O₂'-Methylinosine, a constituent of the ribosomal RNA of Crithidia fasciculata.

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ABSTRACT

A novel nucleoside, O₂'-methylinosine (Im), has been identified as a constituent of the ribosomal RNA of Crithidia fasciculata, a hemoflagellate protozoan. The nucleoside is released as part of an alkali-stable dinucleotide, Im-Up, by alkaline hydrolysis of Crithidia rRNA, and as a 5'-nucleotide, pIm, by snake venom hydrolysis of the same RNA. The Im-containing derivatives isolated from Crithidia rRNA were characterized by comparison with marker compounds prepared by chemical deamination of the corresponding adenosine analogues. O₂'-Methylinosine prepared from either natural Im-Up or natural pIm had the same ultraviolet absorption spectra and chromatographic properties as marker Im. Characterization of the base and sugar components of Im as hypoxanthine and 2-O-methylribose, respectively, provided final confirmation of structure. Control experiments have eliminated the possibility that Im arises from O₂'-methyladenosine (Am), a known constituent of ribosomal RNA, by chemical or enzymatic deamination during hydrolysis of Crithidia rRNA.

INTRODUCTION

A recent report¹ from this laboratory described the isolation of four minor alkali-stable dinucleotides (Nm-Np)², each containing a base modification in addition to sugar methylation, from the ribosomal RNA of Crithidia fasciculata, a hemoflagellate protozoan. One of the dinucleotides was shown to contain uridine and a nucleoside having the properties of O₂'-methylinosine (Im). The identification of this latter compound was based on its ultraviolet absorption spectra, which closely resembled those of inosine, and its chromatographic properties, which suggested the presence of an additional nonpolar substituent and the absence of a cis-diol grouping.

Since the O₂'-methyl derivative of inosine had not previously been identified as a component of either the ribosomal or transfer RNA of any organism, a
more extensive structural characterization of the presumptive Im was undertaken. In particular, it was considered essential to establish that Im did not derive from \(O^{2'-}\text{methyladenosine (Am)}, \) a known constituent of ribosomal RNA, by chemical deamination during alkaline hydrolysis of *Crithidia* rRNA. The present communication describes the isolation of \(O^{2'-}\text{methylinosine 5'-phosphate (pIm)}\) from snake venom hydrolysates of *C. fasciculata* rRNA, and presents further evidence supporting the identity of \(O^{2'-}\text{methylinosine and its existence as a genuine constituent of } *Crithidia* \) rRNA.

**EXPERIMENTAL PROCEDURE**

**Materials**

Ribosomal RNA from *Crithidia fasciculata* grown in culture was prepared as previously described\(^1\). The dinucleoside monophosphates Am-U and Um-A were resolved by two-dimensional paper chromatography of the alkali-stable Nm-N fraction of wheat embryo rRNA\(^3\). \(O^{2'-}\text{Methyladenosine 5'-phosphate (pAm)}\) was isolated by the same chromatographic procedure from the pNm fraction of a snake venom hydrolysate of wheat embryo rRNA\(^4\). \(O^{2'-}\text{Methyladenosine (Am)}\) was prepared by treatment\(^5\) of pAm with *E. coli* alkaline phosphatase (Worthington Biochemicals). The individual Nm-N, pNm, and Nm compounds were recovered by adsorption to and elution from charcoal\(^5\). The corresponding inosine analogues (Im-U, Um-I, pIm, Im) were generated by treatment\(^6\) of the appropriate adenosine derivatives with nitrous acid (5 - 30 A units of compound incubated in 0.2 ml \(0.260^\text{M} \) sodium nitrite + 0.35 ml 2 M acetic acid at room temperature for 18 hr).

The products were desalted on charcoal. Inosine (I) and its 5'-nucleotide (pI) were purchased from Calbiochem, while *Vipera russelli* venom was from Ross Allen Reptile Institute, Silver Springs, Fla.

**Methods**

(a) **Chromatographic and Electrophoretic Techniques**

Solvents employed for descending paper chromatography were: (A) and (A'),
95% ethanol/water, 4/1; (B), saturated ammonium sulfate/propan-2-ol, 40/1; (C), 95% ethanol/1 M ammonium acetate, 7/3, saturated with boric acid and adjusted to pH 9 with ammonium hydroxide; (D), butan-1-ol/95% ethanol/water, 50/18/15; (E), upper phase of ethyl acetate/water/propan-1-ol, 4/2/1; (F), upper phase of ethyl acetate/16% formic acid/2-ethoxyethanol, 4/2/1; (G), propan-2-ol/water/conc. ammonium hydroxide, 7/2/1. Except in the case of system B, tanks were pre-equilibrated with developing solvent. Either untreated (A, C - G) or ammonium sulfate-impregnated7 (A, B) Whatman #1 chromatography paper was used.

Paper electrophoresis was carried out in a Durrum-type electrophoresis apparatus (Beckman).

(b) Isolation of Im-U from an Alkaline Hydrolysate of C. fasciculata rRNA

Alkaline hydrolysis of C. fasciculata rRNA (1 g) and fractionation of the hydrolysis products (on a 4.5 cm X 12 cm column of DEAE-cellulose) were carried out by the procedures of Lane8. The alkali-stable dinucleotide (Nm-Np) fraction was treated with E. coli alkaline phosphatase [544 A units in 0.75 ml 260 water; 0.25 ml 1 M ammonium formate (pH 9.2); 0.05 ml (21 units) alkaline phosphatase; 37°, 2 hr]. For electrophoretic separation of the resultant Nm-N derivatives, the hydrolysate was taken to dryness in vacuo and redissolved in 0.5 ml water. Aliquots (100 ml containing about 100 A units were applied in a strip 28 cm wide to sections (28 cm X 30 cm) of Whatman #1 paper moistened with 1 M formic acid (pH 1.8). Electrophoresis was conducted at 500 volts for 2 hr. By this procedure, Im-U could be obtained free of all other alkali-stable dinucleoside monophosphates (Fig. 1). The Im-U was eluted from the electrophorograms in 0.6 M ammonium hydroxide, which was then removed in vacuo. Final purification of Im-U from non-specific u.v.-absorbing contaminants was achieved by paper chromatography in system A'.

No material corresponding to Im-U could be detected when the above procedure was applied to wheat embryo rRNA.
Ultraviolet contact photograph illustrating the electrophoretic resolution (1 M formic acid, pH 1.8) of the Nm-N fraction isolated from Crithidia fasciculata rRNA. The dinucleoside monophosphates are distributed as follows: (1) Cm-C, Cm-A, Am-C, Am-A; (2) Cm-G, Gm-C, Am-G, Gm-A, Gm-mGA; (3) Gm-G; (4) Cm-U, Um-C, Cm-V, Am-U, Um-A; (5) Gm-U, Um-G, Um-V; (6) Im-U; (7) Um-U. The faint u.v.-absorbing band more cationic than Band 1 contains an unidentified u.v.-fluorescent compound.

(c) Isolation of pUm from a Snake Venom Hydrolysate of C. fasciculata rRNA

Venom hydrolysis of C. fasciculata rRNA (0.5 g) and fractionation of the products (on a 4.5 cm X 5 cm column of DEAE-formate) were carried out as previously described. The mononucleotide fraction (containing 3'-methyl nucleoside 5'-monophosphates, pNm) was further resolved on DEAE-cellulose (formate) in the presence of 1 M formic acid (pH 1.8). Fractions containing pUm as the major component (Subfraction M-4; see ref. 10) were combined, the pool was adjusted to pH 4.5 with pyridine and taken to dryness in vacuo, and the residue was re-evaporated from dilute ammonium hydroxide. Paper chromatography of Subfraction M-4 in system A revealed a diffuse band of u.v.-absorbing material migrating behind the major band of pUm. The section of the chromatogram containing this material was sewn onto a fresh piece of ammonium-sulfate-impregnated Whatman #1 paper. After chromatography in system B, two minor u.v.-absorbing bands were resolved. The slower-moving band had an inosine-like spectrum and
was presumed to be \( \text{p}^{2'-}\text{methyl-pseudouridine (pM)} \) by the methods previously used to characterize the same component isolated from wheat embryo rRNA\(^{10}\). The presence of pM in the venom hydrolysate of \textit{Crithidia} rRNA was expected, since the dinucleotide \( \text{Ym-Gp} \) had previously been isolated from an alkaline hydrolysate of the same RNA\(^1\).

The material corresponding to pM was recovered by charcoal desalting\(^5\) and freed of non-specific u.v.-absorbing contaminants by paper chromatography in system A'.

(d) Preparation of Im from Im-U and pM

Previously-described conditions\(^5\) were used for hydrolysis of Im-U with purified \textit{Viperus russelli} venom phosphodiesterase and dephosphorylation of pM with \textit{E. coli} alkaline phosphatase. Chromatography of the resultant hydrolysates in system A' yielded pure Im, the latter nucleoside migrating well ahead of other components (pU and salts) in this system.

(e) Analysis of the Sugar and Base Components of Im

The methodology of Nichols and Lane\(^11\) was used. Nucleosides were heated in 1 M HCl for 1 hr at 100\(^\circ\) in order to release the base and sugar constituents. After hydrolysis, the HCl was removed in vacuo, and the residues were chromatographed in system A along with various markers. Ultraviolet-absorbing components were located and excised from the chromatograms after recording their positions by making an ultraviolet contact print. Ultraviolet absorption spectra were determined against appropriate blanks. Chromatograms were then sprayed with m-phenylenediamine hydrochloride in 76% ethanol and heated in an oven at 100\(^\circ\) for 5 min, in order to locate the positions of the sugars. Ribose, deoxyribose, and 2-0-methylribose yield readily-distinguishable colored products by this procedure\(^11\).

RESULTS

Previous identification\(^1\) of Im-U isolated from \textit{Crithidia} rRNA was based on enzymic degradation to its constituent nucleosides. This result has been
confirmed by comparison of the properties of Im-U from Crithidia rRNA ("natural Im-U") with those of an Im-U marker generated by chemical deamination of Am-U. The ultraviolet absorption spectra of natural Im-U (not shown) were essentially identical to those of marker Im-U \( \lambda_{\text{max}} \) (nm) = 251 (pH 1), 250 (pH 7), 253.5 (pH 13); \( \lambda_{\text{min}} \) (nm) = 225 (pH 1), 224 (pH 7), 222.5 (pH 13), as were its chromatographic properties (Table 1). It was not possible to distinguish Im-U from its sequence isomer, Um-I, on the basis of u.v. absorption spectra or chromatographic mobility in most systems. However, a distinct separation between Im-U and Um-I was apparent in chromatographic system B (Table 1), which is known to resolve the corresponding adenosine analogues, Am-U and Um-A. The inosine-containing Im-N derivatives displayed the same relative mobility as their adenosine analogues in system B, with Am-U and Im-U migrating more slowly than Um-A and Um-I, respectively.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mobility(^b) in System</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>pI</td>
<td>0.31</td>
</tr>
<tr>
<td>pIm</td>
<td>0.52</td>
</tr>
<tr>
<td>pIm (c.f.)</td>
<td>0.56</td>
</tr>
<tr>
<td>Im-U</td>
<td>0.50</td>
</tr>
<tr>
<td>Im-U (c.f.)</td>
<td>0.48</td>
</tr>
<tr>
<td>Um-I</td>
<td>0.49</td>
</tr>
<tr>
<td>I</td>
<td>0.52</td>
</tr>
</tbody>
</table>

\(^a\) Compounds designated "c.f." were the natural derivatives isolated from Crithidia fasciculata rRNA. Other compounds were commercial or laboratory-prepared markers.

\(^b\) R\(_g\) value, except for system B, where mobilities are expressed relative to that of the inosine marker.
The isolation of Im in the form of an alkali-stable dinucleotide from *Crithidia* rRNA implied that Im should be liberated as a 5'-nucleotide by phosphodiesterase hydrolysis of the same RNA. An attempt was therefore made to prepare O'2'-methylinosine 5'-phosphate (pIm) from *Crithidia* rRNA by a venom hydrolysis procedure which has been developed in this laboratory for the large-scale group isolation of the Im constituents of RNA. Preliminary experiments with marker compounds indicated that during two-dimensional paper chromatography of the entire *Crithidia* rRNA pNm fraction, the relatively large amount of pAm in this fraction would migrate with and undoubtedly obscure any trace amount of plm which might be present. Accordingly, the *Crithidia* rRNA pNm fraction was further resolved by chromatography on DEAE-cellulose at pH 1.8, in order to separate pAm and plm into different subfractions. As expected, material with the properties of plm was found in the subfraction containing pUm as the major component (Subfraction M-4; ref. 10). Significantly, no plm could be detected when wheat embryo rRNA was analyzed under identical conditions, even when the same venom preparation was used for hydrolysis of both *Crithidia* and wheat embryo rRNA.

The natural pIm had the same ultraviolet absorption spectra (not shown) and chromatographic properties (Table 1) as marker pIm. As expected of the O'2'-methyl derivative of pi, pIm had a higher mobility than pi in a nonpolar system (A) and a lower mobility in a polar system (B). In contrast to pi, pIm did not complex with borate during chromatography, as evidenced by the enhanced mobility of the latter nucleotide in system C. Both marker and natural pIm (as well as pi) had an electrophoretic mobility slightly greater than that of a picrate marker in 0.025 M ammonium formate (pH 9.2). Treatment of pIm and pi with *E. coli* alkaline phosphatase resulted in loss of this negative charge, consistent with the removal of a monoester phosphate group.

The presumed Im from natural pIm and from natural Im-U had the same chromatographic mobility as marker Im in six different systems (Table 2). In
the nonpolar solvents used, Im displayed, as expected, an enhanced mobility relative to I (as did m1I). The ultraviolet absorption spectra of natural Im (Fig. 2A) were identical to those of marker Im (Fig. 2B), as well as to published u.v. spectra of inosine12. The spectral properties of Im and its chromatographic mobility in several systems clearly distinguished this nucleoside from m1I. In particular, the inability of Im to complex with borate during chromatography, in contrast to both I and m1I, was clearly evident (system C, Table 2).

Direct evidence of O2'-methylation in Im was obtained by examining the sugar moiety released by mild acid hydrolysis of the nucleoside. As shown by the data in Table 3, the sugars obtained from natural and marker Im were identical and had the properties ascribed to 2-O-methylribose in the analytical system used11. The base constituents of natural and marker Im and of I had identical ultraviolet absorption spectra (Table 4), and the same chromatographic mobility as the natural and marker Im.

![Figure 2](image_url)

**Figure 2**
Ultraviolet absorption spectra of natural (A) and marker (B) Im. Spectra were recorded on a Bausch and Lomb Spectronic 505 spectrophotometer. Compounds were first chromatographed in system A' and eluted in water, along with appropriate blanks. Neutral spectra (— —) were determined on 1 ml aqueous eluate, recorded against 1 ml of blank. Acidic spectra (— —) were recorded after addition of 10 μl conc. HCl to the sample and blank, and alkaline spectra (— —) after a further addition of 20 μl 10 M NaOH to sample and blank.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \lambda_{\text{max}} )</th>
<th>( \lambda_{\text{min}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural Im</td>
<td>238 (neutral) 252.5 (alkaline)</td>
<td>229 (neutral) 230.5 (alkaline)</td>
</tr>
<tr>
<td>Marker Im</td>
<td>238 (neutral) 252 (alkaline)</td>
<td>229 (neutral) 230.5 (alkaline)</td>
</tr>
</tbody>
</table>
Table 2

Chromatographic Identity of Natural and Marker Im

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mobility (Rf Value) in System</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A'</td>
</tr>
<tr>
<td>I</td>
<td>0.53</td>
</tr>
<tr>
<td>Im</td>
<td>0.66</td>
</tr>
<tr>
<td>Im (from C.f. pIm)</td>
<td>0.65</td>
</tr>
<tr>
<td>Im (from C.f. Im-U)</td>
<td>0.66</td>
</tr>
<tr>
<td>m1I</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Compounds designated "C.f." were the natural derivatives isolated from Cnidia fasciculata rRNA. Other compounds were commercial or laboratory-prepared markers.

Table 3

Characterization of the Sugar and Base Components of Inosine Derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Base</th>
<th>Sugar</th>
<th>Colora</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RInosine</td>
<td>RInosine</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>1.11</td>
<td>1.27</td>
</tr>
<tr>
<td>m1I</td>
<td></td>
<td>1.35</td>
<td>1.28</td>
</tr>
<tr>
<td>Im (marker)</td>
<td>1.14</td>
<td>1.66</td>
<td>Pink</td>
</tr>
<tr>
<td>Im (natural)</td>
<td>1.13</td>
<td>1.63</td>
<td>Pink</td>
</tr>
</tbody>
</table>

aAfter reaction on the chromatogram with m-phenylenediamine.

The base released from m1I migrated with a 1-methylhypoxanthine marker (Table 3) and could be distinguished spectrally from the base released from Im by its absorption minima (Table 3), as well as by the absence of the shoulder near 273 nm which is evident in the alkaline spectrum of hypoxanthine13.
Ultraviolet Spectral Constants of Bases Released from Inosine Derivatives

<table>
<thead>
<tr>
<th>Parent Nucleoside</th>
<th>$\lambda_{\text{max}}$ neutral</th>
<th>$\lambda_{\text{max}}$ acidic</th>
<th>$\lambda_{\text{max}}$ alkaline</th>
<th>$\lambda_{\text{min}}$ neutral</th>
<th>$\lambda_{\text{min}}$ acidic</th>
<th>$\lambda_{\text{min}}$ alkaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>248</td>
<td>247</td>
<td>260</td>
<td>221</td>
<td>216</td>
<td>232</td>
</tr>
<tr>
<td>Im (marker)</td>
<td>248.5</td>
<td>247</td>
<td>260</td>
<td>220</td>
<td>215.5</td>
<td>232</td>
</tr>
<tr>
<td>Im (natural)</td>
<td>248</td>
<td>247</td>
<td>259</td>
<td>220</td>
<td>215</td>
<td>231.5</td>
</tr>
<tr>
<td>$\text{mI}^1$</td>
<td>249</td>
<td>247.5</td>
<td>259</td>
<td>225</td>
<td>221.5</td>
<td>236</td>
</tr>
</tbody>
</table>

DISCUSSION

The data presented in this report conclusively identify $O^{2',3'}$-methylinosine (Im) as a constituent of both chemical and enzymic hydrolysates of *Crithidia fasciculata* ribosomal RNA. The following considerations eliminate the possibility that Im might derive artifactually from $O^{2'}$-methyladenosine (Am), a known constituent of ribosomal RNA, by chemical or enzymatic deamination of the latter during RNA hydrolysis:

1. No inosine-containing alkali-stable dinucleotides other than Im-Up have been detected in alkaline hydrolysates of *Crithidia rRNA*, even though all possible adenosine-containing Nm-Np derivatives are present. In particular, no Um-Ip has been found, even though both Am-Up and Um-Ap are present in the Nm-Np fraction of *Crithidia rRNA*. (Although the relative molar proportion of Am-Up to Um-Ap is about 4:1 in the *Crithidia rRNA* Nm-Np fraction, a 20% content of Um-I in the Im-U isolated by the large-scale technique described in this report should have been detected.) On the basis of unpublished measurements of the molar proportions of Am-Up and Im-Up in alkaline hydrolysates of *Crithidia rRNA*, a precursor-product relationship would require the selective conversion of about 10-15% of the total Am-Up sequences to Im-Up in the course of hydrolysis, an untenable proposition.

2. Even though there is about 1.5 times as much Am-Up in wheat embryo rRNA
as in \textit{Crithidia} rRNA, no Im-Up has been detected in alkaline hydrolysates of wheat embryo 18S + 26S rRNA, hydrolyzed and analyzed under conditions identical to those employed for \textit{Crithidia} rRNA hydrolysis.

(3) No pIm has been detected in snake venom hydrolysates of wheat embryo rRNA, even using the same venom preparations which yield pIm from \textit{Crithidia} rRNA. This eliminates the possibility of trace deaminase-catalyzed conversion of pAm to pIm during venom hydrolysis of RNA.

(4) The proportion of Im in hydrolysates of \textit{Crithidia} rRNA is the same (about 0.01 mole %) whether the nucleoside is measured in the form of Im-Up in alkaline hydrolysates or as pIm in snake venom hydrolysates of \textit{Crithidia} rRNA.

A number of the modified nucleoside constituents of transfer RNA are known to be labile during alkaline hydrolysis but to be partially or completely stable during venom hydrolysis\textsuperscript{6,14,15}. The isolation of equimolar amounts of Im from alkaline and venom hydrolysates of \textit{Crithidia} rRNA makes it unlikely that Im arises from some more complex, alkali-labile nucleoside constituent during hydrolysis. Such a constituent would have to undergo quantitative conversion to Im under relatively mild conditions (24 hr at pH 9.2 and 37°).

Allowing, then, that Im exists as such in \textit{Crithidia fasciculata} rRNA, and assuming that it arises by post-transcriptional modification of an adenosine residue during rRNA biosynthesis, its formation must require two distinct steps, methylation and deamination, presumably catalyzed by two separate modification enzymes. As in the case of other hypermodified nucleosides identified as constituents of ribosomal RNA\textsuperscript{1,10,11,16,17}, the functional significance of O\textsuperscript{2'}-methylinosine in \textit{C. fasciculata} rRNA remains to be determined.

\textbf{ACKNOWLEDGEMENTS}

The skillful technical assistance of Carl Thomas throughout this study is greatly appreciated. Financial support was provided by a grant (MA-1224) from
the Medical Research Council of Canada. The assistance of Mr. R. S. Cunningham in preparing the RNA used in this study is also gratefully acknowledged.

REFERENCES


2 Abbreviations: N, general abbreviation for ribonucleoside; A, I, U, Y, adenosine, inosine, uridine, pseudouridine; Nm, O²'-methylnucleoside; mN, base-methylated nucleoside; pN, nucleoside 5'-monophosphate; pNm, O²'-methylnucleoside 5'-monophosphate; Nm-Np, alkali-stable dinucleotide; Nm-N, alkali-stable dinucleoside monophosphate. In defining sites of substitution, a superscript indicates the position on the heterocycle of the particular substituent in question, e.g., m¹I = 1-methylinosine.


8 Lane, B. G. (1965) *Biochemistry* 4, 212-219.


13 tbd, p. 63.


