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Studies on Metalloflavoproteins. IV. The Role of the Metal[1]

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RECEIVED AUGUST 6, 1954

In an attempt to elucidate the function of the metal in metalloflavoproteins two experimental approaches have been used: a study of the interaction of seven different pure flavoproteins, both metal-containing and metal-free, with a series of quinones of widely varying oxido-reduction potentials; and an investigation of some of the properties and reactions of riboflavin–metal chelates, as models of metalloflavoproteins. The first approach has led to the recognition of two characteristic constants for flavoproteins, the optimal interaction potential (ϵ) and the reaction constant (κ). A comparison of metal-free and metal-containing enzymes reveals that the presence of metal leads to a shift in ϵ and a decrease in κ. The study of the model system has disclosed that the oxidation of DPNH by cytochrome-*c* in the presence of diaphorase is specifically stimulated by ferririboflavin. The rate of this reaction is further enhanced if the ferririboflavin is chelated with aromatic, but not with aliphatic, iron-binding agents. Some of the theoretical and practical implication of these findings especially as they relate to electron transport and oxidative phosphorylation are discussed.

Introduction

In previous papers of this series[3–7] we have shown that a number of flavoproteins possess composite prosthetic groups consisting of a metal in association with one of the catalytically active flavin nucleotides. The functional role of the metal appears to be that of the intramolecular channeling of electrons from a two-step oxido-reduction (the reduction of flavin by the substrate) into a one-step process (the reoxidation of the reduced flavin by certain acceptors). Structurally, the metal appears to facilitate the binding of the flavin to the protein by chelation with each of the two partners. Mechanistically, the metal permits the stabilization of the activated complex formed by reduced flavoprotein and acceptor—a stabilization which is necessary for efficient electron transfer from enzyme to oxidizing agent. This is accomplished by actual binding of the acceptor to the metal[5]; thus during the transient existence of this complex, apoenzyme, coenzyme, metal and acceptor all form part of the same resonating system and the transfer of one electron from one part of this mobile system of π-electrons to another is eminently feasible, and both rapid and efficient.

The present investigation was undertaken in an attempt to gain further insight into some of the details of the processes just described. Two experimental approaches will be discussed: a systematic study of the interaction of a number of metalloflavoproteins and metal-free flavoproteins with a series of quinones, and the study of the reactions of metal–riboflavin chelates as models of metalloflavoproteins.

Experimental

Preparation and Purification of Enzymes.—The following enzymes were used in this investigation: DPNH-cytochrome reductase,[8] diaphorase,[9] xanthine oxidase,[4] aldehyde oxidase,[6] L-amino acid oxidase,[10] D-amino acid oxidase,[11] butyryl CoA dehydrogenase.[12] All chemicals used were recrystallized. The various Versenes were gifts of the Bersworth Chemical Co.

Oxidation of DPNH[13] by Quinones.—In a typical experiment 0.13 μmole of DPNH,[14] 0.05 μmole of the quinone indicated and 10 μmoles of buffer of appropriate *p*H were mixed in a total volume of 0.95 ml. in a cuvette of 1.0-ml. capacity and an optical path of 1.00 cm. After the log I_0/I at 340 mμ had been determined,[15] 0.05 ml. of enzyme solution was added and the changes in log I_0/I determined every 15 seconds for 2 minutes. The decrease in optical density between 15 and 75 seconds constitutes the initial rate (V_0). The change in log I_0/I can be converted to μmoles by multiplying by 16.1×10^{-3}.[16] To determine the blank in the absence of enzyme the same system with the enzyme omitted is used.

Reduction of Cytochrome-*c* by Hydroquinones.—In a typical experiment a mixture containing 0.1 to 1.0 μmole of substrate, 0.05 μmole of quinone, 1. mg. of cytochrome-*c* and 10 μmoles of buffer was made up to a total volume of 0.95 ml. for the experimental and 1.00 ml. for the blank. After the log I_0/I at 550 mμ had been determined, 0.05 ml. of enzyme was added to the experimental cuvette and the change in optical density determined every 15 seconds for 2 minutes. Again the initial rate is defined as the change from 15 to 75 seconds. The μmoles of substrate disappearing per minute can be calculated by multiplying the above value by 2.67×10^{-3}.[17]

Riboflavin-catalyzed Oxidation of DPNH.[18]—Similar to the system just described except that the quinone was

(1) Supported by grants-in-aid to H.R.M. by the American Cancer Society (on recommendation by the Committee on Growth of the National Research Council) and by the National Institutes of Health as well as by a grant of the Nutrition Foundation to Dr. D. E. Green. A preliminary report of some of this work was delivered at the 126th Meeting of the American Chemical Society, New York, Sept. 15, 1954.

(2) Post-doctoral trainee of the National Heart Institute, National Institutes of Health.

(3) H. R. Mahler, THIS JOURNAL, **75**, 3288 (1953); *J. Biol. Chem.*, **206**, 13 (1954).

(4) B. Mackler, H. R. Mahler and D. E. Green, *ibid.*, **210**, 149 (1954).

(5) H. R. Mahler and D. G. Elowe, *ibid.*, **210**, 165 (1954).

(6) H. R. Mahler, B. Mackler and D. E. Green, *ibid.*, **210**, 465 (1954).

(7) H. R. Mahler and D. E. Green, *Science*, **120**, 7 (1954).

(8) H. R. Mahler, N. K. Sarkar, L. P. Vernon and R. A. Alberty, *J. Biol. Chem.*, **199**, 585 (1952); L. P. Vernon, H. R. Mahler and N. K. Sarkar, *ibid.*, **199**, 599 (1952).

(9) F. M. Straub, *Biochem. J.*, **33**, 789 (1939).

(10) T. P. Singer and E. B. Kearney, *Arch. Biochem.*, **29**, 190 (1950).

(11) E. Negelein and H. Brömel, *Biochem. Z.*, **300**, 225 (1939).

(12) D. E. Green, S. Mii, H. R. Mahler and R. M. Bock, *J. Biol. Chem.*, **206**, 1 (1954).

(13) The following abbreviations will be used: DPN^+, diphosphopyridine nucleotide; DPNH, reduced DPN; E, enzyme; F, flavin nucleotide; Q, quinone; QH_2, hydroquinone; cytc. or cytc(Fe^{+++}), ferricytochrome-*c*; cyt-c(Fe^{++}), ferrocytochrome-*c*; Versene, ethylenediaminetetraacetic acid; Versene-EDG, N-hydroxyethyliminodiacetic acid; Versene-Fe^3 specific, N,N-dihydroxyethylglycine; Versene-ol-N-hydroxyethylethylenediamine triacetic acid; tris, tris-(hydroxymethyl)-aminomethane; diol, 2-amino-2-methyl-1,3-propanediol; Rb, riboflavin; RbMe or $RbMe_2$, riboflavin–metal chelates; E_0, standard potential compared to the hydrogen electrode at a given *p*H; K_m' = Michaelis–Menten constant at a given set of experimental conditions; V_{max}, extrapolated velocity at infinite substrate concentration. EF, enzyme-bound flavin nucleotide.

(14) 85–90% pure by enzymatic assay; obtained from the Sigma Chemical Co., St. Louis, Mo.

(15) Beckman spectrophotometer, Model DU.

(16) B. L. Horecker and A. Kornberg, *J. Biol. Chem.*, **175**, 385 (1948).

(17) E. C. Slater, *Biochem. J.*, **46**, 484 (1950).

(18) T. P. Singer and E. B. Kearney, *J. Biol. Chem.*, **183**, 409 (1950).

omitted and 0.05 μmole of Rb or $RbMe_2$ chelate[19] was added as the catalyst instead of the enzyme.

Diaphorase Reaction.—Similar to the above, except that 20 μg. of diaphorase was added as well, wherever indicated.

Preparation of Systems Containing Complexes of Rb, Fe and Complexing Agent.—A mixture of 0.25 μmole of $RbFe_2$ or of ferric chloride, 1.0 μmole of complexing agent and 10 μmoles of Tris buffer of *p*H 7.9 made up to 1.00 ml. was incubated for 30 minutes at 38°. At the end of this period 0.2 ml. of the incubation mixture was tested in the DPNH–cytochrome-*c* system described.

Results

Reaction of Flavoproteins with Quinones.—The classical investigations of Wieland[20] and Booth[21] have shown that quinones could be used as electron acceptors for certain flavoproteins. Recently Wosilait and Nason[22,23] have demonstrated the existence of dihydropyridine nucleotide–quinone reductase activities in a wide variety of tissues and species. Two activities have been described: one apparently specific for benzoquinones and one for 2-methyl-1,4-naphthoquinone. It has been our aim to inquire whether quinones in general and a series of structurally related quionones[24] (*i.e.*, substituted naphthoquinones) of widely varying oxido-reduction potential in particular, could function as electron acceptors with seven different highly purified flavoproteins; and whether there exists any functional relationship between the rate of reduction of these quinones and their oxido-reduction potential. Of the flavoproteins studied four were metalloflavoproteins, *viz.*, DPNH–cytochrome reductase, xanthine oxidase (with molybdenum added) aldehyde oxidase, and butyryl CoA dehydrogenase. Four were metal-free flavoproteins, *viz.*, diaphorase, ophio L-amino acid oxidase, D-amino acid oxidase and xanthine oxidase which had been completely freed of molybdenum.[4] Preliminary experiments indicated that quinones could not be used with two of these enzymes, aldehyde oxidase and D-amino acid oxidase, because of the strong inhibitory action of the quinones, presumably on sulfhydryl groups, on the enzymes.[6,25] All the other flavoproteins tested proved to be capable of interaction with quinones. Some of these reactions will now be described in detail.

1. **DPNH-Cytochrome Reductase. Reaction with Oxygen.**—In the presence of an excess of DPNH the reduced enzyme is only very slowly reoxidized by atmospheric oxygen.[8] As shown in Fig. 1 the addition of 5.0×10^{-4} *M* 2-methyl-1,4-naphthoquinone induces a rapid oxidation of DPNH by air. The sequence of reactions is

$$\mathrm{DPNH} + \mathrm{EF} + \mathrm{H^+} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{DPN^+} + \mathrm{EFH_2} \quad (1)$$

$$\mathrm{EFH_2} + \mathrm{Q} \xrightarrow{k_3} \mathrm{QH_2} + \mathrm{EF} \quad (2)$$

$$\mathrm{QH_2} + \mathrm{O_2} \xrightarrow{k_4} \mathrm{Q} + \mathrm{H_2O_2} \quad (3)$$

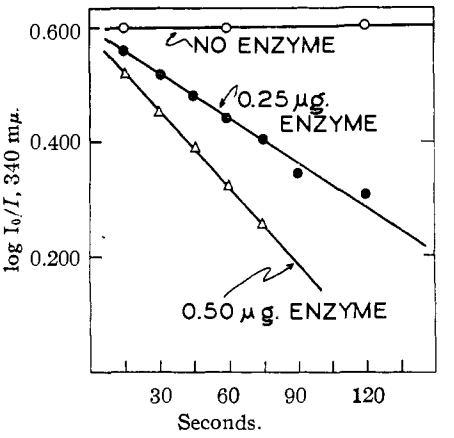


Fig. 1.—The oxidation of DPNH by oxygen in the presence of cytochrome reductase and 2-methyl-1,4-naphthoquinone (5.0×10^{-5} *M*). The curve labeled "no enzyme" is also representative of conditions in the presence of 0.25 μg. of enzyme but with the quinone omitted. All experiments in 0.03 *M* diol buffer, *p*H 8.5.

Under the particular conditions of the experiments, *i.e.*, at high DPNH concentrations k_1, k_2 and k_4 are probably fast and thus k_3 becomes rate limiting and is the rate constant of the reaction actually being studied. Some of the characteristics of this reaction are shown in Fig. 2, which demonstrates the variation of the rate with *p*H, and the effect of various buffers and other anions on the velocity. It can be seen that the *p*H optimum is identical with that previously described for the interaction of the same enzyme with cytochrome-*c* with the difference that the reduction of quinones is insensitive to the presence of such complexing anions as phosphate, pyrophosphate and citrate. Thus qui-

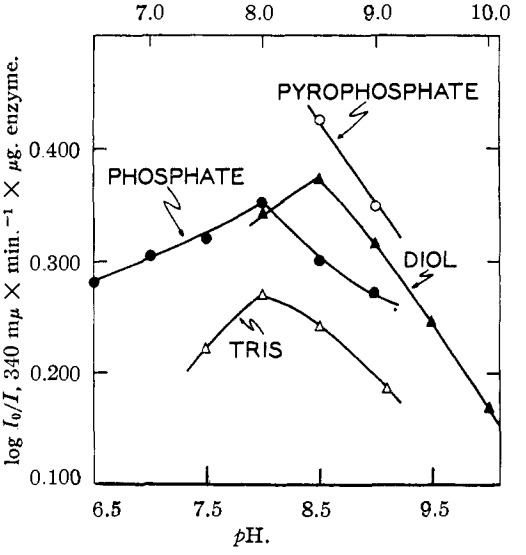


Fig. 2.—*p*H activity curve for the oxidation of DPNH by oxygen: all buffers at 0.01 *M*; 2-methyl-1,4-naphthoquinone as acceptor.

(19) W. O. Foye and W. E. Lange, THIS JOURNAL, **76**, 2199 (1954).

(20) H. Wieland and W. Mitchell, *Ann.*, **483**, 217 (1930).

(21) V. H. Booth, *Biochem. J.*, **29**, 1732 (1935). The use of quinones as electron acceptors is discussed in E. Baumann and K. Myrback, "Die Methoden der Enzymforschung," Georg Thieme, Leipzig, 1941, and Academic Press, Inc., New York, N. Y., 1945, p. 2357.

(22) W. D. Wosilait and A. Nason, *J. Biol. Chem.*, **206**, 255 (1954); W. D. Wosilait, A. Nason and A. J. Terrell, *ibid.*, **206**, 271 (1954).

(23) W. D. Wosilait and A. Nason, *ibid.*, **208**, 785 (1954).

(24) The collection of highly purified quinones was made available to us through the courtesy of Dr. H. Beinert.

(25) L. Hellerman, A. Lindsay and M. R. Bovarnick, *J. Biol. Chem.*, **163**, 553 (1946).

nones, as might be anticipated from their close structural similarity to certain oxidation–reduction dyes such as the indophenols, fall into the class of "two-electron" acceptors for metalloflavoproteins—a conclusion which had already been verified experimentally in the case of xanthine oxidase.[4]

The variation of reaction rate with quinone concentration is shown in Fig. 3 in the form of Lineweaver–Burk[26] plots for 2-chloro-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone. In

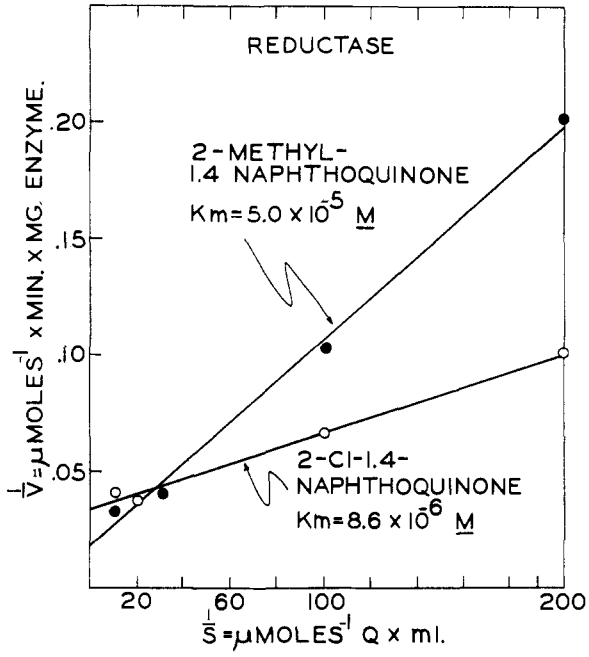


Fig. 3.—Lineweaver–Burk plot for quinones in the air oxidation of DPNH by cytochrome reductase in 0.03 *M* diol buffer, *p*H 8.5, temperature 25°.

Table I the Michaelis–Menten[27] constants and maximal velocities calculated from these data are compared with the analogous constants for the two acceptors previously studied—cytochrome-*c* and 2,6-dichlorophenolindophenol,[26] all derived under the same conditions of buffer *p*H and concentration, substrate concentration, and with the same enzyme preparation. It is apparent that quinones are efficient electron acceptors capable of interacting with the flavoprotein under conditions and at rates quite comparable to those observed with more conventional oxidizing agents.

TABLE I

REACTION CONSTANTS FOR DPNH-CYTOCHROME REDUCTASE

All experiments at 25° in 0.01 *M* diol, *p*H 8.5, at a DPNH concentration of 2×10^{-4} *M*, and using 0.5 μg. of enzyme.

Electron acceptor	K_m, *M* × 10^5	k_3,[a] min.$^{-1}$ × 10^{-2}
Cytochrome-*c*	12	1.6
2,6-Dichlorophenolindophenol	6.4	1.5
2-Methyl-1,4-naphthoquinone	1.0	3.2

[a] Defined as $V_{max}/(E)$ where E, the enzyme concentration, is calculated using a molecular weight of 80,000[5] and V_{max}, the extrapolated initial zero-order rate at infinite acceptor concentration, is expressed as μmoles DPNH oxidized per minute. The data are calculated from the appropriate Lineweaver–Burk plots.

(26) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).
(27) L. Michaelis and M. Menten, *Biochem. Z.*, **49**, 333 (1913).

Reaction with Cytochrome-*c*.—Although the reaction with oxygen is the most convenient for the study of quinone-catalyzed oxidations other devices for the reoxidation of the hydroquinone (equation 3) can be used. One that has been employed by us because of its general applicability to all the enzymes under study and its possible physiological significance is the reduction of ferricytochrome-c according to equation 3a

$$QH_2 + 2\text{cytc (Fe}^{+++}) \xrightarrow{k_5} Q + 2\text{cytc (Fe}^{++}) + 2H^+ \quad (3a)$$

Under aerobic conditions there will, of course, be a competition between oxygen and cytochrome-*c*. Reaction 3 can be eliminated by excluding oxygen and it can then be shown that the rate of disappearance of substrate in the presence of very high concentrations of cytochrome-*c* is identical with that measured previously with oxygen when reaction 3 was being studied. Ordinarily it is unnecessary to maintain anaerobicity. For any particular set of experimental conditions a constant proportion of the total hydroquinone molecules (and thus of the substrate molecules initially) will be oxidized by path 3a as compared to path 3. Therefore the rate of cytochrome-*c* reduction provides an equally valid measure of the over-all reaction rate as does substrate disappearance but the rate constants for the two reactions may be different. This point is borne out by the data of Fig. 5 and Table II. The substrate disappears at exactly the same rate whether cytochrome-*c* is present or not, indicating that k_3 is probably rate limiting.

In the particular case of DPNH as substrate, and cytochrome reductase as the enzyme, special conditions have to be maintained for the study of the quinone–cytochrome reaction. The enzyme is, of course, capable of catalyzing the oxidation of DPNH by cytochrome-*c* even in the complete absence of quinone. By running the reaction in the presence of relatively high levels of phosphate, pyrophosphate or citrate, however, the direct reduction of cytochrome can be completely suppressed.[5,8] On the further addition of quinone, cytochrome-*c* reduction is again observed as shown in Fig. 4.

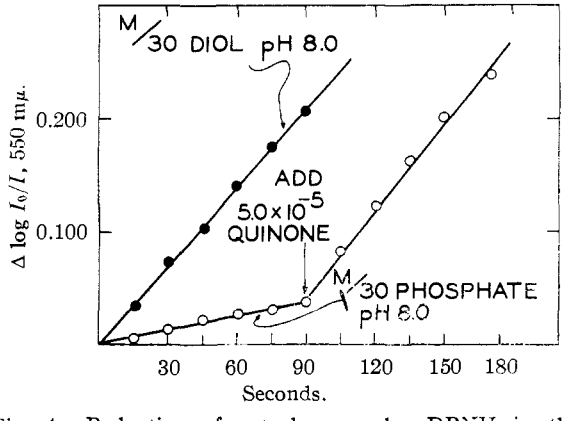


Fig. 4.—Reduction of cytochrome-*c* by DPNH in the presence of cytochrome reductase and quinone.

This reaction can be shown to be proportional to enzyme concentration. In Fig. 5 the effect of quinone concentration on the reaction rate is compared for the three assay systems used (oxidation of DPNH by quinone in the presence and in the absence of cytochrome-*c* and reduction of cytochrome-*c*). The rate of cytochrome-*c* reduction is a constant fraction of the rate of DPNH oxidation (1/2.4) at all concentrations of quinone studied and the saturation levels and K_m's found are identical in all systems.

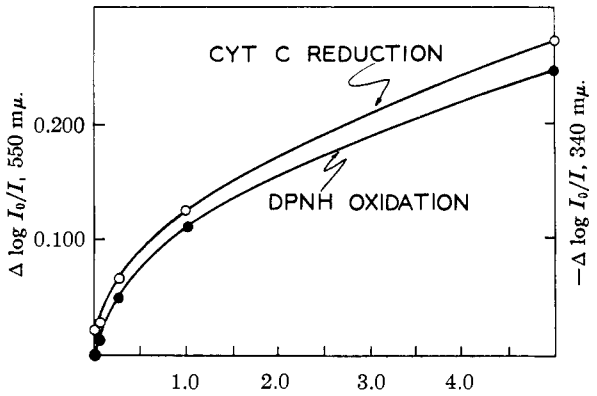


Fig. 5.—Comparison of cytochrome-*c* and oxygen as final acceptors in the oxidation of DPNH by cytochrome reductase and quinone. All experiments in 0.01 M pyrophosphate buffer, *p*H 8.5, and in the presence of 5.0 × 10^{-5} M 2-methyl-1,4-naphthoquinone. The curve for DPNH oxidation is the same whether O_2 or cytochrome-*c* (aerobic) is used as final oxidant.

2. Diaphorase.—The interaction of diaphorase with quinones has been studied in all three of the assay systems described for cytochrome reductase. Figure 6 shows Lineweaver–Burk plots for different quinones in the cytochrome-*c* assay system, while Table II compares the initial rates with different quinones, as measured in the oxygen reaction, with the rates observed when cytochrome-*c* is the final acceptor.

TABLE II

OXIDATION OF DPNH BY QUINONES AND CYTOCHROME-*c* IN THE PRESENCE OF DIAPHORASE

All experiments at 25° and in 0.01 M phosphate buffer *p*H 7.0 in the presence of 2 × 10^{-4} M DPNH, 1.0 mg. of cytochrome-*c* where shown, and 1–5 μg. of enzyme. In the cytochrome experiments aerobic reduction of cytochrome-*c* was used as a measure of the reaction; thus part of the QH_2 molecules were oxidized by O_2 even in the cytochrome system.

	Quinone	k_3' (μmoles DPNH oxidized × $min.^{-1}$ × $mg.^{-1}$ of enzyme) Cytochrome-*c* as acceptor	O_2	$\frac{k_3'O_2}{k_3'cyto.}$
1	2-Hydroxy-1,4-naphthoquinone	0.019	0.030	1.50
2	Anthraquinone-β-sulfonate	.027	.036	1.30
3	2-Ethyl-1,4-naphthoquinone	.150	.240	1.45
4	2-Methyl-1,4-naphthoquinone	.370	.405	1.08
5	*p*-Xylo-*p*-quinone	.588	.780	1.33
6	*p*-Benzoquinone	.900	1.68	1.86
7	1,4-Naphthoquinone	1.23	1.62	1.32
8	2-Cl-1,4-naphthoquinone	1.98	2.10	1.08
9	3-Me-1,2-naphthoquinone	2.40	2.70	1.13
10	2-Me-5,8-dihydroxy-1,4-naphthoquinone	2.88	4.02	1.40
			Mean	1.34

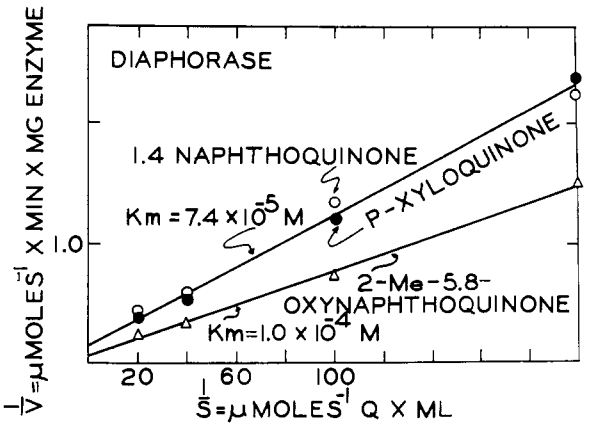


Fig. 6.—Lineweaver–Burk plot for quinones in the air-oxidation of DPNH by diaphorase in 0.01 M phosphate buffer, *p*H 7.5, temperature 25°.

3. Xanthine Oxidase.—Quinones can be employed as carriers to either oxygen or cytochrome-*c* in the presence of any of the possible substrates of this enzyme, *i.e.*, hypoxanthine, DPNH or aldehydes. Typical data obtained in the presence and absence of molybdenum, using an enzyme initially completely freed of the metal, are shown in Table III. The conditions are such (low phosphate concentration) that there is essentially no reduction of cytochrome-*c* in the absence of the various quinones.

TABLE III

THE EFFECT OF MOLYBDENUM ON THE OXIDATION OF HYPOXANTHINE AND DPNH BY OXYGEN IN THE PRESENCE OF XANTHINE OXIDASE AND VARIOUS QUINONES

All reactions at 25° in the presence of 5 × 10^{-3} M phosphate *p*H 7.0 with 20–50 μg. of enzyme.

Quinone	k_3' (μmoles substrate oxidized × $min.^{-1}$ × $mg.^{-1}$ of enzyme) Without molybdenum DPNH[a]	Hypoxanthine[b]	With molybdenum[c] DPNH[a]
Anthraquinone-β-sulfonate	0.096		0.096
2-Hydroxynaphthoquinone	.105		.128
2-Methyl-1,4-naphthoquinone	.280	0.120	.440
2-Ethyl-1,4-naphthoquinone	.305	.100	.555
p-Xylo-*p*-quinone	.320	.120	.455
p-Benzoquinone	.830		.855
3-Methyl-1,2-naphthoquinone	.910	.450	.943
1,4-Naphthoquinone	1.030	.200	.625
2-Me-5,8-dihydroxy-1,4-naphthoquinone	1.15	.225	.855
2-Cl-1,4-naphthoquinone	1.40	.255	.575

[a] Substrate, 2 × 10^{-4} M DPNH, O_2 as acceptor. [b] Substrate, 2 × 10^{-5} M hypoxanthine, 1.0 mg. cytc as acceptor, cytochrome-*c* reduction measured. [c] In the presence of 1 × 10^{-4} M MoO_3.

4. L-Amino Acid Oxidase.—Although oxygen or oxidation–reduction dyes[27] are suitable electron acceptors for this enzyme, cytochrome-*c* is completely inert. This is in line with the fact that

this flavoprotein enzyme is completely devoid of heavy metals.[27] In the presence of quinones cytochrome-*c* is rapidly reduced and this reaction has been used to obtain the data of Table IV, column A.

5. Butyryl CoA Dehydrogenase.—Although this cuproflavoprotein is capable of interacting directly with cytochrome-*c*, the oxidation of the substrate by cytochrome-*c* is known to be exceedingly slow.[3,12] In the presence of quinones the rate of reduction of cytochrome-*c* is increased significantly as the data of Table IV, column B, show.

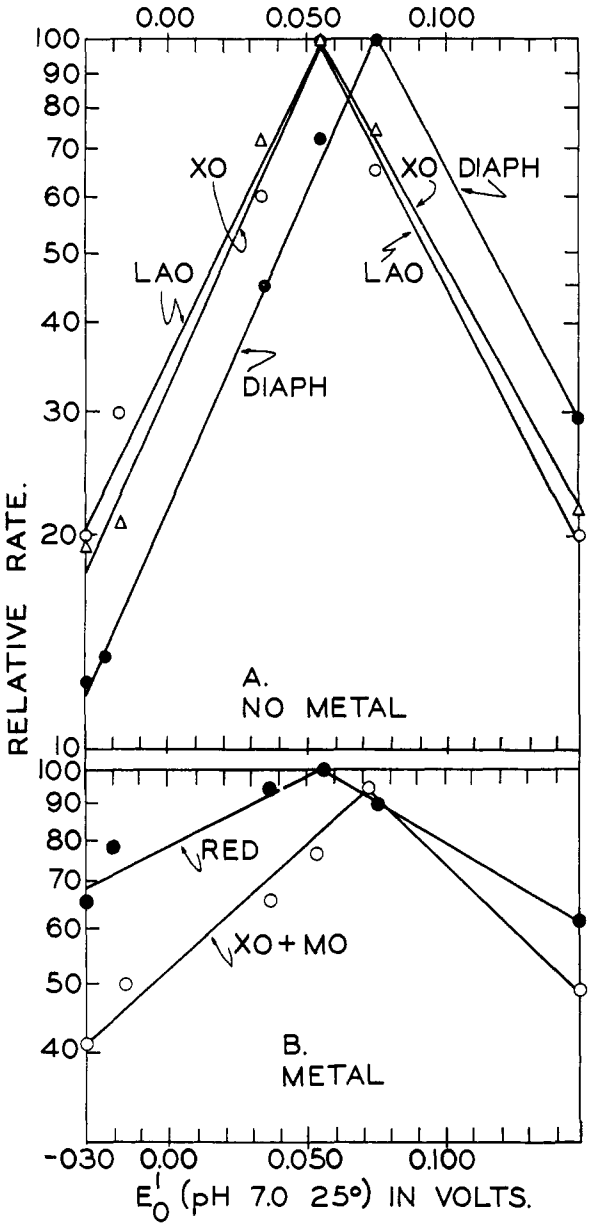


Fig. 7.—Plot of relative cytochrome-*c* reduction rates *vs.* E_0' of substituted naphthoquinones for different flavoproteins. Relative rates calculated on the basis of 100 for most rapid rate (log I_0/I at 550 mμ between 15 and 75 seconds): LAO = l-amino acid oxidase; DIAPH = diaphorase; RED = DPNH-cytochrome reductase; XO = xanthine oxidase. A, flavoproteins not containing metal; B, metalloflavoproteins.

TABLE IV

REDUCTION OF CYTOCHROME-*c* BY L-LEUCINE AND BUTYRYL-CoA IN THE PRESENCE OF APPROPRIATE ENZYMES AND VARIOUS QUINONES

Quinone	Rate = log I_0/I at 550 mμ × 5 min.$^{-1}$ A. *l*-Leucine[a]	B. Butyryl-CoA[b]
p-Xylo-*p*-quinone	0.100	0.100
p-Benzoquinone	.185	.100
1,4-Naphthoquinone	.190	.020
2-Methyl-1,4-naphthoquinone	.100	.005
2-Methyl-5,8-dihydroxy-1,4-naphthoquinone	.265	.080
2-Cl-1,4-Naphthoquinone	.450	.107

[a] In 0.01 *M* phosphate buffer, *p*H 7.0, in the presence of 10^{-3} *M* *l*-leucine at 38°. [b] In 0.01 *M* tris buffer, *p*H 7.0, at 25° in the presence of 10^{-5} *M* butyryl-CoA. Both reactions were catalyzed by 10 μg. of their respective enzymes.

6. The General Reaction with Quinones.—From the foregoing it is apparent that the various flavoproteins are capable of interacting with quinones and that certain regularities exist in this general reaction. This becomes more striking when the data are arranged in the manner of Table V where the various naphthoquinones used (and the closely related *p*-xyloquinone) are arranged according to their E_0' [28] and the reaction rates, all obtained with cytochrome-*c* as final acceptor, are expressed on a relative basis. There appears to be a definite functional relationship, the nature of which emerges when the data are plotted as log relative initial rate (V_0) against E_0' of the naphthoquinones. Figure 7A shows these curves for metalloflavoproteins while Fig. 7B is the analogous one for metal-free flavoproteins. The following characteristic features are observed: (a) the function relating V_0 with E_0' is

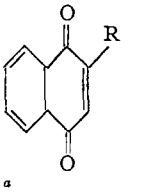
$$\log V_0 = \kappa E_0' \qquad (5)$$

(b) there appears to be an optimum potential (ϵ)

TABLE V

OXIDATION POTENTIALS AND RELATIVE RATES OF DIHYDROFLAVINENZYME OXIDATION BY SUBSTITUTED NAPHTHOQUINONES

All reactions at 25° and *p*H 7.0 with the quinones at a concentration of 5×10^{-5} *M*.



R	Other	E_0' *p*H 7.0	k_2'[a] I[b]	II	III	IV	V	VI
CH_3	.....	−0.030	65	15	20	2	19	42
C_2H_5	.....	− .020	78	17	30		22	50
H	.....	.036	94	45	60	15	72	66
Cl	.....	.056	90	100	66		75	95
CH_3	5,8-Dihydroxy	.075	100	73	100	80	100	76
p-Xylo-*p*-quinone		.166	61	29	20	80	22	49
3-Methyl-1,2-naphthoquinone		.075	70	80		100	62	100

[a] All normalized to 100 for the most rapid rate. [b] I, DPNH–cytochrome reductase; II, diaphorase; III, l-amino acid oxidase; IV, butyryl-CoA dehydrogenase; V, xanthine oxidase freed of molybdenum; VI, xanthine oxidase with molybdenum added.

(28) The potentials used are those given in H. A. Lardy, "Respiratory Enzymes," Burgess Publishing Company, Minneapolis, Minn., 1949, p. 72 ff. and in reference.[32] They have been corrected to *p*H 7.0.

characteristic for each flavoprotein, below this potential κ is positive, above it κ is negative. (c) The absolute value of κ is larger for metal-free than for metal-containing flavoproteins. (d) The value of the optimum potential, also, is influenced by the presence of the metal.

Properties and Reactions of Riboflavin–Metal Chelates

That riboflavin could form stable chelation compounds with metals, notably with iron, was first discovered by Albert[29] on the basis of titration data. He also postulated the possible existence of riboflavin–metal–enzymes. A series of riboflavin–metal chelates all having the general formula $RbMe_2$ have been prepared and isolated by Foye and Lange.[19] These compounds, generously provided by Dr. Foye, formed the subjects of the present study.

Absorption Spectra.—The absorption spectra of the iron–, copper–, cobalt–, nickel–, manganese– and zinc–riboflavin chelates have been determined and their characteristic features are summarized in Table VI. The position of the maxima is shifted slightly, if at all; the effect on the ϵ_{max} is minor except in the case of the Mn compound; there is no appearance of any new (non-flavin) peak either in the spectra of the oxidized compounds or those which had been reduced by hydrosulfite; finally there is increased absorption above 500 mμ in all cases.

TABLE VI

ABSORPTION SPECTRA OF RIBOFLAVIN–METAL CHELATES

All spectra were taken after dissolving 1.00 μmole of the various compounds in 10.0 ml. of 0.005 *M* tris buffer, *p*H 7.0.

Metal	λ_{max}, mμ	ϵ_{max}	ϵ_{ps} (cm.^{2} × μmole^{-1} × 10^{-8})	i_{20}
None	445	11.1	10.5	0.350
Fe	455	11.0	9.20	.870
Cu	455	11.0	9.40	.740
Co	445	9.60	7.90	.620
Ni	450	9.60	7.90	.560
Mn	447	7.50	6.35	.500
Zn	450	10.9	8.80	.950

Catalysis of DPNH-Cytochrome-*c* Reaction.—In an attempt to study the mode of action of riboflavin–metal chelates as possible models of metalloflavoproteins we have investigated their effect on the non-enzymatic riboflavin-catalyzed reduction of cytochrome-*c* by DPNH, discovered by Singer and Kearney.[18] This reaction proceeds by the following steps (reactions 6–7a).

$$H^+ + DPNH + Rb \longrightarrow DPN^+ + RbH_2 \quad (6)$$

$$RbH_2 + 2cytc(Fe^{+++}) \longrightarrow Rb + 2cytc(Fe^{++}) + 2H^+ \quad (7a)$$

In this sequence, unlike the enzymatic one, it is reaction 6 which is rate limiting, as had already been shown by the earlier authors, since other electron acceptors such as dyes or oxygen could be used without affecting the rate of oxidation of DPNH and since the rate of the over-all reaction was identical with reaction 6 when the latter was carried out anaerobically.

(29) A. Albert, *Biochem. J.*, **54**, 646 (1953).

When the various metal–riboflavin chelates were substituted for riboflavin in this reaction sequence no effect on reaction rate could be observed. Thus once again the model bears out the hypothesis that metals have no effect on the reduction of flavin by substrate.

Catalysis of Interaction of Diaphorase with Cytochrome-*c*.—A search for a possible system in which reaction (6) or its equivalent would be rapid, and reaction (7) rate limiting, led us to a study of the reaction of Straub's diaphorase with cytochrome-*c*. This enzyme as already observed by Straub[30] does not interact with cytochrome-*c* in the absence of carriers (Fig. 8). When riboflavin was added to the system the rate of reduction of cytochrome-*c* was that obtained with riboflavin as a catalyst alone. Similar results were observed with any of the riboflavinchelates substituted for riboflavin, with the exception of the iron and copper compound. The $RbCu_2$ gave a slight stimulation while the $RbFe_2$ increased the rate of reduction at all concentrations tested two- to threefold when compared to the rates obtaining at the same concentration of riboflavin. All three components were thus necessary for this effect: diaphorase (with its bound FAD), riboflavin plus iron.

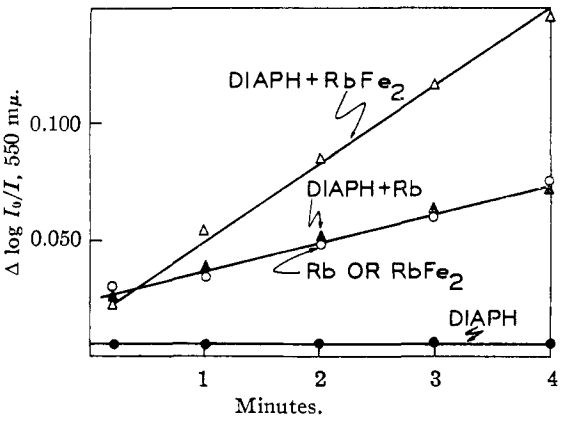


Fig. 8.—Reduction of cytochrome-*c* by DPNH in the presence of diaphorase and ferririboflavin in 0.01 *M* Tris buffer, *p*H 7.9.

When oxygen or 2,6-dichlorophenolindophenol was substituted for cytochrome-*c* no enhancement of the rate over that obtained with enzyme and riboflavin alone could be observed. Similarly, ferric ions alone did not stimulate the reduction of cytochrome-*c* by diaphorase plus riboflavin. The ferricyanide–ferrocyanide couple, on the other hand, could function as a carrier between diaphorase and cytochrome-*c*.

As a working hypothesis we have postulated that the function of the metal in metalloflavoproteins is to increase resonance stabilization in the activated complex by providing a nexus for mobile π-electron interaction between protein, flavin and acceptor. In this manner activation energy would be lowered and rates enhanced. As a test of the hypothesis we have incubated the ferri-riboflavin with a number of compounds known to complex with iron. In some of these complexes the π-electrons of the

(30) F. M. Straub, *Enzymologia*, **9**, 148 (1941).

metal–riboflavin would be capable of interaction with those of aromatic systems, thus giving rise to possible increased resonance stabilization, in others no such interaction is possible. Table VII shows that in the cases of ordinary strong complex-formers such as azide or cyanide, or chelating agents such as the Versenes or citrate no increase in the rate of the diaphorase–riboflavin complex catalyzed reduction of cytochrome-*c* is observed. In the case of α,α'-dipyrydyl, 8-hydroxyquinoline or *o*-phenanthroline, however, there is an added increase in rate. A small part of this increase only can be due to the possible formation of free ferric complexes of the aromatic and iron-binding agents; such iron complexes are capable of enzymatic and non-enzymatic reduction by DPNH, followed by reoxidation by cytochrome-*c*.[31] This reaction is exceedingly slow, and even if all the iron had become detached from the flavin and attached to the chelating agent, the rates actually observed could not be accounted for by this mechanism alone. There would also be no explanation for the qualitative difference between the mode of action of the aromatic and non-aromatic chelating agents.

TABLE VII

REDUCTION OF CYTOCHROME-*c* BY DPNH IN THE PRESENCE OF DIAPHORASE AND VARIOUS IRON-CONTAINING CATALYSTS

Additions	Rate[a] $RbFe_2$ Omitted[b]	Added[c]	Additions	Rate[a] $RbFe_2$ Omitted[b]	Added[c]
None	10	105	Cyanide	28	80
Cysteine	15	90	Versene-Fe^{III}	14	90
Glutathione	15	35	Versene-ol	12	85
Antimycin[d]	24	110	Versene-EDG	13	110
Thyroxine[e]	13	100	*o*-Phenanthroline	100	185
Azide	8	115	8-Hydroxyquinoline	17	300
Pyrophosphate	10	100	α,α'-Dipyridyl	35	183
Citrate	13	95			

[a] Log I_0/I per 4 minutes at 550 mμ in the presence of 25 μg. of diaphorase. [b] With 1.0×10^{-5} *M* $FeCl_3$. [c] With 1.0×10^{-5} *M* Fe_2Rb. [d] 1 γ/ml. [e] 4.0×10^{-6} *M*.

Discussion

The reaction of flavoproteins with quinones here described is of some interest from several points of view. If we consider first some of the theoretical implications we find that the relationship given in equation 5 is one that relates the rate of a reaction to some function of the free energy of one of the reacting species. Although similar correlations are frequently feasible when studying the kinetics and mechanisms of organic reactions,[32,33] this has not heretofore been possible for an isolated, well-characterized enzyme reaction.

For each reaction and each enzyme studied two characteristic constants (κ and ϵ) have been obtained. κ varies most widely if metalloflavoproteins are compared with their metal-free counterpart and can, as a matter of fact, be used as a diagnostic for the presence of metal.[34] ϵ also is affected by the presence of metal (*e.g.*, xanthine oxidase in its two states) but the most significant aspect of this constant is its variation between enzymes. If we assume that the value of ϵ is always higher than the potential of the enzyme, then a determination of the former is tantamount to an indirect and semi-quantitative measurement of the latter. The presence of the metal profoundly influences both the potential and the reaction characteristics of the enzyme. The large value of κ and the very sharp break for metal-free enzymes suggest a requirement in the activated complex for the matching of energy levels of enzyme and acceptor which is best accomplished at just one value of the latter. The presence of metal has the effect of smearing out these levels so that matching can take place at a larger number of values. It must be emphasized that the reaction studied here is one that does not ordinarily require the presence of the metal. The rates for any one acceptor are relatively insensitive to the presence or absence of the metal, as the data for xanthine oxidase show. Only when the different quinones are compared with one another under standard assay conditions does this difference emerge. Thus the metal influences the active site profoundly even in a reaction in which it is not functionally involved. Metalloflavoproteins therefore constitute entities which permit close electronic interaction of their three components with each other and (during the life-time of the activated complex) with the electron acceptor as well.

Quinones, especially naphthoquinones, also are of interest from the point of view of their possible involvement in vital biochemical processes. They have been shown to be antimaterials,[35] inhibitors of electron transport[36] and uncouplers of oxidative phosphorylation.[37] The present investigation has some bearing on the possible effect of quinones on electron transport between DPNH and the cytochrome system.[38] If we take DPNH–cytochrome reductase, a soluble enzyme, as a suitable model of

(31) M. M. Weber, H. M. Lenhoff and N. O. Kaplan, *Biochim. Biophys. Acta*, **14**, 289 (1954).

(32) The well-known Hammett equation is of this type; L. P. Hammett, "Physical Organic Chemistry, McGraw-Hill Book Company," Inc., New York, N. Y., 1940, p. 73 ff., and numerous other references. For earlier attempts at similar correlations in biological systems see the work of E. S. G. Barron and L. A. Hoffman, *J. Gen. Physiol.*, **13**, 483 (1930), and D. E. Green, L. H. Stickland and H. L. A. Tarr, *Biochem. J.*, **20**, 1812 (1934). Investigations bearing on this point have been reviewed by E. S. G. Barron, *Physiol. Rev.*, **19**, 209–210 (1939).

(33) A very interesting correlation of this sort relating the quantum yield in the photo-oxidation of chlorins by certain quinones and oxygen to their E_0 is given by F. M. Huennekens and M. Calvin, THIS JOURNAL, **71**, 4029, 4032 (1949).

(34) Thus a particular highly purified sample of the yellow fatty acyl CoA dehydrogenase of Beinert, *et al.*, (THIS JOURNAL, **75**, 4111 (1953)) behaved like a typical non-metal containing flavoprotein. Subsequent analysis showed that heavy metals were present in trace amounts only.

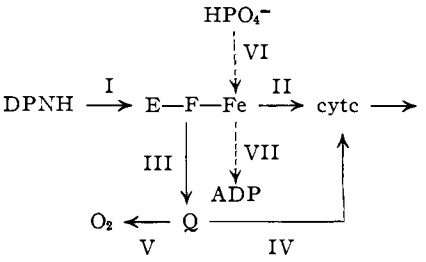
(35) E. G. Ball, C. B. Anfinsen and O. Cooper, *J. Biol. Chem.*, **168**, 257 (1947).

(36) H. Heymann and L. F. Fieser, *ibid.*, **176**, 1359 (1948).

(37) Vitamin K_1 itself appears to stimulate oxidative phosphorylation while the free naphthoquinone is an inhibitor as shown by C. Martius and D. Nitz-Litzow, *Biochim. Biophys. Acta*, **12**, 134 (1953); *ibid.*, **13**, 152 (1954); *ibid.*, **13**, 289 (1954). In the scheme presented here a slight modification is sufficient to account for this observation also. It is only necessary to assume that phosphorylation accompanies reaction *III*[23] as well; then the addition of uncouplers may once again introduce a diversion of electrons through the uncoupler to oxygen or cytochrome-*c*.

(38) For other possible schemes involving quinones in electron transport see.[22,23] Schemes postulating a role of metalloflavoproteins in oxidative phosphorylation have been presented by us,[7] by P. D. Boyer (*Federation Proc.*, **13**, 185 (1954) and by C. B. Anfinsen and W. W. Kielley (*Ann. Rev. Biochem.*, **23**, 38 (1954).

the enzymes involved in at least part of this sequence, it becomes clear that in the interaction of phosphate, quinone, cytochrome-*c* and oxygen we may be presented with a very sensitive device for the channeling of electrons and the conservation or dissipation of oxidative energy



In solution the enzyme ordinarily carries out reactions I and II leading to the oxidation of DPNH by cytochrome-*c*. This reaction is inhibited by phosphate, possibly due to the coördination of this ion with the iron of the enzyme during the catalytic process (reaction VI). If the oxidized phosphoenzyme is thermodynamically unstable with respect to the aquo-enzyme then this process would lead to oxidative phosphorylation, provided that this breakdown is not spontaneous,[39] and that a suitable acceptor system, eventually leading to ADP, is provided (VII). Reaction III would then lead to a diversion of the electrons and attendant uncoupling, especially at high phosphate concentration when reaction VII becomes rate limiting.[40] The electrons may be channeled back into the cytochrome system (IV) and give rise to possible subsequent phosphorylations or else they may be diverted completely to oxygen and thus uncoupling of all subsequent phosphorylations may also be induced.

(39) In the case of xanthine oxidase and aldehyde oxidase which show an absolute requirement for phosphate when catalyzing cytochrome-*c* reduction, this spontaneous breakdown is believed to occur. Thus these enzymes although engaged in phosphate uptake during part of their catalytic cycle are nevertheless capable of functioning in the absence of an *added* system catalyzing reaction VII. For oxidative phosphorylation to be feasible by this mechanism it is only necessary that the negative free energy of hydrolysis of the oxidized phosphoenzyme exceed that of the reduced form by 10,000 cal.

(40) H. A. Lardy, "The Biology of Phosphorus," Michigan State College Press, Lansing, Mich., 1952, p. 134 ff. The hypothesis proposed here would explain uncoupling at the flavin and cytochrome, but not at the DPNH level.

The demonstration of the interaction of diaphorase with the iron–riboflavin chelate raises certain questions concerning the mechanism of this reaction. It may be due to reaction 8 and 9, *i.e.*, an equilibrium between the two flavins followed by a rapid reduction of cytochrome-*c* or to reaction 10 and 11, *i.e.*, the binding of the riboflavin–metal chelate to the enzyme, forming a modified enzyme, now capable of interaction with cytochrome-*c*.

$$EFH_2 + MeRbMe \rightleftharpoons EF + MeRbH_2Me \quad (8)$$

$$MeRbH_2Me + 2cytc\,(Fe^{+++}) \longrightarrow MeRbMe + 2cytc\,(Fe^{++}) + 2H^+ \quad (9)$$

$$EFH_2 + MeRbMe \rightleftharpoons E\begin{matrix}FH_2\\MeRbMe\end{matrix} \rightleftharpoons E\begin{matrix}F\\MeRbH_2Me\end{matrix} \quad (10)$$

$$E\begin{matrix}F\\MeRbH_2Me\end{matrix} + 2cytc\,(Fe^{+++}) \longrightarrow E\begin{matrix}F\\MeRbMe\end{matrix} + 2cytc\,(Fe^{++}) + 2H^+ \quad (11)$$

$$E\begin{matrix}R\\MeRbMe\end{matrix} \rightleftharpoons EF + MeRbMe \quad (12)$$

The first mechanism appears unlikely in view of the great specificity for the iron compound and for diaphorase.[41] It seems more reasonable to assume that diaphorase is indeed a transformed DPNH cytochrome reductase,[5] free of iron, and with some of the flavin–protein bonds modified, but still capable of coördinating iron at the active site, if the metal is complexed in the proper manner. The added increase in rate in the presence of aromatic chelating agents may then be due to the formation of an enzyme which has undergone further modification to include the aromatic ring chelated with one of the two iron atoms as well.

Acknowledgments.—The authors wish to thank Dr. David E. Green for many helpful discussions and Mrs. Dorothee G. Elowe for competent technical assistance.

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(41) When other flavoproteins such as the amino acid oxidases plus their substrates were tested for their ability to provide EFH_2 as the reactant for cytochrome-*c* reduction in the presence of the model compounds no stimulation by the $RbFe_2$ was obtained.