Hadacidin, a New Inhibitor of Purine Biosynthesis*

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(Received for publication, January 9, 1962)

The inhibition of purine biosynthesis has been observed with the use of various types of compounds. The antibiotics O-diazooacetil-L-serine (azaer sine) and 6-diazoo-5-oxo-L-norleucine suppress purine formation by virtue of their antagonistic action toward glutamine in amino acid syntheses (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 (IIAD 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).

OH

OHC—N—CH₂—COOH

FORMULA 1

This new compound, N-formyl hydroxyaminosuccinic 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 of adenylic acid and deoxyadenylic acid.

EXPERIMENTAL PROCEDURE

Materials—The following chemicals were used: Formate-C¹⁴, glycine-2-C¹⁴, adenine-8-C¹⁴, and guanine-8-C¹⁴ (New England Nuclear Corporation); glycine-¹³C¹⁴ and hypoxanthine-8-C¹⁴ (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. Boehm Laboratories). 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—Asctic 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. The supernatant was used for cell-free incubation experiments. The inhibition of purine biosynthesis has been observed with the use of various types of compounds. The antibiotics O-diazoacetil-L-serine (azaer sine) and 6-diazo-5-oxo-L-norleucine suppress purine formation by virtue of their antagonistic action toward glutamine in amino acid syntheses (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).

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 × 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 H₂O 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 without glucose and bicarbonate, and centrifuged at 15,000 × 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 reaction mixtures 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 (9). 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 HClO₄.

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 μe of glycine-2-C¹⁴ and 310 mg of hadacidin dissolved in 1.5 ml of 0.85% NaCl. The experiments were performed with cell suspensions containing 6 to 9 × 10⁷ cells per ml.

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control animal received 1.5 ml of 0.85% NaCl containing 10 μc of glycine-2-C14. 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, 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 bases 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-8-C14 into nucleic acids was inhibited by 80% in the presence of 1 X 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-C14 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-C14 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-C14 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>2</td>
<td>A</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-C₁⁴ and formate-C₁⁴ clearly indicate that the pathway of synthesis de novo common to adenylic acid and guanylic acid (up to and including the formation of inosinic acid) was not inhibited by hadacidin. It was also evident that the formation of acid-insoluble guanylic acid from inosinic acid was also not suppressed. The area of inhibition appeared to lie between IMP and acid-insoluble adenylic acid. This reasoning was supported by the results obtained when hypoxanthine-S-C₁⁴ was used as the precursor (Table II). The formation of acid-insoluble adenylic 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-C¹⁴ 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 KHCO₃; 0.1 ml of hypoxanthine-S-C₁⁴, 0.5 μc, 0.22 μmole, in a final volume of 3.8 ml. Test vessel contained 15 μmoles of hadacidin.

**Experiment 2**: 3.3 ml of cell suspension containing 0.1% glucose and 0.02 M KHCO₃; 0.1 ml of hypoxanthine-S-C₁⁴, 0.5 μc, 0.22 μmole, in a final volume of 3.8 ml. Test vessel contained 3.3 μmoles of hadacidin.

**Experiment 3**: 3.6 ml of cell suspension containing 0.1% glucose and 0.02 M KHCO₃; 0.1 ml of hypoxanthine-S-C₁⁴, 0.5 μc, 0.22 μmole, in a total volume of 3.8 ml. Test vessel contained 1.5 μmoles of adenylic acid. All vessels were incubated with shaking at 37°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>Adenylic acid</th>
<th>Guanylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hadacidin-treated</td>
</tr>
<tr>
<td>1</td>
<td>420*</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>675(1730)</td>
<td>55(120)</td>
</tr>
<tr>
<td>3</td>
<td>1215</td>
<td>130</td>
</tr>
</tbody>
</table>

**Table III**

**Incorporation of hypoxanthine-S-C¹⁴ 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 μmole; 3-phosphoglycerate, 30 μmoles; C₅₀-hypoxanthine, 5 μmoles; hypoxanthine-8-C₁⁴, 0.5 μc, 0.22 μmole; l-aspatic acid, 5 μmoles; GTP, 1 μmole; ribose 5-phosphate, 5 μmoles, in a total volume of 3.48 ml. Test vessel contained 4.1 μmoles of adenylic acid. Incubated for 1 hour at 37°C with shaking.

**Experiment 2**: 9 ml of cell-free extract in Robinson's medium, without glucose and bicarbonate; ATP, 5 μmoles; 3-phosphoglycerate, 30 μmoles; C₅₀-hypoxanthine, 15 μmoles; hypoxanthine-8-C₁⁴, 1 μc, 0.44 μmole; l-aspatic acid, 15 μmoles; GTP, 3 μmoles; ribose 5-phosphate, 15 μmoles, in a total volume of 10.9 ml. Test vessel contained 10 μmoles of adenylic acid. Second test vessel, indicated by plus sign, contained the same components as the first test vessel except 75 μmoles of L-aspartate were added. Incubated for 1 hour at 37°C with shaking.

**Table IV**

**Incorporation of adenine-S-C¹⁴ and guanine-S-C¹⁴ into acid-insoluble AMP and GMP**

Reaction mixture contained 3.5 ml of tumor cell suspension; 0.1 ml of adenine-S-C₁⁴, 0.2 μc, 1.05 μmoles; or 0.2 ml of guanine-S-C₁⁴, 0.4 μc, 0.60 μmole, in a total volume of 3.8 ml. Test vessels contained 8 μmoles of hadacidin. Incubated at 37°C for 45 minutes with shaking.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Precursor</th>
<th>Adenylic acid</th>
<th>Guanylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hadacidin-treated</td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>Adenine-S-C¹⁴</td>
<td>1215*</td>
<td>1825</td>
</tr>
<tr>
<td>2</td>
<td>Guanine-S-C¹⁴</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Specific activity = c.p.m. per μmole.
The conversion of inosinic acid to adenylic acid is catalyzed by the enzymes adenylosuccinate synthetase (21) and adenylosuccinase (22), respectively, via the formation of the intermediate adenylosuccinic acid. Since the specific requirement of L-aspartate implicated Reaction 1 rather than Reaction 2 and adenylosuccinase, the synthesis of guanylic acid as the site of inhibition. The significance of the latter observation is not clear.

An examination of the specific activities of DNA purine bases shows that the formation of deoxycadenosine acid was also inhibited by hadacidin. As in the case of guanylic acid, the synthesis of deoxycadenosine 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 observations that the incorporation of neither glycine nor formate into adenylic acid. There was no inhibition of guanylic acid formation.

The preceding experiments in vivo and in vitro have, however, demonstrated that hadacidin markedly suppressed the biosynthesis of adenylic acid and deoxyadenosine acids, and that the site of inhibition appeared to be localized between imosinc acid and adenylic acid.

The conversion of inosinic acid to adenylic acid is catalysed by the enzymes adenylosuccinate synthetase (21) and adenylosuccinase (22), respectively, via the formation of the intermediate adenylosuccinate acid. Since the specific requirement of L-aspartate in Reaction 1 has been shown by Lieberman (21), the preliminary observation reported here that the inhibition of AMP formation by hadacidin was partially removed in the presence of excess L-aspartate implicated Reaction 1 rather than Reaction 2 as the locus of action of the antibiotic. That Reaction 2 was probably not the inhibited site is consistent with the observation that another reaction catalyzed by adenylosuccinase in purine biosynthesis de novo, namely, the conversion of 5-amino-4-imidazole-N-succinocarboxamidate 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 adenylic 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-mercaptoptopurine, inhibits both Reaction 1 and the conversion of inosinate to xanthylate in enzyme systems obtained from *Streptococcus faecalis* and pigeon liver acetone powder (25); on the other hand, only the formation of adenylic acid is inhibited by 6-mercaptopurine in ascite cells of lymphoid leukemia L1210 (26). Although the nucleotide derivative of 6-mercaptopurine is believed to be the active compound, the exact mode of action is as yet not clearly understood.

**SUMMARY**

Experiments with isotopes have shown that a new antibiotic, hadacidin (N-formyl hydroxyaminoacetic acid), which exhibits some antitumor activity, inhibited the incorporation in vivo of radioactive formate, glycine, and hypoxanthine into adenylic acid. There was no inhibition of guanylic acid formation.

Experiments in vivo showed that in addition to adenylic acid, deoxyadenosine acid synthesis was also suppressed. Hadacidin did not significantly affect the formation of guanylic and deoxyguanylic acids in the spleen and thymus.

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

The observation that the inhibition of incorporation of hypoxanthine into adenylic acid was partially reversed with 6-mercaptopurine as the site of inhibition of adenylosuccinic acid as the site of inhibition.

**REFERENCES**

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