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METABOLISM OF ADENOSINE ANALOGUES BY *SCHISTOSOMA MANSONI* AND THE EFFECT OF NUCLEOSIDE TRANSPORT INHIBITORS*

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Abstract—*Schistosoma mansoni* incorporated tubercidin, nebularine, 9-deazaadenosine, 5'-deoxy-5'-iodo-2-fluoroadenosine, 7,9-dideaza-7-thiaadenosine and toyocamycin but not sangivamycin, 3'-deoxysangivamycin, or 1-methylformycin into their nucleotide pool after a 4-hr incubation *in vitro*. In contrast to mammalian systems, addition of nucleoside transport inhibitors nitrobenzylthioinosine 5'-monophosphate (NBMPR-*P*), dilazep, or benzylacyclouridine had no significant effect on the pattern of incorporation of these adenosine analogues. Dipyridamole, on the other hand, reduced, but did not prevent, the incorporation of these analogues into the nucleoside 5'-triphosphate pool. These results suggest that the transport of purine nucleosides in schistosomes is different from that of their mammalian hosts. Therefore, coadministration of a specific nucleoside transport inhibitor with tubercidin, nebularine, 9-deazaadenosine, 5'-deoxy-5'-iodo-2-fluoroadenosine, toyocamycin, or 7,9-dideaza-7-thiaadenosine may result in high selective toxicity against schistosomes, as was the case with the combination of tubercidin plus NBMPR-*P* [el Kouni *et al., Proc. natn. Acad. Sci. U.S.A.* **80**, 6667 (1983); el Kouni *et al., Biochem. Pharmac.* **34**, 3921 (1985)], by protecting the host but not the parasite from the toxicity of these analogues.

Schistosomes, unlike their mammalian hosts, lack *de novo* purine biosynthesis and depend on the salvage pathways for their purine requirements [for a review see Ref. 1]. Therefore, schistosomes can be selectively deprived of the vital purines by blocking or interfering with the parasite purine salvage pathways by using one or more of the numerous available purine analogues. However, few attempts were made to exploit this striking difference between the schistosomes and their host, and few analogues were tested as antischistosomal drugs [2–9]. Among these analogues, only tubercidin (7-deazaadenosine) showed any promise and was tested *in vivo* by Jaffe and coworkers [see Ref. 10 for review] and el Kouni *et al.* [7, 8]. Unfortunately tubercidin is also very toxic to the host [2, 7, 8]. Recently, however, we were able to make tubercidin selectively toxic against schistosomes [7, 8]. This was achieved by coadministration of nitrobenzylthioinosine 5'-monophosphate (NBMPR-*P*‡), a prodrug of the potent nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR) [11]. We found that, in contrast to mammalian systems, NBMPR-*P* does not interfere significantly with the uptake of tubercidin by *Schistosoma mansoni* [7]. Therefore, when we coadministered NBMPR-*P* with potentially lethal doses of tubercidin to *S. mansoni*- and *S. japonicum*-infected mice, we were able to protect the host but not the parasite from tubercidin toxicity [7, 8]. Although the combination of tubercidin plus NBMPR-*P* was very successful in treating schistosomiasis in mice, there might be some reservation on using it in chemotherapy in humans. First, tubercidin is very toxic [2, 7, 8]. Second, although NBMPR is the best known inhibitor of nucleoside transport in mammalian systems [11, 12], its therapeutic potential may be limited by its breakdown to 6-mercaptopurine [13], which has cytostatic and immunosuppressive actions. There are other nucleoside transport inhibitors which do not have this side-effect and generally have low toxicity in mammals. Among these are dipyridamole [14–19, see also Ref. 12 for a review], dilazep [20–25] and benzylacyclouridine (BAU) [26, 27]. The effects of these compounds on nucleoside uptake by schistosomes have not yet been tested.

In search of other possible combinations of adenosine analogues and nucleoside transport inhibitors that can be used as effectively as the tubercidin plus NBMPR-*P* combination, we studied the *in vitro* uptake by *S. mansoni* of several purine nucleoside analogues, namely 5'-deoxy-5'-iodo-2-fluoroadenosine, nebularine, toyocamycin, sangivamycin, 3'-deoxysangivamycin, 9-deazaadenosine, 7,9-dideaza-7-thiaadenosine and 1-methylformycin. The chemical structures of these analogues are shown in Fig. 1. The effects of the nucleoside transport inhibitors, NBMPR-*P*, dipyridamole, dilazep and BAU (Fig. 2), on the patterns of incorporation of these analogues as well as tubercidin into the parasite nucleotide pool were determined. A preliminary report has been presented [28].

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‡ Abbreviations: BAU, benzylacyclouridine [5-benzyl-1-(2'-hydroxyethoxymethyl)uracil]; 2-FATP, 2-fluoroadenosine 5'-triphosphate; NBMPR, nitrobenzylthioinosine or 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBMPR-*P*, nitrobenzylthioinosine 5'-monophosphate; and TuTP, tubercidin 5'-triphosphate.

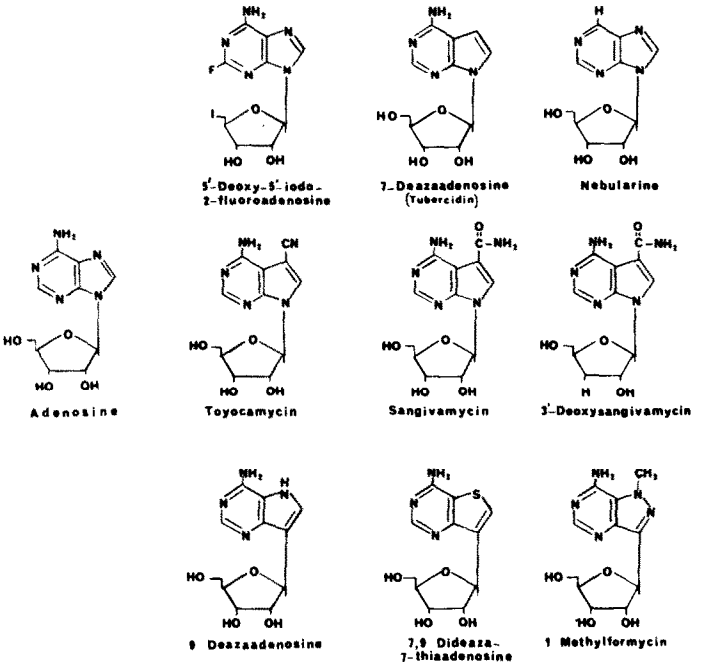

Fig. 1. Chemical structures of adenosine and various adenosine analogues.

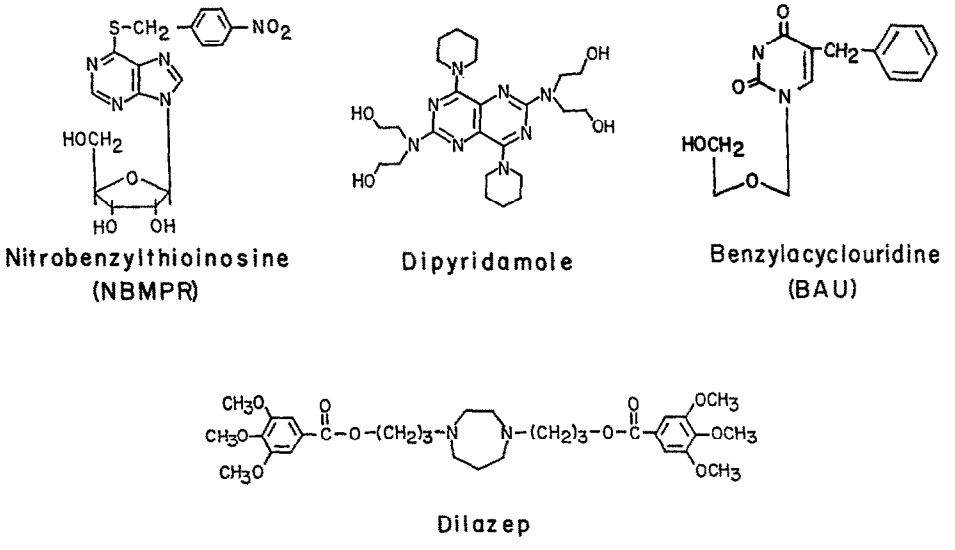

Fig. 2. Chemical structures of nitrobenzylthioinosine (NBMPR), dipyridamole, benzylacyclouridine (BAU) and dilazep.

MATERIALS AND METHODS

Chemicals. NBMPR-*P* was a gift from Dr. A. R. P. Paterson, Cancer Research Unit (McEachern Laboratory), University of Alberta, Edmonton, Alberta, Canada; dilazep from Asta-Werke AG, Frankfurt, F.R.G.; 9-deazaadenosine and 7,9-dideaza-7-thiaadenosine from Dr. R. S. Klein, Sloan-Kettering Institute, Rye, NY; 3′-deoxysangivamycin and 1-methylformycin from Dr. L. B. Townsend, University of Michigan, Ann Arbor, MI; 5′-deoxy-5′-iodo-2-fluoroadenosine from Drs. J. A. Secrist III and J. A. Montgomery, Southern Research Institute, Birmingham, AL; and benzylacyclouridine from Dr. S. H. Chu of this University. Toyocamycin, nebularine and sangivamycin were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Tubercidin and dipyridamole were purchased from the Sigma Chemical Co., St. Louis, MO; Fischer's medium from the Grand Island Biological Co., Grand Island, NY; and a penicillin–streptomycin mixture from M. A. Bioproducts, Walkersville, MD. To increase the solubility of dipyridamole, the chloride salt was made by dissolving dipyridamole in methanol and saturating the solution with HCl.

Animals. Female CD1 mice (20–25 g) were obtained from Charles River Laboratories, Wil-

mington, MA.

Life cycle maintenance. The life cycle of *S. mansoni* (Puerto Rico strain) was maintained using mice as the primary host and *Biomphalaria glabrata* (Puerto Rico Strain 2) as the vector snail. Mice were exposed to 200 cercariae for 30 min by tail immersion [29]. Miracidia were prepared by hatching eggs from livers of 7- to 9-week infected mice [29, 30]. Groups of 60–70 young snails, 5 mm in diameter, were exposed to about 10 miracidia per snail for 3 hr at 26°. Cercariae were collected from shedding snails 5–12 weeks after infection.

In vitro *uptake assay.* The *in vitro* uptake of various adenosine analogues and the effects of nucleoside transport inhibitors, NBMPR-*P*, dipyridamole, dilazep or BAU, on the uptake of these nucleoside analogues *in vitro* were analyzed by HPLC. Worms were collected from 45- to 60-day infected mice by portal perfusion [31]. Fischer's medium supplemented with a penicillin (1 unit/ml)–streptomycin (1 μg/ml) mixture and buffered with sodium bicarbonate was used as the perfusing fluid as well as the incubation medium. The worms were captured on nylon mesh (200 mesh), quickly rinsed *in situ*, and then incubated in Fischer's medium at 37°. Those found *in copula* were transferred in groups of ten pairs into test tubes containing 2 ml of Fischer's medium with or without a nucleoside analogue (10^{-4} M) in the presence or absence of the nucleoside transport inhibitor (10^{-4} M) for 4 hr at 37°. Worms were then washed three times with normal saline (0.9%) and homogenized in 0.5 ml perchloric acid (4%). Denatured proteins were removed by centrifugation, and the supernatant fluid (acid-soluble fraction) was neutralized with 50 μl KOH (5 N) and 50 μl potassium phosphate (0.5 M, pH 7). Insoluble potassium perchlorate was removed by centrifugation, and the supernatant fluid was analyzed for nucleotide content by HPLC using a linear gradient (0.001 to 0.5 M) of potassium phosphate (pH 3.9), at a flow rate of 1.4 or 1.6 ml/min, on a Waters LCS III liquid chromatograph system equipped with a Reeve-Angel Partisil-10 SAX (25 cm × 4.6 mm) column (Whatman, Inc.). When the nucleoside contents were analyzed, a C_{18} μBondapack (30 cm × 3.9 mm) column (Waters Assoc.) was used. The column was eluted with water (0–20 min) followed by 50% methanol in water (20–30 min) at a flow rate of 1 ml/min. Natural nucleosides and nucleotides and those of 2-fluoroadenine (from 5'-deoxy-5'-iodo-2-fluoroadenosine) were monitored at 254 nm, while those of tubercidin at 272 nm, nebularine at 262.5 nm, toyocamycin at 278 nm, sangivamycin at 278 nm, 3'-deoxysangivamycin at 280 nm, 9-deazaadenosine at 274 nm, 7,9-dideaza-7-thiaadenosine at 295 nm, and 1-methylformycin at 301 nm. In addition to absorbance, these compounds were identified by retention time and coelution with authentic samples.

RESULTS

Uptake of various purine nucleoside analogues by schistosomes in vitro *and the effect of NBMPR-*P. When worms were incubated with sangivamycin, 3'-deoxysangivamycin or 1-methylformycin, we could not detect the nucleotide peaks of these analogues on the HPLC nucleotide profiles in spite of the fact that these adenosine analogues do enter the nucleoside pool of the schistosomes. Figure 3 shows the time-dependent accumulation of unmetabolized sangivamycin in the parasite. Similar results were obtained with 3'-deoxysangivamycin and 1-methylformycin as well as other analogues that are metabolized by the worms, i.e. toyocamycin and tubercidin (see below). After a 4-hr incubation, the amounts of unmetabolized sangivamycin, 3'-deoxysangivamycin, 1-methylformycin, toyocamycin and tubercidin accumulated in the worms were: 290, 315, 230, 275 and 60 pmoles/one pair of worms respectively. These results indicate that, although *S. mansoni* can take up sangivamycin, 3'-deoxysangivamycin or 1-methylformycin, it cannot phosphorylate these compounds to their nucleotide derivatives. In contrast, the appearance of the recognizable peaks of the di- and/or triphosphates of 2-fluoroadenosine (from 5'-deoxy-5'-iodo-2-fluoroadenosine), toyocamycin, 9-deazaadenosine, nebularine and 7,9-dideaza-7-thiaadenosine (Fig. 4) demonstrates the uptake and metabolism of these nucleoside analogues by the parasite. Schistosomes incubated with the adenosine analogues had similar nucleotide profiles whether the nucleoside transport inhibitor NBMPR-*P* was present or not (data not shown) as was the case with tubercidin (Fig. 5). These results demonstrate that, contrary to what had been shown in mammalian systems [11, 12, 23, 24], NBMPR-*P* had no significant effect on the uptake of these nucleoside analogues by schistosomes.

Effects of dilazep, dipyridamole and BAU on the in vitro *uptake of purine nucleoside analogues by schistosomes.* Figure 5 shows the HPLC nucleotide profiles from worms incubated with tubercidin in the presence and absence of NBMPR-*P*, dilazep, dipyridamole or BAU. Neither NBMPR-*P* nor dilazep or BAU had a significant effect on the incorporation of tubercidin into the nucleotide pool in schistosomes. Dipyridamole, on the other hand, reduced the incorporation of tubercidin into the nucleotide pool as indicated by the approximately 30% reduction in tubercidin 5'-triphosphate (TuTP)/ATP ratio. Similarly, neither dilazep nor BAU had a significant effect on the uptake of 5'-deoxy-5'-iodo-2-fluoroadenosine, toyocamycin, 9-deazaadenosine, nebularine or 7,9-dideaza-7-thiaadenosine by the parasite *in vitro*. Only dipyridamole reduced the incorporation of these adenosine analogues into the nucleotide pool of the parasite (data not shown) as was the case with tubercidin (Fig. 5). These results indicate that the inhibitors of mammalian nucleoside transport, NBMPR-*P*, dilazep, and BAU, do not inhibit the uptake of these adenosine analogues by schistosomes.

DISCUSSION

The present results demonstrate that *S. mansoni* incorporated tubercidin, nebularine, 9-deazaadenosine, 5'-deoxy-5'-iodo-2-fluoroadenosine, 7,9-dideaza-7-thiaadenosine and toyocamycin but not sangivamycin, 3'-deoxysangivamycin, or 1-methylformycin into their nucleotide pool. The

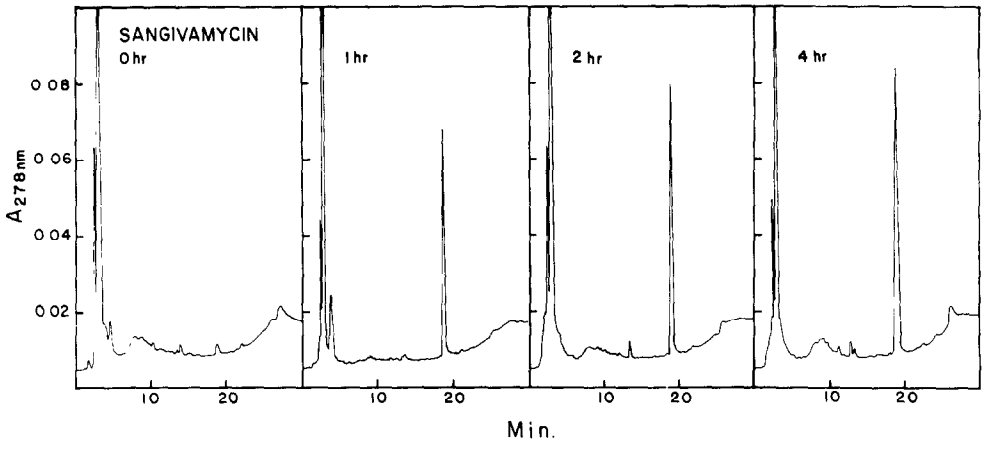

Fig. 3. HPLC nucleoside profiles demonstrating the time-dependent accumulation of sangivamycin in *S. mansoni*. The amounts of sangivamycin accumulated were 0, 210, 270 and 290 pmoles/one pair of worms after 0, 1, 2 and 4 hr, respectively, of *in vitro* incubation. Sangivamycin was identified by 278/254 nm absorbance ratio, retention time (19.6 min), and coelution with authentic samples.

inability of the parasite to metabolize sangivamycin, 3′-deoxysangivamycin and 1-methylformycin was not due to a lack of uptake of these analogues. Only tubercidin was shown previously to be metabolized by schistosomes [4, 6, 7, 9, 32]. 9-Deazaadenosine, on the other hand, has been reported not to be metabolized by schistosomules [9], presumably because a lower concentration of 9-deazaadenosine (10^{-6} M) was used in the latter study [9].

All the analogues tested in this study have been shown to exhibit cytotoxic effects on mammalian cells through a wide range of biological activities. They interfere with numerous cellular processes including *de novo* purine biosynthesis, rRNA processing, methylation of tRNA and nucleic acids and protein synthesis [33–40]. The metabolism of tubercidin, nebularine, sangivamycin, toyocamycin [33–34], 1-methylformycin [39], 9-deazaadenosine ([40, 41], unpublished results), 7,9-dideaza-7-thiaadenosine [40] and 3′-deoxysangivamycin (unpublished results) is somewhat similar. All, at best, are very poor substrates for both mammalian adenosine deaminase (EC 3.5.4.4) and purine nucleoside phosphorylase (EC 2.4.2.1). However, they are phosphorylated by adenosine kinase (EC 2.7.1.20) to their respective nucleoside 5′-monophosphates and further metabolized to the di- and triphosphate levels in mammalian cells.

The present results and those of others [1, 3–5, 9, 42] suggest that the metabolism of these analogues in schistosomes follows the same pathways observed in mammals. The presence of the C–C glycosidic bond in 9-deazaadenosine and 7,9-dideaza-7-thiaadenosine and the absence of the nitrogen on either the 7 or 8 position of the purine ring of tubercidin, toyocamycin and 7,9-dideaza-7-thiaadenosine preclude these analogues from being substrates for the nucleoside phosphorylases [43]. Indeed, tubercidin is not cleaved to 7-deazaadenine by schistosome extracts [4, 9]. Likewise, the absence of N7 and/or the presence of a bulky substituent on N7 impedes the deamination of these analogues by adenosine deaminase in both mammalian cells [39, 44] and schistosomes [3, 4, 9, 42]. Therefore, these analogues can enter the nucleotide pool only after being phosphorylated by adenosine kinase. These results stress the importance of the parasite adenosine kinase in the metabolism of these various adenosine analogues.

The inability of the schistosomes to phosphorylate 1-methylformycin, sangivamycin and 3′-deoxysangivamycin to the nucleotide level is puzzling in view of the fact that the parasite can convert the closely related analogues, formycin A [6, 9] and toyocamycin (Fig. 4), to their respective nucleotides. It is possible that these analogues do enter the parasite nucleotide pool but not in quantities sufficient to be detected by the technique employed in the present study. It may be that the adenosine kinase of the worms does not tolerate a bulky group on N7 of the purine ring as suggested by the relatively small amount of toyocamycin nucleotides formed (Fig. 4). The fact that human erythrocytes readily metabolize 1-methylformycin [39], toyocamycin, sangivamycin [33, 34] and, to a lesser extent, 3′-deoxysangivamycin (S. F. Chen and R. E. Parks Jr., unpublished results) to their respective nucleotides suggests that the schistosomal adenosine kinase may be different from that of their host.

The analogue 5′-deoxy-5′-iodo-2-fluoroadenosine is not a substrate for mammalian adenosine kinase, adenosine deaminase or purine nucleoside phosphorylase [37, 38, 43, 44]. To be metabolized to the toxic 2-fluoroadenosine nucleotides it must first be cleaved by 5′-deoxy-5′-methylthioadenosine phosphorylase (EC none) to yield 2-fluoroadenine [37] which is rapidly metabolized to the corresponding nucleotides via adenine phosphoribosyltransferase (EC 2.4.2.7) [38]. A similar metabolic pathway seems to occur in schistosomes. Figure 4 shows that the parasite was able to synthesize 2-fluoroadenosine 5′-triphosphate from 5′-deoxy-5′-iodo-2-fluoroadenosine. Furthermore, it was shown previously that 2-fluoroadenine compounds are not deaminated in schistosomes [1, 3, 4, 42] but the parasite can synthesize 2-fluoroadenosine 5′-triphos-

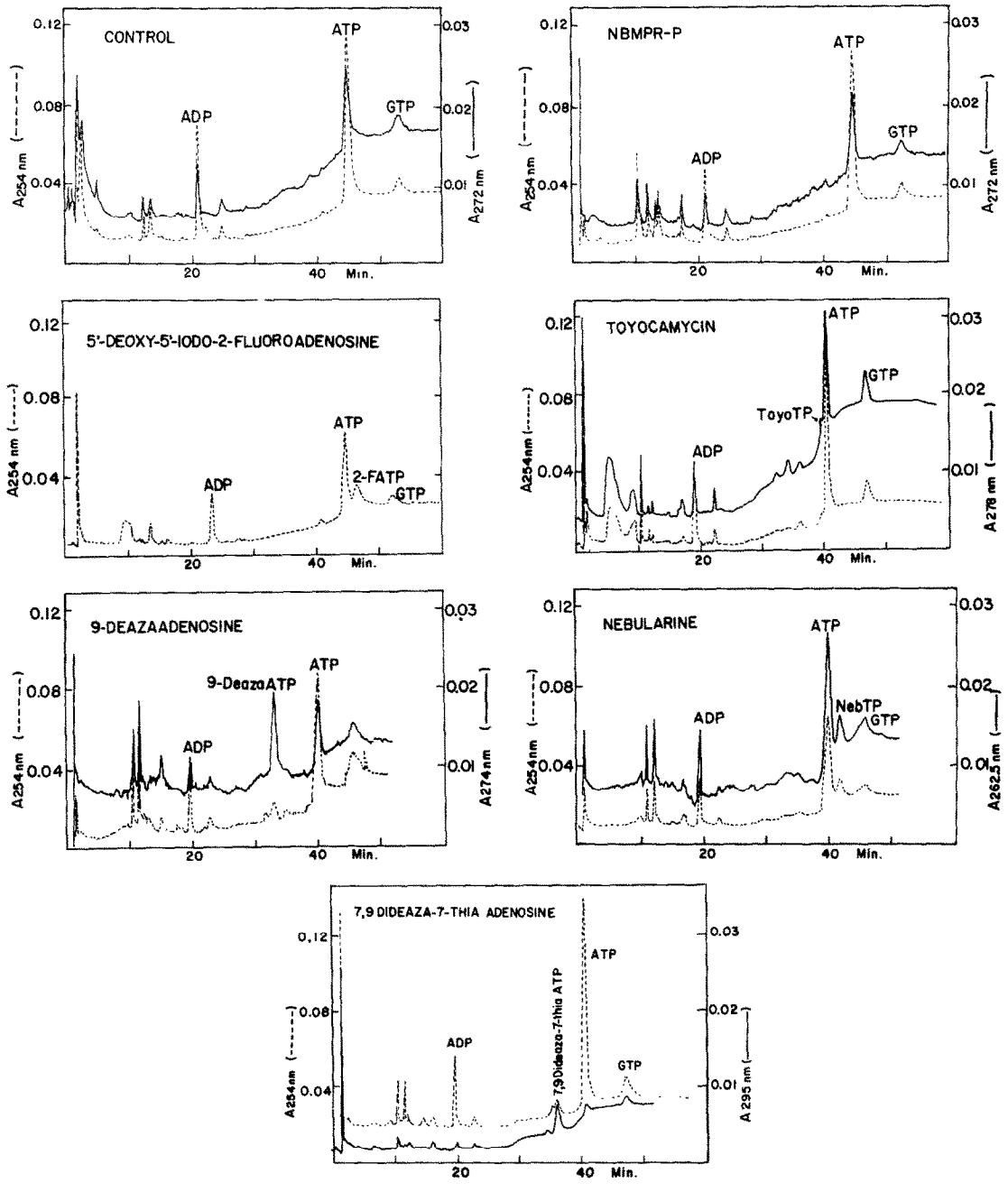

Fig. 4. HPLC nucleotide profiles demonstrating the incorporation of several adenosine analogues into the nucleotide pools of *S. mansoni* after a 4-hr incubation *in vitro* and the lack of effect of NBMPR-*P* on their nucleotide profile.

phate from 2-fluoroadenine by the action of adenine phosphoribosyltransferase [4]. At the present time we do not know whether 5'-deoxy-5'-iodo-2-fluoroadenosine is cleaved in the schistosomes by a yet unidentified 5'-deoxy-5'-methylthioadenosine phosphorylase or by the distinctive adenosine phosphorylase identified in this parasite [42]. Experiments are under way to clarify this point.

NBMPR-*P*, dilazep, dipyridamole [11, 12, 23, 24, 45] and BAU [26] inhibit the transport of various nucleosides, albeit to different degrees, in a variety of animal cells. NBMPR-*P* exerts its effect after dephosphorylation to its active form, NBMPR, by cellular ecto 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) [46]. NBMPR, dilazep, dipyridamole and BAU inhibit nucleoside transport in mammalian cells by binding tightly but reversibly to the transport sites on these cells [11, 12, 23, 24, 26]. However, there seem to be distinct differences in the specificities of NBMPR and dipyridamole inhibition [12, 47–51]. Inhibition of nucleoside transport by dilazep is similar to that by

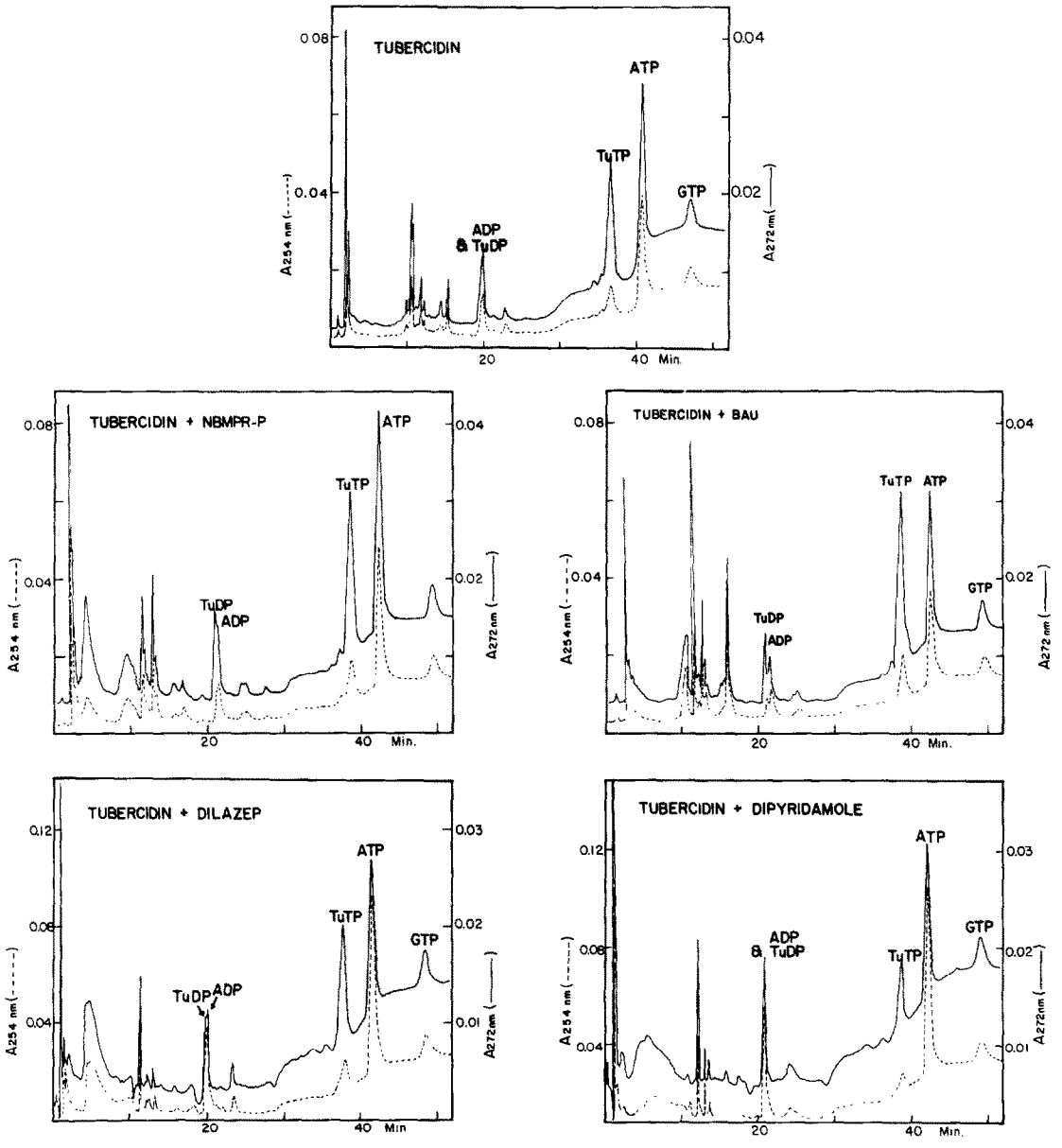

Fig. 5. HPLC nucleotide profiles showing the effects of the nucleoside transport inhibitors, NBMPR-*P*, BAU, dilazep and dipyridamole, on the incorporation of tubercidin into the nucleotide pools of *S. mansoni* after a 4-hr incubation *in vitro*. The ratios of TuTP/ATP peak areas were 0.7, 0.7, 1.3, 0.9 and 0.5 for tubercidin alone and in the presence of NBMPR-*P*, BAU, dilazep and dipyridamole respectively.

NBMPR [51], although there is no obvious structural resemblance between these compounds.

Our present and previous results [7] show that neither NBMPR-*P* nor NBMPR, dilazep or BAU is effective in blocking the uptake of adenosine analogues by the schistosomes. On the other hand, dipyridamole at the concentration employed reduced, but did not block completely, the incorporation of these analogues into the nucleotide pool. Dovey *et al.* [9], however, reported that NBMPR inhibits the transport of adenosine and tubercidin by schistosomules. These contradictory results may be explained by differences between the nucleoside transport mechanisms of adult worms and the younger schistosomules. A more likely explanation is that the NBMPR concentrations (10^{-5} and 10^{-4} M) used by Dovey *et al.* [9] were 10- to 100-fold higher than that of the permeant (10^{-6} M), whereas in the present work the same concentration (10^{-4} M) was used for both the transport inhibitor and the permeant. NBMPR in excess to the permeant may be more effective in inhibiting the uptake of nucleosides by schistosomes for at least two possible reasons. NBMPR may be a weak nucleoside transport inhibitor in schistosomes; therefore, higher concentrations are required to show inhibition of nucleoside uptake. Alternatively, NBMPR, a nucleoside itself, may be transported, albeit poorly, by schistosomes and at higher concentrations competes more successfully with the other nucleosides, preventing

them from entering the parasite cells. Experiments are underway in this laboratory to determine which of the two possibilities may be responsible for the inhibition of nucleoside uptake by excess concentrations of NBMPR.

The lack of inhibition of nucleoside uptake in schistosomes by NBMPR, NBMPR-*P*, dilazep and BAU, along with the fact that coadministration of NBMPR-*P* or dilazep and to a lesser extent dipyridamole with either tubercidin or nebularine to schistosome-infected mice results in high selective toxicity against the parasite ([7, 8, 28] and unpublished results), suggest that the mechanism of nucleoside transport in schistosomes differs from that of their host and resembles that of trypanosomes. Dipyridamole inhibits adenosine uptake in *Trypanosoma brucei* [52]. On the other hand, the combination of tubercidin plus NBMPR was used successfully in the treatment of mice infected with *T. gambiense*, and highly selective toxicity against the parasite was achieved [53]. The lack of effect of NBMPR on the trypanocidal action of tubercidin has been attributed to the absence of NBMPR binding sites on *T. gambiense* [54]. Whether or not this is also the case in schistosomes remains to be determined. Thus, the parasites may have nucleoside transport systems that are distinctly different from those of mammalian cells. Alternatively, the nucleoside transport system in the parasites may resemble the NBMPR-insensitive transporter which is present in various amounts in different animal cells [45, 47–51]. It is interesting that nucleoside transport in the NBMPR-insensitive mammalian cells is sensitive to inhibition by dipyridamole [47–51] as is the case with both schistosomes (the present results) and trypanosomes [52–54].

In conclusion, schistosomes can incorporate several toxic adenosine analogues into their nucleotide pool. In contrast to most mammalian cells, the nucleoside transport inhibitors NBMPR, dilazep, or BAU had no significant effect on the uptake of these analogues. These results suggest that the transport of nucleosides in schistosomes is different from that of their mammalian hosts. Therefore, coadministration of nucleoside transport inhibitors with adenosine analogues may result in high selective toxicity against schistosomes by protecting the host but not the parasite from the toxicity of these analogues, as we have shown previously with the combination of tubercidin plus NBMPR-*P* [7, 8].

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