Hadacidin, a New Inhibitor of Purine Biosynthesis*

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The inhibition of purine biosynthesis has been observed with the use of various types of compounds. The antibiotics O-diazo-acetyl-L-serine (azaerine) and 6-diazo-5-oxo-L-norleucine suppress purine formation by virtue of their antagonistic action toward glutamine in amination reactions (1-3). Several structural analogues of purine and of pteroylglutamic acid are also known to block the biosynthesis of purine nucleotides. The literature pertaining to these antimetabolites has recently been extensively reviewed (4-6).

The fermentation broth of Penicillium frequentans (Westling) was found to show growth-inhibitory activity against human adenocarcinoma (HAD No. 1) growing in the embryonated egg.

A substance possessing the same activity was subsequently isolated from the broth and found to have the structure shown in Formula 1 (7).

\[
\text{OHC} = \text{N} - \text{CH}_2 - \text{COOH}
\]

**FORMULA 1**

This new compound, N-formyl hydroxyaminocetic acid, is also known by the trivial name of hadacidin because of its inhibitory action against human adenocarcinoma.

The effect of this compound on nucleic acid metabolism has been investigated and data obtained from experiments with isotopes in vivo and in vitro indicated that hadacidin markedly suppressed the biosynthesis de novo of adenylc acid and deoxyadenylic acid.

**EXPERIMENTAL PROCEDURE**

Materials—The following chemicals were used: Formate-C\(^{14}\), glycine-2-C\(^{14}\), adenine-8-C\(^{14}\), and guanine-8-C\(^{14}\) (New England Nuclear Corporation); glycine-\(^{13}\)C\(^{14}\) and hypoxanthine-8-C\(^{14}\) (Nuclear-Chicago Corporation); ATP disodium salt (Sigma Chemical Company); GTP sodium salt, AMP sodium salt, and IMP sodium salt (Pabst Laboratories); 3-phosphoglycerate and ribose 5-phosphate barium salt (C. F. Boehringer and Sons).

**Ehrlich Ascites Tumor Cell**—Tumor-bearing Swiss albino mice were used 7 days after transplantation. Two different methods were used for the preparation of cell suspensions.

**Method A**—Ascent fluid obtained from five or six mice was pooled by mixing with about 25 ml of Robinson's medium (8, 9) in a chilled flask. Unless otherwise specified, Robinson's medium contained 0.1% glucose and 0.02 M KHCO\(_3\) (9). The following steps were performed at 4-5°C. After filtration through glass wool and centrifugation at 60 x g for 5 minutes, the pellet was washed free of red blood cells by repeated suspension and centrifugation at 60 x g in Robinson’s medium. The final pellet of ascites cells, observed microscopically to contain only an occasional red blood cell, was suspended in the same medium. Experiments were performed with cell suspensions containing 6 x 10\(^7\) cells per ml.

**Method B**—This method of cell preparation, described by Harbers and Heidelberger (10), permits the study of nucleic acid metabolism under conditions where cell multiplication occurs. The methods of cell preparation and incubation were similar to those described by the authors except that freshly prepared chick embryo extract was used. The extract was prepared as follows. One part of chick embryo (9 days old) was suspended in an equal part of 0.85% NaCl, homogenized in a loosely fitting Teflon homogenizer, and the supernatant obtained upon centrifugation of the homogenate at 15,000 x g for 15 minutes was added to incubation mixtures in the proportions indicated in the legends to the tables.

**Preparation of Cell-free Fractions**—Eight milliliters of packed ascites cells, previously washed and centrifuged, were suspended in 2 ml of distilled H\(_2\)O and homogenized vigorously in a tightly fitting Teflon homogenizer (six to eight up-and-down strokes) as described by Elwood et al. (11). The homogenate was then mixed with 2 ml of Robinson's medium (8) without glucose and bicarbonate, and centrifuged at 15,000 x g for 10 minutes. The supernatant was used for cell-free incubation experiments.

**Incubation**—Incubation experiments with either cell-free preparations or cell suspensions prepared by Method A were performed in 10-ml Erlenmeyer flasks placed in a Dubnoff incubator and shaken constantly under air for a specified time at 37°C. The reactions were terminated by the addition of cold perchloric acid to a final concentration of 0.4 M.

Experiments with viable cell suspensions (Method B) were done in 25-ml Erlenmeyer flasks that were loosely stoppered with cotton and incubated without shaking for 6 hours at 37°C (8). At the end of the incubation period, the reaction mixtures were centrifuged at 4°C, and the pellets were washed once in cold 0.85% NaCl and recentrifuged before being mixed with cold 0.4 M HClO\(_4\).

**Experiments in Vivo**—Two Sprague-Dawley rats of approximately equal weight (155 to 160 g) were used. One rat was treated by intraperitoneal injection of 10 μC of glycine-2-C\(^{14}\) and 310 mg of hadacidin dissolved in 1.5 ml of 0.85% NaCl. The
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control animal received 1.5 ml of 0.55% NaCl containing 10 μc of glycine-2-C⁴. After 4 hours, the animals were killed and spleen, thymus, and intestinal mucosa were removed, homogenized in 0.85% sodium chloride solution and precipitated in cold 0.4 M perchloric acid. RNA and DNA were isolated from the acid-insoluble residues as described below.

Isolation of Purine Nucleotides—The acid-insoluble residues obtained upon the addition of perchloric acid either to incubation mixtures or to tissue homogenates from experiments in vivo were washed three to four times with cold 0.4 M perchloric acid, and total nucleic acids were extracted with hot 10% NaCl by the procedure of Schneider and Potter (12). The partially dried mixed nucleic acid fractions were suspended in about 1 ml of 0.5 M KOH and kept overnight at 30°. After acidification and centrifugation to remove DNA, the supernatant fluids were neutralized and applied to 0.6 x 16-cm Dowex 1-formate columns for the fractionation of the ribonucleotides. The nucleotides were separated by means of gradient elution in formic acid system, the fractionation of the ribonucleotides. The nucleotides were dried, and the bases located under ultraviolet light, eluted with 0.1 M KOH and kept overnight at 30°. After acidification and centrifugation to remove DNA, the supernatant fluids were neutralized and applied to 0.6 x 16-cm Dowex 1-formate columns for the fractionation of the ribonucleotides. The nucleotides were separated by means of gradient elution in formic acid system, Type I (13).

In order to test the reliability of the specific activities of purine ribonucleotides obtained by means of column chromatography, the nucleotides from some experiments were hydrolyzed to their free bases by heating in 1 N HCl for 1 hour at 100°. Adenine and guanine were separated by means of paper chromatography on Whatman No. 1 paper, with the use of water-ammonia-butanol (10:0.7:74) as the eluting solvent (14). The papers were air dried, and the bases located under ultraviolet light, eluted with 0.1 N HCl, and examined spectrophotometrically in preparation for radioactivity determinations.

Isolation of DNA Purine Bases—The DNA purine base were separated only in the experiments in vivo. DNA precipitates obtained as described under “Isolation of Purine Nucleotides” were processed as follows. To ensure the complete removal of ribonucleotides, precipitates containing DNA were dissolved in 0.2 N NaOH and heated at 80° for 20 minutes. After cooling, acidification, and centrifugation, the DNA precipitates were hydrolyzed in 7.5 N HClO₄ at 100° for 1 hour (15). The purine bases were resolved by means of paper chromatography as described above.

Determination of Radioactivity—Amounts of purine nucleotides or free bases measured by absorption spectrophotometry were applied to 5-cm² stainless steel planchets, dried under an infrared lamp, and counted at infinite thinness in a windowless gas flow counter.

In some cases, the radioactivity of the bases was measured in the Packard Tri-Carb liquid scintillation counter. The specific activity was expressed as counts per minute per μmole of nucleotide or base.

RESULTS

The first indication of the activity of hadacidin in nucleic acid metabolism was obtained when a suspension of Ehrlich ascites tumor cells was incubated with radioactive hypoxanthine in the presence of the antibiotic. The specific activity of the mixed nucleic acids isolated from the tumor cells treated with hadacidin was considerably lower than that of the untreated cells. The incorporation of hypoxanthine-S-C⁴ into nucleic acids was inhibited by 50% in the presence of 1 × 10⁻³ M hadacidin. This initial observation led to studies with other precursors of nucleic acids.

Experiments in Vitro—A suspension of washed tumor cells was incubated with glycine-U-C⁴ and hadacidin, and adenylic and guanylic acids from the acid-insoluble fractions were examined. The results as shown in Table I, Experiment 1, indicate that the incorporation of glycine into acid-insoluble adenylic acid was considerably depressed in the presence of hadacidin whereas the synthesis of acid-insoluble guanylic acid was not affected.

The experiment with glycine-U-C⁴ was repeated with growing cell suspensions (Table I, Experiments 2 and 3) and it can be seen that the synthesis of acid-insoluble adenylic acid was again profoundly inhibited whereas that of guanylic acid was not suppressed. The slight increase in the specific activity of guanylic acid may be due to increased utilization of common precursors, such as inosinic acid or precursors of inosinic acid. A block in adenylic acid formation in such a system could conceivably lead to increased incorporation of radioactive glycine into guanylic acid.

Qualitatively similar results were obtained when formate-C⁴ was used as the precursor (Table I, Experiment 4). The numbers in parentheses represent the specific activities of the purine bases obtained from the nucleotides in Experiment 4. The relative specific activities of the bases (control versus hadacidin-treated), as compared to those of the nucleotides, remained essentially unchanged. The higher specific activities recorded for the

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Method of cell preparation</th>
<th>Radioactive precursor</th>
<th>Adenylic acid</th>
<th>Guanylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Hadacidin-treated</td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>Glycine</td>
<td>70*</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>B</td>
<td>Glycine</td>
<td>95</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>Glycine</td>
<td>260</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>Formate</td>
<td>450 (975)</td>
<td>6 (10)</td>
</tr>
</tbody>
</table>

* Specific activity = c.p.m. per μmole. Cell suspensions of Ehrlich ascites cells were prepared as described in text.
bases are due to the greater efficiency of the scintillation spectrometer.

The results obtained with glycine-U-C\textsuperscript{14} and formate-C\textsuperscript{14} clearly indicate that the pathway of synthesis de novo common to adenylate and guanylate (up to and including the formation of inosinic acid) was not inhibited by hadacidin. It was also evi-

dent that the formation of acid-insoluble guanylic acid from inosinic acid was also not suppressed. The area of inhibition appeared thus to lie between IMP and acid-insoluble adenylate. This reasoning was supported by the results obtained when hypoxanthine-S-C\textsuperscript{14} was used as the precursor (Table II). The formation of acid-insoluble adenylate acid was markedly depressed in the presence of hadacidin whereas no inhibition was observed in the synthesis of guanylic acid. The results also indicated that inosinic acid pyrophosphorylase was not affected by hadacidin. The figures in parentheses again represent the specific activities of the purine bases obtained from the corresponding nucleotides in Experiment 2 and counted in the liquid scintillation spectrometer.

**Table II**

**Incorporation of hypoxanthine-S-C\textsuperscript{14} into acid-insoluble AMP and GMP**

The components of the reaction mixtures were as follows.

*Experiment 1:* 3.4 ml of cell suspension containing 0.1% glucose and 0.02 M KHCO\textsubscript{3}; 0.1 ml of hypoxanthine-S-C\textsuperscript{14}, 0.5 \mu c, 0.22 \mu mole, in a final volume of 3.8 ml. Test vessel contained 15 \mu moles of hadacidin.

*Experiment 2:* 3.3 ml of cell suspension containing 0.1% glucose and 0.02 M KHCO\textsubscript{3}; 0.1 ml of hypoxanthine-S-C\textsuperscript{14}, 0.5 \mu c, 0.22 \mu mole, in a final volume of 3.8 ml. Test vessel contained 3.3 \mu moles of hadacidin.

*Experiment 3:* 3.6 ml of cell suspension containing 0.1% glucose and 0.02 M KHCO\textsubscript{3}; 0.1 ml of hypoxanthine-S-C\textsuperscript{14}, 0.5 \mu c, 0.22 \mu mole, in a total volume of 3.8 ml. Test vessel contained 1.5 \mu moles of hadacidin. All vessels were incubated with shaking at 37\textdegree C for 45 minutes.

Figures in parentheses represent specific activities of purine bases obtained from the corresponding purine nucleotides in Experiment 2 and determined in liquid scintillation spectrometer. All other samples were counted in gas flow counter.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control Adenylate</th>
<th>Hadacidin-treated Adenylate</th>
<th>Control Guanylate</th>
<th>Hadacidin-treated Guanylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>420*</td>
<td>20</td>
<td>55</td>
<td>65</td>
</tr>
<tr>
<td>2</td>
<td>675 (1730)</td>
<td>55 (120)</td>
<td>55 (110)</td>
<td>50 (120)</td>
</tr>
<tr>
<td>3</td>
<td>1215</td>
<td>90</td>
<td>85</td>
<td></td>
</tr>
</tbody>
</table>

**Table III**

**Incorporation of hypoxanthine-S-C\textsuperscript{14} into acid-soluble AMP**

The components of the reaction mixtures were as follows.

*Experiment 1:* 3 ml of cell-free extract in Robinson's medium, without glucose and bicarbonate; ATP, 1 \mu mole; 3-phosphoglycerate, 30 \mu moles; C\textsuperscript{14}-hypoxanthine, 5 \mu moles; hypoxanthine-S-C\textsuperscript{14}, 0.5 \mu c, 0.22 \mu mole; l-aspartic acid, 5 \mu moles; ribose 5-phosphate, 5 \mu moles, in a total volume of 3.48 ml. Test vessel contained 4.1 \mu moles of hadacidin. Incubated for 1 hour at 37\textdegree C with shaking.

*Experiment 2:* 3.3 ml of cell suspension containing 0.1% glucose and 0.02 M KHCO\textsubscript{3}; 0.1 ml of adenine-8-C\textsubscript{14}, 0.2 \mu c, 1.05 \mu moles; or 0.2 ml of guanine-8-C\textsubscript{14}, 0.4 \mu c, 0.44 \mu mole; in a total volume of 10.9 ml. Test vessel contained 10 \mu moles of hadacidin. Second test vessel, indicated by plus sign, contained the same components as the first test vessel except 75 \mu moles of L-aspartate were added.

Incubated for 1 hour at 37\textdegree C with shaking.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
<th>Hadacidin-treated</th>
<th>Hadacidin-treated plus L-aspartate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>543*</td>
<td>33</td>
<td>432+</td>
</tr>
<tr>
<td>2</td>
<td>522</td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

* Specific activity = c.p.m. per \mu mole.

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biosynthesis de novo, namely, the conversion of 5-amino-4-imidazole-N-succinocarboxamide ribonucleotide to 5-amino-4-imidazole carboxamide ribonucleotide (23, 24) was apparently also not inhibited by hadacidin.

In spite of its potent inhibitory activity against adenlylic acid synthesis de novo, hadacidin has been found to exhibit relatively weak antitumor activity in several test systems. This may be explained by the availability of preformed adenine and derivatives of adenine to malignant cells or other parasitic cells existing in complex living systems or in chemically undefined media. Cells capable of satisfying most of their purine requirements through the "salvage" or "preformed" pathway would be expected to be resistant to hadacidin.

It has recently been found that the antitumor agent, 6-mercaptopurine, inhibits both Reaction 1 and the conversion of inosinic to adenylic acid. In spite of its potent inhibitory activity against adenlylic acid, hadacidin has been found to exhibit relatively weak antitumor activity in several test systems. This may be explained by the availability of preformed adenine and derivatives of adenine to malignant cells or other parasitic cells existing in complex living systems or in chemically undefined media. Cells capable of satisfying most of their purine requirements through the "salvage" or "preformed" pathway would be expected to be resistant to hadacidin.

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The significance of the latter observation is not clear.

An examination of the specific activities of DNA purine bases shows that the formation of deoxyadenylic acid was also inhibited by hadacidin. As in the case of guanylic acid, the synthesis of deoxyguanylic acid in the intestinal mucosa was also slightly inhibited.

**DISCUSSION**

The antitumor activity of hadacidin and its structural resemblance to either glycine or to a "formylating intermediate" suggested possible action of the antibiotic in the utilization of glycine and formate in nucleic acid synthesis. That hadacidin is not metabolically related to glycine or formate in so far as purine synthesis de novo is concerned was shown by the observation that the incorporation of neither glycine nor formate into guanylic acid was inhibited.

The preceding experiments in vivo and in vitro have, however, demonstrated that hadacidin markedly suppressed the biosynthesis de novo of adenylc and deoxyadenylc acids, and that the site of inhibition appeared to be localized between inosinic acid and adenylc acid.

The observation that the inhibition of incorporation of hypo-
xanthine into adenylc acid was partially reversed with L-aspar-
tate implicated the conversion of inosinic to adenylosuccinic acid and to deoxynosuccinic acid in the spleen and thymus.

Hadacidin did not influence the uptake of the preformed bases, adenine and guanine, into ribonucleic acids.

The observation that the inhibition of incorporation of hypo-
xanthine into adenylc acid was partially reversed with L-aspar-
tate implicated the conversion of inosinic to adenylosuccinic acid and to deoxynosuccinic acid as the site of inhibition.

**REFERENCES**

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