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RELATIONSHIP OF THE SINGLE-ELECTRON REDUCTION POTENTIAL OF QUINONES TO THEIR REDUCTION BY FLAVOPROTEINS

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Abstract—The aerobic and anaerobic metabolism of a series of quinones of known single-electron reduction potential has been studied using flavoenzymes catalyzing single-electron reduction. Metabolism was more closely related to single-electron reduction potential than to structural features or lipid solubility of the quinones studied. The pattern of quinone reduction with purified NADPH-cytochrome P-450 reductase was similar to that seen with NADH : ubiquinone oxidoreductase with NADPH as the cofactor; the lower limit for reduction was a quinone single-electron reduction potential of −240 mV. The lower limit for quinone reduction with purified NADH-cytochrome b_5 reductase and NADH : ubiquinone oxidoreductase with NADH as the cofactor was a single-electron reduction potential of −170 mV. With all three enzymes there was a decreased quinone metabolism at higher single-electron reduction potentials. The same pattern of quinone metabolism was seen using purified or microsomal NADPH-cytochrome P-450 reductase and purified or microsomal NADH-cytochrome b_5 reductase respectively. Microsomal quinone metabolism under aerobic conditions showed an increased V_{max} and an unchanged K_m compared to metabolism under anaerobic conditions.

Flavoproteins catalyze both one- and two-electron reduction. For example, NAD(P)H : (quinone-acceptor) oxidoreductase (EC 1.6.99.2), also called DT-diaphorase, catalyzes two-electron reduction [1], while NADPH cytochrome P-450 reductase (EC 1.6.2.4), NADH-cytochrome b_5 reductase (EC 1.6.22) and NADH : ubiquinone oxidoreductase (EC 1.6.5.3) catalyze single-electron reduction [1–5]. Quinones form an important group of substrates for flavoenzymes and undergo either one- or two-electron reduction [1–5]. Single-electron reduction of quinones can lead to the formation of cytotoxic intermediates [6, 7]. This is important because quinones are widely distributed in nature [8], and a relatively large number of anticancer drugs of current clinical and research interest contain the quinone nucleus [9]. Bachur *et al.* [7, 10] have proposed a general mechanism for the antitumor effect of quinone anticancer agents based upon their single-electron reduction within the cell to the semiquinone free radical. They suggested that the free radical is sufficiently stable to enter the nucleus and bind through intercalation to DNA [10]. Little is known, however, about factors which govern single-electron reduction of quinones. The nonenzymatic, two-electron transfer from soluble reduced flavin to an acceptor has been shown to be dependent upon the reduction potential of the acceptor molecule [11]. The single-electron reduction potential of the quinone acceptor could be a factor in the rate of flavoprotein-mediated reduction, especially since flavin-containing enzymes exhibit little substrate specificity for artificial electron acceptors [2, 3, 12–14]. An attempt to relate the rate of single-electron reduction to reduction potential is complicated by the fact that the reduction potentials normally quoted in the literature are for two-electron reductions. These are usually determined in nonaqueous media [15] and cannot be related directly to the component single-electron reduction potentials [16]. Single-electron reduction potentials obtained by pulse radiolysis have recently become available for a limited number of quinones [16–18]. The purpose of the present investigation was to study the role of the single-electron reduction potential upon the reduction of some of these quinones by NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase and NADH : ubiquinone oxidoreductase.

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MATERIALS AND METHODS

Enzyme preparations

Male rats of the Sprague–Dawley strain (Sprague–Dawley, Madison, WI), weighing between 150 and 200 g, were killed by a blow on the head and exsanguinated; the livers were removed and flushed retrogradely through the hepatic vein with 50 ml of 0.9% NaCl at 4°. Hepatic microsomes were prepared by ultracentrifugation following homogenization in 0.25 M sucrose as described by Ernster *et al.* [19]. The microsomes were washed by suspension in 20 vol. of 0.15 M KCl, collected by centrifugation, and suspended in 0.15 M KCl at a protein concentration of 10 mg/ml. Protein was determined by the method of Lowry *et al.* [20], with crystalline bovine serum albumin as a standard. NADPH-cytochrome P-450 reductase was prepared from hepatic micro-

Table 1. Quinones employed in the study*

	No.	Apparent octanol/H_2O partition coefficient	E_7^1 (mV)
p-Benzoquinone	1	1.29	+99 [16]
2-Methyl-*p*-benzoquinone	2	5.34	+23 [17]
1,4-Naphthoquinone-2-sulfonate	3	0.03	−60 [16]
2,5-Dimethyl-*p*-benzoquinone	4	17.34	−67 [17]
2,3,5-Trimethyl-*p*-benzoquinone	5	29.23	−165 [17]
2-Methyl,1,4-naphthoquinone	6	75.00	−200 [17]
2,3,5,6-Tetramethyl-*p*-benzoquinone	7	130.80	−240 [17, 18]
Indigodisulfonate	8	0.01	−247 [18]
9,10-Anthraquinone-2-sulfonate	9	0.01	−375 [16]

* Octanol/H_2O partition coefficients were determined as described in the text. Single-electron reduction potentials (E_7^1) are literature values. References are shown in parentheses.

somes of phenobarbital-induced rats (three intraperitoneal injections of 80 mg phenobarbital/kg/day) by the method of Yasukochi and Masters [21]. NADH-cytochrome b_5 reductase was prepared from hepatic microsomes of uninduced rats by the method of Spatz and Strittmatter [22]. Mitochondria were isolated from the livers of untreated rats, and NADH : ubiquinone oxidoreductase was prepared by the method of Hatefi and Rieske [23].

Incubations

Metabolism was measured by determining the oxidation of NADPH and NADH at 340 nm in an incubation mixture containing Tris–HCl buffer, pH 7.4 (300 μmoles), $MgCl_2$ (15 μmoles), EDTA (0.3 μmole) and microsomal protein (1 mg), NADPH-cytochrome P-450 reductase (40 μg), NADH-cytochrome b_5 (40 μg) or NADH : ubiquinone reductase (50 or 250 μg), all in a final volume of 3 ml at 37°. The quinones, dissolved in 10 μl dimethylsulfoxide, were added 1 min prior to the addition of NADPH or NADH dissolved in 10 μl water. This amount of dimethylsulfoxide had no effect upon NADPH or NADH oxidation. There was no interaction between NADPH or NADH and any of the quinones in the absence of enzyme. Anaerobic incubations were carried out in sealed cuvettes which were alternatively evacuated and gassed with N_2 prior to temperature equilibration. Traces of oxygen were removed from the N_2 used for gassing, as described by Meites and Meites [24]. Superoxide formation was measured by following the reduction of acetylated ferricytochrome *c* at 550 nm (using an extinction coefficient of 19.6 $mM^{-1}cm^{-1}$) [25]. The incubation mixture contained acetylated ferricytochrome *c* (60 μM), and superoxide formation was measured as the difference in the rate of reduction measured with and without superoxide dismutase (33 μg/ml).

Drugs and chemicals

NADH and NADPH were obtained from the Boehringer Mannheim Co., Indianapolis, IN. Superoxide dimutase was obtained from the Sigma Chemical Co., St. Louis, MO. 2,3,5-Trimethyl-*p*-benzoquinone was prepared by oxidation of 2,3,5-trimethyl-*p*-hydroquinone with potassium ferricyanide under alkaline conditions [26] and was purified by thin-layer chromatography [8]. *p*-Benzoquinone, 2-methyl,1,4-naphthoquinone, 2,3,5,6-tetramethyl-*p*-benzoquinone, indigodisulfonate, 9,10-anthraquinone-2-sulfonate, *p*-hydroquinone, 2,5-dimethyl-*p*-hydroquinone and 2,3,5-trimethyl-*p*-hydroquinone were purchased from the Aldrich Chemical Co., Milwaukee, WI, and 2-methyl-*p*-benzoquinone, 1,4-naphthoquinone-2-sulfonate and 2,5-dimethyl-*p*-benzoquinone from the Eastman Kodak Co., Rochester, NY.

RESULTS

Physicochemical characteristics of the quinones

Table 1 lists the quinones used in this study, their apparent octanol/water partition coefficients determined by their u.v. absorption in the two phases at room temperature (no correction was applied for pK_a), and their single-electron reduction potentials (E_7^1).

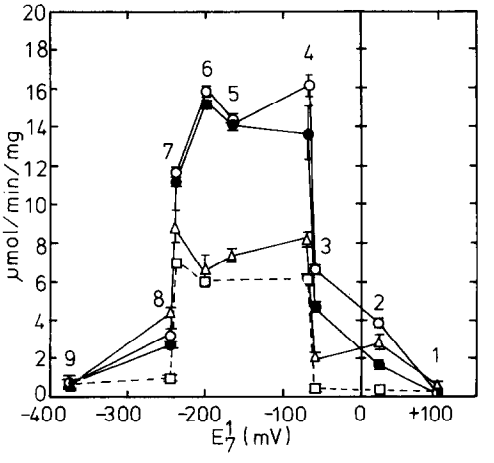

Fig. 1. Quinone reduction by NADPH-cytochrome P-450 reductase. Conditions are described in the text. All quinones were at 10^{-4} M. Key: (□---□) anaerobic NADPH oxidation; (△——△) aerobic NADPH oxidation; (○——○) aerobic acetylated ferricytochrome *c* reduction; and (●——●) aerobic superoxide formation. Each point is the mean of three observations; bars represent S.E.M. Quinones and their single-electron reduction potentials are shown in Table 1.

Quinone metabolism and reduction potential

NADPH-cytochrome P-450 reductase. The metabolism of the various quinones by purified NADPH-cytochrome P-450 reductase is shown in Fig. 1. Quinone metabolism under anaerobic conditions was slower than under aerobic conditions. The rate of NADPH oxidation by NADPH-cytochrome P-450 reductase in the absence of added quinones was 7.1 nmoles·min^{-1}·mg^{-1} under aerobic conditions and was undetectable under anaerobic conditions. NADPH oxidation was stimulated to 8 μmoles·min^{-1}·mg^{-1} by some quinones. The enzymatic reduction of acetylated ferricytochrome *c* under aerobic conditions was 8.0 nmoles·min^{-1}·mg^{-1} in the absence of added quinone and was stimulated over 2000-fold by some quinones. In most cases, acetylated ferricytochrome *c* reduction was inhibited by superoxide dismutase, showing it to be due to the formation of superoxide. Oxygen was reduced only slowly to superoxide by purified NADPH-cytochrome P-450 reductase, and thus the enhanced superoxide formation must have been due to the reduction of the quinone, followed by transfer of an electron from the semiquinone to molecular oxygen. The stoichiometry of superoxide formation to NADPH oxidation, at the higher rates of metabolism, was close to 2:1, suggesting that the increased oxidation of NADPH under aerobic conditions was due to an increased rate of quinone reduction and not to additional pathways of oxidative metabolism. At quinone single-electron reduction potentials more positive than about −100 mV, however, a small proportion of the reduction of acetylated ferricytochrome *c* was not inhibited by superoxide dismutase, probably due to a direct transfer of an electron between the semiquinone and acetylated ferricytochrome *c*. It is interesting that superoxide was formed even at single-electron reduction potentials more positive than −155 mV, the reduction potential of the $O_2/O_2^{\cdot}$ couple under physiological conditions [16].

NADH-cytochrome b_5 *reductase.* A different pattern of quinone stimulated metabolism was seen with NADH-cytochrome b_5 reductase (Fig. 2). Quinone metabolism under aerobic conditions again exceeded that under anaerobic conditions. The rate of NADH oxidation by NADH-cytochrome b_5 reductase in the absence of added quinone was 80.6 nmoles·min^{-1}·mg^{-1} under aerobic conditions and 3.8 nmoles·min^{-1}·mg^{-1} under anaerobic conditions. The relationship between NADH oxidation and acetylated ferricytochrome *c* reduction and superoxide formation was not as straightforward as with NADPH-cytochrome P-450 reductase. The proportion of superoxide formed to acetylated ferricytochrome *c* reduced was lower, probably because of the more positive single-electron reduction potentials of the quinones metabolized by NADH-cytochrome b_5 reductase which would favour a direct electron transfer from the semiquinone to acetylated ferricytochrome *c*. The ratio of total acetylated ferricytochrome *c* reduced to NADH oxidized was, in some cases, less than 2:1, suggesting that the increase in NADH oxidation under aerobic conditions may sometimes be associated with additional pathways of oxidative metabolism. Surprisingly, no acetylated

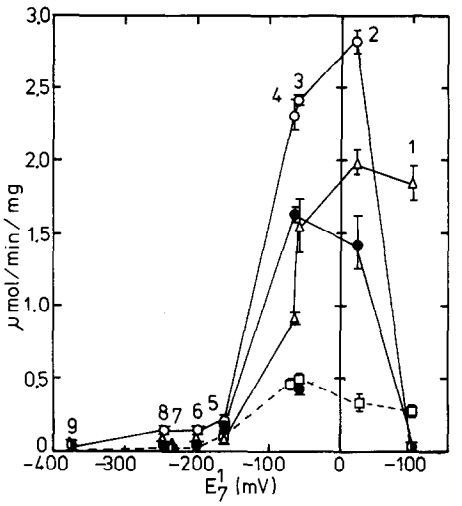

Fig. 2. Quinone reduction by NADH-cytochrome b_5 reductase. Conditions are described in the text. Quinones are as in Table 1 and were at 10^{-4} M. Key: (□---□) anaerobic NADH oxidation; (△——△) aerobic NADH oxidation; (○——○) aerobic acetylated ferricytochrome *c* reduction; and (●——●) aerobic superoxide formation. Each point is the mean of three observations; bars represent S.E.M.

ferricytochrome *c* reduction could be detected with *p*-benzoquinone under aerobic conditions, despite a previous report that *p*-benzoquinone facilitates the reduction of ferricytochrome *c* by NADH-cytochrome b_5 reductase under aerobic conditions [3].

NADH:ubiquinone oxidoreductase. NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase showed an absolute specificity for NADPH and NADH as the respective cofactors. NADH:ubiquinone oxidoreductase, on the other hand, accepted electrons from NADH and NADPH, although at a much slower rate with NADPH. A different pattern of quinone-stimulated metabolism was seen when NADH was the cofactor and when NADPH was the cofactor (Fig. 3). There was no

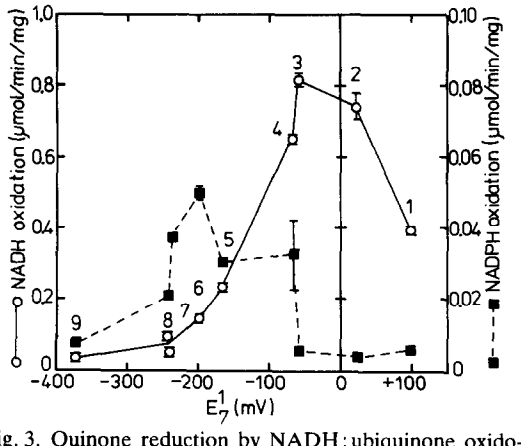

Fig. 3. Quinone reduction by NADH:ubiquinone oxidoreductase under anaerobic conditions. Conditions are described in the text. All quinones were at 10^{-4} M. Key: (○) with NADH as the cofactor; and (■) with NADPH as the cofactor. Quinones are as in Table 1. Each point is the mean of three observations; bars represent ± S.E.M.

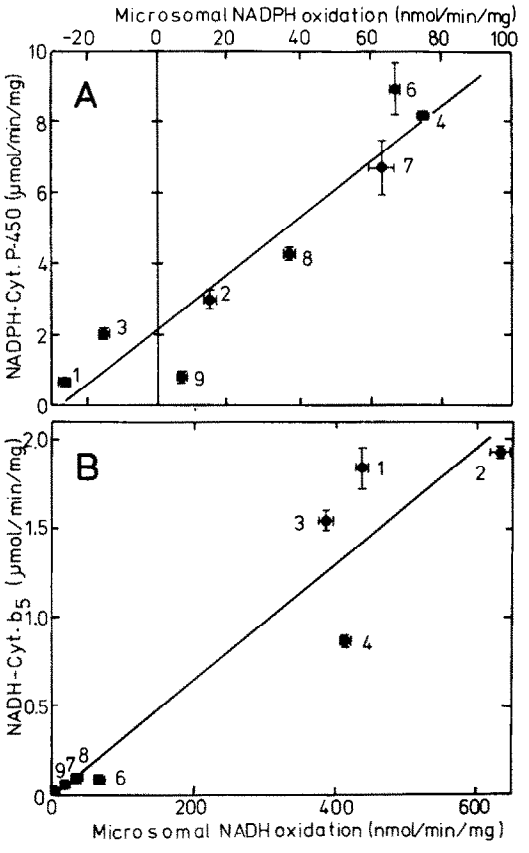

Fig. 4. Quinone metabolism by hepatic microsomal fraction and purified flavoproteins. Conditions are as described in the text. (A) Microsomes with NADPH as the cofactor compared to NADPH-cytochrome P-450 reductase ($r = 0.88$, $P < 0.05$). (B) Microsomes with NADH as the cofactor compared to NADH-cytochrome b_5 reductase ($r = 0.90$, $P < 0.05$). Quinones are as in Table 1. Each point is the mean of three observations for each preparation; bars are S.E.M. Metabolism is expressed as the change in NADH or NADPH oxidation. Endogenous rates of microsomal metabolism were: NADPH, 38.1 nmoles·min^{-1}·mg^{-1}, and NADH, 12.7 nmoles·min^{-1}·mg^{-1}.

obvious relation between the rate of metabolism and the two-electron reduction potential of the quinone/hydroquinone couples with any of the flavoproteins studied (data not shown).

Microsomal quinone metabolism. A linear relation was observed between the metabolism of various quinones by the hepatic microsomal fraction with NADPH as the cofactor and purified microsomal NADPH-cytochrome P-450 reductase, and between the hepatic microsomal fraction with NADH as the cofactor and NADH-cytochrome b_5 reductase (Fig. 4). The kinetic constants for quinone reduction by the hepatic microsomal fraction in the presence of NADPH under aerobic and anaerobic conditions are shown in Table 2. With all the quinones studied, there was a decreased V_{max} under anaerobic conditions and no change in the K_m. The K_m of NADPH for aerobic microsomal quinone metabolism in the presence of 10^{-4} M 2,5-dimethyl-*p*-benzoquinone was 9.5 μM. No evidence was found for inhibition of the microsomal metabolism of *p*-benzoquinone or 2,5-dimethylbenzoquinone under aerobic or anaerobic conditions using *p*-hydroquinone or 2,5-dimethylhydroquinone (results not shown).

DISCUSSION

The products of the single-electron reduction of quinones are relatively unstable semiquinones. It is known that NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase and NADH: ubiquinone oxidoreductase catalyze single-electron transfer [1–5], but it is difficult to measure the rate of formation of semiquinones directly. Electron paramagnetic resonance studies under steady-state conditions have shown that semiquinones are formed during flavoprotein-mediated single-electron reduction of quinones [1, 3] and, in the absence of other electron acceptors, quinones lead to the oxidation of stoichiometric amounts of reduced pyridine nucleotide [1, 3]. Under anaerobic conditions and using purified enzymes, there are no other acceptors present and the oxidation of reduced pyridine nucleotide will accurately reflect quinone reduction. There was, however, an increased quinone-dependent cofactor oxidation under aerobic conditions with both NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase. With NADPH-cytochrome P-450 reductase this cannot be accounted for by additional pathways of oxidative metabolism since at the highest rates of metabolism the ratio of superoxide formed to NADPH oxidized was 2:1. Superoxide is formed by the transfer of an electron from the semiquinone to molecular oxygen and only slowly by the direct reduction of oxygen by the enzyme. The increased rate of NADPH oxidation must, therefore, represent an increased

Table 2. Kinetic constants for microsomal quinone reduction*

	Air		N_2	
	K_m (mM)	V_{max} (nmoles·min^{-1}·mg^{-1})	K_m (mM)	V_{max} (nmoles·min^{-1}·mg^{-1})
2,5-Dimethyl-*p*-benzoquinone	25.0	250	22.9	82
2-Methyl,1,4-naphthoquinone	6.2	152	5.5	95
2,3,5,6-Tetramethyl-*p*-benzoquinone	25.0	125	23.2	69
Indigodisulfonate	12.5	71	11.1	55

* Hepatic microsomal metabolism was determined at 37° as described in the text by following the increase in the oxidation of NADPH, present at an initial concentration of 0.33 mM. The unstimulated rates of microsomal NADPH oxidation were: aerobic 29.3 nmoles·min^{-1}·mg^{-1}; and anaerobic 17.6 nmoles·min^{-1}·mg^{-1}.

flow of electrons through the semiquinone intermediate. The situation with NADH-cytochrome b_5 reductase is not so straightforward, and for some quinones there may be alternative pathways for NADH oxidation under aerobic conditions not involving quinone reduction. A consequence of the reaction of the semiquinone with oxygen is, of course, that the parent quinone is regenerated, and its concentration will not change despite the rapid rates of enzymatic quinone reduction, cofactor oxidation and superoxide formation.

Oxidation reduction potentials are only truly meaningful in determining equilibria, but they can have significance for rates of metabolism [11]. The rate of two-electron transfer from soluble reduced flavin to acceptor, in the case of azo compounds, has been shown to be proportional to the reduction potential of the acceptor [11]. The results of the present study show that there is no simple relation between the rate of reduction of flavoproteins and the single-electron reduction potential of the quinone acceptor. The reason for the decreased rate of metabolism at more positive single-electron reduction potentials, particularly obvious when NADPH is the cofactor, is not clear. A possible explanation is product inhibition by the semiquinone. Substrate free radicals, such as semiquinones, formed by enzyme activity are poor substrates for further reduction [27]. The semiquinone is released from the enzyme and, depending on its reduction potential, reacts with oxygen to form superoxide or dismutates to form the quinone and the hydroquinone [3]. Semiquinones of the more electron affinic quinones may undergo dismutation more slowly than those of less electron affinic quinones [3] and might reach appreciable concentrations in the vicinity of the enzyme. Hydroquinones do not appear to inhibit quinone reduction.

NADPH-cytochrome P-450 reductase differs from most other flavoproteins in that it contains 1 mole each of FMN and FAD per polypeptide chain [28]. Vermilion and Coon [29] have suggested that FAD and FMN act as low and high potential flavins and that FAD is probably involved in the reaction of the enzyme with NADPH whereas FMN transfers electrons to the acceptor. The FMN-depleted enzyme is readily reduced by NADPH but is unable to transfer electrons to the normal acceptors, including 2-methyl-1,4-naphthoquinone [29]. Iyanagi *et al.* [30] have assigned values to the one-equivalent redox couples of NADPH-cytochrome P-450 reductase of −110, −270, −290 and −365 mV, which, according to the work of Vermilion and Coon [29], correspond to the couples FMN/FMNH·, FMNH·/$FMNH_2$, FAD/FADH· and FADH·/$FADH_2$, respectively. Only the three higher-potential redox states are found under physiological conditions with NADPH as the reductant [29]. Under aerobic conditions, NADPH-cytochrome P-450 reductase exists in a one-electron, air stable, semiquinone form [29, 31], probably FAD-FMNH· [31]. This one-electron reduced state of the enzyme is formed slowly under anaerobic conditions but more rapidly under aerobic conditions by autoxidation of the two-electron reduced form of the enzyme [31]. One explanation for the stimulation of quinone reduction by oxygen is that the formation of the one-electron reduced state is necessary for electron transfer to the quinone acceptor and is rate limiting under anaerobic conditions. This is unlikely, however, since the lower limiting single-electron reduction potential of quinones undergoing reduction by NADPH-cytochrome P-450 reductase is −240 mV, and FMH, with a reduction potential of −110 mV [30], is too electron affinic to account for the rapid reduction of several of the quinones. $FMNH_2$ and FADH· are the most likely candidates for electron transfer to the quinone acceptor and, since the FMN-depleted enzyme is unable to catalyze quinone reduction [29], electron transfer to the quinone acceptor is most likely to be via $FMNH_2$. Some other mechanism must account for the effect of oxygen in stimulating quinone metabolism. Oxygen may also stimulate quinone reduction by the FAD-containing NADH-cytochrome b_5 reductase.

A different pattern of quinone reduction is seen with NADH-cytochrome b_5 reductase. NADH-cytochrome b_5 reductase contains only 1 mole of FAD per polypeptide chain [32]. The midpoint potential of the enzyme for two-electron reduction is −258 mV. NAD^+ binds to the reduced enzyme, stabilizing the semiquinone form of the enzyme and causing a shift in the midpoint potential to −160 mV. $NADP^+$ does not bind in this manner to reduced NADPH-cytochrome P-450 reductase [32]. The binding of NAD^+ may explain the higher minimum potential for quinone reduction, around −170 mV, seen with NADH-cytochrome b_5 reductase.

An unexpected finding was that of different patterns of quinone reduction by NADH:ubiquinone oxidoreductase, depending on whether NADH or NADPH was the source of reducing equivalents. The NADPH dehydrogenase activity of the mitochondrial electron transport system fractionates mainly into NADH:ubiquine oxidoreductase [33]. NADH and NADPH reduce the FMN and iron–sulfur centers of the enzyme [34], and it has been suggested that NADH and NADPH bind to the same site on the NADH:ubiquinone oxidoreductase [33]. There is no information on the redox properties of the flavin of NADH:ubiquinone oxidoreductase, although the low free radical content of the enzyme has been taken to indicate that the flavin exists in a fully reduced or fully oxidized form [35]. It appears that with NADH:ubiquinone oxidoreductase the cofactor can imprint a pattern of substrate affinity on the enzyme. The possibility must be considered, however, that the preparation contains two distinct enzymes.

Both NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase are bound to the microsomal membrane and it has been suggested that NADH-cytochrome b_5 reductase is more deeply embedded in the lipid membrane than is NADPH-cytochrome P-450 reductase [36]. Lipid solubility appears, however, to have little effect upon the rate of metabolism. There was no statistical correlation between the apparent octanol/water partition coefficient of the quinones and their rate of reduction. Quinones 4, 5, 6 and 7, which have the highest apparent partition coefficients, were most rapidly metabolized by NADPH-cytochrome P-450 reductase and NADH:ubiquinone oxidoreductase with

NADPH as the cofactor. There was, however, little difference in the rate at which they were metabolized, while there was over a 7-fold range in their apparent partition coefficients. There was no correlation with NADH-cytochrome b_5 reductase and NADH : ubiquinone oxidoreductase with NADH as the cofactor. A similar pattern of quinone reduction was seen with purified microsomal enzymes and the intact microsomal fraction, again suggesting that the lipid solubility of the substrate has little effect on metabolism.

In conclusion, the present studies have shown that single-electron reduction potential can be an important factor in the metabolism of quinones by flavoproteins catalyzing single-electron transfer. A similar pattern of metabolism was observed with NADPH-cytochrome P-450 reductase and NADH : ubiquinone oxidoreductase with NADPH as cofactor, at lower single-electron reduction potentials than with NADH-cytochrome b_5 reductase and NADH: ubiquinone oxidoreductase with NADH as the cofactor. There was a decreased rate of metabolism at higher single-electron reduction potentials. Lipid solubility appears to have little influence upon the rate of metabolism.

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