Studies on Alloxan

III. EFFECT ON THE BIOSYNTHESIS OF URIDINE NUCLEOTIDES FROM URACIL*

Ezzat S. Younathan, Joseph E. Stone, and Thomas S. Harris

From the Department of Biochemistry and the Department of Pharmacology, University of Arkansas Medical Center, Little Rock, Arkansas

(Received for publication, July 20, 1963)

The mechanism of the cytotoxic action of alloxan on the β-cells of the pancreas and on other sensitive cells is not known. Possible modes of action have been reviewed in previous communications (1, 2).

In view of its structural similarity to uracil, the influence of alloxan on the utilization of this base for the formation of uridine nucleotides was investigated. The steps of this biosynthetic pathway (3) are

\[ \text{Uracil} \quad 1 \quad \text{uridine} \quad 2 \quad 5'\text{-UMP} \quad 3 \quad \text{UDP} \quad 4 \quad \text{UTP} \]

These steps are catalyzed by uridine phosphorylase, uridine kinase, ATP:UMP phosphotransferase, and ATP:UDP phosphotransferase, respectively.

In this paper it will be shown that alloxan inhibits UTP formation by blocking Step 3. The inhibition seems to be caused by binding an essential sulfhydryl group on the enzyme rather than by the structural similarity to uracil.

**EXPERIMENTAL PROCEDURE**

**Materials**—Uracil-2-14C was purchased from Calbiochem, Inc. and had a specific activity of 6.5 μC per amole. Alloxan (Eastman Kodak Company) was recrystallized twice from glacial acetic acid. Because of its instability, alloxan solutions were prepared just before use. N-Ethylmaleimide was a chromatographically pure sample from Mann Laboratories. Alloxanic acid was synthesized by the method of Biltz, Heyn, and Bergius (4), and 3',5'-uridine monophosphate by the method of Smith, Drummond, and Khorana (5). 2',3'-Uridine monophosphate was purchased from the Pabst Laboratories.

**Enzyme Source**—Rat brain was chosen as the enzyme source because of its high activity in utilizing free uracil for the synthesis of uridine nucleotides.1 A 20% homogenate was prepared in 0.25 M sucrose, and the particulate material was sedimented by ultracentrifugation at 100,000 × g for 1 hour. The clear supernatant fraction was utilized. Besides having the enzymes needed for the utilization of uracil, this preparation contains also those required for the generation of ATP from ADP and 3-phosphoglyceric acid.

**Incubation Medium**—The composition of the reaction medium and the conditions of incubation used routinely in this study are given in the legend of Fig. 1. The reaction was terminated by the addition of 1.5 ml of 1.5 N perchloric acid to each flask, and the contents were centrifuged. The supernatant solution was neutralized with 2 N KOH. After refrigeration and removal of potassium perchlorate by centrifugation, the supernatant solution was chromatographed on a Dowex 1 (formate) column.

**Chromatography of Acid-soluble Extracts**—In some experiments reported here (e.g. Fig. 1), manually operated columns were used and 21 fractions were collected. The details of the elution scheme and the characterization of each peak have been published elsewhere (6). In other experiments (e.g. Table II), the extended gradient elution scheme of Hurlbert et al. (7) with 200 fractions was employed. The results from both schemes were similar. Since the Dowex 1 columns did not separate uracil from uridine, this mixed peak was further resolved into the free base and the nucleoside on activated charcoal columns by the method of Stambaugh and Wilson (8).

**Measurement of Radioactivity**—The radioactivity in each chromatographic fraction was determined by placing an aliquot at "infinite thinness" on aluminum planchets and counting in a thin window gas flow counter. The total radioactivity in each peak was calculated and used as a measure of the amount of products formed from uracil.

**Paper Chromatography**—This technique was used to identify the nucleotide in Peak "X" (Fig. 1). The solvent systems used (5) are described in Table I. The unknown radioactive sample was detected by a radiochromatographic strip scanner, and the unlabeled authentic nucleotides were localized by an ultraviolet lamp.

**RESULTS**

**Profiles of Nucleotides Produced in the System**—Fig. 1 shows that, in the absence of inhibitor, a major part of the labeled uracil added is transformed into UTP under the experimental conditions used. When alloxan was incorporated in the reaction medium at a final concentration of 1 mM, the UTP peak was greatly diminished, and a new peak (labeled "X" in Fig. 1) made its appearance. Since alloxan isomerizes rapidly at physiological pH values to alloxamic acid (9), the effect of the latter was tested. The profile obtained in this case was very similar to that of the control, indicating that the observed inhibition is due to alloxan per se. Since alloxan inhibits several sulfhydryl-dependent enzymes (10), the influence of N-ethylmaleimide on this system was studied. As shown in Fig. 1, this sulfhydryl inhibitor brought about a nucleotide profile very similar to

---

1 A comparative study of the importance of the uracil and the orotic acid pathways of pyrimidine nucleotide synthesis in various issues of the rat will be published elsewhere.

* This investigation was supported by Grants AM 4050-03 and CA 4084-05 from the National Institutes of Health, United States Public Health Service. A preliminary report appeared in Federation Proc., 22, 522 (1963).
that obtained with alloxan. At a final concentration of 0.1 mM it inhibited almost completely the production of UTP from uracil, and once again led to the appearance of Peak "X".

Identification of Peak "X" as Uridine 5'-Monophosphate—The chromatographic behavior of the nucleotide in Peak "X" was suggestive of a uridine monophosphate. To ascertain the identity of this product, samples from several experiments were pooled and concentrated, and the ammonium formate used in the eluent was removed by treatment with Dowex 50 (acid form) followed by lyophilization. The residue obtained was dissolved in a small volume of water and chromatographed on paper in three different solvent systems. Authentic samples of 5'-UMP, 2',3'-UMP, 3',5'-UMP and UDP-glucose were analyzed concomitantly. The RF values obtained for the unknown sample and the other nucleotides are given in Table I. Based upon the identity of the values obtained in all three solvents, it is concluded that the nucleotide forming Peak "X" is 5'-UMP.

Quantitative Estimation of Nucleotides Produced—For the quantitative determination of the distribution of radioactive uracil into the various products, experiments were conducted in which the extended gradient elution of Hurlbert et al. (7) was used. Table II summarizes the data obtained when alloxan was incorporated in the reaction medium at 1.0 mM final concentration. Whereas alloxan caused 95% inhibition of the synthesis of UTP, it brought about a considerable accumulation of both uridine and 5'-UMP in the system. The synthesis of UDP and UDP-glucose was also completely inhibited. These results indicate that alloxan blocked the step between UMP and UDP in the synthetic scheme. The enzymes catalyzing the two preceding steps, uridine phosphorylase and uridine kinase, do not seem to be sensitive at all to this compound. As shown

![Fig. 1. Effect of alloxan, alloxamic acid, and N-ethylmaleimide on the profiles of uridine nucleotides produced from uracil-2-14C.](http://www.jbc.org/)

#### Table I

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>RF (Solvent I)</th>
<th>RF (Solvent II)</th>
<th>RF (Solvent III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-glucose</td>
<td>0.04</td>
<td>0.08</td>
<td>0.21</td>
</tr>
<tr>
<td>2',3'-UMP</td>
<td>0.36</td>
<td>0.29</td>
<td>0.54</td>
</tr>
<tr>
<td>3',5'-UMP</td>
<td>0.35</td>
<td>0.32</td>
<td>0.49</td>
</tr>
<tr>
<td>5'-UMP</td>
<td>0.05</td>
<td>0.23</td>
<td>0.41</td>
</tr>
<tr>
<td>Nucleotide &quot;X&quot;</td>
<td>0.08</td>
<td>0.28</td>
<td>0.41</td>
</tr>
</tbody>
</table>

#### Table II

<table>
<thead>
<tr>
<th>Bioisotopic product</th>
<th>Control</th>
<th>Alloxan (1.0 mM)</th>
<th>N-Ethylmaleimide (0.1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent of total radioactivity</td>
<td>Per cent of total radioactivity</td>
<td>Change*</td>
</tr>
<tr>
<td>Uricril</td>
<td>47.6</td>
<td>49.6</td>
<td>+3%</td>
</tr>
<tr>
<td>Uridine</td>
<td>2.9</td>
<td>15.8</td>
<td>+445%</td>
</tr>
<tr>
<td>5'-UMP</td>
<td>0</td>
<td>16.3</td>
<td>New peak</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>1.9</td>
<td>0</td>
<td>Complete inhibition</td>
</tr>
<tr>
<td>UDP</td>
<td>1.2</td>
<td>0</td>
<td>-95%</td>
</tr>
<tr>
<td>UTP</td>
<td>46.4</td>
<td>2.3</td>
<td>-95%</td>
</tr>
</tbody>
</table>

*Per cent change = \[(\text{percentage of total radioactivity in experimental minus control}) \times 100\] divided by percentage of total radioactivity in control.

The inhibition proved to be a direct one, i.e. not mediated through the influence on the energy-yielding system. The amount of ATP, as measured by the absorbance at 260 nm of its chromatographic peak after the termination of the reaction, was little affected by the presence of alloxan. Moreover, the possibility that alloxan is bringing about the inhibition of UTP synthesis through binding the essential magnesium ions is not plausible (see "Discussion").
DISCUSSION

The possibility that alloxan might be exerting its cytotoxic action by acting as a pyrimidine antimetabolite has been suggested in the literature (11). The present work, however, provides no support for such a thesis, at least as far as the biosynthesis of uridine nucleotides is concerned. The insensitivity of the first two steps in this biosynthetic scheme to alloxan and to N-ethylmaleimide, as well as the similarity between the action of these two inhibitors in the present system, indicate that the inhibition is brought about by binding a sulfhydryl group essential for the activity of ATP:UMP phosphotransferase.

This inhibition seems also to be a direct one. It is very improbable that it is caused by binding the magnesium ions required for Steps 2, 3, and 4. The following points provide evidence against such a mechanism: (a) magnesium chloride is present in the reaction medium in a large excess, and the molar ratio of \( \text{Mg}^{++} \) to inhibitor is 5:1 in the case of alloxan and 50:1 in the case of N-ethylmaleimide; (b) the data presented here indicate that uridine kinase is insensitive to the two inhibitors tested in spite of its absolute requirement for magnesium (12); (c) although ATP (one of the reactants in the medium) is known to form stable complexes with magnesium (13), alloxan and N-ethylmaleimide have not been shown to chelate this metallic ion.

Since both uridine kinase and ATP:UMP phosphotransferase catalyze the transfer of a phosphate group from ATP to an acceptor, the difference in their sensitivity to alloxan and N-ethylmaleimide is of interest. It should be noted that the product of the former reaction is a low energy phosphate linkage whereas the product of the latter is a high energy one. A role of a reactive sulfhydryl group in ATP:creatinine phosphotransferase has been described recently by Watts and Rabin (14).

The bearing of the present results on the cytotoxic action of alloxan on the \( \beta \)-cells of the pancreas and on other sensitive cells is hard to assess at the present time. Hultquist (15) reported that the two sulfhydryl inhibitors, iodoacetic acid and iodoacetamide, were nondiabetogenic in spite of initiating some nuclear changes in the \( \beta \)-cells. Therefore, in the case of these cells, it seems that the effect of alloxan cannot be correlated with its sulfhydryl-binding capacity alone. In concentrations slightly higher than the diabetogenic dose, this compound causes injury to several tissues, e.g. liver, kidney, lung, and adrenal gland (16). It is possible that the blocking of essential sulfhydryl groups of key enzymes might contribute to the toxic effect of alloxan in these tissues.

Finally, the use of sulfhydryl inhibitors in the present system might have some applications, e.g. the synthesis of labeled uridine and 5'-UMP from labeled uracil, or the assay of uridine phosphorylase and uridine kinase without interference from subsequent steps of the synthetic scheme.

SUMMARY

In a soluble system from rat brain capable of synthesizing uridine triphosphate from uracil-2-\( ^{14} \)C, the presence of alloxan at 1 mM concentration led to 95% inhibition of formation of this nucleotide and the accumulation of both uridine and uridine 5'-monophosphate. N-Ethylmaleimide showed a similar effect at 0.1 mM concentration. The possibility that the inhibition is caused by influencing the energy-yielding system or by binding \( \text{Mg}^{++} \) is excluded. It is concluded that these two inhibitors exert their effect through binding an essential sulfhydryl group on the enzyme adenosine triphosphate:uridine 5'-monophosphate phosphotransferase. Uridine phosphorylase and uridine kinase, on the other hand, are not sensitive to these two compounds.

The bearing of these results on the mechanism of the cytotoxic action of alloxan and their possible application for the synthesis of labeled uridine and uridine 5'-monophosphate from uracil are discussed.

Acknowledgment—The able technical assistance of Miss Norma J. McManus is gratefully acknowledged.

REFERENCES

Studies on Alloxan: III. EFFECT ON THE BIOSYNTHESIS OF URIDINE NUCLEOTIDES FROM URACIL

Ezzat S. Younathan, Joseph E. Stone and Thomas S. Harris