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PATHWAYS OF NUCLEOTIDE METABOLISM IN *SCHISTOSOMA MANSONI*—III

IDENTIFICATION OF ENZYMES IN CELL-FREE EXTRACTS*

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Abstract—A survey of purine anabolic and catabolic enzymes has resulted in identification of major pathways in schistosome nucleotide biosynthesis. It is shown that multiple pathways for the incorporation of purine bases and nucleosides exist. The evidence suggests that adenosine phosphoribosyltransferase (APRT) activity is about ten times greater than adenosine kinase activity. Furthermore adenosine is converted to AMP principally via the pathway of adenosine deaminase, followed by conversion of inosine to hypoxanthine. In this sequence hypoxanthine phosphoribosyltransferase (HPRT) activity is rate limiting.

On the basis of enzyme activities determined, one can suggest candidates of nucleotide analogs which might be useful chemotherapeutic agents.

IN PREVIOUS communications[1,2] *Schistosoma mansoni* has been shown to be incapable of the biosynthesis *de novo* of purine nucleotides. Thus, salvage mechanisms for nucleotides (or their immediate precursors) must represent vital pathways for the maintenance of ATP or GTP energy systems, and for the provision of precursors for coenzyme and nucleic acid synthesis.

It has already been shown that adenine may be taken up and rapidly converted to ATP; adenosine, in contrast, is deaminated to inosine and appears to be processed primarily via hypoxanthine and IMP enroute to ATP.[3] However, little information is available concerning the relative activities of the enzyme systems involved in the metabolism of purines in these worms. The subject of this report is an attempt to identify these pathways in this parasite.

All enzymes named in this and related papers are identified as to official and trivial names (Table 1).

MATERIALS AND METHODS

For the following studies, two sources of schistosome material were used. Fresh worms were obtained from infected CF[1] mice and were washed free of plasma and RBC. Lots of 35, 50 and 100 (about 20 mg, wet wt) pairs were homogenized in 0·5 ml Tris–acetate buffer (0·1 M) at pH 7·5 and containing 0·5 mM EDTA. An all-glass manual microhomogenizer kept at ice temperature was employed to grind the worms

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TABLE 1. NOMENCLATURE OF ENZYMES AND COMPOUNDS

Name used in text	Trivial name	Systematic name	EC No.
APRT	Adenine phosphoribosyltransferase	AMP: Pyrophosphate phosphoribosyl transferase	2.4.2.7
GPRT	Guanine phosphoribosyltransferase	GMP: Pyrophosphate phosphoribosyl transferase	2.4.2.8
HPRT	Hypoxanthine phosphoribosyltransferase	IMP: Pyrophosphate phosphoribosyl transferase	2.4.2.8
	Adenosine kinase	ATP: Adenosine 5'-phosphotransferase	2.7.1.20
AMP kinase	Adenylate kinase	ATP:AMP phosphotransferase	2.7.4.3
GMP kinase	Guanylate kinase	ATP:GMP phosphotransferase	2.7.4.8
IMP kinase		ATP: Nucleoside monophosphate phosphotransferase	2.7.4
CMP kinase		ATP: Nucleoside monophosphate phosphotransferase	2.7.4
UMP kinase		ATP: Nucleoside monophosphate phosphotransferase	2.7.4
NDP kinase	Nucleoside diphosphokinase	ATP: Nucleoside diphosphate phosphotransferase	2.7.4.6
ATPase		ATP: Phosphohydrolase	3.6.1.4
AMP deaminase	AMP aminohydrolase		3.5.4.6
	Adenosine deaminase	Adenosine aminohydrolase	3.5.4.4
	Guanine deaminase	Guanine aminohydrolase	3.5.4.3
	Deoxycytidine deaminase	Deoxycitidine aminohydrolase	3.5.4.
PNPase	Purine nucleoside phosphorylase	Purine nucleoside: Orthophosphate ribosyltransferase	2.4.2.1
	Adenine deaminase	Adenine aminohydrolase	3.5.4.2
	Creatine kinase	ATP: Creatine phosphotransferase	2.7.3.2
AR	Adenosine		
In	Inosine		
Hx	Hypoxanthine		
S-AMP	Adenylsosuccinic acid		

until particulate matter was no longer grossly visible. As an alternate source of enzymes, 20 mg of lyophilized worms (obtained from saline-perfused infected mice through the courtesy of Dr. Shirley Maddison of the Center for Disease Control in Atlanta) were similarly treated.

After homogenization, cellular debris was removed by centrifugation at 18,000 rev/min (39,000 *g*) for 40 min at 4°. Protein and nucleic acid concentrations were determined by the method of Warburg and Christian.[4] For the following studies all unlabeled compounds were obtained in the highest available purity from P–L Biochemicals. Radiochemicals were supplied by Schwarz Bioresearch.

Enzyme assays. In these studies all enzyme assays were expressed in a common unitage which is that amount of enzyme which catalyzes the consumption of the micromole of substrate or the production of 1 μmole of product/min under the assay conditions described. Specific activities are expressed as units per gram of wet weight of intact schistosomes.

Assays for adenine phosphoribosyltransferase (APRT), hypoxanthine phosphoribosyltransferase (HGPRT), and guanine phosphoribosyltransferase (GPRT). The reaction mixtures consist of 50 μl of extracts of lyophilized worms or 25 μl of extracts of fresh *S. mansoni* prepared as above, 5′-phosphoribosyl-1-pyrophosphate (PRPP, 0·5 mM), $MgCl_2$ (5 mM), adenine-8-^{14}C (0·1 mM; sp. act. = 11·0 mCi/m-mole), hypoxanthine-8-^{14}C (0·1 mM; sp. act. = 9·9 mCi/m-mole), or guanine-8-^{14}C (0·1 mM; sp. act. = 6·1 mCi/m-mole) and Tris–HCl buffer, pH 7·4 (0·2 M) in a total volume of 1 ml. The mixtures were incubated at 37° with shaking (approx. 80 oscillations/min) with air as the gas phase. At 0, 5, 15, 30, 60 and 120 min after the addition of isotropic precursor, 100-μl aliquots of the reaction mixtures were transferred to chilled tubes containing 20 μl of 4 M formic acid. The samples were thoroughly stirred and placed in an ice bath for about 1 hr.

Aliquots (25 μl) of the samples were spotted on MN–Polygram Cel 300 plates for thin-layer chromatography (Brinkmann Instruments, Inc., Westbury, N.Y.) on a line 2 cm from the bottom of the plate. Appropriate unlabeled carriers (adenine + AMP + ADP + ATP in the case of adenine-8-^{14}C as precursor; hypoxanthine + IMP + AMP + ADP + ATP + XMP + GMP + GDP + GTP in the case of hypoxanthine-8-^{14}C as precursor); guanine + GMP + GDP + GTP in the case of guanine-8-^{14}C) were used as monitor compounds in 30-nmole amounts. The plates were developed with 5% aqueous disodium hydrogen phosphate for about 2 hr. After the plates were dried, the nucleotides and bases were visualized with u.v. light at 254 nm. The u.v.-absorbing areas were then cut out and placed in counting vials containing 20 ml of toluene-based phosphor solution. The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer model 3000.

Metabolism of adenine and adenosine. Fresh homogenates of *S. mansoni* prepared as above were incubated under conditions which were the same as those for the enzyme assays for APRT, HPRT and GPRT outlined previously. In addition to adenine-8-^{14}C (10^{-4} M, sp. act. = 11·0 mCi/m-mole), adenosine-8-^{14}C (5×10^{-5} M, sp. act. = 5·96 mCi/m-mole) was used as a precursor. For the analysis of radioactivity in the various purine ribonucleotides, the following technique was utilized: after the contents of the tubes had been thoroughly mixed and allowed to sit in the cold for at least 1 hr, 20-μl samples of each mixture were spotted together with the appropriate unlabeled nucleotides (30 nmoles of each) on MN–Polygram Cel PEI sheets for thin-layer

chromatography (Brinkmann Instruments, Inc., Westbury, N.Y.). The resolution of nucleotides in these samples was carried out by the method of Crabtree and Henderson[5] which results in the satisfactory separation of the 5′-mono, di- and triphosphate of adenosine and guanosine, inosine-5′-monophosphate, and xanthosine-5′-monophosphate. After chromatography, spots were visualized and radioactivity was determined as described above.

Adenosine kinase procedures. Adenosine kinase was assayed by a radioactive method which used either ^{14}C-methyl-labeled 6-methylmercaptopurine ribonucleoside (MMPR) or adenosine-8-^{14}C as the substrate. In a final volume of 200 μl, the reaction mixture contained: MMPR-$^{14}CH_3$, 0·5 mM; Tris–acetate, pH 7·0, 100 mM; ATP, 0·5 mM; phosphoenolpyruvate, 3 mM; KCl, 50 mM; $MgCl_2$, 5 mM; and pyruvic kinase 0·22 mg.

The assay was a modification of the procedures of Lindberg *et al.*[6] and Schnebli *et al.*[7] The reaction was started by adding enzyme; the mixture was then incubated for 10 min at 37°. At the end of the incubation period, the assay tubes were immersed in a boiling water bath for 90 sec. After centrifugation to remove precipitated protein, aliquots of the supernatant were spotted on cellulose-coated thin-layer sheets. When the substrate was MMPR, the thin layers were developed in disodium hydrogen phosphate (5 per cent). When adenosine was used as the substrate, ethanol–1·0 M ammonium acetate (7:3), pH 7·5, was employed as the developing solvent.

In either case, the nucleotide areas (MMPR-5′-PO_4 or AMP + ADP + ATP) were cut out of the chromatography sheets for radioactive counting. From the radioactivity determined, the amount of conversion of the nucleoside to the nucleotides could be calculated.

Deaminase and nucleotide kinase assays. Mononucleotide kinases, nucleoside diphosphokinase (NDPK) and creatine kinase were assayed spectrometrically by the coupled pyruvate kinase–lactate dehydrogenase method described by Miech and Parks,[8] with the exception that ATP concentration was set at 4·0 mM.

Adenosine deaminase, guanine deaminase (guanase), AMP deaminase, deoxycytidine deaminase and adenine deaminase (adenase) were also assayed spectrophotometrically. The ammonia produced upon deamination was measured by an α-ketoglutarate-glutamic dehydrogenase system as modified from Goldstein.[9]

The reaction mixture for the deaminase assays contained in 1 ml: potassium phosphate buffer, 100 mM, pH 7·4; EDTA, 1 mM; DPNH, 0·2 mM; α-ketoglutaric acid, 17 mM; and glutamic dehydrogenase, 1 mg (45 units). The substrates were present in the following concentrations: adenosine, 1 mM; guanine, 0·1 mM; AMP, 1 mM; deoxycytidine, 1 mM; and adenine, 0·1 mM.

In the case of guanase and 5′-AMP deaminase, the enzymes were also assayed in the presence of 0·8 mM GTP and ATP respectively.

The reaction was started by the addition of substrate, and was followed by the decrease in absorbance at 340 nm. Proper controls for endogenous ammonia were also run.

Purine nucleoside phosphorylase assay. The assay utilized was a coupled xanthine oxidase method[10] which is based on the measurement of the increase in absorbance at 293 nm due to the formation of uric acid. Conditions for this assay closely followed techniques previously described by Kim *et al.*[11]

Metabolism of hypoxanthine by intact S. mansoni. Intact *S. mansoni* (10 pairs/flask)

were incubated in 25 ml Erlenmeyer flasks with hypoxanthine-8-^{14}C (0·96 mM; sp. act. = 24·8 mCi/m-mole) in 5 ml of Fischer's medium at 37° with occasional very gentle swirling. At appropriate times (15, 30, 60 and 120 min) after addition of the isotope, the reactions were terminated by the addition of perchloric acid (final concentration 2 per cent) to the flasks. The worms were removed from the flasks, homogenized (see above) and the resulting material was resuspended in the incubation medium. After centrifugation (2600 rev/min in a clinical centrifuge) the supernatant was neutralized with KOH. The mixtures were cooled in an ice-bath for 30 min, and the potassium perchlorate was removed by centrifugation.

The incorporation of radioactivity into the various purine nucleotides was determined as outlined above (Materials and Methods "Metabolism of adenine and adenosine") with the exception that 50-μl samples of the supernatant were spotted.

RESULTS

A survey of purine and pyrimidine anabolic and catabolic enzymes is given in Table 2. Some of the experimentally determined values are relatively high when

TABLE 2. ENZYMES IN PURINE METABOLISM IN *Schistosoma mansoni*

	Lyophilized worms (μmoles/min/g)	Fresh worms (μmoles/min/g)
Anabolic enzymes		
APRT	0·34	2·1
GPRT	0·013	0·41
HPRT	0·025	0·76
"Adenosine kinase"*	Trace	0·18
AMP kinase	0·12	60
GMP kinase	1·4	4·5
"IMP kinase"†	0·54	2·25
CMP kinase	Nil	15
UMP kinase	Nil	15
NDP kinase	132	450
Catabolic enzymes		
AMP deaminase (with 0·8 mM ATP)	Nil	Nil
Adenosine deaminase	0·02	1·5
Guanine deaminase	0·01	0·3
Deoxycytidine deaminase	Nil	Nil
PNPase	0·09	3·8
Adenine deaminase	Nil	Nil
Creatine kinase	Nil	Nil

* Adenosine kinase was measured using 6-MMPR as the substrate since this has been found in other systems not to be a substrate for either adenosine deaminase[13] or for PNPase.[14,15]

† As noted in Materials and Methods, this enzyme is measured by means of an indirect assay. The complete substrate specificity of this enzyme activity has not been established. It is possible that GMP kinase can react with IMP with decreased velocity.[16]

compared to the activities measured in various mammalian tissues.[12] For example schistosome NDP kinase (450 μmolar units/g, wet wt) showed the highest activity of many various tissues measured in this laboratory.

A comparison of the data obtained from lyophilized worms to that derived from fresh worm homogenates shows that in all cases considerable inactivation of these enzymes occurs during the freeze-drying step. In particular, the adenosine kinase activity is quite labile, as shown by an almost total loss of activity in the lyophilized extract.

Figures 1 and 2 illustrate key reactions in the metabolism of adenine and adenosine in homogenates of fresh worms. Under the conditions utilized in the experiments depicted in these figures, any di- or tri-phosphate which is formed would be degraded to the monophosphate level. Therefore, radioactivity shown on the figures as "AMP" actually represents AMP + ADP + ATP. It is apparent that when adenine-8-^{14}C is used as precursor, large amounts of radioactivity accumulate as AMP, whereas only a very small amount is present as IMP. In agreement with this finding was the failure to detect measurable amounts of 5'-AMP deaminase even in the presence of high concentrations of ATP, an activator of this enzyme.[17] No other non-adenine purine contained significant amounts of the label, even after 120 min of incubation. This suggests that conversion of adenine nucleotides into guanine nucleotides occurs slowly in *S. mansoni*.

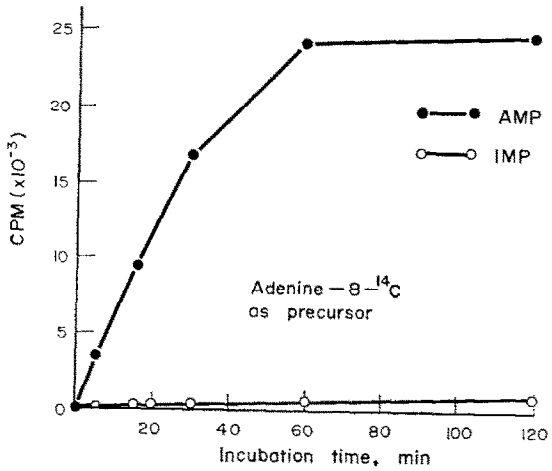

FIG. 1. Conversion of adenine-8-^{14}C to nucleotides *in vitro*. Extracts (25 μl) of fresh *S. mansoni* were incubated with adenine-8-^{14}C (0·1 mM; sp. act. = 11·0 mCi/m-mole); PRPP (0·5 mM) and $MgCl_2$ (5 mM) in Tris–HCl buffer, pH 7·4 (0·2 M) in a final volume of 1 ml at 37° with shaking. At various times, portions were removed for analysis of radioactivity in purine nucleotides. Each point represents the average of three determinations.

In contrast to the results obtained with adenine-8-^{14}C, when adenosine-8-^{14}C was used as a substrate, both AMP and IMP contained significant amounts of the label (Fig. 2). After 5 min of incubation the AMP:IMP ratio was 2·7; this ratio became 0·8 after 15 min and 0·33 after 120 min. The initial lag period apparent in IMP formation from adenosine (Fig. 2) may reflect the time required for completion of the sequence of reactions: AR→In→Hx→IMP.

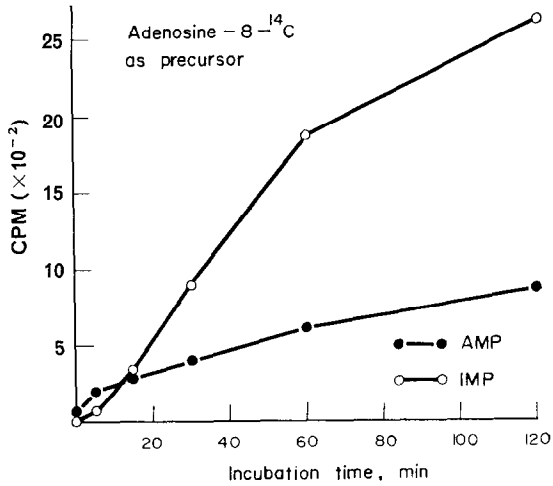

FIG. 2. Conversion of adenosine-8-^{14}C to nucleotides *in vitro*. Extracts of fresh *S. mansoni* were incubated as described in Fig. 1 with adenosine-8-^{14}C (5×10^{-5} M, sp. act. = 5·96 mCi/m-mole). At various times, portions were removed for analysis of radioactivity in purine nucleotides.

When the incorporation of hypoxanthine-8-^{14}C into purine ribonucleotides is examined (Fig. 3), it is apparent that the major portion of radioactivity is present as ATP. Much lower, but significant amounts, of radioactivity are present as GTP, especially after 120 min of incubation. Evidently, the pathway from IMP to AMP is

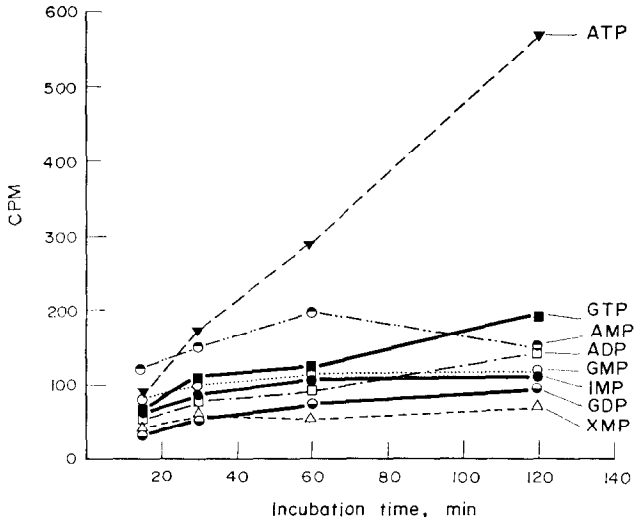

FIG. 3. Metabolism of hypoxanthine-8-^{14}C by intact schistosomes. *S. mansoni* (10 pairs/25 ml Erlenmeyer) were incubated with hypoxanthine-8-^{14}C (0·96 mM, sp. act. = 24·8 mCi/m-mole) in Fischer's medium (5 ml) at 37° with occasional shaking. At the times indicated, reactions were terminated by the addition of perchloric acid (2 per cent final concentration), and the worms were homogenized; portions of the combined homogenate and incubation medium were analyzed to determine the incorporation of radioactivity into the purine nucleotides. Results are averages of duplicate determinations.

preferred over that from IMP to GMP in these worms. Only small amounts of IMP accumulate (in intact worms) reflecting a limited conversion of hypoxanthine into nucleotides (see previous paper) and the presence of very active NDPkinase activity. In the data presented here only 1–2 per cent conversion of hypoxanthine into nucleotides occurred even after 2 hr.

Deamination of adenosine was compared to analog purine nucleosides. Using adenosine as the standard, it can be seen that Formycin A and 8-aza-adenosine are better substrates for deamination than is adenosine itself. However, tubercidin and 2-fluoroadenosine are not deaminated by this enzyme system (Table 3).

TABLE 3. DEAMINATION OF ANALOG PURINE NUCLEOSIDES BY *S. mansoni* ADENOSINE DEAMINASE*

	Activity with adenosine, % $\left(\frac{\text{act. with analog}}{\text{act. with adenosine}} \times 100\right)$
Control (no enzyme)	
+ Adenosine	100
+ Formycin A†	144
+ 8-Aza-adenosine	367
+ 7-Deazaadenosine (tubercidin)‡	0
+ 2-Fluoroadenosine	0

* Source of enzyme: 35 pairs of *S. mansoni* were homogenized in 0·25 ml of Tris–acetate 0·1 M, pH 7·5, containing EDTA (0·5 mM). The homogenate was centrifuged at 3800 *g* for 45 min to remove debris. Twenty μl of the resulting supernatant was taken for each assay. Each measurement is the average of duplicate determinations. Details of the assay procedure were given in Materials and Methods. The nucleosides were present at a concentration of 1 mM.

† Formycin A = 7-amino-3(β-D-ribofuranosyl)pyrazolo-[4,3-*d*]pyrimidine.

‡ Tubercidin = 4-amino-7-(β-D-ribofuranosyl)pyrrolo-[2,3-*d*]pyrimidine.

DISCUSSION

It has been established through studies with ^{14}C-labeled glycine and glucose that adult schistosomes are deficient in the *de novo* pathway of purine biosynthesis.[2] The present study and the preceding papers clearly establish that schistosomes have available multiple mechanisms for incorporating preformed purine bases and nucleosides into the purine nucleotide pool, e.g. adenosine kinase, adenine phosphoribosyl transferase, guanine phosphoribosyltransferase, and hypoxanthine phosphoribosyltransferase. On the basis of comparisons of enzymatic activities (Table 2) it appears that APRT activity is approximately ten times greater than the adenosine kinase activity. Also in the alternative pathway: adenosine deaminase→PNPase→HPRT, it is the latter enzyme that is rate limiting. Its activity is intermediate between that of APRT and adenosine kinase. These observations on the activities of enzymes in worm homogenates are consistent with the results of studies on the incorporation of adenine and adenosine into intact schistosomes (see accompanying papers).

It must be noted that additional alternative pathways for the incorporation of purine nucleosides have been described in other tissues but have not been examined

here. For example, we have not ruled out the possible occurrence of guanosine kinase, inosine kinase and the purine deoxynucleoside kinases. Additionally, in many tissues the same enzyme catalyzes the phosphoribosylation of both hypoxanthine and guanine. It has not yet been established whether in schistosomes one or two phosphoribosyl transferases exist.

Of some interest is the unusually high activity of NDP kinase found in adult schistosomes. This is approximately 5- to 10-fold greater than the activity of NDP kinases found in various mammalian tissues.[18] Recent work in this laboratory has demonstrated that in the human erythrocyte,[12] as well as in other tissues,[19] a striking heterogeneity of NDP kinases exists, with the occurrence of multiple isozymes. It would be of considerable interest to learn whether a similar isozymic pattern of NDP kinase occurs in these parasites as well.

It has been noted (preceding paper) that when live, adult schistosomes are incubated with inosine, significant amounts of adenine nucleotides are formed. Likewise, it is seen (Fig. 3) that when adult worms are incubated with hypoxanthine-8-^{14}C, substantial quantities of ATP and GTP are synthesized. Therefore, although the specific enzymes have not been assayed directly, it is assumed that the pathways of IMP →S-AMP→AMP exist in these worms, as well as IMP→XMP→GMP. Thus, these pathways have been indicated in Fig. 4.

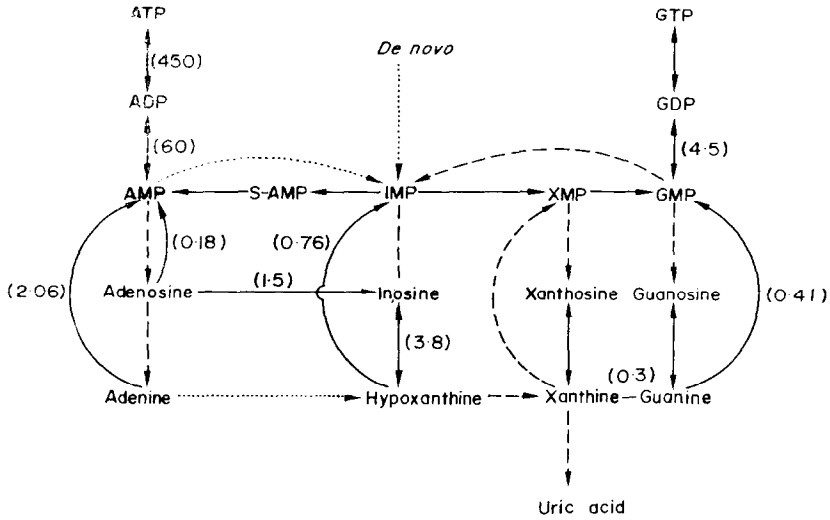

FIG. 4. Pathways of purine nucleotide metabolism in *S. mansoni*. Figures in parentheses refer to micromolar units of enzyme activity per gram of worms. Solid lines indicate reactions believed to be present in the worms. Dashed lines refer to reactions which may or may not be present. Reactions not present in the *S. mansoni* are denoted by dotted lines.

Somewhat surprising is the apparent absence of 5'-AMP deaminase in schistosomes, since this enzyme plays a key role in regulating the interconversions of purine nucleotides in many other tissues.[17]

The question might be asked whether some of the enzymatic activities described in Table 2 actually derive from mouse erythrocytes rather than from schistosomes

since these parasites dwell in the blood and consume substantial amounts of erythrocytes. It should be noted that the worms, prior to homogenization, were thoroughly washed (three times) in Fischer's medium, and the incubation medium was free of detectable erythrocytic contamination. It is possible that red cell slurry in the gut of schistosomes might have contributed some fraction of the measured enzyme activities. However, it seems unlikely that many red cell enzymes would survive the proteolytic environment and presumed low pH of the parasite gut. It should also be noted that the activities of several of the enzymes reported in Table 2 are many fold greater than the activities normally found in erythrocytes.

The fact that adult schistosomes require preformed purines and are devoid of the *de novo* pathway of purine biosynthesis presents a unique opportunity for the rational design of new chemotherapeutic approaches. The vast literature in the field of viral and cancer chemotherapy and the availability of large numbers of thoroughly tested analogues suggest numerous possibilities. The results of this paper have demonstrated that adult schistosomes have present the enzymes, adenosine kinase, APRT, HPRT, and GPRT. This indicates that adenosine, adenine, guanine, and hypoxanthine analogues are all potential candidates for chemotherapy.

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