Effect of Dietary Protein and Iron on the Fractional
Turnover Rate of Rat Liver Xanthine Oxidase

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ABSTRACT Rat liver xanthine oxidase activity is regulated in response to dietary protein and iron. To investigate whether the change in activity was mediated by a change in the rate of protein degradation, we measured the fractional turnover rate using the double-isotope technique with [3H]- and [14C]leucine and calculated the apparent half-life of xanthine oxidase in rats fed diets containing either 20 or 5% casein with either 35 or 5 mg iron/kg diet. Under control conditions, xanthine oxidase had an apparent half-life of 4.8 d and approximately 65% of the enzyme subunits were active. Rats fed diets with low dietary protein had lower xanthine oxidase activity, but the enzyme had a slower fractional turnover rate, resulting in an apparent half-life of 6.4 d, and only 15—20% of the enzyme was active. The apparent half-life of xanthine oxidase increased to 7.5 d in rats fed diets with low dietary iron, but dietary iron did not affect the specific activity of the enzyme or the percentage of active subunits. These results suggest that the loss of enzyme activity is not due to loss of enzyme protein by increased degradation, but rather to inactivation of the enzyme. J. Nutr. 117: 2054—2060, 1987.

INDEXING KEY WORDS:
• xanthine oxidase • enzyme turnover
• dietary protein • dietary iron

Xanthine oxidase catalyzes the conversion of hypoxanthine and xanthine to uric acid, and the activity of this enzyme changes in response to dietary protein [1] and iron [2]. Xanthine oxidase is a complex enzyme and may be subject to many levels of regulation. It is composed of two subunits, each with a pterin-containing Mo cofactor, a molybdenum atom with a terminal sulfide, flavin adenine dinucleotide (FAD), two Fe/S centers and a phosphoserine at the active site [3, 4]. Xanthine oxidase can exist either as an active enzyme in the dehydrogenase or oxidase form or as an inactive enzyme in the desulfo or demolybdo form [3, 5, 6]. There has been much recent interest in xanthine oxidase because the enzyme may be converted to the oxidase form during ischemia and then could generate superoxide radicals, which may initiate tissue damage [7].

We have been studying the mechanisms for regulation of xanthine oxidase in response to diet. A previous study in this laboratory using a radioimmunoassay to measure xanthine oxidase protein [8] showed that in rats fed low protein diets, xanthine oxidase activity was lower than control levels, but the amount of xanthine oxidase protein was unaffected, indicating that a pool of inactive xanthine oxidase protein existed in the livers of these rats.

A number of investigators have measured the half-life of xanthine oxidase activity in rat liver. The turnover of the enzyme activity varied with the stimulus used to produce the change in activity, and the reported values for the half-life of xanthine oxidase activity were 1.58 d [9], 2.9 d [10] and 4.0 d [1, 11] in these studies. Because a pool of inactive xanthine oxidase exists, it is also important to measure the turnover of the total xanthine oxidase in conjunction with enzyme activity and immunologically detectable protein to achieve a better understanding of the regulation of this enzyme.

We used the double-isotope technique of Arias, Doyle and Schimke [12] to measure the turnover of hepatic xanthine oxidase. For this method, a labeled amino acid, usually [14C]leucine, is administered to the animal and allowed to decay a specified time. Then the same amino acid with a different label, usually [3H]leucine, is administered, and the animal is killed 4—6 h later. With the double-isotope technique, each animal is its own control in that the proteins in each animal are labeled at two time points along the decay curve. The ratio


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the $^3$H to $^{14}$C radioactivity in a protein gives an indication of the fractional turnover rate. Proteins that degrade more rapidly will have greater $^3$H/$^{14}$C ratios.

The decay rates of proteins that have been measured, except for hemoglobin, follow exponential kinetics (13). Thus, in theory, the ratio of $^3$H to $^{14}$C in the protein, or the ratio of the initial radioactivity to the radioactivity remaining in the protein after an interval, can be inserted into the equation describing first-order decay to determine the $k_d$, which is the fractional turnover rate. The apparent half-life ($t_{1/2}$) can then be calculated from the $k_d$.

Leucine is most often used with the double-isotope technique but it is reutilized to a considerable extent, so absolute $k_d$'s cannot be determined, but only apparent $k_d$'s or relative differences for different proteins. Glass and Doyle (14) found that the logarithms of the isotope ratios for the proteins of rat liver homogenate, mitochondria, microsomes and supernatant obtained with the double-isotope technique can be linearly correlated to the $k_d$'s for the same fractions obtained by following the decay of [guanidino-$^{14}$C]arginine, a precursor that is reutilized to only a small extent in the liver because the guanidino carbon is hydrolyzed by arginase to form urea. Thus, from this standard curve, one can determine the $k_d$ of a rat liver protein from its $^3$H/$^{14}$C isotope ratio. However, because Swick and Ip (15) showed that some reutilization of [guanidino-$^{14}$C]arginine can occur in liver, the apparent half-lives are probably overestimates.

We measured the fractional turnover rate of rat hepatic xanthine oxidase and found that the apparent half-life increased in rats fed diets with low protein and iron. We also measured the xanthine oxidase activity and immunoprecipitable protein concentration and estimated that in rats fed control diet 65-76% of enzyme subunits were active, but in rats fed low protein diets a large pool of inactive protein existed and only 15-20% of the protein was active.

**MATERIALS AND METHODS**

**Animals and diets.** Male Sprague-Dawley rats, purchased from Bantin and Kingman (Fremont, CA), were housed individually in hanging stainless steel cages. The room temperature was 21 ± 1°C and the lighting was on a 12-h light-dark cycle. Food and deionized water were provided ad libitum. The rats were weighed, fresh food was provided and the amount of food consumed was determined every 3 d. The rats were fed their respective diets for 14 d prior to the isotope injections and throughout the duration of the experiment.

The four diets were modifications of the AIN-76 purified diets for rats (16) (Table 1) and contained either 20 or 5% protein, with casein as the protein source, and either 35 or 5 mg iron/kg diet, with ferric citrate as the iron source. They are designated as 20P35Fe (control), 20P5Fe (low iron), 5P35Fe (low protein) and 5P5Fe (low protein-low iron). Powdered sugar was substituted for the cornstarch in the AIN diet because the cornstarch had iron contamination. Sodium molybdate was added to all the diets in a final concentration of 1 mg/kg of diet to ensure adequate molybdenum.

**Isotope injection protocol: pilot study.** A pilot study was conducted to determine the optimal interval between isotope injections for the double-isotope technique for measuring protein turnover and also to test whether the same interval between isotope injections could be used to measure the enzyme turnover in rats fed different diets. In this study, 12 rats of approximately 89 g initial weight were randomly divided into two groups of 6 rats each and given either the 20P35Fe or 20P5Fe diet. On d 14, the rats were fasted for 16 h, then injected intraperitoneally with 25 µCi of L-[U-$^{14}$C]leucine in 0.5 ml of a sterile aqueous solution (specific activity of 348 mCi/mmol). At 4 h after the injections, the respective diets were returned to the rats. After an interval of either 3 or 10 d, three rats from each diet group were fasted, then injected intraperitoneally with 25 µCi of L-[4,5-$^3$H]leucine in 0.5 ml, diluted to a specific activity of 1044 mCi/mmol with nonradioactive leucine in sterile 0.9% NaCl. The total leucine concentration of both the $^{14}$C- and $^3$H-leucine injection solutions was 72 nmol leucine per 0.5 ml. All rats were killed by asphyxiation with carbon dioxide 4 h after the second injection. The livers were excised, rinsed in 0.25 M sucrose, blotted dry and weighed. A

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**Table 1**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>20P35Fe</th>
<th>20P5Fe</th>
<th>5P35Fe</th>
<th>5P5Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20.00</td>
<td>20.00</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.30</td>
<td>0.30</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Sucrose</td>
<td>62.00</td>
<td>63.71</td>
<td>77.23</td>
<td>78.94</td>
</tr>
<tr>
<td>Corn oil</td>
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<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Fiber</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Choline bitartrate</td>
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<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Mineral mix</td>
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<td>3.50</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1.00</td>
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<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Molybdenum mix</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Iron mix</td>
<td>2.00</td>
<td>0.29</td>
<td>2.00</td>
<td>0.29</td>
</tr>
</tbody>
</table>

1. Vitamin-free casein, Teklad Test Diets, Madison, WI.
2. Sigma Chemical, St. Louis, MO.
4. Mazola Corn Oil, CPC International, Englewood cliffs, NJ.
5. Nonnutritive fiber, cellulose type, Teklad Test Diets.
7. Based on AIN-76 vitamin formulation (16).
8. Provides 63.1 mg Na2MoO4·2H2O/250 g sucrose.
9. Provides 2.1926 g ferric citrate/200 g sucrose.
portion of each liver from the rats fed the 20P35Fe diet was used immediately for the separation of subcellular fractions. The livers were frozen at \(-20^\circ\text{C}\).

**Major experiment.** The results from the pilot study indicated that a 10-d interval between isotope injections was more appropriate than a 3-d interval for measuring the turnover of xanthine oxidase. The protocol for the major experiment proceeded as in the pilot study.

The rats [initial weight 85 g] were randomly divided into four groups: 20P35Fe, eight rats; 20P5Fe, six rats; 5P35Fe, five rats; 5P5Fe, six rats. All the rats consumed their diets for 14 d. On d 15, five rats from each group were injected with the \([^{14}\text{C}]\)leucine. On d 25, the same rats from each group were injected with the \([^{3}\text{H}]\)leucine. The animals were killed 4 h later.

The other three rats in the 20P35Fe diet group were given diet for 24 d, then fasted for 16 h. On the morning of d 25, the rats were injected with 0.5 ml of both the \([^{14}\text{C}]\)- and \([^{3}\text{H}]\)leucine solutions, then killed 4 h later. The purpose of injecting the isotopes simultaneously in these three rats was to test whether the two different isotopic forms of the same amino acid were incorporated into proteins to the same extent. Because we injected 75 \(\mu\text{Ci}\) of L-[4,5-\(^{3}\text{H}\)] leucine and 25 \(\mu\text{Ci}\) of L-[\(^{14}\text{C}\)]leucine, we expected to find a \(^{3}\text{H}/^{14}\text{C}\) ratio of 3.0 in newly synthesized protein.

**Standard curve of \(^{3}\text{H}/^{14}\text{C}\) ratios.** Portions of fresh livers from the rats fed the 20P35Fe diet were homogenized and the subcellular fractions were separated by the methods of Novikoff and Heus [17]. Aliquots of 100 \(\mu\text{l}\) homogenate, 50 \(\mu\text{l}\) mitochondria, 25–55 mg microsomes and 100 \(\mu\text{l}\) of the 114K supernatant were mixed with 400 \(\mu\text{l}\) of 10% trichloroacetic acid to precipitate the proteins, then centrifuged for 1 min in a Brinkmann Eppendorf centrifuge 541 [Westbury, NY]. The precipitated proteins were washed by the method of Siekevitz [18], then counted in a Beckman LS-230 [Palo Alto, CA] beta counter with an error of \(\approx 7\%\). Quench curves were constructed using samples of the \([^{14}\text{C}]\)- and \([^{3}\text{H}]\)leucine solutions, which were quenched with successive additions of chloroform and counted. Efficiencies of the dual channels were determined with an external standard.

The logarithm of the \(^{3}\text{H}/^{14}\text{C}\) ratios obtained for the liver homogenate proteins and subcellular protein fractions [mitochondria, microsomes and 114K supernatant] was plotted versus the \(k_d\) values for those fractions calculated from the decay of \([\text{guanidino-}^{14}\text{C}]\)arginine as described by Glass and Doyle [14]. There was a linear relationship between the logarithm of the \(^{3}\text{H}/^{14}\text{C}\) ratios and the \(k_d\) values for the subcellular fractions from the rats with either the 3-d or the 10-d interval between isotope injections. The \(^{3}\text{H}/^{14}\text{C}\) ratios of the immunoprecipitated hepatic xanthine oxidase were then measured, and the \(k_d\)’s were determined from this standard curve. The apparent half-life of each protein fraction or immunoprecipitated protein was calculated from the \(k_d\)’s by the relationship \(t_{1/2} = \ln 2/k_d\).

**Immunoprecipitation of xanthine oxidase and ornithine transcarbamylase.** The antibody directed against xanthine oxidase was judged monospecific because it gave only a single band corresponding to 150,000 daltons when rat liver homogenates were probed with the anti-xanthine oxidase antibody using the Western blotting procedure. Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.1.3.22) was immunoprecipitated from the tissue homogenates by the addition of 100 \(\mu\text{l}\) of antibody directed against xanthine oxidase to 900 \(\mu\text{l}\) supernatant and incubated for 3 d at 4°C. To immunoprecipitate ornithine transcarbamylase [ornithine carbamoyltransferase, EC 2.1.3.3], portions of the 20% (wt/vol) supernatant samples were further diluted to 4% with distilled water; antibody directed against ornithine transcarbamylase was diluted 2.5 with 0.05 M potassium phosphate buffer, pH 7.4, and 0.5 mM EDTA, and then 0.4 ml of the diluted antiserum was added to 1 ml of the 4% supernatants and incubated for 4 d at 4°C. The immunoprecipitates were centrifuged in a Beckmann Eppendorf centrifuge for 10 min, then washed twice in 100 \(\mu\text{l}\) of a solution containing 2% Triton X-100, 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1 mM EDTA, once in 100 \(\mu\text{l}\) of 10 mM Tris-HCl, pH 8.0, and once in 100 \(\mu\text{l}\) distilled water. Before counting, the immunoprecipitates were solubilized in 100 \(\mu\text{l}\) of the denaturing solution of 0.11 M Tris-HCl, pH 6.8, 1.8% sodium dodecyl sulfate (SDS), 32% glycerol and 4.5% \(\beta\)-mercaptoethanol.

**Assays.** The rats were homogenized in 4 parts (g/ml) of 0.05 M potassium phosphate buffer, pH 7.4, with 0.5 mM EDTA in a Potter-Elvehjem homogenizer with a Teflon pestle, then centrifuged at 30,000 \(\times\) g for 30 min in a Sorval RC-5 centrifuge with an SS-34 rotor [Wilmington, DE]. The supernatants were centrifuged in a Beckman L2-65B ultracentrifuge with a 50Ti rotor at 110,000 \(\times\) g for 1 h, dialyzed at 4°C against two changes of homogenate buffer for 24 h, then frozen at \(-20^\circ\text{C}\) until assayed for enzyme activity or immunologically detectable protein.

Xanthine oxidase activity was measured by spectrophotometric detection of the production of uric acid [5]. Specific activity is expressed as the change in absorbance at 295 nm/[min \(\cdot\) mg protein]. The extinction coefficient for uric acid at 292 nm is \(10.5 \times 10^{-3} \text{M}^{-1} \text{cm}^{-1}\) [19]. The protein concentration of the samples was determined by the method of Lowry et al. [20], with bovine serum albumin as the protein standard.

The amount of immunologically detectable xanthine oxidase was measured by an immunological assay [K. M. Manchester, manuscript in preparation] using monospecific antibody directed against rat liver xanthine oxidase. Briefly, the liver homogenates were diluted to 20 \(\mu\text{g}\) protein/ml, and 100-\(\mu\text{l}\) aliquots were applied in duplicate to nitrocellulose paper using a slot blot apparatus [Schleicher & Schuell, Keene, NH]. The paper was soaked in blocking solution containing albumin to saturate all the binding sites on the nitrocellulose paper with pro-
tein. Next, the paper was washed with a solution containing antibodies specific for rat liver xanthine oxidase, then washed with additional blocking solution. The immunoprecipitates were localized by the binding of 125I-labeled Protein A. The nitrocellulose paper was washed again, cut into pieces and the amount of bound radioactivity measured by counting the paper in a Searle Model 1197 gamma counter (Des Plaines, IL). The concentration of the xanthine oxidase immunologically detectable protein was calculated from a standard curve generated with purified rat liver xanthine oxidase, diluted to between 6.0 and 60 pmol xanthine oxidase.

Concentration of active xanthine oxidase. The concentration of active liver xanthine oxidase was calculated using the turnover number for bovine milk xanthine oxidase, and so we expressed the active xanthine oxidase as micromoles of FAD per subunit of xanthine oxidase, we expressed the active xanthine oxidase in terms of whole enzyme.

Because there is one FAD per subunit of xanthine oxidase, we expressed the concentration of active xanthine oxidase as micromoles of active xanthine oxidase subunit. Nishino and Tsushima (22) have confirmed that there is no interaction in catalytic activity between the subunits of xanthine oxidase in terms of active subunits, rather than in terms of whole enzyme.

We calculated the mol of active xanthine oxidase subunit/ml of supernatant and also the concentration of immunologically reactive xanthine oxidase expressed as mol of xanthine oxidase/ml of supernatant in order to determine the percentage of active xanthine oxidase subunits.

Sources of reagents. Rabbit anti-rat liver ornithine transcarbamylase was a gift of Dr. Janice Harris. The L-[4,5-3H]leucine, specific activity 45 Ci/mmol, and the L-[U-14C]leucine, specific activity 348 mCi/mmol, were from Amersham (Arlington Heights, IL).

Statistical analyses. The data were analyzed by two-way analysis of variance (ANOVA). Tukey's studentized range test, with a procedurewise error rate of 5%, was employed as a follow-up procedure to the ANOVA when statistical significance was indicated. The Dixon test was used to test for outliers.

RESULTS

Pilot study. The pilot study was performed to determine whether a 3-d or a 10-d interval between injections of the isotopes was more suitable for measuring the turnover of xanthine oxidase and whether the turnover of xanthine oxidase from rats fed control and low iron diets could be measured with the same protocol. The 3H/14C ratios for xanthine oxidase from rats that had a 3-d interval between injections of the isotopically labeled amino acid were close to 3.0, suggesting that little degradation of xanthine oxidase had occurred during the 3-d interval. The 3H/14C ratios for xanthine oxidase from rats with a 10-d interval between isotope injections were much greater than 3.0, indicating that extensive degradation had occurred over the 10-d interval. Therefore, we used a 10-d interval between isotope injections in the major experiment. The pilot study also showed that the turnover of xanthine oxidase from rats fed different diets could be measured with the same protocol because the k_d's for xanthine oxidase from rats fed different diets were within the range of the standard curve.

Standard curve. In the major experiment, we measured the 3H/14C ratios of the subcellular fractions, generating a standard curve from the plot of the logarithm of the 3H/14C ratios versus the k_d's for these proteins obtained by Glass and Doyle (13) and calculated the apparent t_1/2 for each cellular fraction (Table 2).

The three rats fed the control diet injected with the two isotopes simultaneously served as isotope incorporation controls to ensure that both isotopes of leucine were utilized in a similar way. Because we administered three times as much 14C as 3H, we were able to incorporate the double isotopes. The k_d's for the subcellular fractions from the isotope incorporation controls ranged from 2.62 to 2.73 for the four protein fractions (Table 2).

Fractional turnover rate of xanthine oxidase. We measured the 3H/14C ratios in the immunoprecipitated xanthine oxidase from the livers of rats injected with the double isotopes. The k_d's were determined from the standard curve generated from the subcellular fractions, and the t_1/2's were calculated (Table 3). The k_d's for xanthine oxidase from rats fed low iron or low protein diets were significantly lower than the k_d's for rats fed the control diet and injected with the two isotopes of leucine simultaneously.

<table>
<thead>
<tr>
<th>Protein fraction</th>
<th>n</th>
<th>3H/14C</th>
<th>k_d²</th>
<th>t_1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>4</td>
<td>11.67 ± 0.53</td>
<td>0.200</td>
<td>3.47</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>4</td>
<td>7.23 ± 0.15</td>
<td>0.100</td>
<td>6.93</td>
</tr>
<tr>
<td>Microsomes</td>
<td>4</td>
<td>17.24 ± 0.70</td>
<td>0.280</td>
<td>2.48</td>
</tr>
<tr>
<td>Supernatant</td>
<td>5</td>
<td>12.18 ± 0.021</td>
<td>0.200</td>
<td>3.47</td>
</tr>
<tr>
<td>Isotope incorporation controls²</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>3</td>
<td>2.65 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>3</td>
<td>2.73 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>3</td>
<td>2.62 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>3</td>
<td>2.67 ± 0.11</td>
<td></td>
<td></td>
</tr>
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</table>

1Values are means ± SD.
²k_d's for subcellular protein fractions are from ref. 14.
³The isotope incorporation control group consisted of three rats fed the control diet and injected with the two isotopes of leucine simultaneously.
TABLE 3

Isotope ratios and apparent half-lives of rat hepatic xanthine oxidase and ornithine transcarbamylase

<table>
<thead>
<tr>
<th>Diet group</th>
<th>n</th>
<th>$^{3}H/^{14}C$</th>
<th>k</th>
<th>$t_{1/2}$</th>
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<tbody>
<tr>
<td>Xanthine oxidase</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>20P35Fe</td>
<td>4</td>
<td>9.05 ± 0.62</td>
<td>0.144 ± 0.014</td>
<td>4.81</td>
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<tr>
<td>20P5Fe</td>
<td>4</td>
<td>7.01 ± 0.06</td>
<td>0.092 ± 0.001</td>
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</tr>
<tr>
<td>5P35Fe</td>
<td>4</td>
<td>7.62 ± 0.33</td>
<td>0.109 ± 0.009</td>
<td>6.36</td>
</tr>
<tr>
<td>5P5Fe</td>
<td>5</td>
<td>7.03 ± 0.35</td>
<td>0.093 ± 0.010</td>
<td>7.45</td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td></td>
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</tr>
<tr>
<td>20P35Fe</td>
<td>4</td>
<td>7.08 ± 0.31</td>
<td>0.094 ± 0.009</td>
<td>7.37</td>
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<tr>
<td>20P5Fe</td>
<td>4</td>
<td>6.91 ± 0.42</td>
<td>0.089 ± 0.013</td>
<td>7.79</td>
</tr>
<tr>
<td>5P35Fe</td>
<td>4</td>
<td>6.21 ± 0.86</td>
<td>0.065 ± 0.029</td>
<td>10.66</td>
</tr>
<tr>
<td>5P5Fe</td>
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<td>5.40 ± 0.29</td>
<td>0.038 ± 0.011</td>
<td>18.24</td>
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<tr>
<td>Xanthine oxidase</td>
<td></td>
<td></td>
<td></td>
<td>3.15 ± 0.13</td>
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<tr>
<td>Ornithine transcarbamylase</td>
<td></td>
<td></td>
<td></td>
<td>2.92 ± 0.24</td>
</tr>
</tbody>
</table>

Values are means ± SD. Values in the same column not sharing a common superscript letter are significantly different (P < 0.05).

The two-way ANOVA indicated that protein but not iron had a significant effect (P < 0.001) with no statistical interaction.

The isotope incorporation control group consisted of three rats fed the control diet and injected with the two isotopes of leucine simultaneously.

Xanthine oxidase from rats fed the 20P35Fe (control) diet. Thus, the apparent half-life of xanthine oxidase was significantly greater in rats fed low protein or low iron diets. The half-life of xanthine oxidase increased from 4.8 d in rats fed the control diet to 6.4 d in rats fed the 5P35Fe diet and to 7.5 d in rats fed low iron diets at either level of dietary protein.

The turnover of ornithine transcarbamylase was measured as a control because the amount of ornithine transcarbamylase protein changes with dietary protein (23) but not with dietary iron (Table 3). The apparent half-life of ornithine transcarbamylase was significantly increased in rats fed low dietary protein but was not affected in rats fed low dietary iron. Thus, ornithine transcarbamylase served as a negative control for the effect of dietary iron.

The $^{3}H/^{14}C$ ratios in immunoprecipitated xanthine oxidase and ornithine transcarbamylase were also measured in the isotope incorporation control rats (Table 3). The isotope ratios in xanthine oxidase and ornithine transcarbamylase were very close to the expected value of 3.0 and showed that the two different isotopes of leucine were incorporated into xanthine oxidase and ornithine transcarbamylase to the same extent.

Xanthine oxidase activity and immunologically detectable protein. The specific activity of xanthine oxidase was significantly lower in rats fed the low protein diets than in the rats fed control diets, but enzyme activity was not affected in the rats fed the low iron diets at either level of dietary protein (Table 4).

The amount of xanthine oxidase protein was measured by an immunoassay (Table 5). When the concentration of xanthine oxidase was expressed as pmol of xanthine oxidase/mg of hepatic protein in the supernatant, both dietary protein and iron exerted significant effects on the amount of xanthine oxidase protein, and there was no statistical interaction between these dietary variables. There was a loss in xanthine oxidase protein in rats fed low dietary protein and an increase in rats fed low dietary iron. When the amount of xanthine oxidase was expressed as pmol of xanthine oxidase/g of liver, only dietary protein had a significant effect.

TABLE 4

Growth, hepatic protein and xanthine oxidase activity

<table>
<thead>
<tr>
<th>Diet group</th>
<th>n</th>
<th>Final body weight g</th>
<th>Liver weight g</th>
<th>Homogenate protein mg/ml</th>
<th>Xanthine oxidase activity u/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>20P35Fe</td>
<td>8</td>
<td>265 ± 11</td>
<td>9.88 ± 0.86</td>
<td>22.5 ± 1.2</td>
<td>0.010 ± 0.002</td>
</tr>
<tr>
<td>20P5Fe</td>
<td>6</td>
<td>225 ± 23</td>
<td>7.47 ± 0.74</td>
<td>22.3 ± 1.2</td>
<td>0.014 ± 0.006</td>
</tr>
<tr>
<td>5P35Fe</td>
<td>5</td>
<td>104 ± 8</td>
<td>3.69 ± 0.35</td>
<td>20.2 ± 2.0</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>5P5Fe</td>
<td>6</td>
<td>106 ± 16</td>
<td>3.78 ± 0.62</td>
<td>14.9 ± 1.2</td>
<td>0.002 ± 0.001</td>
</tr>
</tbody>
</table>

Values are means ± SD. Values in the same column not sharing a common superscript letter are significantly different.

The two-way ANOVA indicated that protein but not iron had a significant effect on liver xanthine oxidase activity (P < 0.0001) with no statistical interaction between the variables.
effect on the amount of xanthine oxidase. The rats fed the low protein diets at both levels of dietary iron had significantly lower amounts of xanthine oxidase. When the results are expressed as pmol of xanthine oxidase/100 g of body weight, the data show the same pattern as when expressed in pmol of xanthine oxidase/g of liver because the livers represented the same percentage of body weight in all diet groups (Table 5).

**Percentage of active xanthine oxidase.** In rats fed the 20% protein diets, approximately 65–76% of the subunits of xanthine oxidase were active (Table 5). Rats fed the 5% protein diets had a significantly lower percentage of active xanthine oxidase subunits, with only 15–20% of the subunits active. There was no significant effect of low dietary iron on the percentage of active xanthine oxidase. Thus, low dietary protein resulted in a decrease in both the amount of xanthine oxidase protein and the fraction of the remaining xanthine oxidase that was active.

**DISCUSSION**

We measured rat liver xanthine oxidase activity, immunologically detectable protein and fractional turnover rate, from which we calculated the apparent half-life of xanthine oxidase subunits that were active. The xanthine oxidase in rats fed the control diet had an apparent half-life of 4.8 d and approximately 65% of the enzyme subunits were active. The xanthine oxidase in rats fed the control diet had an apparent half-life of 4.8 d and approximately 65% of the enzyme subunits were active. Rats fed diets with 5% dietary protein had lower xanthine oxidase activity, but this enzyme had a slower turnover, resulting in a longer apparent half-life (6.4 d) compared with controls. This is an apparently paradoxical situation in which the xanthine oxidase activity decreased, but the lifetime of the protein increased. Only 15–20% of the xanthine oxidase protein was active in rats fed the low protein diets, indicating that these rats had a large pool of inactive xanthine oxidase protein.

In rat liver, xanthine oxidase may be inactive due to lack of molybdenum in the enzyme (demolybdo enzyme) or lack of terminal sulfide on the molybdenum (desulfo enzyme) [24]. It is not yet known whether the demolybdo and desulfo forms are intermediates in the biosynthesis of active enzyme or whether they represent enzyme that has been inactivated.

Several mechanisms may be postulated to explain how xanthine oxidase activity could be decreased while at the same time the half-life could be increased. If the decrease in xanthine oxidase activity were mediated by inactivation of the enzyme, and the inactive xanthine oxidase were more resistant than active enzyme to degradation, then inactive enzyme would accumulate, resulting in a longer apparent half-life of the total xanthine oxidase and a lower percentage of xanthine oxidase that was active. Alternatively, if there was a pool of xanthine oxidase that was not fully assembled (i.e., lacking molybdenum or the terminal sulfide on the molybdenum), this enzyme might not be degraded as rapidly as fully assembled, active xanthine oxidase. From our data, we cannot directly show that the pools of active and inactive enzyme turn over at different rates, but our apparent half-life for total xanthine oxidase protein in rats fed low protein diets (6.4 d) is longer than the half-life for xanthine oxidase activity (4.0 d) in response to low protein diets (1, 10). Therefore, the longer half-life of total enzyme would be due to both the rate of degradation of active enzyme and a very low rate of degradation of inactive enzyme. The function of the pool of inactive xanthine oxidase is not known, but the inactive enzyme may serve as a reserve that could be rapidly activated when needed.

The apparent half-life of xanthine oxidase increased significantly from 4.8 d in control rats to 7.5 d in rats fed diets with low dietary iron. Unlike the low protein diets, the low iron diets did not affect the specific activity of xanthine oxidase or the percentage of active hepatic xanthine oxidase subunits at either level of dietary protein. The longer half-life of xanthine oxidase in rats fed low dietary iron may serve to stabilize the pool of active enzyme and conserve iron (xanthine oxidase has eight iron atoms per enzyme).

The protocol that we used met the criteria of Arias,
Doyle and Schimke (12) and also met the criterion that the two isotopes should be incorporated into proteins in the same proportion (14). The isotope ratios in the immunoprecipitated xanthine oxidase and ornithine transcarbamylase from the control rats injected simultaneously with both isotopes were very close to 3.0. However, the isotope ratios in the subcellular protein fractions from the isotope incorporation control rats were 2.6–2.7; perhaps this was due to quenching of the $^3$H in the protein fractions.

The fractional turnover rate of liver proteins in rats in response to a low protein diet is specific for the individual proteins and does not occur with all proteins (25, 26). We measured the half-life of ornithine transcarbamylase as a control for nonspecific changes in protein turnover in response to dietary iron and found that the apparent half-life did not change in response to low dietary iron.

Our conclusions do not agree with those of Rowe and Wyngaarden (1), who found that the ratio of active to inactive xanthine oxidase was unaffected by low dietary protein. They measured xanthine oxidase protein with a purification procedure, and less stable forms of the enzyme may not withstand the conditions of a purification procedure, so the method may tend to selectively purify active enzyme.

In conclusion, we found that the regulation of hepatic xanthine oxidase by dietary protein and by dietary iron was quite different, but the apparent half-life of the enzyme was increased by both dietary treatments. In future studies, we plan to isolate the inactive xanthine oxidase from rats fed low dietary protein and iron and characterize the properties of the enzyme from these studies to investigate further how these enzymes are inactivated. The turnover of enzymes is regulated by mechanisms for altering the content of enzymes by changes in rates of degradation as well as in rates of synthesis. Thus the amount of xanthine oxidase activity represents a balance between the rates of synthesis and degradation of protein as well as interconversion of active and inactive enzyme.

**LITERATURE CITED**


