# ROLE OF SUPEROXIDE RADICAL IN MITOCHONDRIAL DEHYDROGENASE REACTIONS

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SUMMARY: The reduction of dichlorophenolindophenol by both dihydroorotate dehydrogenase and succinate dehydrogenase in the presence of cyanide is mediated in part by superoxide radical. Qualitative and quantitative distinctions in the mode of dichlorophenolindophenol reduction and in the inhibition by ferrisuperoxide dismutase of this reduction suggest differences in the manner in which the two dehydrogenases are coupled to the electron transport chain.

## INTRODUCTION

Dihydroorotate dehydrogenase, the fourth enzyme in the pathway for synthesis of pyrimidines *de novo*, catalyzes the oxidation of dihydroorotate to orotate. In *Zymobacterium oroticum*, the organism in which oxidation of dihydroorotate was first discovered (1), dihydroorotate dehydrogenase (EC 1.3.3.1) can function as a NAD-dependent dihydroorotate dehydrogenase, a dihydroorotate oxidase or as a NADH oxidase (2). However, mammalian dihydroorotate dehydrogenase, a mitochondrial enzyme (3), does not use NAD but apparently is linked to the electron transport chain through ubiquinone (4,5,6). Miller and Curry (6) provided spectrophotometric evidence which suggested that dihydroorotate dehydrogenase is as effective as succinate dehydrogenase (EC 1.3.99.1) in reducing the cytochrome b content of whole and sonically disrupted mitochondria. In the presence of cyanide, dihydroorotate dehydrogenase has been shown to reduce DCIP[2] (5). This reduction of an artificial electron acceptor and the presence of both flavin and non-heme iron in the partially purified dihydroorotate dehydrogenase complex (5) suggests certain similarities to xanthine oxidase.

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[2]Abbreviations: DCIP, dichlorophenolindophenol; PMS, phenazine methosulfate.

A number of years ago Horecker and Heppel (7) showed that the xanthine-dependent cytochrome c reductase activity of xanthine oxidase is accelerated by oxygen. Subsequently Fridovich and Handler (8) showed that xanthine oxidase reduces oxygen both univalently to superoxide and divalently to peroxide. Both the non-heme iron (9,10) and the flavin (11) prosthetic groups have been proposed as the site of this reduction. The use of superoxide dismutase (12) has provided evidence for the involvement of superoxide in the mechanisms of several enzymes containing a variety of cofactors (11,13,14), in reductive activation of enzymes (15), in non-enzymatic oxidations (16,17,18) and hydroxylation (19) and in the electrolytic reduction of oxygen (20). In studies of the electron transport chain where cyanide is present, the copper-zinc superoxide dismutase cannot be used because it is inhibited by cyanide; however, the more recently discovered manganese containing superoxide dismutases from *Escherichia coli* (21) and mitochondria (22) and the iron containing superoxide dismutase from *E. coli* (23) are not inhibited by cyanide (24). In the present investigation the production of superoxide radical by dihydroorotate dehydrogenase and by succinate dehydrogenase in the presence of cyanide is demonstrated through the use of ferri-superoxide dismutase. Differences between the two dehydrogenases in their mode of electron transfer were observed.

## MATERIALS AND METHODS

Antimycin A, cytochrome c type VI, DCIP, and PMS were obtained from Sigma Chemical Company. Dihydroorotate dehydrogenase was assayed using direct observation of orotate formation at 280 nm (3) and/or DCIP reduction in the presence of cyanide according to Miller (5) except that extra ubiquinone was not added and 0.1 M Tris-HCl buffer, pH 8.5, was substituted for phosphate buffer. Succinate dehydrogenase was assayed by following DCIP reduction at 600 nm in the presence of cyanide according to the method of Arrigoni and Singer (25) or by following fumarate production at 250 nm in the presence of cyanide as in the method of Chance (26). Mitochondria were prepared from rat liver homogenates as previously described (3). After freezing, thawing and washing, the mitochondrial fragments are essentially free of mitochondrial superoxide dismutase. Dihydroorotate dehydrogenase activity was stable during storage at 4° whereas succinate dehydrogenase activity diminished at a rate of approximately 5% per day. Protein was measured by the method of Lowry *et al.* (27).

## RESULTS AND DISCUSSION

When dihydroorotate dehydrogenase is assayed in the presence of cyanide

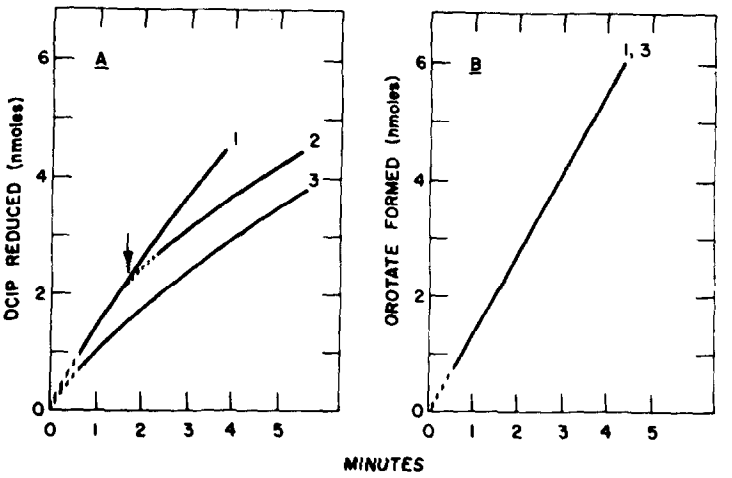


Fig. 1. Effect of superoxide dismutase on DCIP reduction (A) and dihydroorotate oxidation (B) by dihydroorotate dehydrogenase in the presence of cyanide. All reaction mixtures contained: 0.1 M Tris-HCl buffer, pH 8.5; $1x10^{-3}$ M EDTA; $4.3x10^{-5}$ M DCIP; $1x10^{-3}$ M KCN; $1x10^{-4}$ M sodium dihydroorotate and the mitochondrial preparation (0.54 mg of protein) in a final volume of 3 ml. The reaction was started by addition of dihydroorotate and followed at 25° as described under METHODS. Curve 1, superoxide dismutase absent; Curve 2, addition of superoxide dismutase to a concentration of $1x10^{-7}$ M at the time indicated by the arrow; Curve 3, $1x10^{-7}$ M superoxide dismutase present at the beginning of the reaction.

and with DCIP as the terminal electron acceptor, the initial rate of DCIP reduction is equivalent to the rate of conversion of dihydroorotate to orotate (Fig. 1). Addition of ferri-superoxide dismutase from *E. coli* has no effect on the rate of conversion of dihydroorotate to orotate but does affect the reduction of DCIP. Reduction of DCIP is decreased approximately 30%, indicating that superoxide is one product of the reaction. Larger amounts of superoxide dismutase did not enhance the decrease in DCIP reduction. The results with succinate dehydrogenase are quite different. As previously reported by Arrigoni and Singer (25), DCIP reduction in the presence of PMS (Fig. 2A, curve 4) is comparable to fumarate production (Fig. 2B, curve 1). In the absence of PMS (Fig. 2A, curve 1) the magnitude of DCIP reduction is only about 3% of that of the conversion of succinate to fumarate. This small amount of DCIP reduction is totally dependent on superoxide formation (Fig. 2A, curves 2 and 3) whereas in the presence of PMS no superoxide dependent reduction was detected (Fig. 2A, curve 5). A recent report (28) has demonstrated the production of superoxide

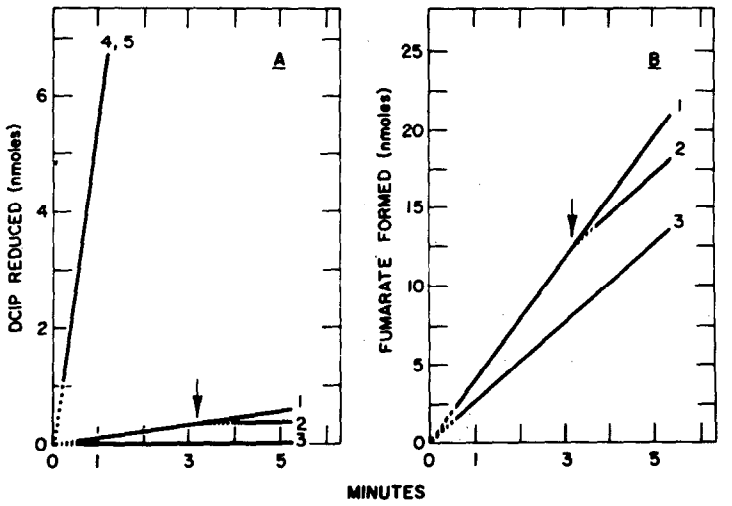


Fig. 2. Effect of superoxide dismutase and PMS on DCIP reduction (A) and succinate oxidation (B) by succinate dehydrogenase in the presence of cyanide. All reaction mixtures contained: 0.1 M sodium phosphate buffer, pH 7.5; $1x10^{-3}$ M EDTA; $4.3x10^{-5}$ M DCIP; $1x10^{-3}$ M KCN; $5x10^{-3}$ M sodium succinate and the mitochondrial preparation (0.27 mg of protein) in a final volume of 3 ml. After addition of substrate, the reaction was followed at 25° as described under METHODS. Where present, the concentrations of superoxide dismutase and PMS were $1x10^{-7}$ M and $3.2x10^{-2}$ M, respectively. Curve 1, superoxide dismutase and PMS absent; Curve 2, superoxide dismutase added at the times indicated by the arrows; Curve 3, superoxide dismutase present initially; Curve 4, PMS present; Curve 5, both superoxide dismutase and PMS present.

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by succinate dehydrogenase in the presence of antimycin A and the absence of PMS. These authors also observed inhibition of peroxide production by superoxide dismutase above pH 7.2. In an attempt to explain what is an apparent contradiction (as the product of superoxide dismutation is peroxide), they suggested that singlet oxygen is an intermediate product which leads to peroxide formation and that superoxide dismutase would quench the singlet oxygen, thus preventing peroxide production. From the results shown in Figure 2, it would seem to be unnecessary to implicate singlet oxygen formation. Superoxide dismutase inhibits not only DCIP reduction in the absence of PMS but conversion of succinate to fumarate as well (Fig. 2B, curves 2 and 3). The mechanism whereby superoxide dismutase inhibits both DCIP reduction and fumarate production is not clear. Inhibition by interaction of superoxide dismutase with superoxide in the active site of the dehydrogenase or removal of "activating" superoxide are two possibilities. Peroxide is a known inhibitor of succinate dehydrogen-

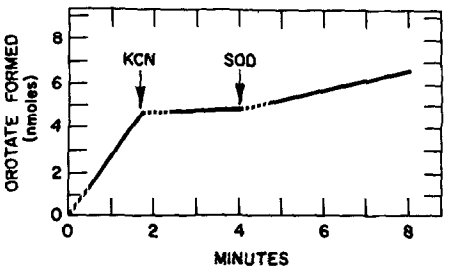


Fig. 3. Effect of superoxide dismutase on dihydroorotate oxidation in the absence of DCIP. The reaction mixture contained 1.08 mg of mitochondrial protein. KCN (final concentration, $3x10^{-4}$ M) and superoxide dismutase (final concentration, $1x10^{-7}$ M) were added at the times indicated. DCIP was not present. Other conditions were those described for Fig. 1. SOD = superoxide dismutase.

ase (29) and apparently superoxide is the precursor for much of the peroxide production in this system (28). In the presence of PMS, no inhibition of DCIP reduction was seen. This suggests that PMS and molecular oxygen compete for the same site on succinate dehydrogenase. An additional problem with singlet oxygen as a product is that oxygen does not accept electrons in going from the ground state to singlet oxygen, and therefore the electrons from succinate would have no acceptor.

If DCIP is omitted from the dihydroorotate dehydrogenase assay, orotate is formed at a rapid rate until addition of KCN, at which point the reaction rate drops almost to zero (Fig. 3). When superoxide dismutase is then added, orotate production resumes, though at a much slower rate than initially. Thus it appears superoxide may cause product inhibition of dihydroorotate dehydrogenase and the inhibition is eliminated by superoxide dismutase (Fig. 3) or by DCIP (Fig. 1A). It is likely that DCIP competes with superoxide dismutase and can also accept electrons through some other channel not involving superoxide, *e.g.*, ubiquinone.

When added cytochrome c is employed in the dihydroorotate dehydrogenase system as the terminal acceptor in the presence of cyanide, only slight (3%) inhibition by superoxide dismutase is seen (Fig. 4, curve 6). Inclusion of antimycin A results in slower reduction of added cytochrome c (Fig. 4, curve 1) and superoxide dismutase now inhibits approximately 25% (Fig. 4, curve 3). This suggests that the site of superoxide formation is within the steps before electron transfer from cytochrome b to cytochrome c.

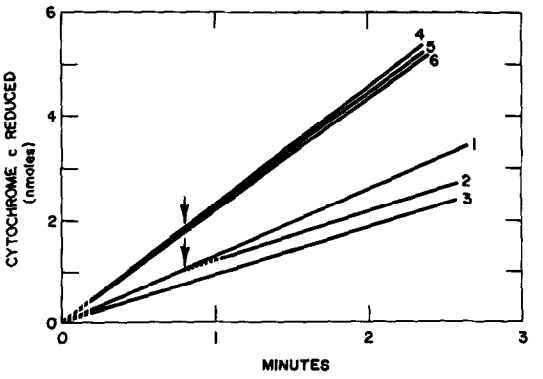


Fig. 4. Effect of superoxide dismutase and antimycin A on cytochrome c reduction by dihydroorotate dehydrogenase in the presence of cyanide. The reaction mixtures (see Fig. 1) contained 0.36 mg of mitochondrial protein and $2 \times 10^{-5}$ M cytochrome c in place of DCIP. Reduction of cytochrome c was followed at 550 nm. When present, the concentration of superoxide dismutase was $1 \times 10^{-7}$ M. Curves 1, 2,3: antimycin A, 0.05 mg per ml, present at the start of incubation. Curves 1 and 4, superoxide dismutase absent; Curves 2 and 5, superoxide dismutase added (arrows); Curves 3 and 6, superoxide dismutase present initially.

Dihydroorotate dehydrogenase and succinate dehydrogenase appear to differ in that the dihydroorotate dehydrogenase complex inherently possesses a substance which acts like PMS or ubiquinone, a substance seemingly lacking in the succinate dehydrogenase complex since DCIP reduction equivalent to fumarate formation requires addition of PMS. It is important to note that the effects of superoxide dismutase were the same whether present initially or added during the course of the reactions. This distinction is necessary as can be seen with the different tryptophan dioxygenases. Superoxide dismutase inhibits superoxide activation but not the on-going reaction of the Pseudomonad and rat liver tryptophan dioxygenases (15) while with the tryptophan dioxygenase of rabbit intestine, superoxide dismutase inhibits whether added before or during the reaction (13); in the former, superoxide is one of several reductants which can activate the enzyme while in the latter, superoxide is apparently an intermediate in the reaction. These preliminary results form the basis for further investigations into the connection of dihydroorotate dehydrogenase with the electron transport chain.

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## REFERENCES

1. Lieberman, I. and Kornberg, A. (1953) Biochim. Biophys. Acta *12*, 223-234.
2. Friedmann, H. C. and Vennesland, B. (1958) J. Biol. Chem. *233*, 1398-1405.
3. Kennedy, J. (1973) Arch. Biochem. Biophys. *157*, 369-373.
4. Kuligowska, E. and Erecinska, M. (1967) Bull. Acad. Polon. Sci. *15*, 187-190.
5. Miller, R. W., Kerr, C. T. and Curry, J. R. (1968) Can. J. Biochem. *46*, 1099-1106.
6. Miller, R. W. and Curry, J. R. (1969) Can. J. Biochem. *47*, 725-734.
7. Horecker, B. L. and Heppel, L. A. (1949) J. Biol. Chem. *178*, 683-690.
8. Fridovich, I. and Handler, P. (1962) J. Biol. Chem. *237*, 916-921.
9. Palmer, G., Bray, R. C. and Beinert, H. (1964) J. Biol. Chem. *239*, 2657-2666.
10. Bray, R. C., Palmer, G. and Beinert, H. (1964) J. Biol. Chem. *239*, 2667-2676.
11. Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. C., Matthews, R. G., Schulman, M. and Sullivan, P. A. (1969) Biochem. Biophys. Res. Commun. *36*, 891-897.
12. McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. *244*, 6049-6055.
13. Hirata, F. and Hayaishi, O. (1971) J. Biol. Chem. *246*, 7825-7826.
14. Strobel, H. W. and Coon, M. J. (1971) J. Biol. Chem. *246*, 7826-7829.
15. Brady, F. O., Forman, H. J. and Feigelson, P. (1971) J. Biol. Chem. *246*, 7119-7124.
16. McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. *244*, 6056-6063.
17. Misra, H. P. and Fridovich, I. (1972) J. Biol. Chem. *247*, 3170-3175.
18. Cohen, G. and Heikkila, R. E. (1974) J. Biol. Chem. *249*, 2447-2452.
19. Goscin, S. A. and Fridovich, I. (1972) Arch. Biochem. Biophys. *153*, 778-783.
20. Forman, H. J. and Fridovich, I. (1972) Science *175*, 339.
21. Keele, B. B., Jr., McCord, J. M. and Fridovich, I. (1970) J. Biol. Chem. *245*, 6176-6181.
22. Weisiger, R. and Fridovich, I. (1973) J. Biol. Chem. *248*, 3582-3592.
23. Yost, F. J., Jr. and Fridovich, I. (1973) J. Biol. Chem. *248*, 4905-4908.
24. Beauchamp, C. O. (1973) Ph.D. Thesis, Duke University.
25. Arrigoni, O. and Singer, T. P. (1962) Nature *193*, 1256-1258.
26. Chance, B. (1952) J. Biol. Chem. *197*, 557-589.
27. Lowry, O. H., Rosebrough, M. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. *193*, 265-275.
28. Loschen, G., Azzi, A., Richter, C. and Flohe, L. (1974) FEBS Letters *42*, 68-72.
29. Singer, T. P. and Kearney, E. B. (1957) Methods of Biochem. Anal. Vol. *IV*, pp. 307-333, Interscience, New York.