Coenzyme Models. Part 39. Synthesis and Properties of a Flavin with a Fused Phenolate Moiety which serves as a Metal Chelation Site†

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A new flavin, sodium 7,9,10,11-tetrahydro-1-hydroxy-7-methyl-9,11-dioxonaphtho[1,2-*g*]pter-idine-3-sulphonate ('sodium 1'-hydroxy-10-methylbenzo[2',3'-*h*]isoalloxazine-5'-sulphonate,' OHFl), which has a fused phenol within the molecular structure, has been synthesized. Measurement of absorption spectra revealed that in contrast to the non-affinity of conventional flavins for most heavy metal ions, OHFl can form complexes with several heavy metal ions in aqueous solution; in particular, Cu^{II} shows very strong affinity. It has been demonstrated through oxidation of 1-benzyl-3-carbamoyl-1,4-dihydroquinoline (BCQH), an acid-stable NADH model compound, that several metal ions (Cu^{II}, Hg^{II}, Mg^{II}, etc.) can 'activate' OHFl as an oxidising agent; Cu^{II} shows the highest co-catalytic effect. Spectroscopic examination established that OHFl forms 1 : 1 complexes with Cu^{II} and Ni^{II} with K (association constant) 26 300 and 806 l mol⁻¹, respectively. The OHFl–Cu^{II} complex in aqueous solution has been found to have square-bipyramidal co-ordination about copper from e.s.r. spectra. In methanol, many heavy metal ions could bind to OHFl and the rate constants for the oxidation of BCQH by OHFl·M^{n+} complexes (M^{n+} = Zn^{II}, Ni^{II}, and Cr^{III}) were enhanced by more than three orders of magnitude. These results suggest that the metal–OHFl complexes are not only useful as biomimetic oxidation catalysts but also may provide a clue to understanding metal–flavin interactions in metalloflavoproteins.

Flavin and $NAD(P)^+$ coenzymes are versatile redox 'catalysts' in many biological systems. In the past, model studies of flavin coenzymes have been of interest, but attention has been paid mainly to the reaction mechanisms.[1-3] It is known that, in contrast to the ability of flavin coenzymes to oxidise a variety of substrates *in vivo*, flavin model compounds *in vitro* exhibit a rather weak oxidation ability. However, little attention has been paid to model studies focussing on the 'activation of flavin models.' The most expeditious method to design activated flavins would be to introduce electron-withdrawing substituents into the flavin skeleton. Examples are cyano-,[4,5] pyridyl (or pyridinio)-,[6-8] and nitro-substituted[9] flavins. It seemed to us, however, that this method has little relevance to the flavo-enzyme systems in which flavin coenzymes are activated through noncovalent interactions with apoenzymes. Further-more, some of these electron-deficient flavins are readily decomposed by hydrolysis. On the other hand, it is known that several flavin-mediated reactions are efficiently catalysed by artificial 'apoenzymes' which can provide microenvironments similar to enzyme active sites,[10] and probably this method has more relevance to flavin activation *in vivo*.

We felt therefore that it would be useful to synthesize a new flavin with metal affinity. It is known that a number of flavoenzymes (*i.e.*, metalloflavoproteins) contain metal ions in the active sites and mediate electron transfers to and from iron–sulphur, molybdenum, and haem sites but there are few precedents for model studies of flavin–metal interactions.[11-13] The difficulty stems from the fact that flavin molecules generally lack affinity with metal ions. We have previously synthesized a flavin with a phenanthroline-like structure within the mole-cule.[14] The molecule showed significant affinity with several metal ions in organic solvents but not in aqueous solution. We here report the synthesis of a new flavin (OHFl) (sodium 7,9,10,11-tetrahydro-1-hydroxy-7-methyl-9,11-dioxonaphtho-[1,2-*g*]pteridine-3-sulphonate) with a fused phenol structure at the 6,7-position of the isoalloxazine moiety. We have found that OHFl forms complexes with several metal ions in aqueous


OHFl   MeLFl
(non-systematic numbering)
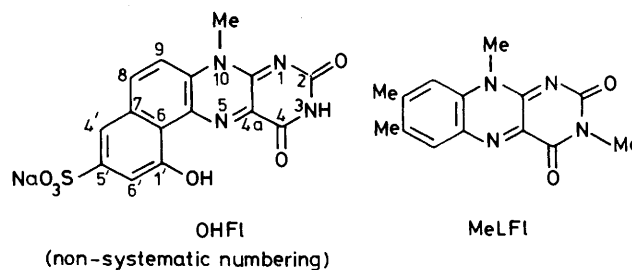
solution and acts as strong oxidation catalyst. For control experiments, we used 3-methyl-lumiflavin (MeLFl).

Experimental

Materials.—OHFl was synthesized from γ-acid (6-amino-4-hydroxynaphthalene-2-sulphonic acid) according to the re-action sequence in Scheme 1. Purifications of sulphonate derivatives were so difficult that we performed detailed analyses on compounds (2), (3), and OHFl. We analysed other intermediates qualitatively (mainly by t.l.c.) and used them directly for the next reaction.

Sodium 6-(p-Tolylsulphonylamino)-4-(p-tolylsulphonyloxy)-naphthalene-2-sulphonate (1).—γ-Acid (40.0 g, 0.167 mol) was treated with toluene-*p*-sulphonyl chloride (TsCl) (70.1 g, 0.367 mol) at 35—40 °C in aqueous solution in the presence of excess of NaOH. The pH of the reaction mixture was maintained at 11 by the addition of NaOH solution. The progress of the reaction was monitored by two different diazo-coupling methods with γ-acid: diazo-coupling with *p*-nitrobenzene diazonium tetra-fluoroborate to show the disappearance of the OH group and reaction with HNO_2-treated 4-hydroxynaphthalene-1-sulphon-ic acid to show the disappearance of the NH_2 group. We added 3.2 g of TsCl five times to complete the reaction. The solution was washed well with benzene and acidified using conc. HCl. The product (1) was a black oil. We dissolved the oil in aqueous alkali and used the solution for the next reaction without further purification.

† Part 38, S. Shinkai, H. Ena, T. Tsuno, and O. Manabe, *Bull. Chem. Soc. Jpn.*, 1984, **57**, 1435.
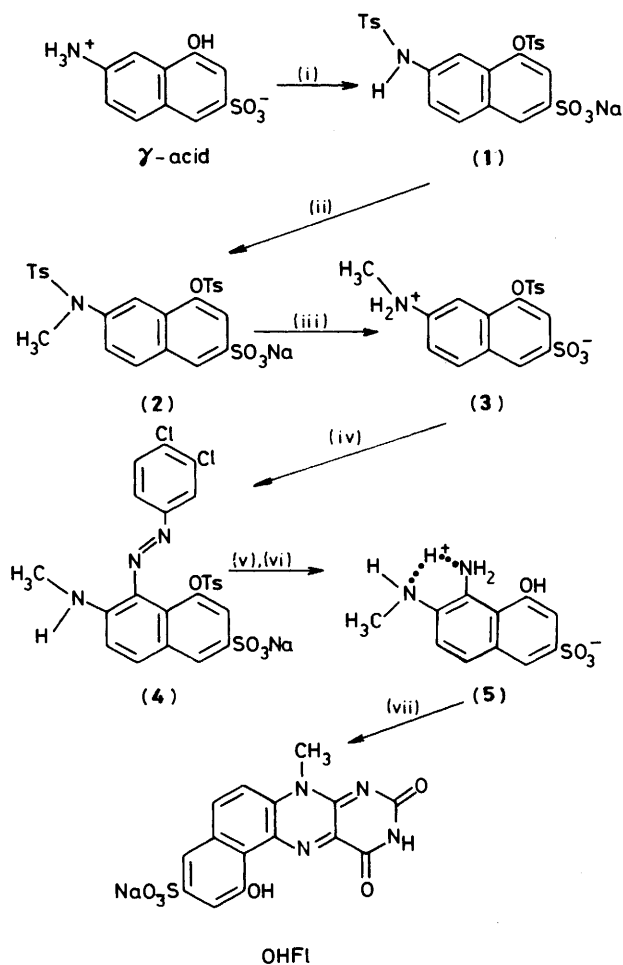
Scheme 1. *Reagents:* (i) TsCl, OH⁻; (ii) Me₂SO₄, OH⁻; (iii) 50% w/w H₂SO₄, heat; (iv) 3,4-dichlorobenzenediazonium chloride; (v) $S_2O_4^{2-}$, OH⁻; (vi) H⁺; (vii) alloxane

Sodium 6-[N-Methyl-N-(p-tolylsulphonyl)amino]-4-(p-tolyl-sulphonyloxy)naphthalene-2-sulphonate (2).—The salt (1) was converted into its methyl derivative (2) by methylation with Me₂SO₄ and NaOH. The method is essentially analogous to that described previously.[14] The product was recovered as a swollen cake. A small portion was purified by acid–base re-precipitation and characterised, m.p. 73—76 °C; δ_H[(CD₃)₂SO] 2.38 (3 H, CH₃ in Ts), 2.42 (3 H, CH₃ in Ts), 3.13 (3 H, NCH₃), and 7.3—8.3 (13 H, ArH); $\nu_{max.}$ (KBr) 1 165 and 1 350, (N–SO₂) and 1 182 and 1 365 cm⁻¹ (O–SO₂) (no ν_{OH}). The spectroscopic data support the assignment of structure (2). The results also indicate that γ-acid was ditosylated in the preparation of (1). Hence, we directly used the caked product in the next reaction.

6-Methylamino-4-(p-tolylsulphonyloxy)naphthalene-2-sulphonic Acid (3).—The salt (2) was refluxed for 1.5 h with 50 wt% H₂SO₄ (400 ml), and the resulting solution was poured into water (1.5 l), the brown precipitate being collected by filtration. The product was washed well successively with butan-1-ol and NN-dimethylformamide (DMF), and was dissolved in aqueous Na₂CO₃ at room temperature and, after treatment with active charcoal, was reprecipitated with conc. HCl. The acid (3) was obtained as white crystals, m.p. 268—271 °C; 42% yield from γ-acid (Found: C, 51.6; H, 4.3; N, 3.4. C₁₈H₁₇NO₆S₂ requires C, 53.06; H, 4.21; N, 3.44%); $\nu_{max.}$(KBr) 1 170 and 1 370 (SO₂) and 2 400—3 000 cm⁻¹ (NH₂⁺) (no ν_{OH}). The analytical data

indicate that a *p*-tolylsulphonyl group was eliminated from the amino group by the above treatment.

Sodium 5-(3,4-Dichlorophenylazo)-6-methylamino-4-p-tolyl-sulphonyloxynaphthalene-2-sulphonate (4).—Compound (3) (28.6 g, 0.0703 mol) was dissolved in an aqueous solution of Na₂CO₃ (8.2 g in 150 ml), and the solution was acidified by the slow addition of 35 wt% HCl (7.6 ml). To this solution was added 3,4-dichlorobenzenediazonium chloride prepared from 3,4-dichloroaniline (12.0 g, 0.0752 mol) and NaNO₂ (5.40 g) in 7 wt% HCl (177 ml). The solution was adjusted to pH 4.0—4.5 with sodium acetate and the reaction was allowed to continue below 10 °C for 24 h. Disappearance of the diazonium salt was monitored by diazo-coupling of an aliquot, withdrawn from the reaction mixture, with 1-hydroxynaphthalene-4-sulphonic acid. On completion of the reaction the precipitate was removed by filtration. The addition of NaOH to the filtrate afforded crystals of the azo compound (4), m.p. 201—202.3 °C; yield 91%; single spot on t.l.c. [silica-gel; butan-1-ol–ethanol–28% NH₃ (5:2:3 v/v)]; $\nu_{max.}$(KBr) 1 170 and 1 390 (SO₂) and 1 610 cm⁻¹ (N=N).

5-Amino-4-hydroxy-6-methylaminonaphthalene-2-sulphonic Acid (5).—The azo salt (4) (16.0 g, 26.5 mmol) was suspended in aqueous NaOH (11.3 g, 0.284 mol in 100 ml). To this solution was added aqueous sodium dithionite (18.5 g, 0.10 mol in 50 ml), and the reaction mixture was refluxed for 1 h. The red colour of the azo compound (4) disappeared gradually and 2,4-dichloroaniline was precipitated. The precipitate was removed by filtration. The fitrate was acidified (pH 1.5) with conc. HCl and the precipitate of the raw product (5) was collected by filtration. The product was dissolved once in an alkaline solution containing a small amount of sodium dithionite and treated with active charcoal. After filtration, the filtrate was acidified (pH 1.5) with conc. HCl. The acid (5) was recovered by filtration and dried *in vacuo*, m.p. >360 °C; yield 77%; single spot on t.l.c.; $\nu_{max.}$(KBr) 3 400—3 500 (OH) and 2 600—2 800 cm⁻¹ (NH₃⁺ or NH₂⁺) (no ν_{SO_2}). The absence of the ν_{SO_2} band in the i.r. spectrum indicates that the remaining *p*-tolylsulphonyl group was eliminated in this step.

OHFl.—Zwitterionic amino acid (5) was sparingly soluble in most solvents, except for DMF saturated with NaClO₄. The acid (5) (0.16 g, 0.596 mmol) was dissolved in NaClO₄-saturated DMF (20 ml) by sonication. Alloxane hydrate (0.12 g, 0.656 mmol) was also dissolved in NaClO₄-saturated DMF (2 ml) saturated with NaClO₄. Two solutions were mixed, heated at 70—75 °C for 2 h, and the solution was then poured into acetonitrile (250 ml), the precipitate being collected by filtration. The solid was dissolved in water (50 ml) and treated with active charcoal, and filtered to give red crystals (OHFl) on addition of NaCl to the filtrate, m.p. >360 °C; yield 42%; single spot on t.l.c. [silica gel; ethanol–water (1:3 v/v)]; $\nu_{max.}$(KBr) 3 447 (OH) and 1 714 and 1 663 cm⁻¹ (C=O); δ_H[(CD₃)₂SO] 4.07 (3 H, CH₃), 7.33 (1 H, 4'- or 6'-H), 7.79 (1 H, 6'- or 4'-H), 7.89 (1 H, 9-H), 8.38 (1 H, 8-H), 11.49 (1 H, NH or OH), and 12.69 (1 H, OH or NH). The two peaks at δ_H 11.49 and 12.69 disappeared on the addition of D₂O, thus they were assigned to NH and OH groups.

OHFl-Cuᴵᴵ *Complex.*—OHFl (50 mg, 8.49 × 10⁻⁵ mol) was mixed with CuCl₂·2H₂O (14.8 mg, 8.49 × 10⁻⁵ mol) in water (60 ml). The reaction mixture immediately turned black. Methanol (20 ml) was added but the complex did not precipitate. The solvent was removed gradually under reduced pressure, and the evaporation was stopped when the solution became slightly turbid. On being cooled in a refrigerator the mixture deposited black crystals. The precipitate was collected by filtration, washed with methanol, and dried *in vacuo*, m.p.

>320 °C; yield 54%; $\nu_{max.}$(KBr) 3 504 and 3 417 (OH) 1 676 and 1 657 cm^{-1} (C=O) (Found: C, 38.0; H, 3.05; N, 9.8. $C_{14}H_8N_4O_6S\cdot Cu\cdot(CH_3OH)_3\cdot H_2O$ requires C, 39.31; H, 4.03; N, 10.19%).

MeLFl was synthesized according to the method of Hemmerich.[15] Preparation of 1-benzyl-3-carbamoyl-1,4-dihydroquinoline was as described previously.[16]

Miscellaneous.—Kinetic measurements of the flavin oxidation of 1-benzyl-3-carbamoyl-1,4-dihydroquinoline were carried out at 30 ° C under aerobic conditions by following the disappearance of the absorption band of 1-benzyl-3-carbamoyl-1,4-dihydroquinoline (340 nm). The water concentration in methanol was determined by a coulometric Karl-Fischer apparatus (Kyoto Electric Co. Ltd., Type MK-AII). E.s.r. spectra were measured with a JEOL-JES-FE spectrometer and simulated by using a computer program made by Dr. M. Iwamoto (Nagasaki University).

Results and Discussion

Absorption Spectra of OHFl *and Reduced* OHFl (OHFlH$_2$).— Flavins usually have two characteristic absorption maxima in the u.v. (*ca.* 330 nm, S$_2$ peak) and visible regions (*ca.* 440 nm, S$_1$ peak) and give a yellow colour in aqueous solution. We measured the absorption spectrum of OHFl in water at 30 °C (Figure 1). The aqueous solution of OHFl was red. The
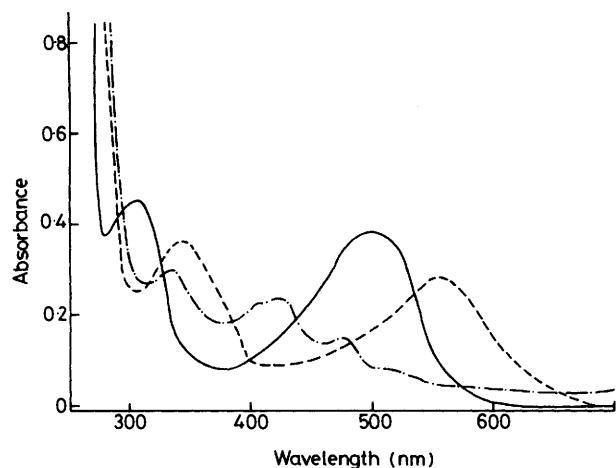

Figure 1. Absorption spectra of OHFl and reduced OHFl in aqueous solutions (30 °C): [Flavin] 2.50 × 10^{-5}M. —— OHFl at pH 6.3 (0.01M phosphate buffer), – – – – OHFl at pH 12.8 (0.01M KOH), – • – • – OHFl reduced by butane-1,4-dithiol (2.50 × 10^{-3}M) at pH 9.28 (0.01M carbonate buffer)

absorption spectrum, which featured a shift of the S$_1$ band to longer wavelength (502 nm, ε 14 900) and the S$_2$ band to shorter wavelength (307 nm, ε 17 300), was rather similar to that of 6-substituted flavins.[17] The absorption spectrum was pH-dependent because of dissociation of the 1'-OH group, and new absorption maxima appeared at 580 nm and 340 nm at high pH. The spectra had several tight isosbestic points. From the absorbance at 580 nm the pK_a of the 1'-OH group was determined to be 10.7.

Generally flavins can be photoreduced by ethylenediamine tetra-acetic acid (EDTA). We found, however, that OHFl (2.50 × 10^{-5}M) is not reduced by visible light even in the presence of EDTA·2Na (2.50 × 10^{-4}M). Probably, this is due to electrostatic repulsion occurring at the excited state between anionic OHFl and EDTA·2Na. Instead, we obtained the

absorption spectrum of reduced OHFl by anaerobic reduction with butane-1,4-dithiol (2.50 × 10^{-3}M): $\lambda_{max.}$ 337 and 410—420br nm. Introduction of molecular oxygen instantaneously regenerated red OHFl.

The absorption peak of the S$_1$ band similarly appeared at around 500 nm in acetonitrile (507 nm) and DMF (511 nm). On the other hand, the absorption spectrum in methanol was markedly different: the solution was blue in very carefully dehydrated methanol ([H$_2$O] = 0.01—0.03M) but became red in wet methanol. The blue colour is due to a new absorption band at 650 nm (Figure 2). The new absorption band disappeared on the addition of trace amounts of water or acetic acid. This peculiar phenomenon is rationalised either by an equilibrium between two isomers or by a solvent-dependent aggregation. As shown in Figure 3, the absorbance at 500 nm in water correlated linearly with the OHFl concentration, whereas the absorbances at 520 and 630 nm in methanol did not show straight lines. The deviation from Beer's law suggests that the peculiar colour change in methanol is probably caused by some aggregation phenomenon. It is known that flavin molecules are prone to stacking aggregations,[18,19] but the origin of the blue colour in OHFl is not clear yet.

Effects of Metal Ions on the Absorption Spectra of OHFl *in Aqueous Systems.*—We measured the absorption spectra of OHFl and MeLFl in aqueous solution at 30 °C. Early spectral studies of model flavin–metal interactions have established that oxidised flavins in aqueous solution exhibit no significant affinity for most metal ions (except a few, *e.g.*, Ag^I and Ru^{III}: they bind to oxidised flavins probably by means of a metal–flavin charge-transfer stabilisation[11–14]). Spectral and X-ray crystallographic studies showed that the metal binds primarily to O(4) or N(5), or both, as shown below, structure (6).[13,20,21]

We found that the absorption spectra of OHFl are affected by several metal ions, giving rise to a new absorption maximum at around 600 nm (Figure 4 and Table 1). These metal ions were Cu^{II}, Ni^{II}, Fe^{II}, and Co^{II}:Cu^{II} induced the strongest spectral change. At a ratio of [Cu(NO$_3$)$_2$]/[OHFl] = 15, the absorption maximum at 502 nm disappeared almost completely, while both peaks were still observable in the presence of equal concentrations of other metal ions. The band almost disappeared at [M^{n+}]/[OHFl] = 150. Zn^{II}, Mg^{II}, Ag^I, Fe^{III}, and Zr^{IV} also induced the spectral shift, but the change was not large enough to produce the new absorption maximum. We also tested other metal ions (Hg^{II}, Cr^{III}, Mn^{II}, Ca^{II}, Sr^{II}, Ca^{II}, Cd^{II}, and Ru^{III}) and found little spectral change. The absorption spectra of MeLFl, taken as a reference, did not change at all in the presence of the same concentration of these metal ions. The results imply that 1'-OH plays a decisive role in binding these metal ions.

The spectral pattern in the presence of Cu^{II} and Ni^{II} is very similar to that of OHFl at high pH (*i.e.*, $^-$OFl). This means that the added metal ions facilitate the dissociation of the 1'-OH group. The absorption maxima (605—644 nm) appeared at wavelengths longer than that of $^-$OFl (580 nm). The red shift of the S$_1$ band observed for conventional flavin–metal complexes is usually attributed to the interaction of metals with O(4).[11–14] Therefore, the red shift of the OHFl–metal complexes relative to $^-$OFl may be also accommodated by a possible interaction with O(4) in OHFl. These results suggest that the most reasonable structure of the OHFl–Cu^{II} complex is (7), which is not incompatible with the fact that Cu^{II} prefers planar co-ordination. This problem is discussed later in more detail on the basis of e.s.r. spectral data.

We studied further the interaction with Cu^{II}, as this gave the most conspicuous spectral change. In Figure 5, continuous variation plots at [OHFl] + [Cu^{II}] = 6.00 × 10^{-5}M are illustrated. The absorption bands employed (610 and 630 nm) were
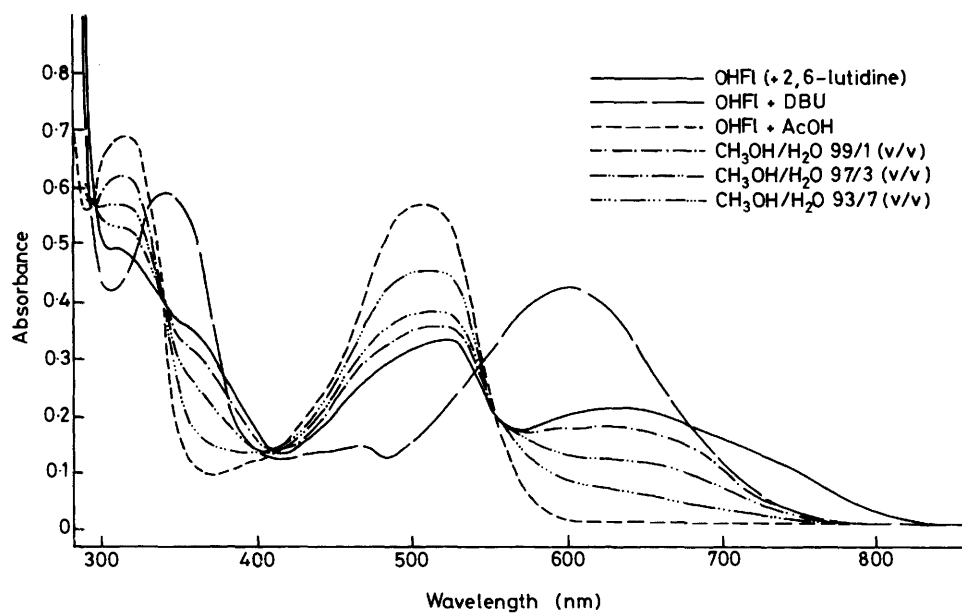
Figure 2. Effect of the water concentration on the absorption spectra of OHFl in methanol (30 °C): [OHFl] 4.00×10^{-5}M, 1.6 vol % DMF. ——— OHFl in very carefully dehydrated methanol [the addition of 2,6-lutidine (2.00×10^{-3}M) did not change the spectrum], ————— OFl (λ_{max} 605 nm) produced by the addition of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (2.00×10^{-3}M), ———— OHFl plus acetic acid (2.00×10^{-3}M)
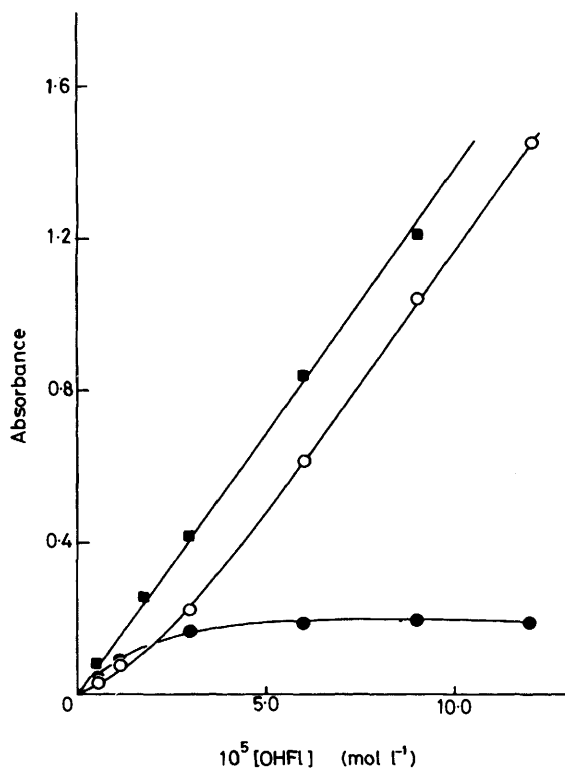

Figure 3. Plots of absorbances against [OHFl] (30 °C): ■ OD_{500} in 10 vol% aqueous ethanol at pH 5.65 with 0.01M acetate buffer, ○ OD_{520} in methanol, ● OD_{630} in methanol
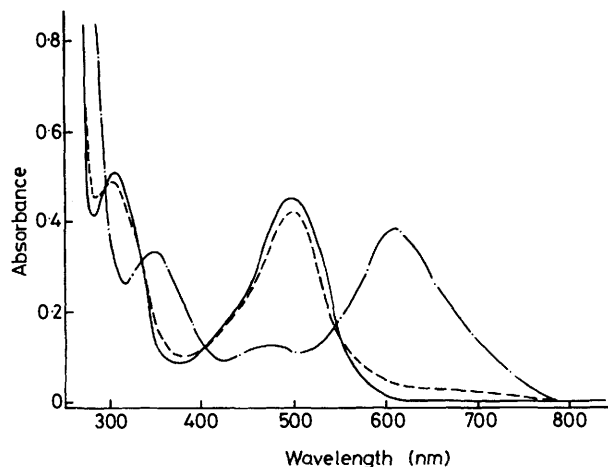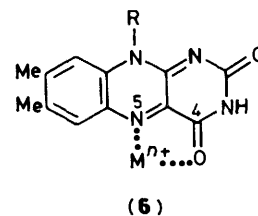

(6)

Figure 4. Effect of metal ions on the absorption spectra of OHFl in aqueous solution (30 °C; pH 5.69 with 0.01M acetate buffer, 10 vol% ethanol). ——— OHFl (3.00×10^{-5}M), —·—·— OHFl +Cu(NO$_3$)$_2$ (4.50×10^{-4}M), ———— OHFl + Zn(NO$_3$)$_2$ (4.50×10^{-4}M)

those of the OHFl–Cu^{II} complex (*vide supra*). Figure 5 clearly shows that a maximum appears at [OHFl]/[Cu^{II}] = 1.0, supporting the formation of the 1:1 flavin–metal complex. Subsequently, we tried to determine the association constant (*K*). It was evaluated from equation (1) which holds for the formation of a 1:1 complex,[22] where OD_0 and OD are the
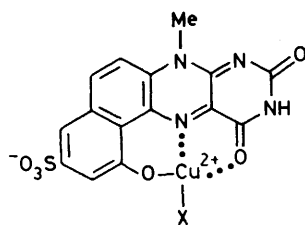
absorbances of OHFl in the absence and the presence of Cu^{II}, respectively, and ε is the apparent molar absorption coefficient of OHFl–Cu^{II}.

$$\frac{OD - OD_0}{[Cu^{II}]_0} = \varepsilon K[OHFl]_0 - K \cdot OD \qquad (1)$$
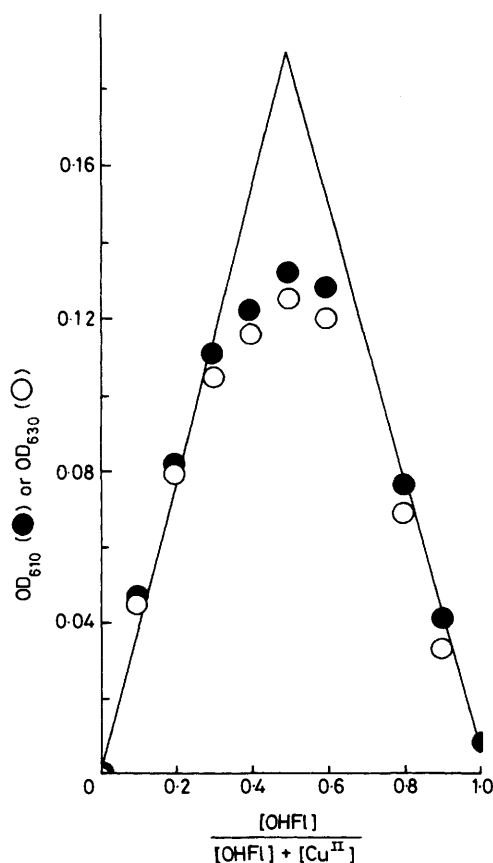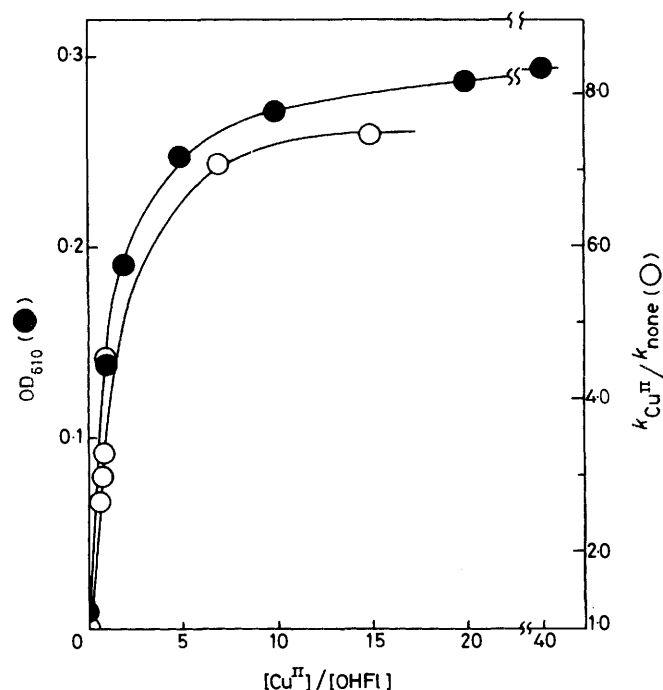
Table 1. Influence of added metal ions on the absorption spectra of OHFl in aqueous solution [a]

Metal salt	$[M^{n+}]/[OHFl]$	$\lambda_{max.}/nm\ (\varepsilon_{max.})$		
		S_2 band	S_1 band	
None		307 (17 300)	502 (14 900)	
$Cu(NO_3)_2$	15	354 (11 200)		605 (10 700)
$Ni(NO_3)_2$	120	366 (9 530)		644 (7 600)
$FeCl_2$	120	b	509 (11 300)	600 (6 400)
$Co(NO_3)_2$	120	305 (14 600)	502 (10 700)	608 (3 600)
$Zn(NO_3)_2$	120	306 (16 700)	503 (13 800)	
$Mg(ClO_4)_2$	120	306 (17 500)	503 (14 200)	
$AgNO_3$	120	306 (16 900)	501 (14 000)	
$FeCl_3$	15	306 (18 800)	502 (14 100)	
$ZrCl_4$	120	306 (16 400)	502 (15 300)	

[a] 30 °C; pH 5.69 with 0.010M acetate buffer, 10 vol% EtOH. The reference cell contains the same concentration of metal salts. [b] Not clear.

(7)

Figure 6. OD_{610} (●) and $k_{Cu^{II}}/k_{none}$ (○) plotted against $[Cu^{II}]/[OHFl]$ in water at 30 °C: [OHFl] 3.00 × 10⁻⁵M; pH 5.61 with 0.01M acetate buffer

Figure 5. Continuous variation plots for the OHFl·Cu^{II} complex in water at 30 °C: [OHFl] + [Cu(NO₃)₂] 6.00 × 10⁻⁵M; pH 6.51 with 0.01M acetate buffer, 1.6 vol% DMF

As shown in Figure 6, OD_{610} plotted against $[Cu^{II}]/[OHFl]$ gave a saturation curve. The plot of $(OD - OD_0)/[Cu^{II}]_0$ against OD gave a straight line with r 0.999. The K value was thus determined from the slope $(-K)$ by the least-squares procedure: $K = 26\ 300\ l\ mol^{-1}$. This value (log K 4.42) is much smaller than the association constants of 8-hydroxyquinoline (log K 15.0) and o-aminophenol (log K 9.25).[23] Based on the same continuous variation method ([OHFl] + [Ni^{II}] 1.50 × 10⁻⁴M, λ 660 nm), we established that OHFl and Ni^{II} also form a 1:1 complex (data not shown). OD_{660} Plotted against $[Ni^{II}]/[OHFl]$ gave a saturation curve ([OHFl] 3.00 × 10⁻⁵M, $[Ni^{II}]$ = (1.5—30) × 10⁻³M). Analysis of the data by using equation (1) afforded $K\ 806\ l\ mol^{-1}$ (r 0.999). This value (log K 2.91) is again smaller than those of analogous ligands (log K 11.65 for 8-hydroxyquinoline and 6.62 for o-aminophenol).[23] The relatively small association constants are ascribed to the weak basicity of N(5) (pK_a 0).[24] To the best of our knowledge, however, this is the first example for flavin–metal complexes with typical heavy metal ions such as Cu^{II}, Ni^{II}, Co^{II}, etc. formed in aqueous solution.

E.s.r. and I.r. Spectra.—Previously, Fritchie reported crystallographic studies of a Cu^{II} complex with the flavin model compound (10-methylisoalloxazine).[13] He has found that (i) the complex is octahedral and bound to two flavins [at O(4) and N(5)] and two water molecules and (ii) Cu^{II} binds to relatively 'hard' O(4) more strongly, unlike Ag^I which rather prefers 'soft' N(5). We prepared the OHFl–Cu^{II} crystals from OHFl and CuCl₂·2H₂O. Elemental analysis of the crystal showed that it consists of 1:1 OHFl/Cu^{II} and does not contain chloride. Hence, the cationic charge of Cu^{II} is apparently neutralised by 1′-O⁻ and 5′-SO₃⁻. The i.r. spectrum (KBr) gave the absorption bands of C(2)=O and C(4)=O at 1 657 and 1 676 cm⁻¹, respectively (corrected by the 1 601.4 cm⁻¹ band of polystyrene). These peaks shift to lower wavenumbers by 6 cm⁻¹ C(2)=O] and 38 cm⁻¹ [C(4)=O]. The shift of C(4)=O is

comparable with those of Cu^{II} complexes with salicylaldehyde (62 cm^{-1}) and acetylacetone (*ca.* 50 cm^{-1}).[25] It is unequivocal, therefore, that C(4)=O interacts with Cu^{II} in the solid state.

E.s.r. spectra of the OHFl–Cu^{II} complex was measured in *order to obtain* further insight into the co-ordination structure. Typical spectra are illustrated in Figure 7 together with its
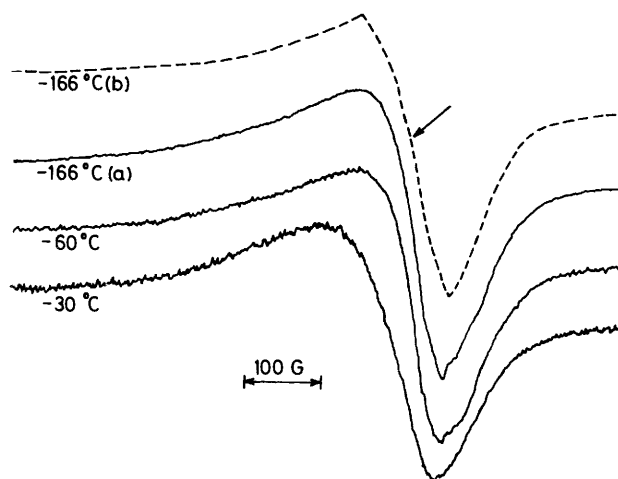

Figure 7. E.s.r. spectra of the OHFl–Cu^{II} complex prepared from OHFl (1.47 mM) and $Cu(NO_3)_2$ (1.03 mM) in 0.0047M acetate buffer (pH 5.7): (*a*) observed spectrum at -166 °C (microwave frequency 9.262 GHz, modulation amplitude 5.0 gauss), (*b*) simulated spectrum for (*a*)

simulation curve. The e.s.r. spectra of Cu^{II} mixed with OHFl in acetate buffer solution suggest the copper has the square-bipyramidal configuration.[26,27] This suggests that, as shown in structure (7), OHFl serves as a planar tridentate ligand in the *xy* plane of the complex. The spectra scarcely changed from -116 to -30 °C but small peaks assignable to free Cu^{II} appeared above -10 °C. The spectrum of aqueous $Cu(NO_3)_2$, taken as a reference, could be simulated by using two *g*-values (Table 2), indicating that it has a neat square-planar structure. In contrast, the third *g*-value was required in order to simulate fully the spectrum of the OHFl–Cu^{II} complex (Table 2). This is due to a shoulder at around 3 190 G (arrow mark in Figure 7). The existence of the shoulder was evidenced by the appearance of two maxima in its first-order differential spectrum. The result means that the square-planar structure of the OHFl–Cu^{II} complex is somewhat distorted and probably close to a lozenge shape. In other words, Cu^{II} cannot nest in the position equidistant from the three heteroatoms.

Oxidation of an NADH *Model Compound in Aqueous Systems.*—It was of interest to investigate the co-catalytic effect of complexed metals on the reaction of OHFl. We evaluated this by the reaction of OHFl with an NADH model compound. It is known that the reaction is first order in flavin and NADH model and the logarithm of the second-order rate constants (k_2) is linearly correlated with polarographic half-wave potentials of flavins.[28] We employed 1-benzyl-3-carbamoyl-1,4-dihydro-quinoline (BCQH: acid-stable NADH model)[16] to carry out the reaction [equation (2)], because conventional NADH models (dihydronicotinamides) are stable only at basic pH (where most metal ions precipitate) and decompose rapidly at acidic pH (where metal ions are soluble). Interaction between metals and BCQH was hardly expected in aqueous solution, and in fact the absorption spectrum of BCQH was not affected by added metal ions. Hence, the change in the rate constants could be attributed solely to OHFl–metal interactions. The results are summarised in Table 3.

**Table 2.** E.s.r. parameters for the OHFl–Cu^{II} complex

Sample	Temperature (°C)	*g*-value[a]		
		g_1	g_2	g_3
OHFl + $Cu(NO_3)_2$[b]	-166	2.361	2.100	2.070
$Cu(NO_3)_2$[c]	-10	2.240	2.135	0

[a] Parameters simulated by a computer program. [b] [OHFl] 1.47 × 10^{-3}M, [$Cu(NO_3)_2$] 1.03 × 10^{-3}M; pH 5.7 with 0.0047M acetate buffer. [c] [$Cu(NO_3)_2$] 1.03 × 10^{-3}M; pH 5.7 with 0.0047M acetate buffer.

Table 3. Effect of added metal ions on the oxidation of BCQH in aqueous solution[a]

Metal salt	[M^{n+}]/ [flavin]	k_2/l mol^{-1} s^{-1}		k_2/k_{none}	
		OHFl	MeLFl	OHFl	MeLFl
None		0.497	0.311	1.00	1.00
$Cu(NO_3)_2$	15	3.70	0.278	7.44	0.89
$FeCl_2$	15	0.581	0.229	1.17	0.74
$Mg(ClO_4)_2$	120	0.820	0.278	1.65	0.89
$FeCl_3$	15	0.580	0.242	1.17	0.78
$HgCl_2$	15	2.40	0.400	4.83	1.29

[a] 30 °C; aerobic; pH 5.69 with 0.010M acetate buffer, 10 vol% EtOH; [flavin] 3.00 × 10^{-5}M, [BCQH] 6.00 × 10^{-5}M
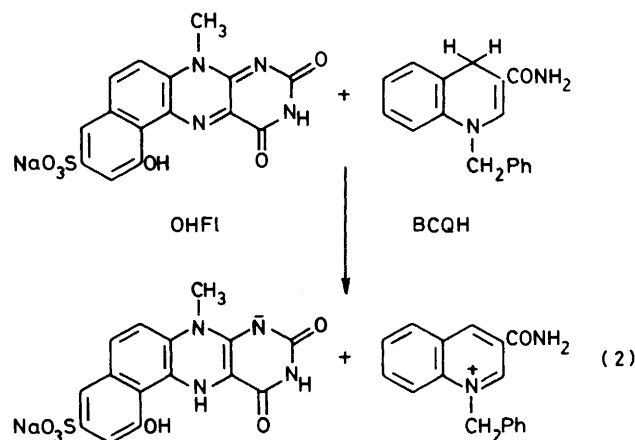
As shown in Table 3, several heavy metal ions were effective in the oxidation of BCQH. In particular, Cu^{II}, which induced the most prominent spectral shift, served again as the most efficient co-catalyst and Hg^{II} was the second most efficient. As NaCl (0.1M) exerted no catalytic effect, the effect of these metal ions cannot be explained by ionic strength. Ni^{II}, Co^{II}, Zn^{II}, and Zr^{IV} showed no or a slightly inhibitory effect on the oxidation rate. In the past, the oxidisability of flavin–metal complexes has been examined by a few groups. For example, Baarda and Metzler[29] reported that Ag^I weakly inhibits the reaction of an NADH model compound with conventional flavins. Also, Clarke *et al.*[30] showed that reduction of the Ru^{II}–flavin complex is significantly more difficult relative to that of free flavin under the same conditions. These are all due to the significantly increased electron-density donated from the metals to the flavin π-system.[30] The results suggest a difficulty in enhancing the oxidisability of flavins: that is, the metals that can interact with flavin rather deactivate flavin because of a metal→flavin charge transfer, whereas the metals that might enhance electron-deficiency of flavin cannot interact with flavin. In the present

metal–OHFl system, on the other hand, some metals can interact with the flavin as well as enhance the oxidisability. This is due solely to the fused phenolate group introduced as an additional chelation site.

We further studied the co-catalytic effect of Cu^{II}. The ratio of the second-order rate constants in the presence and the absence of Cu^{II} ($k_{Cu^{II}}/k_{none}$ is plotted against the concentration of Cu^{II} in Figure 6. The curvature is very similar to that of the plot of OD_{610} vs. [Cu^{II}] illustrated in the same Figure. As the reaction proceeds according to Scheme 2, the plot can be analysed by using equation (3),[31] which holds for the formation of a 1:1 complex.

$$OHFl + Cu^{II} \xrightleftharpoons{K} OHFl \cdot Cu^{II}$$

$$k_{none}[BCQH] \qquad k_{Cu^{II}}[BCQH]$$

$$products \qquad products$$

$$\frac{k_{none}}{k - k_{none}} = \frac{1}{qK} \frac{1}{[Cu^{II}]_0} + \frac{1}{q} \qquad (3)$$
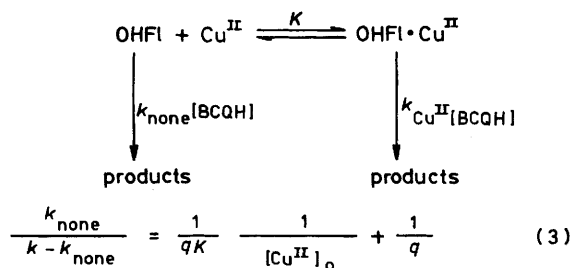
Scheme 2. $q = (k_{Cu^{II}}/(k_{none}) - 1$

From a plot of $k_{none}/(k - k_{none})$ against $[Cu^{II}]_0^{-1}$, we determined $(qK)^{-1}$ (slope) and q^{-1} (intercept) by the least-squares procedure (r 0.99). K and $k_{Cu^{II}}$ were calculated from these two terms: K 22 000 1 mol^{-1} and $k_{Cu^{II}}$ 3.73 1 mol^{-1} s^{-1}. The association constant is in good accord with that determined spectroscopically (26 300 1 mol^{-1}). The $k_{Cu^{II}}$ value thus determined indicates that the OHFl·Cu^{II} complex is 7.5 times more reactive than OHFl as an oxidising reagent.

Flavin–Metal Interactions in Methanol.—The spectroscopic measurements in methanol showed that OHFl can interact with a large number of metal ions. The spectral changes observed in the presence of 2,6-lutidine (2.0 × 10^{-3}M) are summarised in Table 4. Examination of Table 4 reveals that the influence of metal ions on the absorption spectra could be classified into three groups (Figure 8 and Table 5): in type A, the absorption
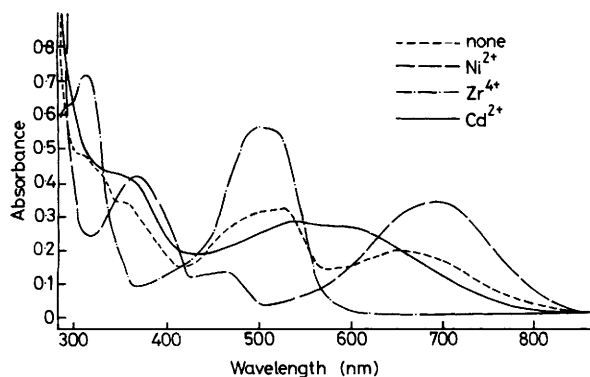

Figure 8. Absorption spectra of OHFl in methanol (1.6 vol% DMF) at 30 °C: [OHFl] 3.00 × 10^{-5}M, [metal salt] 4.50 × 10^{-4}M. Metal salts used are $Ni(NO_3)_2$, $ZrCl_4$, and $Cd(NO_3)_2$

band at the longer wavelength region (636—696 nm), which is similar to that of OHFl–metal complexes in aqueous solution, was enhanced and the absorption maxima of the S_2 bands shifted to longer wavelength. The S_1 band at 520 nm was hardly detectable. In type B, the absorption band at 630 nm in

Table 4. Influence of added metal ions on the absorption spectra of OHFl in methanol[a]

	$\lambda_{max.}$/nm ($\varepsilon_{max.}$)		
		S_1 band	
Metal salt	S_2 band		
None	318(12 500)	520(8 000)	650(4 630)
$Ni(NO_3)_2$	371(10 400)	[b]	696(8 800)
$Cu(NO_3)_2$	368(11 100)	—	662(9 930)
$Zn(NO_3)_2$	362(12 300)	—	648(9 280)
$FeCl_2$	354(11 700)	—	636(9 850)
$Co(NO_3)_2$	364(10 900)	—	666(8 130)
$MnCl_2$	367(9 800)	—	663(7 630)
$Cr(NO_3)_3$	312(19 700)	503(14 100)	—
$SbCl_3$	316(15 500)	504(13 600)	—
$RuCl_3$	315(17 400)	503(14 100)	—
$ZrCl_4$	312(17 600)	503(13 700)	—
$MoCl_5$	315(17 400)	503(14 100)	—
$Cd(NO_3)_2$	357(11 500)	546(6 930)	—

[a] 30 °C; 1.6 vol% DMF; [OHFl] 4.00 × 10^{-5}M, [2,6-lutidine] 2.0 × 10^{-3}M, [metal salt] 6.00 × 10^{-4}M, [H_2O] 0.01—0.04M. [b] The line indicates that the absorption maximum of this band disappeared.

Table 5. Classification of metal ions from change in spectral patterns

	S_1 band at		Metal ions
Type	520 nm	650 nm	
A	decrease	increase	Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}
B	increase	decrease	Cr^{3+}, Sn^{3+}, Ru^{3+}, Zr^{4+}, Mo^{5+}
C	$\lambda_{max.}$ 546 nm		Cd^{2+}

dehydrated methanol disappeared, and an absorption band at around 500 nm developed. This spectral change is analogous to the effect of added water or acetic acid. As the water concentrations determined after mixing with metals were less than 0.05M, the spectral change cannot be attributed to the increase in the water concentration. Interestingly, most of divalent metal ions are classified into type A and high-valent metal ions belong to type B. Probably, type-A metal ions form complexes such as (7) whereas type-B metal ions may induce deaggregation of OHFl through complexation with OHFl. Type-C metal (Cd^{II}) gave a single absorption maximum at 546 nm. $Mg(ClO_4)_2$ provided a spectral change similar to type A, but the absorption band at 520 nm was still observable under the same conditions. On the other hand, Ag^I, Hg^{II}, and Fe^{III} changed the spectral shape only to a smaller extent and Ca^{II}, NH_4^+, and Na^+ had no effect.

We estimated the stoicheiometry and the association constant (K) of the OHFl·Ni^{II} complex in methanol.* Figure 9 shows the continuous variation plot at [OHFl] + [Ni^{II}] = 6.00 × 10^{-5}M. The wavelength employed was 750 nm corresponding to a new band which appeared on addition of Ni^{II}. The water concentration in methanol was maintained at 0.02—0.05M, so that the absorption band at around 650 nm was little affected. It can be seen from Figure 9 that OHFl also forms a 1:1 complex with Ni^{II}.† As shown in Figure 10, a plot of OD_{750} against [Ni^{II}] gave a saturation curve. The plot of (OD − OD_0)/[Ni^{II}]_0 against OD was a good straight line with r

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* The OHFl·Cu^{II} complex was sparingly soluble in methanol and precipitated under some conditions, so that we could not make further examinations.

† When the water concentration was enhanced to 0.3—1.0M, the maximum appeared at 0.6. This implies that the 2:1 OHFl/Ni^{II} complex may be partially formed.
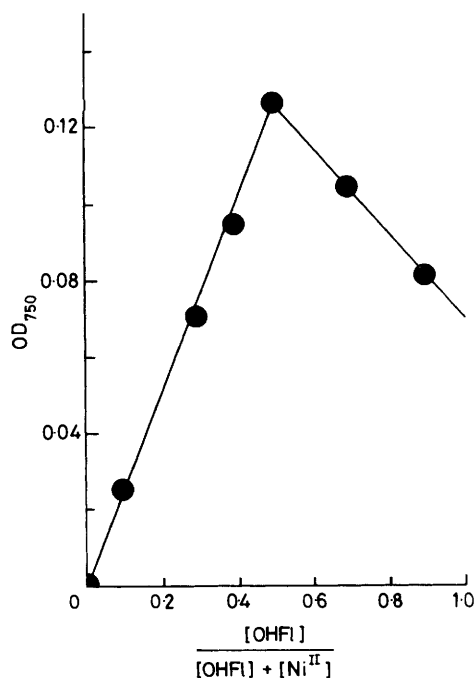
Figure 9. Continuous variation plot for the OHFl·Ni^{II} complex in methanol (1.6 vol% DMF) at 30 °C: [OHFl] + [Ni(NO$_3$)$_2$] 6.00 × 10^{-5}M, [2,6-lutidine] 1.50 × 10^{-3}M
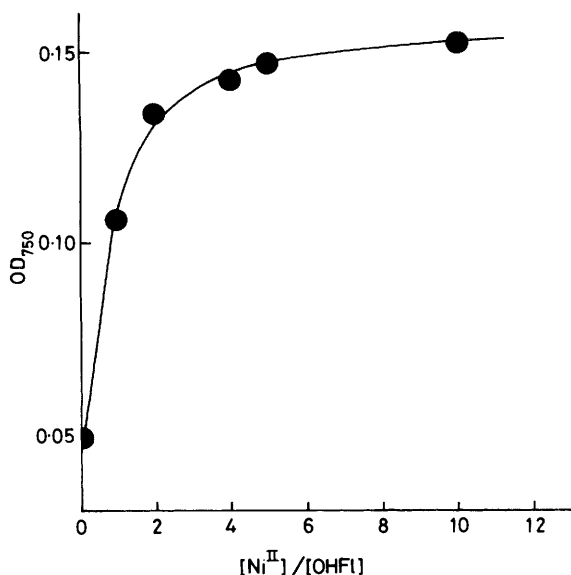

Figure 10. OD$_{750}$ plotted against [Ni^{II}]/[OHFl] in methanol (1.6 vol% DMF) at 30 °C: [OHFl] 3.00 × 10^{-5}M, [2,6-lutidine] 1.50 × 10^{-3}M

0.994, and by least-squares computation we obtained K 58 500 l mol^{-1}. This value is greater by a factor of 73 than that in aqueous solution. Thus, one might expect more marked metal effects on the oxidations by OHFl in methanol.

Oxidation of an NADH *Model Compound in Methanol.*—The metal effect in methanol was again evaluated through the reaction with BCQH. The absorption spectrum of BCQH in methanol was not affected by the addition of metal ions used in the present study. Therefore, one may disregard the interaction between BCQH and metal ions. The reaction of OHFl and

Table 6. Effect of added metal ions on the oxidation of BCQH in methanol (1.6 vol % DMF)^a

| | | 10^2 k_2/l mol^{-1}s^{-1} | |
Metal salt	[M^{n+}]/[flavin]	OHFl	MeLFl
None		0	0
NaClO$_4$	15	0	0
Zn(NO$_3$)$_2$	15	8.77	0
Ni(NO$_3$)$_2$	15	5.20	0
Cr(NO$_3$)$_3$	15	9.12	0

^{a} 30 °C; aerobic; [BCQH] 6.00 × 10^{-3}M, [2,6-lutidine] 1.50 × 10^{-3}M, [flavin] 3.00 × 10^{-5}M. Cu^{II} could not be used because of precipitation.

BCQH did not occur in the absence of metal (k_2 <10^{-5} l mol^{-1} s^{-1}). The addition of NaClO$_4$ (10^{-3}—10^{-2}M) was ineffective. Amongst the metal ions tested we found that Zn^{II}, Ni^{II}, and Cr^{III} effectively catalysed the reaction, the rate constants being enhanced by more than 10^3-fold (Table 6). From the association constant, OHFl almost solely exists as OHFl·M^{n+} at [M]/[OHFl] 15. Hence, the second-order rate constants obtained in Table 6 can be regarded as the rate constants for the oxidation of BCQH by OHFl·M^{n+} complexes. These metal ions exerted no catalytic effect on the reaction of MeLFl and BCQH. Therefore, the markedly improved oxidisability is ascribed to the OHFl–metal interaction.

Conclusions.—The present results indicate that the oxidisability of OHFl can be markedly enhanced through metal complexation in aqueous and methanolic solutions whereas that of conventional flavins cannot. The difference is caused by the fused phenol group introduced to give a new chelation site in the isoalloxazine skeleton [*i.e.* near C(4)=O and N(5)]. As reduced OHFl can be immediately reoxidised to OHFl by oxygen, the finding suggests that metal–OHFl complexes could serve as recycle oxidation catalysts according to a ping-pong mechanism. Also significant is the fact that the absorption spectra of OHFl are sensitively affected by bound metal ions, leading to shifts to longer wavelengths. The changes exhibit similarities to the spectra of some metalloflavoproteins. Therefore, the present study may provide a clue to understanding the spectral properties of metalloflavoproteins.

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