The Preparation, Properties, and Inhibition of Hypoxanthine Dehydrogenase of Avian Kidney

ERWIN J. LANDON* AND CHARLES E. CARTER

From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

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Renal enzymic mechanisms associated with the process of uric acid excretion in avian species have not been characterized. Early work on uric acid synthesis in pigeons established the kidney as the site of the terminal conversion of hypoxanthine to uric acid (1). A detailed study of this reaction in pigeon and chicken kidney here reported shows that uric acid synthesis in these tissues is catalyzed by a diphosphopyridine nucleotide dependent hypoxanthine dehydrogenase.

Chicken liver contains a flavoprotein enzyme which was purified by Remy et al. (2) and characterized as a hypoxanthine dehydrogenase on the basis of its catalyzing uric acid synthesis from hypoxanthine in air at only 1% of the rate found in the presence of methylene blue. In a crude chicken liver system Moroll (3) found uric acid synthesis associated with diphosphopyridine nucleotide reduction. A similar finding in a partially purified chicken liver preparation was reported by Felig and Wiley (4). Pigeon liver, however, does not synthesize uric acid (1). Avian species apparently utilize dehydrogenase mechanisms for uric acid production and in the case of kidney tissue where the reaction may have unique functional importance in terms of tubular secretory processes, the diphosphopyridine nucleotide dependence of the system is readily demonstrated. The purified chicken kidney hypoxanthine dehydrogenase reported herein differs in several respects from mammalian xanthine oxidase and from the chicken liver enzyme. Mahler (5) and De Renzo (6) have discussed the mechanism of action and chemical characterization of mammalian xanthine oxidase.

**EXPERIMENTAL**

Materials and Methods—DPN and TPN were purchased from the Pabst Laboratories; DEAE-cellulose from Eastman Organic Chemicals; DPNH, cytochrome c and p-chloromercuribenzoate from Sigma Chemical Company, sodium pyruvate from C. F. Boehringer Company, and hypoxanthine from the Nutritional Biochemicals Corporation.

Protein was determined by the Sutherland modification (7) of the Folin-Ciocalteau method. Bovine albumin was employed as a protein standard. Iron was determined by the method of Kitzes et al. (8) and molybdenum by the method of Ellis and Olson (9). Flavin adenine dinucleotide was measured fluorometrically by the method of Bessey et al. (10). Microbiological assays for enzyme riboflavin employed the procedure of Strong (11). Fluorometric measurements were made on a Farrand Fluorometer model A or an Amino Bowman Spectrophotofluorometer. Lactic dehydrogenase was assayed spectrophotometrically at 340 mμ (12). Calcium phosphate gel was prepared by the method described by Wood (13).

Purification of Enzyme—Hypoxanthine dehydrogenase activity is greater in pigeon kidney than in chicken kidney, but because the latter tissue is more readily available it is a preferable starting material. The fractionation of pigeon and chicken kidney and the properties of the purified enzyme show no significant differences. Extracts of normal mammalian kidneys contain no detectable hypoxanthine dehydrogenase.

Chicken backs were obtained from the local meat market. If well refrigerated, the tissue yielded active enzyme preparations within 24 hours of slaughtering. The kidneys were dissected from surrounding structures and 500 g of tissue were disrupted in several batches in a glass homogenizer in 1 volume of 0.17 M potassium phosphate buffer, pH 7.4, at 4°. All subsequent operations were conducted at 4° unless otherwise specified. The homogenate was centrifuged at 105,000 X g for 90 minutes at 0° in a Spinco preparative ultracentrifuge. The supernatant fraction was placed in a constant temperature water bath at 56° and stirred constantly for 12 minutes, or until the temperature of the solution reached 56°. The preparation was then quickly chilled in an ice bath and centrifuged for 15 minutes at 15,000 X g at 0°. Acetone, 80 ml, cooled to -10°, was slowly added with constant stirring to 160 ml of supernatant solution at 0°. The temperature of the mixture was kept at 0° to -5° in a Dry-Ice-ethanol bath. The precipitate was removed by centrifugation at 15,000 X g for 15 minutes at 0° and discarded. The supernatant solution was adjusted to 5° and, while maintaining this temperature, another 23 ml of cold acetone were added. The precipitate of the 30 to 40% acetone fraction was collected by centrifugation at 15,000 X g at 0° for 15 minutes and dissolved in 65 ml of 0.05 M potassium phosphate buffer, pH 7.4, which contained 0.001 M sodium ethylenediaminetetraacetate. This fraction was dialysed for 6 hours in 0.005 M PO₄ buffer, pH 7.4, which contained 0.001 M sodium ethylenediaminetetraacetate. The dialyzed fraction contained cytochrome and about 100 times more lactic dehydrogenase than hypoxanthine dehydrogenase.

Several additions (usually 4) of aliquots of 10 ml of calcium phosphate gel suspension containing 30 mg of gel per ml were made to the dialyzed fraction. After each addition the mixture was stirred for 5 minutes and the gel centrifuged. The supernatant solution was assayed for enzyme activity, and the addi-
tion of gel was repeated until the enzyme was adsorbed. The gel fractions were successively eluted with 10 ml each of 0.04 M, 0.1 M, 0.2 M, and 1 M potassium phosphate, pH 7.4. Eluates were assayed and the high specific activity fractions combined and again dialyzed as described above. Some variability in desorption of enzyme from gel was encountered but usually the 0.1 M and 0.2 M fractions contained the highest specific activity. There was about 60% loss of total enzyme in this fractionation step, but much of the supernatant cytochrome and about 95% of the contaminating lactate dehydrogenase was removed. The enzyme was labile after desorption from gel, and it was necessary to accomplish the subsequent step without delay. Solid ammonium sulfate was added to the gel eluate fraction and, by addition of dilute NH₄OH, pH (determined with pH 6.0 to 8.0 indicator paper), was maintained at approximately 7.4. The fraction precipitating between 32 and 58% saturation with ammonium sulfate contained the hypoxanthine dehydrogenase activity. The precipitate was dissolved in 40 ml of 0.05 M phosphate buffer, pH 7.4, which contained sodium ethylenediaminetetraacetate 0.001 M and was dialyzed for 6 hours against 4 liters of 0.005 M buffer, 0.001 M sodium ethylenediaminetetraacetate. The dialyzed fraction was then adsorbed on a DEAE-cellulose column of 2 cm diameter and 40 ml volume which had been previously treated by washing with 0.005 M phosphate buffer, pH 7.4, containing 0.001 M sodium ethylenediaminetetraacetate. Gradient elution from the column was employed with 800 ml of distilled water in the mixing chamber and a solution of 0.2 M potassium phosphate, pH 8.0, in the reservoir. The enzyme was sufficiently stable on the column so that this operation could be conducted at room temperature. Chromatographic separation achieved apparent resolution of cytochrome from hypoxanthine dehydrogenase and further separation of residual lactate dehydrogenase. The resolution of these components on a DEAE-cellulose column is illustrated in Fig. 1. By increasing the buffer gradient to 1 M immediately after the hypoxanthine dehydrogenase enzyme peak (approximately 7 column volumes), the remaining enzyme was concentrated about 10-fold without increasing the elution rate of residual lactate dehydrogenase. This resulted in a fraction with lactate dehydrogenase specific activity of less than 3 and hypoxanthine dehydrogenase specific activity of approximately 150.

Table I summarizes representative data on the yield and purification of hypoxanthine dehydrogenase through several fractionation procedures.

In the absence of sodium ethylenediaminetetraacetate, preparations stored at -15° lost all activity within 2 weeks. By storing preparations in 5 X 10⁻³ M sodium ethylenediaminetetraacetate and employing this compound in the course of purification, it was possible to maintain full activity for 1 to 2 weeks and between 30 and 60% of the original activity for 1 month.

**Enzyme Assay**—One unit of hypoxanthine dehydrogenase is defined as equivalent to the production of 0.01 µmole of DPNH per minute under the following standard assay conditions: 0.75 µmole of hypoxanthine, 3 µmoles of DPN, and enzyme in a total volume of 3.3 ml of 0.01 M phosphate buffer, pH 7.4. Incubations were conducted in silica cuvettes at room temperature and increment absorption at 290 mp and 340 mp followed in the ultraviolet spectrophotometer for determination of the amount of uric acid and DPNH formed in the reaction (14).

Lactate dehydrogenase activity was assayed with 0.5 µmole of sodium pyruvate, 0.5 µmole of DPNH, and enzyme in a total volume of 3.3 ml of 0.01 M phosphate buffer, pH 7.4. For comparative purposes, the units of lactate dehydrogenase activity were those employed for hypoxanthine dehydrogenase.

**Properties of Hypoxanthine Dehydrogenase**—The following reactions are catalyzed by hypoxanthine dehydrogenase: Hypoxanthine + 2 DPN → uric acid + 2 DPNH; and xanthine + DPN → uric acid + DPNH. The optimal rate of reaction was found in phosphate and Tris (hydroxymethyl)aminomethane buffers at pH 8.0. The reactions were not reversible and the dismutation of xanthine to hypoxanthine and uric acid reported by Green (15) to occur with milk xanthine oxidase was not found.

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**Table I**

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Total units</th>
<th>Specific activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>23,400</td>
<td>2.4</td>
</tr>
<tr>
<td>Supernatant</td>
<td>20,400</td>
<td>3.5</td>
</tr>
<tr>
<td>Heat step</td>
<td>14,820</td>
<td>11.6</td>
</tr>
<tr>
<td>Acetone</td>
<td>15,100</td>
<td>50</td>
</tr>
<tr>
<td>Gel and ammonium sulfate</td>
<td>2,200</td>
<td>100-200</td>
</tr>
<tr>
<td>Ammonium sulfate 35 to 55% (gel step omitted)</td>
<td>12,500</td>
<td>30</td>
</tr>
<tr>
<td>DEAE-column (gel step omitted)</td>
<td>10,300</td>
<td>49-80</td>
</tr>
</tbody>
</table>

**Fig. 1. Partial resolution of hypoxanthine dehydrogenase activity from other components on a DEAE-cellulose column employing a dialyzed preparation after gel desorption and ammonium sulfate fractionation.**
with the kidney enzyme. The enzyme had no diaphorase (DPNH oxidase) activity and DPNH was not oxidized in the presence of cytochrome c. In the absence of DPN the rate of uric acid synthesis from hypoxanthine was less than 1% that found in the presence of the coenzyme. Oxidase activity for xanthine was approximately 5% that of the DPN reaction when purified preparations of enzymes were employed, but was undetectable in crude extracts. TPN did not substitute for DPN in the reaction, but methylene blue and hydroxylamine served as hydrogen acceptors. Milk xanthine oxidase is strongly inhibited by 5 X 10⁻³ M hydroxylamine (16); at this concentration hydroxylamine replaced DPN in the chicken kidney hypoxanthine dehydrogenase system. The kidney enzyme was incapable of catalysing uric acid synthesis if DPN was replaced by cytochrome c, ferricyanide, and 2,6-dichlorophenolindophenol. Acetaldehyde was a substrate in the DPN dehydrogenase system but the reaction took place at a very slow rate. The apparent Michaelis constants calculated by the method of Lineweaver and Burk (17) for substrates in the hypoxanthine dehydrogenase reaction recorded in Table II were similar to comparable data reported by by Mackler et al. (18) for milk xanthine oxidase. The turnover of milk xanthine oxidase calculated by Horecker and Heppel (19) for hypoxanthine in the presence of oxygen was 50 mmoles per minute per mmole of flavin. A comparable calculation for chicken kidney hypoxanthine dehydrogenase in the presence of DPN gave a turnover of 270 mmoles per minute per mmole of flavin.

Inhibitors—As in the case of milk xanthine oxidase, compounds which react with sulfhydryl groups inhibit the dehydrogenase enzyme. One unit of enzyme was completely inhibited by 3.5 X 10⁻² M p-chloromercuribenzoate and over 50% inhibited by 5 X 10⁻⁴ M arsenite. Prior addition of 10⁻³ M cysteine protected the enzyme from both inhibitors. Arsenite inhibition was not reversed by subsequent addition of 10⁻⁴ M cysteine, but this concentration of cysteine restored activity to the mercurial-inhibited enzyme. Cyanide at a concentration of 10⁻⁴ M progressively inhibited hypoxanthine dehydrogenase during the course of incubation resulting in over 50% decrease in the rate of reaction after 30 minutes. Preincubation of enzyme with hypoxanthine had no effect on the action of these inhibitors.

Adenine, 6-mercaptopurine, and 6-selenopurine reacted slowly with hypoxanthine dehydrogenase and DPN and at concentrations of 3 X 10⁻⁴ M produced approximately 50% noncompetitive inhibition of uric acid synthesis from hypoxanthine. Chlorothiazide, a potent diuretic agent, competitively inhibited hypoxanthine dehydrogenase as shown in Fig. 2. Milk xanthine oxidase was also inhibited by chlorothiazide under comparable conditions, and the extent of inhibition was similarly related to hypoxanthine concentration. Two other diuretic agents, theophylline and acetazolamide, produced no inhibition of uric acid synthesis by these enzymes.

**FAD Content and Enzyme Spectra**—The flavoprotein character of chicken kidney hypoxanthine dehydrogenase is based on the fluorometric assay of FAD which shows a relatively constant ratio of FAD to enzyme specific activity throughout several steps of purification in different preparations (Table II). Fluorometric emission spectra of the native enzyme in the spectrophotofluorometer did not show the maximum at 520 mλ characteristic of riboflavin. However, this maximum was observed after deproteinizing the enzyme with 20% trichloroacetic acid and neutralizing the supernatant solution. The riboflavin content of.

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**TABLE II**

**FAD and enzyme activity**

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Mg FAD-riboflavin/units of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate</td>
<td>0.9/100,000</td>
</tr>
<tr>
<td>DEAE-column combined fractions</td>
<td>1.48/100,000</td>
</tr>
<tr>
<td>DEAE-column single fractions from the enzyme peak</td>
<td>1.35-1.47/100,000</td>
</tr>
<tr>
<td>Combined fractions from DEAE-column; gel step omitted</td>
<td>1.02/100,000</td>
</tr>
<tr>
<td>DEAE-column after acetone step; single fractions from the enzyme peak</td>
<td>1.3-1.4/100,000</td>
</tr>
</tbody>
</table>

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*FIG. 2. The effect of increasing concentration of hypoxanthine on chlorothiazide inhibition of hypoxanthine dehydrogenase. In the absence of chlorothiazide the enzyme showed maximal activity at all three concentrations of hypoxanthine: O—O, 3 X 10⁻⁴ M chlorothiazide + 3 X 10⁻⁴ M hypoxanthine; Δ···Δ, 3 X 10⁻⁴ M chlorothiazide + 6 X 10⁻⁴ M hypoxanthine; X—X, 3 X 10⁻⁴ M chlorothiazide + 12 X 10⁻⁴ M hypoxanthine, ○—○, hypoxanthine control.*
column fractionation data (Fig. 1) were apparently free of contaminating supernatant cytochromes. However, the cytochrome which occurs abundantly in the chicken kidney supernatant fraction exhibited a prominent $\gamma$ absorption maximum at 405 m$\mu$ and small $\alpha$ and $\beta$ peaks at 570 and 520 m$\mu$. Heat treatment of this cytochrome, followed by solution of the denatured protein in dilute alkali, also resulted in the appearance of a 410 m$\mu$ absorption band. It is possible that the iron protein of hypoxanthine dehydrogenase and the cytochrome fractions show similar spectral characteristics or that an extraneous protein fraction contaminates the enzyme. The absorption spectra of chicken liver xanthine dehydrogenase reported by Remy et al. (2) closely resembles that shown in Fig. 3.

The content of molybdenum and iron was determined afterashing the purified enzyme. The ratio of FAD-molybdenum-

**DISCUSSION**

The excretion of uric acid in avian species (29) entails enzymic dehydrogenation linked to DPN reduction in the terminal conversion of hypoxanthine to uric acid in kidney tissue. The relationship of this enzyme reaction to the renal tubular secretory process for uric acid is not known. In extracts of renal tissue from normal pig, dog, and man hypoxanthine dehydrogenase activity and uric acid formation was not found. The comparative biochemical significance of the dehydrogenase activity in bird kidney may be concerned with the relative economy of the anaerobic mechanism for synthesis or tubular secretion of the large amounts of uric acid excreted by birds. Studies with purified chicken kidney enzymes showed that the rate of the hypoxanthine dehydrogenase reaction was accelerated by the removal of pyruvate and regeneration of DPN during simultaneous incubation with lactic dehydrogenase. The high concentration of iron was 1:1:8. Similar analytical values have been reported for chicken liver xanthine dehydrogenase (2).

**Effect of Associated Lactic Dehydrogenase**—In chicken kidney extracts lactic dehydrogenase activity was associated with hypoxanthine dehydrogenase activity through several stages of purification. The enzyme was resolved by gel adsorption and DEAE-column chromatography. The effect of adding lactic dehydrogenase and pyruvate to the column purified hypoxanthine dehydrogenase is shown in Fig. 4 and B. In the presence of lactic dehydrogenase and $5 \times 10^{-4}$ m pyruvate the pyridine nucleotide was kept in the oxidized state and hypoxanthine dehydrogenase activity was sustained with $2 \times 10^{-4}$ m DPN. In the absence of pyruvate maximal sustained activity was found with $10^{-3}$ m DPN. A 63% increase over this maximal rate was found upon addition of pyruvate and lactic dehydrogenase when $10^{-4}$ m DPN was employed. The increased rate of uric acid synthesis began after an initial 5-minute lag period which was not abolished by preincubation with DPN, hypoxanthine, or DPNH. Five minute preincubation with both hypoxanthine and DPN before adding lactic dehydrogenase and pyruvate abolished the lag.

DPNH inhibits hypoxanthine dehydrogenase and continuous removal of the reduced coenzyme might account for some of the increase in reaction rate produced by lactic dehydrogenase. DPNH, $1.5 \times 10^{-4}$ m, inhibited the enzyme about 5% and DPNH, $5 \times 10^{-4}$ m, about 20%. The inhibition was not reversed by 20-fold excess levels of DPN. After 5 minutes of incubation, in the absence of pyruvate and lactic dehydrogenase, the DPNH level was about $4 \times 10^{-5}$ m. The removal of this amount of free DPNH from the medium would not account for the 63% increase in rate of uric acid formation found when pyruvate and lactic dehydrogenase were added. An alternative explanation is that the effect of lactic dehydrogenase and pyruvate is upon the DPNH bound to hypoxanthine dehydrogenase. The increase in reaction rate would then be due to the regeneration of oxidized pyridine nucleotide on the enzymatic site. Cori et al. (20) and Nygaard and Rutter (21) have demonstrated instances of isolated enzyme bound pyridine nucleotides reacting with other enzyme systems.

The optimal pH of the hypoxanthine dehydrogenase reaction was lowered in the presence of pyruvate and lactic dehydrogenase as shown in Fig. 5 and this new optimum was identical with that for pyruvate reduction.

**TABLE III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acceptor</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine</td>
<td>DPN 0.001 m</td>
<td>$2.37 \times 10^{-3}$ M</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>DPN 0.001 m</td>
<td>$6.6 \times 10^{-3}$ M</td>
</tr>
<tr>
<td>Hypoxanthine $2.2 \times 10^{-4}$ m</td>
<td>DPN</td>
<td>$5.5 \times 10^{-3}$ M</td>
</tr>
<tr>
<td>Hypoxanthine $2.2 \times 10^{-4}$ m</td>
<td>Hydroxylamine</td>
<td>$2.1 \times 10^{-3}$ M</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>DPN 0.001 m</td>
<td>$6.8 \times 10^{-3}$ M</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>Methylene blue</td>
<td>$3.6 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>Xanthine</td>
<td>$O_2$</td>
<td>$5 \times 10^{-2}$ M</td>
</tr>
</tbody>
</table>

* $K_m$ is for italicized compound.
these enzymes in bird kidney suggests that a similar relationship might operate to promote uric acid excretion.

Milk xanthine oxidase and the hypoxanthine dehydrogenase enzymes prepared from avian tissues show absorption in the region of 405 μm. This absorption band is prominent in the spectra of the chicken kidney enzyme where it is partially converted to the spectra of the reduced forms on incubation with substrates. The heat denatured proteins of the mammalian and avian enzymes from which the flavin moiety has been removed, have an absorption maximum at 410 μm in alkaline solution (Fig. 3). Treatment of the purified cytochrome component of the chicken kidney supernatant fraction in the same manner gave a solution which also exhibited an absorption maximum at 410 μm. The latter finding indicates that the 410 μm absorption is associated with the iron protein component of the enzymes.

Mammalian and avian enzymes which convert hypoxanthine to uric acid contain iron, FAD, and molybdenum. The ratio of these components in milk xanthine oxidase is 8:2:1, in mammalian liver xanthine oxidase 4:1:1, and in avian dehydrogenase enzymes, purified from liver or kidney tissue, 8:1:1. All of these preparations are inhibited by cyanide and compounds which react with sulfhydryl groups. The principal difference between the chicken kidney hypoxanthine dehydrogenase and the other enzymes is in respect to the mechanism of hydrogen transfer. Kidney hypoxanthine dehydrogenase utilized hydroxylamine as a hydrogen acceptor, whereas the other enzymes were inhibited by this compound; DPN was the naturally occurring hydrogen acceptor and TPN was inactive. Furthermore, chicken kidney hypoxanthine dehydrogenase showed no DPN oxidase activity in the presence or absence of cytochrome c in contrast to the other preparations. The structural basis for the differences in behavior of the several hypoxanthine oxidizing enzymes is not known, but investigation of mechanism of action of these enzymes should be aided by systematic comparison of the basic features of the hydrogen transfer reactions.

**SUMMARY**

The enzymic synthesis of uric acid from hypoxanthine in chicken kidney is accomplished through a diphosphopyridine nu-
cleotide (DPN)-linked dehydrogenation. The enzyme has been purified several hundred-fold. Like other uric acid-synthesizing enzymes, chicken kidney hypoxanthine dehydrogenase contains flavin adenine dinucleotide, molybdenum, and iron, and is inhibited by cyanide and compounds which react with sulfhydryl groups. Hydroxylamine inhibits mammalian hypoxanthine oxidases and chicken liver hypoxanthine dehydrogenase. The chicken kidney enzyme has the ability to utilize hydroxylamine as a hydrogen acceptor in place of DPN. Chicken kidney hypoxanthine dehydrogenase has no DPNH oxidase activity in the presence or absence of cytochrome c. The rate of uric acid synthesis by the kidney hypoxanthine dehydrogenase is accelerated upon simultaneous pyruvate reduction by lactic dehydrogenase. The kinetics of this system suggest that the regeneration of DPN bound to hypoxanthine dehydrogenase is responsible for the increased reaction rate.

REFERENCES

The Preparation, Properties, and Inhibition of Hypoxanthine Dehydrogenase of Avian Kidney
Erwin J. Landon and Charles E. Carter


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