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PATHWAYS OF NUCLEOTIDE METABOLISM IN *SCHISTOSOMA MANSONI*—VI ADENOSINE PHOSPHORYLASE*

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Abstract—*Schistosoma mansoni* worms have been found to have an adenosine phosphorylase enzyme which requires inorganic phosphate during cleavage and α-ribose-1-phosphate for synthesis of adenosine from adenine. Kinetics of the reaction show a K_m of $5{\cdot}6 \times 10^{-5}$ M for adenosine and a broad pH activity range from 6·0 to 8·0. The adenosine phosphorylase activity can be distinguished from purine nucleoside phosphorylase by substrate specificity and by product inhibition studies.

Schistosoma mansoni worms lack the capability of *de novo* synthesis of purine nucleotides [1]. Adenine and adenosine are readily converted to adenine nucleotides by intact worm pairs [2]. Extracts of worm pairs were shown to contain enzymes associated with purine "salvage" pathways, i.e. adenine phosphoribosyl transferase, adenosine kinase and hypoxanthine-guanine phosphoribosyl transferase [3]. In intact worm pairs, the synthesis of adenine nucleotides from adenosine was shown to proceed primarily by biochemical pathways that did not involve adenosine kinase [2, 3]. The presence of an adenosine cleavage enzyme, i.e. phosphorylase or hydrolase, in *S. mansoni* has been suggested in previous studies [2, 4]. The data reported in this paper show that extracts of *S. mansoni* and vomitus from *S. mansoni* both contain an enzymatic activity that catalyzes the phosphorolysis of adenosine yielding adenine and ribose-1-P.

MATERIALS AND METHODS

Radioactive nucleosides were obtained from Schwartz BioResearch, Inc. Adenine, hypoxanthine, adenosine and inosine were obtained from P-L Biochemicals. Adenosine deaminase and R-1-P† were products of Sigma Chemical Co. Tubercidin (7-deazaadenosine) was a gift of the Upjohn Co. and Formycin A [7-amino-3(β-D-ribofuranosyl)pyrazolo-[4,3-d]pyrimidine] was a gift of Dr. Hamao Umezawa, Institute of Microbiological Chemistry, Tokyo, Japan. Formycin B [7-hydroxy-3(β-D-ribofuranosyl)pyrazolo-[4,3-d]pyrimidine] and 6-thioguanosine were purchased from CalBiochem Corp. N^6-methyl adenosine was obtained from Terra-Marine Bioresearch, La Jolla, Calif. and 2-fluoro-2′-deoxyadenosine was obtained from Drug Research and Development, National Cancer Institute, Bethesda, Md.

Worms used in these studies were obtained originally from a Puerto Rican strain. A complete life cycle is maintained in our laboratory using *Australorbis glabratus* as the vector snail. Adult *S. mansoni* were recovered from infected CF[1] mice and washed free of plasma and erythrocytes with 20 ml Fischer's medium [5]. Forty pairs of worms were homogenized at 4° in 0·5 ml of 0·15 M KCl containing 10 mM EDTA adjusted to pH 6·5 with Tris–(hydroxyamino)methane (THAM). The resulting homogenate was centrifuged at 38,000 *g* for 20 min at 4°. The clear supernatant fluid beneath the floating lipid layer was used as the source of enzyme activity. In other experiments, worms were recovered from infected mice by perfusion [6] and placed in chilled saline to induce regurgitation. The regurgitated gut contents ("vomitus") were concentrated and lyophilized. This lyophilized material was kindly prepared for us in the laboratory of Dr. A. Capron, Lille, France. Four mg of the lyophilized material was added to 100 μl of 0·15 M KCl containing 10 mM EDTA adjusted to pH 6·5 with THAM, homogenized and then centrifuged at 38,000 *g* for 10 min. The supernatant fluid was used as the source of enzyme activity.

The formation of adenosine from adenine and α-D-ribose-1-phosphate was measured in a coupled reaction with adenosine deaminase by following the decrease in absorbance at 265 nm as a function of time. Observations were made at 30°, using a Gilford recording spectrophotometer equipped with a pinhole adaptor. The reaction volume (170 μl) contained 180 mM sodium cacodylate buffer, pH 6·1, 6 mM EDTA,

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† Abbreviations are as follows: EDTA, ethylene diamino tetra-acetic acid; A, adenine; AR, adenosine; HR, inosine; H, hypoxanthine; and R-1-P, α-D-ribose-1-phosphate.

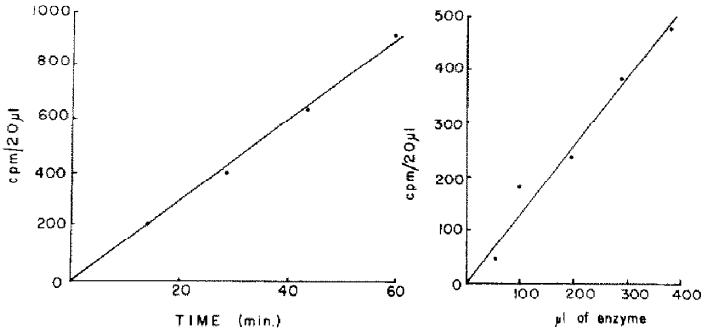

Fig. 1. *Left panel*: linear phosphorolysis of adenosine-8-^{14}C by an extract of *S. mansoni*. Aliquots (20 µl) of a reaction mixture containing adenosine-8-^{14}C, inorganic phosphate and an extract of *S. mansoni* were subjected to paper chromatography (see text for details). The rate of adenine-8-^{14}C production was linear and completely dependent upon the presence of inorganic phosphate. *Right panel*: phosphorolysis of adenosine-8-^{14}C as a function of the amount of *S. mansoni* extract. Aliquots (20 µl) of reaction mixtures containing increasing volumes of extracts of *S. mansoni* were subjected to paper chromatography after a 20-min incubation period. The rate of formation of adenine-8-^{14}C was a linear function of the amount of *S. mansoni* extract added to the reaction mixture.

17 µM units of crystalline calf adenosine deaminase, 1·7 mM α-D-ribose-1-phosphate and 0·06 mM adenine.

The formation of adenine from adenosine and inorganic phosphate was carried out in a 320-µl reaction mixture containing 11 µM adenosine-8-^{14}C (sp. act., 55 µCi/mole), 180 mM potassium phosphate buffer, pH 7·0, and 6 mM EDTA at 25°. Aliquots (20 µl) of the reaction mixtures were spotted on Whatman 3 MM paper after 20-, 40-, 60- and 80-min reaction time and subjected to descending chromatography in water-saturated *n*-butanol–NH_4OH (100:1). In this solvent system, the purine derivatives migrate in the order A > AR > H > HR. Non-radioactive compounds were spotted as ultraviolet markers prior to chromatography. After 55 hr of running time, the sheets were air dried. The ultraviolet-absorbing spots for A, AR, H and HR were cut out, and the radioactivity associated with the marker compounds was measured by a liquid scintillation counting technique [7].

Purine nucleoside phosphorylase activity was measured in a similar fashion to adenosine phosphorylase activity, except that 18 µM inosine-8-^{14}C (sp. act. 34·9 µCi/µmole) was substituted for adenosine-8-^{14}C. The radioactivity associated with hypoxanthine was a measure of the purine nucleoside phosphorylase activity.

The rate of production of adenine-8-^{14}C or of hypoxanthine-8-^{14}C was measured at various time periods up to 1 hr and enzyme concentrations over a final dilution range of 1:100 to 1:500 of the supernatant from homogenized worms.

The substrate specificity of adenosine phosphorylase was determined by chromatography. Worm pairs of *S. mansoni* (80/ml) were homogenized in 0·15 M KCl containing 10 mM EDTA and adjusted to pH 6·5 with THAM and centrifuged at 38,000 *g*. The supernatant fluid was passed through a P-6 polyacrylamide molecular sieving column in order to remove small molecular weight, ultraviolet-absorbing components. The protein peak eluted with 0·15 M KCl, 10 mM EDTA, adjusted to pH 6·5 with THAM, was used as the source of enzyme activity. This enzyme preparation was incubated overnight at room temperature with various nucleoside analogues in the presence and absence of 300 mM phosphate buffer at pH 7·0 and containing 10 mM EDTA. The reaction was stopped by the addition of an equal volume of 8% perchloric acid; $HClO_4$ was removed by addition of K_2CO_3 and centrifugation to remove $KClO_4$.

Twenty µl neutralized extract was spotted on Whatman 3 MM paper. Chromatographic separations were accomplished by using two solvent systems: H_2O-saturated *n*-butanol–NH_4OH (100:1) and boric acid·H_2O·95% ethanol–NH_4OH (1·5 g:67 ml:158 ml:25 ml).

RESULTS

Since adenine is a poor substrate for calf intestinal adenosine deaminase [8], the decrease in absorbance at 265 nm in a reaction mixture containing adenine, α-D-ribose-1-phosphate, excess adenosine deaminase and the supernatant extract of *S. mansoni* is proportional to the rate of adenosine production. In the absence of α-D-ribose-1-phosphate, the rate of decrease in absorbance was less than 0·002 A_{265}/min. Upon the addition of R-1-P, the rate of decline in absorbance increased to 0·010 A_{265}/min in the presence of 6 µl worm supernatant. The decrease in absorbance above background rate of change at 265 nm was not only dependent upon the presence of R-1-P but also was proportional to the amount of worm supernatant or amount of vomitus extract in the reaction mixture. Substitution of THAM for cacodylate buffer in the reaction mixture caused 70–80 per cent inhibition of enzyme activity.

In the presence of 180 mM phosphate buffer (pH 7·0) and 6 mM EDTA, the rate of formation of adenine-8-^{14}C from adenosine-8-^{14}C was linear for 60 min and

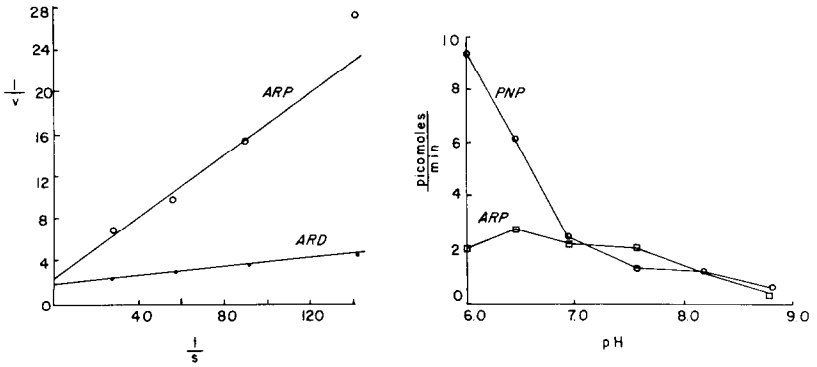

Fig. 2. *Left panel*: Lineweaver–Burk plot of adenosine phosphorylase (ARP) and adenosine deaminase (ARD) in extracts of *S. mansoni*. The Michaelis constant for adenosine was $5{\cdot}6 \times 10^{-5}$ M for adenosine phosphorylase and $1{\cdot}1 \times 10^{-5}$ M for adenosine deaminase. Adenosine deaminase activity was measured by following the rate of formation of inosine-8-^{14}C from adenosine-8-^{14}C in the absence of phosphate. The paper chromatographic technique described for adenosine phosphorylase (see Materials and Methods) was used to separate and quantitate the production of inosine-8-^{14}C from adenosine-8-^{14}C. *Right panel*: adenosine phosphorylase (ARP) activity and purine nucleoside phosphorylase (PNP) activity as a function of pH. The rate of formation of adenine-8-^{14}C from adenosine-8-^{14}C was measured to determine adenosine phosphorylase activity. The rate of formation of hypoxanthine-8-^{14}C from inosine-8-^{14}C was measured to determine purine nucleoside phosphorylase activity.

was proportional to the amount of worm extract present (Fig. 1). When phosphate was eliminated from the reaction mixture, no adenine was formed during a 60-min incubation time. During incubation in phosphate-free buffer, only 10 per cent of the adenosine was deaminated to inosine and no hypoxanthine was formed. Thus, the enzymatic activity which forms adenosine from adenine and R-1-P requires the presence of phosphate to cleave adenosine to adenine, and the term schistosome adenosine phosphorylase should be used to describe this enzyme.

The Michaelis constant for adenosine was $5{\cdot}6 \pm 3{\cdot}7 \times 10^{-5}$ M for adenosine phosphorylase and $1{\cdot}1 \pm 0{\cdot}4 \times 10^{-5}$ M for adenosine deaminase (Fig. 2, left panel). Adenosine phosphorylase and adenosine deaminase activities in *S. mansoni* are 188 pmoles/min/worm pair and 264 pmoles/min/worm pair respectively. The pH-activity curve for adenosine phosphorylase is markedly different from the pH-activity curve of purine nucleoside phosphorylase. Adenosine phosphorylase activity is relatively constant over a broad range from pH 6·0 to 8·0. Purine nucleoside phosphorylase shows high activity at pH 6·0, but this diminishes substantially at pH values greater than 7·0 (Fig. 2, right panel).

Enzyme-catalyzed phosphorolysis of adenosine is completely inhibited by adenine, whereas hypoxanthine causes no inhibition of this reaction (Table 1). In contrast, phosphorolysis of inosine is inhibited about 95 per cent by hypoxanthine but only 20 per cent by adenine. Formycin A, an adenosine analogue, markedly inhibits the phosphorolysis of adenosine, while Formycin B, an inosine analog, markedly inhibits the phosphorolysis of inosine. The formation of adenine from adenosine and the formation of hypoxanthine from inosine are differentially inhibited by various purine nucleoside analogues (Table 1).

Preliminary studies on extracts of *S. mansoni* containing adenosine phosphorylase and purine nucleoside phosphorylase, both of which catalyze the cleavage of nucleoside analogues, indicate that 2-fluoro-adenosine is cleaved, whereas 2-fluoro-2′-deoxyadenosine and 7-deaza-adenosine (tubercidin) are not (Fig. 3). Isolation and separation of adenosine phosphorylase and purine nucleoside phosphorylase from *S. mansoni* are currently being attempted in our laboratory in order to perform more definitive substrate specificity studies.

DISCUSSION

Purine nucleoside phosphorylase (PNPase; purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) has been isolated from a variety of sources and has been shown to catalyze the reversible phosphorolysis of all naturally occurring purine nucleosides except adenosine [9]. However, purine nucleoside phosphorylase from beef liver [10] and from fish muscle [11] has been reported to convert adenine to its ribonucleoside in the presence of R-1-P. Adenosine phosphorylase activity also occurs in membrane vesicles from *Escherichia coli* [12].

This paper reports that enzymatic activity has been demonstrated in extracts of *S. mansoni* which catalyzes the following reaction:

$$AR + P_i \rightleftharpoons A + R\text{-}1\text{-}P$$

The forward reaction is dependent upon phosphate, and the reaction in the reverse direction requires the presence of R-1-P. Differences in pH activity curves

Table 1. Per cent inhibition of *S. mansoni* adenosine phosphorylase (ARP), purine nucleoside phosphorylase (PNP) and adenosine deaminase (ARD) due to the presence of various purine bases and purine nucleosides*

	Concn (mM)	Per cent inhibition ARP	PNP	ARD
Adenine	2·3	100	20	80
	2·2	100	18	80
Hypoxanthine	1·6	0	93	0
	2·2	0	98	0
Formycin A	1·9	88	20	
	1·5	82	21	
Formycin B	1·0	0	93	
	1·2	13	89	
6-Thioguanosine	1·0		100	98
	1·0		94	93
N^6-methyl adenosine	1·4	14	43	100
	1·3	0	24	100
Tubercidin	1·0	29	100	80
	1·2	39	97	72
2-Fluoro-2′-deoxyadenosine	0·56	84	15	100
	1·1	89	0	100

* The data are the results of two separate experiments performed on two different days with freshly prepared extracts of *S. mansoni*.

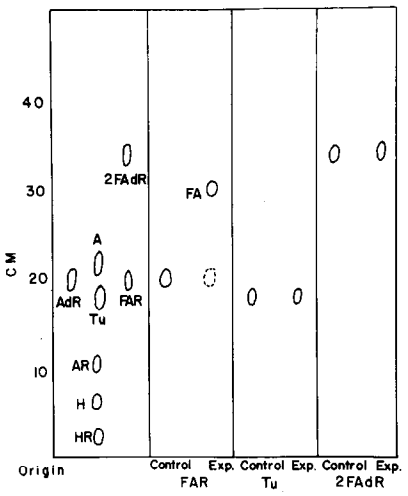

Fig. 3. Substrate specificity of phosphorylase from extracts of *S. mansoni*. The results of chromatography in a water-saturated butanol system (see Methods) are shown. The migration of tubercidin (Tu) and 2-fluoro-2′-deoxyadenosine (2FAdR) after 18 hr of incubation in the presence and absence of an extract of *S. mansoni* did not change. When 2-fluoroadenosine (FAR) was used as a substrate, a marked decrease in intensity of ultraviolet absorption of the 2-fluoroadenosine and the appearance of a new ultraviolet-absorbing spot which migrated further than 2-fluoroadenosine were noted. Chromatography in the borate solvent system showed similar results, i.e. the formation of a second ultraviolet-absorbing spot which migrated further than 2-fluoroadenosine and the absence of cleavage of both tubercidin and 2-fluoro-2′-deoxyadenosine. Abbreviations: FA, fluoroadenine; AdR, 2′-deoxyadenosine.

and studies with product inhibitors and nucleoside analogues as inhibitors all indicate that adenosine phosphorylase activity is a separate entity from purine nucleoside phosphorylase activity in extracts of *S. mansoni*. This observation represents the first report of a nucleoside phosphorylase that is specific for adenosine.

The 20 per cent inhibition by adenine and Formycin A of purine nucleoside phosphorylase in extracts of *S. mansoni* suggests that adenosine also may be an alternative substrate for purine nucleoside phosphorylase. Adenine has been reported to be an alternative substrate having a high Michaelis constant and a low maximal velocity for purine nucleoside phosphorylase isolated from human erythrocytes, rat brain and rat liver [13].

An inhibition of adenosine phosphorolysis by THAM is possibly due to the structural similarity between the ribose portion of adenosine and THAM. Atomic models were used to construct molecular configurations of THAM and adenosine. These showed a marked similarity of the structural positioning of two hydroxy-methyl groups and the nitrogen atom of THAM when compared to the *cis* hydroxyl groups and number 9 nitrogen of adenosine. In addition to this, there is a similar steric compactness of both molecules. Inhibition of human purine nucleoside phosphorylase by THAM has been observed previously.*

Since schistosomes are dependent on salvage pathways to fulfill their requirements for preformed purines, a detailed knowledge of the pathways involved is crucial in the development of new chemotherapeutic agents. Quite likely, a successful therapy will entail use of a combination of drugs, each designed to block a specific route of nucleotide formation.

Recently, a number of adenosine analogues have been considered as candidate antischistosomal drugs

* R. P. Agarwal, personal communication.

[14]. Various combinations of Formycins A and B, arabinosyl-6-mercaptopurine, 2-fluoroadenosine, N^6-phenyladenosine and tubercidin have been tried because of their potential for blocking known adenosine salvage pathways [4]. For the present, only tubercidin, as proposed by Jaffe *et al.* [15], appears to have potential clinical usefulness. Its effectiveness appears to rest on the following characteristics: (1) it is incorporated into the nucleotide pool (probably via the nucleoside kinase enzyme); (2) it is not a substrate for adenosine deaminase and appears to inhibit schistosome purine nucleoside phosphorylase (Table 1); (3) it is a weak blocker of adenosine phosphorylase (Table 1) and is itself probably not a substrate for phosphorolysis (Fig. 3); and (4) it is incorporated into worm nucleotides up to the triphosphate level. Concomitantly, as the tubercidin triphosphate concentration increases, there is a progressive decline in the concentrations of normal adenine nucleotides [14].

It is not known which of the following three sites of action might account for the antischistosomal effect of tubercidin, i.e. the inhibition of purine salvage, interference with nucleotide function, or interference with nucleic acid metabolism [16]. The combination of tubercidin with 2-fluoro-2′-deoxyadenosine should result in marked inhibition of purine salvage, since tubercidin inhibits purine nucleoside phosphorylase and 2-fluoro-2′-deoxyadenosine inhibits adenosine phosphorylase. Since 2-fluoro-2′-deoxyadenosine is converted to the corresponding monophosphate nucleotide and not to the di- and triphosphate nucleotides by human erythrocytes [17], its therapeutic index for treating schistosomiasis may be highly favorable. In the event mammalian tissues do not convert the deoxyadenosine analogue to di- or triphosphates, then incorporation into host nucleic acids would be precluded. In consequence, both toxicity and mutagenic potential might prove to be low. Current studies in our laboratory are in progress to evaluate the therapeutic potential of 2-fluoro-2′-deoxyadenosine. Preliminary findings of the effect of other combinations of multiple blocking agents have been reported [4].

Adenosine deaminase and adenosine phosphorylase have been identified in worm vomitus and thus may represent proteins which are normally present in the gut lumen of this parasite. Because of periodic regurgitation by this intravascular parasite, native enzyme is administered intravenously into the host's circulation. It follows that hypersensitivity to such enzymes might be expected [18]. Considerations of the diagnostic possibilities arising from these events will be presented elsewhere.

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