METABOLISM OF THE NITROFURANS

III. STUDIES WITH XANTHINE OXIDASE IN VITRO

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For a better understanding of the mechanism of action of the nitrofurans in the mammalian organism, studies of their metabolism have been initiated. Excretion studies (1) have revealed that only a small part of most ingested nitrofurans appears unchanged in the urine. Bender and Paul (2) in preliminary studies have shown that 5-nitro-2-furaldehyde semicarbazone (Furacin) undergoes degradation in the presence of various body tissues in vitro and point to possibilities that may include a Furacin-xanthine oxidase relationship in vivo.

That the xanthine oxidase-hypoxanthine system could reduce the nitro group of nitrophenols was shown by Greville and Stern (3), but no reduction products were isolated. Bueding and Jolliffe (4) in their study of trinitrotoluene in vitro found that the xanthine oxidase-hypoxanthine system reduced only one of the nitro groups to form hydroxylaminodinitrotoluene. The reduction to aminodinitrotoluene was accomplished in vitro with tissue slices and extracts.

The metabolic studies of the nitrofurans have been extended to the determination of the mutual effects of xanthine oxidase systems and the nitrofurans. Data will be presented in this paper on the effect of Furacin on the xanthine oxidase-hypoxanthine system and the destruction of Furacin and other nitrofurans by this system under anaerobic conditions.

EXPERIMENTAL

A concentrated xanthine oxidase preparation was made from fresh cream according to the method of Ball (5). The preparation was carried only through Step III and used as such.

For experiments on oxygen uptake, 2.6 ml. of $\frac{1}{15}$ phosphate buffer, pH 7.2, and 0.2 ml. of enzyme solution were pipetted into the main chamber of a Warburg flask. In the side arm was placed 0.2 ml. of a 0.05 $M$ solution of hypoxanthine or xanthine in 0.05 $M$ sodium hydroxide. When Furacin was used, it was dissolved in the buffer solution and placed in the main chamber of the flask with the enzyme. After equilibration at

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1 Brand of nitrofurazone, "New and nonofficial remedies."
38°, the taps were closed, the substrate tipped in from the side arm, and readings taken at 10 minute intervals for 30 minutes. The per cent inhibition was calculated at 30 minutes.

For anaerobic experiments 50 ml. of m/15 phosphate buffer of pH 7.2, containing the desired concentration of dissolved nitrofuran and hypoxanthine or xanthine (1 or 2 mM concentration), was pipetted into a 100 ml. glass-stoppered flask. Purified nitrogen was bubbled through the solution for 10 minutes, the flask in a water bath being maintained at 37°. Depending on the activity, 0.8 to 2.0 ml. of enzyme solution was pipetted into the flask, the contents mixed, and a 2 ml. aliquot removed immedi-

![Fig. 1. Effect of Furacin (5-nitro-2-furaldehyde semicarbazone) on the oxygen uptake of the xanthine oxidase-hypoxanthine system under aerobic conditions.](image)

ately for analysis. The flask was stoppered, placed in the bath, and at intervals aliquots were removed for analysis.

The nitrofuran or the uric acid was estimated by a combination of ultraviolet spectrophotometric methods used by Kalckar (6) and Paul et al. (1). The 2 ml. aliquot was pipetted into a centrifuge tube containing 10 ml. of 95 per cent ethyl alcohol, the contents mixed to stop enzyme activity, and the tube centrifuged to remove the precipitate. The clear supernatant liquid was diluted, and the spectral absorption characteristics were determined on the Beckman spectrophotometer. Owing to the presence of several absorption maxima, readings were made from 420 to 230 mμ. From the known $E_{1\%}^{1\%}$ values of Furacin (786) and uric acid (710), the amounts of each were estimated.

**Oxygen Uptake**—The results of a typical experiment dealing with the effect of Furacin (5-nitro-2-furaldehyde semicarbazone) on the oxygen uptake of the xanthine oxidase-hypoxanthine system in the presence of air are shown in Fig. 1. Since the rates in both the controls and the
Furacin-containing flasks were constant for the first 30 minutes, comparison of the 30 minute values for uptake was used as a measure of inhibition by Furacin.

With several different enzyme preparations (Table I), it was found that Furacin (8.7 × 10⁻⁴ M) inhibited the oxygen uptake of this system by 53 per cent. In the single experiment in which the Furacin concentration was 1.7 × 10⁻⁴ M, the inhibition observed was similar. No significant inhibition was observed with either furaldehyde semicarbazone or with 2,4-dinitrophenol at a concentration of 8.7 × 10⁻⁴ M. In one experiment the Furacin concentration was measured spectrophotometrically at the

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar concentration of compound X 10⁻⁴</th>
<th>Amount of enzyme</th>
<th>Oxygen uptake, 30 min.</th>
<th>Difference in oxygen uptake</th>
<th>Inhibition per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ml.</td>
<td>μl. O₂</td>
<td>μl. O₂</td>
<td>μl. O₂</td>
</tr>
<tr>
<td>Furacin</td>
<td>8.7</td>
<td>0.2</td>
<td>113 ± 12</td>
<td>51 ± 7</td>
<td>62 ± 11</td>
</tr>
<tr>
<td></td>
<td>1.7</td>
<td>0.2</td>
<td>98</td>
<td>40</td>
<td>58</td>
</tr>
<tr>
<td>Furaldehyde semicarbazone</td>
<td>8.7</td>
<td>0.2</td>
<td>109</td>
<td>107</td>
<td>2</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>8.7</td>
<td>0.2</td>
<td>136</td>
<td>130</td>
<td>6</td>
</tr>
</tbody>
</table>

* Average of eight Warburg experiments (duplicate flasks) with standard deviations.
† Single Warburg experiments, duplicate flasks.

start and at the end of the incubation period. The amount of Furacin destroyed was found to be 8 per cent of the initial amount.

Effect of Xanthine Oxidase System on Nitrofurans under Anaerobic Conditions—A typical ultraviolet absorption curve for xanthine oxidase-hypoxanthine-Furacin under anaerobic conditions is shown in Fig. 2. At the start of the experiment (Curve 1, Fig. 2) there were two maxima, one for Furacin at 375 mμ, and another at 252 mμ which was a combination of the 260 mμ maximum of Furacin plus the 250 mμ maximum of hypoxanthine.

At the end of 2 hours the 375 mμ maximum of Furacin had disappeared (Curve 2, Fig. 2) and a new maximum at 335 mμ of "reduced Furacin" (2) had appeared. The maximum at 252 mμ had disappeared and a new maximum in the 290 mμ region (uric acid) (2) had appeared. The reaction was apparently an enzymatic one, since neither hypoxanthine nor
xanthine reacted with Furacin in the absence of an enzymatic source under similar conditions.

The effect of varying amounts of one enzyme preparation (0.2 ml. to

![Graph](image)

**Fig. 2.** Ultraviolet absorption curves of xanthine oxidase-hypoxanthine-Furacin under anaerobic conditions. Furacin concentration approximately $1 \times 10^{-3}$ M; hypoxanthine concentration approximately $2 \times 10^{-3}$ M at start. Samples taken at intervals and diluted 1:30 for reading. Curve 1, sample taken at start; Curve 2, sample taken after 2 hours incubation at 37°.

![Graph](image)

**Fig. 3.** Effect of enzyme concentration on rate of disappearance of Furacin in xanthine oxidase-hypoxanthine system under anaerobic conditions.

0.8 ml.) on the disappearance of Furacin (1 mM) anaerobically with hypoxanthine (2 mM) as a substrate was studied (Fig. 3). Practically no reaction took place with 0.2 ml. of enzyme solution. The rate of destruction of Furacin was a straight line function and directly proportional with
time with 0.6 ml. and 0.8 ml. of enzyme, indicating that the reaction was zero order and independent of the Furacin concentration.

An attempt was also made to measure the amount of uric acid formed by the spectrophotometric method previously described. Hypoxanthine was used in excess as a substrate \((2 \times 10^{-3} \text{ M})\) and Furacin was the limiting reactant at a concentration of 1 or \(0.5 \times 10^{-3} \text{ M}\). When the Furacin was exhausted, the formation of uric acid ceased. That the enzyme system was still active was shown by admitting air to the flask and noting further formation of uric acid. Estimates made on the basis of ultraviolet absorption data indicate that approximately 1 mole of uric acid was formed at the expense of 1 mole of Furacin at either 1.0 or 0.5 mM concentration.

Comparison of the formation of uric acid anaerobically from xanthine \((1.2 \text{ mM})\) or hypoxanthine \((1.8 \text{ mM})\) in the presence of Furacin \((1 \text{ mM})\) is shown in Figs. 4 and 5. When hypoxanthine was used in excess as a substrate (Fig. 4), all the Furacin had disappeared by the end of 2 hours and reduced Furacin \((335 \text{ m} \mu \text{ maximum})\) and uric acid \((290 \text{ m} \mu \text{ maximum})\) had appeared. After 24 hours uric acid was still present at the same concentration, but the labile “reduced Furacin” had disappeared. When xanthine was used as a substrate and was present in a limiting amount the 3 hour curve (Fig. 5) indicates the formation of uric acid and the presence of material with a 350 m\(\mu\) maximum. The 10 hour curve reveals that the 350 m\(\mu\) maximum was probably due to a combination of Furacin.
(375 m\(\mu\)) and "reduced Furacin" (335 m\(\mu\)), for by 10 hours the labile "reduced Furacin" had disappeared, leaving the stable Furacin. In this experiment it may be noted that approximately one-half of the Furacin disappeared, but the uric acid formation was about the same as with the hypoxanthine.

The effect of added cysteine on the rate of disappearance of Furacin was observed in one experiment. The addition of neutralized cysteine hydrochloride in \(5 \times 10^{-4}\) m concentration markedly increased (about 300 per cent) the rate of destruction of Furacin anaerobically.

Other nitrofurans were studied in the anaerobic xanthine oxidase system, with hypoxanthine as a substrate. Some compounds were studied at \(1 \times 10^{-3}\) m and some at \(0.5 \times 10^{-3}\) m concentration. The results are summarized in Table II. Nitrofuroic acid and those compounds studied (5-nitro-2-furaldehyde and methyl 5-nitro-2-furoate) which are metabolized to nitrofuroic acid (1) disappeared and no formation of uric acid was apparent. The more complex derivatives of nitrofuraldehyde disappeared, with a concomitant formation of uric acid. When measurement was possible, disappearance of nitrofuran was found to be accompanied by formation of uric acid in the approximate molar ratio of 1:1. In the studies on the compounds at \(0.5 \times 10^{-3}\) m concentration, absorption due to end-products or other interfering substances was so great that observations in the 290 m\(\mu\) region were somewhat obscured. The presence of uric acid was indicated by a shelf or inflection in the 290 m\(\mu\) region rather than by a true maximum, as in the other experiments.

Most of the compounds studied were destroyed at approximately the
TABLE II

Rate of Destruction of Nitrofurans by Xanthine Oxidase-Hypoxanthine System under Anaerobic Conditions

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Compound</th>
<th>Concentration</th>
<th>Amount destroyed in 30 min.</th>
<th>Uric acid formed</th>
<th>mm uric acid formed</th>
<th>mm nitrofuran destroyed</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>R—CHO</td>
<td>1.0</td>
<td>17</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>R—COOH</td>
<td>1.0</td>
<td>10</td>
<td>Little or none</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>R—COOCH₃</td>
<td>1.0</td>
<td>Little</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>α-R—CH—NOH</td>
<td>1.0</td>
<td>23</td>
<td>Yes</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>R—CH—NNHCONH₂</td>
<td>1.0</td>
<td>20</td>
<td>&quot;</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>R—CH=NNH₂</td>
<td>1.0</td>
<td>29</td>
<td>&quot;</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>R—CH—NNHCOCH₃</td>
<td>1.0</td>
<td>38</td>
<td>&quot;</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>R—CH=NNHCONH₂</td>
<td>0.5</td>
<td>25</td>
<td>Indicated†</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>R—COCH₃</td>
<td>0.5</td>
<td>38</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>R—C(CH₃)=NNHCONH₂</td>
<td>0.5</td>
<td>20</td>
<td>Indicated↑</td>
<td>Not run to completion</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>R—CH=NN(CH₃)CONH₂</td>
<td>0.5</td>
<td>28</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>62</td>
<td>R—CH=NNNHCONH₂</td>
<td>0.5</td>
<td>25</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>67</td>
<td>R—CH=NN(CH₃CH₂OH)CONH₂</td>
<td>0.5</td>
<td>25</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>114</td>
<td>R—CH=NNHOCOCH₃N(CH₃)Cl</td>
<td>0.5</td>
<td>38</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>119</td>
<td>R—C(CH₃)=NN(CH₂)CONH₂</td>
<td>0.5</td>
<td>15—25</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>R—CH=NNHCONH₂</td>
<td>0.5</td>
<td>75</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Compound destroyed; substrate unchanged.
† General absorption obscures 290 μ region. Presence of compound indicated by inflection in curve in 290 μ region and by increase in density at 290 μ with time.
‡ Character of absorption curve of original compound precludes conclusions in 290 μ region.

same rate as Furacin. A significant difference in the rate of destruction of the two isomeric nitrofuraldoximes (7) was noted. The α form (anti) was more slowly destroyed. At the end of the experiment the amount of uric acid formed was the same with both isomers. An isomer of Furacin,
2-nitro-4-furaldehyde semicarbazone (8), was destroyed with much greater rapidity than Furacin.

DISCUSSION

Furacin appears to act as an inhibitor in the xanthine oxidase-hypo-

xanthine system when air is present. Under anaerobic conditions Furacin apparently acts as a hydrogen acceptor. A few calculations have been made to determine whether the lowered oxygen uptake observed aerobically in the presence of Furacin could have been due to the transfer of electrons to Furacin. At normal pressure and 37°, 3 ml. of 0.87 mm Furac-

in could, on complete reduction, account for about 66 c.mm. of "oxygen," with a resultant apparent lowered oxygen uptake. In Table I it can be seen that in five of the nine experiments the difference is of about the same magnitude or smaller, making this appear a plausible explanation. However, in the experiment in which Furacin disappearance was meas-

ured, only 8 per cent of the Furacin was lost. On the basis of present evidence then, the aerobic inhibition cannot be solely accounted for by reduction of the nitro group of the Furacin molecule. Whether the aero-
bic inhibition of oxygen uptake with xanthine oxidase takes place in tivo cannot be said at this time.

However, it has been shown that Furacin is destroyed in the body (1) and that various tissues can destroy Furacin in vitro (2), with the produc-
tion of uric acid and a product absorbing at 335 mμ in a manner similar to that now shown with xanthine oxidase. Also, in the tissue studies in vitro a correlation has been shown between the ability of a given tissue to destroy Furacin with the simultaneous production of uric acid and its xanthine oxidase content (2, 9). Thus it is quite possible that xanthine oxidase contributes to the destruction of Furacin in vivo.

The nature of the nitrofuran end-product of the xanthine oxidase re-
action absorbing at 335 mμ has been studied briefly. Several attempts to isolate this compound have been made, but, owing to its instability, such attempts have not yet been successful.

Oxidation of hypoxanthine to uric acid involves 4 oxidizing equivalents per mole, while oxidation of xanthine to uric acid requires only 2. On the other hand, reduction of Furacin to its corresponding hydroxylamine would require 4 reducing equivalents per mole, and to the amine, 6. The data indicate (Fig. 5) that when xanthine was the substrate and Furacin was present in excess 1 mole of uric acid appeared when 0.5 mole of Furacin disappeared. On the assumption that the point of attack on the Furacin molecule is the nitro group, this indicates that the reduction product is the hydroxylamine. Subtraction of the absorption curves (10 hour curve with stable Furacin from 3 hour curve when both Furacin and labile "reduced Furacin" are suspected) reveals that this reduction
product has the usual peak (335 μm) observed for "reduced Furacin." The reduction product when hypoxanthine was the substrate (Fig. 4) present in excess under conditions such that all the Furacin present had been reduced and an equivalent amount of uric acid formed, likewise is "reduced Furacin," with the characteristic peak at 335 μm. Therefore, it may be concluded tentatively that the reduction product here also is the hydroxylamine. All attempts at reduction of Furacin by such reagents as Raney's nickel-hydrogen2 or sodium hydrosulfite have produced material with an absorption maximum at 335 μm. Until this "reduced Furacin" has been isolated and characterized, the extent of xanthine oxidase reduction cannot be fully determined.

The enhanced destruction of Furacin observed in the presence of cysteine was marked. That the increased rate of Furacin destruction is linked to an enzyme is shown by incubating Furacin with cysteine (no enzyme present). In phosphate buffer (pH 7.2) with added cysteine only 10 per cent of the Furacin was destroyed in 14 hours at 37°. In our enzyme system with cysteine present Furacin had nearly completely disappeared by the end of 40 minutes.

**SUMMARY**

1. The interrelationship between Furacin (5-nitro-2-furaldehyde semicarbazone) and xanthine or hypoxanthine catalyzed by xanthine oxidase prepared from fresh cream was studied in vitro under both aerobic and anaerobic conditions.

2. At 8.7 X 10^{-4} M concentration Furacin significantly inhibited the oxygen uptake of the xanthine oxidase-hypoxanthine system in the presence of air.

3. Under anaerobic conditions Furacin and certain other nitrofurans can apparently act as hydrogen acceptors in the xanthine oxidase-hypoxanthine system. The nitrofuran is reduced, with the simultaneous appearance of uric acid.

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