Coenzyme Models. Part 24.† Micellar Catalysis of Flavin-mediated Reactions. Influence of the Flavin Structure on the Reactivity

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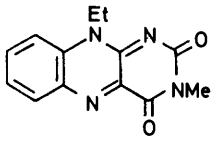
The catalytic effect of a cationic (CTAB) micelle on the flavin-mediated oxidation of 1-benzyl-1,4-dihydronicotinamide (6), nitroethane carbanion (7), and thiophenol (8) is reported. The oxidation of (6) was subject to a small extent to micellar catalysis, whereas the oxidation of (7) and (8) which does not proceed in a simple aqueous solution was efficiently catalysed by the CTAB micelle. The rate of oxidation of (7) was profoundly dependent upon the structure of the flavin: flavins which have either a long alkyl group or a carboxy-group gave rate constants greater by 10^3—10^4 fold than unmodified flavin, and the rate constant for flavin (5) which has both groups was further enhanced (>10^6 fold). On the other hand, the oxidation of (8) was less affected by the change in the flavin structure. The reactivity order for the oxidation of (7) was (1) (unmodified neutral flavin) ≪ (2) (neutral flavin with a hexadecyl group) < (4) (anionic flavin with a carboxy-group) < (5) (anionic flavin with carboxy- and tetradecanoyl groups), whereas that for the oxidation of (8) was (1) < (4) < (5) < (2). The results indicate that the reactivity of flavins is variable, depending not only on the type of reaction but also on the environment. The results provide useful information on the versatile reactivity of flavin coenzymes bound to apoenzymes.

In contrast to the inability of flavins to oxidise all but the most electron-rich substrates, flavoenzymes exhibit a rather broad specificity for carbanions and thiols.[1,2] For example, D-amino-acid oxidase rapidly oxidises nitroalkane carbanions to the corresponding aldehydes and nitrite ion,[3] whereas this reaction does not proceed in nonenzymatic systems.[4] Although aliphatic thiols are slowly oxidised by flavins in nonenzymatic systems,[5-8] the less reactive thiophenol cannot be oxidised under ambient reaction conditions.[4] In 1975, Yokoe and Bruice [4] found that 8-cyano-3,10-dimethylisoalloxazine is able to catalyse these reactions. Since the polarographic half-wave potential ($E_{1/2}$) of the isoalloxazine (−0.317 V) is higher (positively) by 0.221 V than that of unmodified flavin (3-methyl-lumiflavin),[9] the activity of the isoalloxazine stems from the electron deficiency caused by the electron-withdrawing nature of the cyano-group.

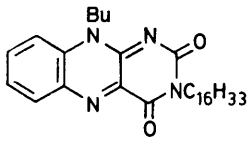
We considered that flavin-mediated reactions may be facilitated by synthetic microheterogeneous catalysts such as micelles and polysoaps. These catalysts can provide both electrostatic and hydrophobic environments [10-12] which are the major driving forces for the binding of small molecules to enzymes and cause activation of adsorbed substrates.[13] Hence, results obtained from a study of these reactions would provide an insight into the mechanisms for the control of coenzyme reactivity by apoenzymes. We have reported that nitroethane carbanion is readily oxidised in the presence of a cationic micelle by a hydrophobic flavin, 10-butyl-3-hexadecylisoalloxazine (2) but not by 10-ethyl-3-methylisoalloxazine (1).[14] The spectral study showed that (2) resides in the hydrophobic domain of the cationic micelle, while (1) is partitioned to a smaller extent to the micellar phase. The result indicates that flavin-mediated reactions are efficiently catalysed by cationic micelles.

† Part 23, S. Shinkai, Y. Kusano, and O. Manabe, *J.C.S. Perkin I*, in the press.
‡ Preliminary communications, refs. 14 and 19.

The object of the present investigation is to search for flavins which act as more efficient oxidizing catalysts in the cationic micellar system. The question arises as to whether the isoalloxazine buried in the hydrophobic domain of the micelle is the reactive centre. Is the electrostatic environment of the micelle surface useless in facilitating the flavin-mediated reactions? In order to



(1)



(2)

find an answer to this, we synthesised 3-methyl- (3), 3-methyl-8α-[N^{α}-acetyl-(N^1-histidinyl)- (4), and 3-methyl-8α-[N^{α}-tetra-decanoyl-(N^1-histidinyl)^6-tetra-*O*-acetyl-riboflavin (5). As expected, the flavin moiety of (5) is on the surface of the cationic micelle because of the effect of the neighbouring carboxylate function, while (4) is adsorbed onto the micelle surface due to electrostatic interactions.

RESULTS

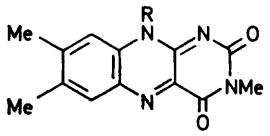
Absorption Spectra of Flavins.—The absorption band of flavins at *ca.* 360 nm is sensitive to the solvent polarity.[15] For example, the $\lambda_{max.}$ of riboflavin in aqueous solution (374 nm) shifts to 344 nm in 98% dioxan solution. Another absorption maximum at *ca.* 440 nm gives distinct shoulders at 420 and 460 nm in organic solvents (MeCN or dioxan). The spectral data of flavins thus become a useful probe for the reaction environment.

The spectral data for flavins (1)—(5) are summarised in Table 1. Flavin (1) (the least hydrophobic flavin) gave absorption maxima at 341 and 433 nm which were not affected by the addition of CTAB § above the critical micelle concentration. On the other hand, the $\lambda_{max.}$ of (2) (a hydro-

§ Hexadecyltrimethylammonium bromide. The critical micelle concentration under the kinetic conditions (μ 0.02) is 8.0×10^{-4}M.

phobic flavin) in the CTAB micelle appeared at 333 nm, and another $\lambda_{max.}$ at 440 nm showed distinct shoulders. The results suggest that the isoalloxazine moiety of (2) exists in the hydrophobic region of the micelle.

A similar spectral shift was observed for (5), but only of 5 nm. The absorption band at 446 nm was relatively broad, but no distinct shoulder was observed. Probably, the flavin moiety of (5) is bound to the Stern layer owing to



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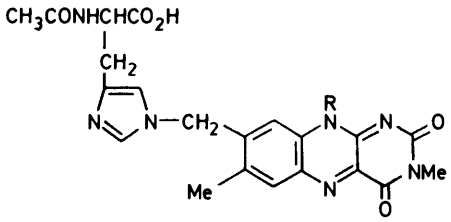

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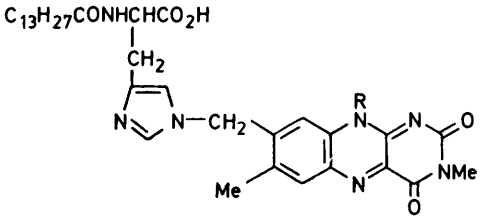


(5)

the electrostatic interaction between the neighbouring carboxy-group and the surfactant head group.

Oxidation of 1-*Benzyl*-1,4-*dihydronicotinamide* (6).—Flavin oxidation of (6) is a typical model reaction for hydrogen transfer between NADH and flavin coenzymes. The reaction was first order in flavin and (6). We have previously determined the second-order rate constants (k_2) for the oxidation of (6) by (1) in micellar systems.[16] A plot of k_2 *versus* [cationic surfactant] showed a rate maximum, and the rate enhancement at maximum concentration was less than two-fold (Table 2). The results show that equation (1) is only weakly subject to micellar catalysis. As shown in Figure 1, a plot for the oxidation of (6) by (4) afforded a similar curve with a rate enhancement relative to the non-micellar system of at most two-fold. This means that anionic flavin (4) also undergoes a weak micellar effect.

The rate constant for (5) in the non-micellar system was

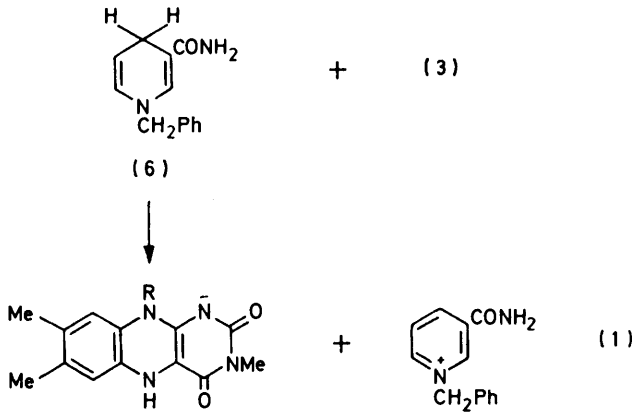
TABLE 1

Absorption maxima of flavins [a]

Flavin	$\lambda_{max.}$/nm: No micelle	$\lambda_{max.}$/nm: 3mM-CTAB	Shoulders at *ca.* 440 nm in the CTAB micelle
(1)	341, 433	341, 433	—
(2)		333, 440	+
(3)	362, 445	362, 445	—
(4)	348, 447	340, 449	—
(5)	343, 446	338, 446	—

[a] 30 °C, μ = 0.02 with KCl.

greater by a factor of 12 than that for (4). The rate constants decreased with increasing CTAB concentration and finally reached the level for (4) (Figure 1). The critical micelle concentrations of anionic C_{14} surfactants are much higher (*e.g.*, 2.1 × 10^{-3} M for sodium tetradecyl sulphate) [11] than the concentration of (5) used (2.0 × 10^{-5}M), so that the



(1)

rate enhancement cannot be simply attributed to micelle formation. We believe, however, that some form of aggregation plays an important role in the rate enhancement since the tetradecanoyl group itself is not a substituent which would affect the reactivity of the flavin. Probably, aggregation between (5) and (6) occurs in a pre-equilibrium step and/or hydrophobic ion-pairing between the carboxylate anion of (5) and the cationic charge developing on the nitrogen of the pyridine ring of (6) is brought about at the transition state.[11,17] The addition of CTAB causes

TABLE 2

Rate constants for flavin oxidation in the absence and the presence of micelles [a]

Flavin	k_2 for (6) [b]/l mol^{-1} s^{-1}: No micelle	CTAB [c]	SDS [d]	Brij-35 [e]	k_2 for (7) [b]/l mol^{-1} s^{-1}: No micelle	CTAB [c]	k_3' for (8) [b]/l^2 mol^{-2} s^{-1}: No micelle	CTAB [c]
(1)	10.0 [f]	11.4 [f]	6.50 [f]	6.13	N.r.	N.r.	N.r.	3 320 [i]
(2)		9.92 [f]	1.16 [f]	7.20		3.63 × 10^{-4} [h]		9 440 [i]
(3)	32.4 [g]				N.r.	N.r.	N.r.	
(4)	48.1	73.0	20.5	42.3	N.r.	9.90 × 10^{-3}	N.r.	4 350
(5)	585	235	91.3	390	1.30 × 10^{-4}	5.75 × 10^{-1}	N.r.	6 650

[a] For reaction conditions see Experimental section. N.r. = 'no reaction' (the reaction rate was not measurable). In the presence of SDS or Brij-35, flavins (1)—(5) could oxidise neither (7) nor (8). [b] For the definition of the rate constants see Experimental section. [c] [CTAB] = 3.00 × 10^{-3}M. [d] [SDS] = 1.00 × 10^{-2}M. [e] [Brij-35] = 1.00 × 10^{-3}M. [f] Cited from ref. 16. [g] Cited from ref. 26. [h] Cited from ref. 14. [i] Cited from ref. 19.

dilution of (5) and (6) in the micellar phase, leading to a gradual rate reduction with increasing CTAB concentration.

Oxidation of Nitroethane Carbanion (7).—In a preliminary communication,[14] we reported that the nonenzymatic

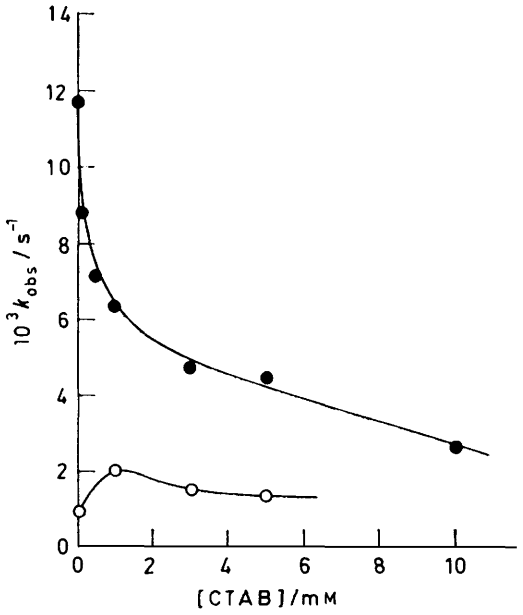
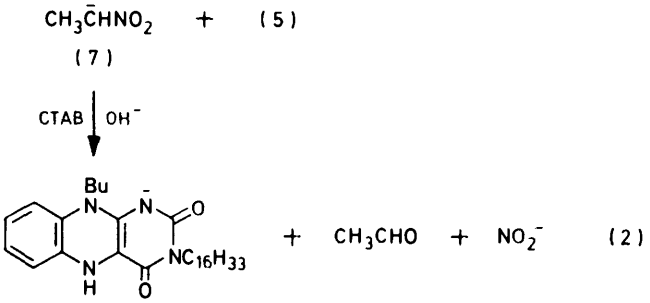


FIGURE 1 Pseudo-first-order rate constant (k_{obs}) for the oxidation of (6). [Flavin] = 2.00×10^{-5}M, [(6)] = 1.00×10^{-4}M, pH 8.5 with 0.01M-borate buffer, μ = 0.02. ○, For (4); ●, for (5)

oxidation of (7) is attained by the use of (5) bound to CTAB micelle. The reaction was first order in flavin and (7). The second-order rate constant (3.63×10^{-4} l mol^{-1} s^{-1}) was smaller by only one order of magnitude than that for electron-deficient 8-cyano-3,10-dimethylisoalloxazine [k_2 = (2.8—5.0) $\times 10^{-3}$ l mol^{-1} s^{-1}].[4]

In Figure 2, pseudo-first-order rate constants (k_{obs}) for the oxidation of (7) are plotted against CTAB concentration. In the non-micellar system, neither (3) nor (4) could oxidise



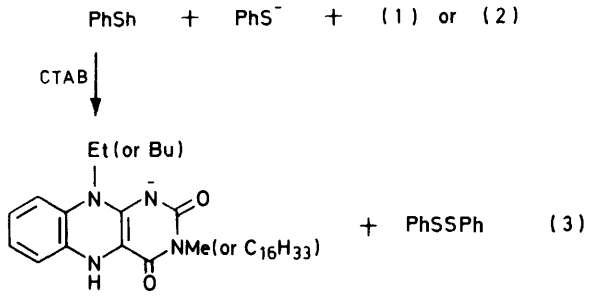
(7). On the other hand, (4) in the CTAB micelle acted as an oxidant for (7), the second-order rate constant (k_2) at 3mM-CTAB being 27 times greater than that for (2) (Table 2). The oxidation rate was further enhanced by the use of the anionic, hydrophobic flavin (5): the rate augmentation relative to (4) in the non-micellar system is greater than 10^6 and that relative to (4) in the micellar system is 58-fold.*

* The rate of oxidation by (4) was not detected in the non-micellar system. The second-order rate constant, which was estimated from the recorder sensitivity, was assumed to be less than 10^{-7} l mol^{-1} s^{-1}.

These results suggest that anionic flavins in cationic micelles are excellent oxidants for carbanions. We thus propose that the flavin oxidation of (7) proceeds more efficiently at the micellar surface than in the micellar core.

On the other hand, anionic (sodium dodecyl sulphate) and nonionic (Brij-35) micelles were totally ineffective as oxidation catalysts.

Oxidation of Thiophenol (8).—It has been established that the flavin oxidation of monothiols is first order in flavin and



second order in monothiol and is not subject to general acid–base catalysis.[4,5,7] Although (8) is hardly oxidised by the usual flavins, Yokoe and Bruice [4] found that 8-cyano-3,10-dimethylisoalloxazine is able to oxidise (8) and the reaction is apparently first order in PhSH and PhS^-. Probably, the reaction consists of general-acid catalysed 4a-addition of PhS^- followed by rate-limiting nucleophilic attack of PhS^- upon the 4a-adduct.[4] The rate-limiting step of equation (3) is thus analogous to the Ellman reaction.[18]

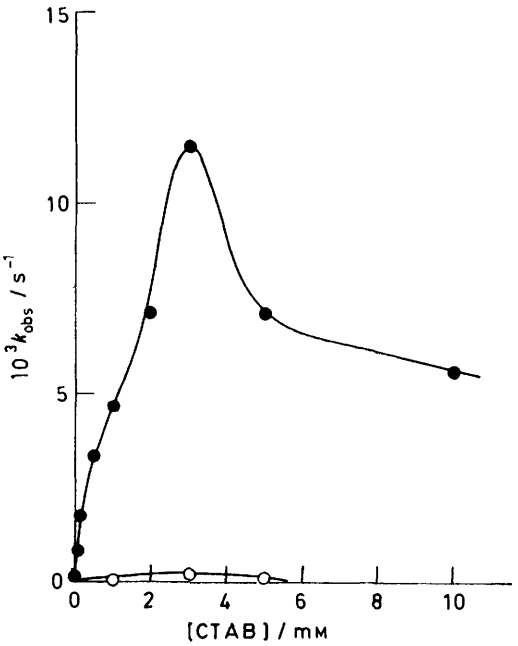


FIGURE 2 Pseudo-first-order rate constant (k_{obs}) for the oxidation of (7). [Flavin] = 2.00×10^{-5}M, [nitroethane]$_{total}$ = 3.00×10^{-2}M, pH = 8.90 with 0.1M-borate buffer, μ = 0.15. ○, For (4); ●, for (5)

We have found that (1) and (2) are able to oxidise (8) to diphenyl disuphide in the presence of the CTAB micelle [equation (3)] and the reaction is again first order in flavin and second order in (8).[19] Here, it should be noted that (7)

is oxidised only by the hydrophobic flavin (2) and not by the less hydrophobic flavin (1),[14] while (8) is oxidized by both flavins (Table 2).[19]

The pseudo-first order rate constants (k_{obs}) for anionic flavins (4) and (5) were plotted as a function of the CTAB concentration (Figure 3). The reaction was not detected at [CTAB] 0mM.* Both flavins were able to oxidise (8) in the CTAB micelle, the rate constants for (5) being slightly greater than those for (4). The apparent third-order rate

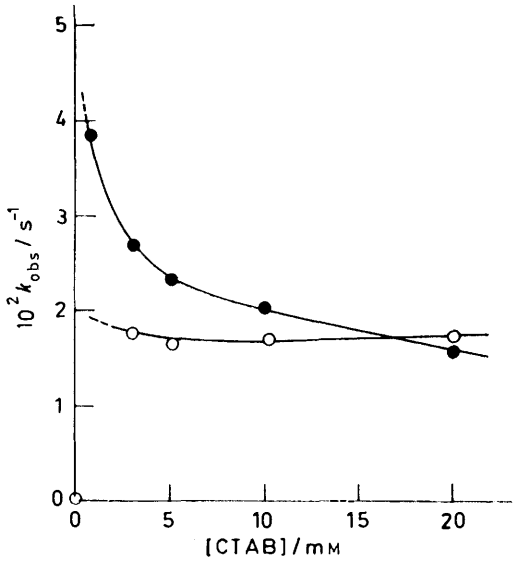


FIGURE 3 Pseudo-first-order rate constant (k_{obs}) for the oxidation of (8). [Flavin] = 2.00×10^{-5}M, $[(8)]_{total} = 2.00 \times 10^{-3}$M, pH 7.0 with 0.05M-phosphate buffer, $\mu = 0.02$, ○, For (4); ●, for (5). The reaction in the non-micellar system was carried out in 40 vol% ethanol to obviate the precipitation of diphenyl disulphide

constants (k_3') at pH 7.0 (Table 2) are comparable with those of (1) and (2). The results suggest that the presence of the CTAB micelle is a prerequisite for the oxidation of both (7) and (8), but the binding position of the flavin moiety in the micelle phase is not an important factor for the oxidation of (8).

SDS and Briji-35 were also added to the reaction system, but these surfactants were again ineffective.

As a summary of the foregoing results, one can describe the following reaction orders: (1) $\ll$ (2) $<$ (4) $<$ (5) for the oxidation of (7) and (1) $<$ (4) $<$ (5) $<$ (2) for the oxidation of (8).

DISCUSSION

The foregoing kinetic results indicate that (i) flavin oxidation of anionic species is profoundly catalysed by cationic micelles, whereas that of (6) (and probably neutral species in general) is much less subject to the micellar effect, (ii) in some cases the micellar environment facilitates flavin oxidation more efficiently than the electron-withdrawing cyano-group, and (iii) most interestingly, the efficiency of the oxidation of (7) changes dramatically depending on the binding position of the flavin moiety in the micelle phase, whereas the oxidation of (8) is much less affected by the structure of the flavin.

Gascoigne and Radda [5] have demonstrated that the rate constants for the reaction of NADH with flavin derivatives correlate reasonably well with their polarographic half-wave potentials. The CTAB micelle accelerated the reaction of (6) with the flavins (1)—(4) by less than two-fold. The fact implies that the polarographic half-wave potentials of the micelle-bound flavins do not greatly change. Hence, the marked rate acceleration observed for the oxidation of (7) and (8) cannot be ascribed to the ' activation ' of the flavins.

In order to account for the marked rate enhancement for (7) and (8), two potential factors come to mind, the concentration effect and the reaction field effect.[10,11] For example, anionic (7) must be adsorbed on the Stern layer of the CTAB micelle. Provided that the flavin moiety of (4) and (5) resides in this layer and that of (2) in the hydrophobic core of the micelle, (4) and (5) are more advantageously placed in entropy terms. Oxidation by (4) and (5) occurring in the Stern layer must also be advantageous in environmental terms, since the ionic reaction generally favours the polar Stern layer rather than the hydrophobic core.[20] Reactant (5) exceeds (4) owing to the favourable partition of the micelle phase. These considerations may accommodate the reactivity order for (7) [(1) $\ll$ (2) $<$ (4) $<$ (5)]. One should note, however, that the reactivity order for (8) cannot be fully accounted for by these two classic micelle effects, because the most hydrophobic reactant (2) is the most reactive (Table 2) and all the rate constants are of the order of 10^3 l^2 mol^{-2} s^{-1}.

We previously proposed that the anion included in the hydrophobic region as a ' hydrophobic ion pair ' attains its high reactivity from dehydration relative to an anion in an aqueous environment.[12,21] The formation of such a ' hydrophobic ion pair ' with thiophenolate ion has been substantiated by spectral data and the change in the apparent pK_a value.[21,22] This proposal is in line with the solvent effect on the nucleophilicity of thiophenolate ion, *i.e.* thiophenolate ion is more nucleophilic in aprotic solvents than in polar protic solvents such as water and alcohol.[23] It is presumed, therefore, that the oxidation of ' activated ' thiophenolate ion proceeds mainly in the hydrophobic region. The flavin (2) which exists in the hydrophobic region is thus a more efficient oxidant than (5) which exists in the Stern layer. The k_3' values for (8) primarily reflect the activation of thiolate ion by the micelle, and the binding position of the flavin moiety plays only a supplementary role.

The importance of this new micelle effect is related to the hydrophobicity of the substrate anion. Substrate (7) is classified as a relatively hydrophilic anion. In fact, no pK_a shift was observed for (7) in the CTAB micelle.[14] This means that (7) is subject to a smaller extent to activation by the hydrophobic environment. We believe, however, that the formation of ' hydrophobic ion pairs ' should not be neglected even in the oxidation

* The reaction in the nonmicellar system was carried out in 40 vol% ethanol to obviate the precipitation of diphenyl disulphide. Precipitation was not observed in the micellar system owing to the solubilisation ability of the micelle. The value of k_3' in the nonmicellar system is assumed to be less than 1 l^2 mol^{-2} s^{-1}.

of (7), since Lapinte and Viout [24] proposed that the most hydrophilic ion, OH^-, is also subject to this effect.

In conclusion, the present study shows that the influence of the cationic micelle on the flavin-mediated reactions changes depending on the hydrophobicity and hydrophilicity of the flavins and substrates employed. That is, a ' surface flavin ' is a good catalyst for the oxidation of hydrophilic anions, while a ' buried flavin ' is a better catalyst for the oxidation of hydrophobic anions. This implies that flavin-mediated reactions can be controlled by environmental factors relatively easily in micellar systems and also in enzymatic systems.

EXPERIMENTAL

Materials.—Preparations of (1)—(4) have been described.[25,26] CTAB was purchased from Wako Pure Chem. Ind. and recrystallised from ethanol before use. Flavin (5) was prepared from N^{α}-tetradecanoyl-L-histidine [27] and 8α-bromo-3-methyltetra-*O*-acetylriboflavin according to the method of Walker *et al.*[28] N^{α}-Tetradecanoyl-L-histidine (1.06 g, 3.0×10^{-3} mol) and 8α-bromo-3-methyltetra-*O*-acetylriboflavin (1.91×10^{-3} g, 3.0×10^{-3} mol) were dissolved in *NN*-dimethylformamide (40 ml) containing powdered potassium carbonate (2.0 g). The mixture was stirred at 50 °C. The progress of the reaction was monitored by t.l.c. (silica gel–ethyl acetate). The spot for 8α-bromo-3-methyltetra-*O*-acetylriboflavin became undetectable after one day. Potassium carbonate was filtered off and the filtrate was evaporated *in vacuo*. The orange residue was recrystallised from acetonitrile, and the orange powder recovered was again recrystallised from acetonitrile–di-isopropyl ether, m.p. 229—232 °C, yield 22% (Found: C, 58.0; H, 6.9; N, 10.0. Calc. for $C_{46}H_{63}N_7O_{13},H_2O$: C, 58.75; H, 6.95; N, 10.45%).

Kinetic Measurements.—Kinetic measurements were carried out at 30 °C at known ionic strengths [0.02 for (6) and (8) and 0.15 for (7)]. The rates for the oxidation of (6) were determined in aerobic solution by following the decrease in the absorption band of (6) (357 nm). The details of the kinetic procedure have been described.[26] On the other hand, the rates for the oxidation of (6) and (7) were determined under anaerobic (N_2) conditions by following the decrease in the absorption band of flavins at *ca.* 440 nm. The decrease of the absorption band as a function of time gives satisfactory first-order plots for up to four half-lives. Under the experimental conditions k_{obs} for equations (1) and (2) is first order in flavin and in (7), respectively, and that for equation (3) second order in (8). The k_2 value for equation (1) was directly determined by $k_2 = k_{obs}/[\text{flavin}]$. As the carbanionic fraction of nitroethane at pH 8.90 is 0.666 (pK_a 8.60),[14] k_2 for equation (2) is given by $k_2 = k_{obs}/[(7)] = k_{obs}/0.666\ [\text{nitroethane}]_{total}$. On the other hand, the reaction rate for equation (3) was simply expressed by the apparent third-order rate constant k_3' ($= k_{obs}/[(8)]^2_{total}$), since the kinetic behaviour is very complicated.[7]

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