

## Coenzyme Models. Part 43. New Metal-stabilised Radicals of a Flavin with a Fused Pyridine Moiety Which Serves also as a Metal Chelation Site in the Oxidised State.†

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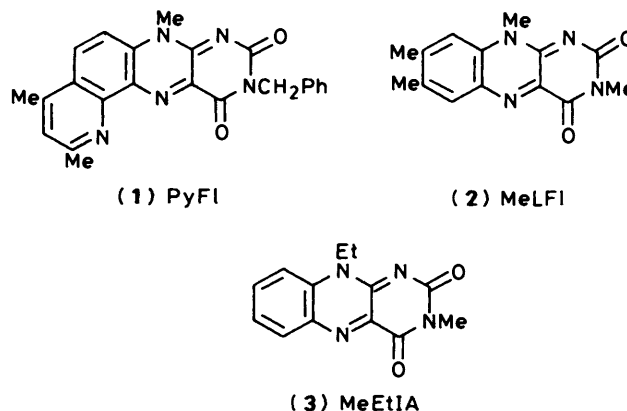
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A new flavin, 2,4,7-trimethyl-10-benzylquino[8,7-*g*]pteridine-9,11(7*H*,10*H*)-dione (PyFI) has been synthesised which has within the molecule a flavin as a catalytic site and a fused pyridine as an additional metal-chelation site. Unlike conventional oxidised flavins lacking affinity with most metal ions, PyFI could associate with a variety of metal ions in acetonitrile. PyFI forms 1:1 complexes with Zn<sup>II</sup>, Cd<sup>II</sup>, and Co<sup>II</sup> ions, the association constants being  $(1.4\text{--}4.4) \times 10^5 \text{ m}^{-1}$ . These are the largest association constants reported for oxidised flavin-*d*-metal complexes. The oxidation of 1-benzyl-1,4-dihydronicotinamide (BNAH) by PyFI in acetonitrile, which did not take place in the absence of metal ion, was efficiently catalysed by several metal ions: [Mg<sup>II</sup>, Zn<sup>II</sup>, Cd<sup>II</sup>, and Sb<sup>III</sup>]. Furthermore, the reaction with BNAH in the presence of Mg<sup>II</sup>, Zn<sup>II</sup>, and Cd<sup>II</sup> resulted in the formation of the flavosemiquinone which could be detected by absorption and e.s.r. spectroscopy. The e.s.r. spectral pattern is identical to that prepared by mixing equimolar amounts of oxidised and reduced PyFI. These results suggest that PyFI becomes even more electron-deficient than flavin owing to a PyFI → metal charge transfer while the semiquinone is stabilised through the interaction with these metal ions. These flavin-metal interactions serve as a model system possibly relevant to the action of metalloflavoenzymes. Such reactions cannot be provided by using conventional flavins.

Flavin coenzymes are versatile redox catalysts in many biological systems and serve as electron carriers, for example, to and from ion-sulphur proteins, molybdenum (xanthine oxidase), and haem proteins.<sup>1-3</sup> Thus, the interactions between metal ions and flavin coenzymes have drawn extensive interest of many flavin chemists. In spite of the potential importance in biological systems, the model investigations have been very limited.<sup>3-13</sup> The limitation stems mainly from the fact that oxidised and reduced flavin molecules generally lack affinity with metal ions. In fact, metal ions which are capable of binding to conventional flavin molecules are limited to several low-valent metal ions, the metal complexes being stabilised in most cases by a metal → flavin charge transfer.<sup>4-9,11</sup> We felt therefore that it would be useful to synthesise new flavins with metal affinity, the metal complexes of which are stabilised, for instance, by a flavin → metal charge transfer.<sup>14-17</sup> By this means, one can expect an increase in the redox potential leading to activation of the flavin as oxidising agent. In this paper, we report on the effect of metal ions on the reactivity of 2,4,7-trimethyl-10-benzylquino[8,7-*g*]pteridine-9,11(7*H*,10*H*)-dione (PyFI) (1) which has within the molecule a flavin as a catalytic site and a fused pyridine ring as an additional metal-chelation site. As expected, the oxidation power was enhanced through complexation with certain metal ions. Furthermore, we have found unexpectedly that the reaction of some metal complexes with an NADH analogue affords the spectroscopically-detectable semiquinone. For control experiments, we used either 3-methyl-lumiflavin (MeLFI) (2) or 3-methyl-10-ethylisalloxazine (MeEtIA) (3).

### Experimental

**Materials.**—PyFI was prepared by the benzylation of 2,4,7-trimethylquino[8,7-*g*]pteridine-9,11(7*H*,10*H*)-dione.<sup>15</sup> 2,4,7-



Trimethylquino[8,7-*g*]pteridine-9,11(7*H*,10*H*)-dione (0.14 g, 1.26 mmol) was treated with benzyl bromide (0.23 ml, 1.95 mmol) in 400 ml of *N,N*-dimethylformamide (DMF) containing powdered potassium carbonate (1.4 g) at room temperature for 3 h. The progress of the reaction was followed by t.l.c. [silica gel; ethanol-chloroform (1:4 v/v)]. The potassium carbonate was filtered off and the filtrate was concentrated under reduced pressure at room temperature and the residue diluted with water. The precipitate thus formed was purified by recrystallisation from ethanol; m.p. 274–276 °C (decomp.); yield 84.3%; single spot on t.l.c.;  $\nu_{\text{max}}$ (KBr) 1648 and 1702  $\text{cm}^{-1}$  (C=O) (Found: C, 63.4; H, 5.1; N, 16.1.  $\text{C}_{23}\text{H}_{19}\text{N}_5\text{O}_2 \cdot 2\text{H}_2\text{O}$  requires C, 63.73; H, 5.35; N, 16.17%).

MeLFI and MeEtIA were prepared according to the method of F. Yoneda *et al.*<sup>18</sup>

Preparation of 1-benzyl-1,4-dihydronicotinamide (BNAH) has been described previously.<sup>19</sup> 4,4-Dideuterio-BNAH (BNAD) was prepared by the repeated oxidation of BNAH with chloranil followed by reduction with  $\text{Na}_2\text{S}_2\text{O}_4$  in  $\text{D}_2\text{O}$ .<sup>20</sup> The isotope purity of BNAD determined by n.m.r. was 98.9%.

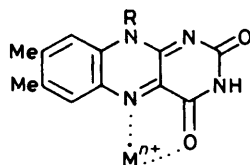
† Preliminary communication: S. Shinkai, N. Honda, Y. Ishikawa, F. Müller, and O. Manabe, *Chem. Lett.*, 1985, 543.

**Kinetic Measurements.**—Oxidation of 1-benzyl-1,4-dihydro-nicotinamide (BNAH) by flavins was carried out at 30 °C in anaerobic acetonitrile (unless otherwise stated). The progress of the reaction was followed spectrophotometrically by monitoring the disappearance of the absorption maxima of the flavins (444 nm for PyFl; 446 nm for MeLFl). The rate was apparently first-order in flavins and BNAH. 2,6-Dimethylpyridine was added (when stated) as a proton scavenger to suppress the acid-catalysed decomposition of BNAH. The h.p.l.c. analysis of the preparative solution established that BNAH is oxidised to 1-benzylnicotinamide quantitatively.

**Miscellaneous.**—As the association constants with metal ions are sensitively affected by a trace amount of water contained in acetonitrile, we maintained the water concentration to  $(1-2) \times 10^{-2} \text{ M}$  (monitored by a Karl-Fischer titration: Kyoto Electric Co. Ltd., Type MK-AII). Some e.s.r. spectra were measured on a JEOL-JES-FE spectrometer and others on a Bruker ER 200D spectrometer at 22 °C. The Bruker instrument was connected to a Data General NOVA 3 computer for storage and handling of the experimental spectra. The spectra were obtained under conditions where no saturation or line broadening occurred.

## Results and Discussion

**Influence of Added Metal Ions on Absorption Spectra of Flavins in Acetonitrile.**—Metal-containing flavoproteins participate widely in biological redox systems, e.g. in the mitochondrial apparatus of the animal cell. The importance of metal ions in flavin catalysis has stimulated model studies of flavin-metal interactions.<sup>4-11</sup> Early investigations on metal-flavin interactions have established, however, that oxidised flavins exhibit no significant affinity with most metal ions except for several low-valent metal ions in dilute aqueous solution: for example,  $\text{Ag}^{\text{I}}$ ,  $\text{Cu}^{\text{I}}$ ,  $\text{Mo}^{\text{V}}$ , and  $\text{Ru}^{\text{II}}$  can bind to conventional oxidised flavins and the complexes are stabilised probably by means of a metal  $\rightarrow$  flavin charge transfer.<sup>2-11</sup> Spectral studies suggested that these metal ions bind to O(4) or N(4), or both as shown below.<sup>2-6</sup> Crystallographic studies of  $\text{Ag}^{\text{I}}$  complexes later verified O(4)-N(5) co-ordination and in addition revealed a second chelating site, namely N(1)-O(2).<sup>21,22</sup> It is expected, however, that such a metal  $\rightarrow$  flavin charge transfer rather induces a negative shift of the redox potential of flavins<sup>6</sup> and decreases the oxidisability. In fact, Suelter and Metzler<sup>9</sup> reported that most metal ions have either no effect or a small inhibitory action on the flavin oxidation of NADH models, while  $\text{Ag}^{\text{I}}$  is a strong inhibitor. On the other hand, Hemmerich *et al.*<sup>1</sup> found spectrophotometrically that conventional flavins interact with redox-inactive  $\text{Mg}^{\text{II}}$  and  $\text{Zn}^{\text{II}}$  only in water-free acetone. However, the effect of the metal ions on the flavin oxidisability has not been studied in detail.



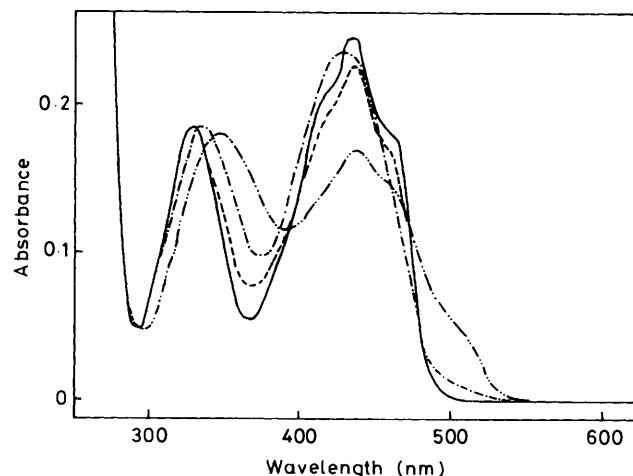
We felt therefore that it would be interesting to introduce an additional chelation site into the isoalloxazine skeleton in order to bind metal ions possibly much more strongly, which then could induce a positive shift of the redox potential by means of a flavin  $\rightarrow$  metal charge transfer. We thus designed PyFl which has a fused pyridine moiety as a metal chelation site.

The influence of added metal ions on the absorption maxima

**Table 1.** Influence of added metal ions on the absorption spectra of PyFl and MeEtIA in the oxidised state<sup>a</sup>

Metal (conc. mM)	$\lambda_{\text{max.}} (\epsilon_{\text{max.}})$	
	PyFl	MeEtIA
None	444 (10 700)	436 (9 660)
$\text{KClO}_4$ (0.40)	444 (10 800)	436 (9 690)
$\text{AgNO}_3$ (0.96)	455 (9 540)	
$\text{Mg}(\text{ClO}_4)_2$ (0.16)	449 (9 380)	436 (9 610)
$\text{Mg}(\text{ClO}_4)_2$ (90.5)		430 (9 400)
$\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.095)	471 (9 390)	
$\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ (2.90)		436 (9 290)
$\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ (29.0)		438 (6 700)
$\text{Cd}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.094)	468 (8 790)	
$\text{Cd}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ (9.15)		436 (9 040)
$\text{Co}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.10)	468 (8 690)	
$\text{Co}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ (14.3)		438 (9 690)
$\text{HgCl}_2$ (0.96)	461 (8 880)	
$\text{CuCl}_2$ (0.96)	468 (9 290)	
$\text{FeCl}_3$ (0.96)	442 (6 010)	
$\text{SbCl}_3$ (2.05)	440 (8 290)	
$\text{ZrCl}_4$ (0.13)	442 (9 290)	

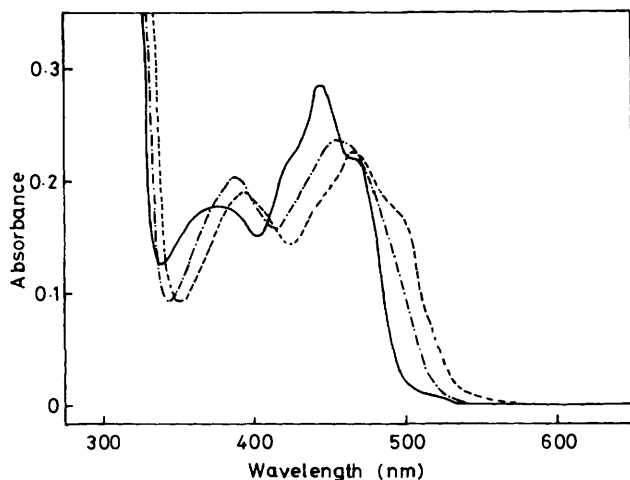
<sup>a</sup> 30 °C, acetonitrile,  $[\text{PyFl}] = 2.53 \times 10^{-5} \text{ M}$ ,  $[\text{MeEtIA}] = 2.51 \times 10^{-5} \text{ M}$ .



**Figure 1.** Absorption spectra of MeEtIA ( $2.51 \times 10^{-5} \text{ M}$ ) at 30 °C in acetonitrile: (—) none and  $\text{KClO}_4$  ( $4.35 \times 10^{-4} \text{ M}$ ); (---)  $\text{Mg}(\text{ClO}_4)_2$  ( $9.05 \times 10^{-2} \text{ M}$ ); (· · · · ·)  $\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$  ( $2.90 \times 10^{-2} \text{ M}$ ); (— — —)  $\text{Cd}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$  ( $9.15 \times 10^{-3} \text{ M}$ ).

(S1 band) of PyFl and MeEtIA in acetonitrile are summarised in Table 1. Perchlorate salts were used when they were accessible and typical spectra are illustrated in Figures 1 and 2. As expected, the absorption spectrum of MeEtIA (Figure 1) changed only when high metal ion concentrations ( $10^{-3}$ – $10^{-2} \text{ M}$ ) were added. An exceptional metal ion was  $\text{Zn}^{\text{II}}$ . The addition of  $\text{Zn}^{\text{II}}$  induced the red shift of the S2 band (329  $\rightarrow$  347 nm) and gave a new broad band at around 500 nm. The fact that  $\text{Zn}^{\text{II}}$  acts as a stronger complexing ion than the isoelectronic  $\text{Cd}^{\text{II}}$  in acetonitrile is in line with Hemmerich's spectral data in water-free acetone.<sup>1</sup> On the other hand, the absorption spectrum of PyFl (Figure 2) was sensitive to the presence of small amounts of metal ions ( $10^{-5}$ – $10^{-4} \text{ M}$ ), indicating the capability of a fused pyrido group as a stabilising chelation site.

Examination of the absorption maxima for PyFl (Table 1)

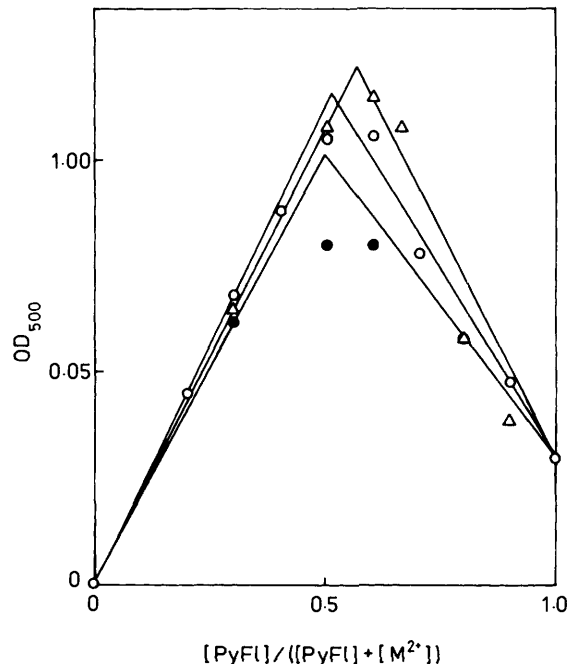
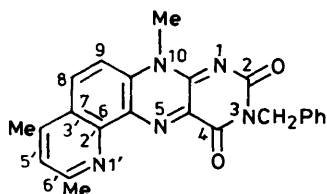


**Figure 2.** Absorption spectra of PyFl ( $2.53 \times 10^{-5} \text{M}$ ) at  $30^\circ \text{C}$  in acetonitrile: (—) none and  $\text{KClO}_4$  ( $4.35 \times 10^{-4} \text{M}$ ); (---)  $\text{Mg}(\text{ClO}_4)_2$  ( $3.21 \times 10^{-2} \text{M}$ ); (····)  $\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$  ( $9.50 \times 10^{-5} \text{M}$ ). The absorption spectra in the presence of  $\text{Cd}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$  and  $\text{Co}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$  were very similar to that in the presence of  $\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ .

reveals that divalent-metal ions induce a red shift of the S1 band, whereas high-valent ions such as  $\text{Fe}^{\text{III}}$ ,  $\text{Sb}^{\text{III}}$ , and  $\text{Zr}^{\text{IV}}$  induce a blue shift. According to theoretical calculations of the isoalloxazine skeleton,<sup>23</sup> the lowest singlet state (S1 band) corresponds to the polarisation along the long axis of the molecule. Presumably, divalent metal ions strongly bind to O(4) in addition to N(5)–N(1') co-ordination, because the interaction with O(4) should further enhance the polarisation along the long axis.\* On the other hand, high valent metal ions probably adopt N(5)–N(1') co-ordination which would offset the polarisation from the benzene moiety to the uracil moiety.

The interaction of PyFl with several metal ions was studied more quantitatively. In Figure 3, continuous variation plots for PyFl at  $[\text{PyFl}] + [\text{metal}] = 3.66 \times 10^{-5} \text{M}$  are illustrated, where metal salts are  $\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{Cd}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ , and  $\text{Co}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ . The absorption bands employed (500 nm) are those of the PyFl–metal complexes (*vide supra*). The maxima for  $\text{Zn}^{\text{II}}$  and  $\text{Co}^{\text{II}}$  appeared at  $[\text{PyFl}]/([\text{PyFl}] + [\text{metal}]) = 0.5$ , supporting that these metal ions form the 1:1 flavin–metal complexes. On the other hand, the maximum for  $\text{Cd}^{\text{II}}$  was little greater than 0.5. Thus, we further confirm the stoichiometry of the PyFl–metal complexes by a mole ratio method. Figure 4 shows the plots of  $\text{OD}_{500}$  vs.  $[\text{metal}]$  at the constant PyFl concentration. The break points at  $[\text{metal}]/[\text{PyFl}] = 1.0$  observed for all metals support again the formation of the 1:1 flavin–metal complexes.  $\text{Mg}(\text{ClO}_4)_2$  did not induce a spectral change large enough to allow to estimate the stoichiometry.

\* This compound may be named as an isoalloxazine derivative 4',6',10-trimethyl-3-benzyl-6,7-(2',3'-pyrido)isoalloxazine. For the sake of simplicity we use this nomenclature when we identify the atom in PyFl in the text.



**Figure 3.** Continuous variation plots of  $\text{OD}_{500}$  for the PyFl–metal complexes at  $30^\circ \text{C}$  in acetonitrile:  $[\text{PyFl}] + [\text{metal}] = 3.66 \times 10^{-5} \text{M}$ ; (○)  $\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ ; (△)  $\text{Cd}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ ; (●)  $\text{Co}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ .

$\text{KClO}_4$  showed no effect on the absorption spectrum of PyFl.

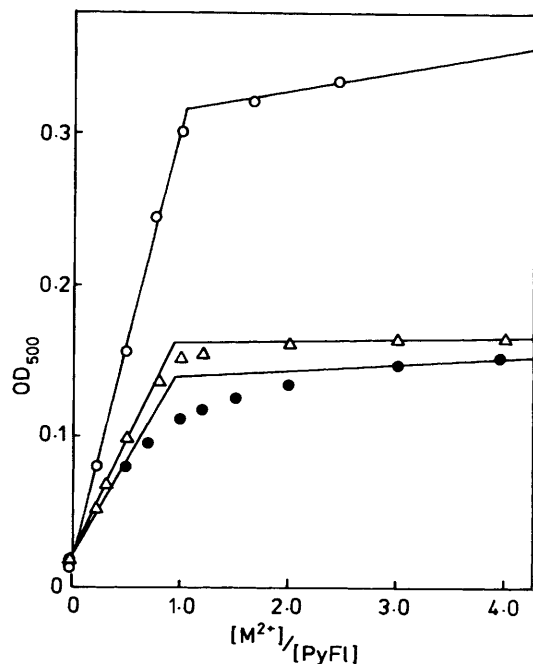
Subsequently, we determined the association constants ( $K$ ) for PyFl and MeEtIA. They can be evaluated by using Equation (1) which holds for the formation of a 1:1 complex and  $[\text{metal}] \gg [\text{flavin}]$ , where  $\text{OD}_0$  and  $\text{OD}$  are the absorbances of flavin in the absence and the presence of metal, respectively, and  $\epsilon$  is the apparent molar absorption coefficient of a flavin–metal complex. The plots of  $(\text{OD} - \text{OD}_0)/[\text{metal}]$  against  $\text{OD}$

$$\frac{\text{OD} - \text{OD}_0}{[\text{metal}]} = \epsilon K [\text{flavin}] - K \text{OD} \quad (1)$$

gave straight lines with  $r > 0.99$ . The  $K$  values were determined from the slopes ( $-K$ ) by the least-squares procedure. In case  $[\text{metal}] \gg [\text{flavin}]$  could not be achieved, we directly computed  $K$  at each complex concentration by using Equation (2) and recorded the average values (Table 2).

$$K = \frac{[\text{complex}]}{([\text{flavin}]_0 - [\text{complex}])([\text{metal}]_0 - [\text{complex}])} \quad (2)$$

Examination of Table 2 reveals that (a) the  $K$  values for conventional flavin MeEtIA lie between  $6.6$ – $60 \text{M}^{-1}$ , (b) PyFl shows the high affinity with transition metal ions, the  $K$  values being  $(1.4$ – $4.4) \times 10^5 \text{M}^{-1}$ , and (c)  $\text{Mg}^{\text{II}}$  gives a similar  $K$  value for PyFl and MeEtIA. The  $K$  values for PyFl and  $\text{Zn}(\text{II})$ ,  $\text{Cd}^{\text{II}}$ , and  $\text{Co}^{\text{II}}$  are greater by factors of  $(2.7$ – $67) \times 10^3$  than those for MeEtIA. The enhanced metal affinity is ascribed to the fused pyrido group introduced near the metal chelation site in the isoalloxazine skeleton [*i.e.*, near  $\text{C}(4)=\text{O}$  and  $\text{N}(5)$ ]. Although PyFl has a phenanthroline-like structure within the molecule, the  $K$  values are smaller than those of *o*-phenanthroline in aqueous solution [*e.g.*,  $\log K = 6.55$  for  $\text{Zn}^{\text{II}}$ ,  $5.93$  for  $\text{Cd}^{\text{II}}$ , and  $7.25$  for  $\text{Co}^{\text{II}}$ ].<sup>24,25</sup> The relatively small  $K$  values are attributed to the weak basicity of  $\text{N}(5)$  ( $\text{p}K_a < 0$ ).<sup>1,2</sup> To the best of our knowledge, however, these are



**Figure 4.** Plots of  $OD_{500}$  for the PyFl-metal complexes against metal concentrations at 30 °C in acetonitrile: [PyFl] =  $2.50 \times 10^{-5} M$  for  $Cd^{II}$  and  $Co^{II}$  and  $3.90 \times 10^{-5} M$  for  $Zn^{II}$ .

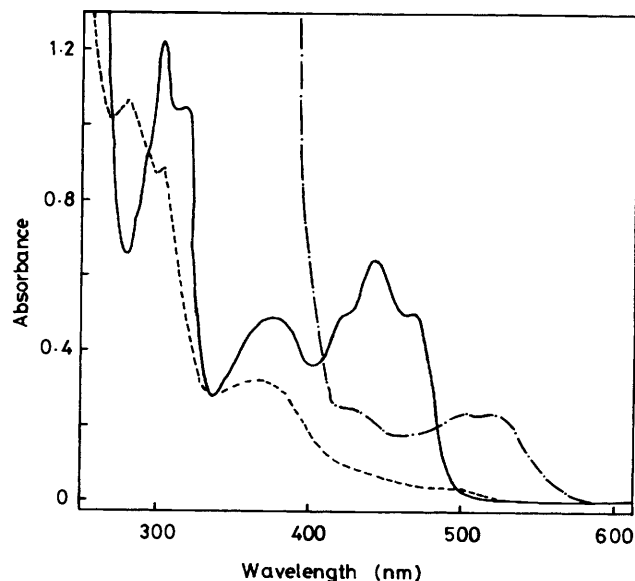
**Table 2.** Association constants ( $K$ ) of flavins with several metal cations in acetonitrile (30 °C)

Metal	$K(M^{-1})$	
	PyFl	MeEtIA
$KClO_4$	0	0
$Mg(ClO_4)_2$	37	16
$Zn(ClO_4)_2 \cdot 6H_2O$	$1.4 \times 10^5$	51
$Cd(ClO_4)_2 \cdot 6H_2O$	$4.4 \times 10^5$	60
$Co(ClO_4)_2 \cdot 6H_2O$	$2.4 \times 10^5$	6.6

the greatest  $K$  values observed for flavin-metal complexes in the oxidised state.<sup>1</sup>

**Effect of Metal Ions on Oxidation of an NADH Model Compound.**—The effect of metal complexation on the oxidation ability of flavins was estimated by the reaction with 1-benzyl-1,4-dihydronicotinamide (BNAH) in acetonitrile [Equation (3)]. The second-order rate constants for the oxidation of BNAH by PyFl and MeLFl in anaerobic acetonitrile are summarised in Table 3. It is known that this reaction easily takes place in water, but neither PyFl nor MeLFl could oxidise BNAH in acetonitrile. The oxidation was not facilitated at all by the addition of  $NaClO_4$  or  $Et_4NBr$ . In contrast, the oxidation by PyFl took place easily in the presence of several metal ions such as  $Mg^{II}$ ,  $Zn^{II}$ ,  $Cd^{II}$ , and  $Sb^{III}$ . It is worth mentioning that the metal effect is observed only for PyFl but not for MeLFl. The difference suggests that the marked metal effect observed for PyFl should be attributed to the PyFl-metal complexation.

It is known that some of these metal ions can associate with BNAH in acetonitrile with  $K = 10^2$ – $10^3 M^{-1}$ .<sup>26,27</sup> The  $K$  value for PyFl +  $Mg^{II}$  ( $37 M^{-1}$ ) is smaller than that for BNAH, so that one may consider that in the reaction system,  $Mg^{II}$  favourably associates with BNAH rather than with PyFl. On the other hand, the  $K$  values for PyFl +  $Zn^{II}$  and PyFl +

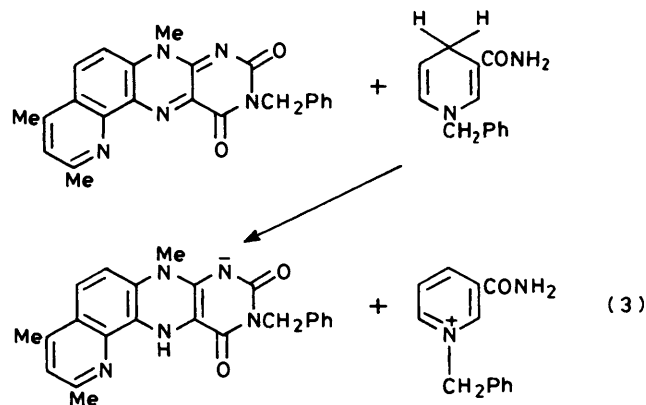


**Figure 5.** Absorption spectra of PyFl (—), fully-reduced PyFl (---), and the semiquinone produced by the reaction with BNAH ( $2.50 \times 10^{-3} M$ ) in the presence of  $Zn(NO_3)_2$  ( $2.19 \times 10^{-4} M$ ) (— · —) in MeCN at 30 °C. [PyFl] =  $5.00 \times 10^{-5} M$ .

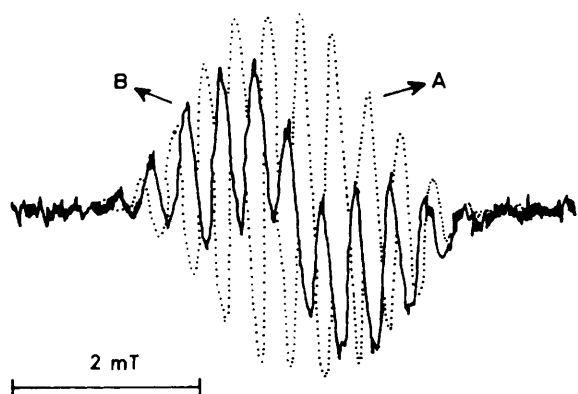
**Table 3.** Second-order rate constants ( $k_2$ ) for oxidation of BNAH by PyFl or MeLFl in acetonitrile at 30 °C<sup>a</sup>

Metal salt	$k_2(M^{-1} s^{-1})$	
	PyFl	MeLFl
None	0	0
$NaClO_4$	0	0
$Mg(ClO_4)_2$	0.486	ca. $4 \times 10^{-3}$
$Zn(NO_3)_2$	4.17	$< 10^{-3}$
$Cd(NO_3)_2$	1.27	ca. $9 \times 10^{-3}$
$SbCl_3^b$	2.86	0.01

<sup>a</sup>  $N_2$ , [flavin] =  $2.98 \times 10^{-5} M$ , [BNAH] =  $1.50 \times 10^{-3} M$ , [metal salt] =  $2.19 \times 10^{-4} M$ , 0.33 vol% DMF. <sup>b</sup> [2,6-dimethylpyridine] =  $1.40 \times 10^{-3} M$ .



$Cd^{II}$  ( $> 10^5 M^{-1}$ ) are much larger than that for BNAH. Therefore, these metal ions should predominantly associate with PyFl. As shown in Table 3, the large rate enhancement was observed for  $Zn^{II}$  and  $Cd^{II}$ . This suggests that the metal ion which is able to chelate with oxidised PyFl is more prone to mediate the hydrogen transfer from BNAH to flavins. As the



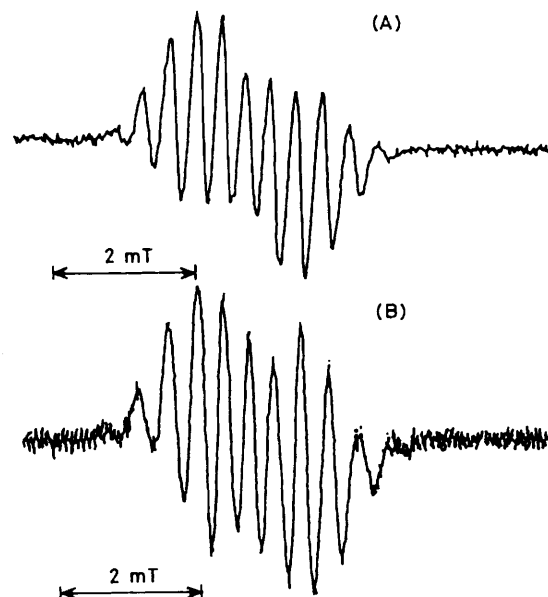
**Figure 6.** E.s.r. spectra of the semiquinones of 3,10-dimethylisoalloxazine (A) and PyFl (B) obtained in DMF by mixing equimolar amounts ( $5.00 \times 10^{-4}\text{M}$ ) of oxidised and reduced flavins in the presence of Bu<sup>o</sup>OK (4-fold).

logarithm of the rate constants between flavins and an NADH model compound is linearly correlated with polarographic half-wave potentials of flavins,<sup>28</sup> the present metal effect indicates that in the PyFl-metal complexes a flavin  $\rightarrow$  metal charge transfer takes place.

The final absorption spectrum obtained by the redox reaction in the presence of Sb<sup>III</sup> was similar to that of reduced PyFl. The authentic spectrum of reduced PyFl was prepared by photoreduction with EDTA or benzyl alcohol (Figure 5). Therefore, the reaction should proceed according to Equation (3). On the other hand, the reaction with BNAH in the presence of Mg<sup>II</sup>, Zn<sup>II</sup>, and Cd<sup>II</sup> produced a red colour. The colour is due to a new absorption band at around 500 nm. When O<sub>2</sub> was introduced into the red solution, oxidised PyFl was regenerated quantitatively. The new absorption spectrum, which is different from either PyFl or reduced PyFl, may be attributed to the flavin radical. Hence, we further investigated this red solution by e.s.r.

**E.s.r. Spectra of PyFl Semiquinone.**—In non-enzymatic systems, flavin semiquinone was prepared by mixing equimolar amounts of oxidised and reduced flavin. To the best of our knowledge, there is no non-enzymatic example that the flavin radical is produced by the reaction with NADH model compounds,\* because the disproportionation reaction is favoured (particularly in aqueous solution).<sup>1-3</sup>

First, we examined the e.s.r. spectrum of PyFl prepared by the conventional method. In mixing equal volumes of a stock solution of reduced and oxidised PyFl under strict anaerobic conditions the anionic semiquinone can be produced almost quantitatively (Figure 6B), employing the special glass equipment and procedure as described previously.<sup>30</sup> The hyperfine coupling constants of the anionic flavosemiquinone have been previously determined from experimental spectra.<sup>29</sup> These coupling constants have been refined using Fourier transformations of the experimental spectra in the simulation.<sup>30</sup> This yielded the following coupling constants (in mT) for the anionic semiquinone of 3,10-dimethylisoalloxazine:  $a(\text{N-5}) = 0.684$ ,  $a(\text{N-10}) = 0.317$ ,  $a(\text{Me-10}) = 0.373$ ,  $a(8\text{-H}) = 0.331$ ,  $a(6\text{-H}) = 0.275$ ,  $a(7\text{-H}) = 0.084$  and a natural line width of 0.66.<sup>31</sup> The experimental spectrum of 3,10-dimethyl-

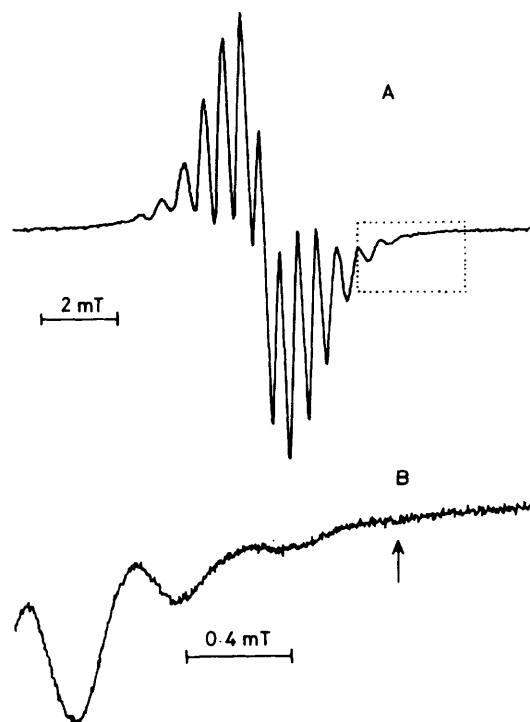


**Figure 7.** E.s.r. spectra of the semiquinone of PyFl obtained (A) by mixing equimolar amounts ( $5.00 \times 10^{-4}\text{M}$ ) of oxidised and reduced PyFl in DMF in the presence of Zn(ClO<sub>4</sub>)<sub>2</sub> (4-fold) and Bu<sup>o</sup>OK (4-fold) and (B) by the reaction of PyFl ( $2.99 \times 10^{-4}\text{M}$ ) and BNAH ( $1.50 \times 10^{-3}\text{M}$ ) in acetonitrile in the presence of Zn(NO<sub>3</sub>)<sub>2</sub> ( $2.20 \times 10^{-3}\text{M}$ ). The spectra were scarcely altered by change of counteranion.

isoalloxazine, which has also been simulated, is shown in Figure 6A. This spectrum is compared with that of PyFl in Figure 6B. It should be noted that the concentration of the radical of 3,10-dimethylisoalloxazine is about twice that of the radical of PyFl, owing to the lesser solubility of the latter compound in DMF in the reduced and radical state. Although the total width of the two spectra is nearly the same, the spectrum of PyFl shows one line less than that of 3,10-dimethylisoalloxazine, *i.e.* 11 and 12 lines respectively. From this comparison the two radicals are very similar. The absence of 6-H in PyFl, as compared to 3,10-dimethylisoalloxazine, causes the disappearance of one line in the spectrum, because the coupling constant of a(6-H) corresponds roughly with the apparent linewidth of about 0.25 mT. However, the apparent linewidth of PyFl is somewhat increased. This is most probably due to a small spin density distribution into the annellated pyridine ring in PyFl. In addition the nitrogen atom in the annellated ring could also carry a small spin density. Nevertheless, this interpretation reasonably explains the experimental spectrum of PyFl and is in agreement with published results on flavosemiquinones.<sup>30</sup> The above interpretation is in full agreement with the observation that deuterium substitution of 8-H or 6-H in 3,10-dimethylisoalloxazine yields a spectrum showing 11 lines, substitution of both protons yields a spectrum of 10 lines, and the apparent linewidth remains unaffected.<sup>31</sup>

In contrast to isoalloxazines, PyFl shows already in the oxidised state a considerable affinity for *d*-metal ions, owing to the additional co-ordination site in the molecule. It could therefore be expected that the semiquinone state of PyFl shows also a higher affinity for these metal ions than the corresponding species of isoalloxazines. Although the affinity constant of the metal-radical chelate has not been determined, we have observed that the chelate is somewhat more stable towards oxidation by dioxygen than the corresponding chelates of isoalloxazines supporting our above-stated notion. At any rate the transformation of the anionic radical PyFl into the Zn<sup>II</sup>-chelate (Figure 7A) leads only to a minor change of the overall

\* A trace amount of flavin radical is formed in every flavin oxidation in aqueous solution *via* a comproportionation step  $\text{Fl}_{\text{ox}} + \text{Fl}_{\text{red}} \rightleftharpoons 2\text{Fl}$ . However, radical yields higher than 60% have never been attained by the reaction with NADH model compounds.



**Figure 8.** E.s.r. spectrum of the semiquinone of PyFl in DMF obtained by mixing equimolar amounts ( $5.00 \times 10^{-4}$  M) of oxidised and reduced PyFl in the presence of  $\text{Cd}(\text{ClO}_4)_2$  (4-fold) and  $\text{Bu}^t\text{OK}$  (4-fold). Spectrum A was obtained by the computer accumulation of 10 spectra. Spectrum B represents the high field part of spectrum A, as indicated. In this case about 500 spectra were accumulated to observe the low intensity outermost lines.

shape of the e.s.r. spectrum, in analogy to the isoalloxazine radicals.<sup>32</sup> The only difference between the two sets of data is that the total width of the spectrum of the chelate of the radical of PyFl remains constant whereas that of the  $\text{Zn}^{\text{II}}$ -chelate of isoalloxazine increases by a few hundredths of a mT as compared to that of the corresponding anionic flavosemiquinone.

The  $\text{Cd}^{\text{II}}$ -chelate of the radical of PyFl shows more lines than the corresponding  $\text{Zn}^{\text{II}}$ -chelate in the e.s.r. spectrum (Figure 8). This has also been observed with isoalloxazines and is due to the content of  $^{113}\text{Cd}$  and  $^{111}\text{Cd}$  in natural cadmium.<sup>32</sup> The detailed analysis in the case of isoalloxazine<sup>32</sup> showed that the presence of these isotopes causes the number of lines in the spectrum of the chelate to increase by 6. Our experimental spectrum shows a total of 15 lines (Spectrum a), *i.e.* the two outermost lines are not observed clearly under the instrumental conditions the spectrum was obtained. Therefore, the spectrum was rerun for many times. The spectrum B demonstrates that the spectrum contains indeed 17 lines as expected from theory. The arrow in the spectrum B indicates the outermost line which just can be seen. These results are in full agreement with the published data on isoalloxazine and support, therefore, our interpretations and also strongly indicate that the spin distribution is not influenced much by the annellation of the pyridine ring to the isoalloxazine ring system *via* the 6 and 7 position.

Figure 6B shows the e.s.r. spectrum of the 'red' solution obtained by mixing PyFl and BNAH in the presence of  $\text{Zn}^{\text{II}}$ . This spectrum is essentially identical to the spectrum in Figure 6A which was obtained by mixing equimolar amounts of PyFl and reduced PyFl in the presence of  $\text{Zn}^{\text{II}}$ . Similarly, the e.s.r. spectrum obtained by mixing PyFl and BNAH in the presence

**Table 4.** Yields of PyFl semiquinone produced by the reaction with BNAH<sup>a</sup>

Metal salt	Concentration (mM)	$\frac{[\text{BNAH}]}{[\text{PyFl}]}$	Radical yield (%) <sup>b</sup>
$\text{Mg}(\text{ClO}_4)_2$	1.51	5	6.5
	7.18	24	12.0
$\text{Zn}(\text{NO}_3)_2$	1.50	5	34.4
	14.9	50	61.5
$\text{Cd}(\text{NO}_3)_2$	1.50	5	60.7

<sup>a</sup> Room temperature,  $\text{N}_2$ , MeCN,  $[\text{PyFl}] = 2.99 \times 10^{-4}$  M. <sup>b</sup> Yield =  $([\text{radical}]/[\text{PyFl}]) \times 100$ .

**Table 5.** Kinetic isotope effects for the reaction of PyFl with BNAH (or BNAD)

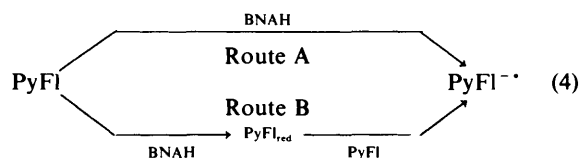
Metal salt	Solvent	$k_2$ for BNAH ( $\text{M}^{-1} \text{s}^{-1}$ )	$k_H/k_D$
None <sup>a</sup>	Water	82.3	$6.2 \pm 0.3$
$\text{Mg}(\text{ClO}_4)_2$ <sup>b</sup>	MeCN	0.486	$14.4 \pm 0.8$
$\text{Zn}(\text{NO}_3)_2$ <sup>b</sup>	MeCN	4.17	$2.7 \pm 0.2$
$\text{Cd}(\text{NO}_3)_2$ <sup>b</sup>	MeCN	1.27	$5.6 \pm 0.2$

<sup>a</sup>  $30^\circ\text{C}$ ,  $\text{O}_2$ ,  $[\text{PyFl}] = 5.92 \times 10^{-5}$  M,  $[\text{BNAH}$  (or  $\text{BNAD})] = 2.96 \times 10^{-5}$  M, pH 7.22 with 0.010 M phosphate. <sup>b</sup>  $30^\circ\text{C}$ ,  $\text{N}_2$ ,  $[\text{PyFl}] = 2.98 \times 10^{-5}$  M,  $[\text{BNAH}$  (or  $\text{BNAD})] = (1.5\text{--}9.3) \times 10^{-3}$  M.

of  $\text{Cd}^{\text{II}}$  is identical to the spectrum in Figure 8B. These findings clearly establish that the new absorption band in Figure 5, which is the origin of the red colour, is ascribed to the semiquinone of PyFl.

The spin concentrations produced by the reaction with BNAH were determined using DPPH as an external standard. Plots of the spin concentration *versus* the reaction time provided a maximum at around 4 h. Similarly, plots of  $\text{OD}_{500}$  *versus* the reaction time in Figure 5 provided a maximum at around 4 h. These complementary data suggest that the reaction with BNAH proceeds in a stepwise manner,  $\text{PyFl} \rightarrow \text{PyFl}^{\cdot-}$  radical  $\rightarrow$  reduced PyFl. In Table 4, the spin concentrations determined at each maximum value are collected. It is seen from Table 4 that the yield is in the order,  $\text{Cd}^{\text{II}} > \text{Zn}^{\text{II}} \gg \text{Mg}^{\text{II}}$ . This trend is exactly in line with the order of the association constants (Table 2). Hence, the result indicates that the metal ion which can strongly associate with oxidised PyFl also strongly stabilises the radicals. We can now estimate the extinction coefficients for the PyFl radicals corrected for the radical concentration: in Figure 5,  $\lambda_{\text{max}}$ , 503 nm ( $\epsilon_{\text{max}}$ ,  $12\,400 \text{ M}^{-1} \text{ cm}^{-1}$ ) and 524 nm ( $13\,500 \text{ M}^{-1} \text{ cm}^{-1}$ ). The extinction coefficients for the conventional flavosemiquinone-metal chelates are between  $(2\text{--}10) \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .<sup>33</sup> Therefore, the  $\epsilon_{\text{max}}$  values for the  $\text{Zn}^{\text{II}}$  chelate of the PyFl radical are somewhat enhanced probably because of the fused pyrido moiety.

**Comments on the Formation of the PyFl Radicals.**—There are two possible routes to reach the semiquinone  $\text{PyFl}^{\cdot-}$  [Equation (4)]: the direct production in which BNAH acts as a one-electron donor (Route A) and the two-step production in which PyFl is in one step converted by BNAH to fully-reduced PyFl ( $\text{PyFl}_{\text{red}}$ ) followed by comproportionation with oxidised PyFl (Route B). The two possible mechanisms can be discriminated



by the reaction of PyFl with 4,4-dideuterio-BNAH (*i.e.*, BNAD), because route B involving a net hydrogen transfer should be subject to the kinetic isotope effect while route A, which involves only the electron transfer, should not.<sup>34</sup> The reaction of PyFl with BNAH (or BNAD, 98.9% isotope purity) in the presence of three metal ions was followed by the appearance of the absorption band at 500 nm. The rate constants for BNAH agreed with those determined by following the disappearance of the absorption maxima of flavin (Table 3), showing several isosbestic points during the first 2–3 h of the reaction. The data were also taken in aqueous solution where fully-reduced PyFl directly resulted without the aid of metal ions. The kinetic isotope effects thus obtained are summarised in Table 5. The fact that the bimolecular reaction is much faster in aqueous solution than in acetonitrile demonstrates that the reaction is not favoured in acetonitrile because the developing negative charge on the flavin is not stabilised by the environment.

Examination of Table 5 reveals that all  $k_H/k_D$  values are significantly greater than unity. This implies that hydrogen transfer is involved (at least partially) in the rate-determining step. One may conclude, therefore, that the radical formation proceeds *via* route B. It is also seen from Table 5 that the  $k_H/k_D$  is inverseley correlated with the  $k_2$ : that is, the greater the  $k_H/k_D$ , the smaller the  $k_2$ . As the comproportionation reaction of reduced and oxidised PyFl to form the semiquinone (second step in route B) is usually fast,  $k_2$  should also reflect the 'hydrogen transfer' from BNAH to PyFl (first step in route B). Therefore it seems that the metal ion which facilitates the 'hydrogen transfer' also loosens the bond order of 4-H leading to a smaller isotope effect. Conceivably,  $Zn^{II}$  and  $Cd^{II}$  which strongly interact with PyFl can delocalise the anionic charge donated by BNAH in the transition state more effectively than  $Mg^{II}$  which only weakly interacts with PyFl.

### Conclusions

In the present paper, we demonstrate that PyFl in the oxidised state efficiently binds metal ions in acetonitrile and that the semiquinone is easily produced by the reaction with an NADH model compound in the presence of certain metal ions. Furthermore, the oxidisability can be markedly enhanced through metal complexation. These results consistently suggest that PyFl becomes electron-deficient owing to a PyFl  $\rightarrow$  metal charge transfer in the oxidised state, whereas the semiquinone state is rather stabilised through the interaction with the metal ions. These metalloflavoenzyme-like phenomena cannot be attained by using conventional flavins. The close imitation can be attained because PyFl has within the molecule a flavin as a catalytic site and a fused pyridine ring as an additional metal chelation site, which are the minimum requisites to constitute an enzyme model system. One may conclude, therefore, that PyFl serves as a well-constructed miniature of metalloflavoenzymes.

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