Nucleoside Hydrolase from Crithidia fasciculata

METABOLIC ROLE, PURIFICATION, SPECIFICITY, AND KINETIC MECHANISM*

(Received for publication, May 6, 1991)

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Crithidia fasciculata cells grown on complex medium with added [8-14C,5'-3H]inosine or [8-14C,5'-3H]adenosine metabolize >50% of the salvaged nucleosides through a pathway involving N-glycoside bond cleavage. Cell extracts contain a substantial nucleoside hydrolase activity but an insignificant purine nucleoside phosphorylase. The nucleoside hydrolase has been purified 1000-fold to >99% homogeneity from kilogram quantities of C. fasciculata. The enzyme is a tetramer of Mr, 34,000 subunits to give an apparent holoenzyme Mr of 143,000 by gel filtration. All of the commonly occurring nucleosides are substrates. The Km values vary from 0.38 to 4.7 mM with purine nucleosides binding more tightly than the pyrimidines. Values of Vmax/Km vary from 3.4 X 106 M⁻¹ s⁻¹ to 1.7 X 10⁶ M⁻¹ s⁻¹ with the pyrimidine nucleosides giving the larger values. The turnover rate for inosine is 32 s⁻¹ at 30 °C. The kinetic mechanism with inosine as substrate is rapid equilibrium with random product release. The hydrolytic reaction can be reversed to give an experimental Keq of 106 M with H2O taken as unity. The product dissociation constants for ribose and hypoxanthine are 0.7 and 6.2 mM, respectively. Deoxynucleosides or 5'-substituted nucleosides are poor substrates or do not react, and are poor inhibitors of the enzyme. The enzyme discriminates against methanol attack from solvent during steady-state catalysis, indicating the participation of an enzyme-directed water nucleophile. The pH profile for inosine hydrolysis gives two apparent pK values of 6.1 with decreasing Vmax/Km values below the pK and a plateau at higher pH values. These effects are due to the pH sensitivity of the Vmax values, since Km is independent of pH. The pH profile implicates two negatively charged groups which stabilize a transition state with oxycyanonium character.

Most species of trypanosomes lack the pathway for de novo synthesis of purines (1–3). Instead, the organisms use a variety of mechanisms to salvage purines from the blood of the host.

*Preliminary studies for this project were supported by Research Grant GM21083 from the National Institutes of Health. The majority of the work was supported by Grant GM41916 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by Postdoctoral Fellowship PG 3298 from the American Cancer Society and by a Mildred and Emil Holland postdoctoral scholarship award from the Albert Einstein College of Medicine.

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Crithidia fasciculata is a trypanosome parasite of the mosquito which does not infect mammals. It is dependent on purine salvage and this provides a convenient biological system for understanding the enzymology of purine salvage in trypanosomes (4). Previous reports have indicated that Crithidia as well as the pathogenic trypanosomes such as Trypanosoma cruzi contain nucleoside hydrolases which cleave the N-glycosidic bond of purine and pyrimidine ribosides to give ribose and the base (5, 6).

In the related organism, Crithidia luciata, a cell-surface associated 3'-nucleotidase/nuclease has been reported which is induced by purine or phosphate starvation (7). The enzyme is proposed to generate nucleosides which are further metabolized by a family of nucleoside hydrolases that convert the nucleosides to the bases (e.g. Ref. 6). Phosphoribosyl transferases are then proposed to form the respective nucleotides by reaction of the bases with 5-phosphoribosyl-1-pyrophosphate (8, 9). Purine nucleoside phosphorylases form the major pathway for nucleoside metabolism in mammalian cells, but are not the dominant salvage pathway in trypanosomes. Trypanosomal nucleoside hydrolases appear to play the role occupied by purine nucleoside phosphorylase in higher organisms.

The purpose of this study was to determine the role of nucleoside hydrolase in the pathway of purine salvage in C. fasciculata, to provide a procedure for obtaining the purified enzyme, to characterize the protein subunit structure, and to establish the catalytic mechanism. The enzyme had not been purified to homogeneity nor had it been well characterized for substrate specificity, inhibitors, metabolic function in purine nucleoside salvage, or for its kinetic mechanism. No equivalent enzyme activity has been documented in mammalian cells. The results have implicated the enzyme as a step in the major pathway for purine nucleoside metabolism. A purification procedure is reported which yields tens of milligrams of highly purified protein. The substrate specificity and kinetic mechanism have been characterized. In other studies, the transition state structure for nucleoside hydrolase has been characterized from kinetic isotope effect studies.1 The metabolic and kinetic features of nucleoside hydrolase make it a potential target for antimetabolites of purine salvage. Since the enzyme is not present in mammals, it may be possible to develop nucleoside hydrolase inhibitors which are specific for trypanosomes and related pathogens.

MATERIALS AND METHODS

Colorimetric Assays for Nucleoside Hydrolase and Purine Nucleoside Phosphorylase—Catalytic activity for nucleoside hydrolase was...
Nucleoside Hydrolase from *C. fasciculata*

The reaction was terminated by the addition of 100 μl of 1.0 M HCl. After the reaction was stopped, the reducing sugar was measured by the addition of 100 μl of 1.0 M NaOH. Zn²⁺ was removed by the addition of 100 μl of 0.1 M NaOH. After centrifugation, 250 μl of supernatant was removed and assayed for reducing sugar formed as described above. Assays for purine nucleoside phosphohydrolase were the same as for nucleoside hydrolase except that 10 mM sodium arsenate was added as the phosphate analogue. Arsenate readily replaces phosphate in the reaction and the product ribose-1-arosonate rapidly hydrolyzes to release ribose which is detected in the reducing sugar assay.

Radioactive Assay for Nucleoside Hydrolase—Catalytic activity was determined by the conversion of [8-³²P]inosine to [8-³²P]hypoxanthine and ribose. Assay mixtures of 19 μl contained the desired concentration of inosine and inhibitor(s) together with approximately 3 × 10⁶ cpm of radioactive inosine in 50 mM Hepes or triethanolamine buffer, pH 7.3. Reactions were initiated by the addition of 1 μl of nucleoside hydrolase in 0.5 M Hepes or triethanolamine, pH 7.3. Samples of 3 μl were removed at appropriate intervals and spotted on polyethyleneimine cellulose thin layer plates which had been previously spotted with 4 μl of a solution containing 2.2 mM each of inosine and hypoxanthine. Control experiments in which the labeled inosine was prespotted on the thin layer plates established that the reaction mixture to the ion-exchange resin stopped the reaction.

HPLC and NMR Assays for Substrate Activity—Substrate activity was determined in incubation mixtures containing nucleosides at 2.5–28 mM in the presence of 50 or 100 mM triethanolamine-HCl, pH 8.0, or 50 mM potassium phosphate, pH 7.5, and 16–400 μg/ml purified nucleoside hydrolase. Reaction mixtures were incubated for 5–27 h and samples analyzed on a 5-μm × 30-cm C18-HPLC column eluted with 5 or 10% methanol in H₂O, 4 ml/min. Samples were detected by the absorbance at 257 nm. Substrate activity with deoxyinosine was also measured at 500 MHz by NMR. The sample contained 14 mM deoxyinosine, 50 mM triethanolamine-HCl, pH 8.0, in D₂O. After collecting the reference proton spectrum, 280 μM/ml nucleoside hydrolyzed was added via a syringe fitted with an extension tube. Proton NMR spectra were collected in sets of eight scans over 15 min at room temperature.

Protein Determination—Protein was routinely estimated by the dye-binding method of Bradford (11). Reagents were purchased from Bio-Rad, and bovine serum albumin was used as the standard. The extinction coefficient and absolute concentration of enzyme was determined by quantitative analysis of samples which had been dialyzed against 1 mM potassium phosphate buffer, pH 7.5, and dried at 80°C under reduced pressure to constant mass. Samples of enzyme and dialyzed were analyzed in triplicate on a Cahn ultrabalance.

**Growth of *C. fasciculata***—*C. fasciculata* were grown in 30°C in small shaken flasks using 10 ml of the medium described above. After reaching an absorbance of 1–2 at 600 nm, approximately 2 μCi of [8-³²P]inosine or [8-³²P]hypoxanthine was added, as ³²P. The amount of labeled inosine or adenine added to the culture medium represented less than 0.6% of the A₂₅₀-absorbing material present in the medium from the yeast extract. Cells were grown to an absorbance of 3–4 at 600 nm in the presence of the labeled nucleosides. Cells were harvested by centrifugation for 10 min at 4°C and washed twice in 10 mM potassium phosphate, pH 7.4, containing 0.9% NaCl. Nucleic acids were extracted by the addition of 1 ml of 10 mM EDTA and 1 ml of 0.1 M sodium acetate, pH 5.0, or 0.5% sodium dodecyl sulfate. Buffer-equilibrated phenol (2 ml) was added followed by 2 min of agitation. Following centrifugation, the aqueous phase was transferred to a tube containing 440 ml of 1 M Tris-Cl, pH 8.0, and 180 ml of 5 M NaCl. Two volumes of ice-cold ether were added, and the nucleic acids collected following centrifugation. The dried pellets were dissolved in 250 ml of 10 mM Tris-Cl, pH 8.0, containing 2 mM EDTA. Samples of 100 ml were counted in 10 ml of scintillation fluid and 100 μl in triplicate, with the growth of cells, cell labeling, and analysis of labeled nucleic acids.

**Purification of Nucleoside Hydrolase**—Freshly harvested or frozen cells were satisfactory for the preparation of nucleoside hydrolase. Approximately 1.5 kg of cells were used for each enzyme preparation. The cell suspension in the buffer and proteinase K was described above was adjusted to pH 9.7 with NaOH. The cell sus-
Nucleoside Hydrolase from C. fasciculata

rupted in a Dyno Mil continuous-flow glass bead mill at 3000 rpm, using 0.4-mm glass beads. The disruption chamber was cooled by circulating external fluid at −20 °C. The disrupted cell suspension was warmed to 55–60 °C for 5–10 min, chilled on ice to 4 °C, and centrifuged for 90 min at 200,000 × g in the cold.

The supernatant was added to 0.5 M ammonium sulfate to give 35% saturation at 4 °C, stirred for 30 min and centrifuged at 5 °C at 20,000 × g for 20 min. The supernatant was brought to 0.57 saturation with additional ammonium sulfate and treated as above. The resulting pellet was suspended in sufficient volume to make a suspension, but less than required to completely dissolve the pellet, using 50 mM Hepes, pH 7.2, and containing 100 mM KCl, 100 mM dithiothreitol, and 100 mM EDTA. The sample was dialyzed overnight against the buffer used to suspend the fraction.

The dialyzed fraction was applied to a 5 × 30-cm column of DEAE-Sephadex A-50 which was equilibrated against the buffer used to suspend the sodium sulfate pellet. The column was washed with 1 liter of the buffer and the enzyme eluted with a 4-liter linear gradient of 0.1–0.3 M KCl in the same buffer. The enzyme elutes near 0.25 M KCl. In some preparations, 2 mM inosine was added to all buffers during ion-exchange chromatography. Only small improvements in recovery were experienced in the presence of inosine. The fraction, which contained nucleoside hydrolase activity was concentrated and concentrated to 70 ml using an Amicon Diaflow ultrafiltration concentrator which retained proteins with Mm greater than 10,000.

The concentrated sample from ion-exchange chromatography was dialyzed against buffer containing 10 mM Hepes and 10 mM Pipes, pH 6.5. The dialyzed fraction was centrifuged to remove precipitated materials and applied to a 2.5 × 100 cm column of Fracton Red HE23 (Mafrex™) Gel Red A, Amicon) previously equilibrated with the Hepes/Pipes buffer. The column was washed with 100 ml of the buffer and the protein was eluted with a 400-ml linear gradient from pH 6.5 to 8.0. The enzyme eluted near pH 7.0. The enzyme-containing fractions were concentrated to 2 ml using a collodion bag with 

M , 25,000 retention and the pH adjusted to 6.5.

The enzyme solution was applied to a second Red A column of 1.2 × 17 cm which had been previously equilibrated with 10 mM Hepes and 10 mM Pipes, pH 6.5. The column was washed with 25 ml of the buffer, and the enzyme was eluted with a 200 ml linear gradient of 0.25 M KCl. The enzyme eluted near 0.7 M KCl. The fractions containing enzyme were concentrated to approximately 1 ml and dialyzed against 0.5 M Hepes, pH 7.5. Purity of the protein was estimated by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Enzyme of adequate purity was rapidly frozen using dry ice ethanol in small glass tubes and stored at −70 °C. In cases where the enzyme was of inadequate purity following this procedure, it was subject to gel filtration on a 2.5 × 65-cm column of Superdex 200 in 50 mM potassium phosphate, pH 7.5, using a flow rate of 2 ml/min.

**Molecular Weight of Nucleoside Hydrolase**—The elution position of active nucleoside hydrolase relative to proteins of known molecular weight was determined by Ultragel AcA34 gel filtration. Bovine serum albumin (Mw = 66,000), β-amyelase (Mw = 200,000), carbonic anhydrase (Mw = 29,000), and alcohol dehydrogenase (Mw = 150,000) were used as protein standards. Approximately 200 ml of a 15 mg/ml solution of each protein was passed through a 2 × 26 cm column equilibrated with 100 mM triethanolamine, pH 7.5, containing 50 mM KCl and 0.05% sodium azide. The column was run at room temperature. The enzyme peaks were monitored at 280 nm, and the elution volume of the protein was determined by gel filtration on a 2.5 × 65-cm column of Superdex 200 in 50 mM potassium phosphate, pH 7.5, using a flow rate of 2 ml/min.

**Analysis of the product was by 'H NMR at 500 MHz.** Only ribose exchanged into D2O by two additional exchanges of 3 ml each.

**RESULTS**

**Metabolism of Labeled Nucleosides**—Enzymatically synthesized [8-14C,5′-3H]AMP was prepared with a 14C/3H ratio of 2.46 ± 0.02 and converted enzymatically to labeled adenosine and inosine with the same specific radioactivity (see "Materials and Methods"). Cultured C. fasciculata readily incorporated labeled inosine and adenosine from the medium. In typical experiments, approximately 5% of the total added label was incorporated in 1–2 cell doublings at cell densities above 1.0 absorbance unit at 600 nm. Isolated nucleic acids demonstrated an increase in the 14C/3H ratio to 5.2 ± 0.9 with [8-14C,5′-3H]inosine and to 5.2 ± 0.3 with [8-14C,5′-3H]adenosine. Salvage of these nucleosides indicated greater than 2-fold enrichment of base relative to ribose in the nucleic acids. The results of nucleoside uptake experiments are summarized in Table I.

**Purine Nucleoside Phosphorylase Determination**—Cell extract C. fasciculata was dialyzed against phosphate-free buffer and assayed for the presence of purine nucleoside phosphorylase by measuring the rate of formation of ribose from 5 mM inosine in assay mixtures with and without inorganic arsenate. In the absence of arsenate the specific activity of inosine hydrolase activity was 87 ± 2 mmol/min/mg protein. In the presence of inorganic arsenate, the total activity for glycoside bond hydrolysis was 58 ± 2 mmol/min/mg protein. Thus, the activity of purine nucleoside phosphorylase was not significant compared with the nucleoside hydrolase activity in dialyzed cell extracts of C. fasciculata.

**Purification of Nucleoside Hydrolase**—Nucleoside hydrolase is resistant to heat denaturation at 55–60 °C. Heat treatment provided a convenient first step and gave 7-fold purification. Although ammonium sulfate fractionation provided only 2-fold purification, it was useful to concentrate the enzyme to a reasonable volume for subsequent dialysis. The major purification resulted from ion-exchange and dye-column chromatography. Purifications of 9- and 6-fold, respectively, were obtained on DEAE ion-exchange chromatography and the first Red A column. The remaining impurity was removed by elution from a second column of Red A gel and, when necessary, by gel-exclusion chromatography. The enzyme exhibits pH dependence of elution on Red A gel. This step was unusual effective for nucleoside hydrolase and often provided enzyme which was nearly pure. The purification following the Red A columns resulted in enzyme which was typically >95% homogeneous as determined by gel electrophoresis on denaturing polyacrylamide (Fig. 1). Following gel-exclusion chromatography, the majority of the fractions were >99% homogeneous. The enzyme was typically purified 1000-fold from cell extracts in 13% yield as summarized in Table II. The

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tbody>
<tr>
<td><strong>Incorporation of labeled inosine and adenosine into nucleic acids of C. fasciculata</strong></td>
</tr>
<tr>
<td><strong>Labelled substrate</strong></td>
</tr>
<tr>
<td><strong>nucleosides</strong></td>
</tr>
<tr>
<td>[8-14C,5′-3H]Inosine</td>
</tr>
<tr>
<td>[8-14C,5′-3H]Adenosine</td>
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</tbody>
</table>

*The ratios of 14C/3H in the nucleosides are identical since labeled inosine and adenosine were synthesized from the same preparation of [8-14C,5′-3H]AMP.*
extinction coefficient for purified enzyme was 0.569 at 280 nm for a 0.1% solution in 1 mM potassium phosphate, pH 7.3. The ultraviolet absorbance spectrum exhibited a maximum at 278 nm and was characteristic of a protein with a low mole-
traction of tryptophan.

**Molecular Weight and Subunit Structure**—The molecular weight of active enzyme was estimated by gel-exclusion chromato-
graphy and by denaturing gel electrophoresis using pro-
tiens of known masses as standards. Nucleoside hydrolase eluted at an apparent molecular weight of 143,000 on gel
exclusion chromatography (Fig. 2). Gel electrophoresis on
denaturing polyacrylamide gave a single molecular weight
species with an apparent subunit molecular weight of 34,000
(Fig. 2). These values are consistent with the active enzyme
being composed of four subunits with identical or highly
similar molecular weight.

**Substrate Specificity of Nucleoside Hydrolase**—The commonly occurring purine and pyrimidine ribonucleosides are
substrates with \( V_{\text{max}}/K_m \) values which range from 3.4 \( \times 10^3 \) to
1.7 \( \times 10^5 \) M \(^{-1}\) s \(^{-1}\) for 5-methyluridine. Purine riboside was a
poor substrate with a \( V_{\text{max}}/K_m \) of 1.5 \( \times 10^2 \) M \(^{-1}\) s \(^{-1}\), a \( K_m \) of
145 \( \mu \)M, and a turnover number of 0.023 s \(^{-1}\). Deoxyribonucleosides
at the 2', 3', or 5'-positions are poor substrates or lack
substrate activity. In several cases the deoxyribonucleosides are
inert at \( <10^{-4} \) the rates for hydrolysis of inosine. An example of
the effect of the 2'-hydroxyl is the comparative reactivity of
5-methyluridine and thymidine, where the apparent \( V_{\text{max}} \) difference is \( >10^4 \), with no activity being detected for thy-
dine. Substitution of the 5'-hydroxyl with phosphate or re-
placement with the thiomethyl group also decreased or elimi-
nated substrate activity. Although the specificity for the aglycone is low for the substrate nucleosides, certain features
of the aglycone are crucial, since tubercidin (7-deazaadenosine)
is not a substrate at a detection level of 3 \( \times 10^{-4} \) the
rate with inosine.

**Inhibitors of Nucleoside Hydrolase**—Product inhibition with both ribose and hypoxanthine resulted in slope-linear,
competitive patterns. The \( K_i \) slope values were 0.70 \( \pm 0.10 \) and
6.2 \( \pm 0.9 \) mM, respectively, for ribose and hypoxanthine.
When both ribose and hypoxanthine were added in combi-
nation and the initial rates estimated using radioactive inosine
as substrate, the slope increased less than expected for the
combination of both inhibitors. These results indicated that
the products combine in an antagonistic manner, with the
combination of one decreasing the affinity for the second.
The dissociation constant for interaction of hypoxanthine
with the enzyme-ribose complex could not be measured ac-
curately because of the limited solubility of hypoxanthine.
However, in the presence of 0.3 mM inosine and 20 mM ribose,
addition of hypoxanthine gave inhibition consistent with a
dissociation constant of approximately 25 mM.

Tubercidin (7-deazaadenosine) was a competitive inhibitor
of nucleoside hydrolase with a \( K_i \) of 2.3 \( \pm 0.4 \) mM. Some inhibitors
were tested in experiments in which inosine was
maintained near the \( K_m \) of 380 \( \mu \)M. Apparent inhibition con-
tants \( (K_{i(app)}) \) were determined from the relationship shown in
the following equation:

\[
v_i/v_0 = (\text{inosine} \pm K_m(1 + I/K_{i(app)}))/(\text{inosine} + K_m)
\]

where \( v_i \) and \( v_0 \) are the initial reaction rates without and with
inhibitor, respectively. \( K_m \) is the Michaelis constant for ino-
sine, \( I \) is inhibitor concentration and \( K_{i(app)} \) is the apparent
compared with the substrates, with most nucleosides having inhibition constants obtained by this method are summarized in Table II. Nucleosides and nucleoside analogues which are not substrates are poor inhibitors of nucleoside hydrolase positions of authentic 1-methyl ribose. Separate experiments were conducted to establish the stability of inosine hydrolase under the reaction conditions.

Equilibrium Constant—The equilibrium constant for nucleoside hydrolase was stable over a wide range of pH values, permitting initial rate studies from pH 5.5 to 9.7 (Fig. 4). The observed $K_m$ for inosine was independent of pH over the entire range. The $V_{max}$ value was independent of pH at values above 7, but decreased rapidly at pH values below 7. The data could not be fit to a single $pK_a$ but was fit well by the equation describing two essential groups with $pK_a$ values of 6.11 $\pm$ 0.01. Both groups must be in the unprotonated form for catalytic activity, but neither

dissociation constant for inhibitor. In cases where the inhibitor is competitive with respect to substrate, $K_{(app)}$ is the dissociation constant for the enzyme-inhibitor complex. Inhibition constants obtained by this method are summarized in Table III. Nucleosides and nucleoside analogues which are not substrates are poor inhibitors of nucleoside hydrolase compared with the substrates, with most nucleosides having inhibition constants in the millimolar range.

Equilibrium Constant—The equilibrium constant for nucleoside hydrolase was established by the equilibrium formation of inosine from relatively high concentrations of ribose and hypoxanthine. The temperature was maintained at 37 °C to increase the solubility of hypoxanthine. At 10 mM hypoxanthine and with ribose concentration varying from 1 to 3 M, inosine was readily detected and quantitated by HPLC. The dissociation constant of 106 $\pm$ 18 M was obtained from three experiments typified by the results of one experiment shown in Fig. 3.

Solvent Reactivity—The ability of methanol to serve as a solvent nucleophile in the nucleoside hydrolase reaction was tested with inosine as the substrate and 20% methanol in water as the solvent. The limit of detection for the product of the methanolysis reaction, 1-methyl ribose, was estimated to be 5% of the total ribose product by NMR (see "Materials and Methods"). There was no detectable resonance at the positions of authentic 1-methyl ribose. Separate experiments established the stability of inosine hydrolase under the reaction conditions.

Effect of pH on the Kinetic Constants of Nucleoside Hydrolase—The activity of nucleoside hydrolase was stable over a

<table>
<thead>
<tr>
<th>Substrate or inhibitor</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$V_{max}/K_m$</th>
<th>$K_a$ or $K_{(app)}$</th>
</tr>
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<tbody>
<tr>
<td>Inosine</td>
<td>50 ± 2</td>
<td>380 ± 30</td>
<td>7.6 $\times$ 10³</td>
<td></td>
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<tr>
<td>Adenosine</td>
<td>7.8 ± 0.2</td>
<td>480 ± 30</td>
<td>9.8 $\times$ 10³</td>
<td></td>
</tr>
<tr>
<td>Guanosine</td>
<td>70 ± 0.04</td>
<td>420 ± 10</td>
<td>3.4 $\times$ 10³</td>
<td></td>
</tr>
<tr>
<td>Purine ribose</td>
<td>0.04 ± 0.01</td>
<td>145 ± 23</td>
<td>1.5 $\times$ 10³</td>
<td></td>
</tr>
<tr>
<td>5'-Methyluridine</td>
<td>380 ± 5</td>
<td>1,300 ± 40</td>
<td>1.7 $\times$ 10³</td>
<td></td>
</tr>
<tr>
<td>Uridine</td>
<td>255 ± 3</td>
<td>1,220 ± 40</td>
<td>1.2 $\times$ 10³</td>
<td></td>
</tr>
<tr>
<td>Cytosine</td>
<td>36 ± 3</td>
<td>4,700 ± 500</td>
<td>4.5 $\times$ 10⁴</td>
<td></td>
</tr>
<tr>
<td>Ribose*</td>
<td>700 ± 40</td>
<td></td>
<td>6,200 ± 900</td>
<td></td>
</tr>
<tr>
<td>Hypoxanthine*</td>
<td>600 ± 40</td>
<td></td>
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</table>

*The inhibition constant is $K_a$ obtained from experiments using at least four inosine concentrations at each of three inhibitor concentrations. The inhibition constant was obtained to the best fit of the data to the equation for competitive inhibition (14).

*The concentration of the nucleoside used in the activity/inhibition determination. The inhibition constant $K_{(app)}$ is an apparent constant determined at a single inosine concentration near the $K_a$ for inosine and three inhibitor concentrations. The value was calculated from the ratio of -inhibitor/+inhibitor initial rates as described under "Results."

Low rates of product formation were observed for both 5'-d-adenosine and 5'-d-inosine. The $K_a$, values were measured with inosine as substrate under conditions where no product would be formed from the 5'-deoxynucleosides. Formycin B is not expected to be a substrate since the compound is a C-glycoside. The N-9 and C-8 atoms are reversed to give formycin B.

![Fig. 3. Determination of the equilibrium constant for nucleoside hydrolase.](image-url)
patterns establish the kinetic mechanism as rapid equilibrium. The rate indicates that much of the substrate binding energy and the relatively low specificity of the enzyme, which includes both purine and pyrimidine nucleosides but does not extend to the deoxynucleosides, nucleotides or nucleosides with substituted riboses. Ribose binds better than hypoxanthine by a factor of approximately 20, consistent with the requirement for the ribose ring for good substrate activity. Both hypoxanthine and ribose bind better to the free enzyme than to the enzyme-product ternary complex. These interactions are shown in the reaction mechanism in Fig. 5. The poor binding of products to the ternary complex predicts a rapid release to the binary complexes which must also release products rapidly relative to the catalytic step. The rapid equilibrium random mechanism is consistent with a step in the conversion of enzyme-bound substrate to enzyme-bound products as the major energy barrier in the overall process of catalysis. Kinetic isotope effects have also established that the formation of an oxycarbonium intermediate transition state is the major energetic barrier on the reaction coordinate. A rapid equilibrium random mechanism has also been established for AMP nucleosidase from Azotobacter vinelandii, the N-glycosidase which performs similar chemistry on AMP (16).

Hydrolytic reactions are often experimentally irreversible because of the unfavorable concentration of water compared to substrate concentrations. The equilibrium position for nucleoside hydrolysis was reached in the reverse reaction by the use of molar concentrations of ribose and saturating hypoxanthine. The experimental $K_m$ of 106 ± 16 M is similar to that of 170 M determined for AMP nucleosidase in similar experiments, and those of 77 and 36 M calculated from the Haldane relationships for AMP nucleosidase (16). The experimental values for $K_{eq}$, $V_{max}$ in the forward direction and the kinetic constants for enzyme-substrate complexes provide the information needed to solve the Haldane relationship for $V_{max}$ in the reverse direction. The expression is given by the equation below.

\[
K_{eq} = \frac{V_{max, forward} \cdot K_{ha} \cdot K_r}{V_{max, reverse} \cdot K_m}
\]

where $K_{ha}$ is the dissociation constant for hypoxanthine from the enzyme-ribose-hypoxanthine complex. The value for $V_{max, reverse}$ is 0.01 s⁻¹ compared with $V_{max, forward}$ of 32 s⁻¹ in Fig. 5. The equilibrium constant for enzyme-bound reactants is thus 3200 in favor of products to give a $\Delta G^0$ of -4.8 kcal mol⁻¹. The overall equilibrium constant for hydrolysis with $H_2O$ at unit molarity is -2.8 kcal mol⁻¹, and is -0.4 kcal mol⁻¹ with water at 55 M. Hydrolysis on the enzyme is the most energetically favored of these reactions. Nucleoside hydrolysis is unlike many transferases which have $\Delta G^0$ values near unity for transfer on the enzyme but exhibit large negative $\Delta G^0$.

**FIG. 4.** Effect of pH on the kinetic constants for nucleoside hydrolase. The right panel demonstrates the independence of $K_m$ value on solution pH. The data points are the best fit of the kinetic data to the Michaelis-Menten equation using the programs of Cleland (14). The line is drawn by eye. The left panel demonstrates the decrease in $V_{max}$ and $V_{max}/K_m$ at low pH values. The experimental points were obtained from the best fits of the data to the Michaelis-Menten equation. The experimental lines are the best fit to the equation for two essential ionizable groups with the indicated $pK_a$ values (14).

influences substrate binding. Response of $V_{max}/K_m$ to pH was parallel to that for $V_{max}$, to give two groups with $pK_a$ values of 6.16 ± 0.08.

**DISCUSSION**

**Nucleoside Salvage in C. fasciculata**—Growing cells readily incorporated labeled inosine and adenosine into cellular nucleic acids, with a 2:1 preference for incorporation of the purine base relative to ribose. N-Glycoside bond cleavage is therefore the major pathway of nucleoside salvage in medium which provides required purines from yeast extract. Purine nucleoside phosphorylase activity is not present at significant levels relative to nucleoside hydrolyase. Thus, nucleoside hydrolysis is implicated as the major catalytic activity of purine salvage in C. fasciculata. The 2.1-fold enrichment of salvaged nucleoside bases with respect to salvaged nucleoside ribose in the isolated nucleic acids represents a lower limit for the nucleoside hydrolyase pathway. The [5-³H]ribose released from the [8-¹⁴C,5-³H]labeled nucleosides can be phosphorylated and converted to 5-phosphoribosyl-1-pyrophosphate. Any reutilization of [5-⁴H]ribose in purine or pyrimidine salvage following its conversion to [5-³H]-5-phosphoribosyl-1-pyrophosphate would be interpreted as part of the direct incorporation of intact nucleoside into nucleic acids by way of the nucleoside kinase pathway.

The broad specificity of nucleoside hydrolase for purine and pyrimidine nucleosides together with the relatively close values of $V_{max}/K_m$ for the natural substrates suggests that this enzyme could play a metabolic role in salvage for pyrimidines, as well as the demonstrated role in inosine and adenosine salvage. The concentration of inosine in mammalian blood is in the low μmolar range, well below the $K_m$ for the C. fasciculata enzyme. The nucleoside levels in mosquito serum, the natural host, are not well established. However, the relatively efficient $V_{max}/K_m$ values, the efficient nucleoside transport systems of trypanosomes (15), and the relatively large amount of the protein (0.1% of extractable protein) permits efficient function of the hydrolase in vivo.

**Kinetic Mechanism**—Two competitive product inhibition patterns establish the kinetic mechanism as rapid equilibrium random, with no significant dead-end complexes forming at the product concentrations used in inhibition studies. In the rapid equilibrium mechanism, all kinetic constants, including that of the $K_m$ for inosine, represent dissociation constants for the enzyme. The weak dissociation constant for hypoxanthine indicates that much of the substrate binding energy comes from recognition of the ribose portion of the substrate. This is consistent with the relatively low specificity of the enzyme, which includes both purine and pyrimidine nucleosides but does not extend to the deoxynucleosides, nucleotides or nucleosides with substituted riboses.

**FIG. 5.** Kinetic mechanism for nucleoside hydrolase. The concentrations shown near the arrows for binding and release of substrate and products are the dissociation constants for each complex. The rates for the interconversion of the enzyme-inosine and enzyme-hypoxanthine-ribose complexes are from $V_{max}$ in the forward direction and calculated from $K_m$ and the Haldane equation in the reverse direction. Free nucleoside hydrolase is abbreviated as E. The dissociation constant for ribose interacting with the E-hypoxanthine complex is estimated from the three experimentally determined dissociation constants and the expression for the thermodynamic box of product release.
values for the overall reaction (17). The relatively large and negative \( \Delta G^0 \) for nucleoside hydrolase on the enzyme, coupled with the poor binding of substrates, indicates that bond-breaking is an irreversible step at the catalytic site during initial rate conditions.

**Substrate Specificity**—Although specificity for the purine and pyrimidine bases is low, nitrogen is essential at N-7 of purines, presumably to permit protonation of the leaving group as proposed for other purine N-glycohydrolases (18). This effect is similar to the protonation of the leaving group in the acid-catalyzed solvolysis of nucleosides, where the reactive species is the protonated dication (19). Substitution(s) on the purine ring is also required for efficient catalysis, since purine riboside has a turnover number of only 0.023 s\(^{-1}\). Of the substrates, purine riboside is the most tightly bound. A pattern of weakly bound substrates with higher turnover numbers suggests that a portion of the normal binding energy is used to distort the sugar as part of the reaction coordinate, the so-called Circe effect (20). A surprising finding for nucleoside hydrolase is that the 2'-deoxyribonucleosides are not substrates. For example, the best \( V_{\text{max}}/K_m \) substrate is 5-methyluridine, while substrate activity was not detected with thymidine. The hydroxyl group at the 2'-carbon of ribose is chemically stabilizing; thus the 2'-deoxyribonucleosides hydrolyze approximately 10\(^2\) times as fast in acid as the corresponding nucleosides (21). Since nucleoside hydrolases apparently protonate the N-7 of purines, acid-catalyzed solvolysis cannot be the primary mode of catalysis, since the 2'-deoxyribonucleosides would then be expected to react rapidly. Catalysis therefore depends on enzymatic action initiated through well matched ribosyl contacts before the reaction can proceed to the point of N-7 protonation.

Although the 5'-deoxynucleosides are similar in chemical stability to the natural nucleosides, they are poor substrates for nucleoside hydrolase. Likewise, 3'-deoxyadenosine (cordycepin) is not a substrate. The poor substrate activity of the three deoxynucleosides suggests that all of the hydroxyl groups on the ribose are critical for efficient catalysis. In x-ray crystal structures of nucleoside-binding proteins such as adenosine deaminase, it is common for the protein to hydrogen-bond all hydroxyls of the sugar (22). An enzyme designed to stabilize a specific conformation of the ribose at the transition state, for example an oxycarbonium ion, would be expected to exhibit a high degree of specificity for ribose. Oxycarbonium ion intermediates are an established mechanism for accomplishing hydrolysis of N-glycosidic bonds (23).

**Inhibitors of Nucleoside Hydrolase**—Inhibition by hypoxanthine is weak with a \( K_i \) of 7.2 \pm 0.9 mM. Solubility of hypoxanthine is approximately 4 mM at 20°C, precluding more accurate estimation of the kinetic constant. The competitive product inhibition by ribose has a \( K_i \) of 700 \pm 40 \mu M. Ribose is a mixture of \( \alpha \) and \( \beta \) anomers at neutral pH, with the \( \beta \) anomer being favored by a ratio of approximately 6:4. If the \( \beta \) anomer is responsible for inhibition, the actual inhibition constant for \( \beta \)-D-ribose would be 420 \mu M. If \( \alpha \)-D-ribose is the inhibitor, the dissociation constant would be 380 \mu M. In either case, the binding of the D-ribose anomer is at least an order of magnitude tighter than that of hypoxanthine, reflecting the more intimate fit of the ribose. A variety of nucleoside analogues which are not substrates bind weakly to the enzyme. Even close structural mimics such as deoxynucleosides, tubercidin, and formycin bind poorly. Tubercidin and formycin often bind well to enzymes which induce distortion in the N-glycosyl torsion angle (24), since the torsion angles differ between these compounds and the naturally occurring purine nucleosides. The lack of strong interactions suggest that nucleoside hydrolase does not depend on torsional strain of the N-glycosyl bond as a major force in formation of the binary complex with substrates.

**Solvent Nucleophiles**—Certain N-glycohydrolases such as NAD\(^+\) glycohydrolase and some O-glucosidases form a covalent intermediate with the carbohydrate moiety which is then displaced by a solvent nucleophile in a second step (25). In covalent mechanisms, displacement of the enzyme is often dependent on the nucleophilicity of the solvent. Methanol is approximately 2 orders of magnitude more reactive toward electron deficient centers than is water (25, 26). For example in NAD\(^+\) glycohydrolase, methanol in the solvent accelerates the reaction and results in methyl-ADP ribose as the major product (25). Nucleoside hydrolase protects the intermediate against methanalysis, indicating that the intermediate is attacked by an enzyme-directed water molecule. This finding does not eliminate the possibility of a covalent ribosyl intermediate. However, if such an intermediate forms, it is not susceptible to attack by bulk solvent, and thus differs considerably from known ribosyl or glucosyl covalent intermediates (27).

**Ionizable Groups in Catalysis**—Two groups with p\( K_a \) values near 6.1 are involved in catalysis but not in substrate binding. The family of glycohydrolases, including lysozyme, has been proposed to stabilize an oxycarbonium type of intermediate by charge stabilization of the developing positive charge with nearby carboxyl groups (27). Nucleoside hydrolase appears to have similar properties in order to stabilize the proposed oxycarbonium intermediate. Since the charged groups do not appear in the \( K_m \) profile, which, for nucleoside hydrolase, is a dissociation constant, the ionizable groups must be moved away from the catalytic site during binding, and move in to stabilize the proposed oxycarbonium transition state.

**Conclusions**—Nucleoside hydrolase from *C. fasciculata* is a nonspecific enzyme with a demonstrated capacity for nucleoside salvage in the trypanosome. Multi-milligram quantities of highly purified protein have been prepared by traditional purification methods. The enzyme catalyzes a rapid equilibrium random kinetic mechanism with the equilibrium for enzyme-bound substrates far in the direction of products. The catalytic mechanism appears to involve an oxycarbonium intermediate which is stabilized by two unprotonated ionizable groups with p\( K_a \) values near 6.1. Catalytic and substrate recognition forces are focused on the ribosyl group, although the leaving group must be capable of being protonated to function as a substrate. The weak binding of non-substrate nucleosides permits the catalytic site to remain free to interact with substrates in the complex mixture of nucleosides found in blood.

**REFERENCES**