Metabolic Effects of 6-Thioguanine

II. Biosynthesis of Nucleic Acid Purines in Vivo and in Vitro

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(McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wis.)

6-Thioguanine (3) has been shown to inhibit a wide spectrum of neoplasms. Its anti-tumor properties are markedly potentiated when combined with azaserine, an inhibitor of the synthesis de novo of purines (10, 13). One of these reports also indicated that, for maximum effect, the anti-metabolites had to be administered simultaneously. These results suggest that thioguanine interferes with some phase of nucleic acid purine metabolism. To investigate this possibility, a study of purine biosynthesis in the presence of 6-thioguanine was made with the Ehrlich ascites carcinoma. Experiments were also carried out with the thioguanine-resistant subline of this tumor to determine whether the resistant population differed in any phase of purine metabolism. In a preliminary report (11), thioguanine was found to inhibit the utilization of guanine-8-C\textsubscript{14} for nucleic acid synthesis. The degree of inhibition was much greater in the sensitive ascites cells.

Brown and Mandel (1) reported that thioguanine decreased the rate of uptake of formate-C\textsubscript{14} into cells of the L1210 leukemia in vitro.

The experiments presented here suggest that 6-thioguanine produces at least three different metabolic blocks in the area of purine biosynthesis. Inhibition of the various pathways occurs to a lesser degree in the resistant tumor; this is correlated with the relatively small quantity of thioguanine in the resistant ascites cells (12).

MATERIALS AND METHODS

Experiments were conducted with the Ehrlich ascites carcinoma (tetraploid line), the TA\textsubscript{3} ascites carcinoma, and the 6-thioguanine-resistant subline of the Ehrlich ascites carcinoma.

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sion in isotonic saline (6–7 volumes) to remove endogenous glycine. The incubations were carried out at 38° C. in War- Burg respirometer vessels of 60-ml. capacity with 80 mg. dry weight of cells in a volume of 12 ml. of Robinson's medium plus bicarbonate and glucose, under 95 per cent N2-5 per cent CO2. The various fractions were isolated and analyzed as previously mentioned.

RESULTS AND DISCUSSION

Metabolic studies in vivo.—The effect of 6-thioguanine on guanine utilization was investigated because of the similarity in structure of these two purines. Experimentally, this was carried out by determining the effect of varying concentrations of thioguanine on the incorporation of guanine-8-C14 into combined nucleic acids. The results obtained are illustrated in Table 1. A marked inhibition of the incorporation of radioactive guanine by Ehrlich ascites carcinoma took place at dosages of thioguanine of 0.5 mg/kg. As the doses of thioguanine were increased, there was a concomitant increase in the percentage inhibition of guanine incorporation. Less inhibition occurred in cells of the thioguanine-resistant subline. The duration of inhibition at a level of 10 mg/kg thioguanine was investigated, with both the sensitive and the resistant sublines, and the results are given in Chart 1. When the guanine-C14 was administered 5 minutes after the injection of the drug, 62 per cent inhibition of guanine incorporation into nucleic acid guanine of the sensitive cells occurred, while there was merely 28 per cent inhibition of the incorporation into the nucleic acid guanine of the resistant tumor. There was no inhibition in either tumor when the radioactive guanine was injected 1 hour after the dose of thioguanine. Two hours or more after the thioguanine dose, there was a steady increase in the amount of guanine-8-C14 incorporated in the 20-minute incubation period. This stimulation of guanine utilization could possibly be due to a compensatory mechanism in response to a depletion of endogenous guanine sources. The conversion of radioactive guanine to nucleic acid adenine was inhibited by 85 per cent in the sensitive tumor when the guanine was injected 5 minutes after the thioguanine dose. There was slight inhibition of the conversion when the radioactive precursor was administered 1 hour after the inhibitor. Essentially no inhibition of guanine incorporation into nu-

<table>
<thead>
<tr>
<th>THIOGUANINE DOSE (mg/kg)</th>
<th>PER CENT INHIBITION</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SENSITIVE</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>49</td>
</tr>
<tr>
<td>5.0</td>
<td>58</td>
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<tr>
<td>10.0</td>
<td>61</td>
</tr>
<tr>
<td>15.0</td>
<td>78</td>
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</table>

Thioguanine-sensitive and -resistant Ehrlich ascites tumor-bearing mice were given injections intraperitoneally of 6-thioguanine; 5 minutes following this dose, 50 μg. of guanine-8-C14 (12.7 × 10⁴ counts/min/mg) was injected, and 20 minutes were allowed for metabolic utilization. Each figure represents the average of results obtained from the separate analyses of two to twelve mice.

**TABLE 1**

**EFFECT OF 6-THIOGUANINE ON GUANINE-8-C14 INCORPORATION INTO NUCLEIC ACID GUANINE**

**CHART 1.**—Duration of inhibition of the incorporation of guanine-8-C14 into nucleic acids of thioguanine-sensitive and -resistant Ehrlich ascites cells. Tumor-bearing mice were given injections of a single intraperitoneal dose of 10 mg of thioguanine/kg. At intervals, 50 μg. of guanine-8-C14 (12.7 × 10⁴ counts/min/mg) was injected, and metabolic utilization was allowed to take place for 20 minutes. The results at zero time are those obtained with control mice receiving no thioguanine. Each bar represents the average of results obtained from the separate analyses of two to ten mice.

**Abbreviations used:** RNA, ribonucleic acid; DNA, deoxyribonucleic acid; FGAR, α-N-formyl glycinamide ribotide; 6-TG, 6-thioguanine; NA, nucleic acid; AS, acid-soluble; AMP, adenosine-5'-phosphate; IMP, inosine-5'-phosphate; GTP, guanosine triphosphate.
6-Thioguanine was used in combination with azaserine to determine the effect of the drug combination on guanine-8-C14 incorporation into nucleic acids, and the results are summarized in Table 3. Azaserine appeared to decrease slightly the inhibition of guanine-8-C14 utilization produced by 6-thioguanine. The probable explanation for this result is that azaserine inhibits de novo synthesis, thereby limiting the pool of acid-soluble precursors in the cell. This results in less dilution of the small amount of labeled guanine which is able to by-pass the thioguanine block.

### Table 2

**Inhibition of the Incorporation of Guanine-8-C14 into DNA and RNA of Ehrlich Ascites Cells by 6-Thioguanine**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA Guanine</th>
<th>DNA Guanine</th>
<th>RNA Guanine</th>
<th>RNA Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.3</td>
<td>15.5</td>
<td>7.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>7.6</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ascites tumor-bearing mice were given injections intraperitoneally of 10 mg of thioguanine/kg; 5 minutes following this dose, 60 µg. of guanine-8-C14 (12.7 X 106 counts/min/mg) was injected, and 30 minutes were allowed for metabolic utilization. Each figure represents the average of results obtained from the separate analyses of two mice.

### Table 3

**Effect of Azaserine Plus Thioguanine on the Incorporation of Guanine-8-C14 into Acid-Soluble Purines and Nucleic Acids**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NA adenine (counts/min/µmole X 10^4)</th>
<th>NA guanine (counts/min/µmole X 10^4)</th>
<th>AS adenine (counts/min/µmole X 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.84 ± 0.3*</td>
<td>32.2 ± 10.5</td>
<td>40.3 ± 15.8</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>0.88 ± 0.1</td>
<td>16.9 ± 1.4</td>
<td>15.9 ± 4.7</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>0.61 ± 0.1</td>
<td>20.1 ± 3.6</td>
<td>22.3 ± 8.5</td>
</tr>
</tbody>
</table>

* Average deviation from mean.

Ehrlich ascites tumor-bearing mice received an intraperitoneal injection of either 2.5 mg of thioguanine/kg or the combination of 2.5 mg of thioguanine/kg and 0.2 mg of azaserine/kg. 5 minutes following this treatment, 50 µg. of guanine-8-C14 (12.7 X 106 counts/min/mg) was injected, and 30 minutes were allowed for metabolic utilization. Each figure represents the average of results obtained from the separate analyses of two to three mice.

A concurrent measurement of the utilization of the preformed purine adenine for nucleic acid synthesis is presented in Chart 2. Thioguanine, at a dose of 10 mg/kg, caused a slight inhibition of the conversion of adenine-8-C14 into nucleic acid-guanine when the inhibitor was injected 5 minutes prior to the radioactive precursor. No inhibition of labeled adenine incorporation into nucleic acid adenine occurred. There was an increased incorporation into the nucleic acids of the cells of the sensitive population at the later times after the thioguanine dose. Since this enhanced adenine-C14 utilization could be due to a decrease in the acid-soluble precursor pool, the total acid-soluble adenine was determined at various times after a dose of thioguanine of 10 mg/kg, and the results are given in Chart 3. There was a slight decrease in the total acid-soluble adenine pool size 4 hours after treatment.
the inhibitor dose; however, this does not appear to be of a magnitude which could completely explain the increased utilization of the radioactive adenine.

The duration of the inhibition, in vivo, of the biosynthesis of purines de novo produced by a single dose of 10 mg/kg of thioguanine was determined for the resistant and sensitive tumor lines.

Chart 4 contains the data obtained from such a study. The incorporation of glycine-2-C\(^{14}\) into the adenine and guanine of nucleic acids was used as a measure of synthesis de novo. Within each subline, the biosynthesis de novo of both nucleic acid adenine and guanine was inhibited to the same extent 5 minutes after the thioguanine dose. However, there was twice as much inhibition of these pathways in the cells of the sensitive tumor. The degree of inhibition was of the same magnitude as that of the incorporation of guanine-8-C\(^{14}\) into nucleic acid-guanine. Injection of the glycine-C\(^{14}\) 1 hour after the thioguanine dose indicated that there was some inhibition of synthesis de novo of nucleic acid adenine in the sensitive cells. Since there was no inhibition of the biosynthesis de novo of nucleic acid guanine at this time, this would suggest that thioguanine is producing at least two different metabolic blocks of varying duration.

Hoagland and Zamecnik (5) reported that guanosine triphosphate mediates the transfer of activated leucine-C\(^{14}\) to a peptide linkage in microsomal protein. The guanosine triphosphate appeared to be a specific component of this reaction. In thioguanine-treated ascites cells, the level of endogenous coenzymes of guanine presumably would be lowered. This prompted an investigation of the effect of thioguanine on glycine-2-C\(^{14}\) incorporation into protein. The results shown in Table 4 indicate that 6-thioguanine had no effect on the incorporation of labeled glycine into proteins of either sensitive or resistant ascites cells.

**TABLE 4**

<table>
<thead>
<tr>
<th>Time after thioguanine</th>
<th>Sensitive (counts/min/mg of protein)</th>
<th>Resistant (counts/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1000 ± 300</td>
<td>1280 ± 110</td>
</tr>
<tr>
<td>5 min.</td>
<td>1780 ± 370</td>
<td>1410 ± 470</td>
</tr>
<tr>
<td>1 hour</td>
<td>1740 ± 540</td>
<td>1970 ± 450</td>
</tr>
<tr>
<td>4 hours</td>
<td>2100 ± 890</td>
<td>1940 ± 710</td>
</tr>
</tbody>
</table>

* Average deviation from mean.

Thioguanine-sensitive and -resistant Ehrlich ascites tumor-bearing mice were given injections intraperitoneally of 10 mg of thioguanine/kg. At intervals, 100 \(\mu\)g. of glycine-2-C\(^{14}\) (25 \(\times\) 10\(^{6}\) counts/min/mg) was injected, and 1 hour was afforded for metabolic utilization. Controls were mice receiving no thioguanine. Each figure represents the average of results obtained from the separate analyses of three to four mice.

Results obtained by studying the effect of thioguanine on the accumulation of \(\alpha\)-N-formyl glycaminide ribotide (FGAR) in TA3 ascites cells pretreated with azaserine indicated that 6-thioguanine imposes a metabolic block in the synthesis de novo of purines prior to the formation of FGAR. These data are summarized in Table 5. Thioguanine inhibited by 73 per cent the accumulation of FGAR in cells pretreated with azaserine. The radioactivity of the acid-soluble glycine of the two groups was the same, suggesting that the endogenous glycine pool was the same size in both cases, and therefore variability in precursor pool size was not responsible for the inhibition of FGAR accumulation. It is conceivable that competition for \(\alpha\)-5'-phosphoribosylpyrophosphate is involved.

**Metabolic studies in vitro.—** To resolve further some of the metabolic effects of thioguanine, experiments were conducted in vitro. The determination of the effect of various concentrations of guanine in the presence of a constant amount of inhibitor is shown in Table 6. The optimal concen-
TABLE 5
ACCUMULATION OF RADIOACTIVITY AS FORMYL GLYCINAMIDE RIBOTIDK (FGAR) IN TA3 ASCITES CELLS
The cells were incubated with glycine-2-C\textsuperscript{14} in the presence of azaserine and thioguanine
\begin{align*}
\text{AS-glycine} & \\
\text{FGAR} & \\
\text{Treatment} & \\
\text{counts/min/120 mg (dry wt cells)} & \\
\text{Azaserine} & 254,000 \quad 92,700 \\
\text{Azaserine} & 263,000 \quad 24,800 \\
\text{+thioguanine} & \\
\end{align*}

Ascites tumor-bearing mice were given injections intraperitoneally of 0.2 mg of azaserine/kg, and 1 hour later one group was given injections of 100 \mu g of glycine-2-C\textsuperscript{14} (25 X 10\textsuperscript{8} counts/min/mg) per kg.; 1 hour was the time afforded for metabolic utilization. A second group received thioguanine intraperitoneally, 10 mg/kg, 55 minutes after the azaserine. Five minutes later, 100 \mu g of glycine-2-C\textsuperscript{14} was injected, and 1 hour was permitted for its utilization. Each figure represents the analysis of pooled cells from three mice.

In an attempt to alter the inhibition of guanine utilization by 6-thioguanine, supplements of ribose, ribose-5'-phosphate, and glutamine were added to the flasks. Table 8 gives the results from such experiments. The addition of these compounds apparently altered the distribution of guanine-C\textsuperscript{14} allotted for nucleic acid synthesis. Supplementation stimulated the conversion of guanine to acid-soluble adenine and decreased the incorporation into nucleic acid guanine. The conversion of guanine to acid-soluble adenine also appeared to be decreased by the supplementation; however, added thioguanine not only abolished this effect but also produced a marked stimulation.

TABLE 6
EFFECT OF 6-THIOGUANINE in Vitro ON THE INCORPORATION OF GUANINE-8-C\textsuperscript{14} INTO EHRlich ASCITES CELLS
\begin{tabular}{|c|c|c|c|c|c|}
\hline
GUANINE-8-C\textsuperscript{14} (\mu g/flask) & NA-ADENINE & NA-GUANINE & AS-ADENINE \\
\hline
Control & TG* & Control & TG* & Control & TG* \\
150 & 1.77 & 1.84 & 19.2 & 16.4 & 12.1 & 13.8 \\
100 & 2.55 & 3.80 & 21.4 & 18.1 & 96.4 & 115.6 \\
50 & 1.84 & 1.10 & 15.3 & 12.6 & 59.5 & 47.7 \\
25 & 0.86 & 0.66 & 8.0 & 5.6 & 22.6 & 30.7 \\
\hline
\end{tabular}

* Denotes flasks containing 6-thioguanine, 200 \mu g.

Cells were incubated with guanine-8-C\textsuperscript{14} (12.7 X 10\textsuperscript{8} counts/min/mg) for 60 minutes. Each figure represents the average of results from duplicate flasks.

The ratio of substrate to inhibitor was lower in vitro, even though the ratio of substrate to inhibitor was lower in vitro. That guanine-8-C\textsuperscript{14} which was not utilized for nucleic acid biosynthesis appeared to be converted both to acid-soluble adenine and to other products, since essentially no acid-soluble guanine could be isolated after incubation. The acid-soluble adenine was of high specific radioactivity but not of sufficient magnitude to account for all the radioactivity from the added guanine-C\textsuperscript{14}. Some variability in incorporation of labeled guanine into nucleic acid guanine occurred in vitro; therefore, an experiment was performed to determine possible reasons for this variability. In these studies, thioguanine-sensitive or resistant ascites cells were incubated with the desired compounds in the presence of ascites fluid. The results obtained are presented in Table 7. Less guanine was utilized for nucleic acid synthesis by thioguanine-resistant cells, and this incorporation was not inhibited by 6-thioguanine at a level of 200 \mu g/flask. The addition of ascites fluid from mice bearing either cell line decreased the uptake of labeled guanine into both sensitive and resistant cells. One possible explanation for the observed result is that the fluid contains enzymes which are capable of catabolizing the radioactive precursor.
The data in Table 9 indicate that the biosynthesis of nucleic acid adenine and guanine in vitro is inhibited by thioguanine.

Experiments in vivo suggested that thioguanine was producing a block in the conversion of inosinic acid to adenyl-3-acid. Table 10 gives the results of experiments which appear to indicate that inhibition of this amination reaction does occur. Thioguanine did not inhibit the incorporation of adenine-8-C\textsuperscript{14} or of AMP-8-C\textsuperscript{14} into the adenine of either nucleic acid or acid-soluble compounds. In contrast, the antimetabolite produced a marked inhibition of the incorporation of IMP-8-C\textsuperscript{14} into both nucleic acid and acid-soluble adenine. It is interesting to note that this conversion in Escherichia coli requires the presence of GTP as a coenzyme (7).

Results in vitro corroborate findings in vivo and demonstrate that the test system studied in vitro can be used to define more specifically the mechanisms of purine inhibition by 6-thioguanine.

**SUMMARY**

The effects of 6-thioguanine on purine metabolism were investigated both in vivo and in vitro in ascites tumor cells. The neoplasms used were the thioguanine-resistant and -sensitive sublines of the Ehrlich carcinoma and the TA\textsubscript{3} carcinoma.

6-Thioguanine was found to inhibit the utilization of guanine-8-C\textsuperscript{14} for the formation of acid-soluble purines and nucleic acids.

Biosynthesis of nucleic acid adenine and guanine, as measured by the incorporation of radioactivity from glycine-2-C\textsuperscript{14}, was also inhibited by thioguanine. The duration of inhibition of the synthesis of nucleic acid adenine was longer than that found for nucleic acid guanine, indicating the presence of at least two sites of inhibition by the drug.

Inhibition by thioguanine of the accumulation of a-N-formyl glycinamide ribotide in azaserine-pretreated cells suggested a block on the pathway of nucleic acid adenine but did inhibit the conversion of inosine-5'-phosphate to nucleic acid adenine.

Incorporation of precursors into nucleic acid purines was inhibited in both thioguanine-sensitive and -resistant Ehrlich ascites cells. The inhibition of the various pathways occurred to a lesser degree in the resistant tumor. This is correlated with the relatively smaller quantity of thioguanine which has been found present in the resistant ascites cells.

**REFERENCES**


### Table 8

<table>
<thead>
<tr>
<th>Addition</th>
<th>NA-adenine</th>
<th>NA-guanine</th>
<th>AS-adenine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.6</td>
<td>20.1</td>
<td>75.9</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>1.2</td>
<td>13.8</td>
<td>86.6</td>
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<tr>
<td>Supplements</td>
<td>2.2</td>
<td>15.7</td>
<td>63.7</td>
</tr>
<tr>
<td>+thioguanine</td>
<td>1.2</td>
<td>12.4</td>
<td>115.3</td>
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</table>

**In Vitro** Effects of Supplements on Guanine-8-C\textsuperscript{14} Incorporation into Ehrlich Ascites Cells

Cells were incubated with 100 pg of guanine-8-C\textsuperscript{14} (12.7 $\times$ $10^4$ counts/min/mg) per flask for 60 minutes; when used, the amount of thioguanine per flask was 200 pg. Each figure represents the average of results from duplicate flasks.

### Table 9

<table>
<thead>
<tr>
<th>Addition</th>
<th>NA-adenine</th>
<th>NA-guanine</th>
<th>AS-adenine</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.20</td>
<td>0.73</td>
<td>6.0</td>
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<tr>
<td>Thioguanine</td>
<td>0.12</td>
<td>0.53</td>
<td>2.9</td>
</tr>
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</table>

**In Vitro** Effect of 6-Thioguanine on the Incorporation of Glycine-2-C\textsuperscript{14} into Ehrlich Ascites Cells

Cells were incubated with 250 pg of glycine-2-C\textsuperscript{14} (25 $\times$ $10^4$ counts/min/mg) per flask for 60 minutes; when used, the amount of thioguanine per flask was 200 pg. Each figure represents the average of results from duplicate flasks.

### Table 10

<table>
<thead>
<tr>
<th>Addition</th>
<th>NA-adenine</th>
<th>NA-guanine</th>
<th>AS-adenine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine-8-C\textsuperscript{14}</td>
<td>99.4</td>
<td>5.0</td>
<td>4,400</td>
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<tr>
<td>Adenine-8-C\textsuperscript{14}</td>
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<td>4,120</td>
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<td>1.8</td>
<td>0.03</td>
<td>100.4</td>
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<td>AMP-8-C\textsuperscript{14}</td>
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<td>0.06</td>
<td>89.3</td>
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<td>1.3</td>
<td>46.4</td>
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<tr>
<td>+thioguanine</td>
<td>0.9</td>
<td>1.1</td>
<td>12.2</td>
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</table>

**In Vitro** Effect of 6-Thioguanine on the Incorporation of Preformed Purines into Ehrlich Ascites Cells

Cells were incubated with 67.5 pg of adenine-8-C\textsuperscript{14} (31.6 $\times$ $10^4$ counts/min/mg) per flask or 174 pg of AMP-8-C\textsuperscript{14} (1.3 $\times$ $10^5$ counts/min/mg) per flask for 50 minutes. IMP-8-C\textsuperscript{14}, 93.5 pg/flask (3.8 $\times$ $10^4$ counts/min/mg), was incubated for 60 minutes. When thioguanine was used, 200 pg was present in each flask. Each figure represents the average of results from duplicate flasks.
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