Coenzyme Models. Part 29.† On the Unusual Spectroscopic Behaviour of 10-Dodecyl-3-methylisoalloxazine (Amphiphilic Flavin Analogue) in Aqueous Solution

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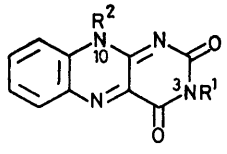
At room temperature in aqueous solution, the visible absorption peak (S1) of the title isoalloxazine(3) gave a well resolved, three-band fine structure which is believed to appear only in apolar solvents or in enzymatic hydrophobic pockets where isoalloxazine is free of hydrogen-bonding effects. On the other hand, 10-ethyl-3-methylisoalloxazine (1) and another amphiphilic isoalloxazine, 10-butyl-3-hexadecylisoalloxazine (2) did not show this fine structure. We have observed that (i) the fine structure disppears upon addition of organic solvents (pyridine or ethanol) or surfactants above the critical micelle concentration, (ii) the blue shift of another absorption peak in the u.v. region (S2) does not occur, and (iii) the fluorescence intensity of aqueous (3) is much smaller than that of aqueous (1) but increases upon addition of organic solvents or surfactant micelles. These results are explicable on the basis of an aggregation–deaggregation equilibrium of (3) in aqueous solution, and the fine structure of aqueous (3) is attributed to the ' stacking ' association of the isoalloxazine molecules. The reactivity of (3) in aqueous solution has been estimated through an investigation of the hydrolytic decomposition and oxidation of *N*-benzyl-1,4-dihydronicotinamide (BzlNH) and several thiols. In most cases, the rate constants for (3) were smaller by a few orders of magnitude than those for (1) but were significantly enhanced by the addition of organic solvents or cetyltriethylammonium bromide micelle. The results indicate that the reactions mediated by isoalloxazine, which mostly involve polarised transition states, are extremely unfavourable in the hydrophobic region of aggregated (3). These novel findings have significant implications for the chemistry of flavoproteins, because the surroundings of the binding sites of flavoproteins have frequently been discussed in terms of the change in the absorption spectra of bound flavin coenzymes.

It is well known that the light-absorption spectra of the flavin (vitamin B_2) family have a characteristic dependence upon the medium polarity. Flavins have usually two characteristic absorption maxima in the u.v. (*ca.* 330 nm, S2 peak) and visible regions (440 nm, S1 peak). It has been established from solvent effects that (i) the absorption maximum of S2 can serve as an indicator of solvent polarity since it shifts to shorter wavelengths in apolar solvents and (ii) the absorption maximum of S1 is scarcely affected by solvent polarity, but the solvent effect is sensitively reflected by the spectral shape: a simple gaussian-type peak in aqueous solution (type A), two or three shoulders in dipolar solvents (dimethylformamide, MeCN, tetrahydrofuran, *etc.*) (type B), and a well resolved three-band fine structure in apolar solvents (benzene, 3-methylpentane, *etc.*).[1-8] The spectral changes have been considered to reflect the degree to which hydrogen bonding to isoalloxazine plays a role in solvent–isoalloxazine interactions.[7] At extremely low temperature (77 K), the fine structure appears even in dipolar solvents (*e.g.*, 2-methyltetrahydrofuran).[4] In apolar solvents, both the blue shift of S2 and the split of S1 occur simultaneously. This basic information on solvent effects has enabled us to use the flavin family as an environmental probe in enzymic and membrane biology.[9-13] In some cases the fine structure of S2 has been found in flavoproteins, suggesting that the binding sites of the flavoproteins are considerably hydrophobic.[9,10] To the best of our knowledge, however, neither a blue shift for S2 nor a split in S1 has ever been reported for aqueous solutions except for those in the flavoproteins. The question thus arises as to whether the fine structure observed in the flavoproteins can be readily rationalised in terms of microsolvent effects in aqueous solution.

We previously reported that 10-butyl-3-hexadecylisoalloxazine (2) bound to a cationic micelle has an absorption spectrum of type B.[14] We here report that 10-dodecyl-3-methylisoalloxazine (3) (amphiphilic flavin analogue) gives the three-band fine structure in aqueous solution at room temperature, while the simple isoalloxazine (1) and (3) with a long alkyl chain at the 3-position give spectra of type A or B. Based on the absorption and fluorescence spectra, we attributed the unusual spectral behaviour of (3) in aqueous solution to the association of isoalloxazine molecules and examined the reactivity of aggregated (3). The results have important implications for the elucidation of the spectral behaviour of flavoproteins and the origin of the fine structure.



(1) R^1 = Me, R^2 = Et
(2) R^1 = n-$C_{16}H_{33}$, R^2 = Bu^n
(3) R^1 = Me, R^2 = n-$C_{12}H_{25}$

RESULTS

Absorption Spectra in Various Solvents.—Typical spectra and absorption maxima are recorded in Figure 1 and Table 1.

† Part 28, S. Shinkai, H. Hamada, H. Kuroda, and O. Manabe, *J. Org. Chem.*, 1981, **46**, 2333.

Table 1 shows that the spectral shape of S1 of (1) is quite dependent upon solvent polarity. The three-band fine structure was seen in benzene and *o*-dichlorobenzene. As has been reported by Koziol [1,2] and others,[3,4,5,6] the $\lambda_{max.}$ of S2 shifted to shorter wavelengths upon lowering the solvent polarity. The spectral behaviour of isoalloxazines (1)—(3)

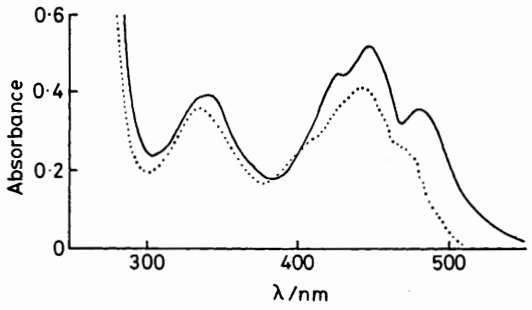


FIGURE 1 Absorption spectra of (3) (5.00×10^{-5}M) at 30 °C: —— in water; . . . . in 10mM-CTAB solution

was very similar in organic solvents, but significant differences were found in aqueous solution. In particular, S1 of (3) in aqueous solution (Figure 1) had the well resolved, three-band fine structure which is similar to that of (1) in benzene. The shape of the absorption spectrum was almost identical at [(3)] = $(0.10—2.40) \times 10^{-4}$M. This is the first example of the observation of the fine structure in aqueous (2) in aqueous solution and (1)—(3) in dipolar solvents; type C, (3) in aqueous solution and (1)—(3) in apolar solvents.

Interestingly, the fine structure of (3) disappeared completely in aqueous solutions containing surfactants above the critical micelle concentration, and the spectra were classified as type B (Figure 1 and Table 1). The fine structure also disappeared upon addition of ethanol (>45 vol %) or pyridine (>20 vol %) but did not disappear upon addition of β-cyclodextrin (20 mmol l^{-1}) or urea (4 mol l^{-1}). These results strongly suggest that the fine structure of aqueous (3) stems from association of the isoalloxazine molecules which is induced by hydrophobic interaction between the 10-dodecyl groups. The disappearance of the fine structure by added organic solvents is accounted for by deaggregation due to the enhanced solubility of (3) in mixed solvents and that in the micellar system is ascribed to the loss of the interaction between isoalloxazine molecules (' dilution effect ') in the micellar phase.

Absorption Spectra in Water–Ethanol and Water–Pyridine Mixed Solvents.—Addition of ethanol or pyridine to the aqueous solution of (1) changes the shape of S1 from type A to B, but the optical density is hardly affected (Tables 2 and 3). $\lambda_{max.}$ of S2 of (1) is shifted from 341 (in water) to 332 (in ethanol) or to 327 nm (in pyridine). These results are typical of solvent effects on the absorption spectra of flavins. On the other hand, the fine structure of (3) gradually disappeared with increasing ethanol concentration, and the spectra in >45 vol % ethanol solution were classified as type

TABLE 1

Absorption maxima (nm) of isoalloxazines in various solvents (30 °C) [a]

Medium	(1) S2	(1) S1	(2) S2	(2) S1	(3) S2	(3) S1
Water	341,	433	334,	426 (S)	340,	427 447 480
CTAB (10mM)	340,	433	336,	440 (S)	338,	440 (S)
Brij-35 (10mM)					340,	445 (S)
SDS (10mM)					341,	440 (S)
Ethanol	332,	435 (S)			332,	435 (S)
DMF	328,	435 (S)			328,	436 (S)
MeCN	328,	433 (S)	328,	436 (S)	328,	435 (S)
Pyridine	327,	438 (S)	328,	439 (S)	328,	438 (S)
o-Dichlorobenzene	332,	420 442 467			332,	420 442 468
Benzene	328,	418 440 466	329,	420 442 466	329,	420 440 467

[a] (S) indicates that S1 has shoulders. Three values given for S1 indicate each absorption maximum for the fine structure split.

solution. Examination of Table 1 shows, however, that the absorption maximum of S2 of (1) in benzene is considerably shifted to shorter wavelength (328 nm) by comparison with that in water (341 nm), whereas that of (3) in aqueous solution scarcely shifts (340 nm). The difference suggests that a split of S1 in aqueous (3) is induced by an effect somewhat different from simple solvent effects. On the other hand, the amphiphilic flavin analogue (2) gave an S1 spectrum of type B with weak shoulders.* Based on the spectral measurements, we have classified the solvent effects as follows: type A, (1) in aqueous solution; type B,

* The flavin concentrations in the measurements of the absorption spectra are: [(1) or (3)] 5.00×10^{-5}M and [(2)] $(1—2) \times 10^{-5}$M. The lower concentration of (2) relative to (1) or (3) is due to the poor solubility in water.

B. The optical density of S1 in ethanol was somewhat smaller than that in water (Table 2 and Figure 2), but the shape of the absorption spectra of (3) in 45—100 vol % ethanol was essentially similar to those of (1) in the same solvent composition. The result suggests that a transition from aggregated (3) to monomeric (3) occurs at *ca.* 45 vol % ethanol.

A dramatic solvent effect was found for the spectra of (3) in water–pyridine mixtures. The fine structure also disappeared on the addition of pyridine (>20 vol %), but the optical density experienced a dramatic change involving a maximum (at 5 vol % pyridine) and minimum (at 20 vol % pyridine). The peculiar behaviour is due to a new absorption band at 500—700 nm. As shown in Figure 3, a new, broad absorption band appeared at longer wavelength on

TABLE 2

Absorption maxima ($\lambda_{max.}$/nm) and $OD_{max.}$ in ethanol–water mixed solvents (30 °C) [a]

Ethanol (vol%)	$\lambda_{max.}$($OD_{max.}$) of (1)		$\lambda_{max.}$($OD_{max.}$) of (3)	
	S2	S1	S2	S1
0	341 (0.482),	433 (0.499)	340 (0.404),	427 (0.445) 447 (0.516) 480 (0.348)
20	340 (0.388),	434 (0.506)	341 (0.404),	427 (0.453) 446 (0.523) 478 (0.342)
40	339 (0.398),	435 (0.512)	341 (0.384),	S (—) 447 (0.479) 478 (0.321)
60	338 (0.397),	435 (0.509)	338 (0.328),	438 (0.417)
80	336 (0.388),	436 (0.505)	336 (0.328),	437 (0.420)
100	332 (0.367),	435 (0.486)	332 (0.312),	435 (0.409)

[a] Three values given for S1 indicate each $\lambda_{max.}$ for the fine structure split. S denotes that the absorption band appears as shoulder. [isoalloxazine] = 5.00×10^{-5}M.

TABLE 3

Absorption maxima ($\lambda_{max.}$/nm) and $OD_{max.}$ in pyridine–water mixed solvents (30 °C) [a]

Pyridine (vol%)	$\lambda_{max.}$($OD_{max.}$) of (1)		$\lambda_{max.}$($OD_{max.}$ × 5) of (2)		$\lambda_{max.}$($OD_{max.}$) of (3)	
	S2	S1	S2	S1	S2	S1
5	346 (0.371),	437 (0.478)	334 (0.325),	426 (0.402)	352 (0.668),	S (—) 451 (0.779) 483 (0.679)
10	345 (0.361),	438 (0.465)	337 (0.361),	423 (0.463)	354 (0.562),	S (—) 452 (0.637) 486 (0.603)
15	346 (0.357),	439 (0.460)			354 (0.542),	S (—) 453 (0.570) 487 (0.540)
18					355 (0.515),	S (—) 453 (0.570) 487 (0.540)
19					358 (0.304),	S (—) 457 (0.331) 493 (0.314)
20	344 (0.360),	439 (0.461)	341 (0.400),	440 (0.497)	346 (0.054),	441 (0.073)
30	343 (0.368),	440 (0.465)	344 (0.450),	442 (0.562)	343 (0.180),	442 (0.240)
40	342 (0.370),	440 (0.467)	344 (0.471),	443 (0.583)	342 (0.291),	442 (0.368)
60	332 (0.380),	440 (0.476)	342 (0.476),	442 (0.595)	340 (0.491),	442 (0.623)
80	331 (0.401),	439 (0.501)	327 (0.478),	441 (0.598)	336 (0.498),	440 (0.636)
100	327 (0.412),	438 (0.508)	328 (0.484),	439 (0.605)	328 (0.522),	438 (0.642)

[a] Three values given for S1 indicate each $\lambda_{max.}$ for the fine structure split. S denotes that the absorption band appears as a shoulder. [(1) or (3)] 5.00×10^{-5}M, [(2)] 1.00×10^{-5}M.

TABLE 4

Medium effects on the fluorescence spectra of (1) and (3) [a]

Medium	(1) at 20 °C		(3) at 7 °C		(3) at 20 °C		(3) at 50 °C	
	$\lambda_{max.}$	R.i.	$\lambda_{max.}$	R.i.	$\lambda_{max.}$	R.i.	$\lambda_{max.}$	R.i.
Water	518	0.337	520	0.333	517	0.155	518	0.332
CTAB (10mM)	514	0.303	505	0.112	505	0.089	504	0.071
Brij-35 (10mM)	516	0.357			516	0.839	516	0.710
SDS (10mM)	515	0.350			518	0.850	517	0.774
Benzene	499	0.428			498	0.986		
o-Dichlorobenzene	501	0.317						
MeCN	505	0.415						
DMF	503	0.505						
Pyridine	502	0.412			504	0.970		

[a] [isoalloxazine] 5.00×10^{-7}M, excitation wavelength 380 nm.

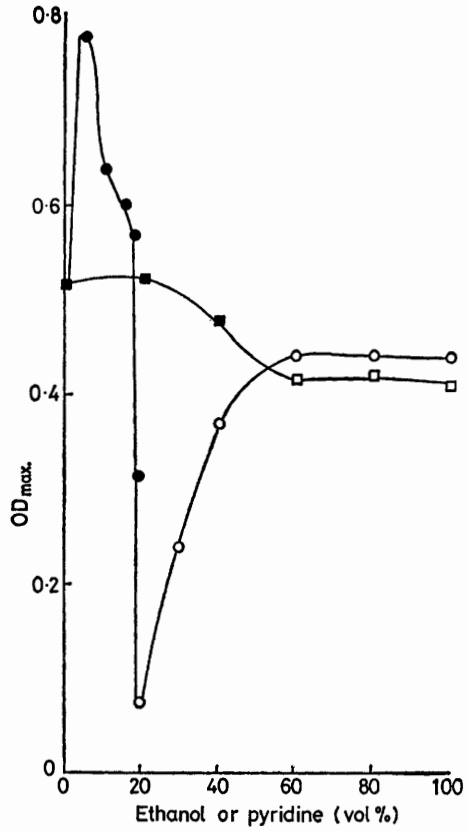


FIGURE 2 $OD_{max.}$ of S1 of (3) as a function of solvent composition: [(3)] 5.00×10^{-5}M; □ and ■ ethanol; ○ and ● pyridine. Filled points indicate that fine structure is observed

the addition of small amounts of pyridine, which apparently pushes up the optical density of S1 at 5—15 vol % pyridine region. A similar absorption band appeared upon addition of 4-dimethylaminopyridine, *o*-aminophenol, and indol-3-ylacetic acid. The dramatic solvent effect is demonstrated by the following fact: the fine structure of S1 was

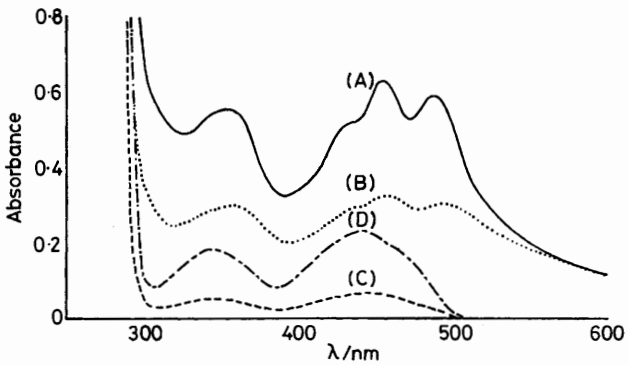


FIGURE 3 Absorption spectra of (3) (5.00×10^{-5}M) in water–pyridine (30 °C). Pyridine content: (A) 10 vol %; (B) 19 vol %; (C) 20 vol %; (D) 30 vol %

still observable in 19 vol % pyridine solution, although much weaker than that in water, while it completely disappeared in 20 vol % pyridine solution. Upon increasing the concentration of pyridine beyond this composition, typical spectra of type B developed. The result suggests that changes in the aggregation mode of (3) are very critical in water–pyridine mixtures. It is also peculiar that the molar extinction coefficient of S1 in 20 vol % pyridine solution is so small ($\varepsilon_{max.}$ 1 460 l mol^{-1} cm^{-1}). The $\varepsilon_{max.}$ of flavin analogues is usually 8 000—15 000 l mol^{-1} cm^{-1}. These strange phenomena were not found for (1). The optical density and the spectral shape for (3) in higher pyridine concentrations were quite analogous to those of (1).

The new absorption band at 500—700 nm is probably ascribable to a charge-transfer band.[7] It has been reported that flavins act as good acceptors, for instance, for indole, purine bases, and 1,4-dihydronicotinamides.[15-18] In Figure 4, the optical density at 550 nm (OD_{550}) as a measure of the

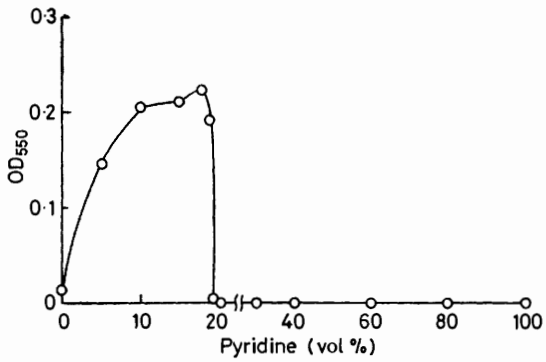


FIGURE 4 OD_{550} of (3) as a function of pyridine content: [(3)] 5.00×10^{-5}M, 30 °C

charge-transfer band strength is plotted against the pyridine concentration. The OD_{550} increased with increasing pyridine concentration and became maximum at 10—18 vol %. The absorption band has suddenly disappeared at 20 vol % where the fine structure of S1 disappeared. These results again suggest that a critical change in the aggregation mode occurs at *ca.* 20 vol % pyridine.

Fluorescence Spectra.—The fluorescence spectra of flavins are also used as probes for medium polarity, since apolar solvents bring about a blue shift of the emission maximum and enhancement of the fluoresence intensity.[19-21] In Table 4, the emission maximum ($\lambda_{max.}$) and the relative intensity (r.i.) of (1) and (3) are summarised. Typical medium effects are seen for $\lambda_{max.}$ and r.i. of (1), and the added micelles have little influence on these values. On the other hand, the r.i. of (3) in aqueous solution was much smaller than those in organic solvents. The increase in r.i. was brought forth by mixing (3) with surfactant micelles [except cetyltriethylammonium bromide (CTAB) which involves Br^- as heavy-atom quencher].[11]

Figure 5 shows the influence of added pyridine on the r.i. of (1) and (3). The r.i. value of (1) was hardly affected by pyridine, slightly increasing in 100% pyridine solvent. On the other hand, the r.i. of (3) drastically increased upon addition of small amounts of pyridine (5—10 vol %).

The foregoing fluorescence behaviour of (3) is explicable on the basis of an aggregation–deaggregation equilibrium. The weak r.i. in aqueous solution is ascribed to concentration quenching due to the association of isoalloxazine molecules. The deaggregation of (3) which may take place in organic solvents would lead to an increase in r.i. more than expected from simple solvent effects. The increase in the micellar system is attributed to the absence of concentration quenching due to the micellar ' dilution effect '.

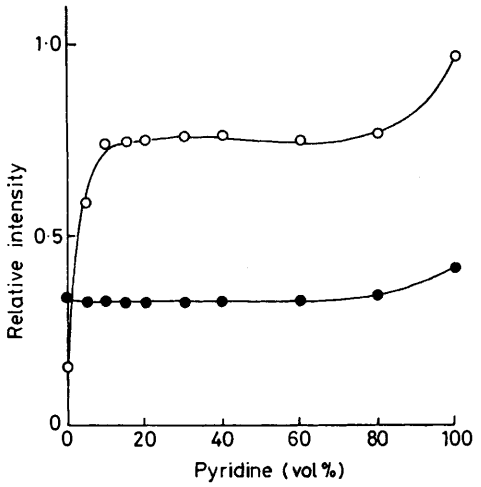
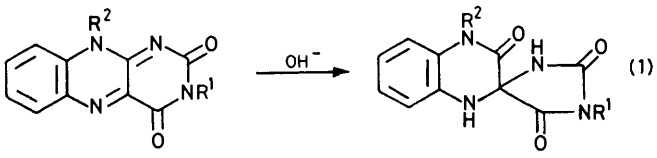


FIGURE 5 Fluorescence intensity as a function of pyridine content (20 °C): ● [(1)] 5.00 × 10^{-7}M; ○ [(3)] 5.00 × 10^{-7}M

Alkaline Hydrolysis of Isoalloxazines.—Alkaline hydrolysis of isoalloxazines had been studied by several groups.[22,23] It is now believed that the hydrolytic decomposition of isoalloxazine at high pH begins with the nucleophilic attack of OH^- on the 4- or 10a-position.



(1)

We determined the rate of the hydrolytic decomposition of the isoalloxazines (1) and (3) by following the disappearance of $\lambda_{max.}$. The rate was first-order in isoalloxazine. The first-order rate constants ($k_{hydr.}$) thus determined are summarised in Table 5. Examination of Table 5 reveals

TABLE 5

First-order rate constants ($k_{hydr.}$) for the hydrolytic decomposition of (1) and (3) [a]

Reaction medium	10^3 $k_{hydr.}$/s^{-1} (1)	10^3 $k_{hydr.}$/s^{-1} (3)	$k_{hydr.}$ for (3) / $k_{hydr.}$ for (1)
3 vol % MeCN	14.4	0.108	7.50 × 10^{-3}
EtOH (40 vol %)	4.90	15.2	3.10
Pyridine (10 vol %)	5.42	0.800	0.148
Pyridine (20 vol %)	4.23	2.38	0.563
Pyridine (30 vol %)	4.25	3.77	0.887
CTAB (10mM)	51.0	411	8.06

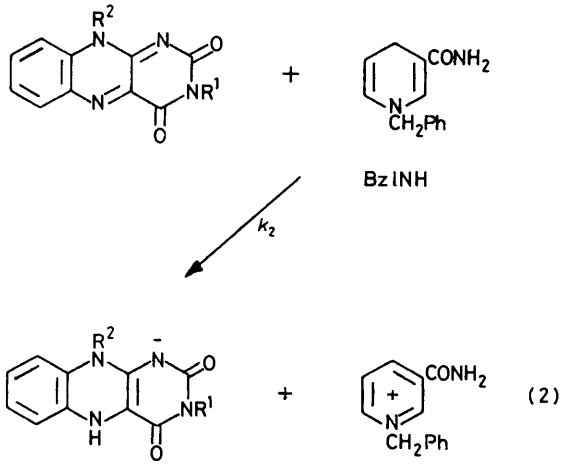
[a] 30 °C, [KOH] 0.020M, [isoalloxazine] 1.00 × 10^{-5}M. All reaction solutions contain 3 vol % acetonitrile which was used to prepare the stock solution of the isoalloxazines.

that the hydrolytic decomposition of aggregated (3) is markedly retarded, the rate constant being smaller by a factor of $>10^2$ than that for (1). This indicates that nucleophilic attack by OH^- is efficiently suppressed by the hydrophobic barrier around the isoalloxazine molecule.

As seen for (1), the hydrolysis rate of isoalloxazines is usually retarded by the addition of organic solvents. On the other hand, the hydrolysis rate for (3) was speeded up by the addition of ethanol or pyridine. This novel trend is rationalised in terms of the deaggregation phenomenon, *i.e.* hydrolysis of the monomeric isoalloxazine should be much faster than that of the aggregated isoalloxazine. Therefore, the addition of organic solvents provides two opposing influences in the reaction rate, rate retardation due to the solvent effect and rate acceleration due to the deaggregative effect. In the present case, the rate constants for (3) were enhanced by the addition of ethanol or pyridine, so that the acceleration due to the deaggregative effect overwhelmed the deceleration due to the solvent effect.

It is well known that a cationic micelle such as CTAB serves as an excellent catalyst for OH^--mediated reactions.[24] As shown in Table 5, the hydrolytic decomposition of (1) is enhanced by a factor of 3.5 in the presence of 10mM-CTAB. On the other hand, the rate constant for (3) in 10mM-CTAB is greater by a factor of 3 800 than that in aqueous solution. The remarkable rate augmentation stems from the concentration effect and the deaggregation effect of the micelle.[25]

Oxidation of N-*Benzyl*-1,4-*dihydronicotinamide* (*BzlNH*).—BzlNH is a typical NADH model compound and is rapidly oxidised by flavins. It has been established that the reaction is first-order in BzlNH and flavin.[26,27] According to Gascoigne and Radda,[28] the logarithm of the second-order rate constants (k_2) is linearly correlated with the polarographic half-wave potentials. Thus, the k_2 values for this reaction should reflect the redox potential of flavins.



(2)

The k_2 values are shown in Table 6. The k_2 values for (1) decreased with increasing the concentration of ethanol or pyridine. The rate constant is related to the highly polarised transition state of this reaction.[27] As shown in Table 6, the rate constant for (3) is smaller by a factor of 11.7 than that for (1). This is probably due to the influence of the hydrophobic region of aggregated (3). Interestingly, a plot of k_2 for (3) *versus* the concentration of ethanol and pyridine experienced a maximum at *ca.* 40 vol % and 20 vol %, respectively. These values are well in accord with the spectroscopically estimated transition concentration from aggregated (3) to monomeric (3). Therefore, two different factors are operative in the oxidation by (3), *i.e.* the rate increase due to deaggregation below the transition concentrations and the rate decrease due to the solvent effect above the transition concentrations.

It is not clear why the rate constant for (3) in 10 vol % ethanol is smaller by a factor of 12.8 than that in aqueous solution. The rate difference between (1) and (3) at this ethanol concentration amounts to 116-fold. Peculiar

kinetic parameters have frequently been observed in ethanol–water and have been attributed to changes in the water structure.[29] The present finding may be also accounted for by this phenomenon.

It is known that the CTAB micelle weakly catalyses the reaction of BzlNH and flavin.[30] The rate constant for (1) was enhanced only by a factor of 1.08 in 10mM-CTAB, whereas that for (3) was enhanced by 16.9-fold. This rate augmentation suggests that the hydrophobic region provided by aggregated (3) is stronger than that of the CTAB micelle. In any case, the hydrophobic environment is not favourable for facilitating the flavin oxidation of BzlNH.

TABLE 6

Second-order rate constants (k_2) for the isoalloxazine oxidation of *N*-benzyl-1,4-dihydronicotinamide (BzlNH) [a]

Reaction medium	k_2/l mol^{-1} s^{-1} (1)	k_2/l mol^{-1} s^{-1} (3)	k_2 for (3) / k_2 for (1)
3 vol % MeCN	14.3	1.22	0.085
EtOH (10 vol %)	11.1	0.095	8.6×10^{-3}
EtOH (20 vol %)	8.52	0.380	0.045
EtOH (40 vol %)	2.78	6.82	2.45
EtOH (60 vol %)	1.47	2.72	1.85
Pyridine (10 vol %)	14.3	2.76	0.193
Pyridine (20 vol %)	5.14	11.4	2.22
Pyridine (30 vol %)	2.76	2.66	0.964
CTAB (10mM)	15.5	20.6	1.33

[a] 30 °C, pH 9.1 with 0.02M borate buffer, [BzlNH] 1.00×10^{-4}M, [isoalloxazine] 1.00×10^{-5}M. All reaction solutions contain 3 vol % acetonitrile which was used to prepare the stock solution of BzlNH and the isoalloxazines.

Oxidation of Thiols.—It has been established that the flavin oxidation of dithiols is first-order in flavin and dithiol, while that of monothiols is first-order in flavin and second-order in monothiol.[31,32] Since the reaction rate is pH dependent,[31,32] the rate constants in this study were determined at constant pH (10.11 for butane-1,4-dithiol, 9.33 for 2-mercaptoethanol, and 8.00 for n-octyl thioglycolate).

The apparent second-order rate constant (k_2') for the oxidation of butane-1,4-dithiol by (1) (Table 7) was enhanced by the addition of ethanol or pyridine, suggesting that apolar solvents are not necessarily unfavourable to this reaction. However, the oxidation of butane-1,4-dithiol by aggregated (3) was very slow in spite of the hydrophobic nature, the rate constant being smaller by a factor of 268 than that for (1). Similarly, the apparent third-order rate constant (k_3') for the oxidation of 2-mercaptoethanol by (1) (30 °C, pH 9.33 with 0.02M-borate buffer) was 0.057 l^2 mol^{-2} s^{-1}, but the rate for the oxidation by (3) was undetectable ($k_3' < 0.001$ l^2 mol^{-2} s^{-1}). Thus, the isoalloxazine buried in the hydrophobic region is classified as a less reactive oxidant for thiols. On the other hand, the k_3' value for the oxidation of n-octyl thioglycolate (30 °C, pH 8.00 with 0.02M-borate) by (3) (0.106 l^2 mol^{-2} s^{-1}) was greater than that by (1) (0.019 l^2 mol^{-2} s^{-1}). The trend is attributed to the enhanced partitioning of n-octyl thioglycolate to the aggregated phase and/or to the activation of the thiolate anion therein.[33] The addition of the CTAB micelle further enhanced the rate constants: 1.80×10^3 l^2 mol^{-2} s^{-1} for (1) and 6.24×10^3 l^2 mol^{-2} s^{-1} for (3).

TABLE 7

Apparent second-order rate constants (k_2') for the oxidation of butane-1,4-dithiol [a]

Reaction medium	k_2'/l mol^{-1} s^{-1} (1)	k_2'/l mol^{-1} s^{-1} (3)	k_2' for (3) / k_2' for (1)
3 vol % MeCN	1.07	4×10^{-3}	3.7×10^{-3}
EtOH (60 vol %)	7.51	9.49	1.26
Pyridine (30 vol %)	1.85	3.84	2.08
CTAB (10mM)	4.23	23.2	5.48

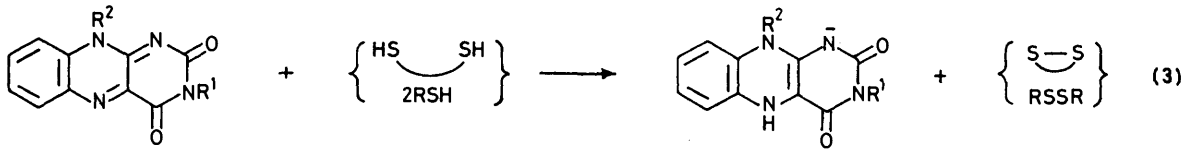
[a] 30 °C, pH 10.11 with 0.02M-carbonate buffer, [$HS(CH_2)_4SH$] 1.97×10^{-3}M, [isoalloxazine] 1.00×10^{-5}M. All reaction solutions contain 3 vol % acetonitrile which was used to prepare the stock solution of the isoalloxazines.

Micellar catalysis of flavin oxidation of thiols has been reported.[14] In the present system, the CTAB micelle gave rise to rate augmentations of 5 750- and 58 800-fold for the oxidation by (3) of butane-1,4-dithiol and n-octyl thioglycolate, respectively. Here again, one should note that the remarkable rate augmentations are caused by two different micellar effects, the deaggregative effect and the binding of thiolate ions to the micellar phase due to electrostatic and/or hydrophobic interactions.

DISCUSSION

The fine structure of S1 has been found for riboflavin-2′,3′,4′,5′-tetrabutyrate in carbon tetrachloride [8] and for 3-methyl-10-octadecylflavin in 3-methylpentane.[4] We noticed, however, that the absorption spectrum of aqueous (3) is different from those in apolar solvents in two essential points: (i) the blue shift of S2 does not occur in aqueous (3) and (ii) the S1 band of aqueous (3) has an weak absorption tail at the longer wavelength region (*e.g.*, ε at 550 nm, 240 l mol^{-1} cm^{-1}), whereas there is no such absorption tail for flavins in apolar solvents.[4,8] The differences strongly suggest that the split of S1 of aqueous (3) is induced by a factor different from that in apolar solvents.[1-8]

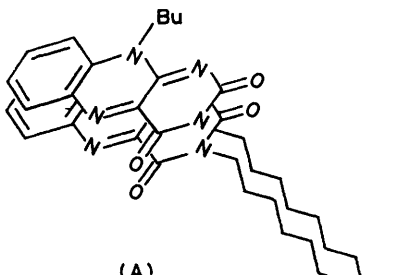
Alternatively, we propose that the fine structure of S1 of aqueous (3) is due to the ' stacking ' association of isoalloxazine head groups. It is frequently seen that ' stacking ' of dye molecules induces a split in the absorption bands in aqueous solution.[34-36] The occurrence of this stacking association is supported by the following findings: (i) the fine structure disappeared in organic solvents, (ii) it also disappeared on the addition of surfactants (above the critical micelle concentrations) which would disperse (3) in the micellar phase, and (iii) the fluorescence intensity of aqueous (3) is suppressed by concentration quenching compared with that in organic



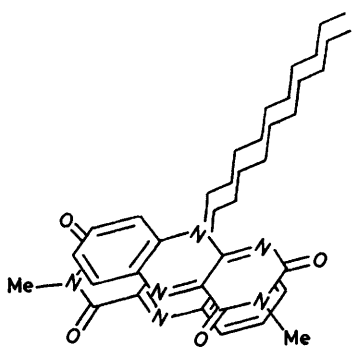
(3)

and micellar media. It is known that surfactant micelles are capable of quenching the fine structure of dye molecules.[25] This phenomenon is attributed to the deaggregation of dye molecules on the addition of the micelles. The fine structure of aqueous (3) might be rationalised in terms of the hydrophobic environment of the aggregate of (3). However, this explanation cannot be the case, because (i) the hydrophobic environment would enhance the fluorescent intensity, as expected from the solvent effect,[1,2,4,5,19-21] but the fluorescence intensity of aqueous (3) is rather suppressed, and (ii) the blue shift of S2 is generally observed for flavins bound to the hydrophobic aggregates,[11,14,37] but S2 of aqueous (3) scarcely shifts to shorter wavelength.

It was more difficult to explain why aqueous (3) has fine structure and (2) has not. However, we may explain the difference between (2) and (3) as follows, although this is still speculative. The driving force of the association is hydrophobic interaction between hydrocarbon chains. Provided that the association of the isoalloxazines occurs so that the hydrocarbon chains are parallel, the two carbonyl groups at the 2- and 4-positions of (2) must always overlap with those of other isoalloxazines as in (A). On the other hand, compound (3) with a hydrocarbon chain at the 10-position can stack in the alternative way (B). In (B), the uracil moiety of the isoalloxazine (3) is over the benzene moiety of another isoalloxazine. The dipole–dipole repulsion between two pairs of carbonyl groups is expected for the association mode (A), while such a disadvantage is absent in (B). Thus, the stacking association of the isoalloxazine nuclei of (3) would be largely facilitated.



(A)



(B)

It is known that the S1 peak of some flavoproteins is partially resolved into the three-band peak.[38] For example, a flavoenzyme, D-amino-acid oxidase gives a split S1 peak which appears upon binding of the enzyme with benzoate and its derivatives.[9,10,39] Massey and Ganther [9] explained that the split is due to the hydrophobic surroundings around the isoalloxazine nucleus of flavin adenine dinucleotide (FAD). The present results suggest that in addition to the hydrophobic surroundings, the isoalloxazine nucleus of FAD (in particular, the uracil moiety) [10] may interact with benzoate in a stacking manner.

Here, we would like to consider why aqueous (3) provides the strong charge-transfer band with pyridine and aqueous (1) does not. Martin and Standing [34] have proposed the formation of pyridine complexes with dye molecules, which is reflected by the increase in the $\epsilon_{max.}$ and the red shift of the absorption maxima. Similarly, it is known that addition of pyridine to an aqueous solution of 3-methyl-lumiflavin reveals a weak but measurable interaction.[7] Probably, the difference between (1) and (3) may be rationalised as (i) the stacking aggregation strengthens the acceptability of the isoalloxazine, for example, owing to the formation of a 1 : 2 pyridine–isoalloxazine sandwich-type complex or (ii) the local concentration of pyridine in the aggregate of (3) is enhanced owing to the hydrophobic properties.

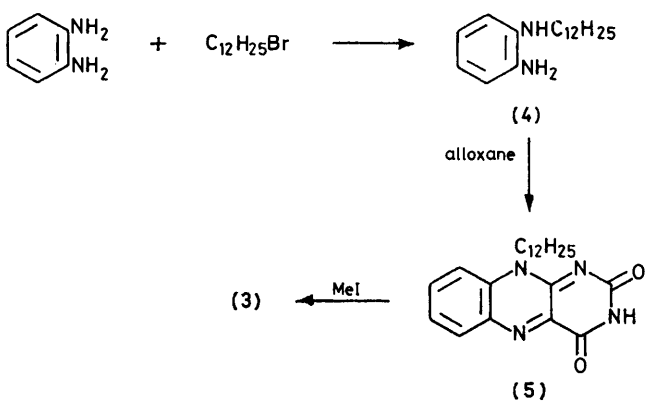
We have evaluated the reactivity of aggregated (3). In all cases examined in the present study, the reactivity of aggregated (3) is surprisingly suppressed. The suppressed reactivity is either due to the stacking of isoalloxazine molecules or due to the hydrophobic environment. In the present system, the stacking aggregation inevitably accompanies the hydrophobic environment, so that we cannot specify these two effects separately. In any case, it is interesting that the reactivities of flavin analogues can be changed readily by controlling the reaction environment around the isoalloxazine molecule.

In conclusion, the present study has demonstrated that fine structure is observed for the S1 peak of the amphiphilic flavin analogue of (3). The results of the spectral measurements support the conclusion that the split of S1 is due to the stacking association of the isoalloxazine head groups of (3). The peculiar spectral behaviour implies that the redox behaviour of amphiphilic flavins should also be different from those of simple flavins.

EXPERIMENTAL

Materials.—The preparations of (1) and (2) were described previously.[14,40] Compound (3) was synthesised by the sequence in the Scheme. *o*-Phenylenediamine (24.0 g, 0.22 mol) was dissolved in absolute methanol (*ca.* 500 ml), and dodecyl bromide (11.1 g, 0.045 mol) dissolved in methanol (300 ml) was added dropwise to the above solution under nitrogen. The mixture was refluxed while addition was continued (4.5 h), and after the completion of addition, the solution was further refluxed for 6 h. Methanol was

evaporated *in vacuo*, the residue being dissolved in benzene (*ca.* 100 ml). The benzene solution was washed with water (5—8 × 300 ml) to remove unchanged *o*-phenylenediamine. The removal of *o*-phenylenediamine was confirmed by the t.l.c. method (silica gel–hexane). T.l.c. gave two spots at R_F 0.5 and 0.8. The spot with R_F 0.8, which was much the smaller, is probably ascribed to *N,N'*- (or *N,N*-) didodecyl-*o*-phenylenediamine. The benzene solution was dried (Na_2SO_4) and concentrated *in vacuo*. The resultant solid (slightly red, 13.5 g) was used without further purification for the synthesis of 10-dodecylisoalloxazine (5).



SCHEME

Raw product (4) (13.5 g) and boric acid (3.6 g) were dissolved in absolute methanol (300 ml), and the solution was stirred at 60 °C. Alloxane monohydrate (8.3 g, 0.059 mol) was added in small portions over 15 min. Heating was continued for an additional 1 h. The solution gave a yellow precipitate which is characteristic of flavins. After cooling in an ice-bath, the precipitate was collected by suction and recrystallised from ethanol; *10-dodecylisoalloxazine* had m.p. 261—263 °C, yield (from dodecyl bromide) 49% (Found: C, 65.6; H, 8.0; N, 14.1. $C_{22}H_{30}N_4O_2$ requires C, 69.1; H, 7.85; N, 14.65%); δ($CDCl_3$) 0.90 (t, CH_3), 1.04—1.48 (m, $C_{10}H_{20}$), 4.74 (t, NCH_2), and 7.6—8.6 (m, 6,7,8,9-aromatic protons).

Compound (3) was synthesised by methylation of (5) with methyl iodide. Compound (5) (2.0 g, 5.2 mmol) was dissolved in *NN*-dimethylformamide (240 ml) containing finely powdered potassium carbonate (7.2 g) and the solution was heated at 45—50 °C. Methyl iodide (7.4 g, 52 mmol) was added to the above solution, and heating was continued for 6 h. The progress of the reaction was followed by t.l.c. (silica gel–ethyl acetate). The spot for (5) had disappeared after the above treatment. After removal of potassium carbonate by suction, the solvent was evaporated *in vacuo*, the residue being recrystallised from ethanol–isopropyl ether; *10-dodecyl-3-methylisoalloxazine* had m.p. 177—179 °C, yield 87% (Found: C, 69.15; H, 8.2; N, 14.05. $C_{23}H_{32}N_4O_2$ requires C, 69.7; H, 8.1; N, 14.15%); δ($CDCl_3$) 0.88 (t, CH_3), 1.18—1.60 (m, $C_{10}H_{20}$), 3.49 (s, NCH_3), 4.68 (t, NCH_2), and 7.5—8.3 (m, 6,7,8,9-aromatic protons).

The preparation of BzlNH was described previously.[26] The purification of butane-1,4-dithiol and 2-mercaptoethanol was described previously.[14] n-Octyl thioglycolate was purchased from Tokyo Kasei Kogyo and used without further purification.

Kinetics.—All the kinetic measurements were carried out at 30 ± 0.1 °C. The rate of the hydrolytic decomposition in 0.02M-KOH was followed spectrophotometrically by monitoring the disappearance of the absorption band of each isoalloxazine [433 nm for (1) and 447 nm for (3)]. The first-order behaviour was observed for at least up to four half-lives. The rates for the oxidation of BzlNH were estimated at pH 9.1 (0.02M-borate) under aerobic conditions where isoalloxazine is used in recycle. The progress of the reaction was monitored by following the disappearance of the absorption maximum of BzlNH (357 nm). Details of the method were described in a previous paper.[27] Under the experimental conditions ([isoalloxazine] 1.00 × 10^{-5}M, [BzlNH] 1.00 × 10^{-4}M), the reaction was first-order in isoalloxazine and BzlNH. The reaction of isoalloxazine and thiols was carried out under anaerobic (N_2) conditions by using a Thunburg cuvette. Since thiolate anions in the CTAB solution are very sensitive to air,[14] the stock solution of thiols was made in ethanol just before the experiment. After bubbling N_2 into the ethanolic solution in the side-arm of the Thunburg cuvette and the buffer solution containing isoalloxazine and CTAB in the bottom, the two solutions were mixed. In the oxidation by (1), the rates which were followed by monitoring the disappearance of the absorption maxima of the isoalloxazine were first-order for up to three half-lives. On the other hand, the oxidation of butane-1,4-dithiol by (3) was so slow that the rate constant was evaluated by analysing the Guggenheim plot for the 15% reaction.

Spectral Measurements.—The absorption spectra of isoalloxazines were taken at 30 ± 0.1 °C on a Hitachi 200 spectrophotometer equipped with a thermostatted cell-holder. The concentration of (1) and (3) was usually 5.00 × 10^{-5}M and that of (2) was 1.00 × 10^{-5}M. The fluorescence spectra were taken at 20 ± 0.1 °C (unless otherwise stated) on a Hitachi 650-10S spectrophotometer equipped with a thermostatted cell-holder. The concentration of (1) and (3) was usually 5.00 × 10^{-7}M. The excitation wavelength was 380 nm and the slit widths for excitation and emission were 3 and 15 nm, respectively.

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