The choice of ΔE_π or ΔE_σ is now the major problem. The first suggestion to come to mind is to choose ΔE_π as the center of gravity of the optical spectrum. For copper etioporphyrin this choice leads to 24,560 cm.$^{-1}$ for ΔE_π.[6] Using the data of ref. 3, we calculate 0.88 for α compared to an experimental value of 0.86.

In the optical spectrum of copper phthalocyanine[7] there are two peaks that could be associated with ΔE_π, one at 33,300 cm.$^{-1}$ and another at 38,000 cm.$^{-1}$. The former leads to an α of 0.73 and the latter to 0.81. The last value is in better agreement with the more reliable value of α obtained from the nitrogen hyperfine splitting. It is of interest to make this comparison of the α's obtained by these two methods since an estimation of this bonding parameter cannot be made if the nitrogen hyperfine splitting is not observed, as was the case in vanadyl porphyrins.[8] Consequently, another method such as the evaluation of α from optical data would be welcome if it were reliable.

We are indebted to Professor A. H. Corwin and the E. I. du Pont de Nemours and Company for a pure sample of copper phthalocyanine.

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Riboflavin as an Electron Donor in Photochemical Reactions[1]

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RECEIVED NOVEMBER 29, 1960

The anaerobic photobleaching of riboflavin, in the absence of added electron donor, has been studied in detail. The photolysis first yields leuco deuteroflavin where the two hydrogens added to the aromatic nucleus have been supplied by the side chain. The leuco deuteroflavin can reduce various substances, being itself oxidized to deuteroflavin. The amount of substrate that can be reduced is limited by the amount of riboflavin initially present. Analysis of the kinetic data indicates that the photoprocess leading to leuco deuteroflavin involves a long-lived excited state which is strongly quenched by the photoproduct itself. The formation of the leuco deuteroflavin involves acid–base catalysis as shown by the increase in quantum yield with increasing buffer concentration. Leuco deuteroflavin is autoxidizable to yield the highly light-sensitive deuteroflavin. The photolysis of this compound also passes through a long-lived state but is not retarded by the presence of its photoproduct, namely, lumichrome.

Introduction

Riboflavin is notoriously unstable under illumination with visible light. Despite the practical importance of this property very few photochemical kinetic studies have been carried out with riboflavin.[3] There exists however a considerable body of work on the examination of the organic chemistry of the photoproducts and of other compounds related to riboflavin (see, for example, ref. 4–6).

Riboflavin, like many other dyes,[7] will undergo photoreduction in the presence of a mild reducing agent or of a tertiary amine. Riboflavin is unique however in that it will also undergo photoreduction even in the absence of an added electron donor for the light excited dye. This behavior has been interpreted to mean that hydrogens are donated by water in the photochemical reaction.[8–10]

Such a reaction occurring under the stimulus of visible light would be energetically unfavorable.[11] Furthermore, the practical implications of the postulated reaction are so far reaching that we cannot leave the question unexamined.

It is the purpose of the present paper to present support for the alternative hypothesis for the source of electrons. We suggest that hydrogens are supplied from within the light excited riboflavin molecule itself.

Experimental

Riboflavin in the form of the 5′ phosphate ester monosodium salt dihydrate was supplied by Hoffmann–LaRoche, Inc. Other reagents were of Reagent Grade obtained from Fisher Scientific Company. Deionized water was used throughout. Prepurified nitrogen (Matheson) with a reported oxygen content of less than 8 p.p.m. was used for flushing the solutions free of oxygen.

The light source used was a 500 w. tungsten projector (TDC) with a heat absorbing filter and usually fitted with a Corning glass No. 3–74 filter which cuts off light below 400 mμ. The reaction cell had an optical path length of 1 cm. and a volume of 20 ml. It had provision for bubbling gases through the solution. From the transmitted light the desired wave length band was isolated with the suitable interference filter (Bausch and Lomb) and the light intensity was continuously determined using a silicon solar cell (Hoffman Electronics, Type 2A) as the detector and recorded on a Varian G-10 recorder with 10 mv. full deflection. It was found that the response of the silicon cell was linear only at low intensities and hence the intensity of the light beam was decreased by means of neutral density filters. Those experi-

(1) (a) Presented at the Meeting-in-Miniature of the American Chemical Society, New York, N. Y., March 11, 1960. (b) This research was supported by the Air Research and Development Command under contract number AF 19(604)-3065.

(2) On leave from the Institute of Physical Chemistry, Uppsala, Sweden, and in part supported by the Swedish Natural Science Research Council.

(3) For review of the photochemistry of riboflavin, see G. Oster and B. Holmström, to be published.

(4) R. Kuhn and Th. Wagner-Jauregg, *Ber.*, **66**, 1577 (1933).

(5) P. Karrer, H. Salomon, K. Schöpp, E. Schlittler and H. Fritsche, *Helv. Chim. Acta*, **17**, 1010 (1934).

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(11) See for example, E. I. Rabinowitch, "Photosynthesis," Vol. I, Interscience Publishers, Inc., New York, N. Y., 1945, Chap. 4, Sec. B5.

ments calling for a detailed wave length–time study utilized the monochromater–photomultiplier-recorder assembly described elsewhere.[12]

Normally the solutions were freed of air by bubbling with nitrogen 15 min. prior to and during the illumination. In some experiments removal of oxygen was carried out by either boiling the solution under vacuum or by repeated freezing and evacuation of the solution. In neither case were the experimental results different from those obtained with flushing.

The spectra were recorded on a Carey Recording Spectrophotometer Model 11.

Actinometry was carried out by means of the photolysis of potassium ferri oxalate.[13]

Results

Unless otherwise stated the experiments were carried out with a riboflavin solution of 6.4×10^{-5} M corresponding at 445 mμ to a transmission for a 1 cm. path length to 10% ($\epsilon_{445} = 1.25 \times 10^4$). Most experiments were carried out in a phosphate buffer at pH 6.8. The buffer concentration was usually 0.12 M but was varied in one set of experiments (see below).

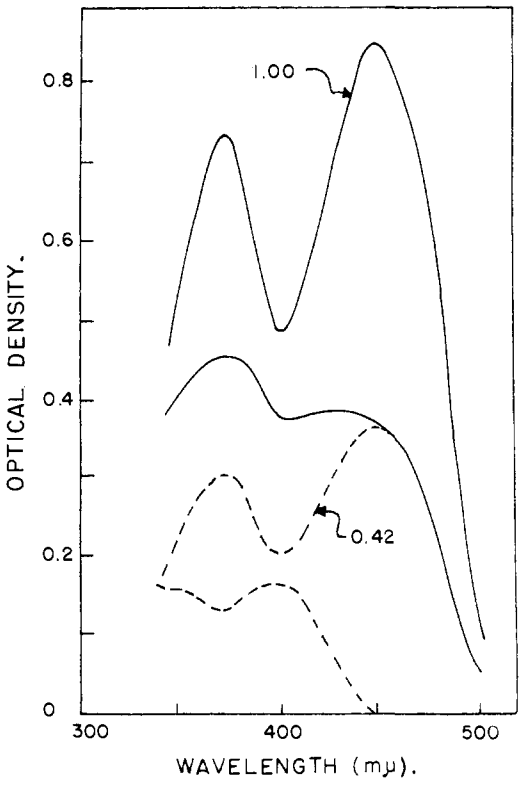

Fig. 1.—Spectra of riboflavin and its photoproduct. Full curves, original solution (rel. conc. 1.00) and partially faded solution; dashed curves, calculated absorption due to riboflavin (rel. conc. 0.42) and to photoproduct.

Using our highest light intensity, it takes 2 min. for an oxygen-free solution to fade to about one-half its original optical density at 445 mμ. The spectrum of the faded solution kept free of oxygen (Fig. 1) shows that the changes in the visible spectrum are greater than those in the ultraviolet. In the region of 450 to 500 mμ the optical density of the faded solution is a constant fraction (0.42 in Fig. 1) of that of the original solution indicating that a certain amount of riboflavin remains. If the absorption below 450 mμ is corrected for the absorption of the remaining dye the resultant is a curve whose maximum is at 400 mμ. Fading under white light was recorded for a series of wave lengths of transmitted light. Analysis of these fading curves indicates that the reaction initially is a simple transformation of riboflavin to the compound having the 400 mμ peak of Fig. 1. Deviations from this behavior become important when the optical density at 445 mμ has decreased to about one half the initial value.

The quantum yield during the early stages of the reaction is not constant but decreases as the reaction proceeds (Fig. 2). The quantum yield Φ is related to the optical density E at any time according to the expression $E = \beta - \alpha \Phi^{-1}$ where α is a constant (equal to 4.8×10^{-4} in our experiments) and the constant $\beta = E_0 + 0.02$, E_0 being the initial optical density.

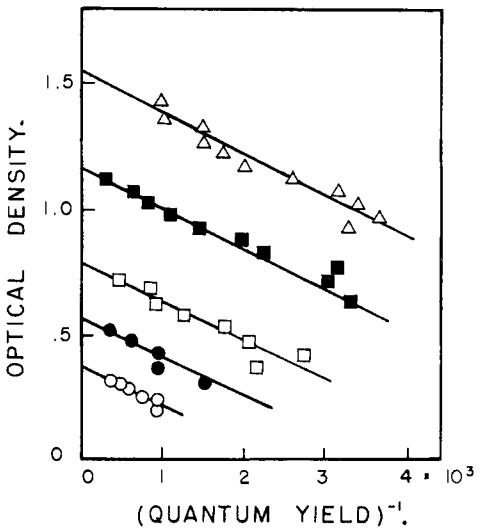

Fig. 2.—Decrease of quantum yield of the photofading of riboflavin as the reaction proceeds. Initial riboflavin concentrations (μM): open circles 32, filled circles 48, open squares 64, filled squares 96, triangles 128.

The reaction is retarded by small amounts of KI according to the Stern–Volmer expression. In the initial part of the fading reaction the rate is decreased to one half its value by the addition of 4.0×10^{-6} M KI. At later stages of the fading reaction, however, the retardation is less pronounced and a higher concentration of KI is needed to reduce the rate. The above relation between Φ and E is retained and the effect of KI is an increase in the constant β according to $\beta = E_0 + 0.8 \times 10^4$ (KI) where the concentration of KI is expressed in moles per liter.

Halwer[14] using various organic buffers observed that the rate of fading of riboflavin under *aerobic*

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conditions increases linearly with concentration of buffer acid or buffer base, that is with the square of the total buffer concentration. We found this also to be the case for the *anaerobic* fading of riboflavin, using phosphate buffer. The constant α described above increases with the square of the phosphate buffer concentration (B) according to the expression $\alpha = 1.2 \times 10^{-4} + 0.025\ (B)$.[2] Thus, the rate for $(B) = 0.065\ M$ is twice that when buffer is absent.

When oxygen is admitted to faded solution, the yellow color is partly restored. Extraction with chloroform yields a pale yellow substance with absorption maxima at 260, 350 and 385 mμ characteristic of lumichome.[5] When this aqueous phase is rendered alkaline and then neutralized subsequent extraction with chloroform yields a yellow compound with absorption peaks at 265, 385 and 445 mμ, characteristic of lumiflavin.[4] The formation of lumiflavin under such conditions has been ascribed[4] to arise from a hypothetical compound named deuteroflavin. We found that even without alkali treatment measurable quantities of lumiflavin are formed when the faded solution is allowed to stand for two days.

When oxygen is removed from the partly restored solution and the illumination is resumed, the dye fades rapidly. In fact, the optical density at 445 mμ decreases more rapidly than that of the original solution (Fig. 3). Subsequently (after about 15 sec. of illumination) the fading curve for the reoxidized solutions follows that observed for an original solution of the same optical density. The phenomenon is less pronounced at wave lengths higher and lower than 445 mμ. In fact, at 470 mμ the fading curves for reoxidized solutions have the same appearance as those for the original solution.

The extent of rapid fading is larger the greater the primary fading. It is also dependent on buffer concentration being more pronounced the higher the phosphate ion concentration for a given extent of primary fading.

In contrast to the above results, we obtain practically complete reversibility on air oxidation of riboflavin photoreduced in the presence of ethylene diamine tetraacetic acid (EDTA-disodium salt) at concentrations about 10 times that of the dye. No chloroform-soluble products are obtained in this reaction. Furthermore, the restored dye fades at practically the same rate as the original dye.

Riboflavin is a sensitizer for photoreductions even in the absence of added electron donors. An oxygen-free solution of silver nitrate ($10^{-4}\ M$) and riboflavin ($10^{-5}\ M$) was illuminated with visible light and colloidal silver was formed. If EDTA is present a silver mirror is deposited on the walls of the reaction vessel.

For quantitative studies of sensitized photoreductions we have employed 2,6-dichlorophenol indophenol (DPIP). This blue dye (absorption maximum at 600 mμ) is not light sensitive and can easily be reduced by chemical means (redox potential at *p*H 7 is + 0.2 v.) to give the leuco form. Oxygen-free solutions of DPIP and varying amounts of riboflavin were illuminated and the transmissions at 440 and 520 mμ were followed. The disappearance of DPIP, as measured at 520 mμ, is arrested after a certain time of illumination and the final extent of its disappearance is directly proportional to the initial riboflavin concentration (Fig. 4). Throughout the course of the fading, the disappearance of DPIP is proportional to the disappearance of riboflavin as determined by the absorption at 440 mμ corrected for the overlap of the DPIP spectrum.

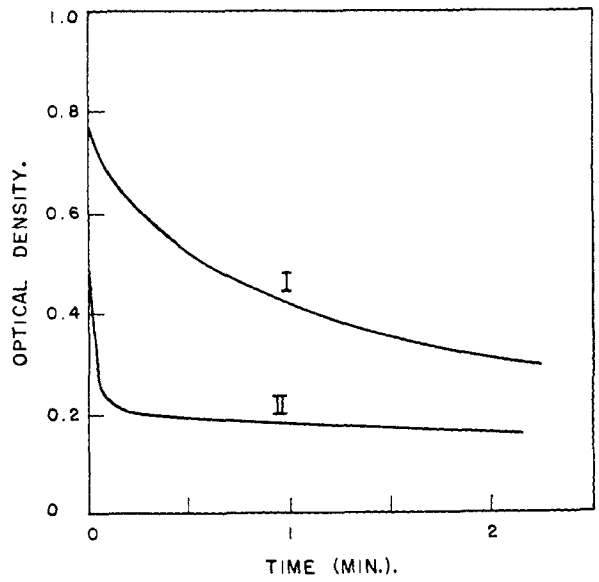

Fig. 3.—Photofading (at 445 mμ) curves: I, riboflavin solution; II, solution I illuminated 15 min. and then autoxidized.

Discussion

The ability of riboflavin to fade by itself when illuminated in the absence of oxygen is manifested in a number of other unusual photochemical properties of this dye. Many dye-electron donor combinations are photosensitizers for the initiation of polymerization of vinyl compounds.[15] Of nearly one hundred dyes examined as photosensitizers for this reaction only riboflavin is effective if no electron donor is added.[16] Potentiometric measurements of a cell containing only riboflavin show that e.m.f. values as high as 700 millivolts are developed when the cell is illuminated.[8] Other photoreducible dyes will exhibit a similar response only if electron donors have been added and the final e.m.f. achieved is determined by the redox potential of the dye.[17] A further unique property of riboflavin is that by itself it will bring about reductions of various substrates (*e.g.* silver ion, DPIP, etc.) when illuminated with visible light. A number of other photoreducible dyes can do this but in all such cases an electron donor for the light excited dye must be present.[18] In these cases the

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amount of substrate which can be reduced is limited by the amount of electron donor present in the system. With riboflavin, however, as we have shown, the amount of substrate reduced is determined by the amount of riboflavin originally present. That is, riboflavin is not a true photocatalyst.

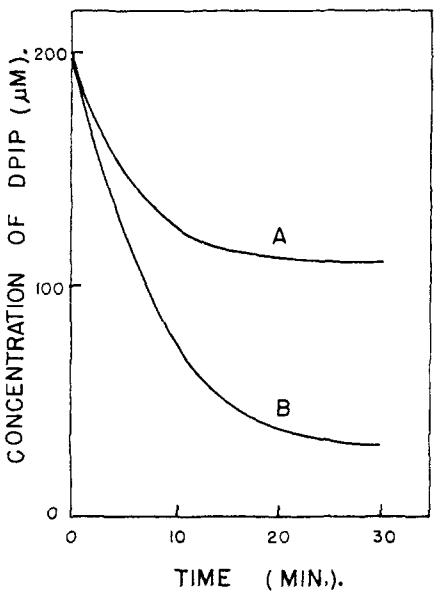

Fig. 4.—Riboflavin-sensitized photoreduction of 2,6-dichorophenol indophenol (disappearance followed at 520 mμ). Initial riboflavin concentration: A, 40 μM and B, 80 μM.

The argument that light-excited riboflavin transfers hydrogen from water to the substrate appears to us to be untenable. Rabinowitch[11] has discussed this general problem in detail and reaches the conclusion, mainly on energetic grounds, that it is unlikely that dye-sensitized oxidation of water can occur using visible light. We prefer to regard the riboflavin molecule itself as the electron donor. This suggestion made earlier[19] is substantiated in our work by our riboflavin-sensitized photoreduction experiments where a reduction of substrate is accompanied by a corresponding disappearance of riboflavin.

Further evidence for the direct chemical involvement of riboflavin is suggested by the nature of the photoproducts. Modifications of the side chain of riboflavin will not be reflected as significant changes in light absorption, fluorescence, e.m.f. or pK values since these properties depend primarily on the oxidation state of the iso-alloxazine nucleus. Chloroform extraction would separate those compounds where the hydrophilic character of the side chain has been removed. Chromatographic analysis is, of course, effective,[20] but in our case the involvement of ambient oxygen would render this technique unusable. The existence of deuteroflavin,[4] which probably is an iso-alloxazine derivative with a slightly altered side chain, is best shown by chemical means. Its presence in the faded and then oxidized solution is demonstrated by the dark reaction with alkali to yield luminflavin. It is probably identical to the highly photolabile product.

Svobodova, *et al.*,[20] carried out *aerobic* illumination of riboflavin on chromatographic paper and were able to distinguish three main products. Their intermediate called 27CX obtained directly from riboflavin appears identical with deuteroflavin. They obtained lumiflavin by prolonged illumination of the spot corresponding to the intermediate but this transformation could, according to our experience, be merely a dark reaction.

A product of the anaerobic fading clearly must contain the reduced form of the isoalloxazine nucleus in which two hydrogens have been added to the nucleus. If water is not involved in this reduction, then the hydrogens must come from the side chain. This product is not reduced (leuco) riboflavin but is rather a reduced form of another flavin which we chose to regard as deuteroflavin. It is significant in this regard that for flavins when the hydrogens on the second side chain carbon and its hydroxyl group are replaced by other groups photofading is suppressed.[5,14]

We now propose a kinetic scheme which is compatible with our observations. Riboflavin (Rf) absorbs light to give the first electronically excited species Rf*: (1) $Rf \xrightarrow{h\nu} Rf^*$. This excited species may fall to the group state with the emission of fluorescence (fluorescence efficiency of 26%[21]) and production of heat: (2) $Rf^* \rightarrow Rf + h\nu$/heat. Alternatively it undergoes transition to a long-lived species (triplet?) Rf′: (3) $Rf^* \rightarrow Rf'$. The long-lived species may fall to the ground state with the evolution of heat and possibly phosphoresce (analogous to its behavior in glasses and with ice[22]): (4) $Rf' \rightarrow Rf + h\ \nu_p$/heat. Internal conversion to the ground state is aided by a quencher Q which can be riboflavin itself (*i.e.*, self-quenching) or the photoproduct. Externally added quenchers, particularly ions of heavy atoms like in KI, are also effective quenchers. Trace amounts of these substances will not noticeably quench the fluorescence but can effect the quantum yield of the reaction by their quenching action on the long-lived species: (5) $Rf' + Q \rightarrow Rf + Q$ + heat. We describe the conversion of excited riboflavin to leuco deuteroflavin DfH_2 as (6) $Rf' + 2B \rightarrow DfH_2 + 2B$ where B is the buffer. This step is compatible with the fact that a long-lived excited species is involved in the fading (since trace amounts of KI retard the reaction) and that buffer accelerates the reaction although it does not affect the fluorescence. Apparently reaction 6 consists of intermediate steps strongly influenced by the buffer which is present in great excess. The rate is proportional to the square of the buffer concentration as would be the case if the reaction was acid-base catalyzed. Reac-

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(22) A. Szent-Györgyi, "Bioenergetics," Academic Press, Inc., New York, N. Y., 1957.

tion 6 implies that hydrogens are transferred within the molecule.

Using steady state assumptions regarding the transient species Rf* and Rf', we obtain for the quantum yield.

$$\Phi = \left[\frac{k_3}{k_2 + k_3}\right] \frac{k_5(B)^2 + k_w}{k_4 + k_5(Q) + k_6(B)^2 + k_w}$$

where $k_5(Q)$ refers to the contributions by the various quenchers (Rf, DfH_2 and KI) and where the constant k_w is included to account for the acid-base catalytic action of water alone (*i.e.*, finite rate for $B = 0$). From the variation of α with (B) we calculate that $k_6 = 210\ k_w$ liter^2 mole^{-2}. Using the rate at zero buffer concentration we calculate that $k_w = 135$ sec.$^{-1}$.

If Q is taken to be the photochemical product then the concentration of Q is proportional to $E_0 - E$ since the dye obeys Beer's law. If the quenching term is dominant in the denominator of the above theoretical equation, then $\Phi = \alpha/\beta - E$ in conformity with the observed result (Fig. 2). From the experimental data it is found that the quenching for the photoproducts $k_5 = 1.06 \times 10^{10}$ mole^{-1} sec.$^{-1}$.

When KI is added to the system there is competition between iodide ion and photoproduct as quenchers. It follows from the theoretical expression for Φ that for a given degree of retardation the KI concentration must be higher for higher product concentration, that is, for greater extent of reaction. From our retardation studies with KI we calculate a quenching constant of $1.5 \times 10^6\ k_w$ liter mole^{-1} scc.$^{-1}$ when extrapolated to zero time (*i.e.*, no product present). If every encounter between Rf' and iodide ion led to quenching, then for KI $k_5 = 6.6 \times 10^9$ liter mole^{-1} sec.$^{-1}$ as shown from diffusional arguments[23] so from this argument $k_w = 4.4 \times 10^3$ sec.$^{-1}$. If it is assumed that all singly excited riboflavin molecules which do not fluoresce are converted to long-lived species, then $k_3/(k_2 + k_3)$ is unity minus the fluorescence yield, namely, 0.26. In pure water $\Phi = 0.006$ so the lifetime of the long-lived species $k_4^{-1} = 1.0$ millisec. or about one hundred thousand times longer than the first excited singlet state.[24] The fact that the photoproduct is a more efficient quencher than KI suggests that the former quenches statically possibly as a semiquinone dimer.

Deviations from the above scheme appear after the reaction has proceeded for some time (points not lying on the straight lines of Fig. 2). This may arise from a dark reaction of leuco deuteroflavin with riboflavin: (7) $DfH_2 + Rf \rightleftarrows Df + RfH_2$ followed by the photolysis of deuteroflavin leading, we assume, to lumichrome Lc plus fission products from the side chain:

(8) $Dt \xrightarrow{h\nu} Lc +$ fragments.

When oxygen is admitted, leuco deuteroflavin is oxidized: (9) $DfH_2 + O_2 \rightarrow Df + H_2O_2$. In a similar way RfH_2 is oxidized: (10) $RfH_2 + O_2 \rightarrow Rf + H_2O_2$. These reactions proceed *via* a free radical since the polymerization of vinyl monomers are initiated under these conditions.[15]

Extraction of the faded and then oxidized solution yields lumichrome formed in step 8. Treatment with alkali yields a second chloroform-soluble compound, lumiflavin Lf: (11) $Df + OH^- \rightarrow Lf +$ fragments. The contribution of this reaction is unimportant unless the *p*H is high (about 9) and/or the intensity of light is low.

The rapid anaerobic fading of the reoxidized solution must be ascribed to a new compound Df since the wave length dependence is different from that of the fading of riboflavin.

The leucoflavin can reduce the substrate S (DPIP): (12) $DfH_2 + S \rightarrow Df + SH_2$. Unlike true photosensitizers, however, the sensitizer riboflavin is destroyed in the over-all reaction.

The fact that H_2O_2 has been detected[10] in the faded solution before oxygen is admitted has been taken[25] to be the proof for the water-splitting hypothesis. On the other hand, H_2O_2 could be produced in the reactions leading to lumichrome (step 8) or lumiflavin (step 11) without the envolvement of water.

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[CONTRIBUTION FROM THE ORGANIC CHEMISTRY DEPARTMENT, UNIVERSITY OF SYDNEY, SYDNEY, N.S.W., AUSTRALIA, THE JOHN CURTIN SCHOOL OF MEDICAL RESEARCH, AUSTRALIAN NATIONAL UNIVERSITY, CANBERRA, AUSTRALIA, AND THE NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND]

Tertiary Amine Oxide Rearrangements. I. Mechanism

BY J. CYMERMAN CRAIG,[1] F. P. DWYER, A. N. GLAZER AND E. C. HORNING

RECEIVED AUGUST 5, 1960

It is proposed that the metal-complex catalyzed rearrangement of trimethylamine N-oxide occurs in two 1-electron steps involving the formation of an intermediate possessing an unpaired electron on a methylene carbon atom which is then attacked by an OH radical to yield the methylolamine known to give dimethylamine and formaldehyde. The N-oxide is bound to the metal through its oxygen, and a hydroxo or aquo group occupies the adjacent (*cis*) coördination position. The necessary ability of the metal complex to exist in a higher oxidation state and to provide the requisite binding sites is satisfied by a number of iron(III), ruthenium(III) and (IV), osmium(III) and (IV) and vanadium(IV) compounds.

Introduction

Although *tert*-amine oxides have been detected in both animals and plants,[2] the smallness of the amounts of these compounds present in biological systems suggests that they are not simply the ter-

(1) Visiting Scientist, National Institutes of Health, 1959.

(2) (a) C. C. J. Culvenor, *Rev. Pure Appl. Chem.*, **3**, 84 (1953); (b) M. S. Fish, N. M. Johnson and E. C. Horning, THIS JOURNAL, **77**, 5892 (1955).