Tetrahedron Vol. 39, No. 20, pp. 3359 to 3366, 1983
Printed in Great Britain.

0040-4020/83 $3.00 + .00
Pergamon Press Ltd.

ACTIVATION AND TRANSFER OF OXYGEN—XVI

SPONTANEOUS ELECTRON TRANSFER IN FLAVINIUM SALT SOLUTIONS. A NEW PRINCIPLE FOR THE DEVELOPMENT OF OXYGEN ACTIVATION MODELS

H. I. X. MAGER* and R. ADDINK
Biochemical and Biophysical Laboratory of the Delft University of Technology, 67 Julianalaan, 2628 BC Delft, The Netherlands

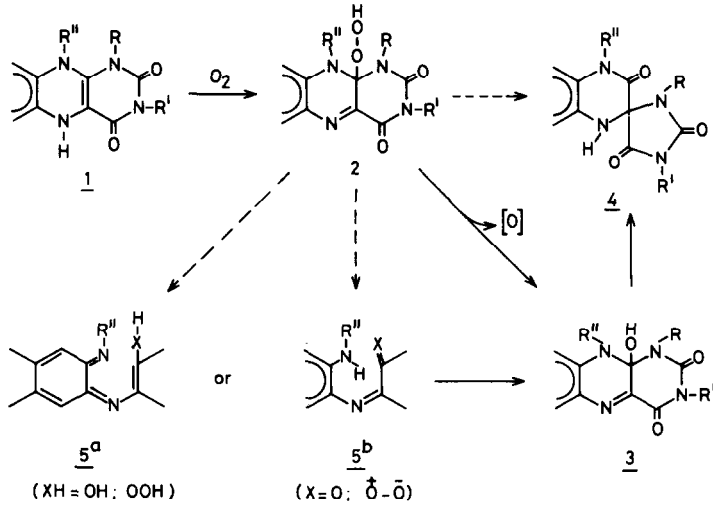
(*Received in UK 4 January 1983*)

Abstract—Blue coloured 10, 10[a]-ring opened intermediates **5**[a], **5**[b] arising in the autoxidation of a dihydroflavin model (Scheme 1) are also formed on proper treatment of some N^1-alkylflavinium salts **7** (Scheme 2). The conditions giving optimal 10,10[a]-ring opening have been determined (Figs. 1–2). The flavinium salts (RFl^+_{ox}, A^-) show spontaneous electron transfer in the dark, producing a flavosemiquinone (RFl·) and a counter-radical, probably a radical cation ($RFl\text{-}A^+$) derived from a flavin adduct (pathway b, Scheme 3). The formation of a neutral, unstable 1-RFl· appeared from the spontaneous N^{10}-dealkylation (pathway d_2) competing with the O_2-activation (pathway c_1). A generation of CO_2 may occur which indicates the formation of an unstable acyloxy radical (A·) by a decomposition of $RFl\text{-}A^+$ (pathway d_3). Cl_3CCOO^- can even be catalytically decomposed by RFl^+_{ox}. This proves that RFl^+_{ox} is recycled from the RFl·-state also (pathway d_1) for which O_2 is not a prerequisite. On the other hand, Cl_3CCOO^- is "repaired" under conditions giving a 10,10[a]-ring opening (Fig. 3). The preservation of the acid anion and the results of the O_2-balance are consistent with the conclusion that the 10,10[a]-ring opening is coupled with or followed by an electron transfer from a peroxy radical to $RFl\text{-}A^+$ giving a generation of O_2 (Scheme 4). A 10,10[a]-ring opened hydroperoxide **5**[a] (XH=OOH; Scheme 1) is proposed to be the result of a similar one-electron transfer reaction (A=OOH; Scheme 4).

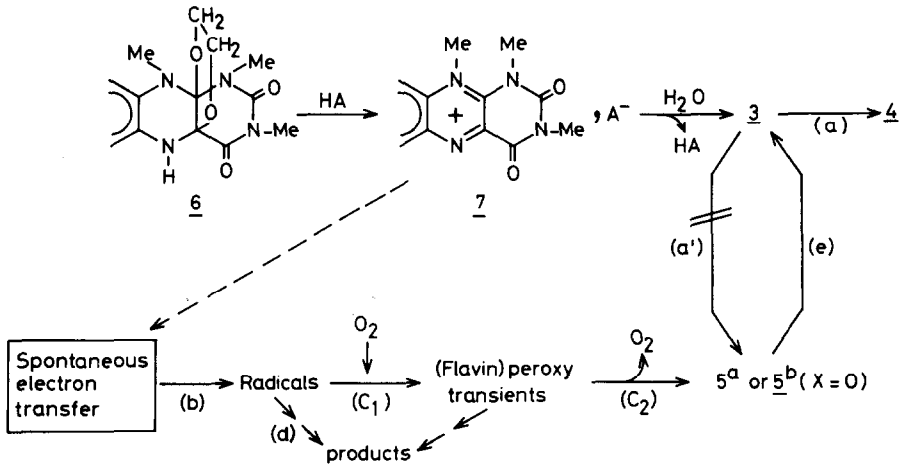
The original concept[1] on the key-role of hydroperoxyflavin transients in dihydroflavin mediated activation and transfer of O has been substantiated by the results of studies on N^1- and N^5-alkylated flavin derivatives. The presence of an alkyl group at N^1 or N^5 has a blocking effect revealing the C^{10a}- and C^{4a}-bridge positions as the reaction sites. The oxidative conversion of the N^1-blocked dihydroflavin model **1** (R=R′=R″=Me) into the C^{4a}-spirohydantoin **4** can occur by different pathways (Scheme 1). In the actual O-transfer step (**2**→**3**) a ring transformation has never been found to be essential. The spirohydantoin **4** is the result of a sequential reaction (**3**→**4**) proving the occurrence of the evasive precursors **2** and **3**.[2,3]

In contrast, ring opening of the flavin hydroperoxide has been postulated[4] to be essential in the O-transfer. A carbonyloxide like **5**[b] (X=O⁺–O⁻) or an isomer arising from a 4[a]-hydroperoxyflavin is supposed to transfer one atom of O to a substrate. For this non-radical ("oxene") mechanism, however, no evidence has ever been presented.

In the early studies[2,3] on the formation of the spirohydantion **4** the occurrence of a blue precursor ($\lambda_{max} \sim$ 600 nm) was already observed when the O_2 activation was carried out in low polar media. A controversy arose on the occurrence of 10[a]-adducts like **2** and on their spectral characteristics.[5–7] After establishing that 10[a]-adducts do occur and that they are definitely not blue,[7]



Scheme 1. 10,10[a]-Ring opened intermediates (**5**[a] or **5**[b]), causing a blue-colouration in the autoxidative process.



Scheme 2. Blue-colouration (formation of 5^a or 5^b) indicating the occurrence of electron transfer and O_2-activation in 1,3,10-trimethylalloxazinium salt solutions.

we proposed[8,9] that 10,10ª-ring opened intermediates 5^a or 5^b are responsible for the blue colouration. The opening of the pyrazine ring is indicated by competitive rearrangements leading to benzimidazoles.[10] In addition, the spectrum[11] of the blue precursor (5^a or 5^b, X=O) of the spirohydantoin is similar to the spectra of products of failed ring closures in flavin syntheses.[12–14]

In contrast with the expectation which may be based on the oxene-theory, the blue colouration (10,10ª-ring opening) was not coupled with an increase but with a drastic disappearance of the ability to transfer O to an aromatic substrate. The mechanistic aspects of this have been subjected to further studies to which an impulse was given by the finding that a blue colouration also takes place on dissolving certain flavinium salts (7, Scheme 2) in some organic solvents. Since the starting compounds 7 are on the level of a completely oxidized flavin it seemed obvious[9] to describe the formation of 5^a or 5^b (X = O) as the result of a non-oxidative pathway a', revealed by a retarding effect of a low polar solvent on the rearrangement or 3 to 4 (pathway a). It will be shown in the present paper that this tentative formulation of pathway a' is incorrect. This is a consequence of the surprising finding that, in spite of the oxidation state of the flavinium salts 7, a blue colouration (pathway c_2) is a sequential step following an *activation of* O_2 (step c_1). This implies that the blue colouration has served as an indicator for the proceeding of some spontaneous electron transfer processes starting with the formation of reduced flavin species (step b).

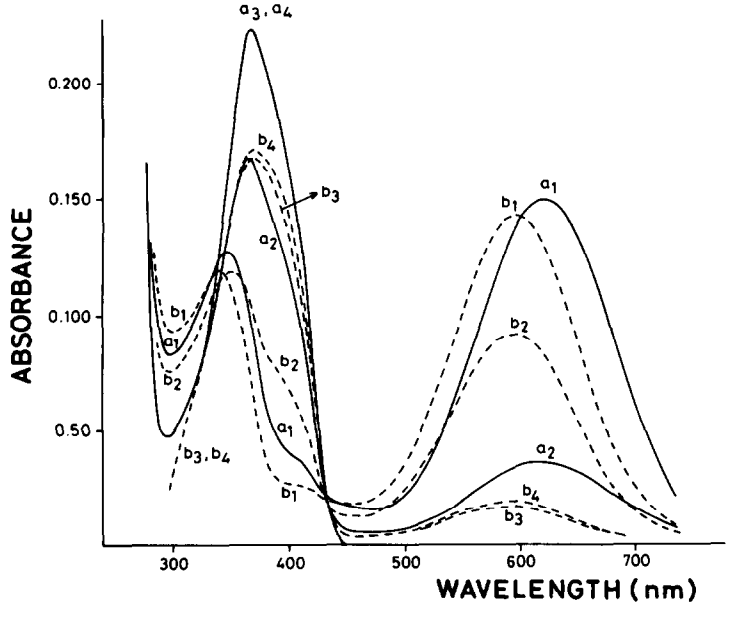


Fig. 1. Spectra recorded at maximal blue colouration of 1.86×10^{-5}M solns of 7 ($A^- = CF_3COO^-$) at 25° in: (a_1) H_2O-satd $CHCl_3$/air; (a_2) $CaCl_2/K_2CO_3$-dried $CHCl_3$/air; (a_3) H_2O-satd $CHCl_3/N_2$; (a_4) $CaCl_2/K_2CO_3$-dried $CHCl_3/N_2$; (b_1) H_2O-satd benzene/air; (b_2) sodium-dried benzene/air; (b_3) H_2O-satd benzene/N_2; (b_4) sodium-dried benzene/N_2. (Procedure: MeCN-stock solns of the salt were diluted with $CHCl_3$ or benzene in the ratio of 1:250).

RESULTS AND DISCUSSION

The 10,10ª-ring opening can be clearly demonstrated using 1,3,10-trimethylalloxazinium salts **7** derived from organic acids. Starting from the ethylenedioxy-bridged adduct **6** the salts can be conveniently prepared *in situ* or isolated like the trifluoroacetate and the trichloroacetate. These were obtained from ethereal solutions as crystals containing one molecule of acid of crystallization. The stability of these salts in crystalline state is in remarkable contrast with the spontaneous behavior in various solutions. In the competition with the conversion of **7**→**3**→**4**, the blue colouration (pathway c_2) has appeared to be dependent on several factors as: (a) the nature of the anion; (b) the nature of the solvent; (c) the presence of small amounts of water and alcohols in the solvent; (d) the presence of molecular oxygen; (e) the concentration of the alloxazinium salts.

(a, b) *The nature of the anion and the solvent.* Solutions of the trifluoroacetate in acetonitrile are reasonably stable, also when the solvent contains slight amounts of water (<0.05%). Stock solutions of both the perchlorate and the trifluoroacetate in acetonitrile ($4 \sim 5 \times 10^{-3}$ M) were used for further analytical work. The absorption spectra of these salts in acetonitrile were identical.

On dissolving the salts in water no differences were observed in the conversion to the spirohydantoin **4** as judged by the appearance of the 300 nm peak, the disappearance of the 370 nm peak of the cation and the fact that no intermediates including the 10ª-pseudobase **3** could be detected (*cf* Fig. 1 in ref. 11). However characteristic differences were observed between the perchlorate and the trifluoroacetate on diluting the respective stock solutions with solvents like benzene, toluene, xylenes, chloroform, ethyl acetate, dioxane, etc. The dilutions of the perchlorate only showed the presence of the cation or its conversion into the spirohydantoin **4** (Schemes 2 and 3; pathway a, R'=H), while the dilutions of the trifluoroacetate could rapidly change to give striking blue colourations (for example, curves a_1 and b_1, Fig. 1). Apparently, there are several pathways in the conversion of flavinium salts (Scheme 3) depending on the available nucleophiles and the nature of the solvent. The solvent determines the conversion of the ionic bond into a covalent one at a bridge C atom, for example with $R'O^-$ (R'=H, alkyl, etc.). The acid anion effect is supposed to be primarily due to a covalent bond formation with A^- to give a (1,10ª- or 4ª,5-)dihydroflavin ester (RFl–A) acting as a key-transient in other pathways.

(c) *The presence of small amounts of water and alcohols in the solvent.* The effects of relatively small amounts of water and alcohols on the rate and the degree of the blue colouration is illustrated in Fig. 2. Starting from a stock solution in reagent grade acetonitrile (H_2O-content <0.05%), a 250-fold dilution with water-saturated chloroform gave rise to a blue colouration as given by curve 1, while the complete spectrum is represented by curve a_1 (Fig. 1). Curves 2–7 (Fig. 2) demonstrate the effects of the stepwise decrease of the water content of the chloroform on the blue colouration. As compared with curve 1 (Fig. 2) and curve a_1 (Fig. 1) a spectacular decrease of 77% is shown by curves 7 and a_2, respectively, while the time to reach the maximal colouration (t_{max}) increased from 8 to 20 min. The final dilutions contained 0.4% of acetonitrile by which water might have been introduced up to a content of 2 ppm. The question arose whether water was still the cause for the blue colouration remaining (curve 7). Likewise, the increase of the relative absorbance of curve 8 as compared with curve 7 might have been caused by less than 1.5 ppm of water, concomitantly introduced on raising the final content of the acetonitrile from 0.4 to 0.7% However, the use of acetonitrile freshly and successively

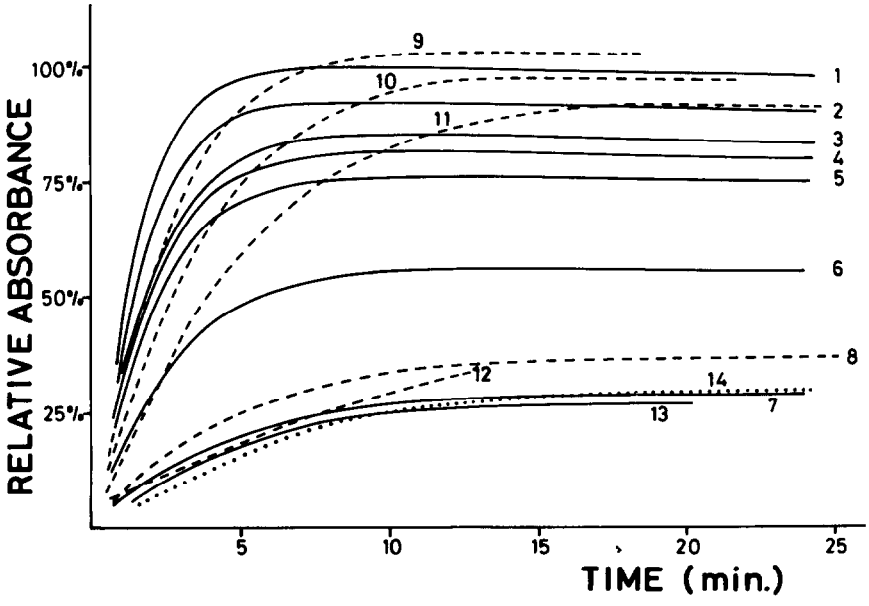


Fig. 2. Relative E_{624} vs time of 1.86×10^{-5}M solns of **7** (A^-=CF_3COO^-)/air at 25° in: (1) H_2O-satd $CHCl_3$; (7) $CaCl_2/K_2CO_3$-dried $CHCl_3$; (2–6) in mixtures of H_2O-satd $CHCl_3$ and $CaCl_2/K_2CO_3$-dried $CHCl_3$ in the ratios of: 5:1 (2); 2:1 (3); 1:1 (4), 1:2 (5); 1:5 (6); (8) in $CaCl_2/K_2CO_3$-dried $CHCl_3$/MeCN (0.7% instead of 0.4%); (9–12) in H_2O-satd $CHCl_3$/EtOH (0.1% (9), 0.2% (10); 0.3% (11); 1.0% (12)). (The final dilutions 1–7 and 9–12 contained 0.4% of reagent grade MeCN (H_2O < 0.05%). Starting from a stock soln of the salt in freshly distilled MeCN and diluting with dried $CHCl_3$ gave curves 13 and 14, which have to be compared with curves 7 and 8, respectively).

distilled over CaH_2 and P_2O_5 gave only slight differences as represented by curves 13 and 14. These results suggest that besides a water-controlled 10,10ª-ring opening, a similar reaction could take place without the aid of water, to an extent of 23% in dried chloroform. In benzene dried over sodium (curve b_2, Fig. 1) a relative blue colouration of 63% was obtained as compared with the experiment in water-saturated benzene (curve b_1).

The presence of 0.05–0.1% of ethanol or methanol in the dried or water-saturated solvents may slightly increase the blue colouration as is illustrated by curve 9 (0.1% of EtOH; t_{max} = 11.5 min). An increase of the t_{max} was found on increasing the content of ethanol to 0.2, 0.3 and 1.0% (curves 10–12). This is due to the formation of the 10ª-ethoxy adduct (RFl–OR': R'=Et) as appeared from the increased absorbance in the 410 nm region and from the fluorescence emission at 529 nm.[15]

It is concluded that in comparison with water, alcohols may have a similar but smaller affect on the 10,10ª-ring opening, but that their main role is in the formation of alkoxy adducts (RFl–OR'; Scheme 3), acting as buffer intermediates for the flavinium salts. The adduct **6** (Scheme 2) can also behave as such a storage compound when an alloxazinium salt is prepared in situ as exemplified in Fig. 3.

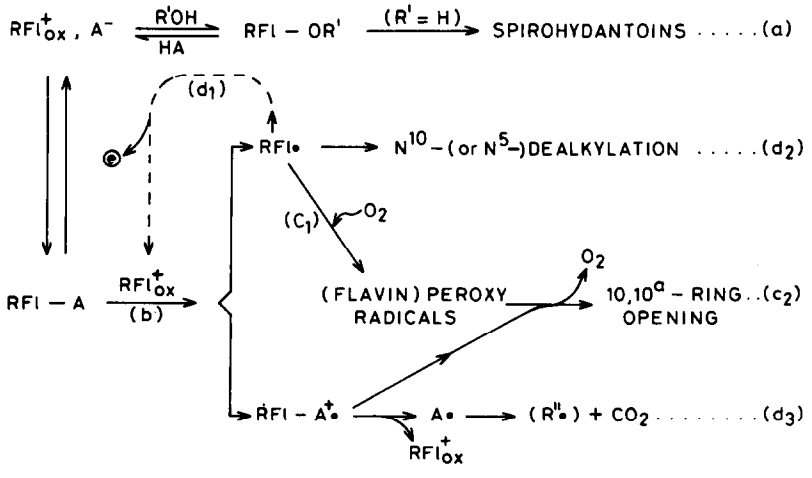
(d) *The presence of molecular oxygen.* Under N_2, the spectra b_3 and b_4 (Fig. 1) were obtained for dilutions made with water-saturated and Na-dried benzene. The relative absorbances at 596 nm were 11% and 13%, respectively, showing that water was not an important factor. Dilutions made anaerobically with water-saturated and dried chloroform gave results even more dramatic than those mentioned in the preliminary paper:[16] *curves a_3 and a_4 show no blue colouration at all.* The requirement for molecular O_2 to obtain a 10,10ª-ring opening was quite unexpected in view of the oxidation level of the starting compound **7**. This new finding proves that in some way the alloxazinium salt has first reacted to give reduced flavin species (pathway b, Scheme 2), subsequently converted to peroxyflavins (step c_1), liable to 10,10ª-ring opening (step c_2).

The occurrence of pathway b (Scheme 2) further appeared from the identity of the products arising on blocking the activation of O_2 (step c_1). Anaerobic conditions should lead either to the accumulation of radicals or to their products of degradation (pathway d). An accumulation of a neutral flavosemiquinone (RFl·) can only be expected to take place in the more stabilized N^5-alkylflavin model series. The evidence indeed found for this is presented in a separate paper.[17,18] On the other hand, the product of a spontaneous N^{10}-dealkylation has served as an indication for the occurrence of a neutral semiquinone in the N^1-alkylflavin model series known from other studies to be unstable. In the experiments of Fig. 1 the formation of 1,3-dimethylalloxazine was revealed by a fluorescence with an emission maximum at 445 and excitation maxima at 324 and 382 nm in chloroform and at 321 and 380 nm in benzene. The 1,3-dimethylalloxazine was also determined by the absorbance spectra for the completely bleached reaction mixtures obtained after standing for some days at room temperature in the dark. After improving the techniques to remove traces of O_2, anaerobic conditions showed a considerable increase of the N^{10}-dealkylation (*cf* Table 1) as appears from the comparison of the experiments: a_3 vs a_1; a_4 vs a_2; b_3 vs b_1; b_4 vs b_2. Some increase of the N^{10}-dealkylation took place on inhibiting the 10,10ª-ring opening (step c_2) by the exclusion of water (*cf* Table, exp, a_2 vs a_1; b_2 vs b_1).

(e) *The concentration of the alloxazinium salts.* In competition with step c_2 (Scheme 3), the radical con-

Table 1. Yields of the spontaneous N^{10}-dealkylation of **7** (A^- = CF_3COO^-) determined in the reaction mixtures of Fig. 1 after complete bleaching had taken place at room temperature in the dark[19]

Number of Exp.	Conditions	Yield of 1,3-dimethylalloxazine
$\underline{a}_1$	$CHCl_3/H_2O/O_2$	4–5%
$\underline{a}_3$	$CHCl_3/H_2O/N_2$	55%
$\underline{a}_2$	$CHCl_3$/ - /O_2	15%
$\underline{a}_4$	$CHCl_3$/ - /N_2	58%
$\underline{b}_1$	$C_6H_6/H_2O/O_2$	2–3%
$\underline{b}_3$	$C_6H_6/H_2O/N_2$	51%
$\underline{b}_2$	C_6H_6/ - /O_2	6%
$\underline{b}_4$	C_6H_6/ - /N_2	51%



Scheme 3. Some competitive pathways in the conversion of alkylflavinium salts.[18]

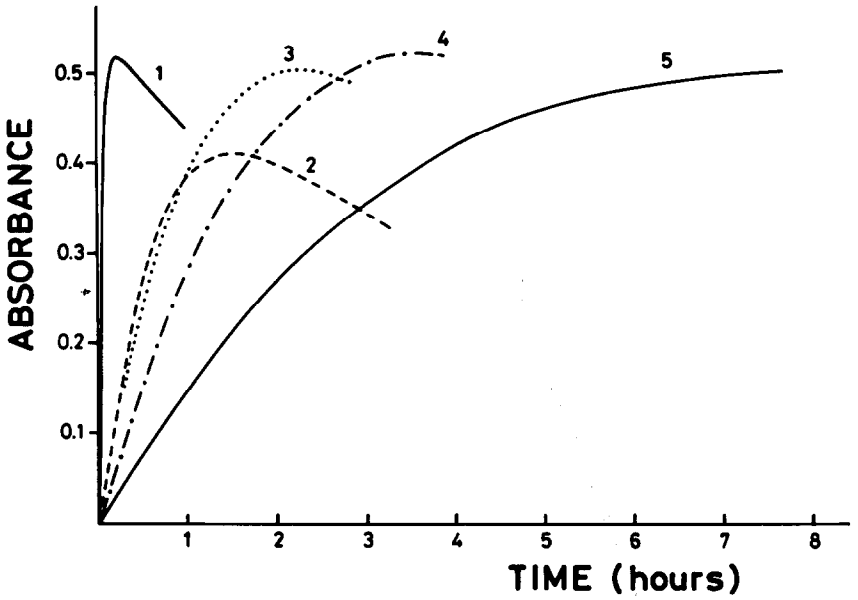
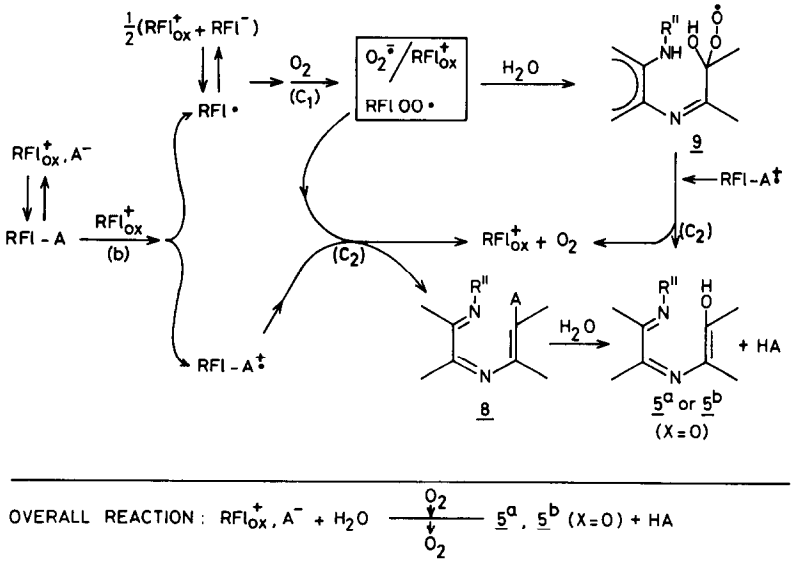


Fig. 3. E_{596} vs time in dependence on the relative amount of acid, in the presence of air, at 25°. H_2O-satd benzene solns (10^{-4}M) of: (1) the salt **7** (A^-=CF_3COO^-; 1 mole of CF_3COOH of crystallization); (2) the adduct **6** + TCA (1:2); (3) the adduct **6** + TCA (1:1); (4) the adduct **6** + TCA (2:1); (5) the adduct **6** + TCA (4:1). (The final dilutions contained 0.4% of MeCN).

suming pathway d_2 occurs more readily in chloroform than in benzene. This is reflected by the maximal concentration of the salt giving an optimal blue colouration. For the trifluoroacetate, it proved to be in the order of $1.5 \sim 2 \times 10^{-5}$ M in chloroform, while twice this concentration is admissible in benzene.

The primary electron transfer occurring in flavinium salt solutions leads to a flavosemiquinone and a counter-radical. We propose that the latter is a radical cation (RFl–$A^{\ddagger}$) derived from a dihydroflavin ester by the loss of one electron to a flavinium cation (pathway b, Scheme 3). Under certain conditions a generation of CO_2 may take place indicating the formation of an unstable acyloxy radical (A·) by a decomposition of RFl–$A^{\ddagger}$ (pathway d_3). This is well illustrated by the behaviour of the more reactive trichloroacetate **7** (A^-=Cl_3CCOO^-). On its dissolving in acetonitrile at room temperature, a rapid evolution of CO_2 occurred either in the absence or presence of O_2. Even a decomposition of an excess of trichloroacetic acid can be accomplished revealing a catalytic role of RFl^+_{ox}. This requires a recycling of RFl^+_{ox} from the RFl·-state also. Since in this step d_1 oxygen is not a prerequisite, the radical $R^{\prime\prime}$(=Cl_3C·) is considered to be the electron-acceptor. Some of the products remain to be characterized.

A decomposition of the trichloroacetate anion in



Scheme 4. The 10,10[a]-ring opening is coupled with or followed by the transfer of an electron from a peroxy radical to the radical cation RFl–$A^{\ddagger}$.

acetonitrile (pathway d_3) is not accompanied by a blue colouration. In the presence of O_2 both a generation of CO_2 and a consumption of O_2 could be established.

In contrast, no generation of CO_2 and no overall consumption of O_2 were found when mixtures had shown a high degree of blue colouration. That the degradation of the anion by pathway d_3 has been displaced at least as a main process is demonstrated by experiments (Fig. 3) performed in solutions of water-saturated benzene, using the degree of the blue colouration as a criterion. Curve 1 shows the blue colouration starting from the crystalline trifluoroacetate (which contains one molecule of CF_3COOH of crystallization). Its maximal absorbance is taken as a ref.[20] Curves 2–5 represent the results of experiments in which the trichloroacetate was prepared *in situ* by adding an excess, 1 equiv and less than 1 equiv of CCl_3COOH to the adduct **6**, respectively. The curves illustrate that in the blue colouration process the scissions of the ether bonds of **6** are the rate-determining steps in dependence on the concentration of the acid. However, the final degree of the blue colouration of curves 4 and 5 is practically not influenced by the presence of less than one equivalent of the acid proving that the acid and the anion had been appreciably preserved.

From the experimental evidence it has become clear that an optimal blue colouration results from blocking the processes d_1–d_3 (Scheme 3). No overall consumptions of O_2 and acid anions occur. These facts imply that in the actual 10,10^a-ring opening step c_2, the acid anion is "repaired" with a formation of O_2 to nullify the O_2-uptake of step c_1. These conclusions provide some insight in the mechanism of the 10,10^a-ring opening. They are most consistent with the assumption that the O_2-activation (step c_1; Scheme 4) is followed by an electron transfer in which a flavinperoxy- or superoxide-radical is the electron-donor. The preservation of the acid anion (A^-) requires the participation of RFl–A^+ as the electron-acceptor. A water-independent blue colouration has to be distinguished from a water-controlled process. The stimulating role of water is supposed to be either in: (a) the formation and participation of RFl–OH^+ as the electron-acceptor or in (b) the formation and participation of HOO· radicals or in (c) a hydrolytic 10,10^a-ring opening of one of the participating organic radicals as exemplified for RFlOO· in Scheme 4.

CONCLUSIONS

The most important result of the present investigations is the establishment of the fact that various electron transfer reactions can take place spontaneously in the dark in solutions of N^1-(N^5-)alkylflavinium salts (RFl^+_{ox}, A^-). The primary products are a flavosemiquinone (RFl·) and a counter-radical, probably a radical cation (RFl–A^+) derived from a dihydroflavin adduct (RFl–A), being a dihydroflavin ester in this particular case. RFl–A^+ can either regain an electron in the preservation of the acid and flavinium ions or act as a precursor of an acyloxy radical (A·) which may decarboxylate as shown for the trichloroacetate model. A catalytic role of RFl^+_{ox} in the decomposition of trichloroacetic acid proves that RFl· has also been recycled to RFl^+_{ox}.

While a neutral flavosemiquinone (5–RFl·) can be accumulated starting from N^5-alkylflavinium salts,[17] such a radical accumulation is not to be accomplished in the N^1-alkylflavin model series, because of the instability of the neutral 1–RFl·-semiquinones already known from other studies. Their occurrence can be derived from subsequent reactions like a typical degradation (N^{10}-dealkylation) or the reaction with O_2. The latter may be followed by a 10,10^a-ring opening, indicated by the appearance of absorbance maxima in the 600 nm region. Such a blue colouration has served as the first means to diagnose the primary and subsequent electron transfer reactions taking place spontaneously in flavinium salt solutions.

Cleavages of the pyrazine ring part of hydroperoxyflavins have been proposed[4] to be essential in flavin-mediated, enzymatic oxygenations. No evidence for this hypothesis has ever been presented. During the activation of O_2 by N^1-alkylflavin models (Scheme 1) a blue colouration may occur which had not been connected with a 10,10^a-bond scission until 1978.[8] The finding that the same blue colouration takes place on proper treatment of some N^1-alkylflavinium salts provided new possibilities to study the mechanistic aspects of the 10,10^a-ring opening. It is concluded that the 10,10^a-ring opening is coupled with or followed by the transfer of an electron from a peroxy radical to RFl–A^+ (Scheme 4).

We now assume that the reactions shown by the dihydroflavin esters (RFl–A; Scheme 4) are not unique, but that these properties are more or less shared with other types of adducts like the alkoxy-, hydroxy- and hydroperoxy-flavins having the same dihydroflavin structure. This implies the possible occurrence of an electron donation giving a radical cation like RFl–OR'^+, RFl–OH^+ or RFl–OOH^+ followed by the return of an electron which may be coupled with a 10,10^a-ring opening. Accordingly, the blue colouration in the autoxidation process (Scheme 1) might be due to a primary 10,10^a-ring opening of either the hydroperoxyflavin **2** or the pseudobase **3** or of both species, but distinguishing between these possibilities will be difficult. Any formation of the ring opened hydroperoxide **5**^{a} (XH=OOH; R″=Me) or the isomeric carbonyloxide **5**^{b} ($X=O^+-O^-$) must have immediately given rise to the loss of half a mole of O_2 or to the coupled oxidation of another reduced flavin species. This is consistent with the early observation[2,3] that starting from this dihydroflavin model **1** (Scheme 1), a blue colouration is accompanied by a nett uptake of half a mole of O_2 and a complete loss of the capacity to transfer O to another substrate.

It is emphasized that a 10,10^a-ring opening of N^1-alkylflavin models occurs in strong dependence on the experimental conditions. A return of an electron to the oxidized adduct RFl–A^+ could have other consequences. Basically, a similar one-electron return to an oxidized adduct (RFl–OH^+) has been claimed by McCapra[21] to be a chemiluminescent reaction. In this connection it would be of interest to study whether suitable flavinium salts could be developed to luminescent model systems.

In some flavoprotein-catalyzed oxidations of acidic substrates (internal monooxygenases, aminoacid oxidases) a proton is supposed to be abstracted from the α-position of the substrate to give a carbanion which reduces the flavin cofactor. A parallel was shown to exist between the enzymes lactate 2-monooxygenase and D-aminoacid oxidase in catalyzing the elimination of halide from β-halogene substrate analogs, being the first results compatible with the carbanion-concept.[22,23] The reduction of flavins by carbanions should be distinguished

from the formation of reduced flavin species as described in the present paper.

EXPERIMENTAL

Materials and methods. UV spectra were recorded on a Perkin–Elmer 505. The cuvets (pathlengths of 10 mm) were thermostated at 25°.

For the aerobic expts of Figs. 1 and 2: a stock soln of **7** (A^-=CF_3COO^-) in MeCN (40 μl; 4.67×10^{-3} M) was injected into the solvent (10.0 ml; benzene, $CHCl_3$, etc.) to give a final concentration of 1.86×10^{-5} M. The conc of the stock soln was checked by measuring the E_{372} of a 100-fold dilution in 1N HCl (ϵ = 14,800). All solvents were reagent grade, but treated before use. Acetonitrile ($H_2O < 0.04\%$) was successively distilled over CaH_2 and P_2O_5. Special care was given to remove all traces of EtOH from the $CHCl_3$ by several washings with equal amounts of H_2O.[15]

The anaerobic expts were carried out in a Thunberg-like apparatus made in this laboratory. It consisted of a 10-mm quartz cuvet fused via an inlet tube to two 40 ml round bottomed flasks, each provided with a rotaflo valve. The contents of these compartments could be magnetically stirred. The apparatus was thrice evacuated by means of an oil pump and filled with N_2. The N_2 (O_2-content < 3 ppm) was further purified over a BASF R3-11 catalyst and led through a washing-bottle with conc H_2SO_4. For the expts of Fig. 1, the solvent (10–12 ml of $CHCl_3$ or benzene) was put into one of the compartments, stirred continuously while N_2 was passed over for 16–20 hr. Half an hr before closing the valves, a stock soln of **7** (A^-=CF_3COO^-) in MeCN (40 μl; $4.5\text{–}5.0 \times 10^{-3}$M) was injected into the 2nd compartment. The valves were closed, the contents of the compartments were mixed and the spectra were recorded at 25°. For comparison, the absorbances were corrected for a conc of 1.86×10^{-5}M. (The volumes of the mixts were derived from their nett weights. A correction was also made for a small, non-dissolved part of the starting material. After leaving the mixts for some days at room temp. in the dark, the solvent was decanted, the ppt (**7**) was twice washed with either $CHCl_3$ or C_6H_6 (5 ml) and dissolved in 1N HCl. The amount was determined from the absorbance at 372 nm).

The yields of the N^{10}-dealkylation (Table 1) were determined by absorbance of fluorescence measurements of the mixts of Fig. 1, after complete bleaching had taken place at room temp in the dark. 1,3-Dimethylalloxazine, UV ($CHCl_3$), λ_{max} (ϵ): 248 (39,600); 324 (7700); 369 (6300; sh); 382 (7400); 398 (5500; sh). UV (benzene), λ_{max} (ϵ): 321 (6900); 366 (6200; sh); 380 (7700); 398 (5900). Fluorescence-measurements were carried out in an adapted Eppendorf digital photometer 6115. Standard curves were determined for 1,3-dimethylalloxazine in different solvents using the Hg-lamp, the 313–366 nm primary filter, and the 430–3000 nm secondary filter.

M.ps were determined in evacuated capilary tubes in a Büchi apparatus.

NMR spectra were recorded on a Varian EM 360 L NMR spectrometer.

Manometric expts (O_2-uptake and/or CO_2-generation) were carried out at atmospheric pressure and at an average temp of 23° in an all-glass manometric apparatus allowing continuous corrections for pressure and temp changes. The mixts were magnetically stirred. The yields of CO_2 were derived from the changes of the gas-volumes and afterwards, checked by absorption in 0.1 N NaOH/$BaCl_2$ (3%) and titration with 0.1 N HCl.

Cl_3CCOO^- was decomposed by the cation of **7** (0.5 mmole) in MeCN (100 ml) giving mixts which were analyzed for:

(a) The remaining amount of the alloxazinium cation by adding a 0.05 M soln of NEt_3 in H_2O (1 vol %) to a 22-fold dilution of the mixt in MeCN. The content of the cation was calculated from the decrease of the E_{364} (ϵ = 12,800) which was due to the immediate formation of the spirohydantion **4**.

(b) An unidentified alloxazine-adduct, which could be oxidized to the alloxazinium cation. To a 22-fold dilution of the mixt (0.25 ml) in 1N HCl a satd soln of $NaNO_2$ in H_2O (10 μl) was added. The mixt was kept at room temp for 1.5–2 hr, aerated for 0.5–1 hr to remove the nitrogen oxides and diluted with 1N HCl to a final volume of 10.0 ml. The increase of the absorbance of the alloxazinium cation at λ_{max} = 372 nm (ϵ = 14,8000) gave the content of the alloxazine-adduct.

(c) The 1,3-dimethylalloxazine formation (N^{10}-dealkylation) by transferring 0.5 ml to a silicagel column (9 cm in length and 0.8 cm in dia.; 2.2 g silicagel 60, product from E. Merck) and carrying out the elution with MeCN. A Hg (366 nm)-lamp was used to check the elution. The blue fluorescent fraction was diluted with MeCN to a volume of 25 ml and subjected to absorbance and fluorescence measurements.

(d) The overall amount of anions (free acids and the anions of quarternized alloxazinium salts) by diluting 12.5 ml with H_2O (50 ml) and titrating with 0.1N NaOH to pH = 7.0. (Note: the amount found may appear to be higher than the value calculated as the difference between the starting and the decomposed amounts of Cl_3CCOO^-, the latter being judged by the CO_2-generation. Such a result is consistent with the assumption that the $Cl_3C\cdot$ radical could act as an electron-acceptor in a reaction following pathway d_3 (Scheme3): $Cl_3C\cdot + \textcircled{e} \rightarrow (CCl_2) + Cl^-$).

Simplified preparation of 1,3,10-*trimethylalloxazinium perchlorate* (**7**; A^-=ClO_4^-)

The starting compound[7] **7**(A^-=ClO_4^-) was used for the prepn of the adduct **6**. In order to obtain larger amounts of **6**, the need arose to prepare **7** (A^-=ClO_4^-) by a more convenient method, evading the tedious procedure to purify 5-aceto-1,3,10-trimethyl-5,10-dihydroalloxazine.[3]

A mixt of 5-aceto-1,3-dimethyl-5,10-dihydroalloxazine[3] (25 g) and anhyd DMF (200 ml) was stirred and heated in a water-bath (80–90°) until a clear soln was obtained. Anhyd K_2CO_3 (40 g) was added, the water-bath was removed and the mixt was allowed to cool off gradually to room temp with continuous stirring. Me_2SO_4 (40 ml) was added dropwise (55–65 min) and the mixt was kept stirred for another 2–3 hr. After standing overnight the colourless ppt was filtered off, washed with $CHCl_3$ (100–500 ml) and discarded. Glacial AcOH was added to the yellow DMF/$CHCl_3$-filtrate until a sample no longer showed an alkaline reaction. The soln was evaporated to dryness *in vacuo*. H_2O (100 ml) and $CHCl_3$ (150 ml) were added to the residue and well mixed. The layers were separated; the H_2O-layer was extracted a few times more with fresh $CHCl_3$. The yellow $CHCl_3$-extracts were combined and evaporated to dryness *in vacuo*. The residue was dissolved in 96% EtOH (500 ml) and 70% $HClO_4$ (50 ml). A soln of $NaNO_2$ (10 g) in H_2O (25 ml) was added drop by drop (30 min) and the mixt was stirred for another hr. The yellow perchlorate was filtered off, washed successively with 96% EtOH and diethyl ether, and dried in a vacuum desiccator over P_2O_5, yield: 28–30 g (89–97%) m.p. 250–251°. No further purification was required for preparative purposes.

Preparation of 4^a,10^a-*ethylenedioxy*-1,3,10-*trimethyl*-4^a,5,10,10^a-*tetrahydroalloxazine* (**6**)

Compound **7** (A^-=ClO_4^-) was converted into **6** in the way as described earlier.

Preparation of 1,3,10-*trimethylalloxazinium trichloroacetate* (**7**; A^-=Cl_3CCOO^- + 1 *molecule of* Cl_3CCOOH *of crystallization*)

Adduct **6** (1.00 g) was dissolved by warming in MeCN (5 ml). The clear soln was stirred and diluted with diethyl ether (150 ml). A soln of Cl_3CCOOH (7.7 g) in diethyl ether (100 ml) was added, after which stirring was continued for 1 hr. The mixt was kept overnight at room temp in the dark. The yellow, crystalline product was filtered off and washed with diethyl ether (50 ml). It contained one molecule of Cl_3CCOOH of crystallization, yield: 1.78 g (97%) m.p. 116° (dec). ($C_{13}H_{13}N_4O_2.Cl_3CCOO.Cl_3CCOOH$ (583.04) Calcd.: C, 35.02; H, 2.42; N, 9.61 Found: C, 35.1; H, 2.4; N, 9.6). UV (1N HCl), λ_{max} (ϵ): 262 nm (27,500); 372 nm (14,800).

Preparation of 1,3,10-*trimethylalloxazinium trifluoroacetate* (**7**; A^-=F_3CCOO^- + 1 *molecule of* F_3CCOOH *of crystallization*)

Using a soln of TFA (3.6 ml) in diethyl ether (100 ml) in a procedure as described above the yellow, crystalline trifluoroacetate was obtained, yield: 1.45 g (95%; MW=484.31) m.p. 154° (dec). PMR (CD_3CN): δ = 3.47 (3, s, N–Me); 3.77 (3, s, N–Me); 4.38 (3, s, N–Me); 8.22 (4, m, Ar–H).

Decomposition of trichloroacetic acid mediated by the 1,3,10-trimethylalloxazinium cation

(a) In a manometric apparatus, 7 (A^-=Cl_3CCOO^-; 1 molecule of Cl_3CCOOH of crystallization; 291.05 mg = 0.5 mmole) was dissolved with stirring in MeCN (100 ml) under pure N_2. The evolution of CO_2 was complete in less than half an hr, yields: (a) CO_2: 0.74 mmole (148% based on the starting amount of alloxazinium cation); (b) the remaining amount of 1,3,10-trimethylalloxazinium cation: 24%; (c) the unidentified alloxazine-adduct: 20%; (d) 1,3-dimethylalloxazine: 19%; (e) the remaining amount of anions: 0.72 mmole (calcd: 0.26 mmole, being the difference of the starting amount and the converted part as derived from the CO_2-generation).

(b) *Decomposition of an additional amount of* Cl_3CCOOH *under* N_2. In a manometric apparatus, 7 ($A^-Cl_3CCOO^-$; 1 mole of Cl_3CCOOH of crystallization; 291.05 mg = 0.5 mmole) was dissolved with stirring in a soln of Cl_3CCOOH (200 mg) in MeCN (100 ml) under N_2. The evolution of CO_2 took about 6 hr. Yields from several expts: (a) CO_2, varying from 1.20 to 1.80 mmole (240–360%); (b) the remaining amount of 1,3,10-trimethylalloxazinium cation: 8–12%; (c) the unidentified alloxazine-adduct: 26–31%; (d) 1,3-dimethylalloxazine: 0%; (e) the remaining amounts of anions were consistent with the calcd values.

(c) *Decomposition of an additional amount of* Cl_3CCOOH *in the presence of air.* In comparison with the similar expts carried out under N_2 (section B), the presence of air affected the yields of: (a) CO_2 by an 1.4-fold increase; (b) the remaining amount of 1,3,10-trimethylalloxazinium cation by a 3-fold increase; (c) the unidentified alloxazine-adduct by a 2-fold decrease.

REFERENCES

[1]H. I. X. Mager and W. Berends, *Rec. Trav. Chim.* **84**, 1329 (1965); In *Flavins and Flavoproteins* (Edited by E. C. Slater), pp. 27–36. Elsevier, Amsterdam (1966).

[2]H. I. X. Mager and W. Berends, *Biochim. Biophys. Acta* **118**, 440 (1966).

[3]H. I. X. Mager, R. Addink and W. Berends, *Rec. Trav. Chim.* **86**, 833 (1967).

[4]G. A. Hamilton, *Progress in Bioorganic Chemistry* (Edited by E. T. Kaiser and F. J. Kézdy), Vol. 1, pp. 134–142 (1971); *Molecular Mechanisms of Oxygen Activation* (Edited by O. Hayaishi), pp. 405–451. Academic Press, New York (1974).

[5]P. Hemmerich and A. Wessiak, *Flavins and Flavoproteins* (Edited by T. P. Singer), pp. 9–20. Elsevier, Amsterdam (1976).

[6]F. Müller, H. J. Grande and T. Jarbandhan, *Flavins and Flavoproteins* (Edited by T. P. Singer), pp. 38–50. Elsevier, Amsterdam (1976).

[7]H. I. X. Mager, *Tetrahedron* **33**, 981 (1977).

[8]H. I. X. Mager, *Flavins and Flavoproteins* (Edited by K. Yagi and T. Yamono), pp. 455–468, Japan Scientific Societies Press (1980).

[9]H. I. X. Mager, *Tetrahedron Letters* 2423 (1979).

[10]H. I. X. Mager, *Ibid* 3549 (1979).

[11]H. I. X. Mager and R. Addink, *Ibid* 3545 (1979).

[12]M. Tishler, J. W. Wellman and K. Ladenburg, *J. Am. Chem. Soc.* **67**, 2165 (1945).

[13]J. W. Clark-Lewis and K. Moody, *Aust. J. Chem.* **23**, 1229, 1249 (1970); **24**, 2593 (1971).

[14]G. E. Trout, A. Wessiak and P. Hemmerich, *FEBS Letters* **120**, 171 (1980).

[15]*Note*: in preliminary experiments[11] on the 10,10^a-ring opening the intermediacy of 10^a-adducts was already revealed by the time course fluorescence curves. It has now appeared that a difference between the chloroform and benzene solns had been caused by the additional presence of the 10^a-ethoxy adduct arisen from the EtOH (0.2–0.3%) still present in the $CHCl_3$.

[16]H. I. X. Mager and R. Addink, *Flavins and Flavoproteins* (Edited by V. Massey and Ch. H. Williams), pp. 248–293. Elsevier, Amsterdam (1982).

[17]H. I. X. Mager and R. Addink, to be published.

[18]*Note*: less complications occurred in the conversions of 5-RFl^+_{ox}: there were no ready formations of spirohydantions in pathway a (Scheme 3) and no 10,10^a-ring openings (step c_2).

[19]*Note*: when bleaching had occurred under ordinary illumination in the laboratory, experiments like a_1 gave 1,3-dimethylalloxazine in yields of 20–24%, in agreement with the results of preliminary experiments.[16]

[20]*Note*: this blue colouration is relatively lower than the one shown by curve b_1 in Fig. 1 because of a 4 times higher concentration. This was deliberately chosen since in more dilute solutions the in situ-preparations of the salt (*cf* curves 2–5, Fig. 3) would require reaction times which would be too long in view of the decolouration process. The latter is accelerated by a higher concentration of the acid as already appears from the comparison of the curves 3 and 2, and 4 with 3.

[21]F. McCapra, *Proc. R. Soc. Lond.* **B215**, 247 (1982).

[22]C. T. Walsh, A. Schonbrunn and R. H. Abeles, *J. Biol. Chem.* **246**, 6855 (1971); C. T. Walsh, O. Lockridge, V. Massey and R. H. Abeles, *Ibid.* **248**, 7049 (1971).

[23]S. Ghisla, *Flavins and Flavoproteins* (Edited by V. Massey and Ch. H. Williams), pp. 133–142. Elsevier, Amsterdam (1982).