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MYELOPEROXIDASE OF THE LEUKOCYTE OF NORMAL BLOOD

II. THE OXIDATION-REDUCTION REACTION MECHANISM OF THE MYELOPEROXIDASE SYSTEM

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SUMMARY

The initial reaction of the aerobic oxidation of NADH in the myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) system was inhibited by superoxide dismutase when the latter had been added to the reaction mixture previously, but the oxidation of NADH was not inhibited when it was added halfway during the reaction. The formation of myeloperoxidase compound III in the aerobic solution containing NADH and 2,4-dichlorophenol was also inhibited by superoxide dismutase. From these results it was concluded that a superoxide anion radical and myeloperoxidase compound III are very important active intermediates for the aerobic myeloperoxidase-catalyzed oxidation of NADH. Myeloperoxidase compound III can be formed by direct reaction between ferric myeloperoxidase and $O_2^-\cdot$, and the electronic structure of myeloperoxidase compound III can be considered to be $Fe^{3+}O_2^-$.

Cytochrome b_5 prepared from thrombocytes was effectively reduced by the NADH–myeloperoxidase system. Myeloperoxidase catalyzed a reaction between epinephrine and H_2O_2. The light absorption maxima of a product of the reaction between epinephrine and H_2O_2 corresponds to those of adrenochrome. Myeloperoxidase also catalyzed the reaction between epinephrine analogues (norepinephrine, dopamine, DOPA, tyrosine and phenylalanine) and H_2O_2.

INTRODUCTION

The role and function of myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) in the leukocyte have not been clarified as yet. The derivatives of the reaction between myeloperoxidase and H_2O_2 were described in a previous paper[1]. Concerning the myeloperoxidase system, we also reported[1] that myeloperoxidase

Abbreviations: Fe^{3+} and Fe^{2+}, ferric and ferrous myeloperoxidase; b_5^{3+} and b_5^{2+}, ferric cytochrome b_5 and ferrous cytochrome b_5; compounds I, II and III, myeloperoxidase compounds I, II and III. Φ–OH, Φ–O· and Φ=O, 2,4-dichlorophenol, its dehydro radical and dehydro form, respectively.

compound III was formed during the aerobic oxidation of NADH in the presence of 2,4-dichlorophenol and that compound III was also formed by direct reaction between ferrous myeloperoxidase and molecular oxygen.

Recently, McCORD AND FRIDOVICH[2] have reported that erythrocuprein (hemocuprein) prepared from bovine erythrocytes catalyzes the dismutation reaction of the superoxide anion radical (O_2^-) and that erythrocuprein should be named "superoxide dismutase". BALLOU *et al.*[3] have also reported that erythrocuprein prepared from beef erythrocytes shows activity with respect to the decomposition of the superoxide anion radical.

In the present paper, the author will mainly describe the oxidation–reduction reaction of the myeloperoxidase system.

MATERIALS AND METHODS

Myeloperoxidase was prepared from leukocytes of normal pig blood by the method reported in a previous paper[1]. The concentration was calculated on the basis of a value of 91 $mM^{-1} \cdot cm^{-1}$ for the extinction coefficient at 430 mμ. The $A_{430\ m\mu}/A_{280\ m\mu}$ ratio for the enzyme used in this experiment was 0.7.

Superoxide dismutase was prepared from pig erythrocytes using a slight modification of the method of McCORD AND FRIDOVICH[2]. The concentration was estimated from the molar extinction coefficient of 350 at 665 mμ (ref. 4).

Cytochrome b_5 of thrombocytes was prepared by DEAE-cellulose column chromatography from thrombocytes of normal pig blood. The concentration of the oxidized and reduced forms of the cytochrome were determined from millimolar absorbance values of 117 at 413 mμ and of 25.6 at 556 mμ, respectively[5].

The recording spectrophotometers used were Hitachi instruments, Types EPS-2, 124 and 356 (two-wavelength and double-beam spectrophotometer). The latter instruments were used for the estimation of absorbance in the expanded scale.

The reaction and estimation were carried out in sodium–potassium phosphate buffer at a solution temperature of 25° except when stated otherwise.

RESULTS

It has been reported[6] that the aerobic oxidation of NADH at neutral pH is catalyzed by horseradish peroxidase in the presence of 2,4-dichlorophenol. The oxidation of NADH was also catalyzed by myeloperoxidase at neutral pH and, during the reaction, most of the NADH was oxidized (Fig. 1, 1b). When superoxide dismutase prepared from pig erythrocytes was added to the reaction mixture prior to the start of the reaction, the oxidation of NADH was completely inhibited (Fig. 1, 1a) and when superoxide dismutase was added at the initial stage of oxidation of NADH, the reaction was also almost completely inhibited (Fig. 1, 2a). However, when superoxide dismutase was added halfway during the reaction, the oxidation of NADH was not inhibited at all (Fig. 1, 2b) but it was inhibited by catalase. From these results it is concluded that the reaction of the aerobic myeloperoxidase-catalyzed oxidation of NADH is initiated by the superoxide anion radical (O_2^-). Fig. 2 shows that the formation of myeloperoxidase compound III during the aerobic oxidation of NADH in the presence of 2,4-dichlorophenol was completely inhibited by super-

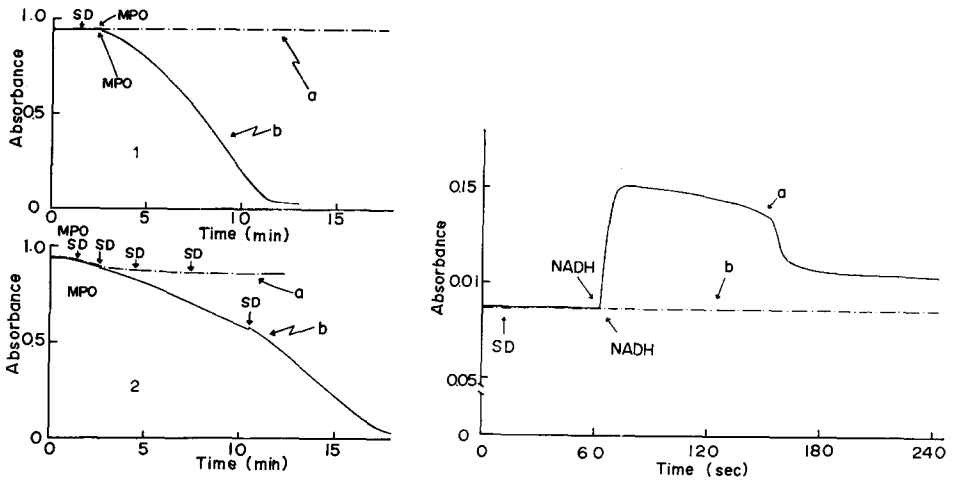

Fig. 1. The inhibition of the aerobic myeloperoxidase-catalyzed oxidation of NADH by superoxide dismutase. (1) Curve b shows the aerobic oxidation of NADH by the myeloperoxidase system in the presence of 2,4-dichlorophenol. The reaction was started by the addition of myeloperoxidase (MPO). Curve a shows the inhibition of the aerobic oxidation of NADH by the myeloperoxidase system in the presence of 2,4-dichlorophenol by superoxide dismutase (SD). Before the oxidation reaction was started by the addition of myeloperoxidase, superoxide dismutase and 2,4-dichlorophenol were added into the reaction mixture. Concentrations: $1.5 \cdot 10^{-4}$ M NADH, $2.8 \cdot 10^{-8}$ M myeloperoxidase, $2 \cdot 10^{-4}$ M 2,4-dichlorophenol, 3.2 μM superoxide dismutase and 0.05 M phosphate (pH 7.0). (2) Curve a shows the inhibition of the initial stage of the aerobic oxidation of NADH by the myeloperoxidase system in the presence of 2,4-dichlorophenol. The reaction of NADH oxidation, in the presence of 2,4-dichlorophenol, was started by the addition of myeloperoxidase (MPO), and then superoxide dismutase (SD) was added. The concentration of superoxide dismutase added each time was $3.2 \cdot 10^{-7}$ M. Concentrations: $1.5 \cdot 10^{-4}$ M NADH, $1.4 \cdot 10^{-8}$ M myeloperoxidase, $2 \cdot 10^{-4}$ M 2,4-dichlorophenol and 0.05 M phosphate (pH 7.0). Curve b shows the oxidation of NADH, in the presence of 2,4-dichlorophenol, started by the addition of myeloperoxidase, followed by addition of 3.2 μM superoxide dismutase halfway during the oxidation reaction. Concentrations: $1.5 \cdot 10^{-4}$ M NADH, $1.4 \cdot 10^{-8}$ M myeloperoxidase, $2 \cdot 10^{-4}$ M 2,4-dichlorophenol and 0.05 M phosphate (pH 7.0). The oxidation of NADH was measured as the change of absorbance at 340 mμ.

Fig. 2. The inhibition of myeloperoxidase compound III formation during the aerobic oxidation of NADH in the presence of 2,4-dichlorophenol by superoxide dismutase (SD). Curve a, changes in the absorbance at 452 mμ as a function of time. The rise in absorbance at 452 mμ is due to the formation of myeloperoxidase compound III. The reaction of myeloperoxidase compound III formation was started by the addition of NADH. 2,4-Dichlorophenol had been added previously. Concentrations: 2 μM myeloperoxidase, $7 \cdot 10^{-4}$ M NADH, $1.7 \cdot 10^{-5}$ M 2,4-dichlorophenol and 0.05 M phosphate (pH 7.0). Curve b, concentrations and conditions correspond to Curve a, except for the previous addition of 3.2 μM superoxide dismutase.

oxide dismutase. From these results, it is also concluded that myeloperoxidase compound III, in the aerobic NADH oxidation system, can be formed from the reaction between ferric myeloperoxidase and O_2^- and that the electronic structure of myeloperoxidase compound III can be considered to be $Fe^{3+}O_2^-$. In the case of horseradish peroxidase it has been suggested[7] that horseradish peroxidase compound III can be formed from the reaction between ferric enzyme and O_2^-, molecular oxygen reduced by a substrate's free radical formed during the peroxidation reaction. In the case of lactoperoxidase, moreover, it has been observed[8] that lactoperoxidase compound III is formed by direct reaction between O_2^- formed by the milk xanthine oxidase system and ferric lactoperoxidase. From the results of Figs. 1 and 2, also, it is

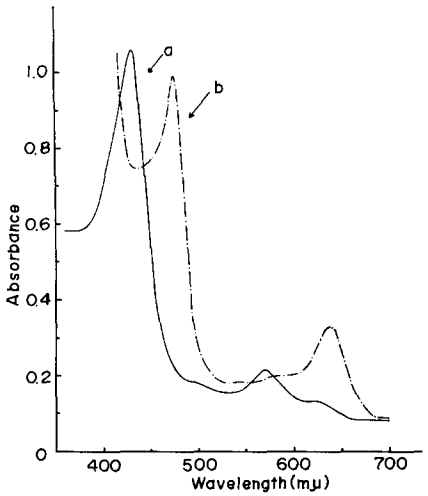

Fig. 3. The formation of ferrous myeloperoxidase during the aerobic oxidation of NADH in the presence of 2,4-dichlorophenol. Curve a, 12 μM myeloperoxidase in 0.05 M phosphate (pH 7.0); Curve b, $3.8 \cdot 10^{-2}$ M NADH and $1.6 \cdot 10^{-4}$ M 2,4-dichlorophenol were added to the reaction mixture of (a). The experiments were carried out at a solution temperature of 10°.

suggested that O_2^- and myeloperoxidase compound III are very important active intermediates. Fig. 3 shows that, at a high concentration of NADH in the presence of 2,4-dichlorophenol, myeloperoxidase converts to ferrous enzyme.

Cytochrome b_5 prepared from thrombocytes of normal peripheral pig blood was effectively reduced by the NADH–myeloperoxidase system in the presence

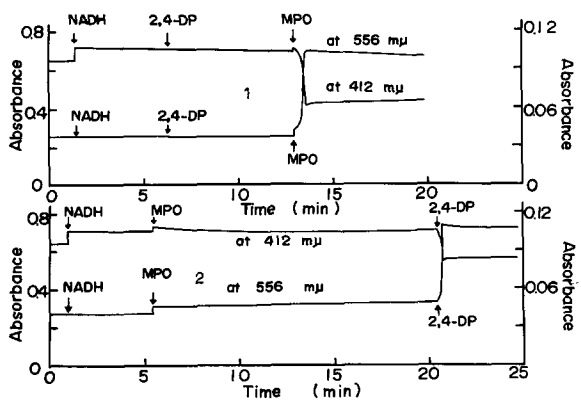

Fig. 4. The reduction of cytochrome b_5 of thrombocytes during the aerobic myeloperoxidase-catalyzed oxidation of NADH in the presence of 2,4-dichlorophenol. The changes in the absorbance at 412 mμ and 556 mμ are due to the formation of ferrous cytochrome b_5. (1) The reduction reaction was started by the addition of myeloperoxidase (MPO), or (2) by the addition of 2,4-dichlorophenol (2,4-DP). Concentrations: 5.6 μM cytochrome b_5, 10.5 mM NADH, $8 \cdot 10^{-8}$ M myeloperoxidase, $2 \cdot 10^{-4}$ M 2,4-dichlorophenol and 0.1 M phosphate (pH 7.0). The experiments were carried out using a two-wavelength spectrophotometer.

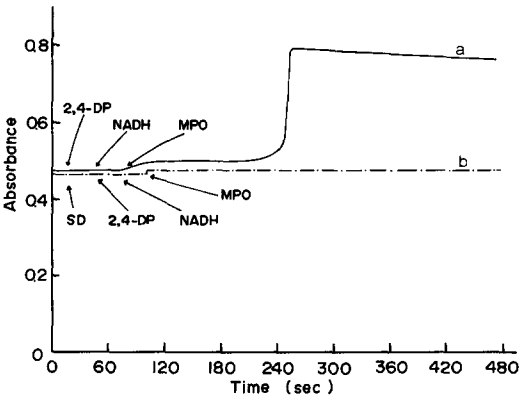

Fig. 5. The inhibition of cytochrome b_5 reduction during the aerobic myeloperoxidase-catalyzed oxidation of the NADH system by superoxide dismutase. Curve a, change in the absorbance at 556 mμ as a function of time. The rise in absorbance at 556 mμ is due to the reduction of cytochrome b_5 by the myeloperoxidase system. The experiments were carried out in aerobic solution. Concentrations: 5.6 μM cytochrome b_5, $8 \cdot 10^{-8}$ M myeloperoxidase (MPO), 1.9 mM NADH, 0.1 mM 2,4-dichlorophenol (2,4-DP) and 0.05 M phosphate (pH 7.0). Curve b, concentrations and conditions correspond to Curve a, except for the previous addition of $6.4 \cdot 10^{-7}$ M superoxide dismutase (SD).

of 2,4-dichlorophenol (Fig. 4). The reaction had a clear lag time (Fig. 5, a) and the reduction reaction of cytochrome b_5 by the NADH–myeloperoxidase system was completely inhibited by the addition of superoxide dismutase (Fig. 5, b). On the other hand, the reaction between ascorbate and H_2O_2 catalyzed by myeloperoxidase in the presence of 2,4-dichlorophenol was not affected by the addition of superoxide dismutase and, in anaerobic solution, myeloperoxidase was directly reduced by ascorbate and ferrous enzyme was rapidly oxidized by the addition of molecular oxygen to the ferric enzyme.

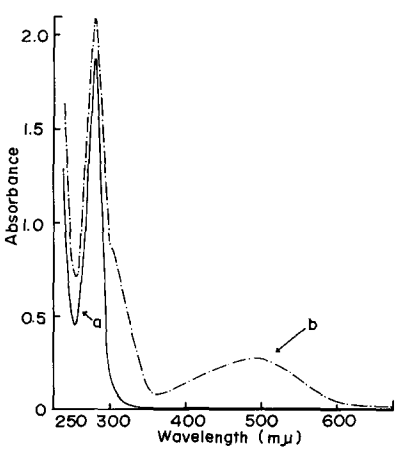

Fig. 6. The spectra of epinephrine and adrenochrome. Curve a, 1 mM L-epinephrine in 0.05 M phosphate (pH 7.0); Curve b, 5 min after the addition of $4 \cdot 10^{-5}$ M H_2O_2 and $1.6 \cdot 10^{-8}$ M myeloperoxidase.

TABLE I

THE INFLUENCE OF SUPEROXIDE DISMUTASE ON THE MYELOPEROXIDASE SYSTEM

Reaction system	*Effect*
Aerobic oxidation of NADH	Inhibition*
Myeloperoxidase compound III formation by the NADH system	Inhibition
Reduction reaction of cytochrome b_5 by the NADH–myeloperoxidase system	Inhibition
Oxidation of ascorbate	No effect
Oxidation of epinephrine	No effect

* The initial stage of aerobic myeloperoxidase-catalyzed oxidation of NADH was inhibited by superoxide dismutase.

Myeloperoxidase catalyzes the reaction between epinephrine (adrenal hormone) and H_2O_2; a pigment formed during the reaction had light absorption peaks at 485 mμ (Fig. 6). The position of the light absorption maxima of the pigment was clearly identical to those of adrenochrome. The formation of the adrenochrome by the myeloperoxidase system was not affected at all by the superoxide dismutase. From these results it is concluded that the peroxidase reaction dependent upon H_2O_2 can not be affected by superoxide dismutase (refer to Table I).

Myeloperoxidase also catalyzed a reaction between the epinephrine analogues norepinephrine, dopamine, DOPA, tyrosine and phenylalanine, and H_2O_2. The

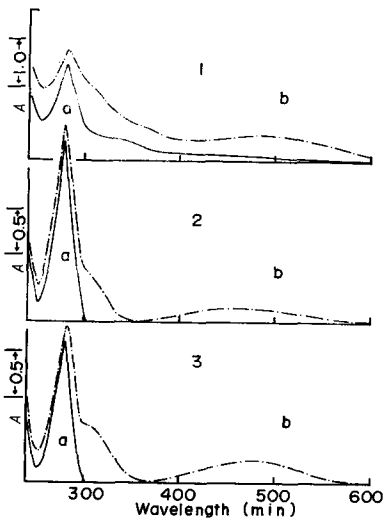

Fig. 7. The spectra of epinephrine analogues and products formed during the reaction between these analogues and H_2O_2 in the presence of myeloperoxidase. (1) Curve a, 4 mM norepinephrine in 0.05 M phosphate (pH 7.0); Curve b, 10 min after addition of 0.1 mM H_2O_2 and $8 \cdot 10^{-8}$ M myeloperoxidase to the reaction mixture of Curve a. (2) Curve a, 1.2 mM dopamine in 0.1 M phosphate (pH 7.0); Curve b, 12 min after addition of 0.1 mM H_2O_2 and $5.5 \cdot 10^{-8}$ M myeloperoxidase to the reaction mixture of Curve a. (3) Curve a, 1 mM DOPA in 0.05 M phosphate (pH 7.0); Curve b, 25 min after addition of 0.1 mM H_2O_2 and $3 \cdot 10^{-8}$ M myeloperoxidase to the reaction mixture of Curve a.

reaction products of norepinephrine, dopamine and DOPA had a light absorption peak in the 480-mμ region (Fig. 7).

DISCUSSION

The mechanism for the oxidation–reduction reaction in the myeloperoxidase system has not yet been elucidated in detail. In the present paper, the mechanisms of the oxidation–reduction of myeloperoxidase in the NADH–myeloperoxidase system and of the reduction of cytochrome b_5 prepared from thrombocytes during the aerobic myeloperoxidase-catalyzed oxidation of NADH, and of the peroxidations of ascorbate, epinephrine and its analogues by the H_2O_2–myeloperoxidase system have been investigated.

The aerobic oxidation of NADH by the horseradish peroxidase system in the presence of 2,4-dichlorophenol was originally described by AKAZAWA AND CONN[7]. It is obvious from the results of Figs. 1 and 2 that, in the myeloperoxidase system, the aerobic oxidation of NADH in the presence of 2,4-dichlorophenol may be caused by the superoxide anion radical (O_2^-). In this reaction, O_2^- and myeloperoxidase compound III are very important active intermediates. O_2^- may be generated from the following reactions:

$$\Phi\text{–OH} + O_2 \rightharpoonup \Phi\text{–O}\cdot + HO_2 \; (HO_2 \rightarrow O_2^- + H^+) \quad (1)$$

$$\Phi\text{–O}\cdot + O_2 \rightharpoonup \Phi{=}O + O_2^- \quad (2)$$

$$NADH + O_2 \rightharpoonup NAD\cdot + HO_2 \; (HO_2 \rightarrow O_2^- + H^+) \quad (3)$$

$$NAD\cdot + O_2 \rightharpoonup NAD^+ + O_2^- \quad (4)$$

Myeloperoxidase compound III can be formed *via* different pathways as follows:

$$Fe^{3+} + O_2^-\cdot \rightharpoonup Fe^{3+}O_2^- \quad (5)$$

$$Fe^{2+} + O_2 \rightharpoonup Fe^{2+}O_2 \quad (6)$$

At a high concentration of NADH in the presence of 2,4-dichlorophenol, when the molecular oxygen in the reaction solution is almost consumed, myeloperoxidase can be reduced (Fig. 3) by NAD· that is formed from the autoxidation reactions of NADH (Eqns. 3 and 4), oxidase reaction by compound III (Eqn. 7) and peroxidase reaction by myeloperoxidase compounds I and II (Eqns. 8 and 9). When molecular oxygen is added exogenously, the ferrous enzyme can be converted to myeloperoxidase compound III (ref. 1). At a low NADH concentration and in the presence of sufficient molecular oxygen, compound III will be formed as represented by Eqn. 5 in accordance with the results of Figs. 1 and 2.

If myeloperoxidase compound III is an intermediate for the NADH–myeloperoxidase system, the reactions of myeloperoxidase compounds with NADH may be written speculatively as follows:

$$Fe^{3+}O_2^-(Fe^{2+}O_2) + NADH \rightharpoonup \text{compound I} + NAD\cdot \quad (7)$$

$$\text{Compound I} + NADH \rightharpoonup \text{compound II} + NAD\cdot \quad (8)$$

$$\text{Compound II} + NADH \rightharpoonup Fe^{3+} + NAD\cdot \quad (9)$$

It has been reported[9,10] that the free radicals of some substrates formed

during one-electron oxidation in the horseradish peroxidase system were detected directly by applying the EPR technique. In the presence of superoxide dismutase, the decomposition of O_2^- causes inhibition of the initial reaction of the aerobic myeloperoxidase-catalyzed oxidation of NADH. However, despite the fact that there are some possible pathways for H_2O_2 production, for example the dismutation and one-electron reduction of O_2^- or two-electron reduction of O_2, this peroxidase reaction did not occur. Therefore, these results may suggest that, for the aerobic myeloperoxidase-catalyzed oxidation of NADH, O_2^- and compound III are not only very important active intermediates but that the formation of myeloperoxidase compound I during one-electron reduction of compound III is more essential than the formation of compound I by direct reaction between the ferric enzyme and H_2O_2. From these results and considerations, the reaction mechanism of an oxidation–reduction of myeloperoxidase during the NADH system is proposed as that represented in Fig. 8.

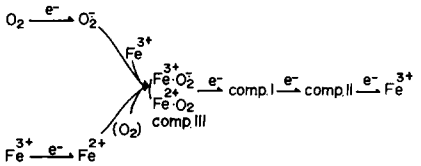

Fig. 8. A possible scheme of the oxidation–reduction mechanism of the NADH– myeloperoxidase system. O_2 in parentheses represents molecular oxygen that was added exogenously.

Cytochrome b_5 prepared from thrombocytes was effectively reduced by the NADH–myeloperoxidase system (Figs. 4 and 5). In the measured reaction time course, a clear lag time was observed (Fig. 5, a). It has been suggested[11] that cytochrome b_5 prepared from pig liver can be reduced by NAD· which is formed by the H_2O_2–horseradish peroxidase system. Our results described in the present paper can be considered to be consistent with those of ref. 11. The lag time observed in our experiment may be suggested to be the time that NAD· is consuming molecular oxygen. The NAD· as reductant in this reaction can be mostly formed during the reactions of Eqns. 7, 8 and 9. Finally, the reaction mechanism of reduction of cytochrome b_5 by the NADH–myeloperoxidase system may be suggested to follow Eqn. 10:

$$NAD\cdot + b_5^{3+} \rightarrow NAD^+ + b_5^{2+} \qquad (10)$$

The oxidation of ascorbate by myeloperoxidase in the presence of H_2O_2 has originally been reported by AGNER[12]. In 1959, SCHULTZ AND ROSENTHAL[13] reported that a compound with light absorption peaks at 457.8 mμ and 624.3 mμ can be observed in difference spectra of 5 mM ascorbate–myeloperoxidase and the myeloperoxidase system. The myeloperoxidase compound is very similar to our myeloperoxidase compound III (ref. 1). In the present paper, it is interesting to note that myeloperoxidase can be reduced directly by ascorbate. If the myeloperoxidase compound of SCHULTZ AND ROSENTHAL[13] corresponds to our myeloperoxidase compound III, it may be suggested that the ferrous myeloperoxidase formed, which was directly

reduced by ascorbate, was oxidized by molecular oxygen to myeloperoxidase compound III.

The difference between the reaction mechanisms of the aerobic myeloperoxidase-catalyzed oxidation of NADH and the simple peroxidations of the H_2O_2–myeloperoxidase system, for example where ascorbate or epinephrine are electron donors, may suggest that, in the former case, O_2^- and compound III are very important active intermediates; in other words, molecular oxygen can be effectively reduced by NAD· to O_2^-. And in the latter case, substrate free radicals formed from ascorbate and epinephrine can reduce molecular oxygen very slowly or not at all.

The peroxidation of epinephrine and its analogues by the H_2O_2–myeloperoxidase system may be suggested to have a function in the biological regulation of metabolism, for example glycolysis.

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