STUDIES ON URIC ACID AND RELATED COMPOUNDS

III. OBSERVATIONS ON THE SPECIFICITY OF MAMMALIAN XANTHINE OXIDASES

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Xanthine oxidase (XO) has been thoroughly studied by many investigators and the range of possible substrates of this enzyme is well known (1, 2). In the early experiments, enzymatic activity was measured by decolorization of a suitable dyestuff such as methylene blue or by oxygen consumption. Therefore, the exact pathway of oxidation remained unknown for many substrates. The spectrophotometric method of Kalckar (3, 4), which is now being widely applied to hypoxanthine and xanthine, is much more specific. This method has been extended in the present and the following papers to identify the oxidation products of various purine derivatives unequivocally. In this way, the substrate specificity and other characteristic properties of xanthine oxidase have been determined. Our results enable us to draw certain conclusions about the mode of attachment of the substrate to the active center of the enzyme and about the mechanism of the dehydrogenation catalyzed by it. We have also found that among all methylated uric acids, which are formed after administration of methylated xanthines to animals, only the L-methyl derivative can be produced by direct action of XO. Thus, all other substituted uric acids must result from a different biochemical pathway. This problem will be dealt with in Paper IV.

EXPERIMENTAL

Materials—Purine was supplied by Dr. Aaron Bendich of the Sloan-Kettering Institute for Cancer Research. The 2- and 8-hydroxypurines, and likewise the 2,8- and 6,8-dioxypurines, were obtained through the courtesy of Professor Adrian Albert of the Australian National University, Canberra. Both 1- and 7-methylhypoxanthines were a gift from Dr. Gertrude B. Elion of the Wellcome Research Laboratories, Tuckahoe, New York. 3-Methylxanthine was given to us by Dr. V. Papesch of G. D. Searle and Company, Chicago, and the 7-methyl and 1,7-dimethyl derivatives by Dr. J. J. Fox of the Sloan-Kettering Institute for Cancer Research.

* Part of a thesis for the degree of Doctor of Philosophy, submitted to the Faculty of Science, The Hebrew University, Jerusalem, 1957.
Research. 1-Methylxanthine was synthesized by us as reported previously (5).

Enzymes—Milk xanthine oxidase was a gift of Professor F. Bergel and Dr. R. C. Bray of the Chester Beatty Institute of Cancer Research, London (6). At pH 8.3, the preparation, when diluted 1:400, produced 1.0 \( \gamma \) of uric acid per ml. per minute when \( 10^{-4} \) M xanthine served as substrate. Human liver xanthine oxidase was purified according to the method of Kielley (7). Under the same conditions as before, a 1:20 dilution of this enzyme produced 0.2 \( \gamma \) of uric acid per ml. per minute.

Methods of Analysis

Uric acid production was measured at 300 m\( \mu \), although the extinction is only about 85 per cent of that at 290 m\( \mu \), where readings are usually taken. Xanthine at pH 8.0, in \( 10^{-4} \) M concentration, shows an optical density of 0.1 at 300 m\( \mu \) as against 0.42 at 290 m\( \mu \). An equimolar solution of uric acid possesses an extinction of 1.0 at 300 m\( \mu \) and of 1.20 at 290 m\( \mu \). Therefore, enzymatic conversion of xanthine into uric acid at 300 m\( \mu \) requires a correction factor of 1.1 only and is thus much more reliable.

In Table I we show the variation of optical density with pH. These figures have been used to calculate the amount of uric acid formed at pH 5.5 and 6.4. At the other pH values the optical density is constant.

2,8-Dioxypurine was determined at 320 m\( \mu \). As is evident from Fig. 1, it is possible to identify this compound unequivocally in the presence of 8-oxypurine and uric acid, which do not absorb at such a high wave length. It is more difficult to analyze a mixture of 2-oxo- and 2,8-dioxypurines because of the similarity of their absorption curves in the region between 280 and 340 m\( \mu \) (Fig. 1). In the experiments on the oxidation of 2-oxypurine readings were taken at 317 m\( \mu \), the isosbestic point of this substrate with 2,8-dioxypurine, where formation of uric acid lowers the optical density.

### Table I

<table>
<thead>
<tr>
<th>pH</th>
<th>Optical density at 290 m( \mu )</th>
<th>Optical density at 300 m( \mu )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>1.15</td>
<td>0.75</td>
</tr>
<tr>
<td>6.4</td>
<td>1.165</td>
<td>0.89</td>
</tr>
<tr>
<td>7.1</td>
<td>1.18</td>
<td>0.99</td>
</tr>
<tr>
<td>8.0</td>
<td>1.20</td>
<td>1.0</td>
</tr>
<tr>
<td>9.0</td>
<td>1.20</td>
<td>1.0</td>
</tr>
<tr>
<td>9.9</td>
<td>1.20</td>
<td>1.0</td>
</tr>
</tbody>
</table>
density considerably, and at 303 μ, where 2,8-dioxypurine and uric acid possess an isosbestic point; hence any increase in optical density measures their sum. Thus the amount of 2,8-dioxypurine and of uric acid, or of both, formed from 2-oxypurine can be determined quantitatively.

Oxidation of 1-methylxanthine was measured as uric acid, since the latter cannot be distinguished spectrophotometrically from its 1-methyl derivative at any pH. However, the nature of the oxidation product
density considerably, and at 303 μ, where 2,8-dioxypurine and uric acid possess an isosbestic point; hence any increase in optical density measures their sum. Thus the amount of 2,8-dioxypurine and of uric acid, or of both, formed from 2-oxypurine can be determined quantitatively.

Oxidation of 1-methylxanthine was measured as uric acid, since the latter cannot be distinguished spectrophotometrically from its 1-methyl derivative at any pH. However, the nature of the oxidation product
could be established unequivocally by paper chromatography (5) (see under "Results").

Purine oxidation was followed up by measuring through the whole spectral range between 270 and 320 μ.

Procedure—An appropriate dilution of the enzyme, 0.1 M phosphate buffer of pH 8.0 (i.e. near the pH optimum), and the substrate, 10⁻⁴ M, were mixed at room temperature (21°C) at zero time in a quartz cell, and the optical density of the mixture was read in a Beckman ultraviolet spectrophotometer, connected to a photomultiplier, at suitable intervals. For substrates with very low activity, the observation time had to be

![Absorption spectra of various purine derivatives at pH 8.0. Concentration used, 10⁻⁴ M. ○, 2-oxypurine; X, 8-oxypurine; △, 2,8-dioxypurine; □, 6,8-dioxypurine; ○, uric acid. The isosbestic points used in this paper are 317 μ, 2-oxypurine and 2,8-dioxypurine; 303 μ, 2,8-dioxypurine and uric acid; 295 μ, 8-oxypurine and 2,8-dioxypurine.](http://www.jbc.org/)

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extended over many hours. In order to avoid bacterial infection, a drop of chloroform was added after we had found that this substance does not affect enzymatic activity.

For the determination of pH-activity curves the following buffers were used: pH 5.5 to 6.6, 0.1 M acetate; pH 7.7 to 8.3, 0.1 M phosphate; pH 8.6 to 10.0, 0.025 M pyrophosphate.

In control experiments it was shown that the above concentration of acetate does not produce measurable inhibition.

![Graph showing the rate of oxidation of xanthine as a function of time for various dilutions of xanthine oxidase.](http://www.jbc.org/)

**Fig. 2.** Rate of oxidation of xanthine as function of time for various dilutions of xanthine oxidase. Substrate concentration, 10^{-4} M. The curves of this graph demonstrate that the rate is linear only for the first 10 minutes.

The formation of uric acid at various enzyme dilutions is shown in Fig. 2. It is apparent that after 10 minutes the rate decreases, probably because of enzyme inactivation by \( \text{H}_2\text{O}_2 \) (8). Therefore, all rates in this paper represent the values obtained by extrapolation to zero time.

**Results**

**Purine Derivatives As Substrates of Milk Xanthine Oxidase**

**Dioxypurines**—We have confirmed the results of Coombs (1) that xanthine and 6,8-dioxypurine react at about the same rate (Table II). This is true over the whole pH range studied (see below). However, 2,8-dioxypurine is oxidized at a much lower rate, indicating that a certain
mutual relationship must exist for the enzymatic attack at positions 2 and 8 in the purine nucleus. The reaction in the first two cases is linear, until about 40 per cent conversion, whereafter the rate decreases.

**Monooxypurines**—In this group, hypoxanthine exceeds the other two isomers by far (Table II). The figures of Table II show that the first oxidation step of hypoxanthine must be slower than the second one. Since 2,6- and 6,8-dioxypurines are converted into uric acid at the same rate, it remains open whether the intermediate is exclusively xanthine, as is generally assumed, or whether 6,8-dioxypurine may also be involved.

**Table II**

**Relative Rates of Oxidation of Purine Derivatives by Xanthine Oxidase**

Xanthine served as a reference compound. Substrate concentration $10^{-4}$ M. Temperature 21°, pH = 8.0. The column "Oxidative pathway" indicates the order in which the oxidizable positions were attacked by the enzyme.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product measured</th>
<th>Oxidative pathway</th>
<th>Relative rate with</th>
<th>Milk XO</th>
<th>Liver XO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine</td>
<td>Uric acid</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>1-Methylxanthine</td>
<td>1-Methyluric acid</td>
<td></td>
<td>0.45</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>6,8-Dioxypurine</td>
<td>Uric acid</td>
<td>$\rightarrow 2,6 \rightarrow 2,6,8$</td>
<td>0.7</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>Xanthine, uric acid (6,8-dioxypurine?)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purine</td>
<td>Uric acid</td>
<td>$\rightarrow 6 \rightarrow 2,6 \rightarrow 2,6,8$</td>
<td>0.2</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>2-Oxypurine</td>
<td>2,8 Dioxypurine</td>
<td>$\rightarrow 2,8 \rightarrow 2,6,8$</td>
<td>0.16</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>8-Oxypurine</td>
<td>&quot;</td>
<td>$\rightarrow 2,8 \rightarrow 2,6,8$</td>
<td>0.015</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>2,8-Dioxypurine</td>
<td>Uric acid</td>
<td>&quot;</td>
<td>0.002</td>
<td>0.0005</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

In order to decide this point, the experiment described in Table III was carried out at pH 5.5, at which the reaction rates are sufficiently low to permit detection of the intermediates spectrophotometrically. Readings were then taken at the following wave lengths (see Fig. 3): At 249 m\(\mu\), the isosbestic point of hypoxanthine and 6,8-dioxypurine, any decrease in optical density represents formation of xanthine or uric acid or both. At 262.5 m\(\mu\), the isosbestic point of hypoxanthine and xanthine, any increase in optical density is due to 6,8-dioxypurine and any decrease to uric acid. At 270 m\(\mu\), the isosbestic point of uric acid and hypoxanthine on the one hand and of xanthine and 6,8-dioxypurine on the other, any increase in optical density represents the sum of the latter two derivatives. At 300 m\(\mu\) mainly uric acid is measured, since 6,8-dioxypurine makes only a minor contribution.
Inspection of Table III shows the following: (a) Hypoxanthine is continuously being used up. (b) A very slight initial rise appears at 262.5 m\(\mu\), while formation of uric acid is still negligible. This may be taken as an indication that some 6,8-dioxypurine appears during the oxidation of hypoxanthine. However, the effect is too small to give conclusive evidence about this point. (c) The readings at 270 m\(\mu\) show that, between the 2nd and 10th minute, xanthine concentration reaches a steady state in which formation and degradation of this intermediate are balanced. (d) Uric acid formation lags behind for the first few minutes and becomes appreciable after about 4 minutes. These observations confirm that the oxidation of hypoxanthine passes almost exclusively through xanthine as intermediate.

Oxidation of 8-oxypurine may give either 2,8- or 6,8-dioxypurine, either of which is converted into uric acid. Booth (2) concluded from kinetic measurements that the 2,8 isomer must be the intermediate. The spectrophotometric method permits us to establish this point beyond doubt. At 320 m\(\mu\), 8-oxypurine, 6,8-dioxypurine, and uric acid do not show absorption. Therefore, any increase in optical density at this wave length measures exclusively 2,8-dioxypurine. Simultaneously, at 295 m\(\mu\) (the isosbestic point of 8-oxo- and 2,8-dioxypurines) the formation of uric acid can be followed (6,8-dioxypurine showing only weak absorption at this wave length) (see Fig. 1). Only very small amounts of the latter are formed during the usual observation time (1 hour), the rate for conversion of 8-oxypurine into uric acid being only one-fifth of that for the

### Table III

**Intermediates in Oxidation of Hypoxanthine to Uric Acid**

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>249 m(\mu)</th>
<th>262.5 m(\mu)</th>
<th>270 m(\mu)</th>
<th>300 m(\mu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.55</td>
<td>0.49</td>
<td>0.36</td>
<td>0.055</td>
</tr>
<tr>
<td>1.3</td>
<td>0.51</td>
<td>0.50</td>
<td>0.38</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>0.48</td>
<td>0.50</td>
<td>0.42</td>
<td>0.075</td>
</tr>
<tr>
<td>3</td>
<td>0.45</td>
<td>0.49</td>
<td>0.425</td>
<td>0.09</td>
</tr>
<tr>
<td>4</td>
<td>0.43</td>
<td>0.48</td>
<td>0.43</td>
<td>0.16</td>
</tr>
<tr>
<td>6</td>
<td>0.40</td>
<td>0.43</td>
<td>0.43</td>
<td>0.22</td>
</tr>
<tr>
<td>10</td>
<td>0.37</td>
<td>0.37</td>
<td>0.41</td>
<td>0.32</td>
</tr>
<tr>
<td>15</td>
<td>0.36</td>
<td>0.32</td>
<td>0.38</td>
<td>0.39</td>
</tr>
<tr>
<td>20</td>
<td>0.36</td>
<td>0.30</td>
<td>0.36</td>
<td>0.40</td>
</tr>
<tr>
<td>25</td>
<td>0.36</td>
<td>0.30</td>
<td>0.36</td>
<td>0.40</td>
</tr>
</tbody>
</table>
reaction 8-oxypurine $\rightarrow$ 2,8-dioxypurine. On the other hand, the rate for the oxidation of 6,8-dioxypurine is about 500 times faster than that of the 2,8 isomer; hence measurable quantities of uric acid should appear in the early part of the reaction, if the alternative pathway 8-oxypurine $\rightarrow$

![Absorption spectra of various purine derivatives at pH 5.5. Concentration used, $10^{-4}$ M. O, hypoxanthine; O, xanthine; X, 6,8-dioxypurine; ●, uric acid. The isosbestic points used in this paper are 249 m$m_\mu$, hypoxanthine and 6,8-dioxypurine; 262.5 m$m_\mu$, hypoxanthine and xanthine; 270 m$m_\mu$, hypoxanthine and uric acid; xanthine and 6,8-dioxypurine.](image)

6,8-dioxypurine were quantitatively important. The *main* route of oxidation is thus the one indicated by Booth.

8-Oxypurine $\rightarrow$ 2,8-dioxypurine $\rightarrow$ uric acid

The rate of oxidation of 2-oxypurine into 2,8-dioxypurine is 30 times faster than conversion of the latter into uric acid (see Table II). At 317 m$m_\mu$, no change in optical density was observed during the first ½ hour, indicating that the sum of 2-oxo- plus 2,8-dioxypurine remained constant. Therefore, the intermediate accumulates, without any measurable quantities of uric acid, during the first ½ hour, and thus permits unequivocal analysis of the enzymatic pathway as follows:

2-Oxypurine $\rightarrow$ 2,8-dioxypurine $\rightarrow$ uric acid

The alternative route via 2,6-dioxypurine is definitely excluded. The
experiments on monooxypurines again demonstrate the mutual relationship
between carbons 2 and 8 in the purine skeleton. Previous oxidation at
either position directs further oxidative attack to the other partner of
this pair, excluding carbon 6.

**Purine**—This substrate is converted into uric acid at about one-third
the speed of hypoxanthine (Table II). If 2- or 8-oxypurine would partici-
pate in the first oxidation step, 2,8-dioxypurine should accumulate (as
shown under "Monooxypurines"). No trace of a substance absorbing

![Optical density vs. wavelength](image)

**Fig. 4.** Enzymatic oxidation of purine into uric acid. ○, initial absorption spec-
trum of purine; ●, absorption spectrum after enzymatic digestion for 45 minutes.
Substrate concentration, $1.75 \times 10^{-4}$ M; pH 8.0; enzyme dilution 1:20. Each curve
represents the spectrum measured against enzyme and buffer, without substrate, as
blank.

at 320 m\(\mu\) was, however, found. In Fig. 4, an experiment is shown in
which a concentrated enzyme solution (1:20) produced about 95 per cent
conversion during 45 minutes. The final spectrum of the reaction mixture,
obtained after subtracting the “blank” value of the control, containing
enzyme without substrate, indicates pure uric acid. These observations
establish the oxidative pathway as follows:

\[
\text{Purine} \rightarrow \text{hypoxanthine} \rightarrow \text{xanthine} \rightarrow \text{uric acid}
\]

**Methylated Purine Derivatives**—Like 7-methylhypoxanthine (2) the
1-methyl derivative also was found to be inactive.

The successful synthesis of 1-methylxanthine (5) enabled us to test
this substrate. It is oxidized by milk xanthine oxidase at one-half the
rate of the mother substance. Since uric acid and its 1-methyl derivative cannot be distinguished spectrophotometrically, identification of the oxidation product as 1-methyluric acid was accomplished by paper chromatography (5). A mixture of 1-methylxanthine (0.1 ml. = 100 γ), phosphate buffer (0.1 ml.), undiluted X0 (0.1 ml.), and a drop of chloroform was incubated at 34° for 24 hours and 0.06 ml. (20 γ) was spotted on paper, together with uric acid, 1-methyluric acid, and 1-methylxanthine for comparison. The descending chromatogram was developed with a mixture of ethanol-water-acetic acid (85:10:5 volumes). The result, given in Fig. 5, identifies the reaction product unequivocally.

None of the other mono-, di-, and trimethylxanthines was attacked by purified xanthine oxidase, as observed already by previous investigators (1, 9).

**Substrate Specificity of Human Liver Xanthine Oxidase**

In view of the interest in the human metabolism of substituted xanthines, it appeared necessary to establish the specificity of the xanthine oxidase of human organs. As a representative example, the liver enzyme, which has been studied previously by Richert and Westerfeld (10), was purified
and tested. The results were in all respects analogous to those obtained with the enzyme from cow's milk (see Table II).

\[ \text{Amount formed} \]

\[ (\mu g/ml) \]

\[ \begin{array}{c|c|c|c}
\text{Amount formed} \\
(\mu g/ml) \\
\hline
\text{pH} & 5 & 6 & 7 & 8 & 9 & 10 & 11 \\
\hline
\text{Substrate} & \text{xanthine} & \text{2-oxypurine} & \text{purine} & \text{2-oxypurine} & \text{2-oxypurine} & \text{purine} \\
\text{pK} & 8.9 & 8.5 & 8.0 & 7.7 & 7.7 & 7.7 \\
\text{Ionization} & 20 & 40 & 66 & 80 & 80 & 80 \\
\text{per cent} & 20 & 40 & 66 & 80 & 80 & 80 \\
\text{Reference} & \text{Albert and Brown (12)} & \text{“ “ “ “ (12)} & \text{Present authors, unpublished} & \text{“ “ “ (13)} & \text{“ “ “ (13)} \\
\end{array} \]

Because of the low activity of our purified preparation, rate figures less than 0.1 are less reliable than those obtained with the milk enzyme. It is of interest that 1-methylxanthine is oxidized as fast as xanthine itself.

 influence of pH changes on activity of milk xanthine oxidase

Since the most active substrates of XO differ in their pK values, the possibility has to be considered that their different rates might result,
The present experiments establish the main oxidative pathways in the purine series unequivocally and demonstrate that the rate and direction of oxidative attack depend on the position of oxygen introduced previously. The first attack in purine itself is at carbon 6. With hypoxanthine, the next step is directed towards carbon 2. But positions 2 and 8 form a closely related pair; hence oxidation at one of these carbons leads always to attack at the other partner in the next step.

All purine derivatives, tested in this investigation, can be divided into three groups with respect to their activity against mammalian xanthine oxidase: (1) Substrates with a rate of oxidation comparable to xanthine are 1-methylxanthine, 6,8-dioxypurine, hypoxanthine, and purine. (2) Substrates which are attacked at about one-tenth to one-ten thousandth the rate of xanthine are 2- and 8-oxypurines and 2,8-dioxypurine. (3) Purine derivatives which are not attacked at a measurable rate are l- and 'I-methylhypoxanthines, 3-, 7-, and 9-methylxanthines, and xanthosine, all dimethylated xanthines, and caffeine.

Our results enable us to draw certain conclusions about the way in which a substrate attaches itself to the active center and about the mechanism of dehydrogenation by xanthine oxidase. We shall start this discussion by comparing purine oxidation with the conversion of aldehydes into acids, a reaction brought about by a very similar enzyme. Here the following scheme applies:

\[
\begin{align*}
H \\
RC=O & \xrightarrow{+H_2O} H \quad OH \\
\end{align*}
\]

In accordance, we may formulate the conversion of purine into hypoxan-
URIC ACID AND RELATED COMPOUNDS. III

Diagram 1. Formation of lactim of hypoxanthine

Diagram 2. Formation of 8-lactim of uric acid
take place. This reaction is prevented, when either N-1 or N-7 is methylated. Thus we may conclude that the enzyme attaches itself not only to position 2,3 in hypoxanthine, but simultaneously to N-1 and N-7. A multiple point attachment of xanthine oxidase to its substrates is indicated.

On the other hand, oxidation of xanthine is prevented by substitution at N-3, N-7, or N-9, but proceeds normally with the N-1-methyl derivative. We conclude that the mode of attachment changes from hypoxanthine, in which N-1, N-3, and N-7 are involved, to xanthine, in which N-3, N-7, and N-9 must be free for combination with the enzyme. This change

![Diagram 3. Formation of 2-lactim of uric acid](http://www.jbc.org/)

makes it probable that dehydrogenation of xanthine proceeds as indicated in Diagram 2. According to the scheme, the decisive step is dehydrogenation of the grouping $\text{HN}(3)-\text{C}(4)==\text{C}(5)-\text{N}(7)\text{H}$ to the corresponding dienic system, which is analogous to the reactive portion in the molecule of riboflavin in the prosthetic group of xanthine oxidase. It becomes apparent at once that oxidation of 6,8-dioxypurine is in complete harmony with this view, the accompanying steps (Diagram 3) being involved. In the same way, the mutual relationship between 2- and 8-oxypurines, which are both converted into 2,8-dioxypurine, is easily explained. It should be noted that a similar mechanism may also apply to the oxidation of hypoxanthine or purine; e.g., the hydrated form of hypoxanthine may undergo dehydrogenation at N-3 and N-7 to give the intermediate (Diagram 4),
URIC ACID AND RELATED COMPOUNDS. III

Diagram 4. Formation of 2-lactim of xanthine which through hydrogen shift is converted into xanthine. This scheme explains in a natural way why inosine, a 9-substituted hypoxanthine, is resistant to enzymatic attack (14). The substituent in position 9 prevents formation of the above intermediate.

Diagram 5. Formation of lactim of hypoxanthine
In an analogous way, purine may add water at the ends of the conjugated system C(6)=N(1)—C(2)=N(3) to give the intermediate (Diagram 5), which again can undergo dehydrogenation in the same fashion as before. This scheme appears preferable, since it explains in a natural way the significance of attachment of purine and hypoxanthine at positions 1, 3, and 7, and brings all reactions, catalyzed by xanthine oxidase, in line with the transformation of riboflavin into its dihydro derivative. The only exception is the oxidation of 2,8-dioxypurine into uric acid. In this case, it is not possible to formulate the reaction as involving the central group N(3)—C(4)=C(5)—N(7). This indeed may be the reason for the extraordinarily low reaction rate of this purine derivative.

The above considerations lead to the hypothesis that two different modes of combination of XO with purine derivatives are possible. Since intermediate oxidation products can accumulate (see e.g. Table III), the enzyme-substrate complex apparently is freely dissociable. We assume that in hypoxanthine (and perhaps also in purine) attachment takes place at N-1, N-3, and N-7, while in xanthine N-3, N-7, and N-9 are involved in the formation of the enzyme-substrate complex. Participation of N-3 and N-7 is thus common to both complexes and probably indicates combination of the substrates with N-1 and N-10 in the isalloxazin portion of XO. The third participant of the purine nucleus may either attach itself to N-3 in the flavin nucleus or may enter the coordination sphere of the molybdenum atom, which is present in mammalian XO (15, 16, 6).

It should be pointed out that the reaction schemes, presented in this paper, involve two consecutive steps: (a) hydration and (b) dehydrogenation, the former determining the carbon atom to be oxidized. The question arises whether these steps can be separated experimentally.

The hypothesis, developed here, permits certain predictions about the activity of methylated derivatives of purine, 2,8-, and 6,8-dioxypurines towards xanthine oxidase. Experiments on the behavior of these substances towards the enzyme are now being carried out in order to test the validity of the above assumptions and to obtain a clearer picture of the enzymatic mechanism.

**SUMMARY**

1. The spectrophotometric method of Kalckar has been extended to a variety of purine derivatives to determine the pathway of their enzymatic oxidation.

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1 It should be noted that the results with methylated hypoxanthises cannot be explained by the alternative combination at N-1, N-7, and N-3, since this would require reaction at a position which is not involved in binding, viz. N-3. However, for 2,8-dioxypurine such a mode of attachment is not excluded by the present experiments.
2. A peculiar relationship between position 2 and 8 in the purine ring has been found. Oxidation at either position directs further attack to the other partner of this pair.

3. Xanthine oxidases from cow's milk or human liver show identical substrate specificity. Among all substituted xanthines only the 1-methyl derivative is attacked by the enzyme and converted into 1-methyluric acid.

4. A hypothesis is advanced for multiple point attachment of purines to xanthine oxidase and for the mechanism of dehydrogenation.

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