# IDENTIFICATION BY ELECTRON SPIN RESONANCE SPECTROSCOPY OF THE PRIMARY PRODUCT OF TYROSINASE - CATALYZED CATECHOL OXIDATION[1]

H. S. Mason, E. Spencer, and I. Yamazaki

Department of Biochemistry, University of Oregon Medical School, Portland

Received February 9, 1961

We wish to report that during the oxidation of catechols in the presence of tyrosinase, the electron spin resonance spectra of o-benzosemiquinones can be observed. Using a flow technique we have measured free radical concentrations as functions of time, pH, enzyme and substrate concentrations, and we have compared them with the corresponding concentrations observed when catechol is peroxidatically oxidized.

Our observations were made with a Varian V-4500 e.s.r. spectrometer using 100 kc modulation. A flat quartz cell was mounted in the resonance cavity and attached through a four-jet lucite mixer to a gas-driven solenoid-controlled flow system through which 10 ml/sec. of reaction mixture could readily be forced. Field strengths were monitored with a Varian F-8 fluxmeter, and klystron frequencies with a Hewlett-Packard frequency meter. Free radical concentrations were measured against fresh titrimetrically standardized solutions of peroxylamine disulfonate prepared in this laboratory by Dr. G. Narni.

Tyrosinase, purified from mushrooms by the Frieden-Ottesen procedure (1) had specific activities in the order of 700-1200 Miller-Dawson units per mg. We used crystalline horseradish peroxidase, R. Z. = 3.0.

The autoxidative formation of o-benzosemiquinone from 0.01 M. catechol, pH 7.6 (0.1 M phosphate), at 0.6 mM $O_2$, could not be detected,

1. This study was supported by the American Cancer Society and PHS.

but in the presence of 15 Miller-Dawson units of tyrosinase per ml. (overall reaction velocity = $2.1 \times 10^{-4}$ M/sec) or $14.4 \times 10^{-8}$ M peroxidase and 1.2 mM $H_2O_2$ (overall reaction velocity = $3.2 \times 10^{-4}$ M/sec) the concentration of o-benzosemiquinone rose to $3.2 \times 10^{-6}$ M, then decreased. An unidentified radical then formed. The maximum concentration of o-benzosemiquinone was dependent upon initial catechol concentration but not (within the limits employed) on enzyme concentration, which, however, influenced the time required to reach the maximum. Up to the point of virtual exhaustion of $O_2$ or $H_2O_2$ respectively, the free radical formation curves appeared to be described by the equilibrium relationship, Equ. 1:

$$1. \qquad \frac{(\text{Free radical})^2}{(\text{Catechol})\ (\text{o-Quinone})} = K_{equ.}$$

During the first 150 msec. at pH 7.6, the peroxidatic reaction was characterized by a steady state free radical concentration proportional to the square root of enzyme concentration, as previously reported for peroxidatic ascorbate oxidation (3). No steady state was observed in the case of tyrosinase acting at a comparable overall velocity (Figure 1).

At pH 5.3 (0.1 M acetate buffer) the equilibrium concentration of o-benzosemiquinone generated from 0.005 M catechol and .005 M o-quinone is below the level of detectability by our e. s. r. spectrometer ($10^{-7}$ M). The generation of o-benzosemiquinone from 0.01 M catechol, 0.6 mM $O_2$, by tyrosinase ($V = 2.6 \times 10^{-4}$ M/sec) could not be detected. Nevertheless, peroxidatic oxidation of 0.01 M catechol at the same pH and overall velocity ($V = 2.3 \times 10^{-4}$ M/sec) produced a typical steady state at $0.4 \times 10^{-6}$ M o-benzosemiquinone.

Accordingly, 94% or more of the primary product of tyrosinase-catalyzed oxidation under these conditions must be o-benzoquinone. The free radical which appears during the reaction at pH 7.6 is very largely if not entirely formed by reverse disumtation. The mechanism of tyrosinase-

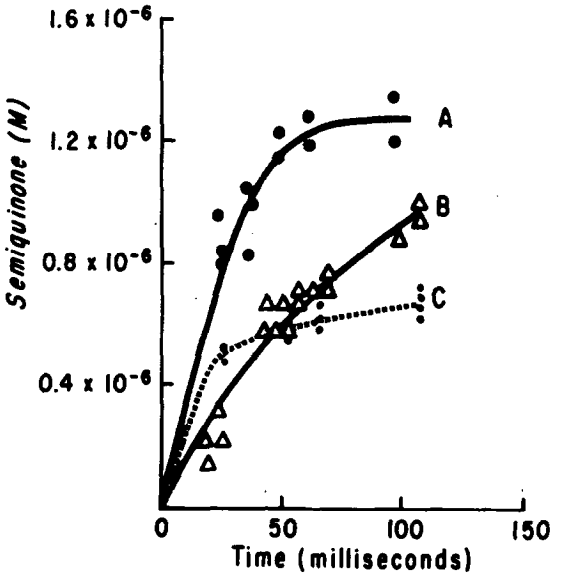


Figure 1. Comparison of o-benzosemiquinone generation by peroxidase and tyrosinase at pH 7.6, 0.1 M. phosphate buffer, 0.01 M. catechol, at 21°. A: peroxidase ($V = 3.2 \times 10^{-4}$ M/sec); C: peroxidase ($V = 0.8 \times 10^{-4}$ M/sec); B, tyrosinase ($V = 2.2 \times 10^{-4}$ M/sec). See text for other details.

catalyzed oxidation therefore involves two two-electron transfers per molecule of oxygen consumed, and the Type III mechanism (2) for the cresolase function of this enzyme becomes probable.

A complete report of this investigation will be published elsewhere. Our e. p. r. work is now being extended to the problem of cresolase mechanism.

References

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