# EVIDENCE FOR ACCELERATED $H_2O_2$ FORMATION BY ERYTHROCUPREIN

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## 1. Introduction

Some definite facts on the chemical and enzymic nature of erythrocuprein have accumulated [1 – 19]. At the moment our knowledge of this bimetallic protein obtained from bovine blood comprises a molecular weight of 32,600, the content of two moles each of $Cu^{2+}$ and $Zn^{2+}$ per mole, two 16,000 molecular weight subunits, the amino acid analysis and proof of the absence of tryptophan. Biochemically its function is unique, the ability to disproportionate anionic peroxide radicals into oxygen and hydrogen peroxide [7, 17 – 19]. Because of this reactivity the metalloprotein was named superoxide dismutase [7].

During the enzymic catalysed oxidation of xanthine, $O_2^-\cdot$ is being formed which can be monitored using the reduction of oxidized cytochrome *c*. In the presence of erythrocuprein the formation of reduced cytochrome *c* was diminished. However, if the diminished initial velocity [7] of the cytochrome *c* reduction is taken as a measure of erythrocuprein concentration, false conclusions are possible. For example, a xanthine oxidase inhibitor would diminish $O_2^-\cdot$ formation with the consequent lower reduction velocity of the cytochrome *c*. A much better proof of the enzymic activity of erythrocuprein would be the measurement of an accelerated $H_2O_2$ formation attributable to the rapid disproportionation of $O_2^-\cdot$.

A ten times excess of xanthine was employed to ensure the complete reduction of cytochrome *c*. Plateau height and reduction velocity were proportional to each other. In the presence of erythrocuprein both evaluation methods gave linear calibration curves. The plateau height as a measure of the concentration of reduced cytochrome *c* was preferred since its determination proved less erratic than the measurement of the reduction velocity. Misleading conclusions due to pseudo erythrocuprein activity were minimized. The accelerated $H_2O_2$ formation catalysed by erythrocuprein was clearly demonstrated using a fluorescence method. *p*-Mercuribenzoic acid used as a specific xanthine oxidase inhibitor diminished the reduction velocity of cytochrome *c* but was unable to accelerate $H_2O_2$ formation.

## 2. Materials and methods

### 2.1. *Chemicals*

Equine cytochrome *c*, milk xanthine oxidase, horseradish peroxidase and catalase were from Boehringer, Mannheim, homovanillic acid and *p*-mercuribenzoic acid from Serva, Heidelberg. All other chemicals employed were of analytical grade purity. Fresh bovine erythrocuprein was prepared according to the method given in [7] and [11].

### 2.2. *Assays*

Protein, Cu and Zn were analysed as described elsewhere [11]. A modified incubation mixture was used to determine the erythrocuprein-catalysed disproportionation of $O_2^-\cdot$. The assay mixture, 0.76 ml, was composed of: 330 $\mu$M xanthine, 27 $\mu$M cytochrome $c_{ox}$, 16 nM catalase, 100 $\mu$M EDTA, 50 mM phosphate buffer, pH 7.8. The reaction was started with 50 $\mu$l of a 3.1 $\mu$M xanthine oxidase solution. The concentration of reduced cytochrome *c* was calculated using a millimolar absorption coefficient ($Cyt_{red}$), $\epsilon_{550} = 29.9$ [20,

A fluorescence method developed by Tipton [22] was used for the determination of $H_2O_2$. In the presence of $H_2O_2$ and peroxidase a fluorescent dimeric product of homovanillic acid is formed. The fluorescence emission was recorded at 425 nm using an Eppendorf photometer. The assay mixture contained: 100 $\mu$M homovanillic acid, 0.5 $\mu$M horseradish peroxidase, 100 $\mu$M EDTA, 330 $\mu$M xanthine, with 50 mM phosphate buffer, pH 7.8, to give 2.25 ml. The reaction was started with 50 $\mu$l xanthine oxidase 9 $\mu$M.

## 3. Results

During the enzymic catalysed oxidation of xanthine, $O_2^-\cdot$ was formed. A proportion of these radicals reduced oxidized cytochrome *c*. Excessive xanthine was necessary to completely reduce the cytochrome *c* (fig. 1). In the presence of catalase a plateau appears after 3 min which remains constant at the 27 $\mu$M $Cyt_{red}c$ level for as long as 30 min, curve 1. In the presence of erythrocuprein the $O_2^-\cdot$ concentration

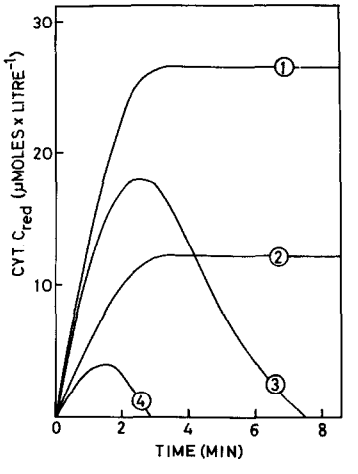


Fig. 1. Formation of reduced cytochrome *c* by $O_2^-\cdot$. 1) In the presence of 16 nM catalase, erythrocuprein omitted; 2) 25 nM erythrocuprein and 16 nM catalase added; 3) both enzymes omitted; 4) in the presence of 25 nM erythrocuprein, catalase omitted. The assay was performed at 25° in a volume of 0.76 ml using 1 cm light path optical glass cells. The concentrations of the components dissolved in 50 mM phosphate buffer, pH 7.8, were: 0.33 mM xanthine, 27 $\mu$M cytochrome $c_{ox}$, 0.1 mM EDTA, 0.21 nM xanthine oxidase. The absorption was recorded at 550 nm.

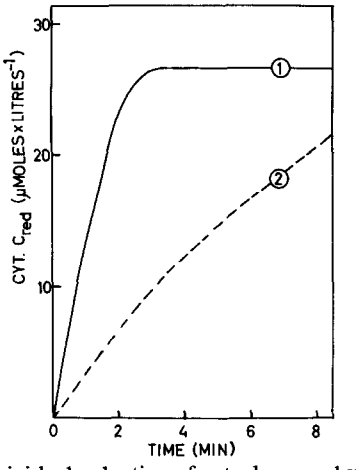


Fig. 2. Diminished reduction of cytochrome *c* by *p*-mercuribenzoic acid. 1) control incubation in the presence of 16 nM catalase, 2) after the addition of 0.4 mM *p*-mercuribenzoic acid. The assay mixture was the same as in fig. 1.

was diminished with the subsequent diminuition of both the reduction velocity of oxidised cytochrome *c* and the $Cyt_{red}$ concentration, curve 2. If the catalase was omitted maximal reduction of cytochrome *c* was not achieved. An apparent reoxidation due to the increasing $H_2O_2$ concentration can be observed, curve 3. In the presence of erythrocuprein the formation of reduced cytochrome *c* was correspondingly diminished, curve 4.

A slower formation rate of $O_2^-\cdot$ was observed with the subsequent diminished reduction velocity of cytochrome *c* if *p*-mercuribenzoic acid was used as a xanthine oxidase inhibitor (fig. 2). It can thus be clearly seen that the reduction velocity is not a reliable measure of the concentration of erythrocuprein.

Probably the best proof of the case would be the actual measurement of erythrocuprein-induced accelerated $H_2O_2$ formation. For the convenient determination of $H_2O_2$ the fluorescent dimeric product of homovanillic acid which is formed in the presence of $H_2O_2$ and peroxidase [22] was employed. In this experiment (table 1) it can be clearly seen that erythrocuprein accelerates $H_2O_2$ production markedly. 10 $\mu$M erythrocuprein is able to stimulate the $H_2O_2$ formation rate after 60 sec by 240% compared to the control. Furthermore, the presence of a pseudo-erythrocuprein activity is clearly excluded. *p*-Mercuribenzoic acid diminished the $H_2O_2$ formation drastically by speci-

Table 1
Hydrogen peroxide formation in the presence of erythrocuprein or *p*-mercuribenzoic acid.

| Time (sec) | $H_2O_2$ concentration ($\mu$moles × litre$^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Control | + 0.1 $\mu$M erythrocuprein | + 0.5 $\mu$M erythrocuprein | + 10 $\mu$M erythrocuprein | + 130 $\mu$M *p*-mercuribenzoic acid |
| 30 | 5.0 | 8.0 | 9.2 | 12.5 | 2.0 |
| 60 | 10.0 | 15.0 | 17.0 | 22.5 | 2.6 |
| 90 | 13.8 | 20.5 | 23.0 | 32.5 | 3.4 |
| 120 | 17.2 | 24.5 | 28.0 | 43.0 | 4.0 |

The incubation mixture, 2.25 ml, contained: 0.9 mM homovanillic acid, 0.5 $\mu$M horseradish peroxidase, 0.1 mM EDTA, 0.33 mM xanthine, 50 mM phosphate buffer, pH 7.8. Fluorescence emission was measured at 425 nm using 1 cm light path quartz cells; the temperature was 22°.

fically inhibiting xanthine oxidase. The specificity of this inhibition was supported by control experiments using horseradish peroxidase and $H_2O_2$ concentrations in the range 7–30 $\mu$M. After addition of 0.2 mM *p*-mercuribenzoic acid the activity of the horseradish peroxidase remained constant.

## 4. Discussion

McCord and Fridovich [7] used a lower xanthine concentration, $5 \times 10^{-5}$ M, compared to $33 \times 10^{-5}$ M used here, probably with the idea of maintaining maximal velocity during the oxidation of xanthine. Furthermore, they used a cytochrome *c* concentration which was only one fifth of the xanthine concentration compared to one tenth used in our experiments. They placed emphasis on the determination of the initial velocity of the reduction of cytochrome *c*.

The lower $O_2^-\cdot$ concentration was probably the reason why McCord and Fridovich were able to demonstrate $H_2O_2$ formation by erythrocuprein [7] but were unable to find differences between the $H_2O_2$ formation rates of the erythrocuprein catalysed and the spontaneous disproportionation [23] of $O_2^-\cdot$.

Thus we thought it worthwhile to examine the question of $H_2O_2$ formation at higher $O_2^-\cdot$ concentrations. A slight decrease of the maximal velocity of the xanthine oxidation, due to limiting amounts of oxygen, was taken into account (94% maximal velocity), in order to ensure the complete reduction of cytochrome *c* in the absence of erythrocuprein. In this system erythrocuprein was still alive and decreased both the plateau height and the reduction velocity of cytochrome *c*. Both parameters were proportional to each other and linear calibration curves of erythrocuprein concentrations against either parameter were obtained. The reliability of this system was tested using different cytochrome *c* concentrations, and in the range 10 – 40 $\mu$M constant relative reduction rates of cytochrome *c* were obtained. The use of the plateau height as a measure of different erythrocuprein concentrations was advantageous in two ways; first its determination proved convenient due to the constancy of plateau for up to 30 min. Second a possibly false evaluation of erythrocuprein activity, if a xanthine oxidase inhibitor was present, was minimized. With the finding that $H_2O_2$ formation is accelerated by erythrocuprein we have good evidence that this enzyme really plays an important, possibly an essential role in oxidative metabolic pathways in which $O_2^-\cdot$ is formed. Furthermore, anionic peroxide radicals induced by radiation may be rapidly decomposed by this enzyme.

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