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Bioorganic Mechanisms of the Formation of Free Radicals Catalyzed by Glucose Oxidase

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In this communication, we have described the activation of several xenobiotics by glucose oxidase from *Aspergillus niger*. The following compounds are readily reduced by D-glucose, in the presence of glucose oxidase: *p*-nitroso-*N*,*N*-dimethylaniline, methyl-1,4-benzoquinone, and 7,7,8,8-tetracyano-quinodimethane. In each case, the products of enzymatic reduction undergo a dismutation reaction with the parent compound and thus afford the formation of free radicals. In some cases, and at an appropriate pH value, the transformation of a parent compound into free radicals is almost quantitative. Under optimal conditions, free radicals are stable for several minutes in aqueous solutions under physiological conditions.

INTRODUCTION

Investigation of the enzyme-catalyzed formation of free radicals, in this communication, was prompted by our early observation that yeast alcohol dehydrogenase is able to catalyze reduction of *p*-nitroso-*N*,*N*-dimethylaniline by NADH (Leskovac *et al.*, 1996); in the course of this enzymatic reaction, a considerable amount of relatively stable quinone diimine cation radicals are formed from NDMA (Leskovac, 1999; Leskovac *et al.*, 2002). In this reaction, NDMA is catalytically activated by an enzymatic reduction with NADH, followed with the formation of free radicals by the process of dismutation.

Further, we have found that a flavoenzyme glucose oxidase from *Aspergillus niger* can catalyze reduction of NDMA with glucose, followed by the same process of radical dismutation. Glucose oxidase is able to catalyze reduction of a large number of substrates with glucose (Wilson and Turner, 1992). In the course of this work, we have found that, among many substrates, quinones and some quinoid compounds are activated by glucose oxidase by the similar process of radical dismutation; considerable amounts of stable free radicals are generated in these reactions.

Stable free radicals, formed by enzymatic activation of xenobiotics, are generally

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toxic in living cells; the chemical and enzymatic reactions of radical dismutation are, therefore, important in toxicology. It appeared to us, that little attention has been paid to this type of reactions, even in the vast scientific literature dealing with the biochemical action of free radicals in living cells. For this reason, we have investigated the activation of various quinoid compounds with glucose oxidase by the chemical process of radical dismutation, which is described in this communication.

MATERIALS AND METHODS

Biochemicals

Kinetic measurements were performed using glucose oxidase preparations from *A. niger* (EC 1.1.3.4), type VII-S, obtained from Sigma. According to Sigma, the lyophilized powder contained approximately 80% protein; balance was phosphate buffer and sodium chloride. The catalase impurity in Sigma preparations was sufficient to remove rapidly the H_2O_2 product from solution. The specific activity of enzyme was 100,000–200,000 units/gram solid (without added oxygen); one unit will oxidize 1.0 μmol of β-D-glucose to D-gluconic acid and H_2O_2 per minute, at pH 5.1 and 35°C. Glucose oxidase is a homodimer, composed of two identical subunits; the molecular weight of enzyme is 150,000 Da. Each subunit carries one molecule of tightly bound coenzyme, FAD, which is acting as a redox carrier in catalysis (Gibson *et al.*, 1964). The concentration of enzyme active sites was determined from its molar extinction coefficient at 450 nm ($\varepsilon = 14100\ M^{-1}\ cm^{-1}$, in 0.1 M sodium acetate buffer, pH 5.5) (Gibson *et al.*, 1964); the concentration of enzyme in solution throughout this work was expressed as the concentration of its active sites.

Cytochrome-c from horse heart was obtained from Sigma (C 7752). Its concentration was determined from the molar extinction coefficient of ferricytochrome-c at 450 nm ($\varepsilon = 105000\ M^{-1}\ cm^{-1}$), or from the difference between the spectrum of ferricytochrome-c and ferrocytochrome-c at 550 nm ($\varepsilon = 21{,}000\ M^{-1}\ cm^{-1}$) (Dickerson and Timkovich, 1975).

Methyl-1,4-benzoquinone was obtained from Aldrich and purified by sublimation; since quinones are sensitive to light, the freshly prepared solutions were kept in dark. 7,7,8,8-Tetracyano-quinodimethane and *p*-nitroso-*N*,*N*-dimethylaniline were obtained from Sigma, and used without further purification.

Throughout this work, 0.1 M McIlvaine citrate-phosphate buffers were used to maintain the pH values from 2.9–6.5, except when stated otherwise.

Methods

The initial velocity studies were carried out and the absorption spectra were recorded in a double-beam spectrophotometer, in thermostated cuvette holders at 25°C. The concentrations of substrates and enzymes were estimated from their molar extinction coefficients (Table 1).

The initial rate of cytochrome-c reduction in Figs. 5, 6, and 7, was measured at 550 nm.

RESULTS

Enzymatic Reduction of TCQD with Glucose and Glucose Oxidase

7,7,8,8-Tetracyano-quinodimethan (TCQD) is readily reduced with D-glucose, under anaerobic conditions, in the presence of glucose oxidase. In the course of this

TABLE 1

Molar Extinction Coefficients in Aqueous Solutions

Compound	λ_{max} (nm)	ε (M^{-1} cm^{-1})	pH	Source
Glucose oxidase	450	14100	5.5	Swoboda and Massey (1966)
TCQD	395	63600	5.5	Acker and Herter (1962)
$TCQDH_2$	476.2	22000	5.5	Svirčević (1987)
TCQD-radical	740.7	23280	5.5	Melby *et al.* (1962)
NDMA	442	35400	7.0	Dunn and Bernhard (1971)
DPD-Radical	550	13450	4.55	This work
MeBQ	332	513	5.5	Leskovac *et al.* (2002)
	253	18563	5.5	Leskovac *et al.* (2002)
Ferri-cytochrome-c	407	105000	7.0	Dickerson and Timkovich (1975)
Ferro/ferri-cytochrome-c	550	21000	7.4	Massey (1959)

enzymatic reaction, characteristic spectral changes are taking place simultaneously: the spectrum of TCQD at 400 nm decreases gradually while the spectra of *p*-phenylenedimalononitrile ($TCQDH_2$) at 476 nm and of TCQD-radical ($TCQDH^{\bullet}$) at 741 nm are increasing, with an isosbestic point at 420 nm. Below pH 6.0, reduction of TCQD does not take place in the absence of glucose oxidase.

Figure 1 shows the reaction progress curves at pH 6.0.

Enzymatic reduction of TCQD with glucose presumably proceeds by the following mechanism:

$$\text{E.FAD} + \text{D-Glucose} \rightarrow \text{E.FADH}_2 + \delta\text{-Gluconolactone} \qquad (1)$$

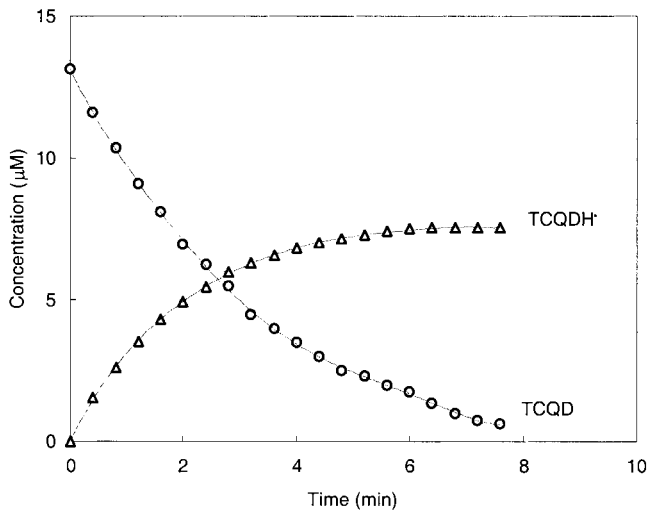


FIG. 1. Reaction progress curves for the enzymatic reduction of TCQD at pH 6.0. Initial concentration of reactants: [GOD] = 16.23 nM; [TCQD] = 13.11 μM; [Glc] = 472 mM.

$$\text{E.FADH}_2 + \text{TCQD} \rightarrow \text{E.FAD} + \text{TCQDH}_2 \quad (2)$$

$$\text{TCQDH}_2 + \text{TCQD} \rightleftharpoons 2\ \text{TCQDH}^{\bullet} \quad (3)$$

$$\text{Total: 2 TCQD} + \text{D-Glucose} \rightarrow 2\ \text{TCQDH}^{\bullet} + \delta\text{-Gluconolactone} \quad (4)$$

where E.FAD is the oxidized form of glucose oxidase, and E.FADH_2 is the reduced form of enzyme.

From Fig. 1, one can calculate that the initial rate of the disappearance of TCQD (4.19 μM/min) is almost identical with the initial rate of appearance of TCQD-radicals (4.05 μM/min). Thus the overall stoichiometry shown by equation (4) holds for the initial phase of reaction, with equation (2) being the rate-limiting step of the overall process; from the above data, one can calculate the bimolecular rate constant for this reaction at pH 6.0, $k_{\text{II}} = 330\ \text{mM}^{-1}\ \text{s}^{-1}$.

Nonenzymatic Reduction of TCQD with NADH

TCQD is readily reduced with NADH, under aerobic conditions (Fig. 2).

Nonenzymatic reduction of TCQD with NADH presumably proceeds by the following mechanism:

$$\text{NADH} + \text{TCQD} + \text{H}^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{NAD}^+ + \text{TCQDH}_2 \quad (5)$$

$$\text{TCQDH}_2 + \text{TCQD} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} 2\ \text{TCQDH}^{\bullet} \quad (6)$$

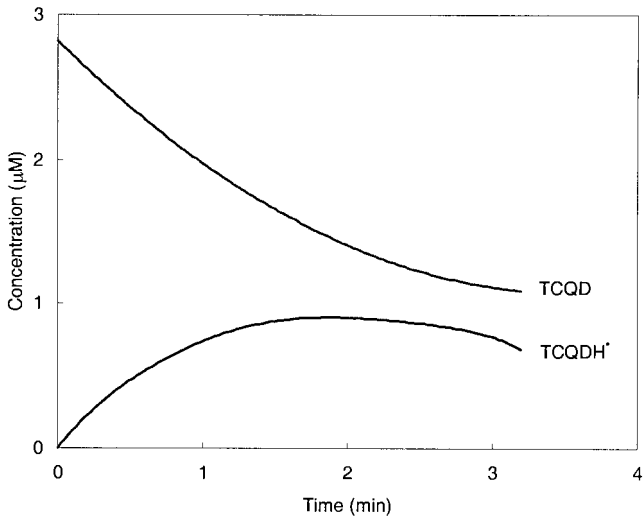


FIG. 2. Reaction progress curve for the nonenzymatic reduction of TCQD at pH 6.0. Initial concentration of reactants: [TCQD] = 2.82 μM; [NADH] = 5.37 μM.

TABLE 2

Rate Constants Obtained by the Simulation of Reaction Progress Curves from Fig. 2 with a FITSIM Program

Rate constant	$mM^{-1}\ s^{-1}$	Rate constant	$mM^{-1}\ s^{-1}$
k_1	0.825 ± 0.026	k_3	63.5 ± 19.3
k_2	1.366 ± 1.833	k_4	49.0 ± 15.8

Reaction progress curves in Fig. 2 were simulated with the KINSIM and FITSIM computer programs (Barshop *et al.*, 1983; Zimmerle and Frieden, 1989; Frieden, 1994), according to the minimal mechanism given by Eqs. (5) and (6). The simulation procedure was performed with two files, one for the disappearance of TCQD and the other for the appearance of the TCQD-radical, affording the following results (Table 2).

Enzymatic Reduction of NDMA with D-Glucose and Glucose Oxidase

p-Nitroso-*N*,*N*-dimethylaniline (NDMA) is readily reduced with D-glucose, under anaerobic conditions, in the presence of glucose oxidase. In the course of this enzymatic reaction, characteristic spectral changes are taking place simultaneously: the spectrum of NDMA at 442 nm decreases gradually, while the spectrum of *N*,*N*-dimethyl-*p*-phenylenediamine radical (DPD-radical) at 550 nm increases.

Figure 3 shows the reaction progress curves at pH. 4.55.

Glucose oxidase-catalyzed reduction of NDMA with D-glucose proceeds by the

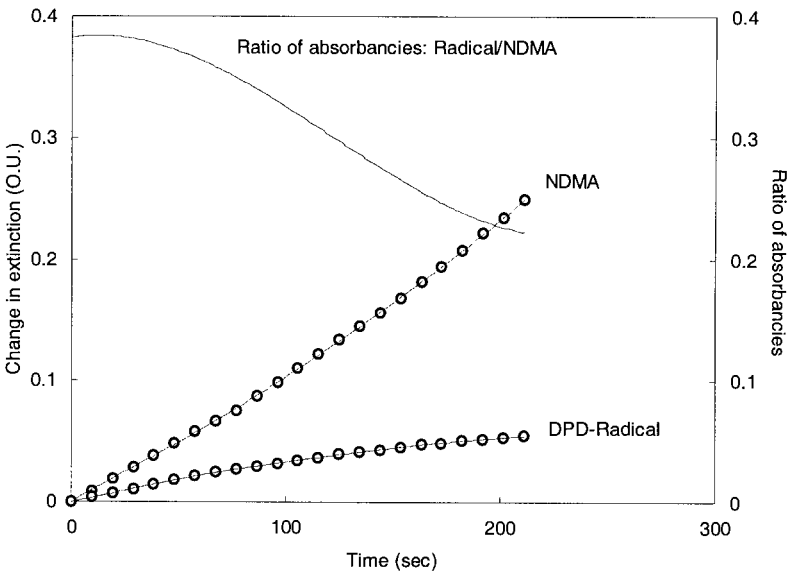


FIG. 3. Reaction progress curves for the enzymatic reduction of NDMA at pH 4.55. Initial concentration of reactants: [GOD] = 25.4 nM; [NDMA] = 32.7 μM; [Glc] = 99.5 mM.

following mechanism (Baetzold and Tong, 1971; Koerber *et al.*, 1980; Leskovac and Trivić 1988; Leskovac *et al.*, 1996).

$$\text{E.FAD} + \text{D-Glucose} \rightarrow \text{E.FADH}_2 + \delta\text{-Gluconolactone} \quad (7)$$

$$\text{NDMA} + \text{E.FADH}_2 \rightarrow \text{QDI}^+ + \text{E.FAD} + \text{HO}^- \quad (8)$$

$$\text{QDI}^+ + \text{E.FADH}_2 \rightleftharpoons \text{ADMA} + \text{E.FAD} + \text{H}^+ \quad (9)$$

$$\text{QDI}^+ + \text{ADMA} + \text{H}^+ \rightleftharpoons 2\ \text{DPD-Radical} \quad (10)$$

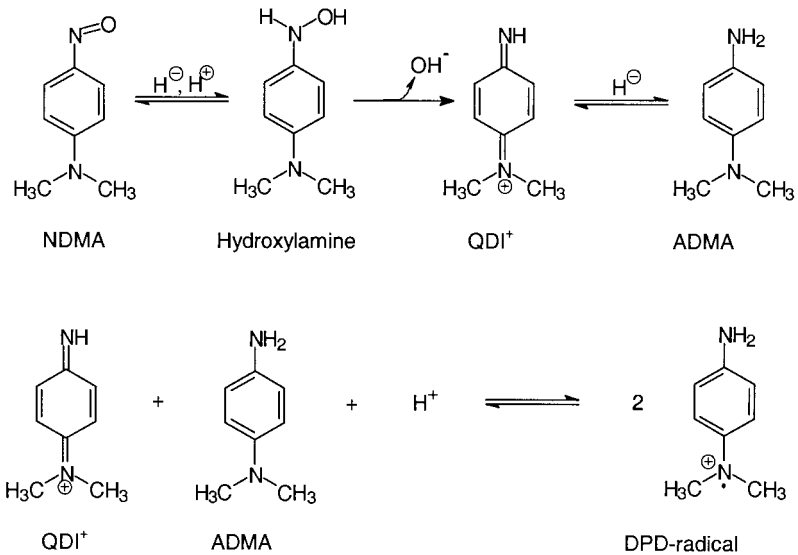
The chemical reactions involved in Eqs. (7)–(10) are shown in Scheme 1.

NDMA becomes protonated in the acid, probably on the amino nitrogen, and the spectral peak at 442 nm is lost; pK_a of this transition is 4.33. The protonated form of NDMA is not reduced by glucose oxidase. Thus,

$$\text{NDMA} + \text{H}^+ \overset{\text{p}K_a=4.3}{\rightleftharpoons} \text{NDMA(H}^+\text{)} \quad (11)$$

The rate of the formation of DPD-radicals is strongly pH-dependent (Fig. 4).

The maximal rate of production of DPD-radicals is at pH 4.55. In the acid, the rate diminishes because NDMA becomes protonated, and in the alkaline because the rate of radical dismutation depends on the concentration of [H^+] [Eq. (10)].



SCHEME 1.