# Syntheses and Reactivity of Bisflavins. Oxidation of *N*-Benzyl-1,4-dihydronicotinamide by Flavins in Aqueous Solution [1]

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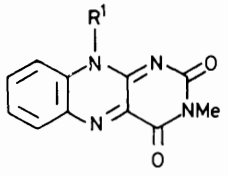
Several bisflavins linked at the 10,10′-positions of isoalloxazine rings have been synthesized. The kinetics of the oxidation of *N*-benzyl-1,4-dihydronicotinamide in aqueous solution have been examined. It has been found that each flavin of a bisflavin is considerably influenced by another intramolecular flavin moiety due to the formation of a charge-transfer complex between intramolecularly reduced and oxidized flavins. The formation of the complex depends on the conformation.

It is known that flavins (F) form charge-transfer complexes with a variety of organic molecules in aqueous solutions, since oxidized flavins (Fox) act as electron acceptors and reduced flavins ($FH_2$) act as electron donors.[2] A mixture of FMN and $FMNH_2$ is known to form a charge-transfer complex at relatively high concentrations of the flavins in aqueous solution.[3]

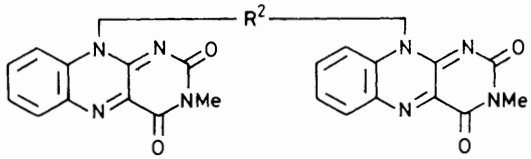
In addition, a flavin can serve as a 'step-down' transforming redox function from a two-electron donor to a one-electron acceptor. This is an essential step in many biological oxidations involving flavins. These flavoproteins often contain a second redox-active group, and both groups are more or less tightly bound proteins close to each other.[4] For example, NADPH-cytochrome p-450 reductase contains one mole each of FAD and FMN per molecule of the enzyme, and it has been suggested that FAD is involved in accepting two electrons from NADPH, and FMN is involved in the subsequent electron transfer to p-450.[5] The second redox-active group seems to function as an electron conduit.

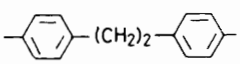
To mimic these systems, first of all, we must employ a system in which both redox groups exist in the same vicinity. Thus, we have chosen bisflavins in which two intramolecular flavin molecules are able to interact with each other.

In this paper, we describe the synthesis of bisflavins and their reactivity towards the oxidation of *N*-benzyl-1,4-dihydronicotinamide (BNAH) in aqueous solution.



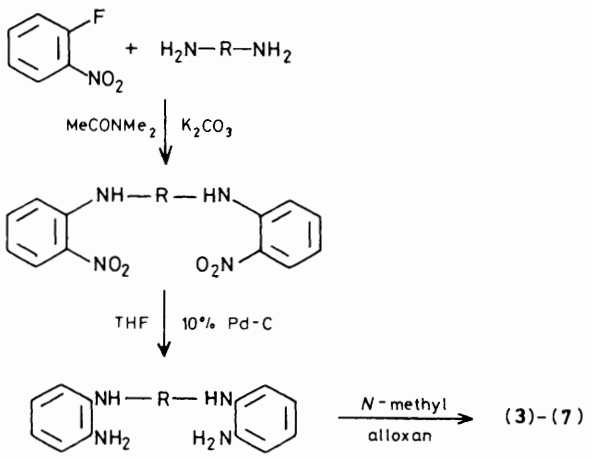
(1) $R^1$ = Me
(2) $R^1$ = Ph



(3) $R^2 = (CH_2)_2$
(4) $R^2 = (CH_2)_3$
(5) $R^2 = (CH_2)_4$
(6) $R^2$ = 
(7) $R^2$ = 

## Results and Discussion

*Synthesis of Flavins.*—Monoflavins were prepared according to the literature of Yoneda *et al.*[6] Bisflavins were synthesized by condensation of the corresponding diamines and *N*-methylalloxan as described below. The bisflavins thus prepared were identified by absorption spectra and elemental analyses. Two characteristic absorption maxima and the molar extinction coefficients are presented in Table 1. Table 1 indicates that all the flavins have similar absorption spectra, probably indicating no special interaction due to flavin–flavin stacking suggested for the absorption spectrum of 10-dodecyl-3-methylisoalloxazine in aqueous solution.[7]



*Oxidation of BNAH.*—Oxidation of *N*-substituted-1,4-dihydronicotinamide by flavins has been extensively investigated.[8] A survey of the literatures suggests that the oxidation proceeds *via* a hydride transfer.

The reactivity of bisflavins towards the oxidation of BNAH was estimated spectrophotometrically by following the decrease of the absorption of Fox (*ca.* 440 nm) in aqueous solution under anaerobic conditions. The formation of 1,5-dihydroflavins was confirmed by quantitative regeneration of Fox by $O_2$ introduction after the reaction. First-order plots [$\ln(OD_0 - OD_\infty)$ $(OD_t - OD_\infty)$ *versus* time] are shown in Figure 1. As can be seen in Figure 1, Fox Fox [(3)—(5)] does not follow a first-order rate equation, whereas (1), (2), (6), and (7) obey first-order kinetics up to more than 90% of reaction.

**Table 1.** The absorption maxima and the molar extinction coefficients of flavins[a]

| Fox | $\lambda_{max.}$/nm (log ε/dm³ mol⁻¹ cm⁻¹) | |
|---|---|---|
| (1) | 341 (3.94) | 434 (4.02) |
| (2) | 348 (4.00) | 438 (4.01) |
| (3) | 345 (4.10) | 440 (4.20) |
| (4) | 342 (4.09) | 436 (4.15) |
| (5) | 336 (4.10) | 438 (4.06) |
| (6) | 347 (4.04) | 437 (4.18) |
| (7) | 347 (4.16) | 438 (4.18) |

[a] In aqueous solution (pH 7.27, 0.1M-phosphate buffer, μ 0.5) containing 7% DMF.

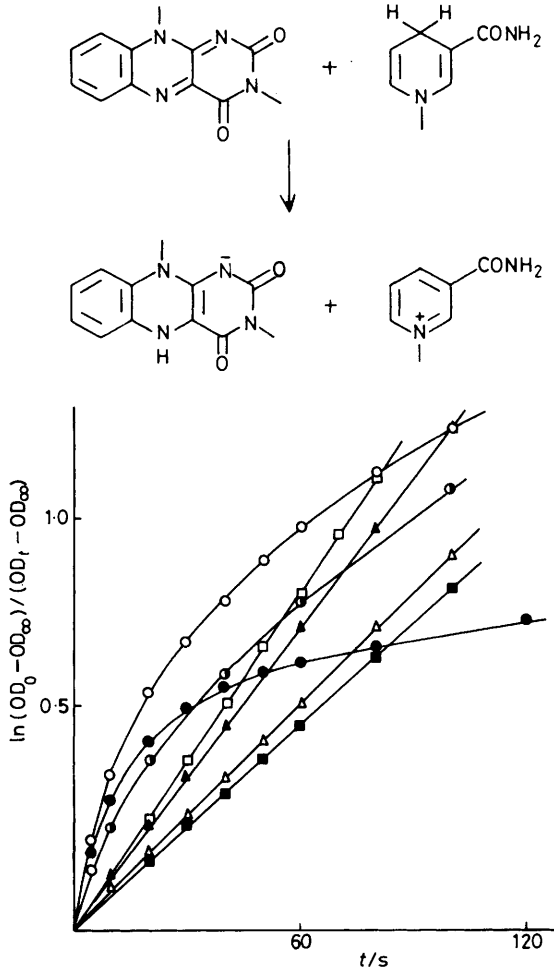



**Figure 1.** First-order plots [$\ln(OD_0 - OD_\infty)/(OD_t - OD_\infty)$ *versus* time], [(1)], [(2)] 1.3 × $10^{-5}$M, [(3)]—[(7)] 6.7 × $10^{-6}$M, [BNAH] 1 × $10^{-3}$M, pH 7.27 (0.1M-phosphate, μ 0.5), 25 °C: ▲; (1), ■; (2), ○; (3), ●; (4), ◑; (5), △; (6), □; (7)

For the non-linear plots in Figure 1, it is assumed that the initial and the subsequent gentle slopes for (3)—(5) correspond to the rate constants of reactions (i) and (ii). During the

$$\overline{\text{Fox}\quad\text{Fox}} + \text{BNAH} \xrightarrow{k_1} \overline{\text{Fox}\quad\text{FH}_2} + \text{BNA}^+ \qquad \text{(i)}$$

$$\overline{\text{Fox}\quad\text{FH}_2} + \text{BNAH} \xrightarrow{k_2} \overline{\text{FH}_2\quad\text{FH}_2} + \text{BNA}^+ \qquad \text{(ii)}$$

**Table 2.** The rate constants of oxidation of BNAH by Fox[a]

| Fox | $10^2 k_{obs}/s^{-1}$ $k_1$[b] | $k_2$[c] | Relative rates |
|---|---|---|---|
| (1) | 1.34 | | 1.0 |
| (2) | 0.880 | | 0.66 |
| (3) | 4.7 | 0.70 | 3.5, 0.52 |
| (4) | 3.8 | 0.18 | 2.8, 0.13 |
| (5) | 3.2 | 0.72 | 2.4, 0.54 |
| (6) | 1.01 | | 0.75 |
| (7) | 1.50 | | 1.1 |

[a] Average of two runs (±5% errors), [(1)], [(2)] 1.3 × $10^{-5}$M, [(3)]—[(7)] 6.7 × $10^{-6}$M, [BNAH] 1 × $10^{-3}$M, pH 7.27 (0.1M-phosphate, μ 0.5), 25 °C. [b] From initial slopes. [c] From the slopes after 50% reaction.

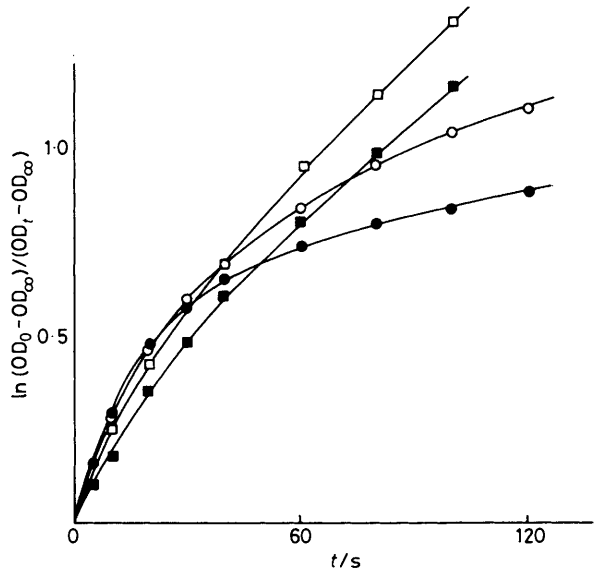



**Figure 2.** Effect of CTABr concentration on first-order plots [$\ln(OD_0 - OD_\infty)/(OD_t - OD_\infty)$ *versus* time]. [(4)] 6.7 × $10^{-6}$M, [BNAH] 1 × $10^{-3}$M, pH 7.54 (0.02M-phosphate buffer, μ 0.06), 25 °C: ●; [CTABr] 0, ○; [CTABr] 5 × $10^{-4}$M, □; [CTABr] 5 × $10^{-3}$M, ■; [CTABr] 1 × $10^{-2}$M

reaction, two processes ($k_1$ and $k_2$) occur simultaneously. Thus, the non-linearity suggests that the reactivity of the oxidized flavin is influenced by a closely located reduced flavin.

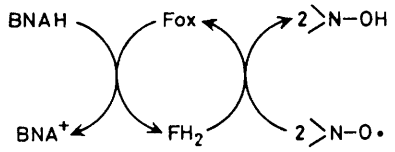
The rate constants ($k_1$ and $k_2$) estimated from the initial and the subsequent slopes together with other data are summarised in Table 2 which indicates that (a) the initial rates of (3)—(5) are a few times larger than those of (1), (2), (6), and (7), and (b) rate retardation with the progress of the reaction is most notable for (4): 1/6.7 for (3), 1/21 for (4), and 1/4.4 for (5).

Examination of CPK molecular models of the bisflavins proved that intramolecular face-to-face interaction of two isoalloxazine rings seems to be possible for (3)—(5), whereas this interaction is geometrically impossible for (6) and (7). Thus, a largest rate retardation for (4) ($k_2/k_1$ = 1/21) suggests that intramolecular electronic interactions in $\overline{\text{FH}_2\quad\text{Fox}}$ are most pronounced for (4).

Murakami *et al.* have reported that reduction of hexachloroacetone by bis-1,4-dihydronicotinamides is remarkably enhanced due to electronic interactions of intramolecular reduced and oxidized nicotinamide rings in $CH_2Cl_2$ and not enhanced in MeCN, indicating that such an interaction is very sensitive to

**Table 3.** The absorption maxima of (**4**) in the presence of CTABr at pH 7.52

| [CTABr]/M | $\lambda_{max.}$/nm | |
|---|---|---|
| 0 | 342 | 436 |
| $2 \times 10^{-3}$ | 341.5 | 436 |
| $5 \times 10^{-3}$ | 340 | 436 |
| $1 \times 10^{-2}$ | 339.5 | 436 |
| $2 \times 10^{-2}$ | 339 | 436 |




**Scheme.**

solvent polarity.[9] Thus, we have examined the effect of a cationic detergent (CTABr) on the oxidation of BNAH by bisflavin (**4**). The first-order plots are shown in Figure 2. As can be seen in Figure 2, the reaction was found to approach first-order kinetics with an increase in CTABr concentration. This may indicate that the electronic interaction of intramolecularly reduced and oxidized flavins is greater in bulk water, suggesting that hydrophobic flavin–flavin association is also important in producing electronic interactions of intramolecular reduced and oxidized flavins in aqueous solution.

Meanwhile, flavins have two characteristic absorption maxima at *ca.* 340 and 440 nm. The former absorption band (340 nm) is known to shift to shorter wavelength in apolar solvents.[7,10] The absorption maxima of (**4**) in the presence of CTABr are shown in Table 3. The shift to shorter wavelength suggests that bisflavin (**4**) exists in hydrophobic region of CTABr micelle. On the other hand, no shift was observed for the absorption spectrum of (**1**) in the presence of CTABr ($1 \times 10^{-2}$M).

*Electron Transfer from BNAH to a Nitroxide Radical* via *Flavins.*—Oxidation of 1,4-dihydronicotinamide by a one-electron acceptor such as a nitroxide radical is known to be catalysed by electron-transforming substances which are able to accept two electrons and transfer one electron to the acceptor.[11]

We have examined the reactivities of the bisflavins in the reaction of electron transport from BNAH (two-electron donor) to 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (one-electron acceptor) in aqueous solution under anaerobic conditions (Scheme).

It was found that BNAH alone does not reduce the nitroxide at pH 9.66 at 25 °C. Addition of a small amount of Fox, however, caused the reaction to proceed smoothly until BNAH was consumed completely. The rate constants were determined by following the decrease of BNAH (357 nm) under anaerobic conditions (Figure 3). The reaction followed first-order kinetics up to more than 90% of the reaction for all the cases examined here. Figure 3 shows that the absorption of Fox (440 nm) does not change throughout the reaction. This indicates that the rate of electron transfer from $FH_2$ to >N–O• is much faster than that of BNAH + Fox. It is known that the reaction of $FH_2$ + >N–O• proceeds on a stopped-flow time-scale.[12] In fact, it has been found that the rates are first-order in the concentration of flavin and independent of the concentration of nitroxide (Figures 4 and 5).

The reactivity of flavins for BNAH reduction is estimated more precisely by this method compared with the direct

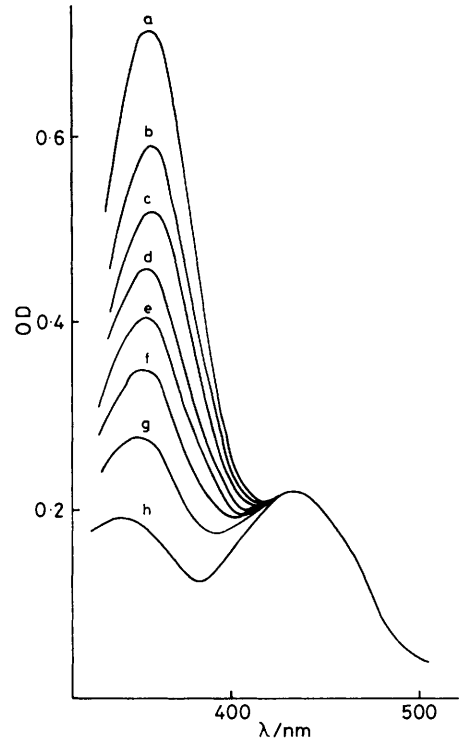



**Figure 3.** Spectral change of the time course for the reaction of BNAH with >N–O• in the presence of Fox, [(**1**)] $2.58 \times 10^{-5}$M, [BNAH] $1 \times 10^{-4}$M, [>N–O•] $1 \times 10^{-3}$M, pH 9.66 (0.02M-carbonate, μ 0.06), 25 °C; (a); 0, (b); 13 min, (c); 23 min, (d); 33 min, (e); 43 min, (f); 58 min, (g); 85 min, (h); ∞

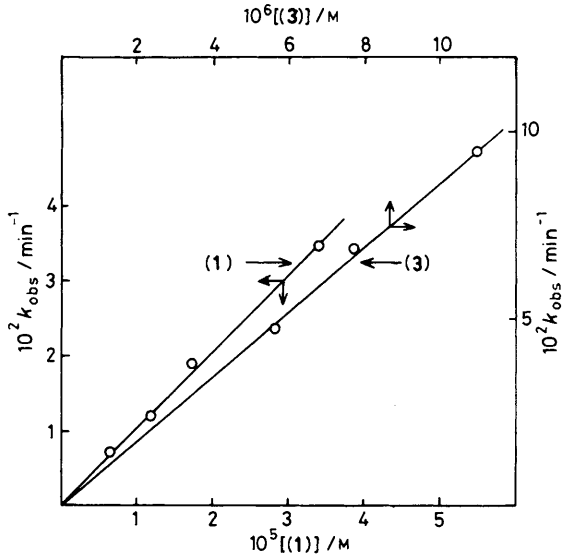



**Figure 4.** Concentration effect of flavin for the reaction of BNAH with nitroxide, [BNAH] $1 \times 10^{-4}$M, [>N–O•] $1 \times 10^{-3}$M, pH 9.66 (0.02M-carbonate, μ 0.06), 25 °C

determination of the rates of Fox + BNAH described earlier, since the decrease of BNAH follows first-order kinetics. It should be noted, however, that this system (Scheme) is not a system which can be used to examine whether Fox in $\overbrace{FH_2\ Fox}$ acts as a conduit of electron transfer from $\overbrace{FH_2\ Fox}$ to >N–O•.

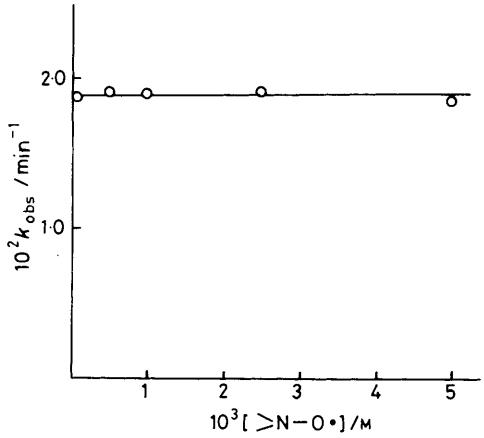



**Figure 5.** Concentration effect of nitroxide, [(**1**)] 1.75 × $10^{-5}$M, [BNAH] 1 × $10^{-4}$M, pH 9.66 (0.02M-carbonate, μ 0.06), 25 °C

**Table 4.** Rate constants and relative rates[a]

| Fox | $10^2 k_{obs}$/min$^{-1}$ | Relative rates |
|---|---|---|
| (**1**) | 1.12 | 1 |
| (**3**) | 4.70 | 4.2 |
| (**4**) | 5.00 | 4.5 |
| (**5**) | 3.41 | 3.0 |

[a] [(**1**)] 1.17 × $10^{-5}$M, [(**3**)]—[(**5**)] 5.88 × $10^{-6}$M, [BNAH] 1 × $10^{-4}$M, [>N–O•] 1 × $10^{-3}$M, pH 9.66 (0.02M-carbonate, μ 0.06), 25 °C.

The rate constants by employing (**1**) and (**3**)—(**5**) are presented in Table 4. Here again, the reactivity of the bisflavins was found to be higher than that of the monoflavin. This enhanced reactivity of the bisflavins may be also accounted for by stabilisation of intramolecular electronic interactions of flavins in aqueous solution.

*Attempt to detect Intramolecular Flavin–Flavin Interactions.*—When oxidized and reduced flavins are close to each other, two electronic interactions are conceivable: (a) charge-transfer complex and (b) flavin radical formation by one-electron transfer [reaction (iii)].

$$\overline{F^1H_2 \quad F^2ox} \rightleftharpoons \overline{F^1H_2 \cdots F^2ox} \rightleftharpoons \overline{F^1H\cdot \quad F^2H\cdot} \qquad \text{(iii)}$$

A charge-transfer absorption band of $FH_2$ and Fox may appear at longer wavelengths, since the charge-transfer band of FMN and $FMNH_2$ is known to appear at *ca.* 700—900 nm in aqueous solution, when a relatively high concentration of flavins is employed.[3] Flavin radicals also possess characteristic absorption spectra, when they are stable in solution.[13]

Thus, we have employed EDTA photoreduction of flavin to detect flavin–flavin interactions, since the rate of BNAH reduction is too fast to scan the whole spectrum during the course of the reaction. EDTA photoreduction has the advantage of recording the whole spectrum at any point in the reaction.

The absorption spectra of (**4**) for photoreduction are shown in Figure 6. The Figure reveals that the reduction progresses smoothly up to *ca.* 50% of the reaction with formation of a new broad band at *ca.* 700—900 nm, and the subsequent reduction was quite slow. Longer irradiation was found to decompose the flavin, which was confirmed as there was no quantitative regeneration of Fox by introduction of $O_2$. Furthermore, the new broad band disappeared when the reduction was completed by addition of $Na_2S_2O_4$. In contrast to this, monoflavin (**1**) and bisflavin (**6**) did not show such a band during the course of photoreduction. It should be noted that the flavin moieties of (**6**) are unable to interact with each other for geometrical reasons. Thus, the broad band at longer wavelength of (**4**) could be reasonably attributed to the 'intramolecular charge-transfer absorption band' of $\overline{FH_2 \cdots Fox}$. These observations suggest that such an intramolecular charge-transfer interaction is also operative in BNAH reduction.

The present finding, that the reactivity of flavin is considerably influenced by a nearby flavin due to the formation of a charge-transfer complex, may suggest that the reactivity of flavin in a biological system is dramatically different from that in a model system especially when a suitable flavin–flavin association is achieved on apoenzymes.

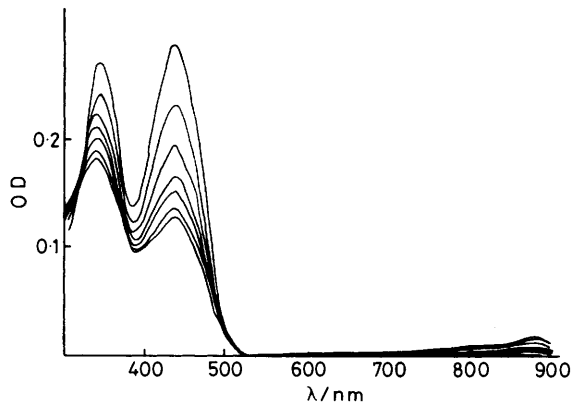



**Figure 6.** Spectral change of EDTA photoreduction, [(**4**)] 1.67 × $10^{-5}$M, EDTA] 3.3 × $10^{-3}$M, ph 7.27 (0.1M-phosphate buffer, μ 0.5), 25 °C.

**Table 5.** Yields and melting points of *NN'*-bis-(2-nitrophenyl)diamines

| Diamines | Yields (%) | M.p. (°C) |
|---|---|---|
| $(CH_2)_2$ | 84 | 192 (lit.,[17] 194) |
| $(CH_2)_3$[a] | 80 | 147 |
| $(CH_2)_4$[b] | 67 | 154—155 |
| *p*-$C_6H_4$[a] | 26 | 235 |
| *p*-$C_6H_4(CH_2)_2C_6H_4$[c] | 80 | 148—149 |

[a] Identification was performed by mass spectra, $M^+$ 316. [b] Found: C, 58.2; H, 5.5; N, 16.9. $C_{16}H_{18}N_4O_4$ requires C, 58.2; H, 5.5; N, 17.0%. [c] Found: C, 68.5; H, 4.9; N, 12.3. $C_{26}H_{22}N_4O_4$ requires C, 68.7; H, 4.9; N, 12.3%.

## Experimental

*Materials.*—*N*-Benzyl-1,4-dihydronicotinamide was prepared according to the literature,[14] and purified by recrystallisation from EtOH, m.p. 122 °C (lit.,[14] 121—122 °C). EDTA (ethylenediaminetetra-acetic acid disodium salt dihydrate) was analytical grade from Wako Chemical Co. 4-Hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl was prepared from 4-hydroxy-2,2,6,6-tetramethylpiperidine by oxidation with 30% $H_2O_2$ in the presence of EDTA and sodium tungstate in aqueous solution. The nitroxide was purified by recrystallisation from diethyl ether–n-hexane, m.p. 71 °C (lit.,[15] 71.5 °C). 3,10-Dimethylisoalloxazine (**1**) and 3-methyl-10-phenylisoalloxazine (**2**) were prepared according to the literature;[6,16] (**1**), m.p. 330 °C (from EtOH) (lit.,[6] 334 °C); (**2**), m.p. >330 °C (from

**Table 6.** Analytical data for bisflavins (3)—(7)

| Bisflavins | Yield (%) | M.p. (°C) | Found (%) (Required) C | H | N |
|---|---|---|---|---|---|
| (3) | 27 | >300 | 57.6 | 3.8 | 22.0) |
| ($C_{24}H_{18}N_8O_4 \cdot H_2O$) | | | (57.6) | (4.0) | (22.4) |
| (4) | 28 | 296 | 57.9 | 4.1 | 21.2 |
| ($C_{25}H_{20}N_8O_4 \cdot 1.2H_2O$) | | | (58.0) | (4.4) | (21.6) |
| (5) | 28 | >300 | 55.8 | 4.75 | 19.55 |
| ($C_{20}H_{22}N_8O_4 \cdot 3H_2O$) | | | (55.3) | (5.0) | (19.85) |
| (6) | 46 | >300 | 61.1 | 3.7 | 20.4 |
| ($C_{28}H_{18}N_8O_4 \cdot H_2O$) | | | (61.3) | (3.7) | (20.4) |
| (7) | 31 | >300 | 66.2 | 4.4 | 16.7 |
| $C_{36}H_{26}N_8O_4 \cdot 1.2H_2O$ | | | (65.9) | (4.4) | (17.05) |

EtOH) (lit.,[16] 360 °C). Bisflavins were prepared by condensation of the corresponding *NN'*-bisdiamines and *N*-methylalloxan. *NN'*-Bis-(2-aminophenyl)diamines were prepared from *NN'*-bis-(2-nitrophenyl)diamines.[17] A mixture of *o*-nitrofluorobenzene (0.02 mol), the appropriate diamine (0.018 mol), and $K_2CO_3$ (0.018 mol) in *NN'*-dimethylacetamide (12 ml) was stirred at 100 °C overnight. After cooling, the reaction mixture was poured into 1N-hydrochloric acid, and the crystals thus formed was collected and recrystallised from THF–light petroleum or ethanol. The yields and physical properties are summarised in Table 5.

*NN'*-Bis-(2-aminophenyl)diamines were obtained by shaking a solution of the corresponding bis-(2-nitrophenyl)diamines (0.3 g) in EtOH or THF (160 ml) under hydrogen in the presence of 10% Pd–C (0.2 g). Hydrogenation was continued until the theoretical amount of hydrogen was absorbed. After filtration of Pd–C and evaporating the solvent to dryness, crude *NN'*-bis-(2-aminophenyl)diamines were obtained and used without purification. Condensation of the diamines (0.2 g) with *N*-methylalloxan (0.4 g) was performed in AcOH (40 ml) in the presence of boric acid (0.5 g).[18] The mixture was stirred at 60 °C for 30 min and at room temperature overnight. After evaporating AcOH, EtOH was added to dissolve the starting materials. The solid undissolved in EtOH was collected and dried. No suitable solvents for recrystallisation were found for any of the bis-flavins. Analytical data are summarised in Table 6.

*Rate Measurements.—Oxidation of BNAH by flavins.* BNAH ($3 \times 10^{-2}$M in EtOH, 0.1 ml) was placed in the upper compartment of a Thunberg cuvette, and Fox (0.2 ml) ($2 \times 10^{-4}$M for monoflavins and $1 \times 10^{-4}$M for bisflavins in DMF) and a buffer solution (2.7 ml) in the lower cell. Each solution was bubbled for 20 min with nitrogen which had been scrubbed with vanadous ion solution[19] and prehumidified with water. After temperature equilibration (15 min), the reaction was initiated by mixing the two solutions. The pseudo-first-order rate constants were determined by following the decrease of the absorptions of Fox (440 nm). In the presence of CTABr, CTABr was added to the cell by a microsyringe after $N_2$ bubbling.

*Electron transfer from BNAH to nitroxide radical* via *flavins.* A typical kinetic run is as follows: BNAH ($1 \times 10^{-2}$M in EtOH, 30 μl) was placed in the upper compartment of a Thunberg cuvette, and Fox ($5 \times 10^{-3}$M in DMF, 10 μl) and the nitroxide (0.1M in EtOH, 10 μl) were placed in the lower cell with buffer solution (2.95 ml). Both solutions were degassed by bubbling vanadous ion-scrubbed $N_2$ prehumidified with $H_2O$ for 20 min. After equilibration at 25 °C, the reaction was initiated by mixing. The absorption decrease of BNAH (350 nm) was monitored spectrophotometrically.

*EDTA Photoreduction.* In a Thunberg cuvette, flavins ($3.3 \times 10^{-5}$M) and (4) ($1.67 \times 10^{-5}$M) were photoreduced by irradiation with a 60 W tungsten lamp in the presence of EDTA ($3.3 \times 10^{-3}$M) in a glass thermocontrolled water-bath (25 °C) under anaerobic conditions.[20] At appropriate intervals of irradiation, the absorption spectra were recorded.

## Acknowledgements

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