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ONE-ELECTRON-TRANSFER REACTIONS IN BIOCHEMICAL SYSTEMS

V. DIFFERENCE IN THE MECHANISM OF QUINONE REDUCTION BY THE NADH DEHYDROGENASE AND THE NAD(P)H DEHYDROGENASE (DT-DIAPHORASE)

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SUMMARY

The mitochondrial NADH dehydrogenase catalyzes a one-electron reduction of quinones. Semiquinones thus formed have the hyperfine structures of their free anion radicals and are suggested to be detached from the enzyme. In the presence of suitable electron acceptors electron transfer occurs from the semiquinone to the acceptor. The mechanism of quinone reduction by spinach ferredoxin–NADP reductase is the same as that by the NADH dehydrogenase.

On the other hand, the NAD(P)H dehydrogenase (DT-diaphorase) prepared from liver soluble fraction catalyzes a typical two-electron reduction of quinones such as *p*-benzoquinone and 2-methyl-1,4-naphthoquinone. The mechanisms of one-electron and two-electron reduction of quinones are readily distinguishable by the use of an electron spin resonance spectrometer equipped with a flow apparatus and also by the use of an appropriate set of electron acceptors.

It is concluded that the reduction of quinones and oxygen by flavoproteins falls into three mechanistic categories: one-electron, two-electron and mixed-type reactions.

INTRODUCTION

The function of quinones in the electron transport systems is probably to act as an electron carrier. It is well known that many flavoproteins catalyze the reduction of quinones. It is of primary importance to differentiate two mechanisms whether the reduction of quinones by flavoproteins occurs by way of a one-electron or a two-electron transfer.

In our laboratory it has been confirmed that microsomal flavoproteins, cytochrome b_5 reductase (EC 1.6.2.2) and NADPH–cytochrome *c* reductase, catalyze a typical one-electron reduction of quinones[1], and that xanthine oxidase catalyzes

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

one-electron and two-electron reduction of *p*-benzoquinone or oxygen, both at the same time, the ratio of the two mechanisms being dependent on the acceptor concentration[2].

It is the purpose of the present paper to summarize mechanisms for electron transfer from flavoproteins to electron acceptors. Three more enzymes we have studied for this purpose are mitochondrial NADH dehydrogenase (EC 1.6.99.3), spinach ferredoxin–NADP reductase (EC 1.6.99.4), and NAD(P)H dehydrogenase (EC 1.6.99.2, DT-diaphorase) prepared from the soluble fraction of pig liver.

MATERIALS AND METHODS

The optical and electron spin resonance (ESR) spectrometers used were the same as described in the previous paper[10].

Mitochondria were prepared from beef heart according to PHARO *et al.*[3]. The NADH dehydrogenase was extracted from sonicated mitochondria with 10% ethanol by the method of PHARO *et al.*[28] and was precipitated from the solution, without the procedure of lyophilization, by ammonium sulfate fractionation between 30 and 70% saturation. The precipitate was dissolved in 10 mM potassium phosphate (pH 7.5) containing 1 mM EDTA and was chromatographed on a column of Sephadex G-100 equilibrated with the same buffer. The fraction containing the dehydrogenase activity was again fractionated by ammonium sulfate between 30 and 50% saturation and was desalted with Sephadex G-25. The enzyme had a very similar absorption spectrum to that reported by PHARO *et al.*[28] and was used without further purification. The concentration of the NADH dehydrogenase was determined from the difference in absorbance at 450 mμ between oxidized and reduced (by NADH) enzymes. The $\Delta\varepsilon_{mM}$ used was 10.3.

NAD(P)H dehydrogenase was partially purified by a slightly modified method of MARKI AND MARTIUS[5] from the soluble fraction of pig liver. The protein concentration was determined by the method of LOWRY *et al.*[6] with the use of serum albumin as standard. Ferredoxin–NADP reductase was prepared from spinach leaves by the method of SHIN *et al.*[7]. The ε_{mM} used was 10.3 at 458 mμ (ref. 4). NADH, NAD^+, NADPH, alcohol dehydrogenase and cytochrome *c* were obtained from Boehringer, Mannheim. Bovine serum albumin was obtained from Daiichi Kagaku Yakuhin Co. Cytochrome b_5 reductase and cytochrome b_5 was prepared from pig liver[1]. The ε_{mM} used for reduced cytochrome b_5 and reduced cytochrome *c* were 28 at 557 mμ and 27.2 at 550 mμ, respectively.

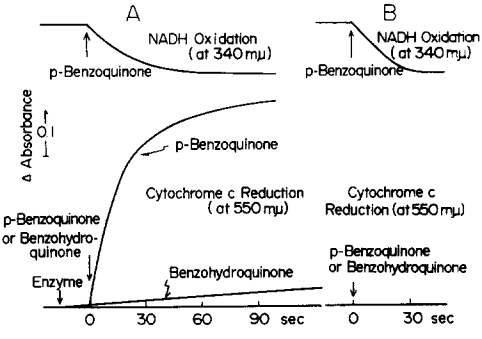
All reactions were carried out at 25°.

RESULTS

Cytochrome *c* is a very slow electron acceptor for the NADH dehydrogenase used in this experiment. Fig. 1A shows that the reduction of cytochrome *c* is stimulated by the addition of *p*-benzoquinone but not of benzohydroquinone. The active molecule which reduces cytochrome *c* is probably *p*-benzosemiquinone formed in the enzymic reaction. Similar reactions have been reported in the systems of microsomal flavoproteins[1] and xanthine oxidase[2]. On the contrary, the NAD(P)H dehydrogenase cannot reduce cytochrome *c* even in the presence of *p*-benzoquinone (Fig. 1B). Since the

reduction of *p*-benzoquinone proceeds rapidly in the presence of the NAD(P)H dehydrogenase, it is suggested that the semiquinone does not occur during the reaction by the enzyme.

Fig. 2 shows that in the presence of 2-methyl-1,4-naphthoquinone (menaquinone-0) (MK) the NADH dehydrogenase can reduce cytochrome b_5. The reduction is more effective in the presence of MK than in the presence of 2-methyl-1,4-naphthoquinone (quinol) (MKH_2). Therefore, the reactive species which causes the rapid reduction of cytochrome b_5 in the presence of MK appears to be 2-methyl-1,4-naphthoquinone (semiquinone) (MKH), as was reported in the reaction of microsomal



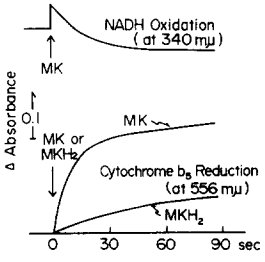


Fig. 1. *p*-Benzoquinone-mediated reduction of cytochrome *c* in the NADH dehydrogenase (A) and the NAD(P)H dehydrogenase (B) systems. Reactions were started by the addition of *p*-benzoquinone. Concentrations: 20 μM *p*-benzoquinone (or benzohydroquinone), 28 μM cytochrome *c* and 0.1 M potassium phosphate (pH 6.5). A, 0.1 μM NADH dehydrogenase; and B, 22 μg NAD(P)H dehydrogenase/ml. NADH oxidations were measured at 340 mμ in the absence of cytochrome *c*. In A, very slow reduction of cytochrome *c* was observed only by the addition of the enzyme and in B, no reduction of cytochrome *c* was observed by the addition of *p*-benzoquinone or benzohydroquinone.

Fig. 2. MK-mediated reduction of cytochrome b_5 in the NADH dehydrogenase. Reactions were started anaerobically by the addition of MK (or MKH_2). Concentrations: 0.1 μM NADH dehydrogenase, 100 μM NADH, 20 μM MK (or MKH_2), 20 μM cytochrome b_5 and 0.1 M potassium phosphate (pH 6.0). NADH oxidation was measured at 340 mμ in the absence of cytochrome b_5.

NADPH–cytochrome *c* reductase[1]. The NAD(P)H dehydrogenase also catalyzes the MK-mediated reduction of cytochromes b_5 and *c*. However, as can be seen in Fig. 3, there are lag phases in the reduction that are not observed with the NADH dehydrogenase. The appearance of the lag phase can be explained by assuming that the reduction of cytochromes occurs after accumulation of MKH_2. Since MKH_2 reduces these cytochromes even at slightly acidic pH, the rates of cytochrome reduction increase with the increase of MKH_2 concentration where the formation of MKH is not involved. The rates of cytochrome reduction observed in Fig. 3 are consistent with those caused by MKH_2 itself. It is known[8,9] that nonenzymic reduction of cytochromes *c* by ascorbate and benzohydroquinone increases with increasing pH. Fig. 4 shows the pH dependence of the rate of cytochrome b_5 reduction by MKH_2. The rates of cytochrome reduction by their semiquinones are very fast. For instance, the rate constant of the reduction of cytochrome b_5 by MKH_2 is $5.0 \cdot 10^2$ $M^{-1} \cdot sec^{-1}$ at pH 6.5 (Fig. 4) and that by MKH has been reported to be $3 \cdot 10^8$ $M^{-1} \cdot sec^{-1}$ and to be almost independent of pH (ref. 10). Thus, the difference in the quinone-mediated

reduction of cytochromes between the NADH dehydrogenase and the NAD(P)H dehydrogenase becomes distinct as the pH decreases.

A similar difference in the reaction pattern arises between two enzymes when oxygen is used as a final electron acceptor. The oxygen-consuming oxidation of NADPH by microsomal NADPH-cytochrome *c* reductase has been observed in the presence of MK, and an active molecule which reduces oxygen has been assumed to be MKH by NISHIBAYASHI *et al.*[11], and IYANAGI AND YAMAZAKI[1]. Fig. 5 shows that the addition of MK causes oxidation of a stoichiometric amount of NADH by the

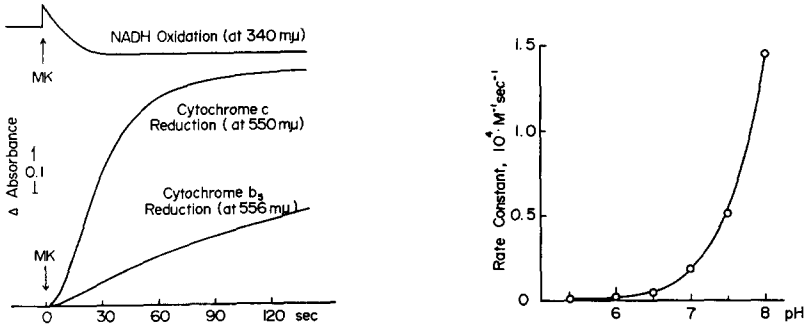


Fig. 3. MK-mediated reduction of cytochromes b_5 and *c* in the NAD(P)H dehydrogenase system. Reactions were started anaerobically by the addition of MK. Concentrations: 42 μg NAD(P)H dehydrogenase/ml, 100 μM NADH, 20 μM MK, 28 μM cytochrome b_5 or 28 μM cytochrome *c* and 0.1 M potassium phosphate (pH 6.0). NADH oxidation was measured at 340 mμ in the absence of cytochromes.

Fig. 4. The pH dependence of cytochrome b_5 reduction by MKH_2. Concentrations: 16 μM cytochrome b_5, 20 μM MKH_2 and 0.1 M potassium phosphate. Reactions were carried out anaerobically.

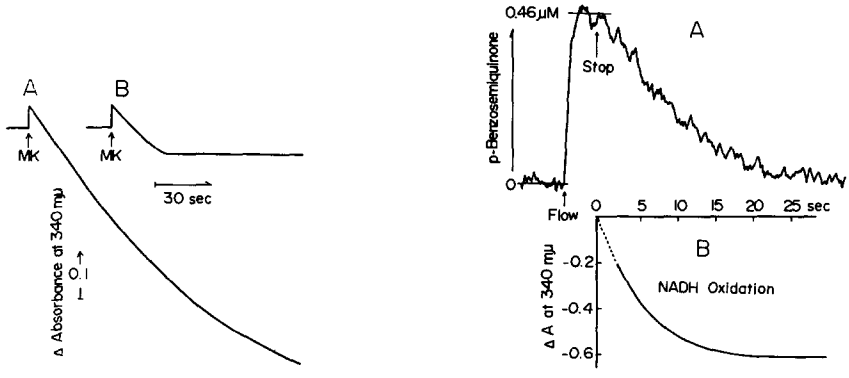


Fig. 5. MK-mediated aerobic oxidation of NADH in the NADH dehydrogenase (A) and the NAD(P)H dehydrogenase (B) systems. Concentrations: 0.15 μM NADH dehydrogenase (A) or 35 μg NAD(P)H dehydrogenase/ml (B), 100 μM NADH, 20 μM MK and 0.1 M potassium phosphate (pH 6.5).

Fig. 6. Time courses of NADH oxidation (B) and *p*-benzosemiquinone decay (A) in the NADH dehydrogenase. In A, the magnetic field was adjusted so as to obtain the maximum of the derivative curve of ESR absorption, and the reaction was started by mixing the solution of NADH and *p*-benzoquinone with the solution of the NADH dehydrogenase[1]. Final concentrations: 0.2 μM NADH dehydrogenase, 200 μM NADH, 100 μM *p*-benzoquinone and 0.1 M potassium phosphate (pH 6.5). In B, NADH oxidation was measured in a different cell under the same conditions.

TABLE I

ESTIMATION OF κ BY ESR AND OPTICAL SPECTROSCOPIC METHODS

ESR method: Reaction rates and semiquinone concentrations were measured under the conditions described for Fig. 6. Each value is the average of 3. The values of κ were calculated from Eqn. 3 assuming that k_d is $7 \cdot 10^7$ $M^{-1} \cdot sec^{-1}$ (ref. 32). Optical method: Expt. 1 was carried out under similar conditions to those described in Fig. 8. Values are averages of 4; Expt. 2, refers to Fig. 8; Expt. 3, values are at pH 7.5 in Fig. 9; Expt. 4, refers to Fig. 1B.

Enzyme	*ESR*			*Expt. No.*	*Optical*		
	Rate of quinone reduction ($\mu M \cdot sec^{-1}$)	*Semiquinone concn. at steady state* (μM)	κ		*Rate of cytochrome c reduction* ($\mu M \cdot sec^{-1}$)	*Rate of quinone reduction* ($\mu M \cdot sec^{-1}$)	κ^*
Cytochrome b_5 reductase	14.3	0.47	2.2	1	1.18	0.59	2.00
NADH dehydrogenase	13.8	0.46	2.1	2	0.70	0.36	1.95
Ferredoxin–NADP reductase				3	0.90	0.46	1.96
NAD(P)H dehydrogenase	13.2	<0.02	<0.004	4	0.00	0.71	0.00

* The ratio of the rate of cytochrome *c* reduction to the rate of quinone reduction.

NAD(P)H dehydrogenase but that MK acts as an electron carrier in the O_2-consuming oxidation of NADH by the NADH dehydrogenase. The result is in accord with the observation by NISHIBAYASHI *et al.*[12] that an addition of MK causes oxidation of a stoichiometric amount of NADPH in the presence of the NAD(P)H dehydrogenase.

It is now clear that there is an essential difference of the reaction mechanism between the NADH dehydrogenase and the NAD(P)H dehydrogenase. The difference is confirmed directly by the use of an ESR spectrometer as shown in Table I. Fig. 6 shows the time courses of NADH oxidation and of *p*-benzosemiquinone formation in the reaction of the NADH dehydrogenase. No ESR signal can be observed in the reaction of the NAD(P)H dehydrogenase at pH 6.5 though the rate of quinone reduction is the same as illustrated in Fig. 6. However, the ESR signal of *p*-benzosemiquinone appears during the reduction of *p*-benzoquinone by the NAD(P)H dehydrogenase when the reaction is carried out at slightly alkaline pH. Fig. 7 shows the time courses of the apparent formation and decay of *p*-benzosemiquinone at pH 8.0. It can be seen from the figure that the concentration of *p*-benzosemiquinone is almost equal to $(K)^{1/2}(Q)^{1/2}(QH)^{1/2}$ at any time of the reaction, where K is the semiquinone formation constant defined by MICHAELIS[13]. Accordingly, *p*-benzosemiquinone will not disappear when *p*-benzoquinone remains after the reaction is over. However, it remains unsolved why *p*-benzosemiquinone appears beyond the calculated value during the flow (reaction time corresponds to about 0.1 sec).

The value of κ, defined by the ratio of the velocity of free radical formation to the velocity of *p*-quinone disappearance at the steady state, can be calculated from the experiment, as shown in Table I. The details of κ estimation have been described in the previous papers[10,14]. These values will contain at least 10 % error, mostly due to the determination of spin concentration by ESR. More accurate estimation of κ can be made by trapping an electron of the semiquinone with a suitable electron

acceptor. When the rate of *p*-semiquinone formation is slow and in the presence of a large amount of cytochrome *c*, a stoichiometric relationship is observed between the rates of NADH oxidation and cytochrome *c* reduction in the NADH dehydrogenase system as well as in the cytochrome b_5 reductase system, which is shown in Fig. 8. The result obtained in the experiment of Fig. 8 suggests the following reaction mechanism, giving 2.0 for the value of κ.

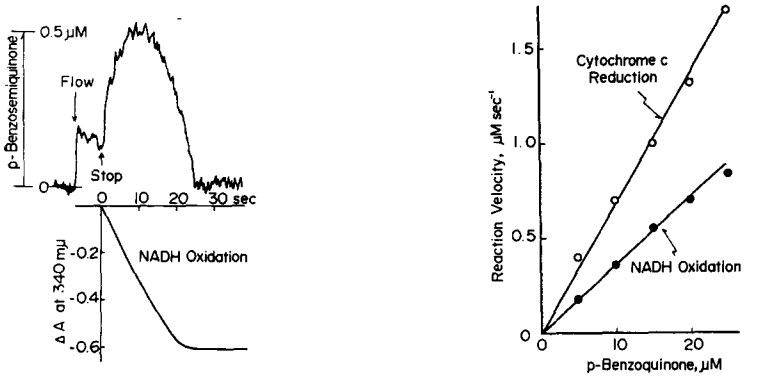


Fig. 7. Apparent formation and decay of *p*-benzosemiquinone during the reaction of the NAD(P)H dehydrogenase. Final concentrations: 35 μg NAD(P)H dehydrogenase/ml, 200 μM NADH, 100 μM *p*-benzoquinone and 0.1 M potassium phosphate (pH 8.0). Experimental procedures were the same as for Fig. 6.

Fig. 8. Relationship between the rates of NADH oxidation and of *p*-benzoquinone-mediated cytochrome *c* reduction in the presence of various *p*-benzoquinone concentrations. The rate of NADH oxidation was measured in the absence of cytochrome *c*. Concentrations: 0.1 μM NADH dehydrogenase, 100 μM NADH, 28 μM cytochrome *c* and 0.1 M potassium phosphate (pH 6.5).

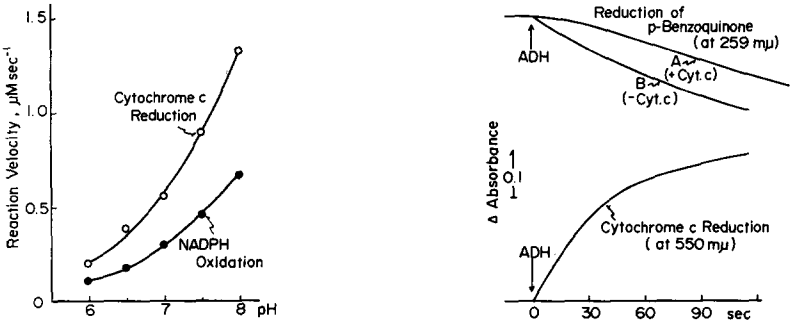


Fig. 9. The pH dependence of the rates of NADPH oxidation and of *p*-benzoquinone-mediated reduction of cytochrome *c* in the reaction of spinach ferredoxin–NADP reductase. The rate of NADPH oxidation was measured in the absence of cytochrome *c*. Concentrations: 0.04 μM ferredoxin–NADP reductase, 100 μM NADPH, 10 μM *p*-benzoquinone, 35 μM cytochrome *c* and 0.1 M potassium phosphate. The ratio of the two reaction rates was calculated to be 2.0 around the pH tested.

Fig. 10. Oxidation–reduction state of *p*-benzoquinone during the rapid reduction of cytochrome *c* by the cytochrome b_5 reductase system. NADH was supplied at a constant rate using an alcohol dehydrogenase system. Concentrations: 0.1 μM cytochrome b_5 reductase, 2 μM NAD^+, alcohol dehydrogenase, 1 % ethanol, 20 μM *p*-benzoquinone, 28 μM cytochrome *c* and 0.1 M potassium phosphate (pH 6.5). The reaction was started by the addition of alcohol dehydrogenase (ADH) and the reduction of *p*-benzoquinone was measured at 259 mμ in the presence (A) and absence (B) of cytochrome *c*.

$$NADH + 2Q \xrightarrow{\text{NADH dehydrogenase}} NAD^+ + 2Q^- + H^+ \quad (1)$$

$$Q^- + \text{cyt. } c^{3+} \xrightarrow{\text{Nonenzymic}} Q + \text{cyt. } c^{2+} \quad (2)$$

Under these conditions the *p*-benzosemiquinone anion (Q^-) is reoxidized to *p*-benzoquinone (Q) only by the reaction with cytochrome *c* but not by dismutation. Similar results are obtained in the reaction of cytochrome b_5 reductase (Table I) and of ferredoxin–NADP reductase (Fig. 9 and Table I). Fig. 10 shows that the formation of benzohydroquinone is not observed in the early stage of the reaction where the rapid reduction of cytochrome *c* occurs in the reaction of cytochrome b_5 reductase. Under the experimental conditions described in Figs. 8 and 9 the rate of cytochrome *c* reduction is proportional to the enzyme concentration. However, when the rate of semiquinone formation is fast and a small amount of cytochrome *c* is present, the semiquinones disappear mostly by dismutation, and the rate of cytochrome *c* reduction is proportional to the square root of the rate of quinone reduction at the steady state[8,15]. This relationship can be observed in the reaction of the NADH dehydrogenase as well as cytochrome b_5 reductase, as shown in Fig. 11. This means that the steady state concentration of *p*-benzosemiquinone is proportional to the square root of the rate of quinone reduction and is not disturbed by the presence of a small amount of elctron acceptor[1,8,10,14].

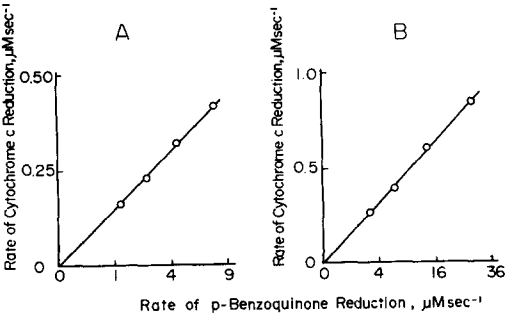


Fig. 11. Relationship between the rate of cytochrome *c* reduction and the square root of the rate of NADH oxidation (or *p*-benzoquinone reduction) when the acceptor concentration is low and the enzyme concentration is high. The reaction rates were measured by the stopped flow method[10]. The rates of NADH oxidation were measured by changing the enzyme concentrations. Concentrations: 200 μM (A) or 100 μM (B) NADH, 100 μM *p*-benzoquinone, 1 μM cytochrome *c* and 0.1 M potassium phosphate (pH 6.5). A, the NADH dehydrogenase and B, cytochrome b_5 reductase.

DISCUSSION

Several flavoproteins are characterized by their catalytic ability to transfer electrons from reduced pyridine nucleotides to suitable quinones, mostly benzoquinones or 1,4-naphthoquinones. These are named quinone reductases by MARTIUS[16] and have been prepared from plants[17,18], animals[19–21] and microorganisms[22–25]. ERNSTER *et al.*[19] studied the enzyme from liver and called it DT-diaphorase. In this paper we have called it the NAD(P)H dehydrogenase according to the Enzyme Commission's nomenclature[26]. The enzyme catalyzes the reduction of quinones and dyes but not the reduction of cytochromes and lipoic acid. ERNSTER *et al.*[19] reported that the enzyme could catalyze the reduction of cytochrome *c* in the presence of

naphthoquinone derivatives but not of benzoquinone derivatives. It was reported by MARKI AND MARTIUS[5] that the mammalian quinone reductase reduced MK very rapidly, and that the autoxidation of MKH_2 became rate-limiting after MK was consumed when the reaction was carried out under aerobic conditions. NISHIBAYASHI *et al.*[12] showed that the amount of NADPH oxidized was equivalent to that of the MK added, even under aerobic conditions. These results suggest that quinone-mediated electron transfer to cytochromes and oxygen is rather sluggish in the presence of the NAD(P)H dehydrogenase, but a clear explanation has not yet been offered.

Several preparations of the NADH dehydrogenase were isolated from mitochondria[27]. The soluble NADH dehydrogenase behaves as a diaphorase and catalyzes the reduction of quinones such as MK and ubiquinones[28–31]. In general it may be said that most of the flavoproteins are able to catalyze the electron transfer to two-electron acceptors such as quinones, dyes or molecular oxygen. It is important to know, quantitatively if possible, whether the enzymes catalyze one- or two-electron transfer to these acceptors. The ESR method seems best for the analysis of the mechanism. A parameter, κ, has been introduced to distinguish the two mechanisms[1,2,10,14].

$$\kappa = \frac{2k_d\,(\text{semiquinone})_s^2}{v} \tag{3}$$

where v is the rate of quinone reduction at the steady state and k_d is the dismutation constant for semiquinones. The value of κ can be estimated by measuring the steady state concentration of the semiquinone with the aid of ESR techniques. The value of κ lies between 2 and 0. The value is 2 for a typical one-electron transfer and is 0 for a typical two-electron transfer mechanism. In order to measure κ according to Eqn. 3 the experimental conditions must be selected so as to avoid nonenzymic formation of the semiquinones, for instance, from the reaction between quinone and quinol[32]. This can be achieved by decreasing the pH of the reaction solution and the concentration of acceptors. It is evident from Eqn. 3 that the value of κ can be estimated only when k_d is known, and failure to observe ESR signals during the reaction does not always mean that $\kappa = 0$. It can be said that, in general, the ESR method is not applicable when k_d is markedly large or the reaction rate (v) is too slow. Even so, there are possibilities that the electron transfer mechanism can be analyzed.

Let us consider the cases where flavoproteins catalyze the reduction of benzoquinone, MK or oxygen. The ESR method is very useful for quantitative analysis of the mechanism when benzoquinone is an acceptor but not when MK or molecular oxygen is an acceptor because MKH and O_2^- have high dismutation constants. The oxygen radical, O_2^-, has been identified by ESR in the frozen state[33,34], but it seems difficult to use the result for the estimation of κ. Fig. 12 shows three reaction systems which may be used for the analysis of the electron transfer mechanism. The experimental conditions can always be so arranged that the electron flows from a flavoprotein to a final acceptor only when the flavoprotein catalyzes one-electron reduction of the carrier molecule. When cytochromes are used as final acceptors the estimation of κ will become possible provided that; (1) the pH of the reaction solution is low so as to avoid the reduction of cytochromes by benzohydroquinone or MKH_2 and (2) the formation of semiquinones or O_2^- is slow and the cytochrome is present in a large amount so that the disappearance of semiquinones or O_2^- by dismutation

may be neglected[1]. Typical examples are shown in Figs. 8 and 9. A clear distinction between the one- and two-electron mechanisms can be observed in the reaction system of MK and O_2 (Fig. 5) but the system may not be used for κ estimation since the O_2 consumption is not stoichiometric. The value of κ will be estimated when cytochrome

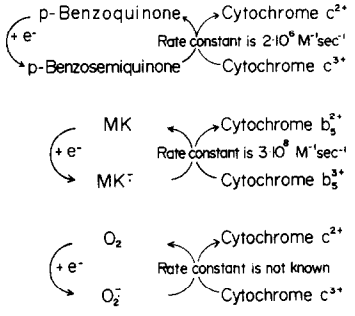


Fig. 12. Three sets of electron acceptors which can be used for the estimation of κ. When the reduction of cytochromes is measured under optimal conditions (Figs. 8 and 9), κ will be calculated as the ratio of the rate of cytochrome reduction to the rate of disappearance of quinone or O_2 in the absence of cytochromes.

b_5 is used as a final acceptor instead of O_2. For this purpose, however, the reaction must be carried out under strictly anaerobic conditions and the exact initial velocity of cytochrome b_5 reduction should be measured. It has been suggested by McCORD AND FRIDOVICH[35] that erythrocuprein is a potent O_2^- dismutase. With the use of erythrocuprein MASSEY *et al.*[36] have distinguished the reduction of cytochrome *c* caused by O_2^- from that by other pathways. Using cytochrome *c* as a final electron acceptor the accurate value of κ will be estimated even when O_2 is a direct acceptor for flavoproteins. The method of trapping O_2^- as Compound III by peroxidase can also be used for this purpose though under restricted conditions[2].

It might be concluded from these observations that flavoproteins fall into three groups according to the mode of electron transfer to acceptors. One group (flavoprotein-1) catalyzes compulsory one-electron reduction of acceptors, forming free radicals of two-electron acceptors ($\kappa = 2$). The other group (flavoprotein-2) catalyzes two-electron reduction of quinones or oxygen, the primary products being the two-equivalent reduced form of the acceptor molecule ($\kappa = 0$). One more group (flavoprotein-1,2) has a mixed mechanism and its κ lies between 0 and 2. These are listed in Table II. Much attention has been given by many workers to the mechanism of electron transfer from flavoprotein to acceptor. The involvement of a one-electron transfer mechanism seems to be demonstrated in the following reactions of flavoproteins. NISHIBAYASHI *et al.*[11] reported that microsomal NADPH cytochrome *c* reductase catalyzed the oxygen-consuming oxidation of NADPH in the presence of MK but not of MKH_2. The reduction of cytochrome *c* by milk xanthine oxidase has been known to be dependent upon the presence of oxygen[37–40]. The formation of an oxygen-free radical during the xanthine oxidase reaction has been suggested by the initiation of sulfite autoxidation[41] and by the induction of chemiluminescence[42]. KNOWLES *et al.*[33] have confirmed the formation of the radical using an ESR technique. Aldehyde oxidase has properties similar to those of xanthine oxidase[43]. The relation between the reduction of cytochrome *c* and the formation of hydrogen peroxide has

TABLE II

CLASSIFICATION OF FLAVOPROTEINS BASED UPON THE MECHANISM OF ELECTRON TRANSFER TO TWO-ELECTRON ACCEPTORS

Group	*Enzyme*	*Ref.*	*Prosthetic group*	*Electron donor*	*Electron acceptor*	
					One-electron	*Two-electron*
Flavoprotein-1 (one-electron type)	Cytochrome b_5 reductase	1	FAD	NADH	Ferricyanide, cyt. b_5	*p*-Benzoquinone, DCIP
	NADPH–cytochrome *c* reductase	1	FAD	NADPH	Ferricyanide, cyt. *c*	*p*-Benzoquinone, MK, DCIP
	NADH dehydrogenase	This paper	FMN, Fe	NADH	Ferricyanide	*p*-Benzoquinone, CoQ, MK, DCIP
	Ferredoxin–NADP reductase	This paper	FAD	Ferredoxin NADPH	Ferricyanide, cyt. *f*	*p*-Benzoquinone, MK, DCIP
	Succinate dehydrogenase	45, 46	FAD, Fe	Succinate	Ferricyanide	DCIP, PMS
Flavoprotein-1,2 (mixed type)	Xanthine oxidase	2, 15, 39, 43	FAD, Mo, Fe	Xanthine	Ferricyanide	*p*-Benzoquinone, O_2, PMS
	Aldehyde oxidase	43	FAD, Mo, Fe	Aldehyde	Ferricyanide	O_2, DCIP, MB
	Dihydroorotate dehydrogenase	44	FAD, FMN, Fe	Dehydroorotate NADH	Fe^{3+}·*o*-phenanthroline	O_2
	Old yellow enzyme	36	FMN	NAD(P)H	Ferricyanide	O_2
Flavoprotein-2 (two-electron type)	NAD(P)H dehydrogenase	This paper	FAD	NAD(P)H	Ferricyanide	*p*-Benzoquinone, MK, DCIP
	D-Amino-acid oxidase	36	FAD	D-Alanine		O_2
	L-Amino-acid oxidase	36	FAD	L-Leucine		O_2
	Glucose oxidase	36	FAD	Glucose		O_2
	Glycollate oxidase	36	FMN	Glycollate		O_2
	Lactate oxidase	36	FMN	Lactate		O_2

Abbreviations: Cyt., cytochrome; DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; MB, methylene blue.

been quantitatively investigated in the dihydroorotate dehydrogenase reaction by MILLER AND MASSEY[44], who suggest a mechanism involving an oxygen-free radical.

Studying reduction of phenazinemethosulfate by the succinic dehydrogenase, KING[45] and SINGER AND KEARNEY[46] suggested a one-electron transfer mechanism. The enzyme catalyzes the reduction of cytochrome *c* in the presence of phenazinemethosulfate as carrier. It seems likely that the enzyme belongs to flavoprotein-1 though crucial evidence has not yet been obtained. There is a group of flavoproteins called oxidases. According to the recent study by MASSEY *et al.*[36] it is very likely that glucose oxidase, D-amino-acid oxidase, L-amino-acid oxidase, glycollate oxidase, and lactate oxidase belong to flavoprotein-2. In the case of the old yellow enzyme the generation of O_2^- was clearly demonstrated by the ability to catalyze the O_2^--mediated reduction of cytochrome *c* and by the inhibition of this reduction by erythrocuprein. However, judged by the low efficiency of cytochrome *c* reduction, O_2^- production seems to be only a side-reaction in the overall reaction of old yellow enzyme. The inability to form an oxygen radical by flavoprotein oxidases may be manifest by the negative sign of the initiation of sulfite oxidation[41] and of the induction of chemiluminescence[42].

Most of the flavoprotein dehydrogenases, such as NADH–cytochrome b_5 reductase[47], NADPH–cytochrome *c* reductase[48], succinic dehydrogenase[49], NADPH–cytochrome *f* reductase[50], and NADPH–cytochrome c_2 reductase[51], have diaphorase activities but they react with one-electron acceptors faster than with two-electron acceptors. MASSEY *et al.*[52] discussed some correlative properties of flavoproteins and suggested that with few exceptions the semiquinoid form of the oxidases is the red (or anionic) form, whereas with the dehydrogenases the blue (or neutral) form is the stable form produced on partial reduction. Table II shows that the typical oxidases will belong to flavoprotein-2 while the dehydrogenases are classified as flavoprotein-1 with the marked exception of the NAD(P)H dehydrogenase. However, further study is needed to discuss the relation between one-electron transfer mechanism of the flavoprotein dehydrogenases and their blue semiquinones as a possible intermediate. It is concluded that quinones behave as a one-electron carrier rather than a two-electron carrier when they are in an electron transport system. Accordingly, when the two-electron acceptor (A) reacts with the reduced flavoprotein (H_2FP) the reaction should be given by the following equation:

$$H_2FP\text{-}1 + 2A \longrightarrow FP\text{-}1 + 2\,HA$$
$$H_2FP\text{-}2 + A \longrightarrow FP\text{-}2 + H_2A$$

KOMAI *et al.*[53] found that the formation of O_2^- requires direct electron transfer from a reduced form of flavin to oxygen in the xanthine oxidase reaction. HATEFI *et al.*[31] and PHARO *et al.*[28] suggested that the labile sulfide–iron system might not be involved in the diaphorase activities of the soluble NADH dehydrogenase. IYANAGI AND YAMAZAKI[1] also reported that microsomal flavoproteins, which have no essential metal ions, catalyze a typical one-electron transfer. From these observations it may be concluded that a metal ion is not necessarily needed for the flavoprotein to catalyze one-electron transfer to acceptors. The problem may be solved by assuming that the enzymes of flavoprotein-1 do yield the stable semiquinones, probably of the flavin on reaction with electron acceptors, but the experimental data we have are not yet sufficient.

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