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PATHWAYS OF NUCLEOTIDE METABOLISM IN *SCHISTOSOMA MANSONI*—VII

INHIBITION OF ADENINE AND GUANINE NUCLEOTIDE SYNTHESIS BY PURINE ANALOGS IN INTACT WORMS*

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Abstract—Twelve analogs of adenosine or its related purines have been tested as single agents or in combinations as possible antischistosomal compounds. Measurements were made of the effect of these drugs on the anabolism and catabolism of [8-^{14}C]adenosine by *Schistosoma mansoni in vitro*. Based on the degree of inhibition of biosynthesis of adenine and guanine nucleotides by intact worms, the best currently available purine analogs are 7-deaza-adenosine (tubercidin) and N^6-phenyladenosine. These drugs reduced the total synthesis of nucleotides to 30 and 25 per cent, respectively, of controls. Blockade of the catabolic pathway (adenosine deaminase) by coformycin resulted in significantly increased synthesis of adenine nucleotides rather than the expected decrease. Thus, adenosine kinase must play a more prominent role in nucleotide synthesis than had been previously estimated. The implications of these findings in the development of new anti-schistosomal drugs are discussed.

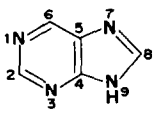
Schistosomes do not have a *de novo* pathway for the formation of IMP [1–3], but do have active salvage pathways for preformed purines [1–5]. Accordingly, various candidate analogs of adenosine or adenine have been proposed as therapeutic drugs. In settling on a suitable agent to be used, the details of the various pathways of adenosine metabolism assume crucial importance. A scheme for the pathways of nucleotide metabolism in terms of activity units has been presented [4]. Recently, Crabtree and Senft [6] and Miech *et al.* [7] have extended these data and have shown that schistosome extracts and intact worm preparations contain adenosine phosphorylase, an enzyme which cleaves adenosine to adenine. Schistosome extracts appear to convert somewhat more than half of an adenosine substrate to inosine and hypoxanthine *in vitro*, using the adenosine deaminase† and purine nucleoside phosphorylase (PNPase) reactions [3, 6]. A considerable fraction of the adenosine is also cleaved to adenine via adenosine phosphorylase, following which, if 5-phosphoribosyl-1-pyrophosphate (PRPP) is present, AMP is formed by phosphoribosylation [6]. Conversion of adenosine directly to AMP by means of adenosine kinase has been previously thought [3, 4, 6] to account for a small percentage of ATP production. Therefore, if one could block adenosine cleavage by the schistosome and interfere with the deaminative pathway as well, one might seriously compromise the parasite's total energy and nucleic acid sources. However, this proposal is valid only in the event that the activity of the adenosine kinase reaction is limited. Evidence presented in this paper will show that this phosphorylation reaction may account for a considerable fraction of adenosine salvage by the schistosomes. A summary of the various pathways by which adenosine is converted into nucleotides in the parasite is given in Fig. 1.

This paper will give results of trials of several adenosine analogs which were examined alone or in combinations of two or three analogs for their effectiveness in inhibiting the conversion of adenosine *in vitro* into nucleotides, using intact, rather than homogenized, worms. The structures of these analogs‡ are shown in Fig. 2, and the major pharmacologic considerations suggesting such drugs are given in Table 1.

* Supported by Grant RF 72066 from the Rockefeller Foundation (A.W.S.) and CA 07340 from the USPHS (G.W.C.).

† Definitions of terms used in the text are: adenosine deaminase, adenosine aminohydrolase (EC 3.5.4.4); purine nucleoside phosphoylase (PNPase), purine-nucleoside: orthophosphate ribosyltransferase (EC 2.4.2.1); adenosine kinase, ATP: adenosine 5'-phosphotransferase (EC 2.7.1.20); adenine phosphoribosyltransferase (APRTase), AMP:pyrophosphate phosphoribosyltransferase (EC 2.4.2.7); hypoxanthine phosphoribosyltransferase (HPRTase), IMP: pyrophosphate phosphoribosyltransferase (EC 2.4.2.8); and adenylate kinase, ATP:AMP phosphotransferase (EC 2.7.4.3). Other abbreviations used are defined in the text.

‡ The numbering sequence used for purines is as follows:



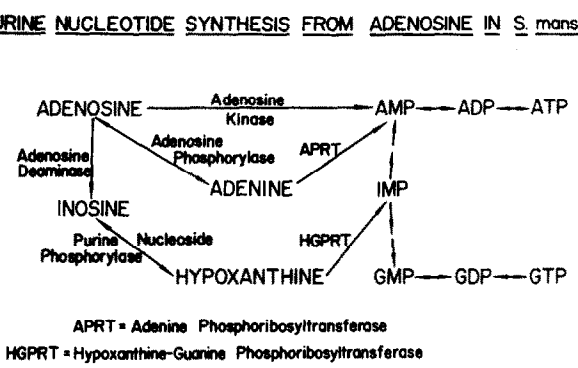


Fig. 1. Pathways for conversion of adenosine into nucleotides in *S. mansoni*. One pathway (adenosine → adenine) is not found in mammalian systems.

METHODS

[8-^{14}C]adenosine (sp. act. = 51 μCi/μmole) was obtained from Schwarz/Mann, Orangeburg, NY. Coformycin (3-β-D-ribofuranosyl-6,7,8-trihydroimidazo-[3,4-d][1,3]diazepin-8-(R)-ol) and formycin [7-amino-3(β-D-ribofuranosyl)pyrazolo-[4,3-d]pyrimidine] were obtained from Dr. H. Umezawa, Institute for Microbial Chemistry, Tokyo. *p*-Nitrobenzyl-6-thioguanosine was a gift from Dr. A. R. P. Paterson, University of Alberta, Edmonton, Alberta. Tubercidin (7-deaza-adenosine) was from the Upjohn Co., Kalamazoo, MI. The carbocyclic nucleosides (carbocyclic-adenosine [9-(β-D,L-2,3-dihydroxy-4-β-(hydroxymethyl)-cyclopentyl)adenine], carbocyclic-inosine and carbocyclic-6-methylmercaptopurine ribonucleoside) were gifts from Dr. L. L. Bennett, Jr., of the Southern Research Institute, Birmingham, Ala. Dr. M. H. Fleyscher of the Roswell Park Memorial Institute, Buffalo, NY, supplied N^6-phenyladenosine. Both 6-thioguanosine and 6-methylmercaptopurine ribonucleoside were purchased from Sigma Chemical Co., St. Louis, MO, and arabinosyl-6-mercaptopurine was from Nutritional Biochemicals Corp., Cleveland, Ohio. Dr. Harry B. Wood, Jr. of the Drug Research and Development, National Cancer Institute, Bethesda, Md., generously supplied us with 2-fluoro-2'-deoxyadenosine.

Mice (CF_1 strain) were exposed percutaneously to cercariae from a laboratory strain of *Schistosoma mansoni* originally obtained from Dr. van der Schalie, Univ. of Michigan, Ann Arbor, MI. This parasite strain was maintained in this laboratory by repeated passage through a mouse–snail (*Australorbis glabratus*) cycle. Female mice of about 25 g were exposed to about 200 cercariae. After 45–60 days, mice were killed by cervical fracture after etherization, and the worms were recovered from portal or mesenteric veins by gentle hook-dissection. The parasites were placed in Fischer's Medium (FM), containing added penicillin (1 unit/ml) and streptomycin (1 μg/ml), and

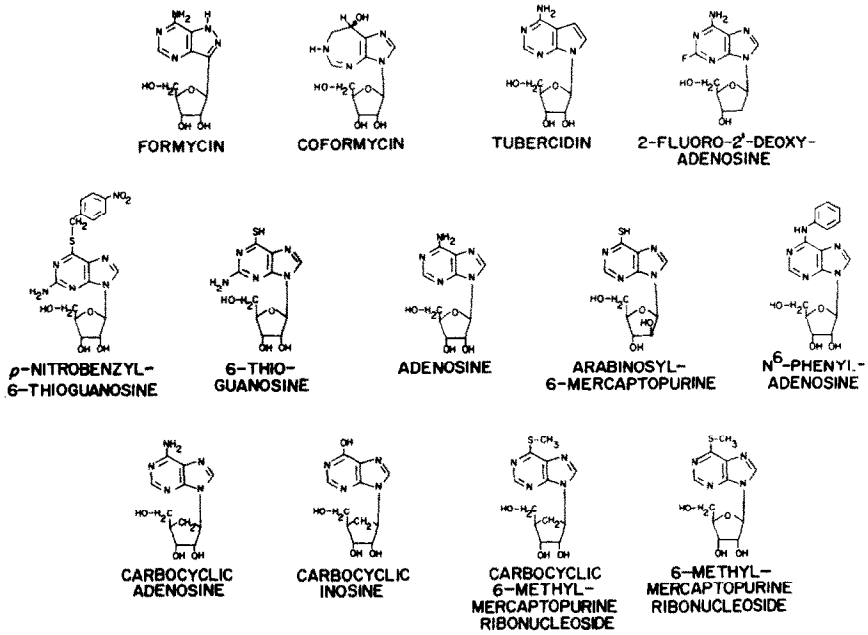


Fig. 2. Structural formulas of adenosine and of analog nucleosides which were examined as inhibitors of nucleotide synthesis from adenosine.

Table 1. Purine analogs tested as inhibitors of nucleotide synthesis

Trivial name	Difference from adenosine	Reasons for use
Formycin	Carbon–carbon glycosidic linkage; altered 5-membered ring	Blocks worm adenosine phosphorylase and PNPase [6]
Tubercidin	7-Nitrogen replaced by carbon	Not a substrate for worm adenosine deaminase [4]; interferes with worm PNPase and adenosine deaminase [6]; forms analog nucleotides in intact worms [5]; antischistosomal agent in monkeys *in vivo* [8]
N^6-phenyladenosine	Phenyl group replaces a hydrogen on 6-amino group of adenosine	Adenosine kinase inhibitor (mammalian enzyme) [9]
2-Fluoro-2′-deoxyadenosine	Fluorine atom on carbon 2, ribose of adenosine replaced by 2′-deoxyribose	In human erythrocytes, accumulates at monophosphate nucleotide level [10]; inhibits worm adenosine phosphorylase and adenosine deaminase [7]
Coformycin	Inosine analog in which 6-membered ring is replaced by a 7-membered ring (methylene group inserted between carbons 5 and 6 of inosine)	Potent inhibitor of human erythrocytic adenosine deaminase ($K_i \simeq 10^{-10}$ M) [11]
p-Nitrobenzyl-6-thioguanosine	6-Amino group of adenosine replaced by *p*-nitrobenzyl-thio group, amino group on carbon 2	Inhibitor of nucleoside transport in human erythrocytes [12]
Arabinosyl-6-mercaptopurine	Ribose replaced by arabinose, 6-amino replaced by thiol	Not a substrate for adenosine deaminase [13], adenosine kinase [14] or PNPase [15]; inhibits worm adenosine deaminase [6]
6-Thioguanosine	6-Amino replaced by thiol, amino group on carbon 2	Inhibits worm adenosine deaminase and PNPase pathways [6, 7]
6-Methylmercaptopurine ribonucleoside	6-amino replaced by methylthio group	Converted to nucleotide via adenosine kinase [16]; may compete with adenosine for this enzyme in worms
Carbocyclic-adenosine	Oxygen atom of ribose moiety replaced by methylene group in the furanose ring	Converted to analog nucleotides via adenosine kinase, adenylate kinase, etc; cytotoxic to H.Ep. no. 2 cells in culture [17]; stable glycosidic bond therefore should not be cleaved by adenosine phosphorylase
Carbocyclic-inosine	As for carbocyclic-adenosine, also 6-amino replaced by hydroxyl group	Stable glycosidic bond [17]
Carbocyclic-6-methyl-mercaptopurine ribonucleoside	As for carbocyclic-adenosine and 6-methylmercaptopurine ribonucleoside	Stable glycosidic bond [17]

were incubated at 37°. After washing with this medium, counted pairs of worms found to be *in copula* were transferred to 13 × 100 mm rubber-stoppered test tubes containing the analog(s) (1×10^{-4} M) under study in FM. Although these high concentrations of analogs are not related in any way to those which may be used for possible chemotherapeutic applications, they were chosen to show maximal biochemical effects. Usually, worms were incubated for 15 min with analog before [8-^{14}C]adenosine (25 mCi/m-mole; 10^{-5} M final concn) was added. In combination studies denoted "sequential" (see Fig. 9), analogs were added to worms in FM at intervals of 10 min. The last analog was added 10 min before [8-^{14}C]adenosine. Routinely, ten worm pairs were incubated at 37° in a total volume of 2 ml.

For the results presented below, the medium was sampled and extracted at various times throughout the incubation period in order to monitor disappearance of adenosine and the appearance of metabolic products derived from adenosine. Methods used here have been fully described previously [6]. At the end of the incubation period, worms (plus incubation medium) were transferred to conical glass microhomogenizer tubes and allowed to settle to the bottom of the tubes. Medium was removed by Pasteur pipet. The worm pairs were washed three times with 2 ml FM; after removal of the final wash solution, worms were blotted with a cotton swab to remove residual FM. FM (2 ml) was then added to the worms followed by 50 μl of 20 per cent perchloric acid. Worms were then extracted by grinding in the microhomogenizer tubes. After removal of denatured proteins by centrifugation at 1350 *g* in a clinical centrifuge for 5 min, 200 μl of the supernatant solutions was neutralized with KOH. Insoluble $KClO_4$ was removed by centrifugation. All manipulations were carried out at 4°. The supernatant solutions were stored (−20°) for analysis.

Aliquots (20 μl) of the neutralized supernatant solutions were subjected to thin-layer chromatography on PEI-cellulose using the method of Crabtree and Henderson [18]. By the use of this method, which employs a stepwise concentration gradient of sodium formate buffer (pH 3.4) as developing solvent, the mono-, di- and triphosphate ribonucleotides of adenine and guanine are well resolved. After chromatography, spots are visualized by ultraviolet light

(2537 Å), cut out and counted in 12 ml of toluene-based scintillation fluid as outlined previously [6].

In a limited number of trials, the supernatants of perchloric acid-extracted worms were analyzed for nucleotide content by means of high pressure liquid chromatography on a Varian LCS-1000 liquid chromatograph equipped with a Reeve–Angel AS-Pellionex-SAX (1 mm × 3 m) column. For these analyses, 0.002 M KH_2PO_4 (pH 4.5) was the low concentrate eluant, and 0.25 M KH_2PO_4) (pH 4.5) in 1.0 M KCl was the high concentrate eluant with a starting volume of 40 ml and flow rates of 14 and 7 ml/hr for the column and gradient pumps respectively. Column effluents were monitored at two wavelengths. The integral detector in the chromatograph, which monitored the effluent at 254 nm, gave a profile of the natural nucleotides. A second variable-wavelength detector (model SF 770 spectroflow monitor, Schoeffel Instruments Corp., Westwood, NJ) was used to detect analog nucleotide formation. The second detector was set at the λ_{max} for the analog under study, i.e. formycin, 295 nm; tubercidin, 272 nm; and N^6-phenyladenosine, 288 nm. In these trials, the worms were incubated only in analogs being tested, but not with adenosine. Conversion of these drugs into analog nucleotides and changes in normal adenine or guanine nucleotide profiles could thus be monitored.

RESULTS

Results shown are presented in three formats: (1) the effect of the addition of drugs on [8-^{14}C]adenosine metabolism as seen in the incubation medium (Figs. 3–6); (2) the influence of the analogs on the total worm adenine and/or guanine nucleotides at the end of an incubation period (Figs. 7–10); and (3) the nucleotide profiles of worms exposed to analogs as seen by high pressure liquid chromatography (Fig. 11). The results presented below represent averages of duplicate or triplicate determinations.

Control (adenosine). From the control graphs in Figs. 3–6, one can note that intact schistosomes metabolize significant amounts of adenosine *in vitro*. Generally, about 30 per cent of the nucleoside is degraded in 2 hr when ten worm pairs are incubated

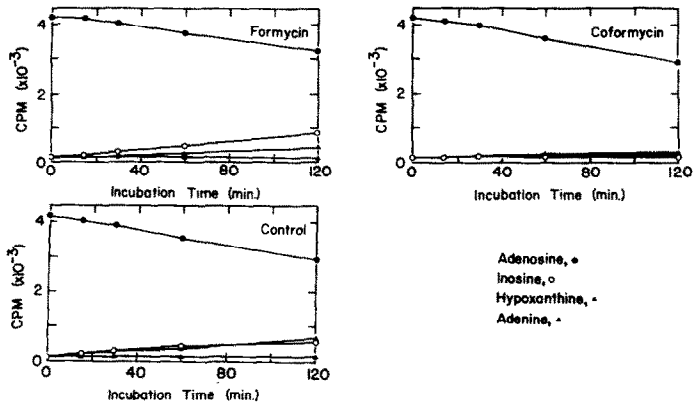


Fig. 3. Effect of formycin and coformycin on the catabolism of adenosine by intact *S. mansoni in vitro*. *S. mansoni* (ten pairs) were incubated in 2 ml of Fischer's Medium containing penicillin (1 unit/ml) and streptomycin (1 μg/ml) at 37°. The analog (10^{-4} M) was present for 15 min before addition of [8-^{14}C]adenosine (sp. act. = 25 μCi/m-mole; 10^{-5} M final concn). Conditions for extraction and analysis are given in Materials and Methods.

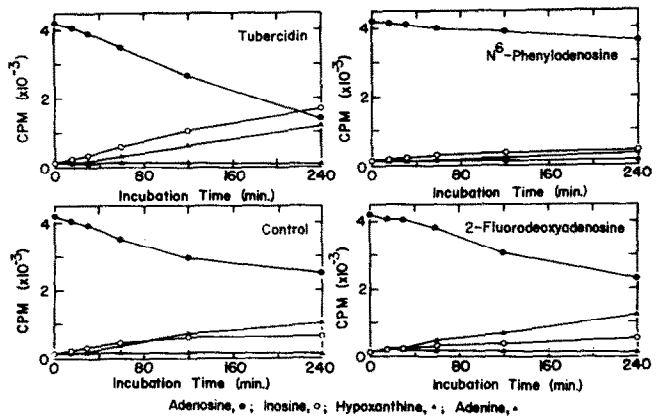


Fig. 4. Effects of tubercidin, N^6-phenyladenosine and 2-fluoro-2′-deoxyadenosine as inhibitors of adenosine catabolism by intact *S. mansoni in vitro*. For incubation and extraction procedures, see Materials and Methods.

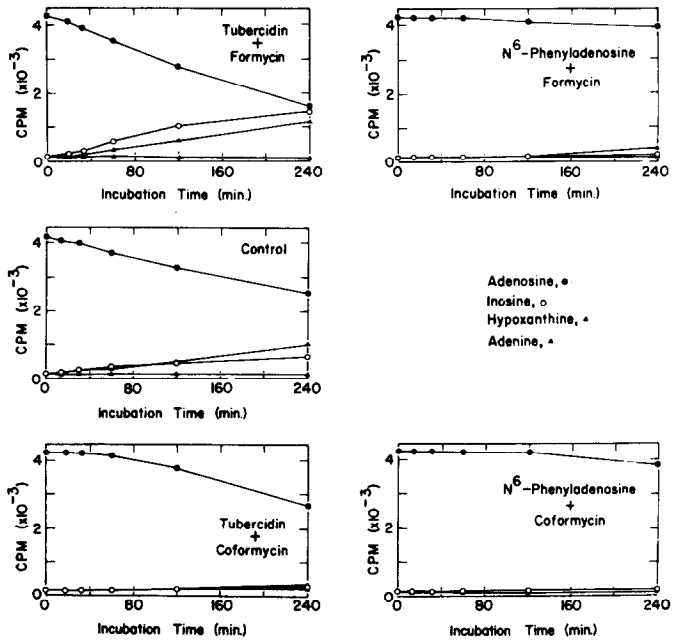


Fig. 5. Effects of combinations of two analog purine nucleosides on the catabolism of adenosine by intact *S. mansoni in vitro*. In each case, both analogs were present for 15 min before addition of [8-^{14}C]-adenosine. Incubation and extraction procedures are given in Materials and Methods.

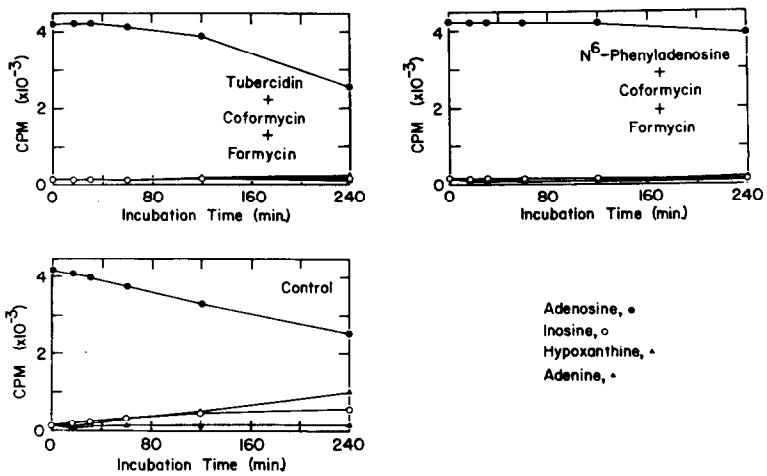


Fig. 6. Effects of combinations of three analog purine nucleosides on the catabolism of adenosine by intact *S. mansoni in vitro*. In each combination, all three analogs were present for 15 min before addition of [8-^{14}C]adenosine. For conditions for incubation, etc., see Materials and Methods.

in 2 ml of 10^{-5} M substrate. Most of the utilized substrate can be accounted for by formation of inosine and hypoxanthine, both of which accumulate in the medium.

Worm extracts have been shown to convert adenosine into nucleotides in the presence of PRPP, or into adenine, inosine and hypoxanthine, in the absence of added PRPP [6]. Typically, reaction rates using worm extracts are significantly greater than those reported here using intact worms.

Formycin. Formycin (Formycin A) has previously been shown [6] to inhibit adenine nucleotide formation from adenosine in worm extract preparations. When whole worms were used (Fig. 3), the rate of utilization of the adenosine substrate was only slightly decreased, compared to the control. Partial blockade of PNPase was noted, resulting in moderate accumulation of inosine in the medium. Measurement of the synthesis of total adenine nucleotides from [8-^{14}C]-adenosine in the worms (Fig. 7) shows a drop of about 37 per cent when formycin, at 10^{-4} M, was utilized to block adenosine metabolism. However, the synthesis of guanine nucleotides from adenosine was not changed when formycin was tested as the inhibiting agent. These data are reflected in the "incorporation ratio" (see legend to Fig. 7) of 1.9 as compared to a control ratio of 2.8.

From Fig. 11, it is apparent that formycin itself

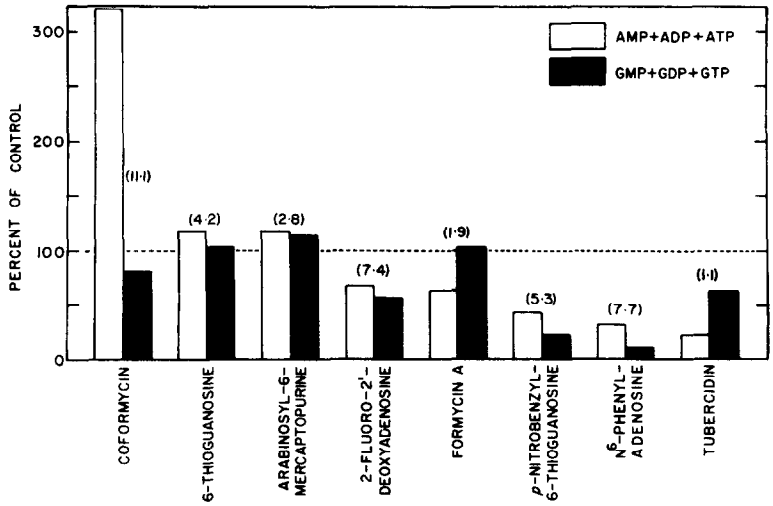


Fig. 7. Effects of single analog purine nucleosides on the synthesis of adenine and guanine nucleotides from adenosine by intact *S. mansoni in vitro*. Conditions for incubation are as for previous figures. Conditions for extraction of worms and analysis of radioactivity in the nucleotides are given in Materials and Methods. Control (100 per cent) levels of radioactivity in nucleotides were approximately: AMP + ADP + ATP, 2800 cpm; GMP + GDP + GTP, 800 cpm. Figures in parenthesis indicate "incorporation ratios," i.e.

$$\frac{\text{cpm in AMP + ADP + ATP}}{\text{cpm in GMP + GDP + GTP}}$$

has several effects in *S. mansoni*: (1) it enters the parasite and is seen in chromatograms as the non-phosphorylated nucleoside; (2) a significant amount of formycin is converted into analog di- and triphosphate nucleotides; formycin monophosphate does not appear to accumulate; and (3) ATP concentration in formycin-treated worms is lower than in control worms.

Coformycin. As shown in Fig 7, the presence of 10^{-4} M coformycin dramatically *increases* the conversion of [8-^{14}C]adenosine into purine nucleotides *in vitro* using intact worms. The amount of adenine nucleotides synthesized is more than triple that observed with control worms. As expected, coformycin effectively blocks the deamination of adenosine (Fig. 3), since both inosine and hypoxanthine fail to accumulate in the medium. Apparently, as a consequence of the failure to deaminate adenosine, more of this substrate is available for insertion into the nucleotide pool via the adenosine kinase reaction, or by an indirect pathway: i.e. adenosine phosphorylase and adenine phosphoribosyltransferase (APRTase). The net amount of adenosine substrate utilized by whole worms *in vitro* is about the same as in the controls (Fig. 3); however, essentially all of the adenosine is converted into adenine nucleotides within the worms. The increased conversion of adenosine into adenine nucleotides coupled with the decreased conversion into guanine nucleotides is reflected in the "incorporation ratio" of 11.1 (control ratio = 2.8).

Tubercidin. When 10^{-4} M tubercidin is used, it can be seen that inosine accumulates in the medium up to a level exceeding hypoxanthine (Fig. 4). This is a reversal of accumulation of catabolic by-products, as compared to the control, and indicates, in agreement with previous reports [7], that one effect of this drug is to inhibit worm PNPase. Tubercidin has been reported not to be a substrate for either adenosine deaminase [2, 4] or PNPase [7] in *S. mansoni*. The effect of tubercidin on the synthesis of nucleotides in the intact worm is shown in Fig. 7. Synthesis of both adenine and guanine nucleotides from adenosine is sharply reduced, the former to about 20 per cent of control levels. These results are emphasized by the "incorporation ratio" of 1.1 (control ratio = 2.8). In confirmation of previous reports [5], Fig. 11 illus-

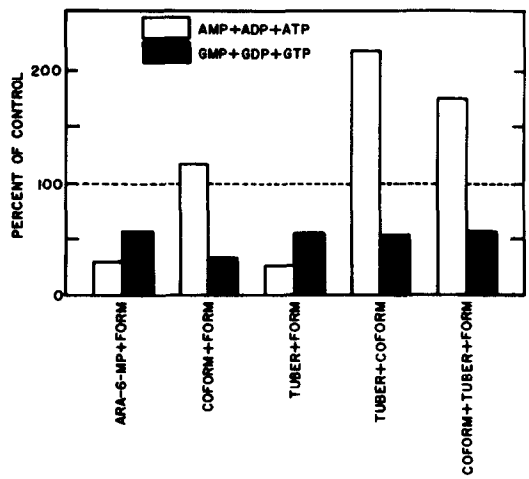


Fig. 8. Effects of combinations of analog purine nucleosides on the synthesis of adenine and guanine nucleotides from adenosine by intact *S. mansoni in vitro*. For conditions for incubation and extraction, see Materials and Methods. In each case, all of the drugs in the various combinations were incubated with worms for 15 min before addition of [8-^{14}C]adenosine. Abbreviations used are: ARA-6-MP, arabinosyl-6-mercaptopurine; FORM, formycin; TUBER, tubercidin; and COFORM, coformycin.

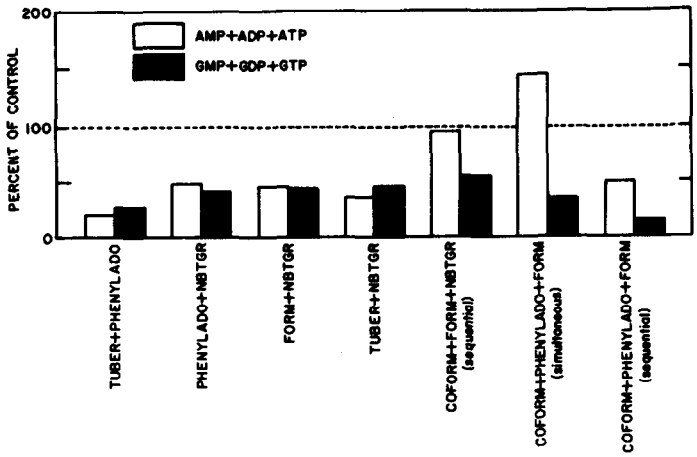


Fig. 9. Effect of combinations of analog purine nucleosides on the synthesis of adenine and guanine nucleotides from adenosine by intact *S. mansoni in vitro*. Conditions for incubation and extraction (and analysis) are given in Materials and Methods. All of the analogs used in the various combinations were incubated with worms for 15 min before addition of labeled precursor except in those cases denoted "sequential." In the sequential combinations, analogs were added to worms in Fischer's Medium in intervals of 10 min. For example, in the COFORM + FORM + NBTGR (sequential) combination, COFORM was incubated with worms for 10 min, FORM was added and incubation continued for 10 min; then NBTGR was added. The last analog of the combination was added 10 min before [8-^{14}C]adenosine. Abbreviations are as for Fig. 8. Others are: PHENYLADO, N^6phenyladenosine; and NBTGR, para-nitrobenzyl-6-thioguanosine.

trates that tubercidin nucleotides produced by whole worms can be identified using high pressure liquid chromatography. Tubercidin triphosphate can be accumulated to levels that actually exceed ATP in the parasites.

2-Fluoro-2'-deoxyadenosine. Figure 4 shows that this analog does not impede the deamination of adenosine, since inosine and hypoxanthine appear in the medium about as rapidly as in the control. Total nucleotide formation from adenosine within the worms is found to be depressed by about 35 per cent, with guanine nucleotide synthesis from adenosine inhibited to a greater extent than adenine nucleotide synthesis. This difference in the degree of inhibition is reflected by the "incorporation ratio" of 7.4 (Fig. 7). High pressure liquid chromatography of worm nucleotides revealed the appearance of a peak after ATP which is consistent with either 2-fluoroATP or 2-fluoro-2'-deoxyATP (data not shown).

p-Nitrobenzyl-6-thioguanosine. This compound is an effective inhibitor of the facilitated transport of nucleosides in human erythrocytes [2]. When tested as an inhibitor of nucleotide synthesis from adenosine in *S. mansoni*, *p*-nitrobenzyl-6-thioguanosine reduced the conversion of adenosine into adenine nucleotides by about 60 per cent and into guanine nucleotides by about 75 per cent (incorporation ratio = 5.3) (Fig. 7). The differences in these two inhibitions cannot be readily explained at the present time. Presumably, *p*-nitrobenzyl-6-thioguanosine has effects other than that of merely inhibiting transport of adenosine into the worms.

6-Thioguanosine. Previously, 6-thioguanosine was shown to be an inhibitor of the adenosine deaminase and purine nucleoside phosphorylase reactions in *S. mansoni* extracts [6]. If similar inhibitions were manifested in intact worms, in the presence of this analog, one might expect a reduction in the incorporation of adenosine into nucleotides. However, when whole worms were incubated with [8-^{14}C]adenosine in the presence of 6-thioguanosine, no inhibition of the synthesis of either adenine or guanine nucleotides was observed (Fig. 7). The slight stimulation of the incorporation of adenosine into adenine nucleotides in the presence of this analog is reflected in the "incorporation ratio" of 4.2 (control = 2.9).

Arabinosyl-6-mercaptopurine. Although this analog has been shown to inhibit the adenosine deaminase reaction in *S. mansoni* extracts [6], no effect on the synthesis of adenine or guanine nucleotides from

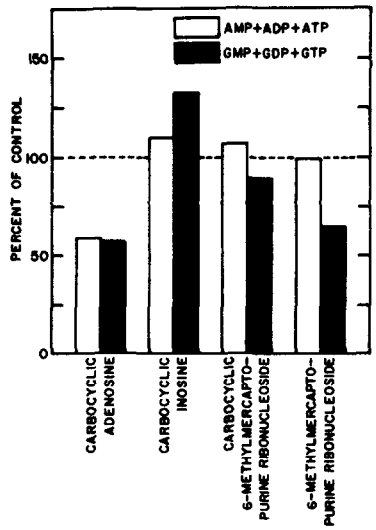


Fig. 10. Effects of carbocyclic nucleosides and 6-methylmercaptopurine ribonucleoside on the synthesis of adenine and guanine nucleotides from adenosine by intact *S. mansoni in vitro*. Conditions for incubation and extraction are given in Materials and Methods.

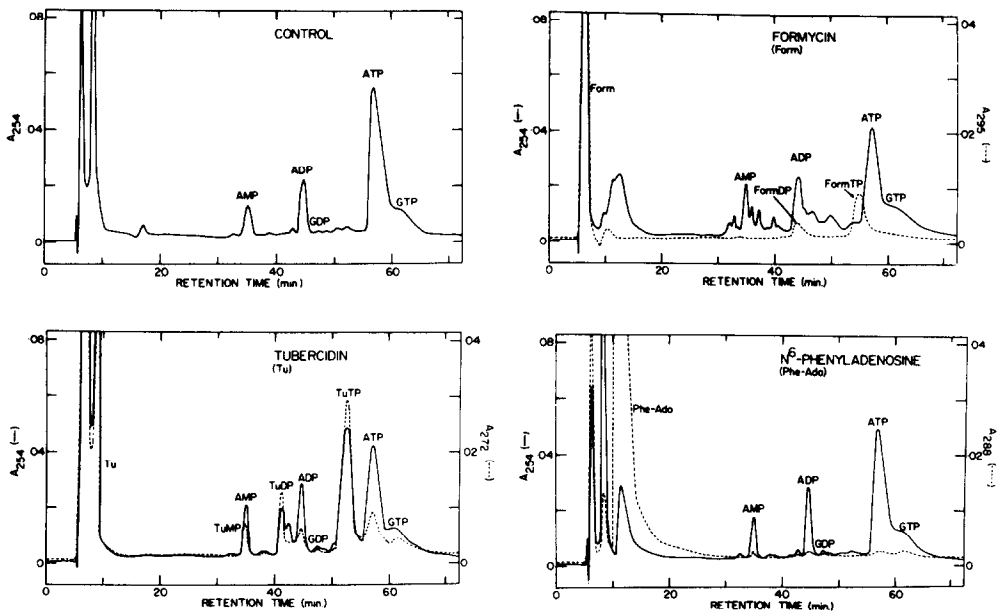


Fig. 11. Nucleotide profiles of intact *S. mansoni* incubated *in vitro* with analog purine nucleosides as determined by high pressure liquid chromatography. Conditions for incubation are as for Fig. 3 except that [8-^{14}C]adenosine was omitted. Conditions for extraction are as for Fig. 7. Aliquots (50 μl) were subjected to high pressure liquid chromatography as outlined in Materials and Methods.

adenosine was apparent with whole worm incubations (Fig. 7). In fact, both processes were slightly elevated over control levels, although the "incorporation ratio" (2.8) was virtually unchanged from the control value (2.9). Whether this lack of inhibition using intact worms indicates poor transport of this analog into the worms is not clear at the present time. Unlike 6-thioguanosine, arabinosyl-6-mercaptopurine does not appear to be anabolized to the nucleotide level in mammalian cells [14] nor is it converted to 6-mercaptopurine [15] and, therefore, arabinosyl-6-mercaptopurine appears to exert its effects as the arabinosyl nucleoside *per se* [15].

N^6-*phenyladenosine.* This agent interferes markedly with utilization of adenosine by intact *S. mansoni* (Fig. 4). It appears to block adenosine deamination partially, since both inosine and hypoxanthine fail to accumulate up to the levels found in the control experiments. Furthermore, it sharply decreases total adenine and guanine nucleotide formation from adenosine in whole worms (Fig. 7). The combined inhibition of adenine and guanine nucleotides, after 4 hr of incubation, is greater with this drug than any others of the group tested. The greater inhibition of adenine nucleotide synthesis from adenosine is shown by the "incorporation ratio" of 7.7 (control = 2.9).

High pressure liquid chromatography of extracted worms exposed to N^6-phenyladenosine reveals that the analog itself is not converted into nucleotides within intact schistosomes although the nucleoside appears to accumulate inside the worms (Fig. 11). In this respect, this analog is substantially different from such drugs as tubercidin, formycin or 2-fluoroadenosine (see Fig. 11 and Ref. 5).

Carbocyclic purine nucleosides. A series of nucleosides which contain a carbon atom in lieu of the bridge oxygen atom in the ribofuranosyl ring of natural nucleosides has been tested as possible inhibitors of nucleotide synthesis from adenosine in intact *S. mansoni.* These compounds include carbocyclic-adenosine, carbocyclic-inosine and carbocyclic-6-methylmercaptopurine ribonucleoside (see Fig. 1). Of these compounds, only carbocyclic-adenosine appeared to be effective as an inhibitor, giving reductions in the conversion of [8-^{14}C]adenosine to adenine and guanine nucleotides of about 40 and 45 per cent respectively (Fig. 10). Carbocyclic-inosine appears to stimulate both processes slightly, whereas carbocyclic-6-methylmercaptopurine ribonucleoside very slightly inhibited guanine nucleotide synthesis but did not inhibit adenine nucleotide synthesis. Similarly, 6-methylmercaptopurine ribonucleoside itself had no effect on adenine nucleotide synthesis but appeared to inhibit guanine nucleotide synthesis by about 35 per cent (Fig. 10). Interestingly, the carbocyclic analogs of inosine and 6-methylmercaptopurine ribonucleoside are not effective as cytotoxic agents against a human epithelial carcinoma cell line in culture, whereas carbocyclic-adenosine is highly cytotoxic [17]. Carbocyclic-adenosine has been shown to be a good substrate for adenosine kinase and is converted to the triphosphate nucleotide level in intact mammalian cells [17].

Combination studies. Various combinations of two or three analogs were tested in an attempt to inhibit the incorporation of adenosine into nucleotides in intact worms. The results of these trials are presented in Figs. 5, 6, 8 and 9.

When coformycin was used in combination with one or more nucleoside analogs, virtually none of the adenosine substrate appeared as purine nucleosides or bases in the medium (Figs. 5 and 6); however, faci-

lhation of formation of nucleotides was noted in the worms (Fig. 7). This undoubtedly is a reflection of the increased availability of adenosine concomitant to the blockade of adenosine deaminase. Sequential use of coformycin + N^6-phenyladenosine + formycin inhibited adenine nucleotide synthesis by about 50 per cent and that of guanine nucleotides by about 80 per cent (Fig. 9).

Several combinations of formycin with other compounds proved to be moderately effective as inhibitors of adenosine anabolism. Arabinosyl-6-mercaptopurine + formycin or tubercidin + formycin reduced adenine nucleotide synthesis by 70 per cent and guanine nucleotide synthesis by about 40 per cent (Fig. 8). The combination of formycin + *p*-nitrobenzyl-6-thioguanosine (a nucleoside transport blocking agent) inhibited adenine and guanine nucleotide synthesis by 50 per cent, and appreciable inhibition was also shown when *p*-nitrobenzyl-6-thioguanosine was combined with N^6-phenyladenosine or tubercidin (Fig. 9).

The most effective combination of agents was tubercidin + N^6-phenyladenosine, which reduced adenine and guanine nucleotide synthesis to 20 and 25 per cent of control values respectively. Interestingly, the combination of tubercidin + formycin (Fig. 5) does not inhibit the conversion of adenosine into other bases and nucleosides in the medium, even though it is relatively inhibitory for synthesis of nucleotides within the worm (Fig. 8).

It seems clear, however, that none of the drug combinations tested in this study gave greater inhibition of adenosine conversion into nucleotides than did the most potent individual drugs themselves.

DISCUSSION

The results presented above demonstrate that none of the various types of adenosine analogs tested in these experiments are capable of completely inhibiting the conversion of adenosine into adenine and guanine nucleotides in intact *S. mansoni in vitro*. From the data reported here, the best candidates appear to be tubercidin and N^6-phenyladenosine, both of which cause a 70–75 per cent decline in nucleotide formation from adenosine. Of these two, tubercidin is slightly more effective in blocking adenine nucleotide synthesis, while N^6-phenyladenosine appears to decrease guanine nucleotide synthesis more effectively. One other compound, *p*-nitrobenzyl-6-thioguanosine, which was chosen for its possible ability to block the uptake of adenosine by worms, caused a considerable reduction of the conversion of adenosine into nucleotides in the worms exposed to this agent.

The appearance of an analog nucleotide peak in the triphosphate region of high pressure liquid chromatographic profiles of extracts of worms treated with 2-fluoro-2′-deoxyadenosine deserves comment. This peak could represent either 2-fluoroATP or 2-fluoro-2′-deoxyATP. Evidence on hand suggests that 2-fluoroATP cannot be derived from 2-fluoro-2′-deoxyadenosine, since this analog nucleoside is not cleaved to the free base, 2′-fluoroadenine (which is capable of being converted to 2-fluoroATP, see Ref. 5) when incubated with *S. mansoni* extracts containing both adenosine phosphorylase and purine nucleoside phosphorylase [7]. Therefore, it appears that 2-fluoro-2′-deoxyadenosine can only be converted into nucleotides via the adenosine kinase reaction. In this regard, it should be noted that, in human erythrocytes, 2-fluoro-2′-deoxyadenosine does not progress past the monophosphate nucleotide level, i.e. 2-fluoro-2′deoxyATP does not accumulate [10]. These results indicate that 2-fluoro-2′-deoxyAMP is not a substrate for human erythrocytic adenylate kinase. The fact that, in schistosomes, analog triphosphate nucleotide is recognized, may indicate that the parasitic adenylate kinase has different substrate specificity than the human erythrocytic enzyme.

Interestingly, one of the most potent inhibitors of the synthesis of nucleotides from adenosine, namely N^6-phenyladenosine, has recently been shown to be a strong inhibitor ($K_i = 6 \times 10^{-7}$ M) of adenosine kinase partially purified from Sarcoma 180 cells in culture [9]. This observation gives support to the belief that adenosine kinase may play a significant role in the conversion of adenosine into nucleotides in these worms. Further support comes from the fact that tubercidin, a substrate for adenosine kinase, is readily converted into analog nucleotides in intact schistosomes ([5] and Fig. 11 of this paper), and is also second in potency only to N^6-phenyladenosine as an inhibitor of the conversion of adenosine into nucleotides in intact worms.

In addition to its effects on the adenosine kinase reaction, N^6-phenyladenosine appears to block worm adenosine deaminase since inosine and hypoxanthine are found in the medium only in small amounts (Fig. 4). These effects of N^6-phenyladenosine presumably are due to the analog nucleoside *per se* since this compound does not appear to form analog nucleotides in intact worms (Fig. 11). Tubercidin, in contrast, does not appear to inhibit the adenosine deaminase reaction to a significant extent. Rather, a site of inhibition by tubercidin appears to be at the purine nucleoside phosphorylase reaction since, in the presence of tubercidin, inosine accumulates to a greater extent than does hypoxanthine, whereas the reverse is true in control experiments.

In a previous paper from this laboratory, the role of the pathway catalyzed by the enzymes adenosine deaminase, purine nucleoside phosphorylase, and hypoxanthine phosphoribosyltransferase in the conversion of adenosine into nucleotides was emphasized. These previous reports on similar studies using worm extracts have tended to minimize the significance of the adenosine kinase reaction [3, 4, 6]. Data presented here, however, modify this belief. Coformycin, an extremely potent inhibitor of adenosine deaminase, does not inhibit adenosine anabolism in intact *S. mansoni in vitro*. Rather, in the presence of coformycin, the synthesis of nucleotides from adenosine is significantly stimulated. Apparently, in the presence of coformycin a larger concentration of the substrate (adenosine) is available for alternative pathways of synthesis (the adenosine kinase pathway, and the adenosine phosphorylase–adenine phosphoribosyltransferase pathway).

The difficulty encountered in abolishing nucleotide formation from adenosine in the intact worms through the use of adenosine analogs, singly or in combination, does not appear to rest in an inability

to block specific pathways, since these can be inhibited *in vitro* using supernatants of homogenates as test systems. In some cases, the problem may lie in the inability to transport such analogs into the parasite itself. As an example, it has already been shown [6] that arabinosyl-6-mercaptopurine can prevent practically all adenosine metabolism in a homogenate preparation. However, within the intact worm (Fig. 8), inhibition is much less complete using the same drug. Likewise, the triple approach of coformycin + N^6-phenyladenosine + formycin (Fig. 6) virtually abolishes catabolism of adenosine in the medium, but does not succeed in eliminating the conversion of adenosine into nucleotides, although this conversion is decreased by about 50 per cent (Fig. 9). As noted previously [6], other factors which should also be considered in attempting to explain the lack of effect of some of these analogs on intact worms include possible drug metabolism within the worms, compartmentation of enzymes within worms and metabolic regulation of enzymes within worms.

Because the importance of the adenosine kinase reaction for the synthesis of nucleotides from adenosine in intact worms has been re-evaluated as a result of the studies reported here, analogs which are inhibitors of adenosine kinase should be closely scrutinized as potentially useful antischistosomal agents. One unstudied candidate is 7-iodo-tubercidin [4-amino-5-iodo-7-β-D-ribofuranosyl-7-H-pyrrolo(2,3,d)pyrimidine]. In studies by Henderson *et al.* [19], this compound was found to be the most potent inhibitor of adenosine kinase (from Ehrlich ascites cells) of many purine analogs examined. In addition, our colleagues have shown that this analog is not converted into analog nucleotides in human erythrocytes (G. W. Crabtree, R. E. Parks, Jr. and L. B. Townsend, unpublished data).

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