Flavoenzyme Models. II. Effect of Added Components on the Reduced Nicotinamide–Adenine Dinucleotide–Flavin Mononucleotide System*

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ABSTRACT: The effects of the addition of a variety of compounds, which have been proposed or shown to complex with flavins or to function as electron acceptors from flavin, on the reduced nicotinamide–adenine dinucleotide–flavin mononucleotide (NADH–FMN) model system were examined. A decrease in reaction rates commensurate with ability to complex flavins was found.

Sulfur-containing redox systems failed to react with the NADH–FMN system. Quinones either did not react or were directly reduced by NADH. A non-heme iron model compound gave a three-component redox system that was successfully examined. Flavodehydrogenase mechanisms are discussed.

Weber (1948, 1950) first reported complex formation with flavins from fluorescence studies. He concluded that an interaction of the adenine and isoalloxazine moieties in FAD[1] was responsible for the decreased absorption and fluorescence of FAD relative to FMN. The structural requirements for purine–isoalloxazine complex formation have been examined by Tsibris *et al.* (1965) and Chassy and McCormick (1965). Optical rotatory dispersion has also been used to demonstrate this interaction (Gascoigne and Radda, 1965; Simpson and Vallee, 1966).

Weber (1950) proposed that the quenching of flavin fluorescence observed upon binding of flavin by a number of enzymes, *e.g.*, old yellow enzyme (Åkeson, *et al.*, 1963), might be due to interaction of flavin with an aromatic amino acid. Nygaard and Theorell (1955) reported that the iodination of a single tyrosine residue in old yellow enzyme led to a loss of flavin binding. Strittmatter (1965) has reported the same observation for NADH–cytochrome b_5 reductase.

Sulfhydryl groups have been implicated in flavin binding in heart muscle NADH dehydrogenase (Mackler, 1965). They also have been proposed to be active in oxidation–reduction reactions for lipoyl dehydrogenase (Massey, 1963; Massey and Veeger, 1963), yeast glutathione reductase (Colman and Black, 1965; Massey and Williams, 1965), and dihydroorotic acid dehydrogenase (Miller and Massey, 1965).

Barbiturates have been shown to be very potent inhibitors of respiratory NADH dehydrogenase (Estabrook, 1957; Ernster *et al.*, 1955). The addition of barbiturates to an NADH–FMN system was found to enhance the decreasing rate of reduction of FMN at higher pH values by Giuditta and Vitale-Neugebauer (1965). No enhancement was seen at neutral pH values, however.

Electron acceptors for flavoenzymes include quinones (Martius, 1963), cytochromes (Hatefi, 1963), and metal ions such as non-heme iron (San Pietro, 1965), found in such enzymes as NADH dehydrogenase from respiratory systems, and molybdenum (Nason, 1963), as is found in nitrate reductase.

The first simple iron compound to be reported that possessed the low epr g value characteristic of biological non-heme iron was sodium nitrosopentacyanoferrate (Beinert *et al.*, 1965). The oxidized diamagnetic form of this compound will accept one electron to form a paramagnetic species which possesses a three-line epr spectrum centered at $g = 2.0253$ at room temperature (Bernal and Hockings, 1962). The reduced form possesses an appreciable optical absorption with a maximum around 600 nm. Ferric ion in the presence of *o*-phenanthroline has been shown by Weber *et al.* (1956) to be capable of reoxidizing reduced FMN.

This report examines the effects of the addition of a variety of compounds, which have been shown to

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[1] Abbreviations used are: epr, electron paramagnetic resonance; FMN, flavin mononucleotide; $FMNH_2$, fully reduced flavin mononucleotide; FMNH·, flavin mononucleotide free radical; FAD, flavin–adenine dinucleotide; NAD, nicotinamide–adenine dinucleotide; NADH, reduced nicotinamide–adenine dinucleotide; NADPH, reduced nicotinamide–adenine dinucleotide phosphate; and NMNH, reduced nicotinamide mononucleotide.

TABLE I: The Effect of Complexing Agents on Reaction Rates.[a]

Component	Initial Rates (OD/min) 340 nm	445 nm	570 nm	900 nm	Epr (arbitrary units)
FMN and NADH only	0.031	0.038	0.340	0.250	1.40
+adenine	0.031	0.038	0.325	0.195	
+adenosine	0.032	0.036	0.350	0.188	
+adenosine monophosphate	0.032	0.038	0.360	0.200	
+adenosine diphosphate	0.034	0.039	0.360	0.180	
+adenosine triphosphate	0.029	0.039	0.360	0.190	
+barbituric acid		0.037	0.345	0.220	1.35
+2,3-naphthalenediol		0.020	0.200	0.085	0.80
+sodium barbital		0.039	0.240	0.250	
+sodium phenobarbital		0.039	0.345	0.250	
+tryptophan		0.033	0.240	0.195	1.15
+tyrosine ($7/3 \times 10^{-3}$ M)		0.038	0.310	0.205	1.30

[a] 7×10^{-3} M FMN, 2×10^{-3} M NADH, and 7×10^{-3} M tertiary component, unless otherwise stated. Error is ±0.002 at 340 and 445 nm, ±0.012 at 570 nm, ±0.006 at 900 nm, and ±0.05 by epr as the maximum values.

complex flavin or have been shown or proposed to undergo oxidation–reduction reactions with flavin, upon the NADH–FMN system (Fox and Tollin, 1966b). Flavoenzyme reaction mechanisms are discussed.

Materials and Methods

NADH and FMN were obtained from Calbiochem (Fox and Tollin, 1966b). All of the chemicals used were in the chromatographically pure state with the exception of the Eastman Organic Chemicals products which were either purified by sublimation or by recrystallization, and the DL-lipoic acid obtained from Calbiochem which was 99.93% pure. Adenine, adenosine, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, cysteine, cystine, cytochrome *c*, oxidized glutathione, DL-lipoic acid, tryptophan, and tyrosine were obtained from Calbiochem. *p*-Benzoquinone, 2,3-naphthalenediol, and 1,4-naphthoquinone were obtained from Eastman Organic Chemicals. Barbituric acid was from J. T. Baker. Ammonium molybdate tetrahydrate, sodium nitrosopentacyanoferrate, and sodium phenobarbital were obtained from Mallinckrodt. Haemin, menadione, menadione bisulfite, and sodium barbital were obtained from Nutritional Biochemicals Corp. Thiamine hydrochloride and research grade argon were obtained from Matheson Coleman and Bell. Acid proteinoid 2:2:1 was kindly supplied by Drs. S. W. Fox and K. Harada of the University of Miami, Fla. It possesses a mean molecular weight of 4000 (Fox and Harada, 1960; Fox, 1965). Measurements and experiments were conducted as previously elaborated by Fox and Tollin (1966a) and as described in paper I (Fox and Tollin, 1966b).

Results

Complexing (Nonredox) Agents. Compounds which will not directly undergo oxidation–reduction reactions with flavins, but would complex them to varying degrees, were examined for their effect on the binary system reaction rates. Generally, compounds which form better complexes exhibited the greatest effect. 2,3-Naphthalenediol forms a complex with FMN in neutral solution with a stability constant of 242 M^{-1} (Fleischman and Tollin, 1965). The L-tryptophan complex with FMN has a stability constant of 0.002–0.10 M^{-1} in neutral solution, and the L-tyrosine–ethyl ester complex has a value of 0.002 M^{-1} (Harbury and Foley, 1958).

The results of the addition of a variety of compounds which complex flavins are shown in Table I. The effects of the addition of an acidic proteinoid on the NADH–FMN reaction are shown in Table II.

Redox Components. No effect upon the binary system was noted upon the addition of possibly redox-active components such as ammonium molybdate, cysteine, cystine, oxidized glutathione, haemin hydrochloride, DL-lipoic acid, menadione, menadione bisulfite, or thiamine hydrochloride. *p*-Benzoquinone and 1,4-naphthoquinone exhibited a direct reduction by NADH, thus obviating a study of this system. Cytochrome *c* could not be obtained in solution in sufficient quantities to give an observable effect. It also clogged the stopped-flow apparatus, making it nonoperative quickly.

A redox-active tertiary system was achieved with the

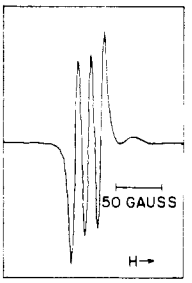

FIGURE 1: Epr spectrum of reduced nitrosopentacyanoferrate ion.

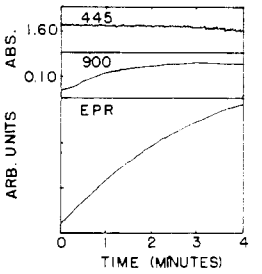

FIGURE 2: Relative kinetics of epr, 445, and 900 nm during nitrosopentacyanoferrate ion reduction. 7×10^{-3} M FMN, 2×10^{-3} M NADH, 2×10^{-3} M $Na_2NOFe(CN)_5$.

TABLE II: Effect of 2:2:1 Acid Proteinoid on Initial Reaction Rates.

System	Initial Rates (OD/min)		
	445 nm	570 nm	900 nm
2×10^{-3} M FMN, 7×10^{-3} M NADH	0.058	0.135	0.250
$+5 \times 10^{-4}$ proteinoid	0.054	0.050	0.240
1×10^{-3} M FMN, 7×10^{-3} M NADH	0.025	0.080	0.155
$+1 \times 10^{-3}$ M proteinoid	0.024	0.010	0.145

Error ranges are ±0.001 at 445 nm, ±0.010 at 570 nm, and ±0.005 at 900 nm.

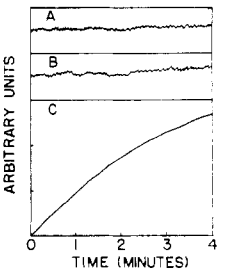

FIGURE 3: An epr comparison of iron reduction requirements. (A) 7×10^{-3} M FMN, and 2×10^{-3} M $Na_2NOFe(CN)_5$. Gain = 500. (B) 2×10^{-3} M NADH and 2×10^{-3} M $Na_2NOFe(CN)_5$. Gain = 500. (C) 7×10^{-3} M FMN, 2×10^{-3} M NADH, and 2×10^{-3} M $Na_2NOFe(CN)_5$. Gain = 25.

addition of sodium nitrosopentacyanoferrate, the first simple iron compound to be reported with an epr g value characteristic of enzyme non-heme iron (Beinert *et al.*, 1965). The nitrosopentacyanoferrate ion undergoes a one-electron-reduction step giving rise to a paramagnetic species which shows a three line epr spectrum centered at $g = 2.026$ (it is diamagnetic in the oxidized form). The reduced form also possesses an absorption maximum at about 600 nm, so appreciable absorption for this species is seen at 570 nm. Upon mixing a solution of NADH with one containing FMN and nitrosopentacyanoferrate ion in the epr spectrometer, one observes the production of the one-electron-reduction product of the iron. The epr spectrum is shown in Figure 1. The kinetics are seen in Figure 2. Until nearly all of the iron is reduced (see Figure 2), essentially no change was noted at 445 nm and only a relatively small change at 900 nm. At very high concentrations of NADH and FMN, a small flavin free-radical signal is observed (about 15% of the value found in the absence of iron). The initial rate at 900 nm is 0.23 its value in the absence of the iron compound and the initial rate for the formation of flavin free radical, as measured by epr, is 0.19 its value in the absence of the iron compound. Changes were noted throughout at 340 nm, corresponding to an oxidation of NADH. Iron reduction occurs only in the presence of all three components, as is shown in Figure 3. With limiting concentrations of iron, reduction of the iron would reach a plateau due to depletion in a few minutes. When this was then correlated with the amount of NADH oxidized, as measured by changes at 340 nm, a ratio of NADH:Fe of 1:2 was obtained.

Kinetic reactions order was determined for this system. Figure 4 shows the initial rate of iron reduction to be zero order with respect to the iron compound and first order with respect to FMN and NADH, whether determined optically or by epr. As essentially no flavin reduction is occurring, as measured at 445 and 900 nm for the appearance of a reduced species, changes at 570 nm reflect the reduction of the iron compound, not flavin free-radical formation.

Discussion

Complexing Agents. The changes which these compounds produced on the binary system might have significance for flavin binding by functional groups in protein, for hormone action, or for intramolecular effects in FAD. The strongest known complexing agent to date for FMN is 2,3-naphthalenediol (Fleisch-

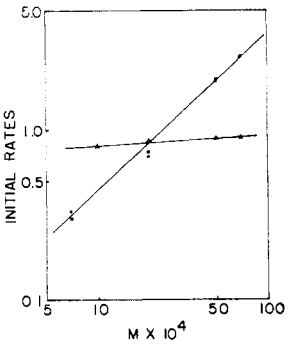

FIGURE 4: Epr reaction orders with respect to NADH, FMN, and $Na_2NOFe(CN)_5$. □ FMN, slope = 0.94; ○ NADH, slope = 0.94; △ $Na_2NOFe(CN)_5$, slope = 0.05.

man and Tollin, 1965). Corresponding decreases in all of the rates of reaction were noted upon the addition of this compound. This is expected if the initial step is rate limiting and if an inactivation of flavin occurs through complex formation. A correspondence between complex stability and reduction rate decreases is noted for the compounds examined.

At the pH used here, no effect of the presence of barbiturates upon reaction rates was noted, except for sodium barbital where the rate of appearance at 570 nm was singularly affected. Tyrosine did not greatly alter the rates of reaction. Tryptophan is seen to have an effect on the reaction rates which is similar to, but smaller than, that produced by naphthalenediol.

Addition of adenine analogs led to some enhancement of free-radical production in a somewhat analogous fashion to the effects observed in FAD (Fox and Tollin, 1966b). This is indubitably of biological significance. The effect in the tertiary systems was not as great as that observed for FAD itself. The structural limitations in FAD greatly increase the possibility of flavin–adenine interaction. It is interesting that little effect on the rate of reduction was observed. The active complexing state for flavin with metal ions has been shown to be the free radical. This appears to be the case for adenine analogs as well. The differences in rates observed with different adenine analogs may be partially due to solubility problems and also to electrostatic charge repulsions or attractions.

Acid proteinoid 2:2:1 was used as a low molecular weight protein analog to examine the effect of flavin binding on the reaction rates. The slight decrease at 900 nm is quite similar to the slight decrease observed at 445 nm when proteinoid is added. The large decrease in the rate at 570 nm is remarkable and can not be interpreted clearly on the basis of the experiments performed here. Under the conditions used here, one obtains the type of kinetic curve at 900 nm shown in Figure 5a of paper I (Fox and Tollin, 1966b). Thus, it is clear that the 900-nm species is being consumed in some reaction at a rate comparable to that for the NADH–FMN binary system. However, the 570-nm species is formed at rates far below those of the binary system, indicating an interaction between the flavin free radical and the proteinoid molecule.

Redox Components. The majority of compounds tested which were capable, in principle, of undergoing oxidation–reduction reactions with reduced flavin failed to have an observable effect on the binary system. An examination of the reported oxidation potentials in Clark (1960) gives −0.21 v for FMN at pH 7, −0.32 v for NADH at pH 7, and a variety of values for the sulfur-containing species ranging from −0.14 to −0.35 v at pH 7. The "sluggishness" of sulfur-containing compounds makes accurate determination of the oxidation potential very difficult. From such values, it is not really possible to tell *a priori* whether a particular reduction scheme can occur thermodynamically. However, the most commonly quoted value for disulfides was −0.32 v. An equilibrium calculation for the reduction of a disulfide bond using this value shows that only about 1% would be reduced by the reduced form of flavin. This is below the level that can be significantly detected in the present system. This apparently is the cause of the nonreactivity in the case of cysteine, cystine, oxidized glutathione, DL-lipoic acid, and thiamine hydrochloride in the present work. The reverse reaction, between dihydrolipoic acid and oxidized flavin, has been followed by Gasciogne and Radda (1965), and it was concluded that a hydride-transfer mechanism was operative, although the evidence was minimal.

The oxidation potentials for 1,4-benzoquinone and 1,4-naphthoquinone allowed direct reduction by NADH to occur, thus obviating examination of the flavin-catalyzed reaction. Menadione and menadione bisulfite failed to react at all. The question of quinone reductases is thus beyond the capability of this model system.

Non-Heme Iron Model System. The reoxidation of reduced flavin by a non-heme iron model, nitrosopentacyanoferrate ion, has been successfully investigated. The reaction was seen to be zero order with respect to iron concentration, indicating that the reoxidation step is considerably more rapid than the reduction step and that the concentration of reduced flavin species is always limiting. Two iron atoms were found to be reduced per NADH oxidized. The reaction orders with respect to FMN and NADH were both first order, implying that reduction primarily occurred from the fully reduced state, rather than from the free-radical state (in the latter case, one would expect to find a one-half-order dependence for NADH). The question is now raised whether reoxidation occurs first from the fully reduced state and then sequentially from the semiquinone state. It was not possible to detect two reoxidation steps under the current experimental conditions (the instrumental limitations are considerable), and so this question cannot be answered with certainty.

The flavin oxidation–reduction disproportionation reaction is known to lie far to the side of oxidized and fully reduced flavin. Thus, on these grounds alone,

it is likely that the oxidized iron compound can react to a greater extent with fully reduced flavin than with the semiquinone. As the iron reduction is probably very rapid, it can compete more successfully than the flavin reactions for $FMNH_2$ (such as charge-transfer complex and free-radical formation). Thus, the $FMNH_2$ level is kept low and no changes are observed at 900 nm and for flavin free radical until all of the iron is consumed. This type of mechanism is consistent with the observation of a parallel reduction in the initial rates of appearance of the 900 nm and epr species by the presence of the iron. The mechanism is probably initially a two-electron-reduction step, followed by a one-electron oxidation to free radical, and a disproportionation to oxidized and reduced flavin producing a cyclic oxidation from the flavin fully reduced state.

$$NADH + FMN + H^+ \dashrightarrow FMNH_2 + NAD^+$$

$$FMNH_2 + Fe(III) \dashrightarrow FMNH\cdot + Fe(II) + H^+$$

$$2FMNH\cdot \dashrightarrow FMNH_2 + FMN$$

This may not necessarily be the biological mechanism. For example, NADH dehydrogenases are known to consume two ferricyanide ions per NADH oxidized (Dixon and Webb, 1964). However, it is noteworthy that the microsomal NADPH–cytochrome *c* reductase has been claimed to function by shuttling between the fully reduced and semiquinone forms of FAD (Masters *et al.*, 1965) in analogy with the above mechanism.

Enzyme Mechanisms. The evidence acquired in the study of tertiary systems is consistent with the mechanism proposed for the NADH–FMN binary system (Fox and Tollin, 1966b). It can thus be suggested that flavoenzymes functioning as reduced pyridine nucleotide dehydrogenases are reduced through a direct hydrogen transfer concomitant with a two-electron transfer in the rate-limiting step. Then, either two one-electron- or one two-electron-reoxidation steps may ensue. Flavoenzymes functioning with two-electron acceptors appear to generally be metal free; their reoxidations are probably hydride transfers. The non-heme iron-containing flavoenzymes, in general, function with one-electron acceptors. The ratio of non-heme iron to flavin is often greater than 1:1 (see San Pietro, 1965). The operative mechanism is likely to be a single two-electron-reduction step followed by two rapid reoxidation steps by interaction with the non-heme iron, which in turn reduces two one-electron acceptors. This has been previously proposed in a somewhat similar fashion by Mahler and Glenn (1956). It would appear that the flavin free radical is at best a very transient species, if it is at all functional in flavoenzymes. Xanthine oxidase (Bray, 1963; Bray *et al.*, 1965) may well be an exception to this, but its diversity of prosthetic groups places it in its own category.

It is apparent that no ubiquitous mechanism can be invoked for the great variety of flavoenzymes that are found. Attempts to extrapolate results from one system to another are often unwarranted and appear to have complicated the whole picture. The results of model system studies such as this one may serve as a guide toward design of experiments for the corroboration or refutation of proposed flavodehydrogenase mechanisms. The relevancy of this study to other flavoenzyme mechanisms can not yet be assessed.

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Tetrahydrofolate Cofactors in Tissues Sensitive and Refractory to Amethopterin*

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ABSTRACT: Tetrahydrofolate cofactors in extracts of two tumors, liver, and intestinal mucosa were separated by column chromatography using DEAE-cellulose and were measured by microbial assays. These tissues were studied because of differences in their sensitivity to treatment with amethopterin and to dietary deficiency of folic acid. The distribution pattern of the tetrahydrofolate cofactors differed in each tissue. The ratio of 10-formyltetrahydrofolate:5-methyltetrahydrofolate was reversed in the two tumors (7:1 in the Murphy Sturm lymphosarcoma and 1:4 in the Walker carcinoma 256). Also, the Murphy Sturm lymphosarcoma, which is sensitive to amethopterin, was characterized by a higher content of total free folic acid activity than the Walker carcinoma 256 which is quite refractory to amethopterin. The major derivative found in liver was 5-methyltetrahydrofolate whereas the elution profile of intestinal mucosa showed almost equivalent amounts of 10-formyltetrahydrofolate and 5-methyltetrahydrofolate.

Derivatives of tetrahydrofolate function as cofactors essential for the biosynthesis of nucleic acids (Friedkin, 1963), yet little is known about their relative concentrations in normal and neoplastic tissues. Such compounds have been identified and assayed in several mammalian tissues (Donaldson and Keresztesy, 1959; Usdin, 1959; Herbert *et al.*, 1962; Silverman *et al.*, 1961; Noronha and Silverman, 1962) as well as in plants (Iwai and Nakagawa, 1959). Variation in the dependence of different tissues on the availability of folic acid cofactors is indicated by the leucopenia and desquamation of mucosal membranes in animals depleted of this vitamin (Delmonte and Jukes, 1962). Also, some selective impairment of different cells is recognized by the remission of acute lymphocytic leukemia in patients treated with amethopterin (Methotrexate) and by the erosion of mucosal surfaces in some subjects treated with this drug (Holland, 1961).

A difference in nutritional requirement for the growth of two tissues was shown by the failure of Walker carcinoma 256 to grow in rats only moderately depleted of folic acid (Rosen and Nichol, 1962), in contrast to the normal growth rate of Murphy Sturm lymphosarcoma even in severely depleted animals (Rosen *et al.*, 1964). It was of interest, therefore, to determine whether there was any difference between these experimental tumors with regard to their content of tetrahydrofolate derivatives. Also, the sensitivity of intestinal mucosa to amethopterin can be contrasted to the lack of any hepatotoxicity of this drug. Consequently, these four tissues were selected for the comparative study described herein. A preliminary report of this work has been presented (Sotobayashi *et al.*, 1965).

Materials and Methods

Reference Compounds. Folic acid and calcium leucovorin were purchased from the American Cyanamid Co., and tetrahydrofolic acid (H_4-folate) from General Biochemicals, Inc. 10-Formyl-H_4-folate was prepared by isomerization of 5-formyl-H_4-folate with dilute HCl at 0° and neutralization to pH 6.0 (May *et al.*, 1951; Cosulich *et al.*, 1952). 5-Methyl-H_4-folate was

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