Excited States of Flavin Coenzymes

INFLUENCE OF STRUCTURAL FACTORS ON THE REACTIVITY OF EXCITED FLAVINS

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Flavin oxidases function by means of component flavin coenzymes that have the ability to accept hydrogen in the presence of oxidizable substrate. A highly reactive state of the flavin appears to be involved, but the nature of the activation process is not clearly understood (Theorell, 1958).

The present paper is concerned with the reactivity of flavins in the excited state; these are found to become hydrogen acceptors in the presence of a substrate. This process is critically dependent on the structure and configuration of the flavin molecule. Flavin derivatives were excited with light of selected wavelengths in the presence of a substrate as hydrogen donor; in these circumstances the substrate is dehydrogenated at a rate that gives a measure of the oxidative activity of the excited flavin. The diene ergosterol (Scheme 1: I) proved to be a suitable substrate. Ergosterol is known to undergo dehydrogenation in sunlight in the presence of eosin (Windaus & Borgeaud, 1928), yielding the sparingly soluble dimer, bisergostadienol (II); oxygen must be excluded to prevent formation of the peroxide (III) (Windaus & Brunken, 1928).

We found that ergosterol in solution under

nitrogen was quantitatively dehydrogenated to bisergostadienol when illuminated with blue light in the presence of flavins. The rate of reaction was followed by estimation of the dimer, formation of which from the diene was quantitative. Lumiflavin, riboflavin, riboflavin 5'-phosphate and flavin-adenine dinucleotide were compared in these experiments.

EXPERIMENTAL

Materials

Lumiflavin was prepared by the method of McNutt (1961). λ_{max} 223 m μ , ϵ 3×10^4 ; λ_{max} 268 m μ , ϵ 3.1×10^4 ; λ_{max} 354 m μ , ϵ 0.8×10^4 ; λ_{max} 442 m μ , ϵ 1.12×10^4 .

Riboflavin (Roche), riboflavin 5'-phosphate sodium salt (FMN) (Roche), and FAD (Fluka) were used. Ergosterol was purified by repeated recrystallization from ethanol (m.p. 160–163°), $[\alpha]_D^{20} - 130^\circ$ (chloroform), λ_{max} 271 m μ , ϵ 10.6×10^3 ; λ_{max} 282 m μ , ϵ 11.2×10^3 (Hudgell, Turnbull & Wilson, 1952).

Apparatus

The apparatus consisted of a light-source comprising three pairs of Mazda MCF/U (blue) 80 w fluorescent tubes, mounted horizontally in a trough, two pairs lying along opposite sides of the trough and the third pair along the

Scheme 1

base. The tubes were used in conjunction with filters of cellulose acetate (Strand Electric and Engineering Co. Ltd. Cinemoid filter Medium Blue no. 32), made by sandwiching the filter between two sheets of glass approximately 5 ft. $\times$ 5 in. The reaction was carried out simultaneously in a set of 10 identical 100 ml. Pyrex flasks, each fitted with a nitrogen-inlet tube and condenser. In each flask the nitrogen-inlet tube terminated in a sintered-glass disk to provide even agitation of the solution. The flasks were placed symmetrically in the trough to ensure uniform illumination.

Method

All experiments were carried out in methanol (AnalaR) solution at room temperature. It was necessary to add 10% (by vol.) of water with FAD and FMN; this did not affect the rate of reaction. Freshly prepared solutions were used, and were screened from daylight to prevent photolysis.

Before each experiment the apparatus was flushed with oxygen-free nitrogen for approximately 5 min. Passage of nitrogen was continued throughout the experiment to ensure brisk agitation of the solution. Reproducible results were obtained under these conditions.

Portions (10 ml.) of the two reactant solutions were irradiated in each flask and the dimer was precipitated as a highly insoluble solid (m.p. 205°, with no absorption in the range 220–300 $m\mu$). Each flask in turn was then removed from the apparatus, the contents were filtered through a weighed Gooch crucible (porosity no. 3) and dried for 6 hr. in a desiccator.

To show that the dimer was formed quantitatively from ergosterol, solutions were irradiated and the dimer was isolated. The weight of dimer was recorded and a sample (2 ml.) of the filtrate was partitioned with 5 ml. of cyclohexane. The amount of unchanged diene in the cyclohexane layer was then determined spectrophotometrically.

The wavelength of light used to excite the flavins was varied by replacing the blue-pigmented tubes with other tubes coated with pigments and phosphors and using Cinemoid filters of various colours. The following combinations of tubes and filters were used: red, Mazda MCF/U, coated with cadmium borate phosphor and red pigment, Cinemoid filter Ruby no. 14; yellow, Mazda MCF/U, coated with yellow pigment, and Cinemoid filter Deep Orange no. 54; green, Mazda MCF/U, coated with zinc silicate phosphor, and Cinemoid filter Primary Green no. 39. The spectral distributions for these combinations of filters and tubes is described by Vince, Clarke, Ruff & Stoughton (1956).

The overall strength of solution (dilution) was varied by (a) diluting a stock ergosterol solution (1.5 mM) with methanol and (b) preparing FMN solutions so that the ratio ergosterol:FMN remained constant. Concentration effects were studied by using (a) 1.5 mM-ergosterol solution and varying the FMN concentration, and (b) 0.2 mM-FMN solution and varying the ergosterol concentration.

Oxidative activities of the flavins were compared by using equimolar (0.2 mM) solutions of lumiflavin, riboflavin, FMN and FAD. The oxidative activity of FAD was also determined in the presence of an equimolar concentration of urea. These experiments were carried out at pH 6.5, and zero ionic strength.

To determine the effect of H^+ ion concentration on the rate of reaction, aqueous 10% methanol was used as solvent in the presence of sodium hydroxide, whose concentration

determined the pH of the solution. External buffers were excluded, since some have been shown to quench excited states (Kavanagh & Goodwin, 1949).

The experiment was repeated with ionic strength constant. This was achieved by adding calculated amounts of sodium chloride to the solution. In a similar manner the ionic strength was also varied at a fixed pH (8.2).

The effect of inhibitors on the reaction rate was studied with 2,4-dinitrophenol, thiourea and β -mercaptoethylamine in concentrations of 10^{-6} – 10^{-2} M.

For optical-rotation measurements aqueous solutions of FMN (concn. 200 mg./l.) were used, the pH being varied as required by addition of sodium hydroxide. All measurements were carried out in a micropolarimeter tube, with a Hilger polarimeter.

The dielectric constant of the reaction medium was varied by replacing the solvent medium successively by aqueous ethanol, propan-1-ol, propan-2-ol and *tert*.-butanol. The dielectric constants of these media were obtained from data given by Akerlöf (1932).

RESULTS

General characteristics of the reaction

When dilute methanolic solutions of FMN and riboflavin were irradiated with blue light (λ 430–450 $m\mu$) in a nitrogen atmosphere in the presence of the substrate ergosterol, the dimer was rapidly formed and the flavin was decolorized. A typical rate curve for FMN is shown in Fig. 1.

Factors affecting the reaction

Wavelength of the exciting light. Only blue light (λ 430–450 $m\mu$) was effective in causing dimer production in FMN- and riboflavin-ergosterol solutions. Red light ($\lambda_{\max}$. 650 $m\mu$), yellow light ($\lambda_{\max}$. 610 $m\mu$) and green light ($\lambda_{\max}$. 530 $m\mu$) yielded no dimer.

Fig. 1. Typical rate curve (methanolic solution, [FMN] 0.48 mM, [ergosterol] 1.5 mM).

Dilution. Rates of dimer formation (k) at different overall dilutions were measured at a constant FMN:substrate ratio of 1:7.5. Over the range of concentrations employed, rate constants decreased linearly with decreasing concentrations according to the equation $k = (3.03 \times 10^{-4})c$, where k is the rate of reaction in moles/l./sec. and c is the concentration of substrate in moles/l.

Relative concentrations of FMN and substrate. The rate of dimer formation at constant substrate concentration was determined as a function of flavin concentration (Fig. 2). The rate showed a rapid linear rise to a sharp maximum with increasing flavin concentration, and a fall abruptly to a minimum value. Measurements of the absorption spectra of flavin solutions (0.1 mm) showed that Beer's law was followed over this range.

The rate of dimer formation determined as a function of substrate concentration (Fig. 3) gave a bell-shaped curve. Combination of these functions in the form of a contour map (Fig. 4) indicates that a maximum rate is obtained at a molar ratio of 1:2 (flavin:substrate).

Structure of the flavin. In comparative experiments FMN was the most effective oxidant ($k = 3.1 \times 10^{-7}$ moles/l./sec.), riboflavin less effective ($k = 1.5 \times 10^{-7}$ moles/l./sec.) and lumiflavin and FAD showed no oxidative activity. Furthermore, excited FAD showed oxidative activity in the presence of urea ($k = 1.9 \times 10^{-7}$ moles/l./sec.). Urea itself was shown to have no effect on the oxidative activity of the other excited flavins.

Effect of hydrogen-ion concentration on reaction. The rates of dimer formation in the presence of FMN and riboflavin respectively were measured as a function of H^+ ion concentration over the range

pH 6–12 (Fig. 5). Both flavins showed a linear decrease of rate with increasing pH, tending to zero at high pH values. It proved difficult to extend these rate measurements below pH 6 because of the low solubility of the flavins in acid solution.

Over the range 0.1–0.3 corresponding to sodium chloride solution of strengths 0.1–0.3 M it was found that the rate of reaction was not affected by changes in ionic strength of the solution.

Presence of inhibitors. The dependence of the rate of dimer formation on the concentration of added inhibitors in the form of a logarithmic plot (Fig. 6)

Fig. 3. Dependence of rate of reaction on concentration of ergosterol ([FMN] 0.3 mm).

Fig. 2. Dependence of rate of reaction on concentration of FMN ([ergosterol] 1.5 mm).

Fig. 4. Contour map of the reaction. Contours B and C represent reaction rates $k \times 10^{-7}$ moles/l./sec. and $k \times 10^{-7}$ moles/l./sec. respectively. The point A represents a rate $k \times 10^{-7}$ moles/l./sec. at [FMN] 0.4 mm and [ergosterol] 0.9 mm.

Fig. 5. Effect of pH on the rate of reaction: ○, FMN, △, riboflavin in the reaction ([flavin] 0.2 mM, [ergosterol] 1.5 mM).

Fig. 6. Effect of inhibitors, ○, 2,4-dinitrophenol, △, thiourea and □, β-mercaptoethylamine, on the rate of reaction ([FMN] 0.2 mM, [ergosterol] 1.5 mM).

shows that the reaction is inhibited sharply by critical concentrations of 2,4-dinitrophenol, thiourea and β-mercaptoethylamine. 2,4-Dinitrophenol proved to be the most effective inhibitor of 0.2 mM-flavin at a concentration of 50 μM.

Effect of hydrogen-ion concentration on optical rotation of FMN. $[\alpha]_D^{20}$ for FMN was found to increase from -5° at pH 6 to -46° at pH 12.

Optical rotation of FAD and FAD+urea. Concentrations: FAD 200 mg./l., urea 200 mg./l. in aqueous 10% methanol.

FAD $[\alpha]_D^{19} = +4 \pm 1^\circ$

FAD + urea $[\alpha]_D^{19} = -3 \pm 1^\circ$.

Effect of dielectric constant (ϵ) of medium on rate of reaction. In the aqueous alcohols employed no change in the rate of reaction was observed over the range $\epsilon = 11.0$ –37.0.

DISCUSSION

The results show that flavin systems are activated by excitation with visible light (cf. Frisell, Chung & Mackenzie, 1959). The nature of this process will now be considered.

The electronic absorption spectra of the flavins exhibit maxima in the ultraviolet and visible range characteristic of the isoalloxazine system. Within the visible portion of the spectrum (400–700 mμ) the flavins show a common maximum ($\lambda_{\max}$ 445 mμ) in the blue region, to which their yellow colour is due (Whitby, 1953). This maximum completely overlaps the emission band (λ 430–450 mμ) of the blue light used as the illuminating source. The blue light must therefore be absorbed specifically by the flavin in the region 430–450 mμ, thereby exciting the molecule. The excited flavin (R^*) is in an activated state, which can accept hydrogen from the diene substrate (E–H) oxidizing it to the dimer (E–E), with the formation of the dihydroflavin (RH_2). No excitation of the flavin and consequently no oxidation of the substrate takes place when green, yellow or red light-sources were used, since their emission bands lie beyond the region where the flavins absorb.

The nature of the excited intermediate R^* is of particular interest since this represents the reactive state of the flavin. Excitation of the flavin by blue light at 445 mμ will raise the molecule initially to the first excited singlet R^{*sing} (lifetime 1×10^{-8} sec.) from which the characteristic green fluorescent emission ($\lambda_{\max}$ 520 mμ) of flavins takes place (Weber, 1950). From the singlet the excited flavin may pass by a change of multiplicity to the longer-lived triplet state (lifetime $\sim 10^{-2}$ sec.), which gives rise to the orange phosphorescent emission ($\lambda_{\max}$ 605 mμ) of flavins (Dhr & Castelli, 1938; Szent-Gyrgyi, 1957). Whereas excited triplet states of simple molecules are usually more stable in viscous media at very low temperatures, where collisional depopulation of the triplet level is small (Freed, Turnbull & Salmre, 1958), in complex molecules structural factors can contribute to the stability of the triplet state under normal conditions. This appears to be the case with riboflavin, which has a low quantum yield of fluorescence ($q = 0.26$) in aqueous solutions (Weber & Teale, 1957), and can pass over into the phosphorescent triplet state on illumination in ice (Szent-Gyrgyi, 1957).

The triplet state, because of its lower energy, longer lifetime and uncoupled spin character, tends to be preferred to the singlet as the reactive inter-

mediate in many reactions (Reid, 1957; Schenck, 1948). We propose (Tether & Turnbull, 1959*a*, *b*; cf. Holmstrom & Oster, 1961) that the excited intermediate (R^*) in the present work corresponds to the chemically reactive triplet state of the flavin.

Comparison of riboflavin with lumiflavin in our experiments shows that riboflavin has oxidative activity in the excited state, whereas lumiflavin is inactive. Since these flavins have identical absorption maxima in the blue region where excitation occurs, and both emit fluorescence from the same excited singlet level (λ_{max} 520 $\mu\mu$), the excited state responsible for oxidative activity cannot be the singlet. The activity shown by excited riboflavin must represent transition from unreactive singlet to a reactive triplet state favoured by the high singlet to triplet transition probability (q 0.26) of the flavin. In lumiflavin, on the other hand, the singlet to triplet transition probability is low, and the excited molecule reverts to the ground state by direct emission from the singlet level with a much higher quantum yield of fluorescence $q \gg 0.5$ (cf. Yagi, Tabata, Kotaki & Arakawa, 1955). Structurally riboflavin differs from lumiflavin only in possessing the carbohydrate side chain, the function of which may well be to promote singlet to triplet transitions in the excited isoalloxazine system. Carbohydrate structures are known to have this property by virtue of their high proportion of oxygen atoms, and their tendency to stabilize the triplet state by protective 'caging'.

Riboflavin phosphate (FMN) shows greater oxidative activity in the excited state than excited riboflavin. This indicates that the terminal phosphate group may itself facilitate singlet to triplet transitions by interacting with the isoalloxazine system. A folding of the FMN molecule (cf. Baughan, 1959) would permit this, thereby influencing singlet to triplet transition probability in the excited state by spin-orbit perturbation (McClure, 1949), and restriction of degrees of freedom. The experimental data are consistent with this view. Added phosphate has a slight catalytic effect on the reaction. It is significant that phosphate has a quenching effect on the fluorescence of riboflavin (Kavanagh & Goodwin, 1949). Furthermore, when the pH of solutions of FMN is increased from 6 to 11 a marked rise in optical rotation is observed, which one would expect on the basis of unfolding by a dissociation process. Both FMN and riboflavin give oxidation rates which decrease with pH over this range (Fig. 5), the rate falling to zero at pH 11.6.

In marked contrast with the behaviour of FMN and riboflavin, excited FAD showed no oxidative activity in our experiments. This effect cannot be due to differences in redox potential as FMN, riboflavin and FAD have virtually the same redox

potential (E'_0 -0.187 at pH 7) (Kuhn & Boulanger, 1936). Furthermore, the identity of the absorption spectrum of FAD with that of riboflavin in the blue region implies that excitation to the same singlet level occurs in the dinucleotide. Singlet FAD has the same lifetime as singlet riboflavin, but has a much smaller quantum yield of fluorescence. This indicates strong singlet to triplet transition probability, but the transition does not result in a chemically reactive state, because the triplet state is quenched by formation of an internal complex between the flavin and adenine parts of the molecule (Weber, 1950). In this form the FAD molecule would be folded up to give, in effect, a closed system. When urea is added to dissociate the complex and unfold the molecule, we find that the specific rotation falls and oxidative activity in the excited state appears.

The oxidative activity of excited FMN was inhibited by low concentrations of 2,4-dinitrophenol, thiourea and β -mercaptoethylamine (Fig. 6). A significant feature of this process is the critical dependence of the rate of oxidation of diene on inhibitor concentration. The sharp decrease in the rate of reaction at inhibitor concentrations in the range 10^{-4} to 10^{-2} M suggests that inhibition is due to deactivation of an excited state of the flavin (Weber, 1950). The most effective inhibitor was 2,4-dinitrophenol, which prevented oxidation of substrate at a concentration of 50 μM . Since this concentration of dinitrophenol has been shown to quench the phosphorescence of riboflavin (Szent-Györgyi, 1957), inhibition must be the result of direct quenching of the reactive triplet state of the flavin. It is significant that the flavin-system xanthine oxidase is similarly inhibited by dinitrophenol at a concentration of 10^{-4} M (Fridovich & Handler, 1962).

If the triplet excited flavin (R^*), with its uncoupled electrons, is represented by the diradical (IV), oxidation of substrate by the flavin may be depicted by the radical process in Scheme 2, involving the transient semiquinone intermediate (V) and yielding the leucoflavin (VI) as the final product (cf. Michaelis, 1940; Beinert, 1960).

In support of this scheme the rate of reaction was found to be independent of ionic strength and dielectric constant, indicating a radical mechanism. The dependence of reaction rate on flavin concentration (Fig. 2) is difficult to interpret; this may be due to quenching by flavin dimers, although Beer's law is followed in this range of concentrations. The dependence of rate on substrate concentration (Fig. 3) shows a marked resemblance to the Michaelis & Menten (1913) function in enzyme-substrate kinetics. A maximum in the contour map of the dependence of rate on the concentration of reactants (Fig. 4) shows that the rate is a maximum

at a molar ratio of flavin:substrate of 1:2. This suggests that a flavin-substrate complex is formed in this ratio, implying that 1 molecule of flavin, represented by the diradical (IV), can oxidize 2 molecules of the substrate by removal of 2 hydrogen atoms.

It is probable that the substrate itself is raised to the triplet state (Schenck, 1948) as a result of indirect excitation by the flavin with spin conservation. Direct (singlet) excitation of ergosterol gives rise to the ergocalciferol change (Havinga, de Kock & Rappoldt, 1960) and not to the dimer.

The foregoing work indicates that flavin co-enzymes are readily activated by excitation to a triplet state, which is capable of accepting hydrogen from an oxidizable substrate. Reactivity of the flavin is strongly dependent on structural and configurational factors. Further work is in progress to determine to what extent the triplet state is involved in the activation of flavins in complete flavin-oxidase systems.

SUMMARY

1. The oxidative activities of lumiflavin, riboflavin, flavin mononucleotide and flavin-adenine dinucleotide in the excited state are compared by excitation with blue light in the presence of a substrate as hydrogen donor.

2. The order of oxidative activity is flavin mononucleotide > riboflavin > lumiflavin = flavin-adenine dinucleotide = 0. Excited flavin-adenine dinucleotide shows oxidative activity in the presence of urea.

3. A general mechanism is proposed for the oxidation process, which is ascribed to structural

factors affecting singlet to triplet transitions in the excited flavins.

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Studies in the Biochemistry of Micro-organisms

111. THE PRODUCTION OF L-PHENYLALANINE ANHYDRIDE (*cis*-L-3,6-DIBENZYL-2,5-DIOXOPIPERAZINE) BY *PENICILLIUM NIGRICANS* (BAINIER) THOM*

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From the culture filtrates of certain strains of *Penicillium nigricans* (Bainier) Thom [= *P. janczewskii* Zaleski] the metabolites griseofulvin and dechlorogriseofulvin, of known structure, have been isolated (MacMillan, 1953). By substituting potassium bromide for potassium chloride in the Czapek-Dox medium the bromo analogue of griseofulvin was obtained (MacMillan, 1954). A further metabolite of the same mould, extractable from the culture fluid by chloroform, was isolated in the form of an antibiotic red pigment, ($C_{16}H_8O_4$)_n, of undetermined structure (Curtis & Grove, 1947).

In the present study of *P. nigricans*, directed more particularly to the mycelial constituents, we have obtained, in addition to griseofulvin and the frequently recorded mould metabolites mannitol and mesoerythritol, a new optically active nitrogen-containing product. The yield of this product reached a maximum after 7 days' incubation. The yield had fallen considerably at 20 days when griseofulvin was beginning to accumulate. The new product, obtained by acetone extraction of the dried mycelium after removal of lipids by light petroleum, possessed the molecular formula $C_{18}H_{18}N_2O_2$. From a study of its derivatives and breakdown products, the structure of this substance is *cis*-L(-)-3,6-dibenzyl-2,5-dioxopiperazine (L-phenylalanine anhydride) (I). This structure was confirmed by synthesis from L-phenylalanine ethyl ester. Being a substituted dioxopiperazine, the molecule could exist as a *trans*-isomer (*meso* form) or as a *cis*-isomer; only the *cis*-isomer could exist in two optically active forms which together give a

racemic modification. The 3,6-dibenzyl-2,5-dioxopiperazine, m.p. 296°, obtained by heating DL-phenylalanine or its ethyl ester, has long been known and may be either the *trans*- or the racemic *cis*-modification, or a mixture of these forms. Vejdělek (1951) has reported the synthesis of the *trans*-isomer from D-phenylalanyl-L-phenylalanine, and of the D-*cis*-isomer by heating D-phenylalanyl-D-phenylalanine for 3 hr. at 200–210°. He records m.p. 315–316°, $[\alpha]_D^{25} + 107^\circ$. No synthesis of the L-form has been described. There is reason to believe that Vejdělek's product was partly racemized by the heat treatment, since our synthetic L-*cis*-isomer produced under much milder conditions had the rotation $[\alpha]_{5461}^{20} - 258 \pm 9^\circ$, in agreement with the natural product, $[\alpha]_{5461}^{20} - 267 \pm 3^\circ$. The optical rotations of the D- and L-forms should be opposite in direction but numerically equal.

The following reactions led to identification of the new fungal product as L-phenylalanine anhydride.

Under drastic conditions *cis*-L-3,6-dibenzyl-2,5-dioxopiperazine gave (+)-diacetyl-3,6-dibenzyl-2,5-dioxopiperazine, which on hydrolysis with methanolic potassium hydroxide yielded two acidic monoacetylphenylalanylphenylalanines. Oxidation

(I)

* Part 110: Birkinshaw & Gowlland (1962).