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PATHWAYS OF NUCLEOTIDE METABOLISM IN *SCHISTOSOMA MANSONI*—II

DISPOSITION OF ADENOSINE BY WHOLE WORMS*

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Abstract—Worm pairs of *Schistosoma mansoni* were incubated *in vitro* with uniformly labeled adenosine-^{14}C in order to determine the adenine–ribose ratio of newly synthesized adenine nucleotides. It was found that A–R of the ATP averaged 2·7.

During incubation of live worms in the presence of 8-^{14}C adenosine or 8-^{14}C inosine, large amounts of hypoxanthine accumulated in the medium. In addition, both hypoxanthine and a considerable concentration of inosine 5′-monophosphate (IMP) were detected in the worm supernatant.

These results suggest that the principal pathway of utilization of adenosine is not mediated by adenosine kinase. An alternative, involving deamination of adenosine to inosine, cleavage to hypoxanthine, conversion to IMP and subsequent synthesis of ATP is the principal metabolic route used by *S. mansoni*.

Worms incubated *in vitro* were shown to release adenosine deaminase into the medium. A comparison of enzyme stability and pH optimal activity curves of the parasite enzyme with host erythrocyte deaminase shows the former to be quite stable at pH 6·0 and almost inactive at pH 8·5. The red cell enzyme, in contrast, showed a broad pH maximum which extends well past pH 8·5.

THE RECENT disclosure concerning the absence or limited activity of a *de novo* pathway for the synthesis of purines in schistosomes[1] necessitates the need for exact knowledge of the pathways of purine and pyrimidine metabolism in these worms. Large quantities of red cells are ingested by the schistosome; thus the nucleotide content of these erythrocytes may supply the parasite with the necessary preformed purines. Since nucleotides are not known to penetrate cell membranes, presumably they are obligatorily converted to nucleosides or bases before they can be utilized by the worm.

It has already been shown that adenine is rapidly taken up *in vitro* and converted into ATP and that APRT† enzyme can be demonstrated *in vitro* in schistosomes.[2] It was, therefore, important that the pathways of adenosine metabolism be investigated. We posed the question whether reactions [1], [2] or [3] of the following scheme were active in the flukes:

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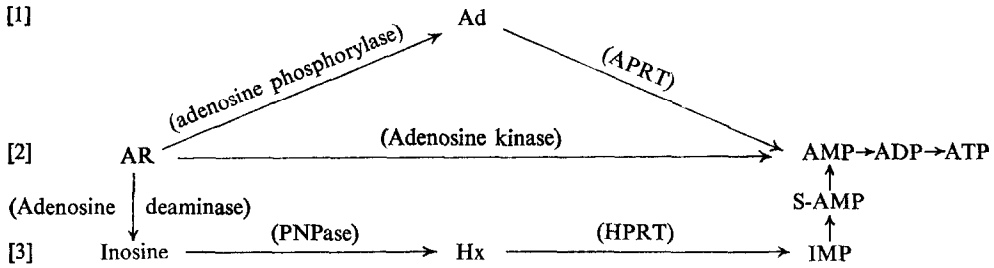
† Abbreviations used in this paper are as follows: Ad, adenine; AR, adenosine; AMP, adenosine monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; APRT, adenine phosphoribosyltransferase; HPRT, hypoxanthine phosphoribosyltransferase; PNPase, purine nucleoside phosphorylase; In, inosine; Hx, hypoxanthine; IMP, inosine 5′-monophosphate and FM, Fischer's medium.



MATERIALS AND METHODS

Maintenance of worm in vitro. *S. mansoni* infected mice were made available through the kindness of Dr. Dan Ostlind at the Merck Institute. These mice were exposed to approximately 300 cercariae 10 weeks prior to recovery of worms. The worms were customarily incubated in Fischer's medium[3] (FM) to which the appropriate labeled nucleotide precursor was added. Fischer's medium, being protein free, is not satisfactory for the maintenance of worms for protracted periods. However, it is able to keep the worms in good condition for a minimum of 24 hr and has the advantage of being totally defined.

The condition of worms maintained *in vitro* can be judged by a number of relatively objective criteria: (a) the worms should stay *in copula* during all or most of the experimental incubation time: (b) they should appear vigorous and move actively; (c) peristalsis should be observable in some section of the gut almost continually; (d) depletion of glucose in the medium and the production of lactic acid should be easily measurable and (e) an amino acid analysis of FM should indicate considerable production and excretion of proline and alanine by the worms during the experimental period.[4] It was found that with frequent gentle agitation during incubation the pH remained between 7·3 and 7·4. For incubation times longer than 1 hr, the addition of a few micrograms of sodium bicarbonate at periodic intervals was necessary to overcome the pH shift caused by accumulation of lactate. Penicillin (100 U/ml) plus streptomycin (100 μg/ml) were added to FM as antibiotic agents. At this concentration no gross contamination of the medium could be observed within 24 hr.

Adenosine and inosine metabolism. Worms (22 pairs of adults) were incubated for 120 min in 2·5 ml of FM to which had been added additional glucose to a final concentration of 2 mg/ml. To this medium was added 2·5 μCi of adenosine-8-^{14}C (sp. act. 47·7 μCi/μmole) or 2·5 μCi of inosine-8-^{14}C (sp. act. 34·9 μCi/μmole). Aliquot samples of the medium (20 μl) were obtained at 30-min intervals for 2 hr. These were spotted on paper, chromatographed with water-saturated butanol solvent (*n*-butanol–NH_4OH, 100:1). The radioactivity associated with AR, In, Ad and Hx was counted. Acid-soluble material was extracted from the worms by homogenization in cold trichloracetic acid (TCA) and nucleotides were separated by paper chromatography on Whatman 3 MM paper with boric acid–95% ethanol–H_2O–NH_4OH (1·5:158:67:25 ml) as the solvent system.[5] The nucleotide regions were eluted and the samples divided into halves; one was hydrolyzed while the other was untreated. These two fractions were then rechromatographed using the water-saturated *n*-butanol solvent system. In

this solvent system nucleotides remain at the origin while inosine, hypoxanthine, adenosine and adenine are easily separated. Thus, the radiopurity of the nucleotides (nonhydrolyzed sample) was determined and the extent of incorporation of radioactivity into adenine nucleotides and inosinic acid was determined with the hydrolyzed sample.

Adenine to ribose ratio of radioactivity in adenine nucleotides. Uniformly labeled adenosine-^{14}C was obtained from Amersham Searle and had a sp. act. of 566 mCi/m-mole. However, this product was found to contain a number of radioactive contaminants. In order to obtain pure adenosine-^{14}C (uniformly labeled), 50-μl aliquots of the starting material were chromatographed in NH_4OH at pH 10 on Whatman 3 MM paper. The adenosine spot was cut out of the chromatogram and eluted with 1:10 dilution of NH_4OH. The technique of Miech and Santos[5] was used to determine the distribution of label between the purine base and the ribose moiety. An acid hydrolyzed aliquot of the purified adenosine when rechromatographed yielded an adenine:ribose radioactivity ratio of 0·96, indicating an equivalent labeling in both moieties of this substrate.

Twenty-two worm pairs were incubated for 60 min at 37° in 2 ml of FM to which about 600,000 counts/min (0·60 nmole) of purified uniformly labeled AR was added. During the incubation, 20-μl samples of the medium were removed at 10-min intervals and spotted for descending paper chromatography using the previously described butanol as the eluant. The origins were previously spotted with 10 μl of 1 mM adenosine, adenine, inosine and hypoxanthine to facilitate detection when using an u.v. lamp.

At the end of the incubation period, the worms were washed twice in fresh FM and then were homogenized in 150 μl cold 10% TCA. The supernatant (120 μl) was recovered and a 10-μl aliquot was counted directly for estimation of total radioactivity. The remaining supernatant (110 μl) was used for the determination of the adenine:ribose ratio in the newly synthesized adenine nucleotides according to previously described techniques.[5]

Determination of schistosome adenosine deaminase in Fischer's medium after the incubation with worms. Relatively large amounts of inosine and hypoxanthine were noted to accumulate in the medium containing adenosine-8-^{14}C. It was therefore necessary to determine if the enzymatic conversion of adenosine to inosine and hypoxanthine occurred in the worm or whether adenosine deaminase and purine nucleoside phosphorylase were secreted into the medium. The following set of control experiments were undertaken: (a) medium (plus radioactive adenosine) was incubated for 4 hr in the absence of worms to rule out the possibility of bacterial contamination; (b) medium (plus labeled adenosine) was incubated for 1 hr in the absence of worms; then 22 pairs of well-washed schistosomes were added for 1 hr; after this the worms were removed and the medium was incubated for an additional 120 min (see Fig. 3); (c) medium (without purine substrate) plus washed worms was incubated for 1 hr; then worms were removed and labeled substrate was added; the mixtures were incubated for 2 additional hr (see Fig. 4). Aliquot samples of each of these three media were taken at 10- or 30-min intervals, and these were chromatographed and counted.

Adult schistosomal worms (30 pairs) were washed free of external erythrocytes by passing the worms repeatedly through Fischer's medium. The 30 pairs of worms were placed in 2 ml of Fischer's medium at pH 7·4 and placed on ice for 1 hr. The worms

and medium were then allowed to warm up to room temperature and the worms removed. No pellet was visualized upon centrifugation of the medium. An aliquot of the medium (500 μl) was adjusted to pH 6·8 with dilute acetic acid and another aliquot (500 μl) was adjusted to pH 8·9 with a saturated solution of Tris. The reaction was initiated by the addition of 0·1 μCi of adenosine-8-^{14}C (sp. act. 55 μCi/μmole). Ten-μl samples were spotted as a function of time on Whatman 3 MM paper. The butanol solvent system was used to separate adenosine, inosine, adenine and hypoxanthine. The disappearance of adenosine was determined by measuring the radioactivity of the adenosine spot on the developed paper chromatogram.

Preparation of adenosine deaminase from fresh schistosomes and mouse erythrocytes. Mature *S. mansoni* (80 worm pairs) were homogenized in 1 ml isotonic NaCl containing 10 mM EDTA. The homogenate was centrifuged at 18,000 *g* for 20 min and the resultant supernatant was used as the source of schistosomal enzyme.

Erythrocytes from mice infected with *S. mansoni* were isolated by centrifugation of heparinized whole blood. The erythrocytes were hemolyzed by diluting with 9 vol. of water and then centrifuged at 18,000 *g* for 20 min. The resultant supernatant was used as the source of erythrocytic enzyme.

Determination of adenosine deaminase activity and stability as a function of pH. The differences in the molar absorptivity coefficients at 265 nm between adenosine and inosine were determined spectrophotometrically after the enzymatic conversion of 0·05 mM adenosine to inosine (Fig. 1). An excess of crystalline calf intestinal adenosine deaminase (190 μmoles/min/mg) was used in the determination of the Δa_M. The buffer for pH values below 7·0 was composed of 0·1 mM EDTA, 190 mM Tris, 190 mM cacodylic acid, 525 mM acetic acid and NaOH to appropriate pH values. The

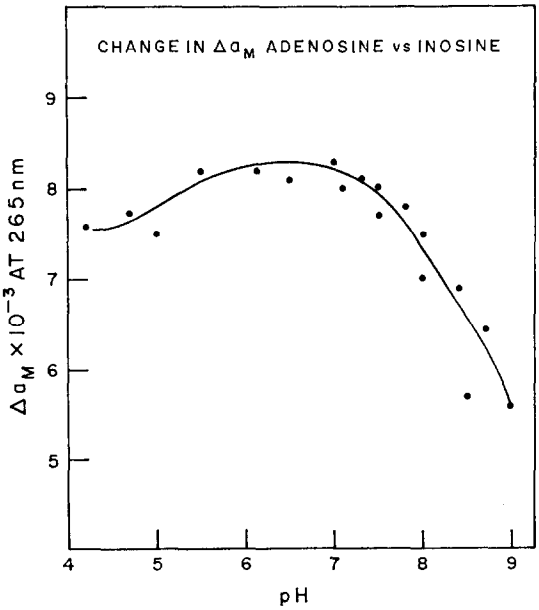


FIG. 1. Differences of the molar absorptivity coefficients between adenosine and inosine are plotted as a function of pH.

buffer for pH values above 7·0 contained 10 mM EDTA, 400 mM Tris and acetic acid to appropriate pH values. Adenosine deaminase activity was measured spectrophotometrically by following the decrease in absorbance at 265 nm as a function of time.

To determine the stability of adenosine deaminase to pH, the enzymes were incubated at various pH values at 0° for 3·5 hr.

RESULTS

In experiments using uniformly labeled ^{14}C-adenosine as a nucleotide precursor, the average ratio of A–R in the adenine nucleotides newly synthesized by schistosomes was 2·7 (Table 1). This ratio suggests that between 60 and 65 per cent of the adenosine is

TABLE 1. CONVERSION OF ^{14}C-ADENOSINE INTO ADENINE NUCLEOTIDES AND ESTIMATION OF ADENINE–RIBOSE RATIO*

	Adenine (counts/min)	Ribose (counts/min)	Adenine–Ribose
Purified adenosine-UL-^{14}C	8650	9040	0·96
Adenosine from ATP pool of			
Exp. 1	370	170	2·14
Exp. 2	510	150	3·31
Exp. 3	1430	560	2·56

* *S. mansoni* (22 pairs) were incubated in 2 ml of Fischer's medium with about 300,000 counts/min/ml or 0·31 nmole/ml, uniformly labeled adenosine added. This substrate had been purified by paper chromatography prior to use (see Materials and Methods). After 60 min 62 per cent of the substrate had been converted into inosine (37 per cent) and hypoxanthine (11·6 per cent). About 8 per cent of the substrate was converted into adenine nucleotides. The average ratio of A–R of three similar experiments was 2·7.

first converted to inosine and hypoxanthine, or to adenine, prior to its anabolism to ATP. The results of incubating live worm pairs in Fischer's medium containing labeled adenosine or inosine are shown in Figs. 2A and B. The adenosine in the medium was converted to inosine (85 per cent) and hypoxanthine (15 per cent). About 4·5 per cent of the label was found in the adenine nucleotide fraction of the worm supernatant. When inosine was used as the substrate, 25·4 per cent was converted to Hx in the medium and 8·8 per cent was taken up by the worms into the nucleotide pool. Hydrolysis of the aliquots of the nucleotide fraction of both adenosine- or inosine-incubated worms showed the major portion of the radioactivity in the form of adenine nucleotides. Approximately 25 per cent of the radioactivity was present as IMP (Table 2). These findings indicate that schistosomes do not rely on the adenosine kinase pathway for the bulk of the synthesis of adenine nucleotides from adenosine. Such synthesis appears, instead, to proceed preferentially via IMP.

Adenosine deaminase and purine nucleoside phosphorylase activities are demonstrable in the medium after the worms are removed (Fig. 3). However, the conversion of adenosine to inosine and hypoxanthine occurs 4–5 times faster in the presence of live worms. The presence of adenosine or inosine in the medium is not necessary for the liberation of these enzymes into the medium (Fig. 4).

There is a marked difference in the pH activity curve and pH stability of adenosine deaminase from mouse erythrocytes and that from fresh schistosomae worm pairs (Figs. 5 and 6). Both enzymes have a pH optimum at pH 7·5. However at pH values above 8·5, the schistosomal adenosine deaminase activity rapidly approaches zero; in contrast, there is little change in activity at this pH of the mouse erythrocytic adenosine deaminase. This difference is presumably due to instability of schistosomal adenosine deaminase at alkaline pH values (Fig. 6).

Fischer's medium which had been used to collect and incubate schistosome worm pairs contains adenosine deaminase activity which is highly active when assayed at pH 6·8 and shows markedly reduced activity when assayed at pH 8·9 (Fig. 7).

DISCUSSION

Since *S. mansoni* are dependent on salvage mechanisms for their supply of purine bases, it is very likely that their nucleotide pathways could be compromised by various purine analogs. Thus the choice of the proper analog is determined by the nature of the preferred pathways which the worms use during nucleotide anabolism. The results of this study and those on enzyme measurements given in a related paper[6] suggest various targets of opportunity in the design of chemotherapeutic agents. For example, it appears that the formation of AMP from IMP is one of the rate-limiting steps in the conversion of adenosine or inosine to adenine nucleotides. One could,

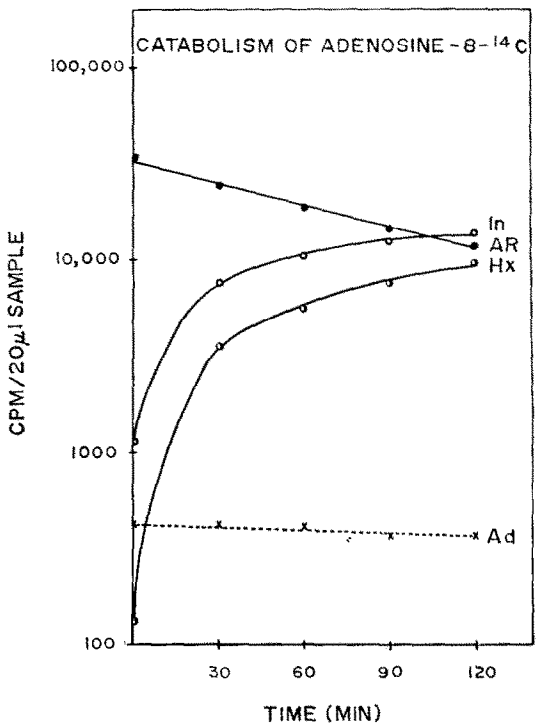


FIG. 2A.

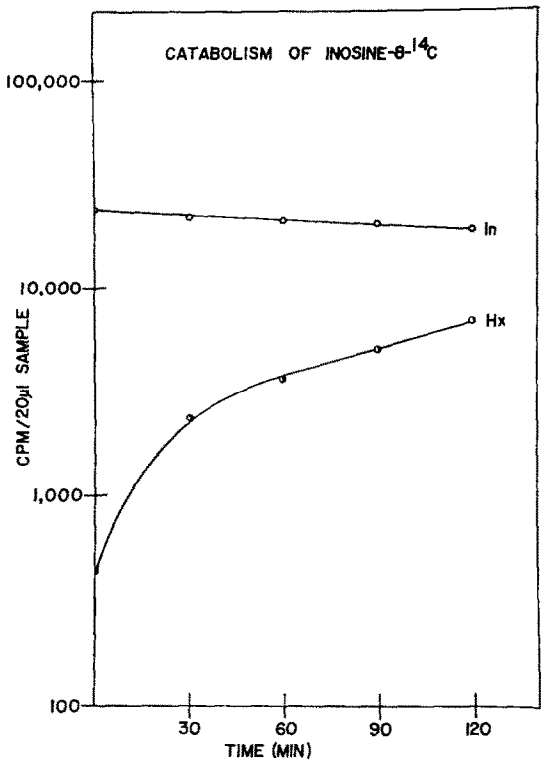


FIG. 2B.

FIG. 2. *S. mansoni* (22 pairs) were incubated in 2·5 ml of Fischer's medium supplemented with glucose for 2 hr at 37°. About 2·5 μCi adenosine (sp. act. 47·2 μCi/μmole) or inosine (34·9 μCi/μmole) was added to each tube. Samples of the medium (20 μl) were taken at 30-min intervals for chromatography.

TABLE 2. NUCLEOTIDES RECOVERED FROM WORMS INCUBATED IN ADENOSINE AND INOSINE (counts/min/10-μl SAMPLE)*

	Adenosine 8-^{14}C		Inosine 8-^{14}C	
	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
Nucleotides	5990	90	4530	70
In	0	25	4	16
Hx	44	840	18	760
AR	0	0	0	0
Ad	25	3650	55	1850

* The worms pairs from the experiments described in Fig. 2A and B were homogenized in 10% TCA. The supernatant from the worms incubated with adenosine-8-^{14}C contained 185,500 counts/min and the supernatant from those incubated with inosine-8-^{14}C contained 61,900 counts/min. The nucleotide pool was isolated and hydrolyzed as described in the Materials and Methods section.

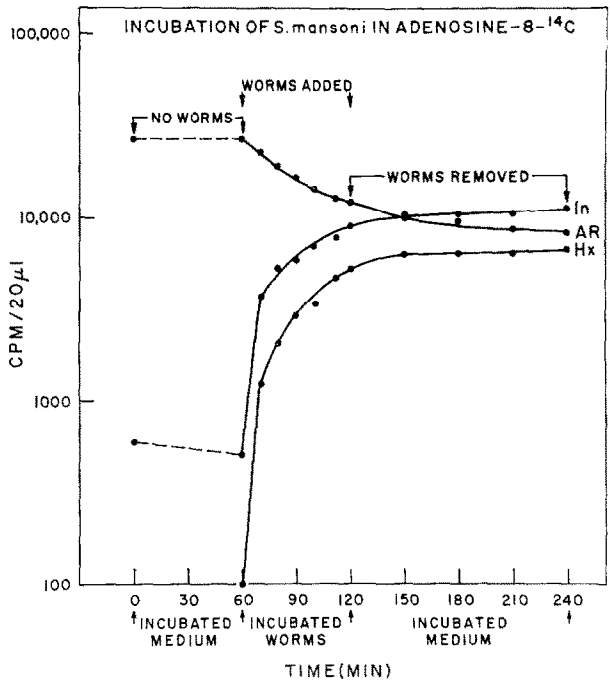


FIG. 3. Fischer's medium (2·5 ml) was supplemented with about 2·5 μl of 8-^{14}C adenosine (sp. act. 47·2 μCi/μmole). Twenty-μl samples were taken prior to the addition of worms, during incubation with *S. mansoni* (22 pairs), and in the period after worms were removed. These samples were chromatographed and then counted to determine the relative concentrations of adenosine, inosine and hypoxanthine.

therefore, postulate that this pathway is a prime candidate for antischistosome agents.

It was surprising to note that under conditions *in vitro*, worms release or excrete active adenosine deaminase into the medium. The source of this excreted enzyme is presumed to be the schistosomes, since pH activity is similar to that found in extracts of fresh worms and is different from deaminase extracted from host red cells. The acid stability of schistosomal adenosine deaminase is consistent with the acid character of the gut contents (Senft, unpublished data) of schistosomes.

If one accepts the presence of multiple enzymes in the medium as representing normal worm products released by cecal regurgitation, and also that these enzymes are excreted within the host plasma, one could expect that multiple enzyme sensitization would be a feature of schistosomiasis. Hypersensitivity to a globin-splitting protease[7] has already been detected in animals and humans infected with this parasite. One could imagine similar sensitization to adenosine deaminase or to purine nucleoside phosphorylase. Thus, active immunization using specific schistosome enzymes as an antigen would thus appear to be theoretically possible as a protective defense procedure.

The significance of the small but constant rate of production of adenine from adenosine (Fig. 4) is presently not understood. It has been shown that adenine is a poor alternative substrate for purine nucleoside phosphorylase from several tissues.[8]

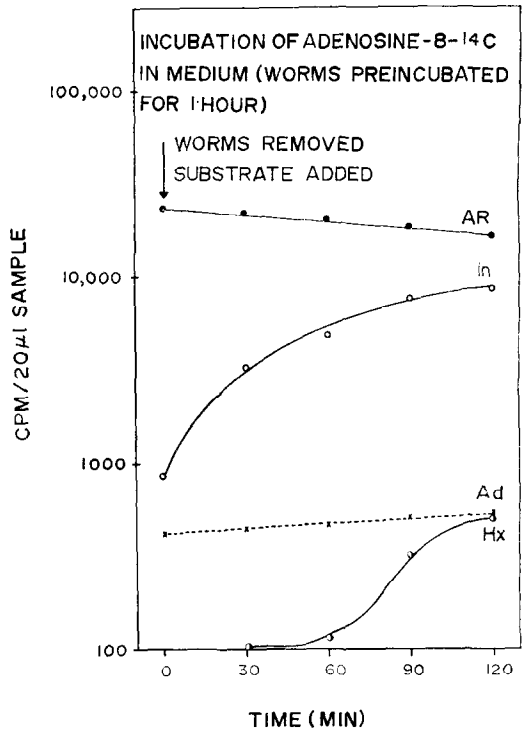


FIG. 4. *S. mansoni* (22 pairs) were incubated for 1 hr in 2·5 ml of Fischer's medium in the absence of labeled substrate. Then the medium was removed and added to a fresh test tube containing 2·5 μCi of dried adenosine-8-^{14}C. Aliquots (20 μl) were removed at 30-min intervals for chromatography.

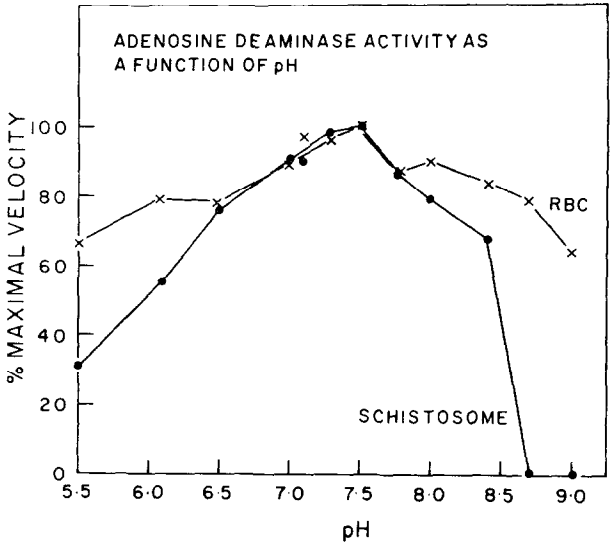


FIG. 5. Aliquots from homogenized *S. mansoni* supernatant or lysed mouse red cell supernatant were tested for adenosine deaminase activity at various pH values. In the pH range of 5·5–7·0 a cacodylate–acetic acid buffer was used. Above pH 7·0, a Tris–acetic acid buffer was employed.

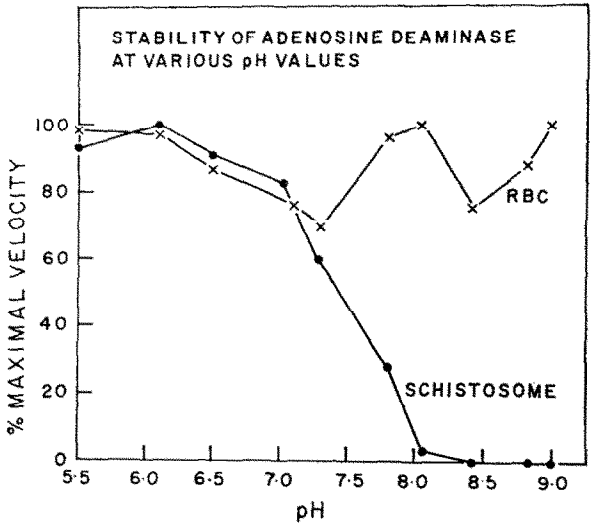


FIG. 6. Homogenized schistosome supernatant or mouse red cell lysate was used as sources of enzyme. The enzymes were incubated for 3·5 hr at various pH ranges using buffers as described in Fig. 5. Assay of activity was made at pH 7·5.

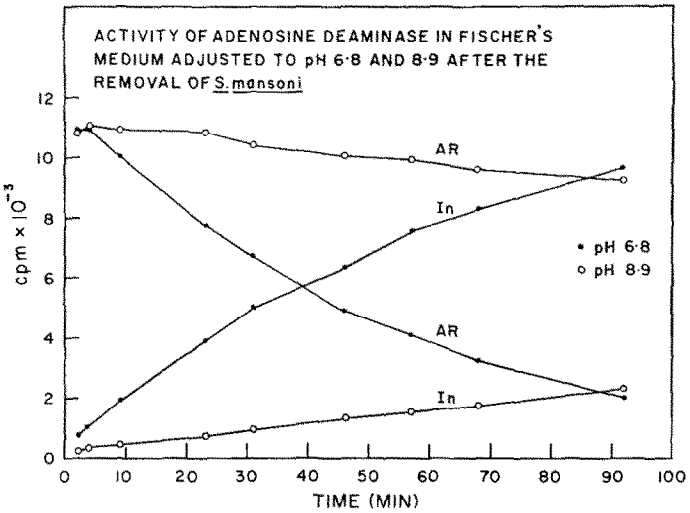


FIG. 7. Whole schistosomes (30 pairs) were incubated at 4° in 2 ml of Fischer's medium for 1 hr at pH 7·4. After warming, the worms were removed and a 500-μl sample of the centrifuged medium was tested at two pH values for adenosine deaminase activity using labeled adenosine as the substrate.

If adenosine is likewise a poor alternative substrate for purine nucleoside phosphorylase, this may account for the small rate of adenine formation from adenosine. The presence of an enzyme which will split adenosine to yield free adenine has not been reported for *S. mansoni*. On the other hand, the adenine may have originated from the adenosine via a membrane transport system for adenosine. Such a transport system has been reported in *Escherichia coli* membranes.[9]

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