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PATHWAYS OF NUCLEOTIDE METABOLISM IN *SCHISTOSOMA MANSONI*—IV

INCORPORATION OF ADENOSINE ANALOGS *IN VITRO**

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Abstract—The incorporation *in vitro* of adenine or adenosine analogs into schistosome nucleotides is demonstrated. Tubercidin, 2-fluoroadenosine and 2-fluoroadenine were all shown to be converted into analog triphosphate nucleotides. Since tubercidin and 2-fluoroadenosine are not substrates for adenosine deaminase or purine nucleoside phosphorylase and are not susceptible to degradation to the free base level, it is assumed that they are converted to nucleotides by reaction with adenosine kinase. The incorporation of 2-fluoroadenine into the nucleotide pools indicates that it serves as a substrate for adenine phosphoribosyltransferase.

Tubercidin, added to the culture medium, interferes with the maintenance of normal ATP levels. When the concentration of the analog greatly exceeded that of adenine or adenosine in the medium, virtual shutdown of adenosine triphosphate synthesis followed. It is suggested that stoichiometric competition for enzyme sites may determine the relative amounts of nucleotides formed.

PREVIOUS communications[1,2] have indicated that the human blood fluke, *Schistosoma mansoni*, lacks (or is severely deficient in) the ability to synthesize purines *de novo*. In consequence, this parasite must resort to salvage mechanisms to sustain nucleotide production. It has been shown[3,4] that adenine, adenosine, inosine, and hypoxanthine are suitable precursors for ATP productions *in vitro*. Adenine is anabolized to AMP via adenine phosphoribosyltransferase (APRT).‡ Adenosine, on the other hand, can be processed by two mechanisms: the adenosine kinase reaction to yield AMP, and the indirect pathway: adenosine → inosine → hypoxanthine → IMP → AMP. The indirect pathway is much more active than the direct kinase route.

This paper will give evidence that analogs of adenine and adenosine are incorporated into schistosomal nucleotide pools *in vitro*. Thus, these studies suggest that a chemotherapeutic approach to the treatment of this parasite disease may lie in the use of such analogs.

MATERIALS AND METHODS

1. *Incorporation of adenine or adenosine analogs into nucleotide pools of* Schistosoma mansoni

Determination of normal nucleotide spectra. Four pairs of worms were recovered from the mesenteric veins of CF[1] mice which had previously been infected with *S. mansoni* at the Merck Institute (Rahway, N.J.). After briefly washing twice in

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‡ Abbreviations used in this communication are identified in paper III of this series.[4]

Fischer's medium (FM) at 37°, the worms were immediately homogenized in 20 μl of ice-cold 10% trichloroacetic acid (TCA) in a 1-ml glass tissue grinder. The suspension was centrifuged (2900 rev/min in a clinical centrifuge) at 4° and about 15 μl of clear supernatant fluid was recovered. TCA was extracted with water-saturated diethyl ether. A 10-μl aliquot sample was then analyzed for nucleotide content by means of a Varian LCS-1000 high pressure liquid chromatography apparatus. Details of the elution procedure have been published elsewhere.[5]

Incorporation of 7-deaza-adenosine ("tubercidin") into worm nucleotides. Worms were washed briefly in FM and then placed in 8 × 118 mm test tubes, each containing a measured amount of substrate in 2 ml of FM. Four pairs of worms taken from a single mouse were incubated in each test tube for 90, 240 and 480 min. Worms were incubated in FM containing no purine substrate, FM plus tubercidin (0·75 mM), FM plus adenine (0·75 mM) plus tubercidin (0·75 mM), FM plus adenosine (0·75 mM) plus tubercidin (0·75 mM), and FM plus adenine or adenosine (also at 0·75 mM) without analog antagonist.

Immediately after the incubation, the wet weight of each worm sample was determined. Schistosomes were removed from the test tubes with a dental pick and then placed on a paper towel in order to absorb excess fluid. Worms were then transferred to 1 cm^2 pre-weighed boats constructed of Lilly weighing paper. These were then weighed on a micro-analytical balance. Analysis for nucleotide content followed procedures outlined above.

Identification of adenine and guanine nucleotides was made by referring to elution patterns of known compounds. In some cases the addition of authentic ATP to the sample was used as a means for corroboration. Since the amount of specific nucleotide represented by its peak is proportional to the area under the curve, calculation of total nucleotide content and ratios per milligram of worm tissue could be made. In these calculations, allowances for different extinction coefficients of adenine, guanine and analog-triphosphate nucleotides were made.

Incorporation of labeled tubercidin into worm nucleotides. Uniformly labeled ^{3}H-tubercidin (sp. act. 970 Ci/mg) was obtained from the Upjohn Company, courtesy of Dr. G. S. Fonken. In order to assure purity of the compound, an aliquot was chromatographed on Whatman No. 3 paper, using NH_4OH–H_2O (1:10, pH 10) as the solvent. The drug position was identified by means of light absorption from a Camag u.v. lamp at 254 nm. The tubercidin was eluted from this spot, using NH_4OH–H_2O (1:10). Fifty μl of the eluent was found to contain 4·9 μCi (6 μg). This was added to 1·5 ml of FM. Twenty-six worm pairs were washed with several millilitres of warm FM and then transferred to the radioactive medium. The parasites were incubated 240 min at 37°. After incubation, worms were washed briefly twice and then homogenized in 100 μl cold TCA. Analysis for nucleotide content was as given above except that the effluent from the chromatography column was collected in 2-min fractions by means of a Gilson model MF Escargot fractionator. Thirty-five to thirty-seven 0·2-ml fractions were collected from each sample. These samples were transferred to glass scintillation vials containing 9·0 ml of Aquasol (New England Nuclear). After a 30-min storage in the dark, the samples were counted for 10 min, using a Packard Tri-Carb Spectrometer.

Incorporation of 2-fluoroadenosine or 2-fluoroadenine into schistosome nucleotides. Worms (22 pairs) were incubated in 2 ml of FM to which was added 10^{-3} M 2-fluoro-

adenosine or 2-fluoroadenine as substrate. After a 60-min incubation at 37°, the schistosomes were removed, washed in FM and then analyzed for nucleotide content using procedures described in Section I.

2. *Incorporation of labeled adenine into schistosome nucleotides in the presence and absence of tubercidin*

Four ml of FM was added to a weighed amount of adenine (P–L Biochemicals) and adenine-8-^{14}C (sp. act. 46 mCi/m-mole) such that the final concentration was 0·75 mM and contained about 1·0 μCi/ml. This solution was then dispensed into two 8 × 118 mm test tubes. To one of the tubes tubercidin was added to make the final analog concentration 1·5 mM, or about twice that of the natural substrate. Six to twelve pairs of washed worms/tube were incubated in the media at 37° for from 4 to 8 hr. The schistosomes were removed, blotted, and their wet weight was determined as above. Nucleotides were extracted by homogenization in cold 10% TCA, after which they were analyzed for nucleotide content and radioactivity, using techniques described above.

RESULTS

The typical spectrum of nucleotides found in normal *S. mansoni* freshly recovered from CF^1 mice is seen in Fig. 1A. A predominance of ATP and an ATP–ADP ratio of 5:1 can be noted. AMP is usually found in very low concentration. GTP is most often seen as a peak along the trailing edge of ATP, although by modifying the elution conditions the GTP can be cleanly separated. A sharp, prominent peak that follows AMP at about 27 min in Fig. 1A has not yet been rigorously identified; however, it has a retention time equivalent to NADP and UDPG.

Incorporation of 7-deaza-adenosine, 2-fluoroadenosine or 2-fluoroadenine into worm nucleotides is shown in several ways. When unlabeled analog compounds at levels of 0·7 to 1·5 mM are used, the appearance of new nucleotide species can be noted on the chromatograms, especially of the di- and triphosphate variety (Figs. 1, 2, 3 and 4). Tubercidin triphosphate (TuTP) generally elutes slightly ahead of ATP and thus can be seen as a peak which appears at about 2–4 min before ATP.*

The 2-fluoroadenosine triphosphate (2-FATP) is delayed under our elution procedures and appears considerably after GTP on the liquid chromatogram (Figs. 2 and 3). Although both 2-fluoroadenine (2-FAd) and 2-fluoroadenosine (2-FAR) yield the same end product (2-FATP), it appears that 2-FAR enters the parasite or is converted to the triphosphate compound more quickly than the base. Worms were observed to have greatly increased activity characterized by writhing and whipping movements shortly after exposure to both of these fluoro compounds.

When ^{3}H-labeled tubercidin was employed as a substrate it was possible to identify a spectrum of phosphorylated analog nucleotides whose ratio of distribution was very similar to normal adenine nucleotides. In addition, a large peak of radioactivity appearing with the nucleoside fraction suggests that tubercidin has little difficulty entering into the worm nucleoside pool (Fig. 5).

The effect of these analogs on schistosome purine nucleotide metabolism over a short term is difficult to assess. It is known that incubation of worms in FM in the

* Considerable care must be exercised in the identification of elution peaks, especially those of tubercidin phosphates. We have recently noted the appearance of TuTP following ATP when the only altered conditions was the routine change of a high pressure pellicular column on the chromatography apparatus.

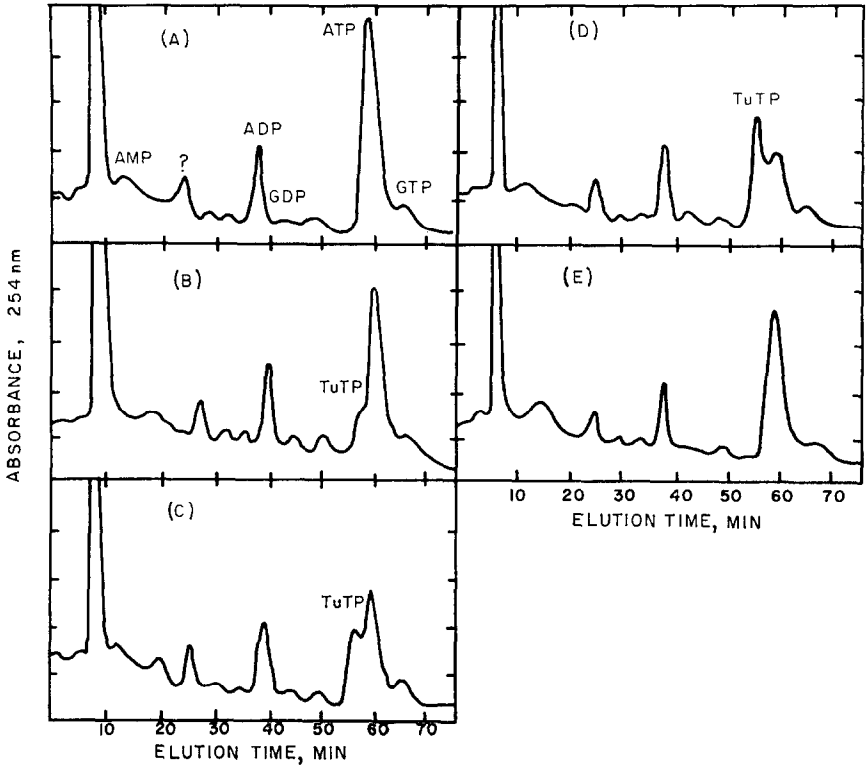

FIG. 1. Incorporation of tubercidin into schistosome nucleotides. (A) Spectrum of nucleotides in normal *S. mansoni* worm pairs freshly recovered from CF[1] mouse host. Four worm pairs were homogenized in 10% trichloroacetic acid and an aliquot of ether-extracted supernatant was analyzed on a Varian LCS high pressure liquid chromatograph. Full scale at 254 nm was 0·04 o.d. Samples were eluted off the column with a KH_2PO_4 buffer gradient in increasing molarity from 0·015 to 0·25 M. KCl, 2·2 M, is present in the high concentrate buffer. (B–D) Worms were incubated in FM plus added adenine (0·75 mM), plus tubercidin (0·75 mM). B: 90 min; C: 240 min; D: 480 min. (E) Worms were incubated for 480 min in FM plus adenine (0·75 mM), but without tubercidin.

absence of purines leads to a gradual loss of ATP (Fig. 6A). However, when tubercidin is added alone as a substrate, the ATP concentration in the worms is drastically suppressed (Fig. 6B) after 400 min of incubation. When adenine or adenosine plus tubercidin was used in the medium (Fig. 7), a more gradual decrease of ATP and a more pronounced rise in the analog triphosphate nucleotide was observed (Fig. 1). Normal adenine nucleotide levels in *S. mansoni* are generally found to be 5–7 nmoles/mg of worm pairs. In the presence of 0·75 mM adenine, nucleotide levels will more than double in 100 min. This high level is gradually reduced with continued incubation (Fig. 7). The addition of tubercidin (0·75 mM) effectively blocks this rapid synthesis of adenine nucleotides.

When tubercidin is added to the medium at twice the concentration of adenine (Fig. 8), only a small amount of the normal substrate is converted to ATP at the end of 4 hr. After 8 hr of exposure to tubercidin (Fig. 9), virtually none of the labeled adenine was incorporated in ATP. This suppression is not due to failure of entry of the normal substrate into the worm, since one can detect labeled adenine (or its riboside) as the initial peak eluted during chromatography.

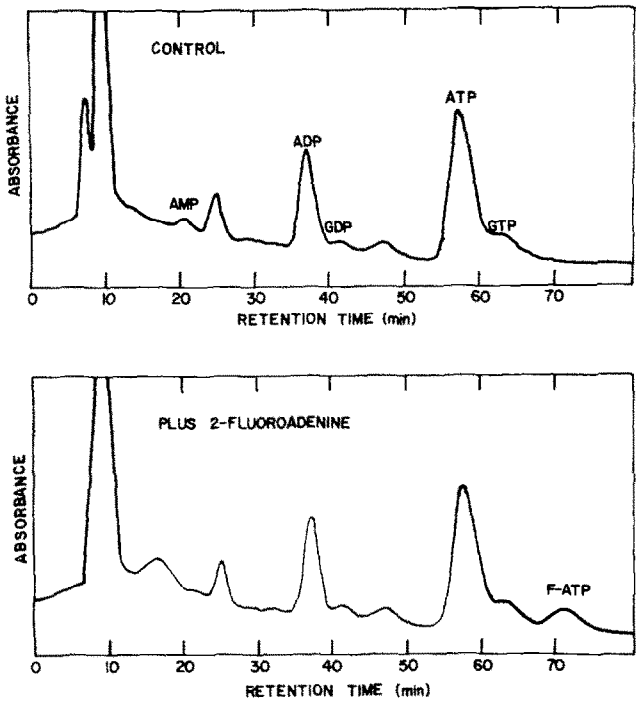

FIG. 2. Formation of analog-ATP using 2-fluoroadenine as substrate. Six worm pairs were incubated in 2 ml FM plus 2-fluoroadenine (1 mM) for 120 min. Preparation and measurement of nucleotides as in Fig. 1.

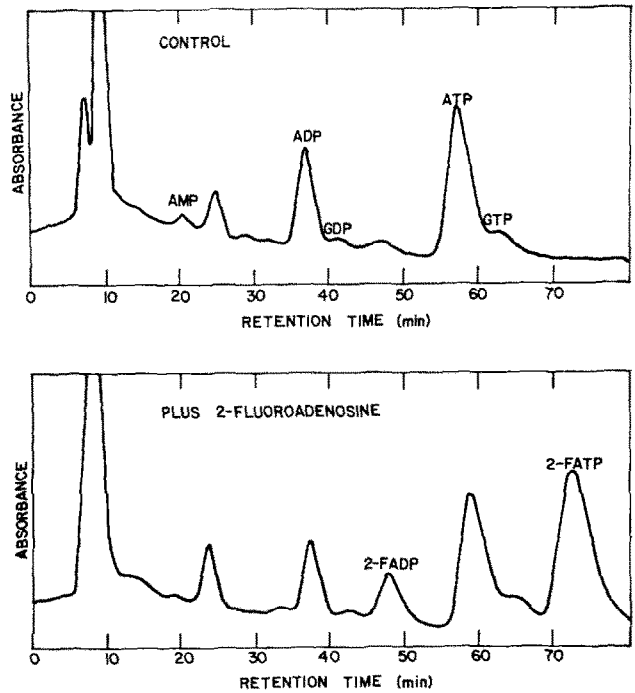

FIG. 3. Conversion of 2-F-adenosine into 2-FATP by schistosomes *in vitro*. Conditions as in Fig. 2 with the exception that the riboside was used as the substrate.

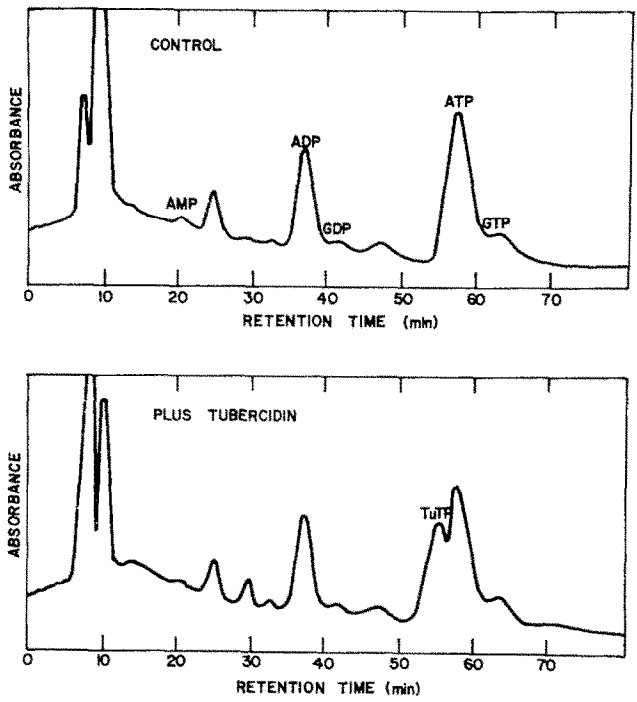

FIG. 4. Conversion of tubercidin into TuTP by *S. mansoni*. Conditions as in Fig. 2.

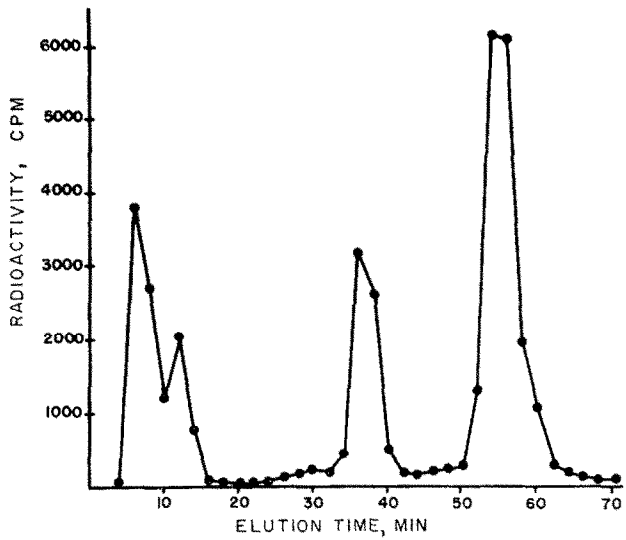

FIG. 5. Distribution of ^{3}H tubercidin nucleotides in *S. mansoni* after incubation. Worms (26 pairs) were incubated for 4 hr in tritiated tubercidin (4 mg/ml; sp. act. 0·8 μCi/μg). Samples of the effluent from the chromatography apparatus were collected for scintillation counting. The spectrum shows that most of the nucleotide activity is present as TuTP. The ratios of TuTP:TuDP:TuMP approximate those of normal purine nucleotides. Elution times of the analog nucleotides are very slightly accelerated in comparison to normal physiological adenine nucleotides.

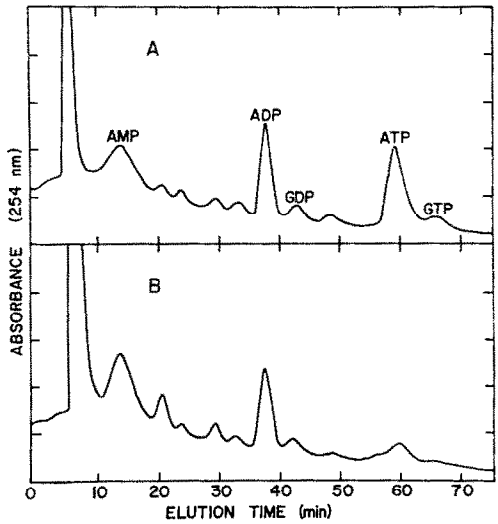

FIG. 6. Depression of ATP in *S. mansoni* worms incubated in the absence of purine substrate. (A) Incubation of worms in FM for 480 min. Conditions of extraction and measurement as in Fig. 1. (B) Schistosomes incubated in FM plus tubercidin (200 μg/ml) for 480 min. ATP and GTP levels are profoundly depressed. AMP concentration is unusually large.

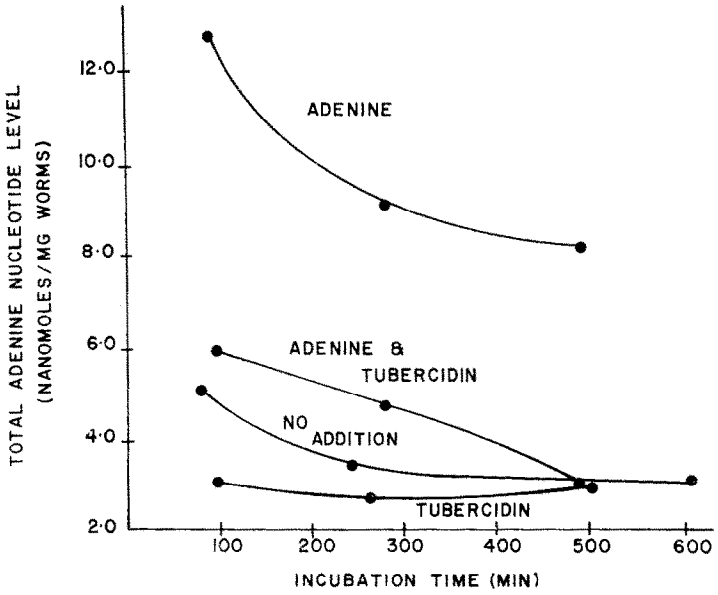

FIG. 7. Total adenine nucleotide levels in *S. mansoni* after incubation with adenine and tubercidin. For each point, 4 pairs of *S. mansoni* worms were incubated in 2 ml of Fisher's medium. All substrates were present at a level of $0{\cdot}75 \times 10^{-3}$ M. Similar data were obtained when adenosine was substituted for adenine.

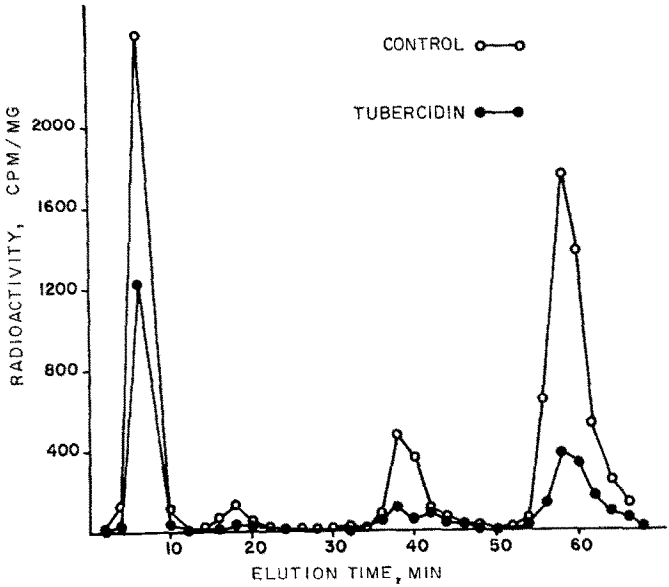

FIG. 8. Incorporation of adenine-8-^{14}C into schistosome nucleotides in the presence of tubercidin. Two groups of schistosomes were incubated with radioactive adenine (2 ml of 0·75 mM; sp. act. 1·3 mCi/m-mole) for 4 hr. Tubercidin was added to one tube at a level of 1·5 mM. Effluent fractions were collected at 2-min intervals in 0·2-ml fractions. Specific radioactivity of each nucleotide species was determined on the basis of wet weight of the schistosome sample.

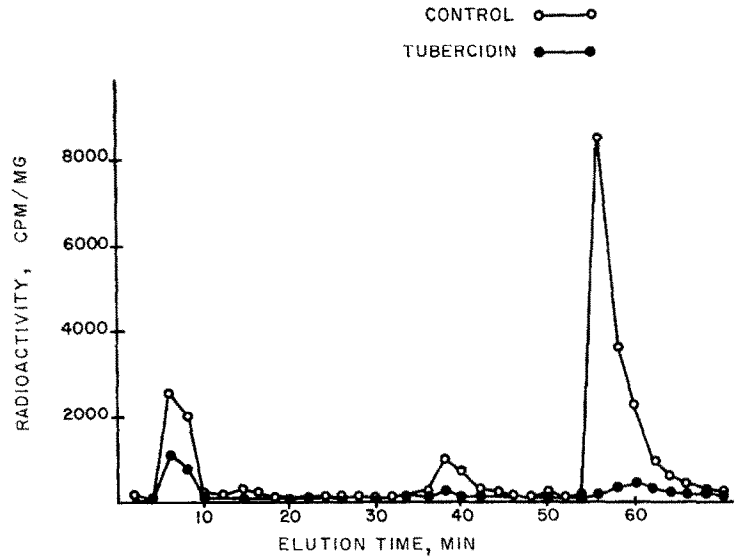

FIG. 9. Incorporation of labeled adenine in schistosome purine nucleotides in the presence of tubercidin. Same experiment as described in Fig. 8, except that the worms were incubated for 8 hr. Almost no adenine is converted into purine nucleotides after prolonged exposure to tubercidin.

DISCUSSION

These experiments indicate that a number of adenine or adenosine analogs are useful candidates in the search for new schistosome chemotherapeutic drugs. Biochemical evidence for entry into the worm's metabolic pool can be shown to be an early event following incubation with an analog. However, the net effect of the incorporation of these analogs is more difficult to assess during short-term experiments. It is possible to show depression of synthesis of ATP, as well as a shift in the ATP:ADP ratio in the presence of tubercidin.

Since tubercidin and 2-fluoroadenosine are not deaminated by schistosome adenosine deaminase and also are not substrates for purine nucleoside phosphorylase,[4] they must be converted to the 5'-monophosphate nucleotides by the adenosine kinase route. Previous papers[3,4] have suggested that the direct kinase reaction accounts for only about 30 per cent of adenosine conversion to AMP. Thus, analogs may be anabolized only by the less active of two routes. It is therefore likely that the rate of formation of fluoroATP from 2-fluoroadenosine or TuTP from tubercidin might be less rapid than the conversion of an equimolar concentration of adenosine to ATP.

It also appears from this data that tubercidin and 2-fluoroadenosine are substrates for AMP kinase and nucleoside diphospholkinase. Since the ratio of analog-ATP to analog-ADP closely simulates the normal distribution of ATP:ADP (see Fig. 4), one is tempted to believe that these analogs and their derivatives do not inactivate the purine anabolic enzymes. They may, instead, be competitive, alternative substrates. Their efficiency as antischistosome agents would, therefore, rest on how effectively the drug could be concentrated in the schistosome. Evidence presented by Jaffe[6] suggests that therapeutic activity could be seen by exteriorizing erythrocytes, loading them with tubercidin, and then reinjecting the TuTP-rich red cells into the host for ingestion by these parasites. A similar approach might be considered for other purine analogs such as F-Ad or F-AR which are also readily incorporated into the nucleotide pools of erythrocytes.

This study did not address itself to the mechanisms by which the worms take up adenine, adenosine, or their anaolgs from the medium. Thus it has not been determined whether this is a transintegumental or gut-mediated phenomenon. Numerous previous studies have suggested that transintegumental adsorption may be the mechanism of uptake for various small molecules such as carbohydrates and amino acids. The gut itself may be used principally for the uptake of larger compounds.[7–9] Therefore, one additional area for investigation must be to determine what proportion, if any, of purine bases or nucleosides can be acquired through the parasitic tegument, since this information is likely to influence the direction of a putative antischistosome therapy.

This study did not examine the capacity of analogs of guanine or hypoxanthine (such as 6-mercaptopurine and 6-thioguanine) to enter the nucleotide pools of schistosomes. This laboratory has shown that the worms have an active purine nucleoside phosphorylase and have, as well, one or more enzymes related to hypoxanthine guanine phosphoribosyltransferase. Thus this approach to trematode chemotherapy should be a fruitful area for study.

Although incorporation of 7-deazadenosine or fluorinated purines can proceed to the triphosphate nucleotide stage, such metabolism is not necessarily associated with toxic manifestations in the schistosome. It has not yet been established whether

2F-ATP, for instance, can or cannot fulfill the biochemical functions of natural ATP. In the event that uptake of analogs results in a metabolic lesion for the schistosome, some time might be required before the gross effect on the worm's physiology could be recognized. Thus, an important topic for future study is the effect of analog compounds on nucleic acid function and metabolism and on enzymic reactions which normally utilize ATP.

REFERENCES

1. A. W. SENFT, R. P. MIECH, P. R. BROWN and D. G. SENFT, *Int. J. Parasit.* **2,** 249 (1972).
2. G. W. CRABTREE, K. C. AGARWAL, R. P. AGARWAL, P. R. BROWN, E. M. SCHOLAR, D. G. SENFT, A. W. SENFT and R. E. PARKS, JR., *Pharmacologist* **13**(2), 311 (1971).
3. A. W. SENFT, D. G. SENFT and R. P. MIECH, *Biochem. Pharmac.* **22,** 437 (1973).
4. A. W. SENFT, G. W. CRABTREE, K. C. AGARWAL, E. M. SCHOLAR, R. P. AGARWAL and R. E. PARKS, JR., *Biochem. Pharmac.* **22,** 449 (1973).
5. P. R. BROWN, *J. Chromat.* **52,** 257 (1970).
6. J. J. JAFFE, *Nature, Lond.* **230,** 408 (1971).
7. P. J. FRIPP, *Comp. Biochem. Physiol.* **23,** 893 (1967).
8. A. W. SENFT, *Ann. N.Y. Acad. Sci.* **113,** 272 (1963).
9. A. W. SENFT, *Ann. N.Y. Acad. Sci.* **160,** 571 (1969).