PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **147**, 140–143 (1974)

Indirect Evidence for Superoxide Anion and Singlet Oxygen Generated by NADPH—NADPH-dependent Cytochrome c Reductase and by L-α-hydroxyacid—L-amino Acid Oxidase at High pH (38298)

MINORU NAKANO, TOSHIKAZU NOGUCHI, YACHIYO TSUTSUMI,
KATSUAKI SUGIOKA, YOSHIAKI SHIMIZU, YASUHIRO TSUJI,
AND HUMIO INABA
(Introduced by T. S. Danowski)

Department of Biochemistry, School of Medicine, Gunma University, Maebashi, 371 Japan; and Institute of Electrical Communication, Tohoku University, Sendai, 980 Japan

Xanthine oxidase can oxidize xanthine and hypoxanthine by means of molecular oxygen. The oxygen is reduced to H_2O_2 via an intermediate superoxide anion ($O_2^{\cdot}$), with concomitant formation of singlet oxygen ($^1O_2$) during the reaction (1). This system induces the oxidation of luminol (2, 3) and of lucigenin (2) with light emission. Similar activation of luminol with light emission is observed during the oxidation of NADPH in the bovine adrenodoxine reductase–adrenodoxine–cytochrome c system (4). This chemiluminescence is completely inhibited in the presence of superoxide dismutase which catalyzes the reaction:

$$2O_2^{\cdot} \rightarrow H_2O_2 + O_2 \qquad (5)$$

This enzyme also acts as a scavenger of $^1O_2$ (6). Since superoxide dismutase apparently only reacts with $O_2^{\cdot}$ which is free in solution (7), chemiluminescence, which can be abolished by the dismutase, could be responsible for $O_2^{\cdot}$ and $^1O_2$ but not for enzyme-bound O_2 (E...$O_2^{\cdot}$). Weiss (8) has proposed the following mechanism for the reaction between $O_2^{\cdot}$ and ferric (or ferrous) ion:

$$Fe^{2+} + O_2 \rightleftarrows Fe^{3+} + O_2^{\cdot}$$

A plausible reaction sequence has also been proposed by several workers (1, 3, 9):

$$2O_2^{\cdot} \rightleftarrows O^{2-}{}_4 \rightarrow O^{2-}{}_2 + {}^1O_2$$

However, $^1O_2$ would not be expected to react with Fe^{3+}. Thus, if activation of luminol induced during substrate–enzyme–O_2 interaction were inhibited both by Fe^{3+} and superoxide dismutase, this would involve both $O_2^{\cdot}$ and $^1O_2$. This may well be true of the xanthine–xanthine oxidase system.

Even though there is no direct evidence that pure $O_2^{\cdot}$ oxidized luminol as well as chemically produced $^1O_2$ exist in aqueous solution (10, 11), application of the luminol reaction based on the above hypothesis should prove useful as indirect evidence for the existence of both $^1O_2$ and $O_2^{\cdot}$.

Materials and Method. Superoxide dismutase (2650 units/mg protein) (12), xanthine oxidase (1.9 units/mg protein) (13), NADPH-dependent cytochrome c reductase (12–20 units/mg protein) (14), and L-amino acid oxidase (10,000 units by α-ketoisocaproate formation/mg protein) (15) were prepared and their activities determined by established methods. Chemiluminescence of the incubation media was measured as previously described (16) at 37° by the single photoelectron counting method in the dark.

Results. NADPH–NADPH-dependent cytochrome c reductase system. The time course of NADPH oxidation either in the presence or absence of superoxide dismutase and the effect of pH on the cytochrome c reductase activity are shown in Fig. 1A. The addition of luminol to the NADPH-enzyme system at near-optimum pH (pH 9.5) produced light. This chemiluminescence was completely abolished by the addition of the Fe^{3+}-ADP complex. The presence of superoxide dismutase prior to the incubation did not affect the initial activity of NADPH-oxidation, but did completely inhibit the chemiluminescence involving activation of luminol (Fig. 1B) and support the view that each system produces both $O_2^{\cdot}$ and $^1O_2$. Even though

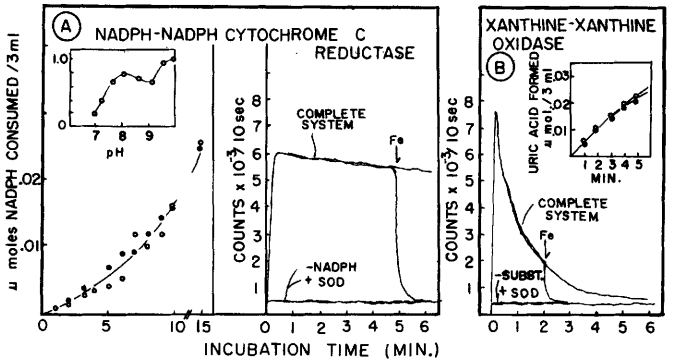

FIG. 1. (A) Consumption of NADPH in NADPH-dependent cytochrome c reductase system at pH 9.5 with (●—●) or without (○—○) superoxide dismutase (left). Incubation media consisted of 0.16 m*M* NADPH, 0.56 units of NADPH-dependent cytochrome c reductase (13 units/mg protein), 40 μg (6000 units) of catalase, 20 m*M* glycine–NaOH buffer (pH 9.5) and 2940 units of superoxide dismutase (SOD) or none, in a total volume of 3 ml. The consumption of NADPH was followed at 340 nm ($\epsilon = 6.2 \times 10^3$) and at 37°. Inset: Activities were determined by increased absorbance at 550 nm in the presence of both cytochrome c and NADPH at different pH, and the results are shown relative to specific activity at pH 10, taken as unity.

Chemiluminescence produced by NADPH–NADPH-dependent cytochrome c reductase system (right). The reaction mixture was the same as that described in (A), save that 0.33 m*M* luminol was added as a fluorophor. A mixture of 1×10^{-4}*M* Fe^{3+} and 1.67 m*M* ADP (Fe) was added at arrow. (B) Time course of xanthine oxidase activity (inset) and chemiluminescence production in the xanthine–xanthine oxidase system. Incubation media contained 0.33 m*M* xanthine, 0.19 units of xanthine oxidase, 40 μg (6000 units) of catalase, 20 m*M* glycine–NaOH buffer (pH 8.6), superoxide dismutase (2940 units) (●) or none (○), and 0.33 m*M* luminol (only for chemiluminescence), in a total volume of 3 ml. Conversion of xanthine to uric acid was followed at 290 nm ($\epsilon = 9.5 \times 10^3$) and at 37°. A mixture of 1×10^{-4}*M* Fe^{3+} and 1.67 m*M* ADP (Fe) was added at the arrow.

some workers have observed a spontaneous chemiluminescence during the incubation of xanthine and xanthine oxidase (1, 6), no significant light emission was detected under our experimental conditions in the absence of luminol.

L-α-Hydroxyisocaproate–L-amino acid oxidase system. L-Amino acid oxidase, which is known to produce H_2O_2 during the oxidation of L-α-hydroxyacids and L-α-amino acids (15), under specified conditions, also activated luminol with emission of light in the presence of L-α-hydroxyisocaproate. As shown in Figs. 2A and 2B, the effects of superoxide dismutase and Fe^{3+} on chemiluminescence are similar to those demonstrated with the above two systems.

Effect of pH on O_2^- and $^1O_2$ generating system. As shown in Fig. 3, the light emission and intensity were partially dependent upon the pH of the incubation media. In these experiments, the lowest pH at which light was emitted increased in the following order: xanthine oxidase, cytochrome c reductase, and L-amino acid oxidase systems.

When the luminol was oxidized with H_2O_2, the light could be detected at pH higher than 8.0.

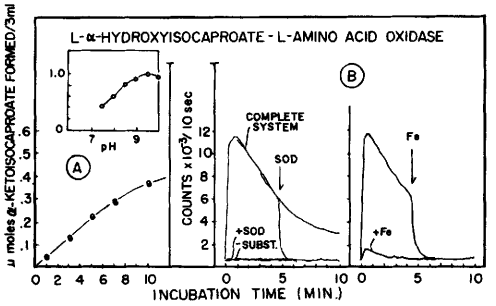

FIG. 2. (A) Formation of α-ketoisocaproate by L-α-hydroxyisocaproate–L-amino acid oxidase system at pH 10.0 with (●—●) or without (○—○) superoxide dismutase and effect of pH on enzyme activity (inset). Incubation media consisted of 1.68 m*M* L-α-hydroxyisocaproate, 640 units of L-amino acid oxidase (10,000 units/mg protein), 40 μg (6400 units) of catalase, 20 m*M* glycine–NaOH buffer (pH 10.0), 2940 units of superoxide dismutase (SOD) or none, in a total volume of 3 ml. α-Ketoisocaproate was determined by the 3-hydrazinoquinoline method. Inset: Enzyme activities were measured at different pH values relative to specific activity at pH 10.0, taken as unity. (B) Chemiluminescence of L-α-hydroxyisocaproate–L-amino acid oxidase system. The reaction mixture was the same as that described in (A), save that 0.33 m*M* luminol was added as a fluorophor. A mixture of 1×10^{-4}*M* Fe^{3+} and 1.67 m*M* ADP (Fe) or 2940 units of superoxide dismutase (SOD) was added at the arrow.

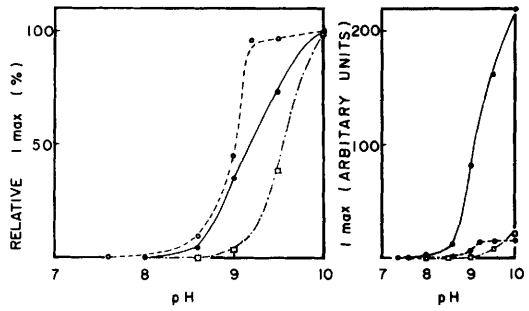

FIG. 3. Effect of pH on the oxidation of luminol by NADPH–NADPH cytochrome c reductase (○—○), xanthine–xanthine oxidase (●—●) and L-α-hydroxyisocaproate–L-amino acid oxidase (□- · -□). Incubation conditions and the measurements of light intensity are described in the legends for Figs. 1 and 2, but the pH of the incubation media was varied. The ordinate represents percent intensity maximum (I_{max}) to that at pH 10.0 (left) and the arbitary units of I_{max} (right) at the pH cited. I_{max} = highest counts per 10 sec of the reaction. The reaction systemwas the same as that described in Figs. 1, and 2, except that 40 m*M* tris–HCl buffer were used with pH ranging from 7.2–8.0.

Therefore, even though the xanthine oxidase and cytochrome c reductase systems generate singlet oxygen at a pH lower than 8.0, the luminol could not be activated to emit light. In terms of the efficiency of the enzyme in producing both $O_2^{\cdot}$ and $^1O_2$ (defined as total light emitted in 1 min/nmoles metabolite accumulated (or substrate degraded) after 1 min) at pH 10.0, both the L-amino acid oxidase and NADPH cytochrome c reductase systems were distinctly less effective than xanthine oxidase system (Table I).

Discussion. Nakamura and Yamazaki (17) have shown that the formation of the perhydroxyl radical ($^{\cdot}OOH$) can be detected by the accumulation of lactoperoxidase complex III from lactoperoxidase added to the system during the oxidation of NADPH by NADPH-dependent cytochrome c reductase at pH 6.0. They postulated that the reaction represents the transfer of one electron from flavoprotein (probably semiquinone of the flavin moiety in the enzyme) to O_2. Our studies herein presented not only confirm their findings with respect to one electron transfer to O_2 in the same system (but at different pH), but also clarify indirectly the formation of $O_2^{\cdot}$ in the system. On the other hand, single flavoprotein oxidases such as glucose oxidase, D-amino acid oxidase, L-amino acid oxidase, glycolate oxidase, and lactate oxidase have been found to not produce $O_2^{\cdot}$ at pH 8.5 (18). However, our data indicate that the mammalian L-amino acid oxidase–L-α-hydroxyisocaproate system produces $O_2^{\cdot}$ and $^1O_2$ at a pH higher than 9.0.

The possibility exists in the cases of such flavoproteins that $O_2^{\cdot}$ might indeed be produced in the reaction of a reduced flavoprotein with O_2 at lower pH values, but that $O_2^{\cdot}$ is so tightly bound to the enzyme that both Fe^{3+} and the superoxide dismutase are ineffective scavengers of the $O_2^{\cdot}$. It may well be that the liberation of free $O_2^{\cdot}$ from mammalian L-amino acid oxidase is dependent upon the pH of the incubation media.

$$FMN^{\cdot} + O_2 \rightarrow FMN\ldots O_2^{\cdot} \quad (1)$$

$$FMN\ldots O_2^{\cdot} + {}^{-}OH \rightleftarrows FMN\ldots {}^{-}OH + O_2^{\cdot} \quad (2)$$

As pointed by Micheleson and Isambert (19), the equilibrium of the second process may be displaced to the right in the alkaline media.

Summary. The generation of singlet oxygen and superoxide anion during a flavoprotein (NADPH cytochrome c reductase, xanthine oxidase, or L-amino acid oxidase)–substrate-O_2 interaction has been proved indirectly by activation of luminol, which could be inhibited by the addition of either superoxide dismutase or Fe^{3+}-ADP complex. Catalase was always added to the system to exclude hydrogen peroxide which probably generates in the system and also activates luminol at pH greater than 8.0. The lowest pH at which light was emitted increased progressively in the xanthine oxidase, cytochrome c reductase, and L-amino acid oxidase system.

In terms of the efficiency of an enzyme in producing both singlet oxygen and superoxide anion at pH 10.0, both L-amino acid oxidase and

Table I.[a]

Enzyme system	Efficiency units
L-α-Hydroxyisocaproate–L-amino acid oxidase	125
NADPH–NADPH-cytochrome c reductase	31,000
Xanthine–xanthine oxidase	500,450

[a] The incubation was carried out under the conditions described in the legend to Figs. 1 and 2, except that the pH of the incubation media was fixed at 10.0. Efficiency units were calculated as described in text.

NADPH cytochrome c reductase systems were distinctly less effective than the xanthine oxidase.

1. Arneson, R. M., Arch. Biochem. Biophys. **136,** 352 (1970).

2. Greenlee, L., Fridovich, I., and Handler, P., Biochemistry **1,** 779 (1962).

3. Henry, J. P., and Michelson, A. M., Biochimie **55,** 75 (1973).

4. Goda, K., Chu, J. W., and Shaap, A. P., Biochem. Biophys. Res. Comm. **52,** 1300 (1973).

5. McCord, J. M., and Fridovich, I., J. Biol. Chem. **243,** 5753 (1968).

6. Agro, F., Giovagnoli, C., de Sole, P., Calabrese, L., Rotilio, G., and Mondovi, V., FEBS Lett. **21,** 183 (1972).

7. Rapp, V., Adams, W. C., and Miller, R. W., Can. J. Biochem. **51,** 158 (1973).

8. Weiss, J., Experientia **9,** 61 (1953).

9. Khan, A. V., Science **168,** 476 (1970).

10. Seliger, H., and McElroy, W. D., "Light Physical and Biological Action," p. 155. Academic Press, New York (1965).

11. Hodgson, E. K., and Fridovich, I., Photochem. Photobiol. **18,** 451 (1973).

12. McCord, J. M., and Fridovich, I., J. Biol. Chem. **244,**6049 (1969).

13. Horecker, B. L., and Heppel, L. A., *in* "Method in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, p. 482. Academic Press, New York (1955).

14. Omura, T., and Takesue, S., J. Biochem. (Tokyo) **67,** 249 (1970).

15. Nakano, M., and Danowski, T. S., *in* "Method in Enzymology" (H. Tabor and C. W. Tabor, eds.), Vol. 17 B, p. 601. Academic Press, New York (1971).

16. Shimizu, Y., Inaba, H., Kumaki, K., Mizuno, K., Hata, S., and Tomioka, S., IEEE Trans. Instrum. Measurem. **IM-22,** 153 (1973).

17. Nakamura, S., and Yamazaki, I., Biochim. Biophys. Acta **189,** 29 (1969).

18. Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. G., Mathews, R. G., Schuman, M., and Sullivan, P. A., Biochem. Biophys. Res. Comm. **36,** 891 (1969).

19. Michelson, A. M., and Isambert, M. F., Biochimie **55,** 619 (1973).

Received May 3, 1974. P.S.E.B.M., 1974, Vol. 147.