Enhancement by Nitrite of Peroxide-induced Degradation of Uric Acid and 3-N-Ribosyluric Acid

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Abstract

The oxidation of uric acid and 3-N-ribosyluric acid by hydrogen peroxide and methemoglobin was stimulated by the addition of sodium nitrite, which alone had no effect on the urates. The urates were not oxidized by either hydrogen peroxide alone or hydrogen peroxide and sodium nitrite unless methemoglobin was present. t-Butyl hydroperoxide also oxidized the urates in the presence of methemoglobin, but the reaction was not stimulated by sodium nitrite. The addition of either sodium azide or potassium cyanide reduced the rate of the reaction with either hydrogen peroxide or t-butyl hydroperoxide both in the presence and absence of sodium nitrite. Possible explanations for the stimulation by nitrite of peroxide-induced degradation of urates are presented.

Introduction

Urates with at least one unsubstituted imidazole nitrogen were peroxidized by hydrogen peroxide in the presence of hemoprotein or hematin [1, 2]. If no hemoprotein was added, or if catalase was added to the hemoprotein, the urates were not degraded. The peroxidation of uric acid by hydrogen peroxide and hematin was inhibited by cyanide, sulfide, carbon monoxide, and mercuric chloride, but not by fluoride; azide augmented the rate of uric acid peroxidation [1]. The oxidation of oxyhemoglobin to methemoglobin by nitrite was stimulated by hydrogen peroxide at concentrations of hydrogen peroxide that had no effect on the oxyhemoglobin [3]. Uric acid has been proposed to be an important antioxidant and free radical scavenger in humans [4] and 3-ribosyluric acid has been proposed to have the same role in the bovine erythrocyte [2]. Uric acid and 3-ribosyluric acid protected oxyhemoglobin from oxidation to methemoglobin by nitrite or nitrite plus hydrogen peroxide [5]. During the course of these studies, it was observed that the degradation of urates by peroxide and methemoglobin was stimulated by nitrite. This paper is a report of these results.

Experimental

Blood was collected from cattle immediately after slaughter into heparin. The blood was centrifuged at 3200 × g for 20 min at room temperature. The plasma and buffy coat of white cells were removed by aspiration and the erythrocytes washed three times with three volumes of 0.9% sodium chloride. The cells were lysed by the addition of 19 volumes of 20 mM phosphate, pH 7.4, and the hemolysate centrifuged at 25,000 × g for 30 min at 0 °C to remove the membranes. The lysate (5 ml) was dialyzed against 1 l of 20 mM phosphate buffer (pH 7.4) for 24 h to remove low molecular weight compounds such as 3-ribosyluric acid and glutathione. This was the source of the oxyhemoglobin. The methemoglobin was prepared by treating the oxyhemoglobin with 1 mM sodium nitrite until it was completely oxidized and the nitrite removed by dialysis as described above. The crystalline bovine (type IV) and human methemoglobin (type I) were purchased from Sigma Chemical Co., St. Louis, Mo. Sigma stated that these preparations might have up to 75% methemoglobin, and were found to contain at least 95% methemoglobin. All of these preparations were assayed for catalase by the method described by Beers and Sizer [6]. The dialyzed hemolysate contained about 78 ± 6 Sigma units of catalase per mg of hemoglobin and the crystalline preparations had no catalase activity. The uric acid, 1-methyluric acid, 3,7-dimethyluric acid, 1,3,7-trimethyluric acid, and 2,8-dihydroxyadenine were purchased from Sigma Chemical Co. The 3-ribosyluric acid was prepared as described by Forrest *et al.* [7].

The degradation of the urates and the 2,8-dihydroxyadenine was followed by measuring the decrease

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TABLE I. Effect of Sodium Azide and Potassium Cyanide on the Degradation of Uric Acid and 3-Ribosyluric Acid.

Additions	Uric Acid ΔA_{292}/5 min	3-Ribosyluric Acid ΔA_{297}/5 min
None	0.00	0.00
1.0 mM H_2O_2	0.173 ± 0.024	0.263 ± 0.010
+0.3 mM NaN_3	0.072 ± 0.012	0.098 ± 0.008
+1.0 mM NaN_3	0.039 ± 0.007	0.060 ± 0.005
+0.3 mM KCN	0.017 ± 0.006	0.022 ± 0.004
+1.0 mM KCN	0.010 ± 0.003	0.010 ± 0.004
1.0 mM H_2O_2 + 1.0 mM $NaNO_2$	0.559 ± 0.095	0.772 ± 0.062
+0.3 mM NaN_3	0.090 ± 0.014	0.194 ± 0.018
+1.0 mM NaN_3	0.047 ± 0.010	0.088 ± 0.008
+0.3 mM KCN	0.020 ± 0.002	0.018 ± 0.005
+1.0 mM KCN	0.002 ± 0.001	0.006 ± 0.003

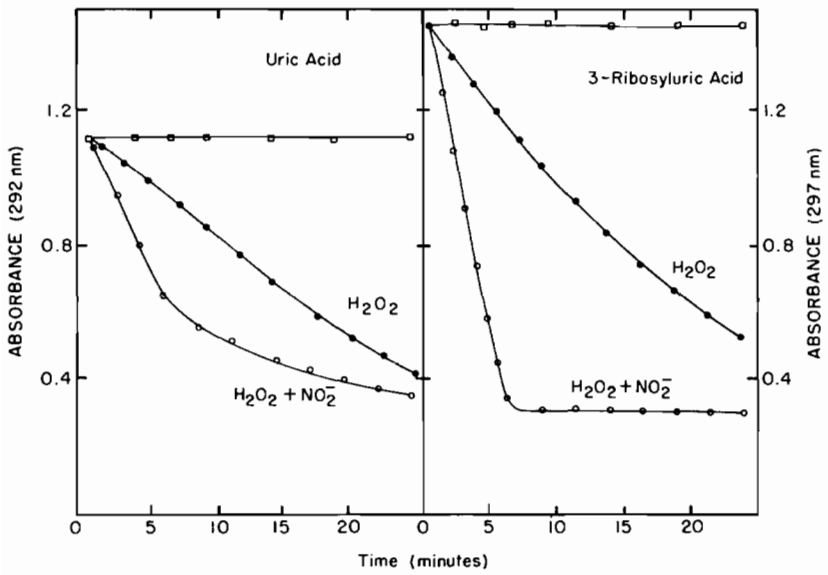

Fig. 1. Degradation of uric acid and 3-ribosyluric acid with 4 μM methemoglobin in the presence of: (•) 1.0 mM hydrogen peroxide; (○) 1.0 mM hydrogen peroxide and 1.0 mM sodium nitrite; and (□) 1.0 mM sodium nitrite.

in absorbance at their wavelength of maximum absorbance [1, 8]. They were incubated in 33 mM phosphate buffer, pH 7.4, in a total volume of 3 ml in a cuvette with a 1 cm light path in a Beckman model 25 recording spectrophotometer equipped with an automatic sample changer. The samples were incubated at 25 °C and the samples read every 50 s. The reactions were initiated by the addition of the hydrogen peroxide.

Results

The addition of either bovine or human methemoglobin and hydrogen peroxide to a solution of uric acid or 3-ribosyluric acid resulted in the degradation of the urates (Fig. 1). The addition of sodium nitrite to these samples resulted in a marked increase in the rate of degradation of the urates. The data shown are from experiments using bovine methemoglobin, but similar results were obtained with human methemoglobin. The data from 5 experiments showed that the rate of degradation of uric acid and 3-ribosyluric acid was over 3 times faster with hydrogen peroxide plus sodium nitrite than with hydrogen peroxide alone. None of the additions alone or methemoglobin and nitrite or hydrogen peroxide and nitrite resulted in the degradation of the urates. 1-Methyluric acid, 3,7-dimethyluric acid, 1,3,7-trimethyluric acid, and 2,8-dihydroxyadenine were also degraded by peroxide, as reported previously [1], and the rates of degradation of these 4 com-

pounds were also stimulated by nitrite. The addition of either sodium azide or potassium cyanide decreased the rate of degradation of the urates both with and without sodium nitrite (Table I). When the concentration of cyanide or azide was lowered from 1.0 mM to 0.001 mM, the inhibition was reduced until it approached the rate characteristic of that of the peroxide or peroxide plus nitrite. The rate of degradation of uric acid by hydrogen peroxide, both with and without sodium nitrite, was dependent on the concentration of methemoglobin (Fig. 2).

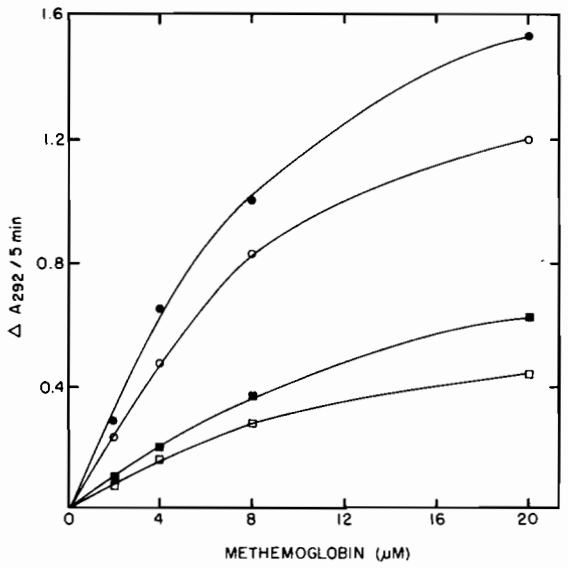

Fig. 2. Effect of the concentration of bovine or human methemoglobin on the degradation of uric acid. (▪) Bovine methemoglobin and 1.0 mM hydrogen peroxide; (•) bovine methemoglobin, 1.0 mM hydrogen peroxide and 1.0 mM sodium nitrite; (□) human methemoglobin and 1.0 mM hydrogen peroxide; (○) human methemoglobin, 1.0 mM hydrogen peroxide and 1.0 mM sodium nitrite.

In addition, preparations of bovine oxyhemoglobin and methemoglobin with catalase activity were also effective in promoting uric acid degradation in the presence of hydrogen peroxide. However, increased concentrations of these hemoglobins resulted in a decrease in the rate of degradation of uric acid (Fig. 3), probably because of the addition of the catalase present in these preparations. With concentrations of oxyhemoglobin that gave very low rates of uric acid degradation, the rate of degradation of uric acid with peroxide plus nitrite was still rapid. If the degradation of either uric acid or 3-ribosyluric acid by hydrogen peroxide was started and then the nitrite, azide, or cyanide added to the reaction mixture, the results obtained were similar to those obtained when these compounds were added before the peroxide (Fig. 4). The data shown are from experiments with 3-ribosyluric acid, but a

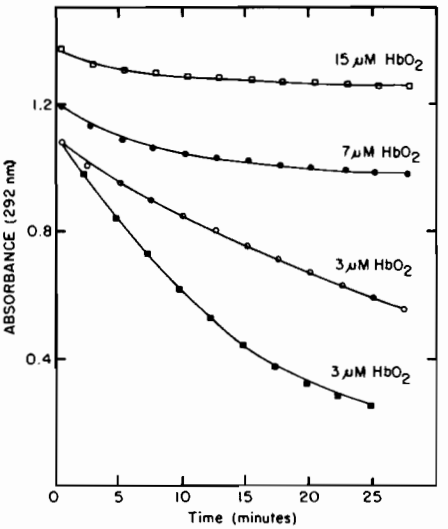

Fig. 3. Degradation of uric acid with 1.0 mM hydrogen peroxide and: (○) 3 μM oxyhemoglobin; (•) 7 μM oxyhemoglobin; (□) 15 μM oxyhemoglobin. The degradation of uric acid by 3 μM oxyhemoglobin, 1.0 mM hydrogen peroxide, and 1.0 mM sodium nitrite (▪) is also given.

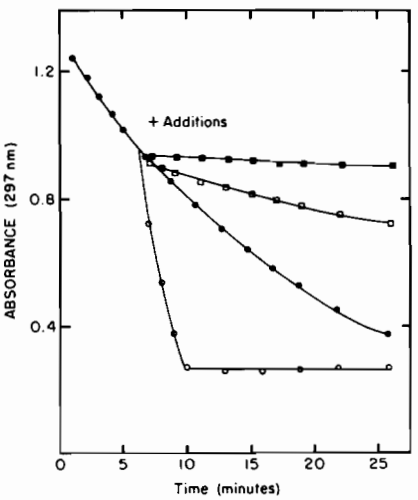

Fig. 4. Degradation of 3-ribosyluric acid with 4.0 μM methemoglobin and 1.0 mM hydrogen peroxide (•). 1.0 mM sodium nitrite, (○); 1.0 mM sodium azide, (□); and 1.0 mM potassium cyanide, (▪) were added after the reaction had proceeded for 6 min.

similar result was observed with uric acid. In this experiment about 25% of the 3-ribosyluric acid was degraded in 6 min and then the additions were made. The stimulation of the breakdown of 3-ribosyluric acid by nitrite and the inhibition of breakdown of uric acid by azide and cyanide began within one min after the compounds were added.

Since t-butyl hydroperoxide also oxidizes oxyhemoglobin to methemoglobin [9], its effect on the

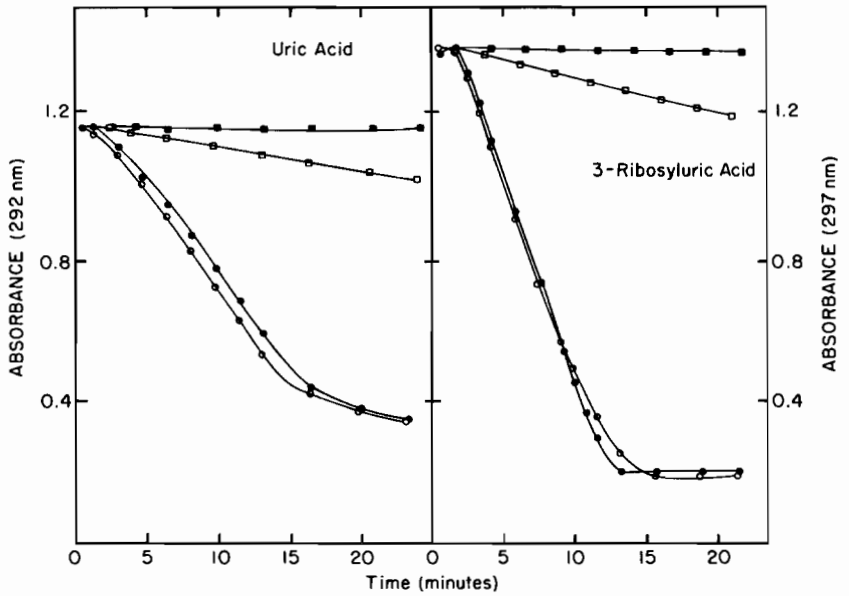

Fig. 5. Degradation of uric acid and 3-ribosyluric acid with methemoglobin and: (○) 1.0 mM t-butyl hydroperoxide; (●) 1.0 mM t-butyl hydroperoxide and 1.0 mM sodium nitrite; (□) 1.0 mM t-butyl hydroperoxide, 1.0 mM sodium nitrite, and 1.0 mM sodium azide; (■) 1.0 mM t-butyl hydroperoxide, 1.0 mM sodium nitrite, and 1.0 mM potassium cyanide.

degradation of urates both with and without sodium nitrite was determined (Fig. 5). When uric acid or 3-ribosyluric acid was treated with t-butyl hydroperoxide and bovine methemoglobin, there was a lag period of 2 to 3 min before the degradation began. This lag was not seen when hydrogen peroxide was used. The degradation of the urates in the presence of t-butyl hydroperoxide was not affected by the addition of sodium nitrite. t-Butyl hydroperoxide without methemoglobin had no effect on the urates. The addition of either sodium azide or potassium cyanide to the t-butyl hydroperoxide and methemoglobin inhibited the degradation of uric acid both in the presence (Fig. 5) and absence (not shown) of nitrite.

Discussion

Howell and Wyngaarden [1] showed that urates with at least one unsubstituted imidazole nitrogen were peroxidized by hydrogen peroxide if methemoglobin or hematin were present. They reported that oxyhemoglobin or hemolysates of human erythrocytes had no uricolytic activity. Their results with uric acid were confirmed in the present study, although it was found that hemolysates and dialyzed preparations of bovine oxyhemoglobin did promote uricolysis. However, this activity decreased as the concentration of oxyhemoglobin was increased, probably because of the increase in catalase added. The difference between the results reported here and those of Howell and Wyngaarden [1] could be because of differences in the concentration of catalase in the red cell preparations. The catalase activity of human erythrocytes has been reported to be 2 to 3 times greater than that of bovine red cells [10, 11]. Garbutt [8] also reported that bovine oxyhemoglobin preparations containing catalase promoted the degradation of uric acid by peroxide.

It was also shown in the present study that nitrite stimulates the degradation of uric acid with methemoglobin, although nitrite itself does not degrade urates. This effect was observed at all concentrations of nitrite and peroxide used. t-Butyl hydroperoxide and methemoglobin also degraded urates, but this activity was not affected by the addition of nitrite. Both azide and cyanide inhibited the peroxidation of urates caused by hydrogen peroxide and t-butyl hydroperoxide in the presence and absence of nitrite. Howell and Wyngaarden [1] reported that the peroxidation of uric acid by hydrogen peroxide and hematin was inhibited by cyanide, but that azide augmented the rate of uric acid peroxidation. It is possible that this difference is because of the hematin, but since they did not state the concentration of the components that were used, there may be other explanations. Griffiths [12] reported that the oxidation of uric acid catalyzed by copper was inhibited by potassium cyanide, but when the concentration of copper was increased, cyanide caused an enhancement of the degradation. The results that we observed are prob-

ably caused by the binding of the azide and cyanide to the iron in the methemoglobin [13] to prevent it from participating in the peroxidation reaction.

The oxidation of oxyhemoglobin by nitrite appears to be a two stage process. There is an initial slow phase which is followed by a rapid autocatalytic phase [3, 13]. Doyle *et al.* [3] reported that hydrogen peroxide stimulated the nitrite ion oxidation of oxyhemoglobin to methemoglobin at concentrations of hydrogen peroxide which alone did not oxidize the oxyhemoglobin. They postulated that the nitrite oxidation of oxyhemoglobin resulted in the formation of peroxide and nitrogen dioxide, which were converted to peroxynitrate and superoxide. They suggested that the addition of the peroxide does not oxidize the oxyhemoglobin directly, but that it is required for the autocatalytic transformation that is the dominant process in the oxidation by nitrite.

The enhanced oxidation of urates by hydrogen peroxide in the presence of sodium nitrite could be caused by the generation of some very active intermediate compound as occurs during the oxidation of oxyhemoglobin by nitrite or by the inhibition of some process that impedes the activity of the peroxide. This could explain the results obtained with preparations of oxyhemoglobin with catalase present, which Cohen *et al.* [14] have reported to be inhibited by nitrite. This could not explain the results obtained with the crystalline hemoglobins since they had no catalase activity. It is also possible that nitrite reacts with peroxide under the conditions used in the present experiments to form some more active intermediate compound such as peroxynitrate or superoxide. Although nitrite has been reported to react with peroxide in the presence of methemoglobin to form nitrate [15], there does not seem to be any known reaction between nitrite and peroxide that would lead to the formation of a more reactive product. Nitrate did not affect the rate of uric acid degradation by peroxide under the experimental procedures used here. Dooley and Pryor [16] reported that human α-1-proteinase inhibitor was not affected when exposed to either nitrogen dioxide or hydrogen peroxide alone, but there was a 65% loss in activity when the protein was incubated with both nitrogen dioxide and hydrogen peroxide. They suggested that superoxide and the hydroxyl radical might be formed in a reaction between nitrogen dioxide and hydrogen peroxide. We have previously shown that the addition of sodium nitrite to washed bovine erythrocytes resulted in a decrease in the concentration of 3-ribosyluric acid in the red cells [5]. It was postulated that the action of the nitrite was not directly on the 3-ribosyluric acid, but was caused by the peroxide which is generated during the oxidation of oxyhemoglobin to methemoglobin [14]. The results reported here show that nitrite alone either with oxyhemoglobin or methemoglobin did not degrade urates and demonstrate that it may, however, enhance the degradation of urates caused by peroxide and methemoglobin.

Acknowledgements

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