RELATION OF URIC ACID METABOLISM TO RELEASE OF IRON FROM HEPATIC FERRITIN*

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In a previous study (1) we observed a marked increase in plasma iron of dogs subjected to drastic hypotension in the course of fatal experimental hemorrhagic shock. It was suggested that the origin of the increased plasma iron was storage ferritin of the liver and that the stimulus for its release was liver hypoxia. Anaerobic incubation of ferritin with liver slices resulted in an increase of its ferrous iron content which was now capable of dissociation for combination with iron-binding agents such as α,α'-dipyridyl or the plasma iron-binding protein.

In the present study the mechanism of ferritin iron reduction and the nature of the compounds involved in this reaction have been investigated. It has been found that anaerobic rat liver slices produce large quantities of uric acid, hypoxanthine, and xanthine, which are freely diffusible into the medium. Of these compounds, only uric acid reduces ferritin iron directly. However, in the presence of ferritin, the oxidation of hypoxanthine or of xanthine by xanthine oxidase takes place anaerobically; in this reaction ferritin acts as an electron acceptor and its iron is reduced to the ferrous state. The reduction of ferritin iron is, therefore, brought about both by the dehydrogenase activity of xanthine oxidase and by the accumulated uric acid formed by this enzyme.

EXPERIMENTAL

Crystalline horse spleen ferritin was prepared by a method described in previous studies (2). All tissues used were from Wistar strain female rats, weighing 150 to 200 gm. Tissue slices were prepared as for the usual microrespiration studies, with the exception that the organs were first perfused in situ with ice-cold Ringer-phosphate solution. In the case of the small intestine, the contents were washed with cold Ringer-phosphate solution, the intestine was cut longitudinally and washed again, and then cut into small strips before being weighed. For incubation experiments, 1.0 gm.

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of liver slices, 0.5 gm. of kidney cortex slices, 0.25 gm. of spleen slices, or 0.5 gm. of intestinal strips was incubated in 50 ml. Erlenmeyer flasks with 5 ml. of Ringer's phosphate at 37° with continuous shaking.

Uric acid, xanthine (sodium), and hypoxanthine were obtained from the Schwarz Laboratories, Inc. Uricase (assaying 125 units per ml.) and milk xanthine oxidase (assaying 15,000 units per ml.) were partially purified preparations from the Worthington Biochemical Corporation, and catalase was a crystalline preparation from the same source and assayed 5000 units per ml. Reduced triphosphopyridine nucleotide (TPNH) cytochrome c reductase was a gift from Dr. B. Horecker. TPN, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Company. Calf liver xanthine oxidase was a partially purified preparation made according to Kielley (3). 2-Amino-4-hydroxy-6-pteridine aldehyde was a gift from the American Cyanamid Company.

**Measurements of Fe**++ **in Ferritin**—Fe**++** in ferritin was not measured quantitatively, but instead the amount of Fe**++** bound by α,α′-dipyridyl was determined colorimetrically. The amount of Fe**++** of ferritin which is bound by dipyridyl represents a comparative measure of its Fe**++** content and varies with the concentration of the ferritin and the pH of the solution (1); more Fe**++** is bound at acid pH than at neutral or slightly alkaline pH. In order to conserve ferritin during the following fractionation studies, the dipyridyl reaction was carried out at pH 4.6. For measurement of the reduction of ferritin iron, 1 ml. of a stock ferritin solution containing 6.0 mg. of total iron was added to 1 ml. of 1 M acetate buffer, pH 4.6, and 1 ml. of 0.2 per cent α,α′-dipyridyl dissolved in water. 1 ml. of the solution to be tested for reducing activity was then added and the mixture incubated for 1 hour at 37°. 4 ml. of a saturated solution of ammonium sulfate, previously adjusted to pH 4.6 with sulfuric acid, were then added in order to precipitate the ferritin, and the mixture was centrifuged. The clear, pink-colored supernatant solution, containing the Fe**++**-dipyridyl complex, was read in a Klett photocolorimeter, with use of a No. 52 filter, against a blank solution which contained all the reagents except ferritin. Fe**++** content was calculated by comparison with a standard solution of Fe**++** treated with the same reagents. Reduction of ferritin iron at pH 7.4 was also measured by incubating 1 ml. of ferritin with 1 ml. of 0.5 M phosphate buffer, pH 7.4, 1 ml. of dipyridyl, and 1 ml. of the solution to be tested for reducing activity. After 1 hour the reaction mixture was quickly treated with 6 ml. of an ice-cold solution containing 1 part acetate buffer, pH 4.6, and 5 parts saturated ammonium sulfate, and the entire mixture was centrifuged. The clear supernatant fluid was read in a colorimeter as above.
Preparation of Ferritin Iron-Reducing Substance from Rat Liver—Rat liver slices were incubated in the proportion of 1 gm. of slices to 5 ml. of Ringer-phosphate solution for varying periods of time in 100 per cent oxygen or nitrogen. The media were freed from particulate matter by centrifugation, heated in a water bath to 80°, and again clarified by centrifugation. 1 ml. aliquots were added to 1 ml. of stock ferritin solution, and the mixture was assayed for Fe++ at pH 4.6. The results (Table I) demonstrate that no reducing substance is present in the medium in which slices are incubated in oxygen, whereas increasing quantities of ferritin iron-reducing substance appear in the medium in which slices are incubated under anaerobic conditions. Most of the reducing activity appears within 1 hour.

**Table I**

**Formation of Ferritin Iron-Reducing Substance by Anaerobic Rat Liver Slices**

1 gm. liver slices were incubated with 5 ml. of Ringer-phosphate solution, pH 7.4, at 37°. Ferritin-ferrous iron measured at pH 4.6 after mixing 1 ml. of ferritin with 1 ml. of supernatant solution. Values for ferritin iron-reducing activity have been corrected by subtracting Fe++ content of ferritin in the absence of reducing substance.

<table>
<thead>
<tr>
<th>Incubation time (min.)</th>
<th>Gas phase</th>
<th>Ferritin iron reduced by supernatant solution (µmoles Fe++ per mmole total ferritin iron)</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>Oxygen</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>Nitrogen</td>
<td>3.2</td>
</tr>
<tr>
<td>30</td>
<td>&quot;</td>
<td>5.0</td>
</tr>
<tr>
<td>60</td>
<td>&quot;</td>
<td>7.0</td>
</tr>
<tr>
<td>120</td>
<td>&quot;</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Purification of Ferritin Iron-Reducing Substance—35 gm. of pooled rat liver slices were incubated in several flasks, with a total of 175 ml. of Ringer-phosphate solution, for 90 minutes in nitrogen. The cell-free supernatant fluid was heated to 80° and the coagulated proteins were removed by centrifugation. The clear solution (146 ml.) is called Fraction A in Table II, which lists the ferritin iron-reducing activity of this and subsequent fractions in terms of mg. of Fe++ formed per mg. of total N in the fraction. Corrections were made in the calculations of total activity for aliquots removed for analyses. A recovery of more than 100 per cent in subsequent fractions suggests the presence of impurities in Fraction A which resulted in low values for ferritin iron reduction.

Fraction A was treated with a 25 per cent solution of basic lead acetate (about 30 ml.), added dropwise, with constant stirring, until precipitation ceased. The mixture was allowed to stand overnight in the refrigerator,
was centrifuged, and the precipitate washed twice with 10 ml. each of 1 per cent lead acetate solution. The solution and washings were discarded, since no ferritin iron-reducing activity could be demonstrated after removal of the lead with H₂S and removal of the latter by concentration of the solution. The lead precipitate was extracted five times with 20 ml. each of 0.2 N HCl and the combined extracts were centrifuged. The supernatant fluid was then treated with H₂S, the PbS was filtered and the precipitate washed with 0.2 N HCl, and excess H₂S was removed from the filtrate by a stream of nitrogen. The filtrate was evaporated in vacuo to dryness at 35–45°, and the residue was taken up in a minimum of water. After neutralization with dilute NaOH and adjustment of the volume to 10 ml., this fraction was labeled Fraction B.

Fraction B was placed on a 2 × 12 cm. column of low porosity cation exchange resin (Dowex 50-X12, 50 to 100 mesh) on the H⁺ cycle, and the

| Table II |
| Ferritin Iron-Reducing Activity and Uric Acid Content of Fractions Obtained during Purification Procedure |

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ferritin iron-reducing activity</th>
<th>Uric acid content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg. Fe³⁺ formed per mg. total N</td>
<td>total mg. Fe³⁺ formed</td>
</tr>
<tr>
<td>A</td>
<td>0.038</td>
<td>1.1</td>
</tr>
<tr>
<td>B</td>
<td>0.410</td>
<td>1.6</td>
</tr>
<tr>
<td>C</td>
<td>1.220</td>
<td>1.3</td>
</tr>
</tbody>
</table>

column was washed with water (100 ml.) until the effluent was neutral to litmus paper. The solution was evaporated in vacuo as before, neutralized, and made to 10 ml. with water. The material adsorbed on the resin was eluted with 100 ml. of N NH₄OH, evaporated to dryness, and dissolved in water. The first effluent, containing material not adsorbed by the resin, contained all of the reducing activity and is referred to as Fraction C.

**Identification of Ferritin Iron-Reducing Substance with Uric Acid**—The reducing activity of Fraction C, calculated from the total nitrogen content, had been concentrated thirty times. In qualitative tests, Fraction C gave a positive reaction with silver nitrate in alkaline solution and a blue color with the Folin phosphotungstic acid reagent, commonly used for the estimation of uric acid (4). The solution had an absorption spectrum with a maximum identical with that of pure uric acid (Fig. 1). Treatment of aliquots of Fraction C with excess uricase resulted in the complete disappearance of the color reaction with the uric acid reagent, of the ferritin iron-reducing activity, and of the absorption maximum at 292 μμ.
To make certain that the purification procedure had not eliminated another reducing substance present in the original solution, Fraction A was treated with uricase with the same results as those given above for Fraction C. The values for each fraction in Table II therefore represent "true" uric acid content.

Reduction of Ferritin Iron by Uric Acid—Although, during the isolation procedure, ferritin iron-reducing activity was measured at pH 4.6, reduction by pure uric acid was now measured at both pH 4.6 and 7.4. With 50 μ of uric acid, at pH 7.4, the Fe^{++} content of ferritin rose from 1.3 to 2.1 μmoles per mmole of total ferritin iron; at pH 4.6, the increase was from 2.3 to 6.5. This difference reflects the greater availability, at the more acid pH, of ferritin-ferric iron for reduction. At both pH values the extent of reduction was found to vary with the quantity of uric acid added.

Uric Acid Accumulation in Anaerobic Liver—To determine whether an over-all synthesis of uric acid had occurred in the anaerobic liver slice or whether that normally present in the tissue had merely diffused out of the slice under the influence of lowered oxygen tension (lowered permeability), aliquots of liver slices were prepared and incubated, one in nitrogen and another in oxygen, for 1 hour, as described above. In addition, a third (control) aliquot of slices was placed immediately in 10 ml. of boiling water for 1 minute. The heated tissue suspension was homogenized and the
cooled homogenate diluted with 0.1 M phosphate buffer, pH 7.4, to 25 ml. The incubated tissues were separated from their media, and each was treated as above. The media were also heated to boiling and aliquots of all specimens analyzed for uric acid as follows: Two equal aliquots of each sample were mixed separately with 2 ml. of 0.1 M phosphate buffer, pH 7.4. One aliquot was treated with 0.3 ml. of a 1:10 uricase solution and the other with an equal quantity of water. (This quantity of uricase had been shown in preliminary experiments to be far in excess of that required, at pH 7.4, to destroy all uric acid present.) Both solutions were made to 10 ml. with water and incubated for 1 hour with shaking at 37°. After

### Table III

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Fraction</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Small intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no incubation)</td>
<td>Tissue</td>
<td>12.4</td>
<td>4.1</td>
<td>25.0</td>
<td>32.2</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Tissue</td>
<td>8.1</td>
<td>14.6</td>
<td>26.2</td>
<td>51.0</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>0.0</td>
<td>142.0</td>
<td>155.5</td>
<td>377.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>8.1</td>
<td>156.6</td>
<td>181.7</td>
<td>428.0</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Tissue</td>
<td>14.5</td>
<td>7.0</td>
<td>26.8</td>
<td>23.7</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>72.7</td>
<td>30.7</td>
<td>78.5</td>
<td>171.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>87.2</td>
<td>37.7</td>
<td>105.3</td>
<td>194.7</td>
</tr>
</tbody>
</table>

incubation, each solution was treated with 2 ml. of 10 per cent sodium tungstate and 1.8 ml. of N sulfuric acid, to stop the reaction and to remove protein. 10 ml. aliquots of the clear supernatant solutions obtained by centrifugation were mixed with 2.5 ml. of urea-cyanide reagent and 1 ml. of uric acid reagent, and the colors were compared after 30 minutes, in the Klett photocolorimeter, with that produced by a known quantity of uric acid treated in a similar fashion. A No. 66 filter was used. The concentrations of "true" uric acid were calculated by subtracting the values due to reducing non-uric material remaining after uricase treatment from the totals. In this way the values were obtained for the uric acid content of control tissue, tissue and medium after oxygen incubation, and tissue and medium after nitrogen incubation. The results (Table III) demonstrate for the liver a marked accumulation of uric acid in the anaerobic slices. The extra uric acid was diffusible into the medium.
Effect of Oxygen Tension on Uric Acid Accumulation in Liver Slices—
The relationship between oxygen tension and uric acid production by the liver in vitro was studied in a manner similar to that described above, except that the oxygen content of the gas phase was varied. After the incubation mixtures were heated to 80°, the clear supernatant media were analyzed. Accumulation of uric acid in liver slices occurred at oxygen tension of air (20 per cent) and increased markedly at lower tensions. Amounts of uric acid formed per 5 ml. of medium were 7 γ at 20 per cent, 35 γ at 10 per cent, and 100 γ at zero per cent oxygen (tank nitrogen).

**Table IV**

*Formation of Uric Acid and Hypoxanthine Plus Xanthine during Aerobic or Anaerobic Incubation*

The conditions are as described in the text. The results are expressed as micromoles per organ in the rat; wet weights of organs were 8.5 gm. of liver, 1.0 gm. of spleen, 2.2 gm. of kidneys, and 6.6 gm. of small intestine.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No incubation</th>
<th>Oxygen</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>1.05</td>
<td>0.69</td>
<td>7.40</td>
</tr>
<tr>
<td>Hypoxanthine + xanthine</td>
<td>1.62</td>
<td>0.76</td>
<td>25.60</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.20</td>
<td>1.38</td>
<td>0.71</td>
</tr>
<tr>
<td>Hypoxanthine + xanthine</td>
<td>0.22</td>
<td>0.29</td>
<td>2.18</td>
</tr>
<tr>
<td>Kidneys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.09</td>
<td>3.45</td>
<td>0.83</td>
</tr>
<tr>
<td>Hypoxanthine + xanthine</td>
<td>0.53</td>
<td>3.88</td>
<td>9.74</td>
</tr>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>2.13</td>
<td>28.25</td>
<td>12.85</td>
</tr>
<tr>
<td>Hypoxanthine + xanthine</td>
<td>3.26</td>
<td>3.46</td>
<td>23.90</td>
</tr>
</tbody>
</table>

Uric Acid Production in Other Rat Tissues—The accumulation of uric acid in kidney cortex, spleen, and small intestine was measured in both oxygen and nitrogen in a manner similar to that described above for liver. The data (Table III) show that uric acid is formed in these three tissues in the presence and the absence of oxygen. However, only in the liver does more uric acid accumulate anaerobically than aerobically. These results also reveal that aerobic conditions produce high uric acid levels per gm. of kidney, spleen, and especially intestine. To show the relationship of these values in the whole animal, the results in Table IV have been calculated in terms of the contributions of whole wet weight organs.

Anaerobic Accumulation of Hypoxanthine and Xanthine in Rat Tissues—Since, so far as is known, the formation of uric acid in rats is dependent upon oxidation, by xanthine oxidase, of hypoxanthine and xanthine, these
compounds were estimated in tissues treated as above. For these experiments, instead of analysis of the incubated tissue and medium separately, the entire incubation mixture was heated and homogenized, and aliquots were removed for analyses. The "true" uric acid content of one aliquot of the homogenate was determined by the uricase procedure. Two additional aliquots were treated with excess xanthine oxidase to convert all of the xanthine and hypoxanthine to uric acid. After this, one sample was treated with excess uricase to destroy the uric acid present originally, as well as that formed from hypoxanthine and xanthine by the action of xanthine oxidase. The quantity of uric acid formed from the xanthine and hypoxanthine could now be calculated by subtraction of the original uric acid content and correction for any reducing material remaining after uricase treatment. Table IV shows that in all tissues anaerobiosis caused a marked increase in concentration of xanthine and hypoxanthine. To determine the relative amounts of hypoxanthine and xanthine present in liver, the spectrophotometric method of Kalckar (5) was employed in one experiment. They were found to be present in approximately equal quantities.

Of the four tissues studied in the rat, uricase was found to be present only in the liver. Uricase activity was determined by the method of Leone (6) and was equal to 162 μl. of O₂ per 100 mg. of wet weight of tissue per hour at 37°.

Effect of Oxygen Tension on Uricase and Xanthine Oxidase Activities—In order to explore the factors influencing uric acid accumulation in anaerobic liver, the sensitivities of the enzymes involved in uric acid metabolism to lowered oxygen tension were measured with use of partially purified enzymes. In the uricase experiments, each flask contained 2 ml. of 0.1 M glycine buffer, pH 9.4, 0.5 ml. of a 1:10 uricase solution, and 2.45 ml. of water. A neutralized solution of 60 μ of uric acid in a volume of 0.5 ml. was suspended, in a small plastic cup, from a rubber stopper which was also fitted with inlet and outlet tubes used for the appropriate gas mixture. After preliminary incubation for temperature and gas equilibration, the inlet and outlet tubes were clamped and the cup containing uric acid was tipped into the reaction mixture. After 15 minutes, the reaction was stopped by addition of tungstate and sulfuric acid and the solution analyzed for uric acid, as described previously. In xanthine oxidase experiments, xanthine or hypoxanthine was used as substrate. Reaction mixtures contained 2 ml. of 0.1 per cent albumin in 0.1 M phosphate buffer, pH 7.4, 0.03 ml. of a 1:25 xanthine oxidase solution, and 2.72 ml. of water. The substrate, containing 250 μ of hypoxanthine, or an equivalent quantity of xanthine, in 0.25 ml., was suspended in the plastic cup. The xanthine reaction was allowed to continue for 15 minutes and the hypoxanthine
reaction for 75 minutes before analyses for uric acid were made. Fig. 2 shows the relative activities of the enzymes at various oxygen tensions, with an activity of 100 representing that of the respective enzyme in 100 per cent oxygen. Uricase is affected to a greater extent than xanthine oxidase as the oxygen tension is lowered. When xanthine is used as a substrate, xanthine oxidase activity is somewhat inhibited by oxygen tensions above 60 per cent. A similar inhibition of xanthine oxidase activity by high oxygen pressure has been reported by Stadie and Haugaard (7).

Ferritin As Electron Acceptor for Xanthine Oxidase—Xanthine oxidase can act as a dehydrogenase in the presence of electron acceptors other than molecular oxygen, such as methylene blue (8) or ferricytochrome c (9). In liver slice experiments, uric acid diffuses out of the anaerobic cells and reduces ferritin in the medium, but, in the intact liver, uric acid would be synthesized inside the cell in the presence of ferritin. We therefore explored the possibility that the process of uric acid formation, involving the action of xanthine oxidase, might bring about ferritin iron reduction, in which case the ferric iron in ferritin would act as the electron acceptor for the enzyme. For this purpose, reaction mixtures were prepared consisting of 1 ml. of ferritin previously adjusted to pH 7.4 with dilute alkali and containing 6.13 mg. of total iron, 1 ml. of 0.2 per cent α,α'-dipyridyl to act as a trapping agent for any Fe^{++} formed, 1 ml. of 0.1 M phosphate buffer, pH 7.4, 0.03 ml. of 1:25 milk xanthine oxidase, and water to make a final volume of 4 ml. A suspended plastic cup contained 250 γ of xan-
thine in 0.25 ml. or an equivalent quantity of hypoxanthine. After temperature and gas equilibration, the reaction was started by tipping the cup and its contents into the reaction mixture. At appropriate intervals, 6 ml. of a chilled mixture, containing 1 part of 1 M acetate buffer, pH 4.6, and 5 parts of saturated ammonium sulfate previously adjusted to pH 4.6, were added to each flask. The mixture was centrifuged and the supernatant fluid compared in the colorimeter with a Fe^{++} standard treated in the same manner.

![Graph]

FIG. 3. Action of ferritin iron as electron acceptor in the xanthine oxidase system
Curve A, ferritin reduction by enzyme in absence of oxygen; Curve B, ferritin reduction by enzyme in presence of 100 per cent oxygen; Curve C, same as Curve B plus 0.05 mg. of crystalline catalase; Curve D, same as Curve B plus 0.5 mg. of crystalline catalase; Curve E, same as Curve A or Curve B plus 2.25 \gamma per ml. of 2-amino-4-hydroxy-6-pteridine aldehyde.

The results obtained with hypoxanthine as a substrate are shown in Fig. 3. Appreciable reduction of ferritin iron occurs in the complete absence of oxygen as indicated by Curve A. The direct reaction of ferritin with xanthine oxidase is apparent from the fact that, in the absence of ferritin, no uric acid is formed anaerobically. More ferritin iron was reduced when the reaction was carried out in the presence of 100 per cent oxygen (Curve B). In addition, experiments carried out with hypoxanthine as well as xanthine as substrates, in the presence of oxygen tensions between zero and 100 per cent, yielded values for ferritin iron reduction intermediate between those obtained in 100 per cent nitrogen and in 100 per cent oxygen. Similar results were also obtained with xanthine ox-
idase prepared from calf's liver by the method of Kielley (3). Curves C and D in Fig. 3 demonstrate that the aerobic reduction of ferritin iron is increased by the addition of crystalline catalase over a 10-fold range of concentration (0.05 mg. and 0.5 mg., respectively, of crystalline enzyme), suggesting that xanthine oxidase activity is inhibited by peroxide formed during the reaction (10). That xanthine oxidase was responsible for ferritin iron reduction was made more certain by our finding that this reduction was completely inhibited (Curve E) in the presence of 2.25 γ per ml. of 2-amino-4-hydroxy-6-pteridine aldehyde (11). This inhibition occurred in both nitrogen and oxygen.

Reduction of Ferritin Iron by TPNH Cytochrome c Reductase—Weber et al. (12) have shown that several flavoprotein enzymes are capable of reducing inorganic ferric iron in the presence of citrate, among them xanthine oxidase and TPNH cytochrome c reductase. Since cytochrome c reductase is also found in liver, experiments were performed in vitro to investigate its ability to reduce ferritin iron. The reaction mixture contained 1 ml. of ferritin, 1 ml. of 0.2 per cent dipyridyl, 1 ml. of 0.1 M phosphate buffer, pH 7.55, 0.1 ml. of glucose 6-phosphate (1.65 mg.), 0.02 ml. of glucose-6-phosphate dehydrogenase (0.2 mg.), 0.05 ml. of triphosphopyridine nucleotide (25 γ), and water to make a final volume of 4 ml. The reaction was started by the addition of 0.05 ml. of a partially purified cytochrome c reductase preparation. The purpose of the glucose 6-phosphate and its dehydrogenase was to insure a constant source of TPNH, as in an assay method described by Haas (13). The original ferritin, assayed at pH 7.4, contained 0.45 μmole of Fe++ per mmole of total iron. In the absence of the cytochrome c reductase, the TPNH generated by the dehydrogenase system itself caused an increase in Fe++ to 0.56 μmole. Upon addition of the reductase, the Fe++ content rose to 1.22, in 100 per cent oxygen as well as in 100 per cent nitrogen. However, TPNH in rat liver slices was found to be lower after anaerobic incubation (16 γ per gm. of wet weight) than after aerobic incubation (57 γ per gm.), decreasing the likelihood that cytochrome c reductase is important for ferritin iron reduction.

Plasma Uric Acid of Rats in Hemorrhagic Shock—The results of our experiments performed in vitro suggest that the plasma uric acid level should increase as a result of liver hypoxia in animals subjected to hemorrhagic shock. Zweifach et al. (14) and Van Slyke (15) reported increased plasma uric acid concentrations in dogs in hemorrhagic shock. In a series of experiments, to be reported in detail at a later date, we have confirmed these findings for the rat in hemorrhagic as well as in traumatic (drum) (16) shock, and for the dog in hemorrhagic shock. Since the drastic hypo-

1 We are indebted to Dr. Baer, Dr. Srikantia, and Anne Carleton for providing us with blood samples from their rats and dogs in hemorrhagic shock for these uric acid analyses.
tension induced in the hemorrhagic shock experiment could be presumed to result not only in liver hypoxia but in renal failure as well, it was necessary to determine whether the observed rise in plasma uric acid could be attributed to renal failure. For this purpose plasma was collected 3 hours after bilateral nephrectomy of twelve control rats. Plasma uric acid, expressed as mg. per 100 ml., averaged 0.6 (range 0.3 to 1.1) in eleven normal control rats; in the twelve renal controls the average was 2.4 (range 1.1 to 2.8), and in eleven normal rats subjected to hemorrhagic shock the values averaged 8.7 (range 4.9 to 14.5).

**DISCUSSION**

In our earlier experiments, designed to study the mechanism by which ferritin iron is reduced, liver slices were incubated anaerobically in a medium containing ferritin (1). Under these conditions, reduction of ferritin iron could only be a result of diffusion into the medium of a compound of low molecular weight, since it would not be reasonable to expect that ferritin would enter the cell, be reduced, and then diffuse out into the medium. The reducing compound produced by anaerobic liver slices has now been identified as uric acid, and its concentration has been found to increase as oxygen tension is decreased to 20 per cent and below. *In vivo*, reduction of ferritin iron would take place inside the hepatic cell in the presence of hypoxanthine, xanthine, and the enzyme, xanthine oxidase, which converts these substrates to uric acid.

Xanthine oxidase is an iron molybdoflavoprotein (17) classified as an aerobic dehydrogenase, since it can utilize molecular oxygen as an electron acceptor. In addition, methylene blue and ferricytochrome c react with this enzyme and are reduced. Ferritin iron can also act as an electron acceptor for reduced xanthine oxidase, and does so even in the complete absence of oxygen. The anaerobic reduction of ferritin iron is therefore primarily due to the enzyme and only secondarily to the uric acid which is formed. The presence of oxygen in the xanthine oxidase system increases ferritin iron reduction, but it is not possible to estimate how much of the reduction is due to reaction with the reduced enzyme and how much to the increased quantity of uric acid formed aerobically. Although it is assumed that ferritin iron is reduced by reaction with reduced flavin adenine dinucleotide of the enzyme, the possibility of reaction with the Fe or Mo of the enzyme still exists. In fact, Mackler et al. (18) have demonstrated that, although removal of Mo from the enzyme does not inhibit the reaction of the enzyme with molecular oxygen, it does inhibit its reaction with 1 electron acceptors.

Our results for the reduction of ferritin iron parallel those of Weber et al. (12) for the reduction of ferric citrate by xanthine oxidase, since in both
cases reduction is stimulated by the addition of oxygen, but Weber’s re-
action is inhibited in the presence of catalase whereas that of ferritin iron
is stimulated, suggesting that in the latter reaction peroxide acts as a xan-
thine oxidase inhibitor. A further difference in the two reactions is that
under anaerobic conditions reduction of ferritin iron is considerable,
whereas Weber et al. find that little reduction of ferric citrate occurs anaer-
obically. Both ferric citrate and ferritin iron are reduced by TPNH cyto-
chrome c reductase; however, it appears unlikely that this flavoprotein
enzyme plays a significant role in the reduction of ferritin iron \emph{in vivo} since
TPNH levels in rat liver slices were found to be lower after anaerobic
than aerobic incubation. The possibility of reduction by other flavopro-
tein enzymes is not ruled out.

Tanaka (19) also reported that, in the presence of hypoxanthine, xan-
thine oxidase reduced ferritin iron anaerobically. Ferrous iron was not
measured directly in this study. Instead, measurements were made of
changes in magnetic susceptibility of ferritin. However, the data given
by this author are contradictory in that the values reported for suscepti-
bility increased after treatment of ferritin with ascorbic acid and cysteine
and decreased after its incubation with xanthine oxidase. We have re-
peated these experiments\footnote{We are indebted to Dr. D. Rittenberg, Department of Biochemistry, Columbia University, for the use of his Gouy balance for the susceptibility measurements.} and have found that treatment of ferritin with
the xanthine oxidase system produces a rise in specific susceptibility of
ferritin iron, a result to be expected from the studies of Michaelis et al.
(20), who first pointed out the unusual state of iron in the ferritin mole-
cule and the effect of reducing agents such as Na$_2$S$_2$O$_4$.

Several factors apparently influence the accumulation of hypoxanthine
plus xanthine and of uric acid in the various tissues subjected to aerobic
or anaerobic incubation \emph{in vitro}. In all four tissues examined, liver, spleen,
kidney, and small intestine, the concentrations of hypoxanthine plus xan-
thine increased markedly in response to lowered oxygen tension. These
increases can be attributed to the progressive loss of high energy com-
pounds needed for synthetic reactions involving the purines (21) and to
an increased effectiveness of the catabolic enzymes acting on nucleotides
and nucleosides (22). Thus, the anaerobic sources of hypoxanthine and
xanthine may be purine derivatives, not as yet incorporated into nucleic
acids, as well as degraded nucleic acids. Jorgensen and Poulsen (23) have
reported a sharp rise in hypoxanthine and xanthine in stored red cells; in
our experiments this source was eliminated by thorough perfusion of the
organs before use.

In the liver, uric acid accumulates during anaerobic incubation and de-
creases slightly upon aerobic incubation as compared with unincubated
RELEASE OF IRON FROM HEPATIC FERRITIN

controls. Similar results have been reported by Bernheim and Bernheim (24). The anaerobic increase can be accounted for by the action of xanthine oxidase on the higher concentration of uric acid precursors together with anaerobic inhibition of the enzyme uricase. In the aerobic rat liver slice the decrease in uric acid is doubtless due to uricase action. In the non-hepatic tissues, on the other hand, we found less anaerobic than aerobic accumulation of uric acid. A possible explanation for this difference may be found in the experiments of Westerfeld and Richert (25), who have shown that the dehydrogenase activity (as measured by the increase in O2 uptake in the presence of methylene blue) of the xanthine oxidases in these non-hepatic tissues is lower than that in rat liver.

The results of our experiments in vitro, together with those which demonstrate increased concentrations of both uric acid and iron (1) in the plasma of animals in hemorrhagic shock, support the hypothesis that the xanthine oxidase system plays an important role in the process of iron reduction and release from hepatic ferritin. When the liver is supplied with adequately oxygenated blood, iron incorporation is accomplished by the withdrawal of iron from the plasma, as shown by experiments with radioactive iron (26). Under the same conditions (as demonstrated by our experiments in vitro at 20 per cent oxygen tension) small amounts of ferritin iron can be reduced and released into the plasma, at a rate consistent with the requirements of the hematopoietic system.

Our results emphasize lowered oxygen tension as a stimulus for increased formation of hypoxanthine, xanthine, and uric acid in the liver. In addition, tissues such as the spleen and small intestine also yield relatively large quantities of uric acid precursors which can be metabolized by the liver. These increases are reflected by an increased reduction and release of ferritin iron to the plasma via combination with the plasma iron-binding globulin, and explain the mechanism of release of extra iron to the plasma of animals in hemorrhagic shock. The response of the animal to lowered oxygen tension, outlined above, helps to explain the origin and mechanism for the release of extra iron needed by the bone marrow during the development of polycythemia at high altitudes. Since the life span of the red cell in a polycythemic animal is normal, the rate of iron release from ferritin stores must be increased in order to maintain a flow of iron, via the plasma, to the activated bone marrow. Our findings are substantiated by the frequency with which elevated plasma uric acid values occur in patients with polycythemia (27) and by the occasional occurrence of gout, secondary to prolonged polycythemia (28). Here, the increased uric acid, which must arise by virtue of the action of xanthine oxidase, may be a result of the degradation of large quantities of red cell nucleic acids, and of nuclei which are removed from the preerythrocyte cells of the bone
marrow. Now under investigation is the occurrence of the xanthine oxidase-ferritin system in bone marrow, spleen, and human placenta, organs which are known to contain ferritin, as well as the implications of these findings for various derangements of iron metabolism in animals.

SUMMARY

The reduction (and release) of ferritin iron during anaerobic incubation of ferritin with rat liver slices is due to the accumulation of uric acid in the tissue and its diffusion into the medium. Accumulation in anaerobic liver of uric acid precursors, hypoxanthine and xanthine, together with the marked sensitivity to low oxygen tensions of uricase as compared with xanthine oxidase, accounts for the elevated levels of uric acid.

Xanthine oxidase, prepared from milk or calf liver, is also capable of reducing ferritin iron under anaerobic conditions in the presence of hypoxanthine or xanthine. Reduction is increased in the presence of oxygen and by addition of catalase. Ferritin iron reduction is due to the activity of xanthine dehydrogenase, the iron of ferritin acting as an electron acceptor.

Although other flavoprotein enzymes can reduce ferritin iron, e.g. reduced triphosphopyridine nucleotide cytochrome c reductase, the role of xanthine dehydrogenase in the release of ferritin iron in vivo is substantiated by findings obtained with intact animals. Rats subjected to hemorrhagic hypotension show abnormally high concentrations of uric acid in the plasma. These results, together with the increases in plasma iron, reported previously for dogs in hemorrhagic shock, serve to relate the xanthine dehydrogenase system with the iron release mechanism.

The relationship of liver xanthine dehydrogenase, acting as a reducing agent for ferritin iron, to the release of iron into the plasma for extra hemoglobin synthesis by the bone marrow, under conditions of low oxygen tension, is discussed.

BIBLIOGRAPHY
