Marked Metal Ion Effects in Electron Transfer from Reduced Flavin to Aromatic Nitro Compounds in Ethanol

Yumihiko Yano,* Terukiyo Sakaguchi, and Michiaki Nakazato
Department of Chemistry, Gunma University, Kiryu, Gunma 376, Japan

The reduction of *p*-chloronitrobenzene by a reduced flavin has been investigated kinetically in the presence of divalent metal ions in EtOH under anaerobic conditions. A large rate acceleration due to the metal ions was observed; 1 (none) : 32(Zn^{2+}) : 420(Ni^{2+}) : 1 000(Co^{2+}) : 12(Mn^{2+}). The role of the metal ions is proposed. The reducing ability of metal-chelated flavin radicals has been briefly examined by employing 2,4-dinitrochlorobenzene as an electron acceptor.

Some flavoproteins are known which contain a metal ion as a prosthetic group that participates in electron transfer reactions.[1] Thus, investigations of the interactions between flavins and metal ions are of primary importance in understanding the functions of metalloflavoproteins. For instance, McCreery *et al.* have shown on the basis of electrochemical studies that the oxidation–reduction potential of riboflavin is significantly affected by complexation with a variety of metal ions.[2] The stability constants for complexes between transition metal ions and riboflavin and FMN have been determined by potentiometric titration.[3] These studies suggest that metal ions interact with the isoalloxazine ring at the C=O(4) and N(5) positions. In fact, *X*-ray analysis of the bis-(10-methylisoalloxazine)–copper(II) complex has confirmed that Cu^{II} complexes with the C=O(4) and N(5) positions of the isoalloxazine nucleus.[4] Flavin radicals (flavosemiquinones) are known to be stabilized by metal co-ordination giving metal-chelated flavin radicals, which have been extensively investigated by means of u.v.–visible and e.s.r. spectroscopy.[5]

To the best of our knowledge, however, there have been no investigations of the interaction of metal ions with a *reduced flavin*. Furthermore, kinetic studies of the effects of metal ions on flavin-mediated oxidation–reduction seem to be very limited.†

In this paper, we describe kinetic investigations on the reactions of (i) *p*-chloronitrobenzene with a reduced flavin in the presence of metal ions (Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+}), and (ii) 2,4-dinitrochlorobenzene with metal-chelated flavin radicals in absolute ethanol under anaerobic conditions (Scheme 1).

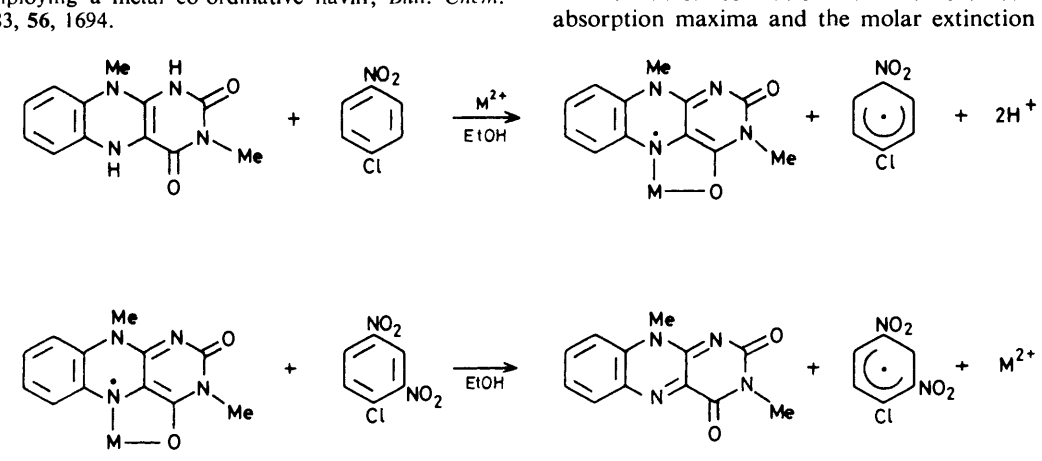
Aromatic nitro compounds were chosen as substrates because: (a) they are good electron scavengers;[6] (b) a nitro group is considered not to be activated by the co-ordination of a metal ion; (c) the reduction of a nitro group by a reduced flavin is of interest in connection with a flavin-containing nitrite reductase;[7] (d) aromatic nitro compounds are known to be reduced by 1,5-dihydroflavin in organic[8] and aqueous[9] solutions, although the effects of metal ions have not been examined.

Results and Discussion

Prior to rate measurements on the reactions in Scheme 1, it is necessary to know the spectroscopic properties and stability of the metal-chelated flavin radicals under the reaction conditions.

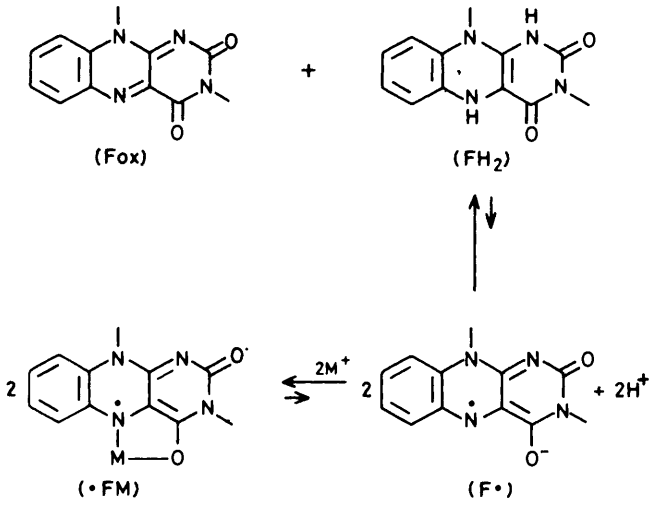
Formation and Stability of Metal-chelated Flavin Radicals.— It is known that oxidized (Fox) and reduced (FH_2) flavins afford a flavin radical (F·), the equilibrium being slightly in favour of the reactants under conventional conditions.[10] The presence of transition metal ions, however, stabilizes the flavin radical (F·) by chelation of the metal ions to afford metal-chelated flavin radicals (·FM) as shown in Scheme 2.

The radical (·FM) was prepared as follows: in a Thunberg cuvette, FH_2 (2.5×10^{-5}M) prepared by photoreduction in absolute EtOH containing *N*-ethylmorpholine (1×10^{-3}M) was mixed with Fox (2.5×10^{-5}M) containing various concentrations of metal ions under anaerobic conditions. The dependence of the amounts of the radicals (·FM) formed on the concentrations of the metal ions is shown in Figure 1. Figure 1 indicates that radical formation was complete with almost equimolar amounts of the flavin and the metal ions for Ni^{2+} and Co^{2+}, and with about a five molar excess of Zn^{2+} and Mn^{2+}. Radical formation was not observed for Mg^{2+}. The absorption maxima and the molar extinction coefficients of



Scheme 1.

† *Note added in proof:* S. Shinkai *et al.* have reported kinetic studies by employing a metal co-ordinative flavin; *Bull. Chem. Soc. Jpn.*, 1983, **56**, 1694.



Scheme 2.

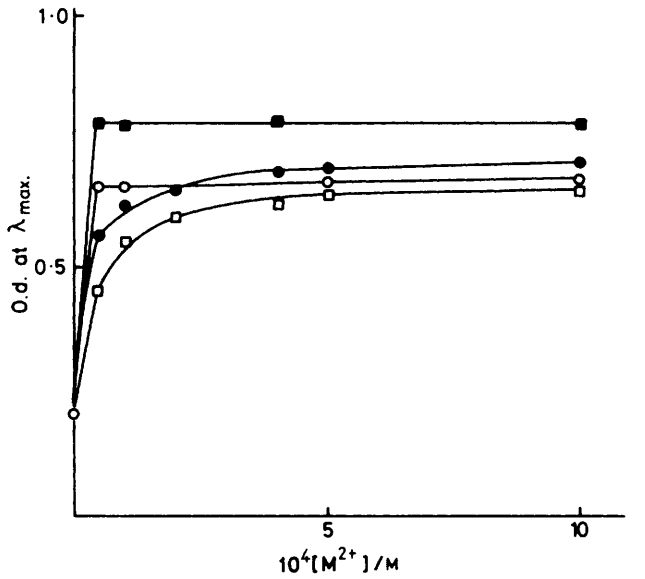


Figure 1. Relationship between metal ion concentrations and extent of metal-chelated flavin radical formation at [flavin] 5 × 10^{-5}M (25 °C): ■; Ni^{2+}, ○; Co^{2+}, ●; Zn^{2+}, □; Mn^{2+}

the metal-chelated flavin radicals were obtained for a ten molar excess of the metal ions over the flavin concentration (Table 1).

The metal-chelated flavin radicals were found to be decomposed gradually to the oxidized flavin by molecular oxygen dissolved in the ethanol. Degassing of the ethanol was found to be crucial to prevent the decomposition. When ethanol degassed by a freeze–thaw method was employed (see Experimental section), all the metal-chelated flavin radicals were quite stable: <10% decomposition occurred after 20 h under the conditions employed in the following experiments. This allows us to perform rate measurements by following the absorption maxima of the metal-chelated flavin radicals shown in Table 1.

Reduction of p-*Chloronitrobenzene by* 3,10-*Dimethyl*-1,5-*dihydroisoalloxazine*.—1,5-Dihydroflavin (FH_2) was prepared in the cell part of a Thunberg cuvette by photoreduction in

Table 1. The absorption maxima and the molar extinction coefficients of metal-chelated flavin radicals [a]

M^{2+}	$\lambda_{max.}$/nm (ε/dm³ mol⁻¹ cm⁻¹)
Ni^{2+}	371 (1.6 × 10^4), 480 (4.4 × 10^3)
Co^{2+}	365 (1.4 × 10^4), 460 (4.4 × 10^3)
Zn^{2+}	371 (1.5 × 10^4), 475 (4.2 × 10^3)
Mn^{2+}	374 (1.4 × 10^4), 460 (4.6 × 10^3)

[a] These values are determined at [flavin] 5 × 10^{-5}M and [M^{2+}] 5 × 10^{-4}M in EtOH (25 °C)

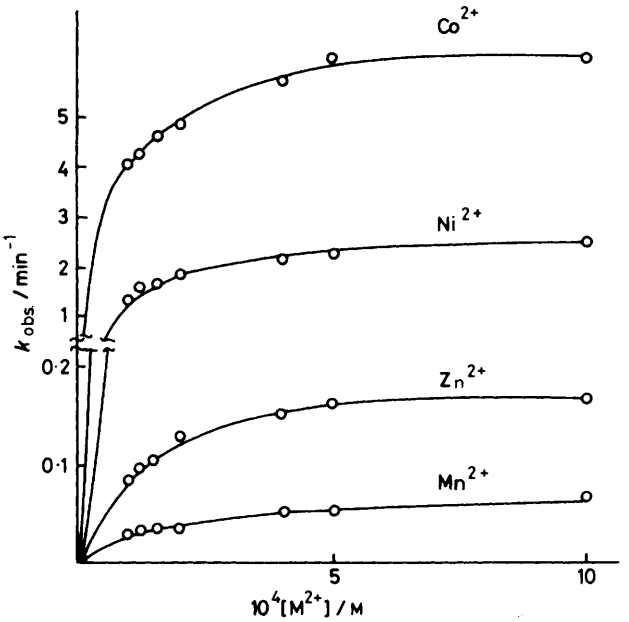


Figure 2. Plot of $k_{obs.}$ *vs.* metal ion concentrations in the reaction of FH_2 and *p*-chloronitrobenzene in EtOH at 25 °C: [FH_2] 5 × 10^{-5}M, [*p*-chloronitrobenzene] 1 × 10^{-3}M

absolute EtOH containing *N*-ethylmorpholine as a photoreductant under anaerobic conditions. The pseudo-first-order rate constants were determined by following the increase of the absorptions of the oxidized flavin (436 nm) in the absence of metal ions except for Mg^{2+}, and of metal-chelated flavin radicals (*ca.* 370 nm) in the presence of the metal ions. Both the reactions followed good first-order kinetics up to more than two half-lives.

(a) *The concentration effects of metal ions and* p-*chloronitrobenzene on the rates.* The rates were found to be markedly accelerated by the addition of metal ions, and to reach saturation as the concentrations of the metal ions increased (Figure 2). However, the rates increased linearly with increase of the concentration of *p*-chloronitrobenzene in the presence of Zn^{2+} (5 × 10^{-4}M) as shown in Figure 3. This linear relation indicates that the rate is first-order in concentration of the substrate, and Zn^{2+} does not activate the substrate as an electron acceptor. Therefore, the rate-accelerating effect of metal ions may be explained by the metal ion co-ordinating to the reduced flavin to form a more reactive metal–flavin complex which is probably a better electron donor than a metal-free reduced flavin.

(b) *The reaction scheme and the rate equation in the presence of metal ions.* The foregoing kinetic observations allows the mechanism in Scheme 3 to be proposed. Namely, the metal (M^{2+}) co-ordinates to the dihydroflavin ($F\bar{H}$) to form a reactive

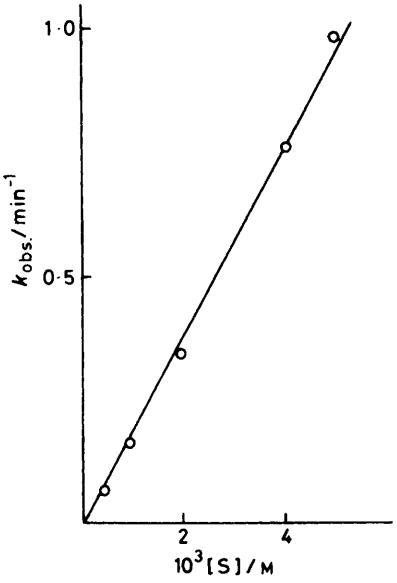
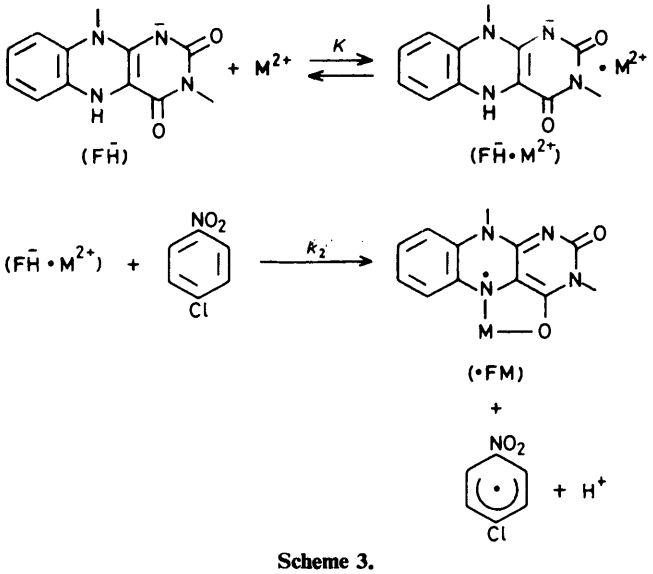


Figure 3. Effect of concentration of *p*-chloronitrobenzene (S) in the reaction with FH_2 in the presence of Zn^{2+} (5×10^{-4}M) in EtOH at 25 °C: $[FH_2]$ 5×10^{-5}M



Scheme 3.

species ($F\bar{H}\cdot M^{2+}$), and this is labile, releasing an electron to *p*-chloronitrobenzene (S) to yield a metal-chelated flavin radical (·FM) and *p*-chloronitrobenzene radical. 1,5-Dihydroflavin (FH_2) may exist in the proton-dissociated form ($F\bar{H}$) in the presence of a large excess of *N*-ethylmorpholine in solution.*

Based on Scheme 3, the observed rate constants ($k_{obs.}$) can be expressed by equation (1),[12] where $[FH_2]_0$, $[S]_0$, and $[M^{2+}]_0$ represent the initial concentrations of the reduced flavin, the substrate, and the metal ion, respectively. By assuming $[M^{2+}]_0 > [FH_2]_0\{1 - k_{obs.}/(k_2[S]_0)\}$, equation (1) can be simplified to equation (2). Rearrangement of equation (2) to the

* Hydrogen at N(1) of FH_2 must be dissociated under the present conditions, since the pK_a values of FH_2 and *N*-ethylmorpholine are 6.7 [10] and 7.7 respectively.[11]

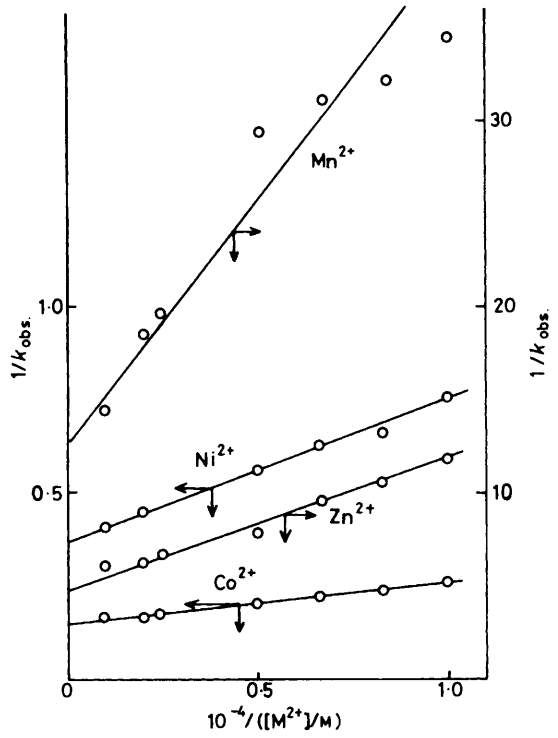


Figure 4. Reciprocal plot of $1/k_{obs.}$ *vs.* $1/[M^{2+}]$

Table 2. *K* and k_2 values [a]

M^{2+}	K/dm³ mol⁻¹	k_2/ dm³ mol⁻¹ min⁻¹	Rel. rate
None	—	6.5	1
Ni^{2+}	9.7×10^3	2.7×10^3	420
Co^{2+}	1.0×10^4	6.7×10^3	1 000
Zn^{2+}	6.8×10^3	2.1×10^2	32
Mn^{2+}	4.3×10^3	8.1×10	12
Mg^{2+}	—	6.0	0.9

[a] The concentrations of FH_2 and *p*-chloronitrobenzene are 5×10^{-5}M and 1×10^{-3}M in EtOH containing *N*-ethylmorpholine (1×10^{-3}M) at 25 °C under anaerobic conditions.

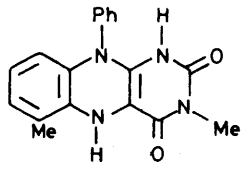
$$k_{obs.} = \frac{k_2K[S]_0[M^{2+}]_0}{1 + K[M^{2+}]_0 + K[FH_2]_0\{1 - k_{obs.}/(k_2[S]_0)\}} \quad (1)$$

$$k_{obs.} = \frac{k_2K[S]_0[M^{2+}]_0}{1 + K[M^{2+}]_0} \quad (2)$$

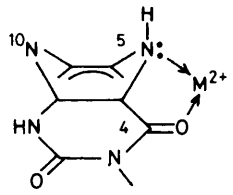
$$\frac{1}{k_{obs.}} = \frac{1}{k_2[S]_0} + \frac{1}{k_2K[S]_0[M^{2+}]_0} \quad (3)$$

reciprocal expression (3) gives a linear relation between $1/k_{obs.}$ and $1/[M^{2+}]_0$. From the slopes and the intercepts, *K* and k_2 can be calculated. The double reciprocal plots are shown in Figure 4. The values of *K* and k_2 thus obtained are listed in Table 2. Table 2 indicates that (a) rate acceleration due to metal ions is in the order of $Co^{2+} > Ni^{2+} > Zn^{2+} > Mn^{2+}$; Mg^{2+} does not influence the rate, and (b) that the metal ion exhibiting the larger rate enhancement has a larger binding constant (*K*) for the dihydroflavin.

Two questions arise: (i) what position of the 1,5-dihydroflavin is the binding site of the metal ions; (ii) why does



(1)



(2)

stronger binding of the metal ion to the dihydroflavin bring about a higher reactivity?

To obtain information on the binding site of the metal ions to 1,5-dihydroflavins, we have employed 3,6-dimethyl-10-phenyl-1,5-dihydroisoalloxazine (6-methyl-1,5-dihydroflavin) (1), since Corey–Pauling–Koltun (CPK) molecular models show that the metal ions are unable to co-ordinate to C=O(4) and N(5) owing to steric hindrance from the 6-methyl group. The kinetic results employing 6-methyl-1,5-dihydroflavin showed that metal ions (Ni^{2+} and Co^{2+}) had no effect on the rate of reduction of *p*-chloronitrobenzene, yielding not the metal-chelated flavin radical, but the oxidized flavin. It was also found that a mixture of equimolar amounts of oxidized and reduced 6-methylflavins does not produce the metal-chelated flavin radical in the presence of Ni^{2+} and Co^{2+}. This may be best accounted for by steric hindrance of the 6-methyl group around the N(5) position. This suggests the metal-co-ordination mode in structure (2) for reduced flavins. Such a metal-co-ordination to C=O(4) and N(5) requires that the lone electron pair is equatorial.

The absence of a metal ion effect in the reduction of *p*-chloronitrobenzene by 6-methyl-1,5-dihydroflavin also indicates that metal ions do not activate the substrate as electron acceptors. Therefore, the question as to why such a metal-co-ordinated dihydroflavin is more reactive than a metal-free dihydroflavin requires an answer.

Oxidized flavins are planar molecules, and reduced flavins are known to be bent, like a butterfly.[10] The conformation of a metal-free flavin radicals has not been established in model systems. In an enzymatic system, however, the conformation of an air-stable flavodoxin semiquinone has been proposed to be planar rather than bent on the basis of *X*-ray analysis.[13] Further it is known that in aminium cation radicals there is considerable flattening at nitrogen.[14] Thus, we assume, based on these facts, that metal-chelated flavin radicals are close to being planar. If so, a metal-co-ordination to C=O(4) and N(5) of 1,5-dihydroflavin may cause destabilization due to enforced flattening. This may make release of an electron from the slightly strained metal-co-ordinated dihydroflavin easy, giving a strain-free metal-chelated flavin radical. This is our proposal to interpret the rate enhancement due to metal ions. In other words, stronger metal-co-ordination to 1,5-dihydroflavin at the C=O(4) and N(5) positions brings about more destabilization due to the strain which may be alleviated by electron release.

Reducing Activity of Metal-chelated Flavin Radicals.—The redox property of the metal-chelated flavin radical (·FM) was examined briefly by employing a more reactive aromatic nitro compound, 2,4-dinitrochlorobenzene, under the same conditions as for the FH_2 reduction of *p*-chloronitrobenzene, since ·FM reduction of *p*-chloronitrobenzene was found to be too slow for accurate rates to be obtained.

The pseudo-first-order rate constants were determined by following the decrease in the absorptions of the radicals ·FM shown in Table 1 in absolute ethanol under anaerobic conditions. The rate data are presented in Table 3. Table 3 shows

Table 3. Rate constants for the reaction of metal-chelated flavin radicals (·FM) with 2,4-dinitrochlorobenzene at 25 °C [a]

M^{2+} in (·FM)	$k_{obs.}$/min^{-1}	Rel. rate
Ni^{2+}	3.5×10^{-3}	1
Co^{2+}	6.5×10^{-3}	1.8
Zn^{2+}	0.29	83
Mn^{2+}	0.74	210

[a] The concentrations of FM and 2,4-dinitrochlorobenzene are 5×10^{-5}M and 1×10^{-3}M in EtOH containing *N*-ethylmorpholine (1×10^{-3}M).

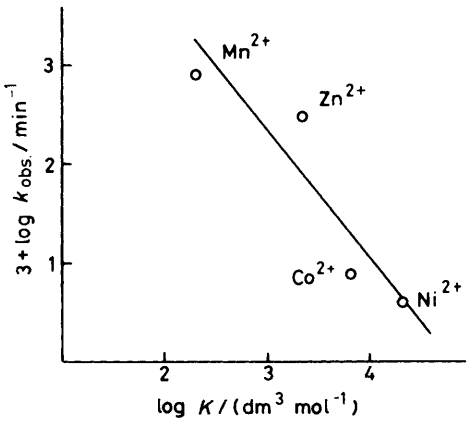


Figure 5. Relationship between log $k_{obs.}$ (2,4-dinitrochlorobenzene reduction by ·FM) and the stability constants of M^{2+}–4-hydroxypteridine complexes

that the reducing reactivity of ·FM is highly dependent on the metal ion. Namely, the reactivity order $Mn^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+}$ appears to be related to the stability constants of the metal ions for the metal-free flavin radical. This was examined by using the stability constants of the metal ions for an analogous ligand, 4-hydroxypteridine,[15] since the stability constants for the radicals are not available. A good relation was obtained (Figure 5). Thus, we presume that the reducing activity of ·FM is higher in the case of weaker binding of the metal ion to the flavin radical (F·).

Conclusion.—It has been shown for the first time that electron transfer from 1,5-dihydroflavin to an aromatic nitro compound is markedly facilitated by metal ions such as Ni^{2+} and Co^{2+}. The metal ion is proposed to act by flattening the bent 1,5-dihydroflavin molecule by co-ordination at the C=O(4) and N(5) positions. It has also been demonstrated that the reducing activity of metal-chelated flavin radicals is dependent on the metal ion.

Experimental

Materials.—3,10-Dimethylisoalloxazine was prepared according to the literature procedure,[16] m.p. 330 °C (from EtOH) (lit.,[16] m.p. 334 °C). 3,6-*Dimethyl*-10-*phenylisoalloxazine* was prepared from 3-methyl-10-anilinouracil and *o*-methylnitrosobenzene in AcOH,[16] m.p. >330 °C (from dimethylformamide, DMF) (Found: C, 67.8; H, 4.6; N, 18.0. $C_{18}H_{14}N_4O_2$ requires C, 67.9; H, 4.4; N, 17.6%). *p*-Chloronitrobenzene and 2,4-dinitrochlorobenzene were purified by recrystallization from EtOH. Commercial $Ni(NO_3)_2 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$, $Mn(NO_3)_2 \cdot 6H_2O$, and $Mg(ClO_4)_2 \cdot nH_2O$ were used without further purification. *N*-

Ethylmorpholine was purified by distillation from KOH pellets under N_2. Absolute ethanol was obtained by distillation from CaO twice under N_2, degassed by several freeze–thaw cycles, and stored under N_2.

Rate Measurements.—In a Thunberg cuvette, 30 μl of flavin stock solution (5×10^{-3}M in DMF), 30 μl of *N*-ethylmorpholine (1×10^{-1}M in EtOH), and EtOH (2.9 ml) were placed in the cell part, and 30 μl of *p*-chloronitrobenzene (1×10^{-1}M in EtOH) and 0—60 μl of metal ions (5×10^{-2}M in EtOH) were placed in the upper part. Both the solutions were degassed by bubbling vanadous ion-scrubbed N_2 prehumidified with EtOH for 20 min. The flavin in the cell part was reduced by irradiation with a 60 W tungsten lamp in a glass, temperature-controlled water-bath (25 °C). After confirmation that the reduction was complete from the absorption spectra, the reaction was initiated by mixing. The pseudo-first-order rate constants were determined by following the increase in the absorptions of the metal-chelated flavin radicals (*ca.* 370 nm).

The kinetics of the reaction of the metal-chelated flavin radicals and 2,4-dinitrochlorobenzene were measured in a similar manner to the 1,5-dihydroflavin reduction. Namely, the radical (·FM) was prepared by photoreduction of oxidized flavin (5×10^{-5}M) in the presence of the metal ion (5×10^{-4}M) in the cell part of a Thunberg cuvette, recording the formation of ·FM at *ca.* 370 nm. The rate constants were determined by following the decrease in the absorption at *ca.* 370 nm.

Product Analysis.—Qualitative product analysis was carried out by employing nitrobenzene instead of *p*-chloronitrobenzene, since authentic samples of the expected products are commercially available. Namely, 3,10-dimethyl-1,5-dihydroisoalloxazine (1×10^{-3}M) in EtOH (20 ml) prepared by photoreduction in the presence of *N*-ethylmorpholine (1×10^{-2}M) in a Thunberg tube under anaerobic conditions, and a mixture of nitrobenzene (5×10^{-3}M) and $Co(NO_3)_2 \cdot 6H_2O$ (5×10^{-4}M) was stirred for 3 days in the dark. After evaporation of EtOH, the residue was analysed by t.l.c. (silica gel; $CHCl_3$). Azoxybenzene was detected in addition to starting materials. It should be noted that nitrobenzene anion radical is known to afford azoxybenzene in alcohol.[6]

Acknowledgement

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