A STUDY OF XANTHINE METABOLISM IN THE RAT*

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For many years it has been known that purine catabolism in the rat results in the formation and subsequent urinary excretion of allantoin as the main end-product. Individual enzymes catalyzing the steps in purine catabolism have been identified and methods for assaying these enzymes in vitro have been developed. With the discovery that xanthine oxidase activity in vitro is influenced greatly by the level of dietary protein (1-3) a means was offered for directly comparing the activity in vitro of the enzymes involved in xanthine catabolism with activity of those enzymes in the intact animal.

In rats on low protein diets it has been observed that liver xanthine oxidase activity as measured in vitro is greatly decreased, in many cases disappearing entirely, if the dietary protein level is reduced sufficiently. If xanthine oxidase rather than uricase limited the rate of breakdown of xanthine to allantoin, animals with low xanthine oxidase activity should convert xanthine to allantoin at a slower rate than animals with normal xanthine oxidase activity. If not, then measurements in vitro of xanthine oxidase activity may not be taken as an indication of the actual rate of xanthine oxidation in the intact animal.

This problem was studied by placing animals on a ration known to decrease xanthine oxidase activity in vitro, by injecting them with a known amount of xanthine, measuring the rate of allantoin excretion in the urine, and finally assaying the tissues of the rats for xanthine oxidase and uricase activity. The urinary excretion of other purines from the animals with xanthine injected was also measured in an attempt to account for all of the injected xanthine either as allantoin or purine. The xanthine was injected rather than fed, since dietary xanthine would probably be incompletely absorbed from the intestinal tract because of its relatively low solubility. In that case quantitative relationships between the amount of xanthine ingested and catabolites excreted could not have been made.

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EXPERIMENTAL

Two groups of male rats of the Holtzman strain, weighing 60 to 70 gm., were given synthetic rations for 3 weeks before use in the enzyme studies. One group of ten animals received a 6 per cent casein ration and the other group of nine animals an 18 per cent casein ration plus 0.25 per cent DL-methionine. The common components of the two rations were as follows: vitamin mixture previously described (1) 2 per cent, Salts IV (4) 4 per cent, corn oil 5 per cent, respective casein level, and sucrose to make 100 per cent. In addition, 2 drops of fortified haliver oil per week were given orally to each animal. At the end of the 3 week feeding period one animal from each group was sacrificed for determinations of liver xanthine oxidase to make certain that the expected drop in liver activity in the rats on the low protein ration had occurred. The remaining animals of both groups were then placed in metabolism cages, two animals per cage, for urine collections. Basal urine excretions were collected under toluene for a 93 hour period. At the end of this time each animal was injected intraperitoneally with a 3 ml. suspension of 25 mg. of xanthine per 100 gm. of body weight. The xanthine was dissolved first in equivalent sodium hydroxide and then brought to pH 7.3 in order to obtain a finely divided suspension. The rats were replaced in the metabolism cages and fed their respective synthetic rations and given water ad libitum. Urine collections were made thereafter at the following intervals from the time of injection: 3, 6.5, 11, 22, 27, 31, 46.5, 53, 70.5, 77, 94.5, and 143 hours.

The urine samples were neutralized to pH 7.0, evaporated to dryness, extracted with small portions of hot water, filtered, and made to 10 ml. Allantoin was determined in each sample by the method of Young and Conway (5). Because of the somewhat unstable nature of allantoin, recoveries of allantoin added to the urines were made in several cases. These averaged 103 ± 0.1 per cent recovery.1 Xanthine and guanine were determined in the urines by the method recently developed by one of the authors (6). Uric acid was determined in the urines by a modification of Brown's method (7). Adenine was not determined because of lack of an adequate micromethod for adenine and because adenase has not been observed in rat tissues, making it doubtful that the injected xanthine would be converted to adenine.

After the final urine collections were made, the rats were sacrificed and xanthine oxidase activity of the liver determined according to the method of Axelrod and Elvehjem (8). It also appeared necessary to measure the xanthine oxidase activity of the kidney as well as of the liver, since considerable xanthine oxidation is believed to occur in kidney as well as in

1 Standard error of the mean.
liver. The manometric method used for measuring liver xanthine oxidase activity, however, could not be employed for measuring kidney activity (8). Therefore, a new method was devised by which kidney xanthine oxidase activity was readily measured.

The following procedure was adopted for assaying kidney xanthine oxidase: 2 ml. of a 1:6 kidney homogenate in 0.1 M sodium phosphate buffer (pH 7.3) were mixed with 4 ml. of a salicylaldehyde solution (1 mg. per ml.) and 2 ml. of the phosphate buffer in a 125 ml. Erlenmeyer flask. A blank was also prepared containing 4 ml. of water in place of the salicylaldehyde. 1 ml. of the mixture was withdrawn immediately and pipetted into a solution of 2 ml. of water, 1 ml. of 0.6 N sulfuric acid, and 1 ml. of 10 per cent sodium tungstate. The rest of the enzyme mixture was lightly stoppered and incubated at 37° for 90 minutes. At the end of that time a 1 ml. aliquot was withdrawn and treated similarly to the pre-incubation aliquot.

The tungstate precipitates were centrifuged and the supernatant was filtered. 2 ml. aliquots of the filtrate were treated with 0.1 ml. of Folin-Ciocalteu phenol reagent (9) and 1 ml. of 1 N sodium hydroxide. Color was allowed to develop for 5 minutes, 6 ml. of water were added, and the tubes were read in an Evelyn colorimeter with a 620 mμ filter. A series of salicylic acid standards (0 to 100 μ) was treated in a similar manner. Under the conditions employed salicylic acid gives a deep blue color, while salicylaldehyde gives only an insignificant response.

It was considered necessary to observe the effects of a low protein ration upon uricase activity in vitro as well as xanthine oxidase activity, since both of these enzymes are involved in the oxidation of xanthine to allantoin. Therefore, two groups of rats of four animals each were given the 6 per cent casein and 18 per cent casein + 0.25 per cent DL-methionine rations, respectively, for the same length of time as the animals previously used in the xanthine oxidase studies. Uricase activity was determined in the liver of each animal with lithium urate as substrate (8). Kidney uricase activity could not be adequately determined by this method.

RESULTS AND DISCUSSION

The results for the enzyme activities as measured in vitro are presented in Table I. Enzyme activities of both xanthine oxidase and uricase of liver are expressed as microliters of oxygen uptake at 30° due to added substrate per hour per gm. of wet liver. Kidney xanthine oxidase activity is expressed as microliters of oxygen uptake equivalent to the amount of salicylaldehyde oxidized to salicylic acid per hour per gm. of wet tissue at 37°.

From these results it may be seen that a diet containing 6 per cent pro-
tein is not adequate to maintain either liver or kidney xanthine oxidase activity at a normal level. In fact, liver xanthine oxidase activity is decreased almost to zero, while kidney xanthine oxidase activity is decreased to less than half that found in animals fed an 18 per cent casein ration supplemented with methionine. These results verify previous work reported by two of the authors (1) in which it was suggested that liver xanthine oxidase activity is a sensitive index of protein balance. The method used for the estimation in vitro of liver xanthine oxidase has been checked against other methods for assaying that enzyme by Richert et al. (10) and the results were in good agreement with those obtained by other methods reported in the literature. Therefore, it is believed that the decrease in liver xanthine oxidase observed in these experiments is not a function of the method employed but a true function of the levels of dietary protein.

**TABLE I**

<table>
<thead>
<tr>
<th>Ration</th>
<th>No. of animals</th>
<th>Enzyme determined</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>6% casein</td>
<td>9</td>
<td>Xanthine oxidase</td>
<td>1.30 (0-13)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Uricase</td>
<td>390 (246-530)</td>
</tr>
<tr>
<td>18% casein + 0.25% DL-methionine</td>
<td>8</td>
<td>Xanthine oxidase</td>
<td>110 (90-143)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Uricase</td>
<td>600 (420-780)</td>
</tr>
</tbody>
</table>

* See the text for units of enzyme activity.
† Range.

The salicylaldehyde method for kidney xanthine oxidase outlined in the foregoing was checked with the livers of the same animals in which the values for kidney activity are reported. This method, when employed for liver, was found to correlate very well with the manometric method.

From Table I liver uricase activity was observed to be decreased by the low protein diet, although not as much as xanthine oxidase. The fact that the actual activity of uricase in either group of animals is much greater than xanthine oxidase activity in oxygen uptake per gm. of liver leads one to believe that, at least in vitro, uricase is not the limiting step in the catabolism of xanthine. Xanthine oxidase appears to be much more limiting than uricase. This same conclusion was drawn by Axelrod and Elvehjem in other deficiency studies in the rat (8).

In Fig. 1 are reported the values obtained for excretion of allantoin, uric acid, xanthine, and guanine after the single intraperitoneal injection of...
xanthine. To obtain the curves presented in Fig. 1 the excretion values for all the animals of each group were averaged and plotted against the time of the urine collections, each value being added to the previous value to give curves analogous to "oxygen uptake curves." In every case the basal excretion per hour, obtained prior to the xanthine injected, was multiplied by the time in hours of each collection and subtracted from the total excretion obtained over that time interval. Consequently, the excretions depicted in Fig. 1 are due only to metabolism of the injected xanthine. The ordinates of the curves represent excretion of the various catabolites in mg. per 100 gm. of body weight of rat. It was necessary to make all animals to the same weight by this means, since at the time of injection the rats receiving the higher protein level were considerably
heavier than those receiving the 6 per cent level. Even if this had not been done, the curves would still lie in the same order with respect to each other.

It can be seen from the allantoin excretion curves, that the animals with low liver and kidney xanthine oxidase as measured in vitro are able to convert injected xanthine to allantoin at a rate nearly double that of the animals with normal xanthine oxidase activity in vitro. Moreover, from Table I it may be observed that uricase activity is not limiting in either group of animals when compared to xanthine oxidase activity. Therefore, it appears that activity of the enzymes investigated in this study when measured in vitro may not be a true indication of the actual rate of xanthine oxidation in the intact animal.

From the excretion curves of uric acid from the two groups of animals, it appears that uric acid is spilled over more easily into the urine of the rats on the higher protein ration than of those fed the 6 per cent protein ration; this is also true in the case of xanthine and guanine. The signifi-

<table>
<thead>
<tr>
<th>Ration</th>
<th>Xanthine injected</th>
<th>Allantoin excreted</th>
<th>Uric acid excreted</th>
<th>Xanthine + guanine excreted</th>
<th>Total metabolites excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>6% casein</td>
<td>164</td>
<td>69.5</td>
<td>5.95</td>
<td>5.60</td>
<td>81.1</td>
</tr>
<tr>
<td>18% casein + 0.25% DL-methionine</td>
<td>164</td>
<td>35.4</td>
<td>13.4</td>
<td>19.5</td>
<td>68.3</td>
</tr>
</tbody>
</table>

cance of these findings is not clear unless the level of dietary protein produces a change in the renal thresholds of these substances. The uricase estimations in vitro do not appear to be directly involved in the results obtained for the excretion of uric acid. The rats receiving the higher protein level excrete more uric acid even though their liver uricase activity is also higher than that of the other group of rats. These results appear to be related to the well known observation that the liver of the Dalmatian dog is fairly rich in uricase, although it excretes most of its uric acid unchanged (11).

At the end of 143 hours the excretion curves have leveled off almost completely, except for allantoin from the animals fed 6 per cent casein. This curve, however, if continued with the same curvature, becomes asymptotic with a level of about 11 mg. of allantoin. If one adds up the total excretion of allantoin and the purines measured, the results shown in Table II are obtained. In Table II the values are expressed in micromoles in order to place all of the substances on an equivalent basis. It thus ap-
pears that only 49 per cent of the injected xanthine can be accounted for by the total excretion from the rats given 6 per cent protein and only 41 per cent from the group receiving 18 per cent protein. These somewhat unusual results cannot be explained by possible destruction of the catabolites by the treatment given the urines, since the only fairly unstable one, allantoin, was found to give excellent recovery values when added directly to samples of the urines (see "Experimental"). One possible explanation for our results is that the xanthine entered some metabolic pathway other than those it is known to enter. For example, the relatively large concentration of xanthine given the animals in the single injection may have reversed by mass action the reactions by which purines are synthesized. Another possible explanation is that some of the injected xanthine was incorporated via guanine into the nucleic acids of the animal. However, Brown, Roll, and Plentl (12) have shown in this respect that dietary guanine is not incorporated into body nucleic acids, although dietary adenine may be. This point remains to be elucidated. Probably the best attack would be through the use of isotopically labeled xanthine.

SUMMARY

Results have been presented which demonstrate that activity of an enzyme (xanthine oxidase) measured in vitro is not a true indication of rate of xanthine catabolism in the intact animal. Moreover, less than half of the xanthine injected intraperitoneally in a single large dose can be accounted for as conventionally expected catabolites.

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