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# ONE-ELECTRON-TRANSFER REACTIONS IN BIOCHEMICAL SYSTEMS

## III. ONE-ELECTRON REDUCTION OF QUINONES BY MICROSOMAL FLAVIN ENZYMES

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SUMMARY

The microsomal flavin enzymes cytochrome $b_5$ reductase (NADH: cytochrome $b_5$ oxidoreductase, EC 1.6.2.2) and NADPH–cytochrome *c* reductase (NADPH: cytochrome *c* oxidoreductase) catalyze one-electron reduction of quinones. Semiquinones thus formed are detached from the enzymes and undergo dismutation. In the presence of suitable electron acceptors, such as cytochromes and molecular oxygen, electron transfer occurs from semiquinone to electron acceptor. In the presence of 2-methyl-1,4-naphthoquinone, for instance, NADPH–cytochrome *c* reductase catalyzes the reduction of cytochrome $b_5$ and molecular oxygen. Reduction of cytochrome *c* is catalyzed by cytochrome $b_5$ reductase in the presence of *p*-benzoquinone. Electron transfer from reduced cytochrome $b_5$ reductase to monodehydroascorbate occurs at a considerable rate. These one-electron-transfer reactions catalyzed by the microsomal flavin enzymes are investigated quantitatively with electron spin resonance spectroscopy.

## INTRODUCTION

Two flavin enzymes which catalyze electron transfer from reduced pyridine nucleotides to hemoproteins and which have different specificities for pyridine nucleotides and cytochromes, have been isolated from liver microsomes. The enzymes are cytochrome $b_5$ reductase (NADH: cytochrome $b_5$ oxidoreductase, EC 1.6.2.2) and NADPH–cytochrome *c* reductase (NADPH: cytochrome *c* oxidoreductase). Cytochrome $b_5$ reductase has been extensively studied by STRITTMATTER[1] since he isolated a NADH-specific microsomal cytochrome reductase in 1956 (ref. 2). NADPH–cytochrome *c* reductase was isolated by HORECKER[3] in 1950 and recently its study has been greatly advanced by KAMIN, MASTERS AND GIBSON[4]. It has been found that both enzymes have FAD as prosthetic group and are free of heavy metals. Besides cytochrome $b_5$, cytochrome $b_5$ reductase catalyzes the reduction of ferricyanide and dichlorophenolindophenol[1]. NADPH–cytochrome *c* reductase also catalyzes the re-

Abbreviations: ESR, electron spin resonance; MK, MKH, $MKH_2$ are 2-methyl-1,4-naphthoquinone (menaquinone-0), its semiquinone and its fully reduced form (quinol), respectively.

duction of ferricyanide[5], dichlorophenolindophenol[5], neotetrazolium[6], and various quinones[7]. Consequently, it can be said that these enzymes catalyze the reduction of two-electron acceptors as well as one-electron acceptors. There is very little doubt that a one-electron-transfer mechanism is involved in these flavoprotein reactions when cytochromes or ferricyanide are used as electron acceptors.

Recently, experimental evidence has been reported which suggests the mechanism of one-electron transfer from flavin to two-electron acceptor in the microsomal electron transport systems[8–10,31]. NISHIBAYASHI, OMURA AND SATO[9] have postulated that the stimulation of the NADPH oxidase activity of NADPH–cytochrome *c* reductase by MK resides in the ability of that compound to serve as a one-electron carrier to oxygen. This assumption, however, has not been fully accepted by KAMIN, MASTERS AND GIBSON[4] and MASTERS *et al.*[5]. We have studied the reduction process of quinones by the microsomal flavin enzymes using electron spin resonance (ESR) spectroscopy and the redox reactions mediated by quinones in the presence of these enzymes. The results obtained are reported in this paper.

## MATERIALS AND METHODS

The optical and ESR spectrometers used were the same as those described in the previous paper[11].

*p*-Benzoquinone, 1,4-naphthoquinone and MK were purified from commercial supplies by sublimation. Benzohydroquinone was recrystallized from ethanol–benzene solution. $MKH_2$ was prepared from MK according to the method of FIESER[12] and crystallized from ethanol–light petroleum solution.

Ferricytochrome *c*, NADH and $NADP^+$ were obtained from Boehringer. NADPH was prepared from $NADP^+$ by enzymic reduction with glucose-6-phosphate dehydrogenase.

Microsome preparation from pig liver was performed essentially according to STRITTMATTER[15]. Cytochrome $b_5$ was solubilized from pig liver microsome by the method of OMURA, SIEKEVITZ AND PALADE[13] and purified by the procedure of KAJIHARA AND HAGIHARA[14] with some modifications. Cytochrome $b_5$ reductase was solubilized by the method of TAKESUE AND OMURA[16] and purified by the procedure of STRITTMATTER[15] except that DEAE-cellulose was used instead of $C_\gamma$-gel. NADPH–cytochrome *c* reductase was prepared by the slightly modified method of OMURA,

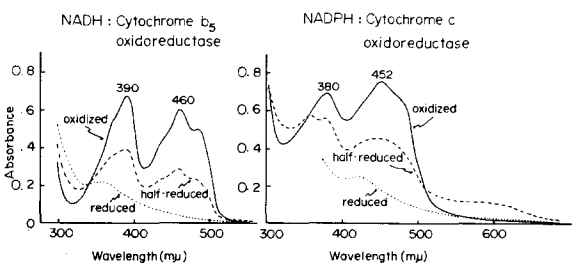


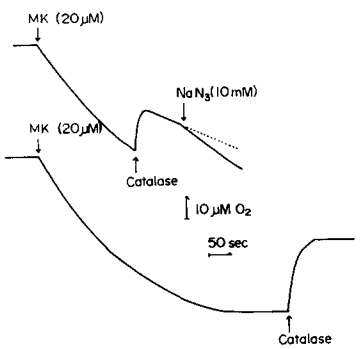

Fig. 1. Absorption spectra of pig liver microsomal flavin enzymes used in the present study. Left, NADH:cytochrome $b_5$ oxidoreductase; right, NADPH:cytochrome *c* oxidoreductase. Both enzymes were reduced with $Na_2S_2O_4$ solution in an anaerobic cell. 0.1 M phosphate (pH 7.5) containing 1 mM EDTA.

SIEKEVITZ AND PALADE[13]. Fig. 1 shows the absorption spectra of cytochrome $b_5$ reductase and NADPH–cytochrome *c* reductase used in the present experiment. The concentrations of cytochrome $b_5$ reductase and NADPH–cytochrome *c* reductase were determined from the absorbance at 460 mμ and 452 mμ, respectively. $\varepsilon_{mM}$ used for cytochrome $b_5$ reductase was 10.2 (ref. 2) and for NADPH–cytochrome *c* reductase was 11.3 (ref. 5). $\varepsilon_{mM}$ used for cytochromes $b_5$ and *c* were 28 at 557 mμ and 27.2 at 550 mμ, respectively.

Ascorbate oxidase was purified according to the method of NAKAMURA, MAKINO AND OGURA[30]. $O_2$ consumption was measured with a Clark electrode, and all experiments were carried out at 25°.

## RESULTS

NADPH oxidation by NADPH–cytochrome *c* reductase is greatly stimulated by the addition of MK as reported by NISHIBAYASHI, OMURA AND SATO[9]. Fig. 2 indicates that the reduction product of $O_2$ is $H_2O_2$, since the addition of catalase after the reaction causes the evolution of almost half the amount of $O_2$ consumed. When catalase is added during the reaction, the rate of $O_2$ consumption decreases to half and the catalase effect is removed by the addition of sodium azide. Cytochrome $b_5$ does not react with NADPH–cytochrome *c* reductase, but MK stimulates the reduction of cytochrome $b_5$ in the presence of NADPH–cytochrome *c* reductase, as shown in Fig. 3. Fig. 3 shows that MK is more effective than $MKH_2$, and the MK-stimulated reduction of cytochrome $b_5$ is inhibited by the presence of $O_2$. It is also shown in Fig. 3 that under anaerobic conditions, the rate of electron flow to MK is almost the same as that of MK-mediated electron flow to cytochrome $b_5$.



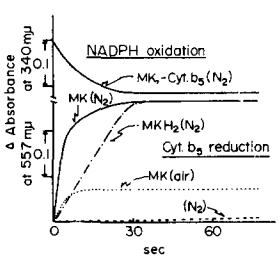
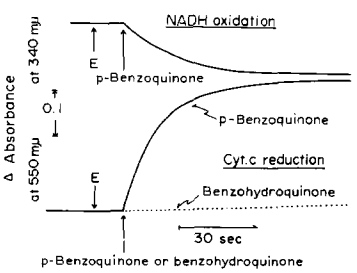




Fig. 2. MK-mediated $O_2$ consumption in the NADPH–cytochrome *c* reductase system. Reactions were started by the addition of MK. 0.22 μM NADPH–cytochrome *c* reductase, 100 μM NADPH, 20 μM MK and 0.1 M potassium phosphate (pH 6.5). Catalase and sodium azide were added at arrows. In the upper trace glucose-6-phosphate dehydrogenase system was coupled. About 20% deviation from the stoichiometry of $O_2$ consumption by NADPH in the lower trace is ascribed to the slow diffusion of $O_2$ into the reaction solution.

Fig. 3. MK-mediated reduction of cytochrome $b_5$ in the NADPH–cytochrome *c* reductase system. Reactions were started by the addition of MK or $MKH_2$. 0.22 μM NADPH–cytochrome *c* reductase, 100 μM NADPH, 20 μM MK or $MKH_2$, 16 μM cytochrome $b_5$ and 0.1 M potassium phosphate (pH 6.5). Experiments of cytochrome $b_5$ reduction were carried out under the aerobic conditions with MK (·····) and under anaerobic conditions with MK (——) or $MKH_2$ (–·—·–) and without both (– – – – –). NADPH oxidation was measured in the presence of MK without cytochrome $b_5$ under anaerobic conditions.

A similar reaction is observed in cytochrome $b_5$ reductase. Fig. 4 shows that cytochrome $b_5$ reductase catalyzes the electron transfer from NADH to cytochrome *c* in the presence of *p*-benzoquinone but not in the presence of benzohydroquinone. Unlike the MK-mediated reduction of cytochrome $b_5$ in NADPH–cytochrome *c* reductase, this reaction is not affected by the presence of $O_2$. Comparison between the rate of NADH oxidation in the absence of cytochrome *c* and the rate of cytochrome *c* reduction indicates that an electron from NADH is transferred effectively to cytochrome *c* without accumulation of benzohydroquinone.

It can be postulated on the basis of these results that active intermediates in the quinone-mediated reactions of the microsomal flavin enzymes are semiquinones as suggested by NISHIBAYASHI, OMURA AND SATO[9] in the MK-mediated NADPH oxidase reaction of NADPH–cytochrome *c* reductase. There is sufficient evidence to indicate the significance of such semiquinones as active intermediates in the biochemical electron transfer reactions[11,17–19]. ESR spectroscopy is the most powerful method to clarify the one-electron-transfer mechanism in the reactions involving two-electron acceptors such as quinones. Of the various quinones, *p*-benzoquinone seems to be the best for this purpose, since a great deal of quantitative information about *p*-benzosemiquinone reactivity is available[11,19], and *p*-benzoquinone has been found to be a common electron acceptor of cytochrome $b_5$ reductase and NADPH–cytochrome *c* reductase. Fig. 5A shows a clear ESR spectrum of *p*-benzosemiquinone formed in the steady state of *p*-benzoquinone reduction by cytochrome $b_5$ reductase. By allowing the reaction solution to flow at a constant rate, the reaction time at which the spectrum is observed is kept constant at about 60 msec. The typical hyperfine structure of the ESR signal indicates that the *p*-benzosemiquinone observed is free in solution. The ESR signal disappears soon after the flow stops (Fig. 5B). A similar experiment is carried out with NADPH–cytochrome *c* reductase. The spectrum is shown in Fig. 6A. *p*-Benzoquinone reduction is slow in this enzyme system, and the spectrum is observed in the presence of concentrated NADPH–cytochrome *c* re-



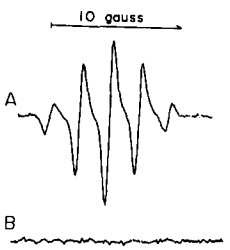




Fig. 4. *p*-Benzoquinone-mediated reduction of cytochrome *c* in the cytochrome $b_5$ reductase system. Reactions were started by the addition of *p*-benzoquinone (———) or benzohydroquinone (·····). Concentrations: 0.02 μM cytochrome $b_5$ reductase, 100 μM NADH, 20 μM *p*-benzoquinone or benzohydroquinone, 16 μM cytochrome *c* and 0.1 M potassium phosphate (pH 6.0). NADH oxidation was measured in the absence of cytochrome *c*.

Fig. 5. ESR spectrum of *p*-benzosemiquinone (A) formed in the steady state of cytochrome $b_5$ reductase reaction during a continuous flow (2 ml/sec). The solution of cytochrome $b_5$ reductase and NADH was mixed with the solution of *p*-benzoquinone. Final concentrations: 0.5 μM cytochrome $b_5$ reductase, 200 μM NADH, 100 μM *p*-benzoquinone and 0.1 M potassium phosphate (pH 6.0). The same magnetic field was scanned in B soon after the flow stopped. The concentration of *p*-benzosemiquinone (A) was found to be 0.81 μM.

ductase with the use of a highly sensitive spectrometer. The spectrum also disappears soon after the flow stops (Fig. 6B). This experiment is carried out at pH 7.0, and there is a possibility that *p*-benzosemiquinone is formed from the mixture of *p*-benzoquinone and benzohydroquinone. The results shown in Fig. 6C, however, exclude this possibility. It can be concluded from these results that the *p*-benzosemiquinone observed is produced only by the enzymic reactions. Dependence of the steady-state concentration of *p*-benzosemiquinone upon the velocity of the enzymic reaction has been described in the previous paper[11,20] for the oxidative reactions, and the same will apply to this case.

$$(\text{Benzosemiquinone})_s = \left(\frac{\kappa \cdot v}{2\, k_d}\right)^{\frac{1}{2}} \qquad (1)$$

Where $k_d$ is the dismutation constant for *p*-benzosemiquinone, and $\kappa$ is constant specific for the enzymic reaction which has been discussed previously[11,20]. When the magnetic field is adjusted so as to obtain the maximum of the derivative curve of ESR absorption, apparent decay curves of *p*-benzosemiquinone can be observed, as in Figs. 7 and 8. Overall reactions are measured by observing absorbance decreases at 340 mμ which indicate the oxidation of NADH and NADPH. Almost 30 times more concentrated enzyme is used for the reaction of NADPH–cytochrome *c* reductase to obtain the same initial oxidation velocity in both reactions. Consequently, the initial *p*-benzosemiquinone concentrations at the steady state are almost the same

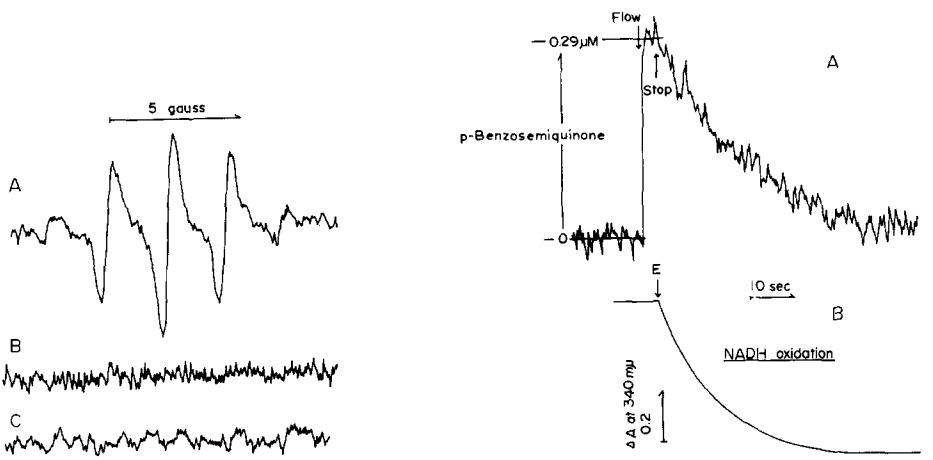



Fig. 6. ESR spectrum of *p*-benzosemiquinone (A) formed in the steady state of NADPH–cytochrome *c* reductase reaction during a continuous flow (2 ml/sec). The solution of NADPH–cytochrome *c* reductase and NADPH was mixed with the solution of *p*-benzoquinone. Final concentrations: 1.2 μM NADPH–cytochrome *c* reductase, 200 μM NADPH, 100 μM *p*-benzoquinone and 0.1 M potassium phosphate (pH 7.0). Sensitivity of the spectrometer was raised to 5 times more than in the previous experiments (Fig. 5). The same magnetic field was scanned in B soon after the flow stopped. A mixture of 50 μM *p*-benzoquinone and 50 μM benzohydroquinone gave no signal (C) under the same conditions. The concentration of *p*-benzosemiquinone (A) was found to be 0.28 μM.

Fig. 7. Time courses of NADH oxidation (B) and *p*-benzosemiquinone decay (A) in the cytochrome $b_5$ reductase system. In A the magnetic field was adjusted so as to obtain the maximum of the derivative curve of ESR absorption. Final concentrations: 0.045 μM cytochrome $b_5$ reductase, 200 μM NADH, 100 μM *p*-benzoquinone and 0.1 M potassium phosphate (pH 6.0). NADH oxidation was measured in a different cell under the same conditions.

in both cases, as can be seen in Figs. 7A and 8A. The reaction patterns, however, are different from each other. In the case of cytochrome $b_5$ reductase (Fig. 7B) the reaction agrees with first-order kinetics, but it is close to a zero-order reaction in the case of NADPH–cytochrome *c* reductase (Fig. 8B). This difference in the reaction

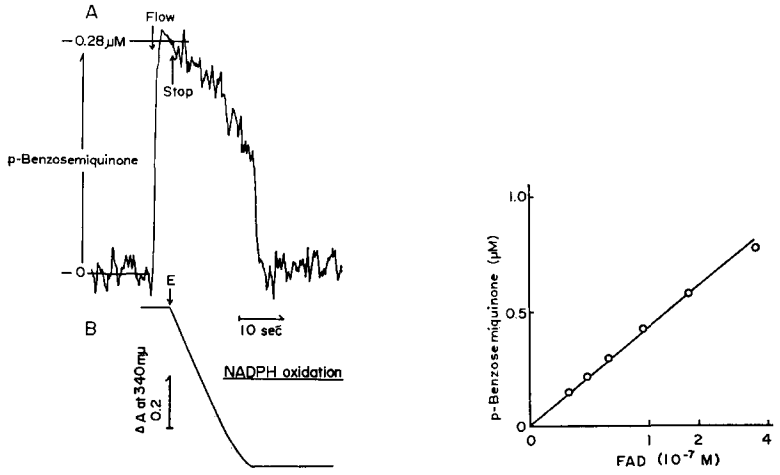



Fig. 8. Time courses of NADPH oxidation (B) and *p*-benzosemiquinone decay (A) in the NADPH–cytochrome *c* reductase system. Final concentrations: 1.2 μM NADPH–cytochrome *c* reductase, 200 μM NADPH, 100 μM *p*-benzoquinone and 0.1 M potassium phosphate (pH 7.0). The other was the same in Fig. 7.

Fig. 9. Relationship between the concentrations of cytochrome $b_5$ reductase and *p*-benzosemiquinone at the steady state. Abscissa is square root of the cytochrome $b_5$ reductase concentration. Concentrations: 200 μM NADH, 100 μM *p*-benzoquinone and 0.1 M potassium phosphate (pH 6.0).

kinetics is reflected in the difference of time course of *p*-benzosemiquinone disappearance between Figs. 7A and 8A. Eqn. 1 also suggests that a linear relationship will be obtained when the initial concentrations of *p*-benzosemiquinone at the steady state are plotted against the square root of the enzyme concentrations, and the result is shown in Fig. 9. These results are very similar to those obtained with the oxidative enzymes[20,21]. As described in the previous paper[11], the rate constant of the reaction of *p*-benzosemiquinone with cytochrome *c* is measured directly, using an ESR spectrometer and a sensitive spectrophotometer, both equipped with a flow apparatus (Fig. 10). The experimental conditions of Fig. 10 differ greatly from those of Fig. 4 in the concentrations of cytochrome $b_5$ reductase and cytochrome *c*. The present experiment gives a value of $1.5 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$ for $k_r$, which is consistent with that obtained in the different reaction systems reported previously[11,19].

Naphthoquinone derivatives are known to be good electron acceptors for NADPH–cytochrome *c* reductase[7]. In the NADPH–NADPH–cytochrome *c* reductase acceptor systems, however, the rate-limiting step is the reduction of flavin by NADPH, and the velocity of quinone reduction by NADPH–cytochrome *c* reductase is saturated with quinones. Furthermore, MKH dismutates at a remarkably high rate ($k_d = 8 \cdot 10^9$ $M^{-1} \cdot sec^{-1}$)[11], it is difficult to produce a detectable amount of MKH in the steady state of the NADPH–NADPH–cytochrome *c* reductase–MK reaction. Dismutation of 1,4-seminaphthoquinone, on the other hand, seems to be

slow, though not estimated, and the ESR spectrum of 1,4-seminaphthoquinone is observed in the presence of a large amount of NADPH–cytochrome *c* reductase as shown in Fig. 11B. This signal has been identified as that of 1,4-seminaphthoquinone by comparison with the ESR spectrum of equilibrated 1,4-seminaphthoquinone (Fig. 11A) at alkaline pH measured with same modulation amplitude of the magnetic field. A kinetic study has not yet been completed for the NADPH–NADPH–cytochrome *c* reductase–1,4-naphthoquinone reaction, but it is very likely that the reduction process of 1,4-naphthoquinone is the same as that of *p*-benzoquinone.

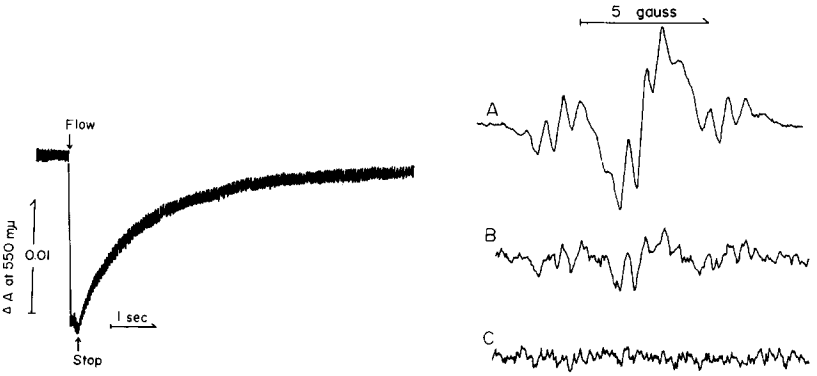



Fig. 10. The rate of cytochrome *c* reduction in the NADH–cytochrome $b_5$ reductase–*p*-benzoquinone system using a stopped-flow method. The steady-state concentration of *p*-benzosemiquinone was found to be 0.57 μM by a separate ESR experiment. Concentrations: 0.18 μM cytochrome $b_5$ reductase, 200 μM NADH, 100 μM *p*-benzoquinone, 1 μM cytochrome *c* and 0.1 M potassium phosphate (pH 6.0).

Fig. 11. ESR spectrum of 1,4-seminaphthoquinone (B) formed in the steady state of NADPH–cytochrome *c* reductase reaction during a continuous flow (1.2 ml/sec) under anaerobic conditions. Final concentrations: 200 μM NADPH, 100 μM 1,4-naphthoquinone and 0.1 M potassium phosphate (pH 7.0). NADPH–cytochrome *c* reductase used reduced 1,4-naphthoquinone at a rate of 21 μM/sec. The same magnetic field was scanned in C soon after the flow stopped. A is an ESR spectrum of 1,4-seminaphthoquinone formed in the partially reduced 1,4-naphthoquinone (1 mM) solution at pH 9.0.

MK is a very slow electron acceptor for cytochrome $b_5$ reductase, and the addition of MK induces only a slight consumption of oxygen in the NADH–cytochrome $b_5$ reductase–$O_2$ system (Fig. 12, dotted line). In the presence of cytochrome $b_5$ $O_2$ consumption is greatly stimulated by the addition of MK, as shown in Fig. 12. Since MK oxidizes cytochrome $b_5$ at a considerable rate, it can be concluded that MK accepts an electron from cytochrome $b_5$ and donates it to molecular oxygen. The role of MK in this sense seems to be the same as in the NADPH–NADPH–cytochrome *c* reductase–$O_2$ system.

SCHNEIDER, STAUDINGER AND WEIS[22], SCHNEIDER AND STAUDINGER[23] and LUMPER, SCHNEIDER AND STAUDINGER[24] have reported that NADH oxidation by liver microsomes is accelerated by the system forming monodehydroascorbate but not by dehydroascorbate. In this case monodehydroascorbate appears to act as an oxidant. Their results in the microsomal system are now reproduced in the pure enzyme system as shown in Fig. 13A. NADH oxidation can be observed only during the oxidation of ascorbate by ascorbate oxidase but not after the oxidation ends (Fig. 13B). It has

been established that ascorbate oxidase catalyzes one-electron oxidation and produces monodehydroascorbate quantitatively ($\kappa = 2$)[18,20]. Fig. 14 is the direct demonstration of the reaction of monodehydroascorbate with reduced cytochrome $b_5$ reductase. Fig. 14B shows the time course of monodehydroascorbate concentration during the oxidation of ascorbate by ascorbate oxidase. At this pH, $k_d$ for monodehydroascorbate is relatively small[18], and its concentration during continuous flow is about half of the steady-state one[20]. The concentration reaches its maximum immediately after the flow stops. The apparent decay curve of monodehydroascorbate is a typical one

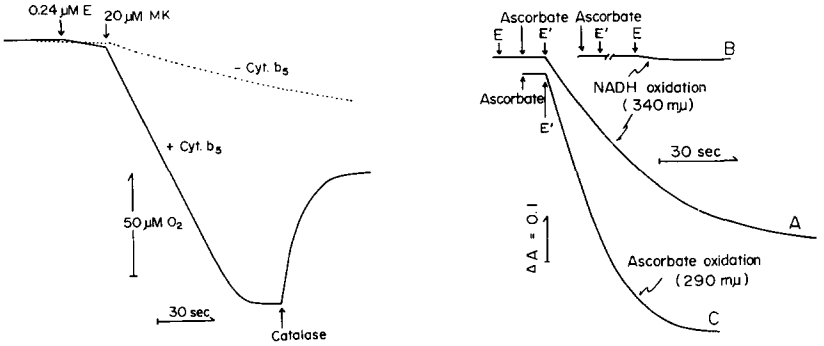



Fig. 12. MK-mediated $O_2$ consumption in the NADH–cytochrome $b_5$ reductase–cytochrome $b_5$ system. Concentrations: 0.24 $\mu$M cytochrome $b_5$ reductase (E), 180 $\mu$M NADH, 20 $\mu$M MK, 8 $\mu$M cytochrome $b_5$ and 0.1 M potassium phosphate (pH 6.0). The reaction started from an air-equilibrated solution. Slow $O_2$ consumption was observed in the absence of cytochrome $b_5$ (·····). Catalase was added at arrow.

Fig. 13. NADH oxidation induced by the addition of ascorbate and its oxidase in the cytochrome $b_5$ reductase system (A). Concentrations: 0.6 $\mu$M cytochrome $b_5$ reductase (E), 100 $\mu$M NADH, 200 $\mu$M ascorbate, 0.078 $\mu$M ascorbate oxidase (E') (on the basis of copper) and 0.1 M potassium phosphate (pH 7.5). In B, cytochrome $b_5$ reductase was added after ascorbate oxidation was over. C shows the time course of the ascorbate oxidation by ascorbate oxidase in the absence of cytochrome $b_5$ reductase and NADH.

which can be predicted on the basis of Eqn. 1 from the reaction curve of ascorbate oxidation (Fig. 14A). When cytochrome $b_5$ reductase and NADH are present in the reaction mixture, the process of monodehydroascorbate reaching its maximum is greatly modified. Monodehydroascorbate stays at a lower level for a considerable time after the flow stops (Fig. 14C). The period is almost twice as long in the presence of twice as much NADH (Fig. 14D). Fig. 14E shows that a stimulated oxidation of NADH is observed during that period (compare with Fig. 14D). It can be concluded from these results that monodehydroascorbate formed in the ascorbate oxidase reaction receives an electron from reduced cytochrome $b_5$ reductase. Approximate rate constant of the reaction between reduced cytochrome $b_5$ reductase and monodehydroascorbate is estimated to be about $4 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$ by rough calculation. The enzyme of LUMPER, SCHNEIDER AND STAUDINGER[24], which catalyzes the electron transfer from NADH to monodehydroascorbate in liver microsomes, is very probably cytochrome $b_5$ reductase itself.

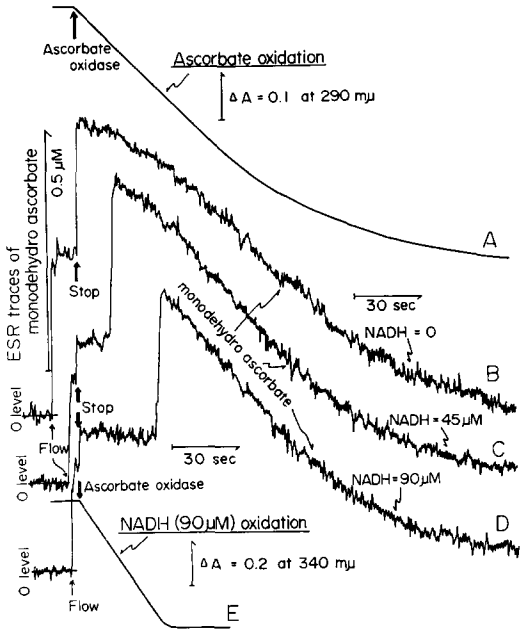



Fig. 14. Time course of the appearance and disappearance of monodehydroascorbate during the ascorbate oxidase reaction in the absence (B) and presence (C, D) of NADH and cytochrome $b_5$ reductase. Final concentrations: 7.5 $\mu$M cytochrome $b_5$ reductase, 200 $\mu$M ascorbate, 8 m$\mu$M ascorbate oxidase (on the basis of copper) and 0.1 M potassium phosphate (pH 7.0). NADH concentrations are indicated in the figure. A shows the time course of the ascorbate oxidation by ascorbate oxidase in the absence of cytochrome $b_5$ reductase and NADH. E shows NADH oxidation under the same conditions as for Expt. D. From Expts. D and E it is obvious that the steady-state concentration of monodehydroascorbate rose to a maximum immediately after NADH oxidation was over. Reaction time at continuous flow in the ESR experiments was about 60 msec.

## DISCUSSION

It is believed beyond doubt that reduced flavin enzymes of liver microsomes are oxidized in the two-step reactions, since ferricyanide and cytochromes serve as electron acceptors for the enzymes. This mechanism suggests the appearance of a semi-oxidized intermediate of the flavin enzymes during the reaction. STRITTMATTER[1] has demonstrated such an intermediate in the cytochrome $b_5$ reductase reaction with ferricyanide as an electron acceptor. MASTERS *et al.*[25] have recently indicated that NADPH–cytochrome *c* reductase contains 2 moles of FAD per mole of enzyme, and the overall reaction involves the operation of a 2 FADH $\longleftrightarrow$ 2 $FADH_2$ "shuttle" for ferricyanide as well as cytochrome *c* reduction[4]. KAMIN, MASTERS AND GIBSON[4] and MASTERS *et al.*[5] have suggested that MK utilizes the same 2 FADH $\longleftrightarrow$ 2 $FADH_2$ shuttle for its oxidation as do the one-electron acceptors, but these investigators have not accepted the mechanism of semiquinone-mediated NADPH oxidation proposed by NISHIBAYASHI *et al.*[9]

When the two-electron acceptor (A) reacts with reduced flavin enzyme ($H_2F$), the reaction has usually been given by the following equation.

$$H_2F + A \longrightarrow F + H_2A \qquad (2)$$

The results of the present studies led to the formulation of the mechanism proposed in Fig. 15. This mechanism emphasizes that a quinone molecule receives one electron from the flavin enzymes and produces a semiquinone which is freed from the enzymes.

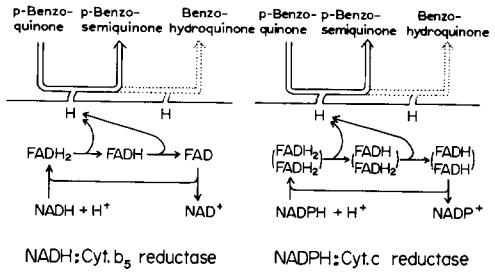



Fig. 15. Tentative mechanism of quinone reduction by the microsomal flavin enzymes. The reaction paths indicated by dotted lines are negligible. The redox states of flavins during the reaction have been described by STRITTMATTER[1] for cytochrome $b_5$ reductase and by KAMIN, MASTERS AND GIBSON[4] for NADPH–cytochrome $c$ reductase.

The mechanism is very similar to that of the oxidative enzyme reported previously[11,20]. When a one-electron transfer mechanism is established in the reaction between a flavin enzyme and a two-electron acceptor, Eqn. 2 should be replaced with the following equation.

$$H_2F + 2\,A \longrightarrow F + 2\,HA\cdot \qquad (3)$$

Semiquinone is much more reactive than the fully reduced form of the corresponding quinone and may play an important role in the electron transfer system, ordinarily as an electron donor. Cytochromes and molecular oxygen are common electron acceptors for semiquinone, and the major paths of semiquinone decay are dismutation and reaction with such electron acceptors. Fig. 16 shows a calculated

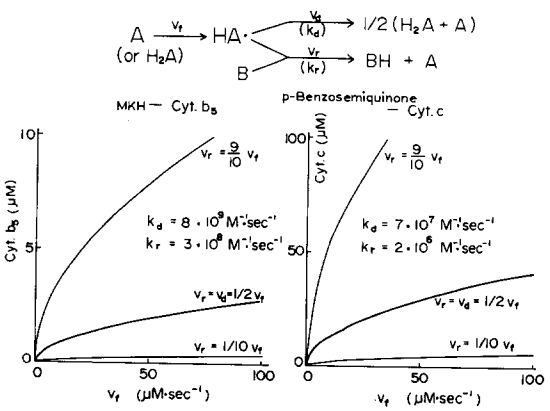



Fig. 16. Dependence of the efficiency of the electron-flow from semiquinone (HA·) to cytochromes (B) upon the cytochrome concentrations and the rate of semiquinone formation. Efficiency is defined by the ratio of rate of cytochrome reduction ($v_r$) to rate of semiquinone formation ($v_f$). For instance, $^9/_{10}$ means that 90% of semiquinone formed donate an electron to cytochrome and the other 10% of semiquinone dismutates. These diagrams may also be applied to the cases where the semiquinones are produced by the oxidative enzyme. Left is the MKH–cytochrome $b_5$ system and right are $p$-benzosemiquinone–cytochrome $c$ systems. Rate constants are cited from the previous paper[11].

diagram of efficiency of cytochrome reduction by semiquinones. The efficiency depends upon the velocity of semiquinone formation and the concentration of cytochrome. This diagram makes it easy to select the experimental conditions under which semiquinone transfers an electron to cytochrome without dismutation.

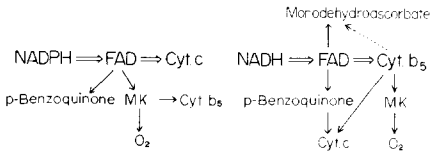



Fig. 17. One-electron transfer paths mediated by two-electron systems in the microsomal flavin enzymes. Left is cytochrome $b_5$ reductase and right is NADPH–cytochrome *c* reductase.

The paths of one-electron transfer in the microsomal flavin enzymes are summarized in Fig. 17. In Fig. 17 MK and *p*-benzoquinone serve as one-electron carriers. MKH can reduce molecular oxygen and cytochrome $b_5$ but *p*-benzosemiquinone cannot[11]. *p*-Benzosemiquinone reduces cytochrome *c* at a considerable rate[11,17,19]. Cytochrome $b_5$ autoxidizes slowly and a catalytic amount of MK stimulates the $O_2$-consuming oxidation of the cytochrome. Monodehydroascorbate-stimulating oxidation of NADH by liver microsomes may be explained by assuming the electron-flow from cytochrome $b_5$ reductase to monodehydroascorbate, but there may be some electron-flow from cytochrome $b_5$ to monodehydroascorbate as suggested by HARA AND MINAKAMI[26].

Most flavin enzymes catalyze the reduction of two-electron acceptors, including molecular oxygen. However, it is not yet established how these acceptor molecules are reduced by the enzymes except for the present enzymes. Besides the results of NISHIBAYASHI, OMURA AND SATO[9], it has been reported that an electron transfer is mediated by two-electron acceptors in the reaction of flavin enzymes. For instance, ROSSI-FANELLI, ANTONINI AND MONDOVI[27] found that NADH–cytochrome *c* reductase (pig heart) catalyzes the reduction of metmyoglobin and methemoglobin only in the presence of methylene blue. ERNSTER, DANIELSON AND LJUNGGREN[28] reported that 1,4-naphthoquinone induced the reduction of cytochrome *c* in the DT-diaphorase (EC 1.6.99.2) –NADH system. MURAOKA *et al.*[29] have recently reported a quinone-mediated reduction of cytochrome *c* in the xanthine oxidase system. These results, however, do not always mean that these mediators serve as one-electron carriers, since the fully reduced molecules of the mediators may also reduce the final electron acceptor. A number of quinone reductases have been reported and these were reviewed by MARTIUS[32]. These enzymes are characterized by their catalytic ability to transfer hydrogen from reduced pyridine nucleotide to suitable quinones, mostly 1,4-naphthoquinones or *p*-benzoquinones. A very interesting point which is still unknown is whether a parameter, $\kappa$ in Eqn. 1, is 0 or 2 in the reactions between these flavin enzymes and two-electron acceptors.

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