THE INHIBITION OF URICASE BY XANTHINE

By JOHN F. VAN PILSUM

(From the Department of Biological Chemistry, University of Utah College of Medicine, Salt Lake City, Utah, and the Department of Biochemistry, State University of New York, College of Medicine at New York City, New York)

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The activity of rat liver xanthine oxidase (*in vitro*) rapidly decreases when the animals are placed on a protein-deficient diet (1–8). Bass, Tepperman, Richert, and Westerfeld (3) reported that such a depletion of the xanthine oxidase activity did not interfere with the formation and excretion of normal amounts of uric acid and allantoin by the intact animal. Williams, Feigelson, and Elvehjem (2) reported that rats maintained on a diet known to cause complete disappearance of liver xanthine oxidase, as measured *in vitro*, continued to excrete uric acid after receiving injections of xanthine. These authors suggested either that "activity of an enzyme (xanthine oxidase) measured *in vitro* is not a true indication of the rate of xanthine catabolism in the intact animal" or that "xanthine entered some metabolic pathway other than those it is known to enter."

The two general methods employed for liver xanthine oxidase activity are the manometric measurement of oxygen consumption (9) and the determination of decolorization time of methylene blue (10, 11). Results by the two methods have been compared and good correlation found (12). The spectrophotometric determination of the enzymatic oxidation of xanthine to uric acid (13) has not been used with rat liver because of the presence of uricase.

Protein-depleted rats excrete uric acid and allantoin, but there is no evidence *in vitro* of xanthine oxidase activity in their livers; therefore it seemed desirable to develop another type of *in vitro* assay. Accordingly, a method was developed for the determination of xanthine oxidase in rat liver which is more direct than previous methods in that the amount of uric acid produced by oxidation of xanthine serves as a basis of measurement of enzymatic activity. This procedure was made possible by the finding that xanthine itself is an inhibitor of the enzyme uricase.

Method

The rats were killed by a blow on the head and exsanguinated, and the livers were removed and cooled on ice. The liver was then blotted dry on filter paper, and a 1.0 gm. sample was homogenized in cold 0.0666 M phosphate buffer, pH 7.4, and brought to a final volume of 10 ml. with the

buffer. 1 ml. of this homogenate was added to a 20 ml. beaker containing 6 ml. of aqueous 4.02×10^{-2} M monosodium xanthine[1] solution (7 mg. per ml.) and 3 ml. of water. The mixture was incubated for 2 hours in a Dubnoff type metabolic shaker (14); the temperature was kept at 37°, an air phase was used over the vessels, and the oscillation rate was 96 per minute. 1 ml. aliquots were withdrawn prior to and at the conclusion of the incubation period and Folin-Wu filtrates were prepared with tungstic acid. The uric acid was determined colorimetrically (15) on 2 ml. portions of the filtrate. The per cent transmission was determined with a Coleman junior spectrophotometer at a wave-length of 540 mμ. The xanthine oxidase activity is expressed on the basis of uric acid produced per gm. of liver (wet weight) per hour.

Development of Method

When a homogenate of liver from a normal rat or a mixture of homogenate plus a small amount of added xanthine (26 μM per gm. of liver) was incubated, there was no increase in uric acid. This, in all probability, was attributable to the rapid destruction of the uric acid by the uricase in the homogenate. It was found, however, that the incubation of rat liver homogenate with larger amounts of xanthine (1.0 to 4.0 mM per gm. of liver) resulted in an easily measurable accumulation of uric acid. That the compound formed was uric acid was shown by its destruction upon further incubation with an added excess of purified uricase.

Effect of Varying Xanthine Concentrations on Uric Acid Accumulation in Normal Rat Liver Homogenates and in Purified Rat Liver Xanthine Oxidase Preparations

1 ml. aliquots of the rat liver homogenate were incubated with increasing amounts of 4.02×10^{-2} M monosodium xanthine solution. The data (Fig. 1) show that the uric acid production per gm. of liver gradually increased to a maximum, leveled off, and finally decreased as the concentration of xanthine in the incubation mixture increased.

With a uricase-free preparation of xanthine oxidase from rat liver (16), slightly different results were obtained (Fig. 1). The gradual rise to a maximum of uric acid production was not observed. Rather with the increasing amounts of xanthine in the mixtures there was a rapid rise to a maximal level of uric acid production. It therefore appeared probable that this difference might be the result of the inhibition by xanthine of the uricase activity in the liver homogenate. To investigate this possibility, the simultaneous effect of the added xanthine on both the xanthine oxidase

[1] Monosodium xanthine was purchased from the Schwarz Laboratories, Inc. The lot was analyzed by M. R. Heinreich, Department of Biology, Amherst College, Amherst, Massachusetts, and found to be free of purine or pyrimidine impurities.

and the uricase activities of the homogenate was determined. The mixtures of homogenate and xanthine were divided into equal portions. One set of the mixtures was incubated as such and the uric acid *appearance* determined. To each beaker in the second set was added an equal amount of uric acid; the reaction mixtures were incubated, and the extent of the *disappearance* of added urate determined. The results are plotted in Fig. 2.

It is seen that the added uric acid was rapidly destroyed by the homog-

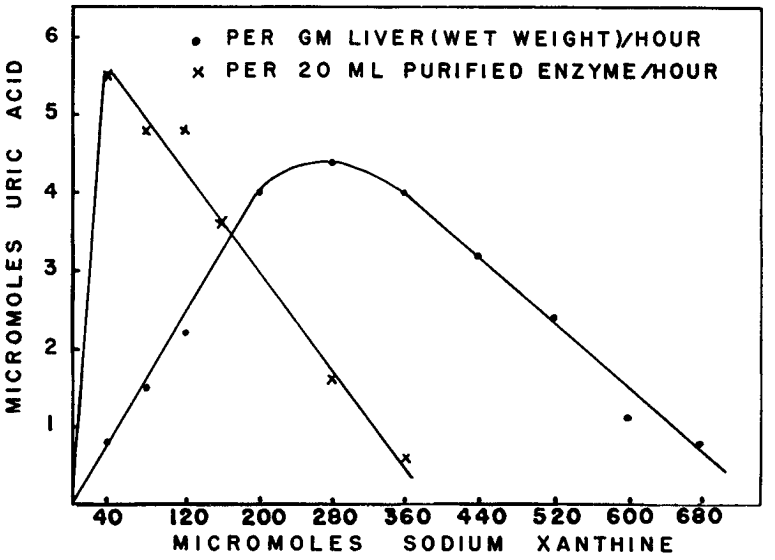

FIG. 1. Effect of varying amounts of xanthine on the uric acid production of a normal rat liver homogenate and on a purified preparation of xanthine oxidase from normal rat liver. 1 ml. aliquots of normal rat liver homogenate, containing 100 mg. of liver in 0.066 M phosphate buffer, pH 7.4, and 2 ml. aliquots of purified xanthine oxidase (devoid of any uricase activity) were added to 20 ml. beakers containing varying amounts of 4.02×10^{-2} M sodium xanthine solution. Water was added to make a total volume of 10 ml. Incubation procedure and uric acid determination as under "Method."

enate in the absence of added xanthine. A separate experiment showed that as high as 180 μM of uric acid could be destroyed per gm. of liver per hour. The rate of urate destruction decreased as the xanthine concentration increased until no added uric acid was catabolized. The appearance of uric acid in the mixtures containing no exogenous uric acid reached a maximal level at this same concentration of xanthine. It seemed, then, from the data in Fig. 2 that the production of uric acid by the liver homogenate was the result of the inhibition of uricase by xanthine.

The possibility, however, still remained that the production of uric acid was the result of the inability of the uricase present to oxidize an increased

amount of uric acid that had been formed from the large amount of the added xanthine. This seemed unlikely, since Axelrod and Elvehjem (9) report that in the oxidation of xanthine to allantoin by rat liver homogenate "the uricase activity never becomes a limiting factor." This is also indicated in Fig. 2. An analysis of these incubation mixtures for allantoin

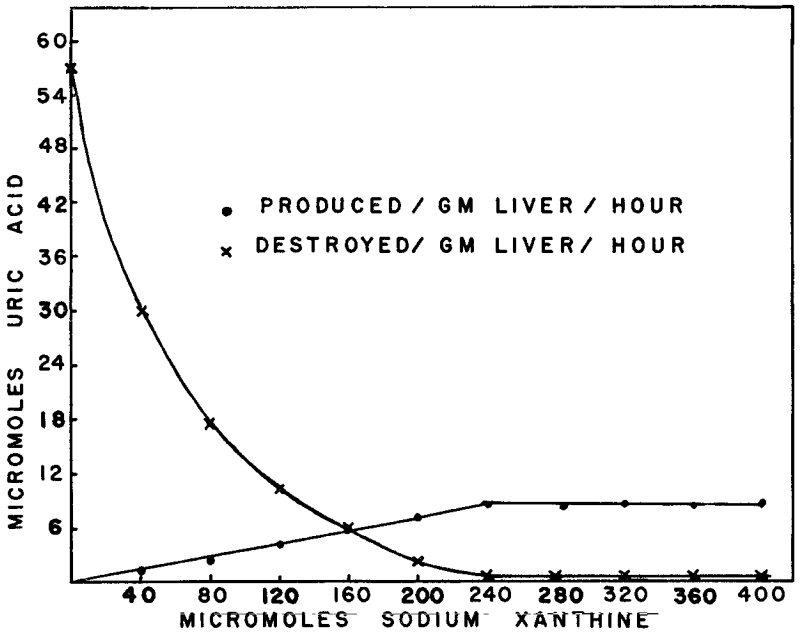

FIG. 2. The effect of varying amounts of xanthine on the uric acid *appearance* and *disappearance* in a normal rat liver homogenate. 1 ml. aliquots of homogenate, containing 100 mg. of liver in 0.066 M phosphate buffer, pH 7.4, were mixed with varying amounts of 4.02×10^{-2} M sodium xanthine solution and water to make 10 ml. Each mixture was divided into two equal portions. One portion was incubated as such and the uric acid *appearance* measured. To the other portion was added 1 ml. of 6×10^{-3} M lithium urate and the uric acid *disappearance* measured. Incubation and uric acid determination as under "Method."

eliminated the possibility. The homogenate-uric acid incubation mixtures produced allantoin, while the homogenate, uric acid, and xanthine (240 μM and up) mixtures produced no allantoin. Young and Conway's method for allantoin was used (17).

Effect of Varying Xanthine Concentrations on Uric Acid Accumulation in Liver Homogenates from Protein-Depleted Rats

It was possible to determine a concentration of xanthine to be used in an incubation medium of normal rat liver homogenate which would completely inhibit the uricase, but which would not inhibit xanthine oxidase.

However, in protein-depleted rats the xanthine oxidase decreases more rapidly than does the uricase (2, 9). An experiment was set up to determine whether in such protein-depleted animals the xanthine levels necessary to inhibit the uricase would or would not inhibit the lowered xanthine oxidase activity.

The dietary regimen was that reported by Litwack *et al.* (8). A group of adult, female, Wistar rats was placed on a protein-free diet and *urate appearance-urate disappearance* determined on the livers, as previously described, every 24 hours for 6 days. The addition of 240 μM of monosodium xanthine was necessary to inhibit effectively the uricase activity of all the livers tested. This indicated that the uricase activity remained essentially unchanged when the animals were subjected to the protein-free diet. The addition of 240 to 400 μM of xanthine seemed not to inhibit the lowered xanthine oxidase activities, since the rate of uric acid production remained constant, as it did with normal livers (Figs. 1 and 2).

The uric acid production *in vitro* rapidly decreased upon protein depletion. At the end of 6 days on the protein-free diet the activity fell to zero. The livers from this group of rats were not assayed for xanthine oxidase activity by the manometric procedure. Consequently, the experiment was repeated on another group of rats and the livers assayed by the oxygen uptake method. The activity rapidly decreased, as reported by Litwack *et al.* (8).

Determination of Some Optimal Conditions for Uric Acid Production by Rat Liver Homogenates

When the amounts of liver tissue in the reaction system were varied and the xanthine in the mixture was kept constant, the urate formed per gm. of liver per hour was not a constant value, but decreased with an increase of tissue in the medium. This was probably caused by the increased amounts of uricase in the medium. If, however, the amount of xanthine was increased in the medium along with the increase of tissue, the urate production per gm. of liver remained constant.

A 2 hour incubation period was found to develop sufficient uric acid for colorimetric measurement. In this period a constant increment of urate was produced per unit of time. The pH for optimal activity was found to be between 8 and 9, as reported previously (18); the pH of the media used was approximately 8.4. The maximal urate production was obtained when the given quantities of liver homogenate and xanthine were contained in a final volume of 10 ml.

Studies with Purified Enzymes

Purified hog liver uricase was prepared according to the method of Holmberg (19). Mixtures of 0.5 ml. portions of the purified uricase suspension,

1.0 ml. of 6.03×10^{-3} M uric acid, 1.0 ml. of 0.2 M borate buffer, pH 9, varying amounts of monosodium xanthine, and water to make 4 ml. were incubated. The uricase activity was measured *both* by oxygen uptake and by the colorimetric determination of uric acid disappearance. It was possible to attain xanthine concentrations that would inhibit the uricase to

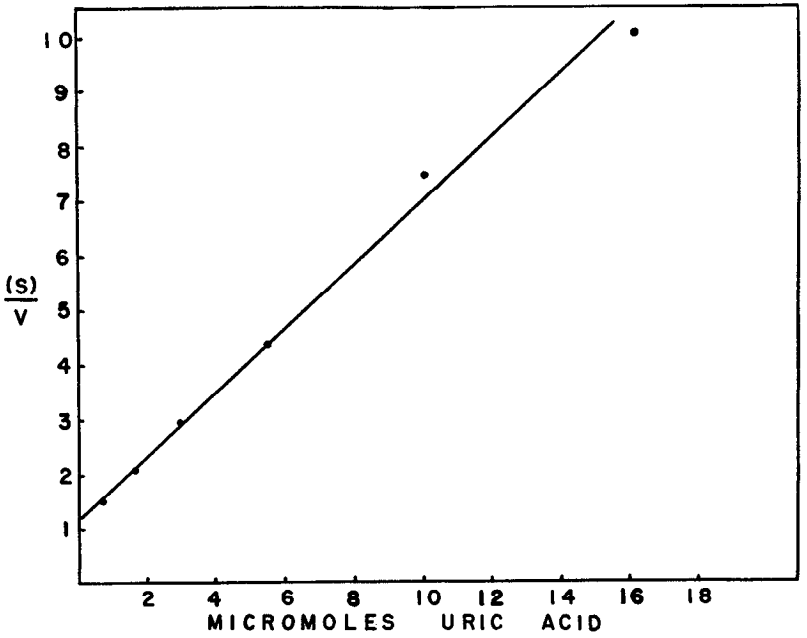

FIG. 3. Determination of the Michaelis constant of the uricase-uric acid complex. 0.2 ml. portions of the purified uricase, suspended in water, were incubated with varying amounts of 6×10^{-3} M lithium urate solution, 2 ml. of 0.2 M borate buffer, pH 9, and water to make a total volume of 10 ml. The incubation periods were 5 minutes at 37° in a Dubnoff type shaker, with an air phase over the vessels. Reactions stopped with tungstic acid and uric acid determined as under "Method."

the extent of 95 per cent. The amount of xanthine required for this inhibition varied with the activity of the uricase preparation.

The Michaelis constant (K_m) of the uricase-uric acid complex was determined by plotting (substrate concentration)/(velocity) against substrate concentration (20). 5 minute incubation periods were used to insure initial and constant velocities, expressed as micromoles of uric acid decomposed per 5 minute interval. The K_m was calculated to be 160 μM per liter (Fig. 3).

The inhibition of xanthine was shown to be competitive by its reversal with increasing amounts of uric acid in the incubation mixture (Fig. 4)

(21). From the data in Fig. 4 the dissociation constant for the xanthine-uricase complex was calculated to be 120 μM per liter.

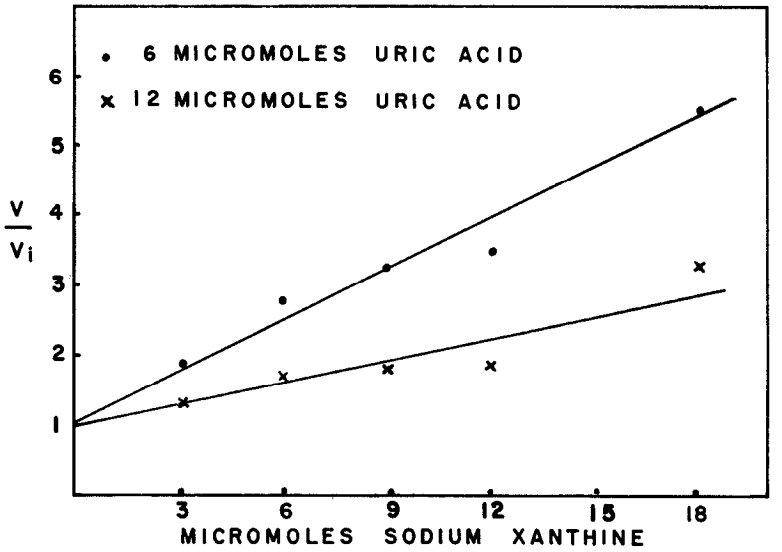

FIG. 4. Competitive inhibition of uricase by xanthine. 1.0 ml. portions of the uricase-water suspension were mixed with increasing amounts of 6.0×10^{-3} M sodium xanthine solution, 2.0 ml. of 0.2 M borate buffer, pH 9.0, 6.0×10^{-3} M lithium urate solution, and water to make a total volume of 10 ml. Incubation for 10 minutes at 37° with an air phase over the vessels.

DISCUSSION

The method of assay presented in this article is valid only if it can be shown that the large amount of added xanthine effectively inhibits the uricase activity, but does not inhibit the xanthine oxidase activity of the liver homogenate. It has been established that concentrations of xanthine can be used which prevent the destruction of exogenous uric acid. Under these conditions the xanthine oxidase is thought not to be inhibited for the following reasons. First, the rate of uric acid production by rat liver homogenate is constant at concentrations of sodium xanthine from 200 to 400 μM per 100 mg. of liver. Above 400 μM the rate decreased (Fig. 1). It would seem that if the xanthine oxidase was inhibited at 240 μM, the concentration used in the actual determination, then the rate would have decreased steadily with increasing xanthine. This did not occur until a concentration of 400 μM was reached. As the rate is constant in this range of substrate concentration, the xanthine oxidase is saturated with its substrate and the xanthine concentration has little or no effect on the enzyme

activity. It is assumed that the reaction rate has become of zero order. The urate production is dependent only upon the enzyme concentration, a condition particularly favorable for assay of enzymic activity. Secondly, the uric acid production of normal rat liver homogenate is equivalent to the oxygen uptake in the manometric method of Axelrod and Elvehjem. The average oxygen consumption attributable to the oxidation of xanthine to uric acid is approximately 200 μl. per gm. of liver (wet weight) per hour (8, 22–25). The calculated amount of oxygen required for the formation of 7.7 μM uric acid from the added xanthine is 180 μl. The activities are similar by both methods, even though 90 to 150 times as much xanthine is added per unit of liver in the uric acid production method. Accordingly, xanthine oxidase of the rat liver is assumed not to be inhibited by the large amount of xanthine used.

The finding that livers from protein-depleted rats produced no uric acid *in vitro* does not explain why such animals could still excrete uric acid and allantoin (2, 3). Perhaps this has been explained with data reported by Westerfeld and Richert (4). They reported that "as the liver xanthine oxidase was depleted by a purified low protein diet, the small intestine lost about two-thirds of its activity, and lung lost about one-half. Losses from the spleen and kidney were small. The entire rat lost three-fourths or more of its xanthine oxidase activity." Westerfeld and Richert postulate that the residual activity is sufficient to maintain an excretion of allantoin and uric acid (3).

SUMMARY

1. It has been demonstrated with both rat liver homogenate and purified hog liver uricase that xanthine is a powerful inhibitor of uricase. The accumulation of uric acid by rat liver homogenate is attributed to an inhibition of the uricase by xanthine.

2. Some optimal conditions for uric acid production by rat liver homogenate were determined. A method is presented for determination of the activity of rat liver xanthine oxidase by incubation with xanthine and measurement of the uric acid formed.

3. Livers from rats placed on a protein-free diet rapidly lost the ability to produce uric acid *in vitro*. At the end of 6 days on the diet, the activity fell to zero. This confirms the results obtained by the manometric and methylene blue methods of xanthine oxidase assay.

4. The inhibition of purified hog liver uricase by xanthine is competitive. The Michaelis constant for the uricase-uric acid complex was calculated to be 160 μM per liter, and the dissociation constant for the uricase-xanthine complex was 120 μM per liter. These constants indicate that xanthine has an affinity for uricase of the same order of magnitude as has uric acid.

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