazolopyrimidine 5′-ribonucleoside monophosphate; h.p.l.c., high performance liquid chromatography; AS, perchloric acid soluble fraction; AIS, perchloric acid insoluble fraction; CFM, cell free incubation media; and PRT, phosphoribosyltransferase.

analysis of the resulting fractions for ^{14}C-nucleotides by liquid scintillation counting [1].

The CFM was deproteinized by extraction in cold 0.5 N perchloric acid followed by neutralization with fresh 3 N KOH. The neutralized extracts were then analyzed for ^{14}C-labeled purine bases and nucleosides by paper chromatography as previously described [10].

The AIS fraction was analyzed to determine the pattern of incorporation of radiolabeled purine metabolites into RNA. The acid insoluble pellet was washed with cold 70% ethanol until radioactivity of the wash reached background levels. The washed pellet was dissolved by incubation in 5 ml of 0.3 N KOH overnight at ambient temperature. This step converted the RNA to a mixture of 2′ and 3′ ribonucleoside monophosphates while not affecting the DNA or protein. Concentrated perchloric acid (0.4 ml) was added to adjust the mixture to pH 1. The mixture was cooled in an ice bath and the $KClO_4$ as well as the DNA were sedimented by centrifugation. The supernatant fraction, which contained the RNA hydrolysate, was decanted and the radioactivity and 260 nm u.v. absorbance were measured. The remainder of the supernatant fraction (4.9 ml) was heated in a boiling water bath for 60 min to convert the purine nucleoside monophosphates to free bases. The supernatant fraction was neutralized with fresh 3 N KOH to pH 7.0–8.0, and the $KClO_4$ precipitate was removed by centrifugation. A 0.05-ml aliquot of the RNA hydrolysate was spotted on Whatman 3 mm paper along with 0.01 ml of a 10 mM solution of adenine and guanine as carriers. The chromatogram was developed overnight in a descending solvent system, 2-propanol–2 N HCl (65:35). The u.v.-absorbing spots corresponding to adenine and guanine were cut out and the radioactivity was measured in ACS counting solution (Amersham/Searle, Arlington Heights, IL) in a Beckman 230 liquid scintillation counter.

Analysis of extracts from pyrimidine metabolism experiments. The analysis of the AS and the AIS fractions of *L. donovani* incubated with [2-^{14}C]orotic acid was performed as described for the purine metabolism experiments. The CFM was analyzed by paper chromatography on Whatman No. 1 paper using the following as standards: orotidine, orotic acid, uracil, and uridine [11]. The ascending chromatogram was developed overnight with a solvent system of 2-butanol–water (86:14). After the chromatogram was allowed to air-dry, the u.v.-absorbing spots were cut out for radioactivity measurements.

Both the AS fraction and the CFM fraction obtained when [6-^{14}C]thymidine was used as precursor were analyzed using paper chromatography. A 0.05-ml aliquot of the sample was spotted on Whatman No. 1 paper. Also applied to the paper was 0.01 ml of a 10 mM solution of thymine, thymidine dTMP, dTDP, and dTTP. The ascending chromatogram was developed overnight with a solvent system of 2-butyric acid–water–concentrated NH_4OH (66:33:1). The u.v.-absorbing spots were cut out for radioactivity measurements. Nucleotide pool levels were measured as previously described [1]. The levels of thymidine deoxynucleotide pools were not measurable by this technique. The RNA was separated from the DNA in the AIS fraction as previously described for the purine metabolism experiments. The DNA and protein pellet was resuspended in 1 N KOH, and the $KClO_4$ was removed by centrifugation. An aliquot of the supernatant fraction was counted by liquid scintillation counting, and the concentration of the DNA in the sample was determined by the diphenylamine colorimetric assay for DNA [12].

Calculation of the metabolic flux of radiolabeled purine and pyrimidines in L. donovani. The flux of the radiolabeled metabolites through the various metabolic pathways was calculated according to the method dsecribed in Refs. 7 and 8. Briefly, the various apparent enzyme activities were calculated as described in Table 1. To determine the metabolic flux through a particular pathway, the following equation was used:

$$\text{Metabolic flux} = \frac{\text{Apparent activity of step considered}}{\text{Apparent activity of first committed step}} \times 100\%$$

For example, to determine the metabolic flux through adenylosuccinate synthetase/lyase (ASS) with [8-^{14}C]hypoxanthine as the precursor:

$$\text{Flux ASS} = \frac{\text{Activity ASS}}{\text{Activity HPRT}} \times 100 = \frac{151.1}{258.3} \times 100\% = 58.5\%$$

So, 58.5 per cent of the metabolized [8-^{14}C] hypoxanthine passes through the adenylosuccinate synthetase/lyase step during the incubation.

RESULTS

Distribution of radiolabeled purines in L. donovani. Table 2 shows the levels of the various metabolites of the radiolabeled purines in *L. donovani.* These metabolites were measured in the three different extraction fractions: the perchloric acid soluble supernatant fraction (AS), the acid insoluble pellet (AIP) and the cell free incubation media (CFM). Table 3 summarizes the distribution of these metabolites in the three fractions. When [8-^{14}C] adenine was incubated with *L. donovani*, only 0.9 per cent of the adenine remained after 1 hr. Purine bases and ribonucleosides made up 83 per cent of the total radiolabeled compounds (intra- and extracellular), with most of the radiolabeled precursor in the form of [8-^{14}C]hypoxanthine. Most of the [8-^{14}C]adenine metabolites in the cells (AS fraction) were in the form of nucleotides, with a small amount present as purine bases and nucleosides. Radiolabeled ATP and GTP were both incorporated into the RNA of *L. donovani* with 83 pmoles/10^6 cells of the original radiolabeled adenine incorporated into the RNA after 1 hr.

[8-^{14}C]Hypoxanthine metabolism was similar to that of [8-^{14}C]adenine; 18 per cent of the [8-^{14}C] hypoxantine was metabolized after the 1-hr incubation as compared to 19 per cent conversion of [8-^{14}C]adenine to metabolites other than hypoxanthine in 1 hr. The levels of labeled nucleotides in the

Table 1. Calculations of apparent enzymatic activities*

Enzymes	Activity Calculations†
Hypoxanthine metabolism	
Hypoxanthine phosphoribosyl-transferase	HPRT = Total − Hyp
IMP phosphatase	IMPD = Ino
Adenylosuccinate synthetase	ASS = Sum Ade
AMP phosphatase	AMPD = Ade + Ado
ADO phosphorylase	ADOP = Ade
AMP kinase	AMPK = Sum Ade − Ado − AMP − Ade
ADP kinase	ADPK = AMPK − ADP
IMP dehydrogenase	XIDH = Xan + Xao + XMP + Sum Gua
XMP aminase	XMPAM = Sum Gua
XMP phosphatase	XMPD = Xan + Xao
XAO phosphorylase	XAOP = Xan
GMP kinase	GMPK = GDP + GTP + RNA/Gua
GDP kinase	GDPK = GMPK − GDP
RNA polymerase	RPOL = RNA/Ade + RNA/Gua
RNA polymerase/adenine	RPOLA = RNA/Ade
RNA polymerase/guanine	RPOLG = RNA/Gua
Xanthine metabolism	
Xanthine phosphoribosyl-transferase	XPRT = Total − Xan
XMP phosphatase	XMPD = Xao
XMP aminase	XMPAM = XPRT − XMP
GMP phosphatase	GMPD = Gua + Guo
GUO phosphorylase	GUOP = Gua
GMP reductase	GMPRE = Sum Ade + Hyp + Ino + IMP
IMP phosphatase	IMPD = Ino + Hyp
INO phosphorylase	INOP = Hyp
Adenylosuccinate synthetase	ASS = Sum Ade
AMP phosphatase	AMPD = Ade + Ado
ADO phosphorylase	ADOP = Ade
AMP kinase	AMPK = Sum Ade − Ado − AMP − Ade
ADP kinase	ADPK = AMPK − ADP
GMP kinase	GMPK = GMP + GTP + RNA/Gua
GDP kinase	GDPK = GMPK − GDP
RNA polymerase	RPOL = RNA/Ade + RNA/Gua
RNA polymerase/adenine	RPOLA = RNA/Ade
RNA polymerase/guanine	RPOLG = RNA/Gua
Orotic acid metabolism	
Orotate phosphoribosyl-transferase	OPRT = Total − Oro
OMP decarboxylase	OMPD = OPRT–OMP
UMP kinase	UMPK = OMPD–UMP–Ura–Urd
UMP phosphatase	UMPD = Ura + Urd
URD phosphorylase	URDP = Ura
UDP kinase	UDPK = UMPK–UDP
RNA polymerase	RPOL = RNA
UDP-glucose pyrophosphorylase	UDPG = UDP-glucose
CTP synthetase	CTPS = CTP
Thymidine metabolism	
Thymidine kinase	Thym. Kin. = dTMP + dTDP + dTTP + DNA
dTMP kinase	dTMPK = Thym. Kin. − dTMP
dTDP kinase	dTDPK = dTTP + DNA
DNA polymerase	DPOL = DNA
Thymidine phosphorylase	Thym. Phos. = Thymine

* These apparent enzyme activities are defined as the sum of the products of the various enzymes listed. The apparent enzyme activities are calculated using the parameters described under Activity Calculations. These apparent enzyme activities were used to calculate the metabolic flux of the various radiolabeled metabolites through the biosynthetic pathways as described in Methods.

† Abbreviations: Hyp, hypoxanthine; Ino, inosine; Ade, adenine; Ado, adenosine; Xan, xanthine; Xao, xanthosine; Gua, guanine; Guo, guanosine; Oro, orotate; Ura, uracil; and Urd, uridine. Sum Ade or Sum Gua is defined as all of the adenine or guanine bases, nucleosides and nucleotides.

Table 2. Levels of radiolabeled purine metabolites in *Leishmania donovani* following a 1-hr incubation with 100 μM [8-^{14}C]purine precursors*

Metabolite	Purine precursor			
	[8-^{14}C]Adenine	[8-^{14}C]Hypoxanthine	[8-^{14}C]Guanine	[8-^{14}C]Xanthine
Adenine	13.1	3.6	0.1	3.5
Adenosine	6.0	5.8	0.0	13.5
AMP	19.5	12.8	2.1	7.8
ADP	33.6	27.2	5.6	19.5
ATP	59.0	52.0	12.1	33.1
Nuc–Ad	54.6	49.3	10.0	16.8
NAD + NADP	1.0	0.4	0.1	0.6
Hypoxanthine	1164.7	1173.7	67.1	136.7
Inosine	10.7	18.2	0.1	9.5
IMP	0.2	0.0	0.0	0.0
Guanine			5.4	7.2
Guanosine			0.1	16.1
GMP	6.7	5.4	1.2	10.8
GDP	5.9	3.8	5.1	15.4
GTP	6.3	5.3	13.8	29.8
Nuc–Gu	28.8	33.6	34.2	83.0
Xanthine	42.0	20.1	536.6	229.3
Xanthosine	4.5	19.7	0.4	3.1
XMP	1.7	1.1	1.8	0.0
Cell density ($\times 10^6$/ml)	64.0	66.0	110.0	110.0
Specific activity of precursor (mCi/mmole)	8.7	8.4	10	5

* Values represent the levels of the various radiolabeled metabolites of the respective [8-^{14}C]purine precursor measured from the AS, AIP and CFM fractions as described in Methods. These values are expressed in pmoles/10^6 cells. Nuc–Ad and Nuc–Gu represent the levels of radiolabeled adenine and guanine incorporated in the RNA of these cells.

cells were lower with [8-^{14}C]hypoxanthine than with [8-^{14}C]adenine. Despite this decreased incorporation into the nucleotides, the levels of radiolabeled RNA were equal for the [8-^{14}C]hypoxanthine experiment and the [8-^{14}C]adenine experiment.

[8-^{14}C]Guanine was almost completely metabolized to xanthine in 1 hr; xanthine accounted for 77 per cent of the total radioactivity recovered after that time. The remaining 23 per cent of the radioactivity was in the form of nucleotides, RNA or other bases and nucleosides (Table 3). [8-^{14}C]Guanine metabolites attained levels of 42 pmoles/10^6 cells in the cellular nucleotides and 44 pmoles/10^6 cells in the RNA, indicating a rapid incorporation of guanine into the RNA.

The metabolism of [8-^{14}C]xanthine was similar to that of [8-^{14}C]guanine although the levels of radiolabeled nucleotides and RNA were much higher in the xanthine experiment. Approximately 64 per cent of the [8-^{14}C]xanthine was metabolized in 1 hr as compared to 22 per cent for guanine. Xanthine was not converted to uric acid, indicating the lack of xanthine oxidase (EC 1.2.3.2). Both cellular nucleotides and RNA were labeled in 1 hr to approximately

Table 3. Distribution of ^{14}C-labeled purines and pyrimidines in *L. donovani**

Radiolabeled precursor	Percent metabolized	Percent of label in CFM	Percent of label in cells	Distribution of radiolabeled metabolites in cells (pmoles/10^6 cells)		
				Nucleotides	Bases and nucleosides	Acid insoluble material
Adenine	19	83	17	134	37	83
Hypoxanthine	18	86	14	108	17	83
Guanine	22	87	13	42	5	44
Xanthine	64	64	36	116	10	100
Orotic acid	1.2	99.7	0.3	1.6	1.3	0.7
Thymidine	26	98.7	1.3	1.5	7.3	0.8

* The distribution of radiolabeled precursors in the CFM and the cellular fractions is presented along with the percent of the precursor metabolized by the parasite. The distribution of the radiolabeled metabolites in the cells is also indicated. These values are expressed in pmoles/10^6 cells. All of the radioactivity added to the cell cultures was accounted for in the various fractions. The first column, indicating the percent of the precursors metabolized, represents those metabolites in the form of bases, nucleosides, nucleotides and nucleic acids. Since adenine is very rapidly converted to hypoxanthine and guanine to xanthine by *L. donovani*, the values for percent metabolized represent the total radioactivity minus the precursor and its immediate metabolite, i.e. total–Ad–Hx or total–Gu–Xa, for these precursors.

Table 4. Nucleotide pool sizes of *L. donovani* incubated with 100 μM ^{14}C-labeled purines*

Cellular nucleotides	Nucleotide pool (pmoles/10^6 cells)				
		Radiolabeled purine precursors			
	Control	[8-^{14}C]Adenine	[8-^{14}C]Hypoxanthine	[8-^{14}C]Guanine	[8-^{14}C]Xanthine
AMP + NAD	19.8	28.3 (0.69)	20.6 (0.62)	7.8 (0.27)	24.0 (0.32)
NADP	1.1	4.0 (0.24)	4.8 (0.07)	2.9 (0.04)	3.3 (0.16)
ADP	36.2	36.0 (0.93)	33.8 (0.80)	17.4 (0.33)	35.5 (0.54)
GDP	14.7	17.8 (0.33)	13.8 (0.28)	12.0 (0.43)	21.8 (0.70)
CTP	2.2	2.1	1.6	1.9	1.9
UTP	14.4	16.2	14.8	14.6	13.6
ATP	51.2	75.9 (0.78)	73.9 (0.70)	48.8 (0.25)	66.4 (0.49)
GTP	10.8	19.5 (0.32)	16.4 (0.32)	24.9 (0.56)	31.5 (0.93)
Final cell density ($\times$ 10^6 cells/ml)	7.3	64	66	110	110
Specific activity of precursor (μCi/μmole)		8.7	8.4	10	5

* The cold nucleotide pools were measured from the AS fraction of *L. donovani* after a 1-hr incubation with the various labeled purines. The numbers in parentheses represent the fraction of the nucleotide pool which was labeled by the precursor.

twice the level observed with [8-^{14}C]guanine.

Incorporation of radiolabeled purine metabolites into cellular nucleotide pools. All four of the radiolabeled purines studied were incorporated into the cellular nucleotide pools (Table 4). When *L. donovani* was incubated with either [8-^{14}C]adenine or [8-^{14}C]hypoxanthine, the purine nucleotides were extensively labeled. Large amounts of the ATP pool (78 per cent) and the GTP pool (32 per cent) were derived from [8-^{14}C]adenine, while [8-^{14}C] hypoxanthine constituted 70 per cent of the ATP pool and 32 per cent of the GTP pool. This slight increase in radiolabeled ATP with [8-^{14}C]adenine as the precursor was not reflected in the levels of radiolabeled metabolites in the RNA of these cells as compared to those treated with [8-^{14}C]hypoxanthine (Table 3). The purine mono- and diphosphates were also labeled by the two radiolabeled precursors. However, in all cases, [8-^{14}C]adenine was incorporated into the adenine nucleotide pools to a slightly greater extent than was [8-^{14}C]hypoxanthine. The guanine nucleotide pools were labeled to a similar

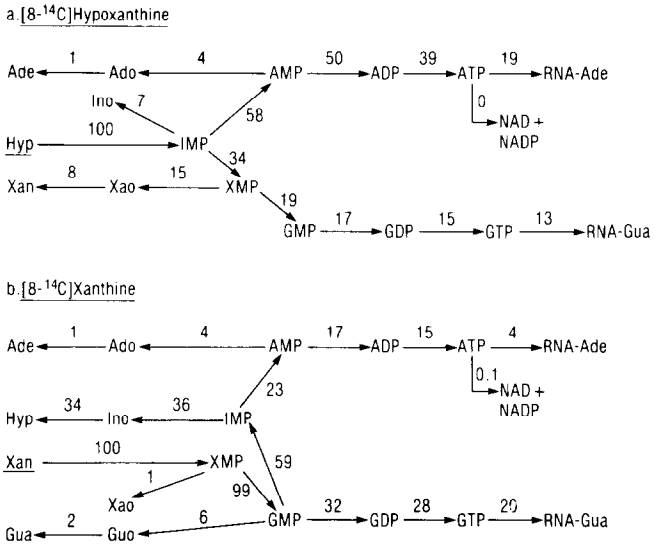

Fig. 1. Flux of radiolabeled purine metabolites through the purine salvage pathway of *L. donovani*. These figures represent the metabolic fate of both [8-^{14}C]hypoxanthine and [8-^{14}C]xanthine after a 1-hr incubation with *L. donovani*. The numbers represent the flow of metabolized radiolabeled precursors along the alternative pathways of purine metabolism. The amount of radiolabeled precursor which passes through the first committed step is set at 100; all other numbers were adjusted to this as described in Methods. Some established metabolic pathways were omitted for the purpose of clarity since they did not contribute significantly to the metabolism of these radiolabeled precursors.

Table 5. Levels of radiolabeled pyrimidine metabolites in *L. donovani* following a 1-hr incubation with 100 μM radiolabeled pyrimidine precursor*

[2-^{14}C]Orotic acid		[6-^{14}C]Thymidine	
Metabolite	Level (pmoles/10^6 cells)	Metabolite	Level (pmoles/10^6 cells)
Orotic acid + Orotidine	1078.6	Thymine	199.9
		Thymidine	564.7
OMP	0.0	dTMP	0.7
Uracil	1.38	dTDP	0.4
Uridine	9.70	dTTP	0.4
UMP	0.20	DNA	0.8
UDP	0.33		
UTP	0.59		
RNA	0.73		
UDP-glucose	0.47		
CTP	0.01		
Cell density ($\times 10^6$/ml)	92		120
Specific activity of precursor (mCi/mmole)	10		9.8

* Values represent the levels of the radiolabeled metabolites of orotic acid or thymidine found in the AS, AIP and CFM fractions. These values were used to calculate the metabolic flux of these metabolites through the pyrimidine biosynthetic pathways.

extent by both precursors. The addition of exogenous [8-^{14}C]adenine and [8-^{14}C]hypoxanthine at these levels elevated the cellular ATP and GTP pool size while not affecting the pyrimidine pool size.

When the cells were incubated with [8-^{14}C]guanine or [8-^{14}C]xanthine, the guanine nucleotides were labeled to a greater extent than the adenine nucleotides. Large portions of the ATP (25 per cent) and the GTP pool (56 per cent) were derived from [8-^{14}C]guanine, and 49 and 93 per cent of these pools, respectively, were derived from [8-^{14}C]xanthine. [8-^{14}C]Xanthine was incorporated into all of the purine nucleotides at a higher rate than was [8-^{14}C]guanine. This was also reflected in the RNA data (Table 3). Both [8-^{14}C]guanine and [8-^{14}C]xanthine elevated the GTP pool size while [8-^{14}C]xanthine alone elevated the ATP pool. The pyrimidine pool sizes were not affected.

Metabolic flux of radiolabeled purines in L. donovani. Figure 1 outlines the flux of [8-^{14}C] hypoxanthine and [8-^{14}C]xanthine metabolites through the purine salvage pathway of *L. donovani*. Since adenine was converted so rapidly to hypoxanthine and guanine to xanthine, the results of these two precursors are not shown.

Hypoxanthine was converted exclusively to IMP which, in turn, was rapidly metabolized. There was a preferential synthesis of adenine nucleotides over guanine nucleotides, with 58 per cent of the radiolabeled metabolites present in the cell as adenine nucleotide and 19 per cent as guanine nucleotides. Large amounts of the metabolized hypoxanthine (39 per cent) were converted to ATP while only 15 per cent was converted to GTP. Despite this, approximately equal amounts of the metabolized hypoxanthine were incorporated into RNA as ATP (19 per cent) and GTP (13 per cent), indicating a preferential incorporation of GTP into the RNA of these cells.

When [8-^{14}C]xanthine was the metabolic precursor, it was converted exclusively to XMP which, in turn, was converted primarily to GMP. XMP, like IMP, did not accumulate in these cells. GMP was converted to IMP with 59 per cent of the metabolized xanthine following this route, thus labeling the adenine and hypoxanthine nucleotides. Large amounts of labeled hypoxanthine were found in the medium after 1 hr, indicating the rapid degradation of IMP. The RNA of these cells was labeled by both ATP and GTP, with 20 per cent of the metabolized xanthine incorporated into RNA as GTP and 4 per cent as ATP.

Distribution of radiolabeled pyrimidines in L. donovani. Table 5 shows the levels of the various [2-^{14}C]orotic acid and [6-^{14}C]thymidine metabolites found in *L. donovani*. These metabolites were measured in the AS, AIP and CFM fractions of the cells following a 1-hr incubation with the precursors. Table 3 indicates the distribution of the precursors and their metabolites in these fractions.

L. donovani salvaged [2-^{14}C]orotic acid very poorly, with only 1.2 per cent of the available orotic acid metabolized after 1 hr. The radiolabeled orotic acid was, however, converted to pyrimidine nucleotides. Of the 13.3 pmoles/10^6 cells of orotic acid metabolized, 11 pmoles/10^6 cells was identified as uridine and uracil. Another 1.6 pmoles/10^6 cells was converted to acid soluble nucleotides and 0.7 pmoles/10^6 cell was incorporated into the RNA.

[^{14}C]Thymidine was also poorly salvaged from the medium. A much greater percentage of the [^{14}C]thymidine (26.4 per cent) was metabolized as compared to [^{14}C]orotic acid (1.2 per cent), but most was found to be thymine. Only 1.5 pmoles/10^6 cells of [^{14}C]thymidine found its way into the nucleotide pools, while 0.8 pmole/10^6 cells was incorporated into DNA.

Incorporation of radiolabeled pyrimidines into the cellular nucleotide pools. [2-^{14}C]Orotic acid was incorporated into the cellular pyrimidine nucleotide pool at rather low levels (Table 6). Only 1 per cent of the cellular CTP pool and 1.9 per cent of the UTP pool was derived from [2-^{14}C]orotic acid. Radio-

Table 6. Nucleotide pool sizes of *L. donovani* incubated with 100 μM ^{14}C-labeled pyrimidines*

Cellular nucleotides	Nucleotide pool (pmoles/10^6 cells)		
	Control	Radiolabeled pyrimidine precursors	
		[2-^{14}C]Orotic acid	[6-^{14}C]Thymidine
NAD + AMP	46.4	34.8	41.5
UMP	1.1	5.8 (0.035)	3.2
UDP-glucose	12.5	21.9 (0.021)	11.3
NADP	8.9	11.4	4.0
UDP	3.2	1.7 (0.194)	2.1
ADP	42.4	40.1	25.9
GDP	16.6	21.6	14.0
CTP	3.7	2.9 (0.010)	1.5 (<0.003)
UTP	26.0	31.0 (0.019)	15.7 (<0.001)
ATP	69.1	65.0	34.0
GTP	23.4	18.5	24.0
Final cell density ($\times 10^6$ cells/ml)	12.5	92	120
Specific activity of precursor (μCi/μmole)		10.0	9.8

* The cold nucleotide pools were measured from the AS fraction of cells incubated for 1 hr with radiolabeled pyrimidine precursors. The numbers in parentheses represent the fraction of the nucleotide pool which was labeled by the precursor.

labeled OMP does not accumulate in these cells. Also, the level of the cellular OMP pool in this parasite is below the limits of detection (<0.01 pmole/10^6 cells). The addition of exogenous orotic acid did not have any effect on the cellular nucleotide pool sizes.

Thymidine was converted to dTMP, dTDP and dTTP, but the cellular pool sizes of these three nucleotides could not be measured. Thymidine was not converted to uridine or cytidine or their corresponding bases since the pyrimidine nucleotide pools were not labeled (Table 6). Also, the addition of the exogenous thymidine caused a decrease in the cellular pyrimidine triphosphate and ATP pool sizes.

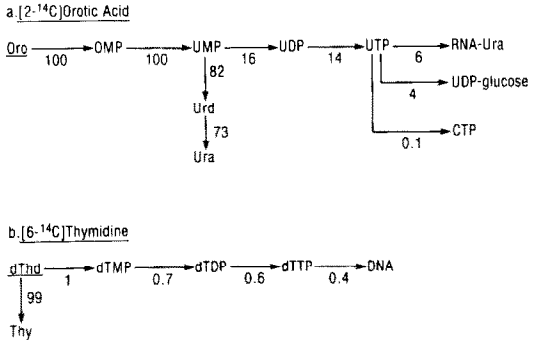

Fig. 2. Flow of radiolabeled pyrimidine metabolites through the pyrimidine salvage pathways of *L. donovani*. These figures represent the metabolic fate of [2-^{14}C]orotic acid and [6-^{14}C]thymidine after a 1-hr incubation with *L. donovani*. The numbers represent the relative amounts of metabolized precursor which flowed through the various pyrimidine pathways in 1 hr. The levels of the radiolabeled precursor which passed through the first committed step are set at 100; all other numbers are relative to this as described in Methods. Some established metabolic steps were omitted for clarity. These steps did not contribute significantly to the metabolism of these radiolabeled precursors.

Metabolic flux of radiolabeled pyrimidines in L. donovani. Figure 2 shows the flow of radiolabeled pyrimidine metabolites through the metabolic pathways of orotic acid and thymidine. [2-^{14}C]Orotic acid is converted to OMP, which is rapidly converted to UMP. Only 16 per cent of the radiolabeled UMP was phosphorylated to UDP, while 82 per cent was converted to uridine and uracil. Only 0.1 per cent of the metabolized orotic acid was eventually converted to CTP, while 6 per cent was incorporated into the RNA, primarily as UTP.

[6-^{14}C]Thymidine was converted primarily to thymine with 99 per cent of the metabolized [6-^{14}C]thymidine present as thymine. The remaining 1 per cent of the metabolized thymidine existed in the form of nucleotides and DNA. A very small amount of the metabolized [6-^{14}C]thymidine (0.4 per cent) was incorporated into DNA.

DISCUSSION

These studies support previous work which demonstrated an active salvage pathway for purines in *L. donovani* and *L. braziliensis* [1]. The inability of leishmania to form purines *de novo* is similar to that in another hemoflagellate, *T. cruzi* [13, 14]. This lack of *de novo* purine metabolism is in accord with the parasitic nature of these organisms since their natural environment within the host cell is abundant with preformed purines.

The purine salvage pathways of these parasites are very similar to those of other organisms. *L. donovani* possesses a very active adenine aminohydrolase but lacks adenosine deaminase (EC 3.5.4.4). It also contains an active guanase, which converts guanine to xanthine, but lacks the xanthine oxidase to convert

this to uric acid. This lack of xanthine oxidase has been demonstrated previously [15]. The absence of xanthine oxidase and the presence of adenine aminohydrolase and guanase in these parasites lead to the rapid production of hypoxanthine and xanthine, which have no route of metabolism in this parasite other than conversion to their respective nucleotides.

All four purine bases tested were converted to their respective nucleotides. The major route of adenine metabolism was deamination to hypoxanthine and conversion to IMP. The major route of guanine metabolism was deamination to xanthine followed by conversion to XMP. These two nucleotides were further metabolized to both the adenine and guanine nucleotide pools. The direct phosphoribosylation of adenine could not be separated from its deamination and metabolism to AMP via IMP in these experiments. An active adenine phosphoribosyltransferase (PRT) does exist, as indicated by the increased labeling of the nucleotide pools when [8-^{14}C]adenine served as the precursor instead of [8-^{14}C]hypoxanthine. Previous studies have demonstrated the existence of active adenine, hypoxanthine-guanine and xanthine phosphoribosyltransferases [1, 5]. The direct phosphoribosylation of guanine could not be supported in this study since [8-^{14}C]xanthine labeled the nucleotide pools to a greater degree than did [8-^{14}C]guanine.

The salvage of pyrimidines does exist in *L. donovani* as demonstrated by the metabolism of orotic acid and thymidine. The enzymes required for *de novo* synthesis from the orotate PRT step on have been shown to exist although the presence of enzymes preceding this step has not been demonstrated. *De novo* pyrimidine synthesis has also been demonstrated in the hemoflagellate, *Trypanosoma cruzi* [16, 17]. The uptake and metabolism of [2-^{14}C]orotic acid and [6-^{14}C]thymidine occurred at a much slower rate than those of the purine precursors. Orotic acid was converted to OMP via an orotate phosphoribosyltransferase. This was then rapidly converted to UMP via OMP decarboxylase. The activity of this enzyme appears to be quite high, as indicated by the lack of OMP accumulation in these cells. UMP was converted rapidly to uridine and uracil, thus indicating the existence of a phosphatase or nucleotidase active on UMP as well as a uridine phosphorylase or hydrolase. The remainder of the UMP not cleaved to uridine was converted to UDP. The radioactivity of [2-^{14}C]orotic acid was eventually incorporated in the CTP and UTP pools, with 1 per cent of CTP being labeled and 1.9 per cent of the UTP labeled.

Thymidine was metabolized primarily to thymine, indicating an active thymidine phosphorylase. A small amount of thymidine was also converted to dTMP. It is not clear whether this came about by direct phosphorylation of thymidine via a kinase or phosphotransferase, or if dTMP was produced from thymine or other labeled metabolites, i.e. uracil or cytosine. The latter possibilities do not seem feasible since no measurable amounts of labeled uracil or cytosine were produced by these parasites, and a thymine phospho-2′-deoxyribosyltransferase is not known to exist. Only 0.4 per cent of the metabolized [6-^{14}C]thymidine was incorporated into DNA; however, considering the fact that only 0.6 per cent of the metabolized thymidine was converted to dTTP, this amount actually indicates a rapid incorporation of [6-^{14}C]dTTP into DNA.

In summary, *L. donovani* has been shown to salvage preformed purines effectively and, to a lesser extent, to utilize preformed pyrimidines. *De novo* purine biosynthesis has been shown previously not to exist in this parasite [1]. Some of the latter enzymes in *de novo* synthesis of pyrimidines by these parasites have been demonstrated by their ability to convert orotic acid to pyrimidine nucleotides. Adenine and guanine entered the purine metabolic pathways primarily as hypoxanthine and xanthine, the latter activated by their respective phosphoribosyltransferases. Orotic acid was activated by conversion to OMP via the action of orotate phosphoribosyltransferase. The OMP was then rapidly converted to UMP by OMP decarboxylase. Thymidine appears to be directly phosphorylated to dTMP by thymidine kinase although large amounts were cleaved to thymine by these parasites.

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