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THE ELECTROCHEMICAL REDUCTION OF RIBOFLAVIN IN DIMETHYLSULFOXIDE

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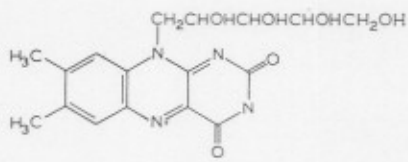
INTRODUCTION

Interest in the possible involvement of intermediate free radicals (semiquinones) of flavin compounds in biological oxidation processes has been evident since the pioneering studies of MICHAELIS and coworkers[1]. Confirming evidence of intermediate radicals has been obtained by numerous electron spin resonance (ESR) spectroscopic studies of chemically-reduced riboflavin (6,7-dimethyl-9-[D-1'-ribityl]-isoalloxazine)



and related compounds in aqueous solutions[2]. Electrochemical studies in aqueous solutions have also provided evidence of a reduction involving two successive one-electron steps to the intermediate radical and a completely reduced (leuco) form[3]. However, studies in aqueous solutions are complicated by the existence of a number of protonated and deprotonated forms of the oxidized, semiquinone, and reduced forms at different pH-values and by the strong adsorption of the various forms at the mercury–solution interface[3,4]. Furthermore, the potentials for the two reduction steps are very near one another, so that clear differentiation of the two waves is difficult and preparation of the radical in the absence of appreciable amounts of either the oxidized or leuco-form is not possible. Finally, the low solubility of riboflavin in aqueous solutions (about 0.3 mM at 25°) makes many types of electrochemical studies difficult.

This study of the electrochemistry of riboflavin in the aprotic solvent dimethylsulfoxide (DMSO) was undertaken with the hope that the reduction path would be similar to that found with aromatic hydrocarbons and related compounds, where a clear separation of the reduction waves is obtained in the absence of a protonation reaction following the first electron-transfer step. Electrochemistry in DMSO, in which riboflavin is easily soluble, has been reviewed recently[5].

RESULTS

Voltammetric methods

The polarographic reduction of riboflavin in DMSO solutions containing $NaClO_4$ as a supporting electrolyte showed three reduction waves (Fig. 1). The first wave was very well-defined with a half-wave potential ($E_{\frac{1}{2}}$) of -0.71 V *vs.* an aqueous saturated calomel electrode (SCE). The height of this wave was directly proportional to con-

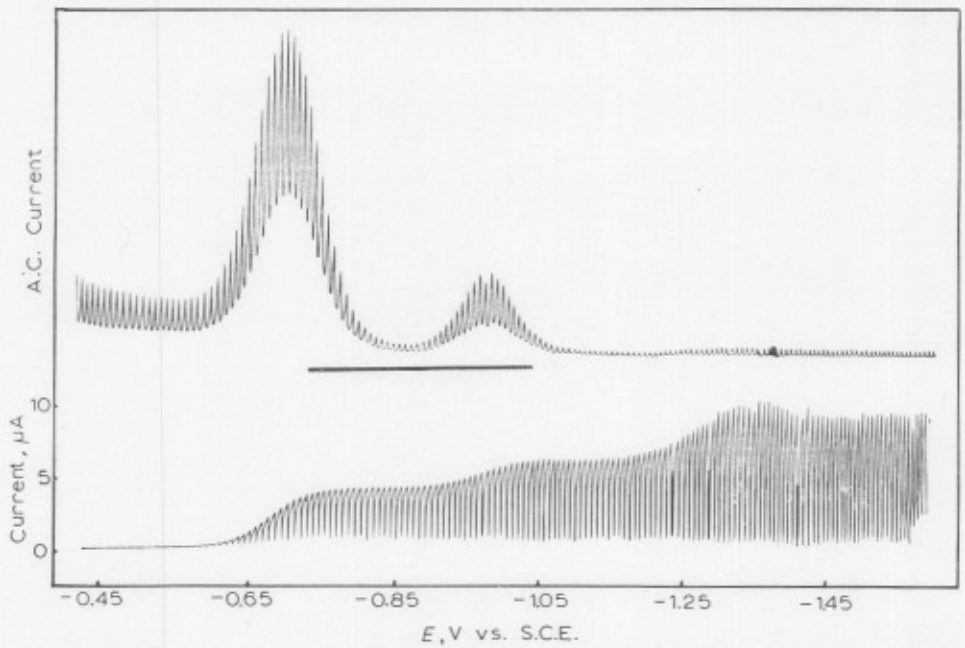


Fig. 1. Polarograms for the reduction of riboflavin (3 mM) in DMSO containing 0.8 M $NaClO_4$. Upper curve, a.c. polarogram, with 10 mV, 200 c/sec superimposed; lower curve, d.c. polarogram.

centration (0.2–4 mM) and varied as the square root of the head of the dropping mercury electrode (DME). The diffusion current constant for this wave was 0.89 in 0.8 M $NaClO_4$, yielding a polarographic diffusion coefficient, (Ilkovic equation), D, of about $1.6 \cdot 10^{-6}$ cm^2/sec, assuming this step to be a one-electron reduction. This value agrees with the D-value obtained in aqueous solutions, $4 \cdot 10^{-6}$ cm^2/sec[3,4], since the viscosity of DMSO is about twice that of water ($\eta_{DMSO} = 1.96$ cps). The second and third waves were smaller and less well-defined than the first, with $E_{\frac{1}{2}}$-values of -1.02 and -1.25 V *vs.* SCE. The second wave became more pronounced and relatively larger at higher riboflavin concentrations, with the total height of waves 2 and 3 about equal to wave 1 at all concentrations. Because wave 2 was rather drawn out, and wave 3 was obscured by a maximum, often with rather erratic current fluctuations on its plateau, reliable quantitative data on these waves by polarography was difficult.

The adsorption prewave which occurs in aqueous solutions is absent in DMSO. Electrocapillary curves, taken by measuring the drop-time at various potentials and

converting these to surface tension measurements, show no appreciable changes in surface tension of the mercury–DMSO solution interface at potentials of -0.3 and -1.0 V when increasing amounts of riboflavin are added to a DMSO–0.1 M $NaClO_4$ solution; a slight increase in surface tension is noticed on going from DMSO–0.1 M $NaClO_4$ to a solution containing 1 mM riboflavin.

When a.c. polarography was carried out by imposing a small a.c. voltage on the d.c. voltage scan, a.c. peaks at potentials corresponding to waves 1 and 2 were obtained (Fig. 1). Clear a.c. peaks were given for wave 2 even under conditions where its d.c. wave was very drawn out and hard to observe.

The cyclic voltammogram of a 1.4 mM riboflavin solution in a 0.1 M $NaClO_4$–DMSO solution at a hanging mercury drop electrode (HMDE) shown in Fig. 2, is

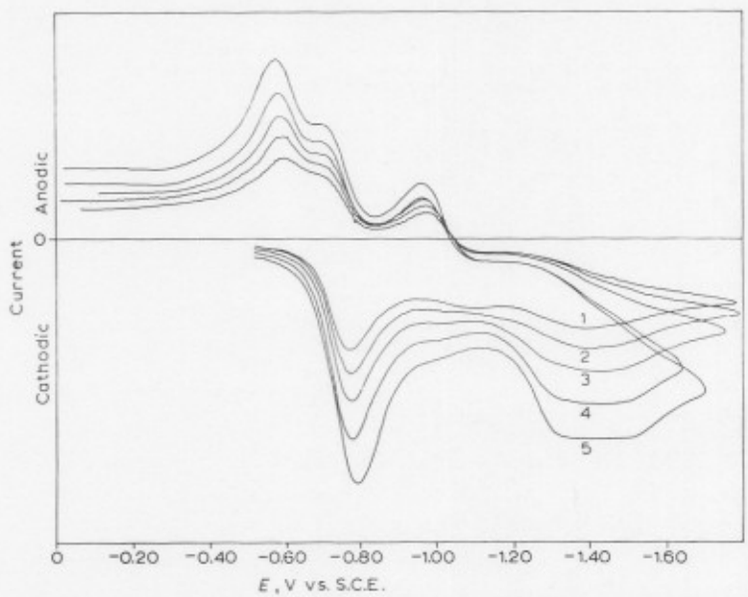


Fig. 2. Cyclic voltammetry of riboflavin. The soln. contained 0.1 M $NaClO_4$ and 1.4 mM riboflavin in DMSO. Scan rates: (1), 152; (2), 222; (3), 312; (4), 476; (5), 714 mV/sec.

characterized by a well-defined reduction peak, with a cathodic peak potential (E_{pc}) of -0.77 V and a rather broad peak with an E_{pc} of -1.3 V. At higher concentrations of riboflavin, *e.g.*, 3.2 mM, another peak, corresponding to the second polarographic wave, with an E_{pc} of about -1.07 V, becomes more distinct. Typical cyclic voltammetric data for these reductions are given in Table 1. The constancy of the current function (proportional to $i_p/v^{\frac{1}{2}}$, where i_p is the peak current and v is the scan rate) for the first peak suggests a diffusion-controlled limiting current, in agreement with the polarographic results. The current function of the second peak is strongly dependent upon scan rate, decreasing with increasing scan rate. This suggests that this wave is caused by reduction of a substance formed by a homogeneous chemical reaction of a product produced during the first reduction wave.

When the scan direction is reversed 80–90 mV past the first cathodic peak, two anodic peaks, with E_{pa}-values of -0.72 V and -0.57 V, appeared (Fig. 3). Typical data for these reversal peaks is given in Table 2. Because the anodic peaks

TABLE 1

CYCLIC VOLTAMMETRIC DATA FOR THE REDUCTION OF RIBOFLAVIN[a]

Sweep rate (mV/sec)	*Peak I*		*Peak II*		*Peak III*	
	$-E_{pc}$ [b]	$i_{pc}/v^{\frac{1}{2}}$ [c]	$-E_{pc}$ [b]	$i_{pc}/v^{\frac{1}{2}}$ [c]	$-E_{pc}$ [b]	$i_{pc}/v^{\frac{1}{2}}$ [c]
Concn. = 1.06 m*M*						
67	0.77	0.38	1.08	0.03	1.30	0.15
152	0.77	0.39	—	—	1.30	0.23
222	0.77	0.38	—	—	1.31	0.23
312	0.77	0.41	—	—	1.31	0.25
476	0.77	0.40	—	—	1.32	0.24
714	0.77	0.39	—	—	1.32	0.23
Concn. = 3.2 m*M*						
67	0.77	1.10	1.05	0.17	1.35	0.13
152	0.78	1.10	1.07	0.15	1.37	0.23
222	0.78	1.10	1.07	0.12	1.38	0.24
312	0.78	1.10	1.07	0.07	1.38	0.23
476	0.78	1.20	1.07	0.01	1.39	0.23
714	0.79	1.10	—	—	1.39	0.30

[a] The soln. contained 0.1 *M* $NaClO_4$ in DMSO.
[b] Potentials in V *vs.* aq. SCE.
[c] In μA sec$^{\frac{1}{2}}$ mV$^{-\frac{1}{2}}$.

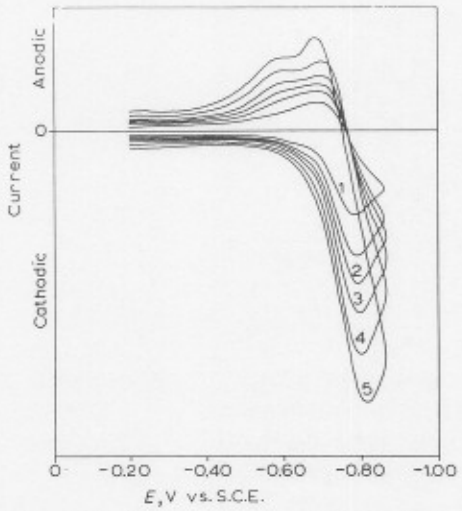


Fig. 3. Cyclic voltammetry of riboflavin for reversal following first peak. Scan rates: (1), 67; (2), 222; (3), 312; (4), 476; (5), 714 mV/sec.

are not well-resolved and it is rather difficult to make adequate correction for the baseline of these peaks (the decaying cathodic current, corrected for residual and charging current), the reported magnitudes must be considered as somewhat arbitrary. The data indicate that the current function of the first anodic peak obtained on scan reversal is almost independent of scan rate and that i_{pa}/i_{pc} is slightly less than one. The second anodic peak increases with increasing scan rate, and is almost absent at

TABLE 2

CYCLIC VOLTAMMETRIC DATA FOR REVERSAL AT FIRST PEAK OF RIBOFLAVIN[a]

Sweep rate (mV/sec)	i_{pc}[b] / $v^{\frac{1}{2}}$	$i_{pa_{II}}$[b,c] / $v^{\frac{1}{2}}$	$i_{pa_{III}}$[b,d] / $v^{\frac{1}{2}}$
Concn. = 1.06 m*M*			
67	0.38	0.33	0.02
152	0.40	0.33	0.02
222	0.41	0.33	0.06
312	0.41	0.34	0.08
476	0.43	0.36	0.10
714	0.42	0.37	0.11
Concn. = 3.2 m*M*			
67	1.26	1.1	—
152	1.26	1.0	0.02
222	1.27	1.0	0.03
312	1.28	1.0	0.04
476	1.28	1.0	0.08
714	1.27	1.1	0.13

[a] The soln. contained 0.1 *M* $NaClO_4$ in DMSO.
[b] μA $sec^{\frac{1}{2}}$ $mV^{-\frac{1}{2}}$.
[c] Scan reversed 80–90 mV more negative than E_{pc}. Measured using procedure of NICHOLSON[6].
[d] Measured from baseline obtained by approximate extrapolation of current from $i_{pa_{II}}$.

very slow scan rates; this suggests that the substance giving rise to this wave is decomposing by a homogeneous chemical reaction.

The addition of a large excess of the proton donor, hydroquinone (HQ) (4.4 m*M* riboflavin–0.57 *M* HQ), resulted in the height of the first cathodic wave doubling and the disappearance of the third cathodic wave. The second wave, or a new wave in the same potential region, was present. The anodic waves, which appeared to be at about the same potentials as before HQ addition, were very distorted by a stirring effect in the presence of HQ.

Controlled potential coulometry

Coulometric experiments at a mercury pool electrode controlled at a potential on the diffusion plateau of the first wave (−0.80 V *vs.* SCE) showed that one (0.99 ± 0.01) faraday per mole of electroactive material is consumed in a complete reduction. Examination of the solution after reduction, by polarographic or cyclic voltammetric techniques, showed an anodic wave (sometimes complicated by stirring at the electrode surface[7]) at about −0.7 V. The polarographic wave height for this anodic wave was about 30–40% smaller than the original height of the first cathodic polarographic wave before reduction. For a cathodic cyclic voltammetric sweep in this solution, two reduction peaks with E_p-values at −1.05 V and −1.37 V, appear. These peaks are better defined than the corresponding peaks obtained during cyclic voltammetry of the original solution. The current functions of these peaks were constant with varying scan rate. Examination of the solution after reduction, by ESR spectroscopy, shows a rather poorly resolved spectrum (Fig. 4) which is stable with time, and which is generally very similar to that observed from riboflavin semiquinone species in aqueous media[2].

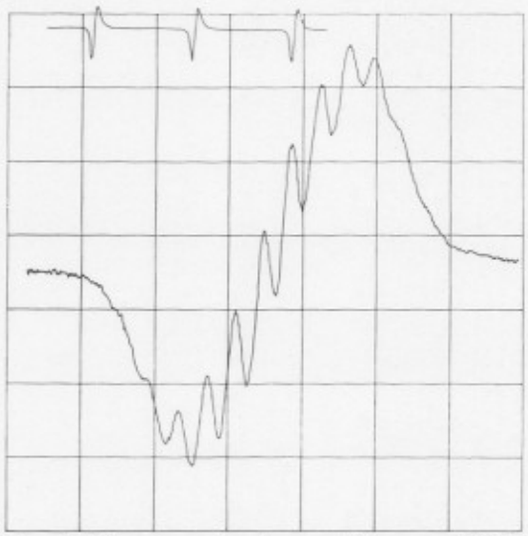


Fig. 4. ESR spectrum for soln. containing 1.1 mM riboflavin and 0.8 M $NaClO_4$ in DMSO following reduction. Upper signal is of potassium peroxylamine disulfonate with 13.0 gauss splitting.

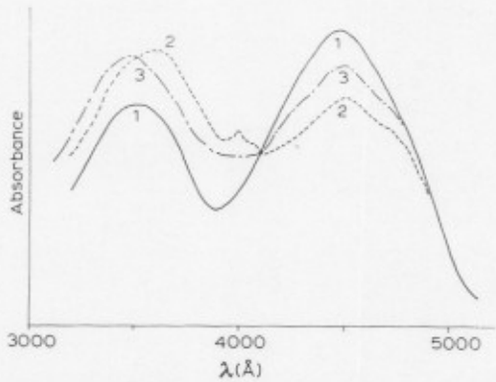


Fig. 5. Visible spectra during riboflavin reduction. The soln. contained 4.5 mM riboflavin and 0.8 M $NaClO_4$ in DMSO. Reduction carried out at −0.8 V *vs.* SCE. (1), Before reduction; (2), partially reduced; (3), almost complete reduction.

Reversal coulometry[8] was employed to obtain information about the stability of the reduced species. The number of coulombs recovered on the oxidation (Q_b) was always less than that for the reduction (Q_f). For short electrolysis times (2 min reduction followed by complete oxidation), Q_b/Q_f was about 0.7. For longer electrolysis times (5–15 min), Q_b/Q_f was 0.4–0.6. The values of Q_b/Q_f did not depend upon the initial concentration of riboflavin, but were not reproducible enough to gain any quantitative kinetic data about the reduction product. When the solution resulting from the oxidation was examined by voltammetric techniques, voltammetric waves at the same potentials as the original solution were observed, with the second cathodic wave ($E_p = 1.05$ V) being somewhat more prominent than the one found in the original solution.

Continued coulometric reduction of the solution at -1.3 V *vs.* SCE following exhaustive reduction at the first wave, showed the consumption of approximatley two additional faradays per mole, and the disappearance of all anodic and cathodic waves on polarographic examination of the reduced solution.

A spectrophotometric analysis of the solution during the course of the reduction was also performed. This was accomplished by pumping solution from the electrolysis cell through a quartz flow cell contained in a spectrophotometer and then back into the electrolysis cell. Typical curves during the course of the electrolysis are shown in Fig. 5. Initially, the riboflavin solution shows two absorption maxima, one at 448 mμ and another at 350 mμ, with a ratio of peak heights of about 1 to 0.78. These values are very close to those found in other non-aqueous solvents[9]. During the reduction, at -0.80 V, the band at 448 mμ decreased in height, while that at 350 mμ increased and shifted to about 360 mμ. A new small peak appeared at about 400 mμ. With continued electrolysis at the same potential, however, the band at 450 mμ increases again while that at 360 mμ shifts back to 350 mμ, with a ratio of peak heights of the 450 mμ to 350 mμ bands of about 1 to 1.1; the peak at 400 mμ has disappeared. Visually, the electroreduction is characterized by a change in the solution from a bright yellow to a deep orange-red. All solutions show an isosbestic point at 410 mμ. These results tend to confirm the previous electrochemical data and suggest the formation of an intermediate upon electroreduction which undergoes a following chemical reaction.

DISCUSSION

Clearly the mechanism of the reduction of riboflavin in DMSO is more complicated than that of simpler organic compounds (*e.g.*, aromatic hydrocarbons, azocompounds, quinones) in aprotic solvents. The first step (the reduction wave with $E_p = -0.78$ V *vs.* SCE) is clearly a one-electron reduction to the riboflavin anion radical:

$$Rf + e \rightarrow Rf^{\cdot -} \qquad (1)$$

All the results indicate, however, that this anion radical, $Rf^{\cdot -}$, is *not stable*. The experimental results are best explained by assuming that the decomposition of $Rf^{\cdot -}$ occurs by parallel reactions such as

$$Rf^{\cdot -} \rightarrow A \qquad (2)$$

$$Rf^{\cdot -} \rightarrow B^{\cdot -} \qquad (3)$$

where A is a substance which is reduced at potentials of the second cathodic wave ($E_p \sim -1.07$ V) and $B^{\cdot -}$ is an anion radical of a substance B whose electrochemical properties are very similar to that of riboflavin itself. This explanation then implies that the radical observed by ESR spectroscopy is $B^{\cdot -}$ rather than $Rf^{\cdot -}$. The third cathodic wave during the reduction may be composed of the reductions of Rf, Rf^-, B, and $B^{\cdot -}$ to the dianions, followed by some following reaction, *e.g.*, protonation. In fact, close observation of the third cathodic wave (Fig. 2) shows that it may be composed of two closely spaced waves.

The anodic waves on reversal of scan in voltammetry may be ascribed to

oxidation of $B^{\cdot-}$ and $Rf^{\cdot-}$, and perhaps another species produced as a product of reactions (2) or (3).

This mechanism also satisfactorily explains the bulk electrolysis, coulometric results. In these long duration experiments, the solution resulting from coulometric reduction will contain A and $B^{\cdot-}$, although $Rf^{\cdot-}$ may appear for a time and may be responsible for the transient spectrophotometric peak at 400 mμ. The solution upon oxidation will show a Q_b/Q_f less than one, since only $B^{\cdot-}$ will be oxidized, and it will be present in smaller concentrations than the amount of riboflavin originally present. For very short electrolysis times, the oxidation is of $Rf^{\cdot-}$ and $B^{\cdot-}$ and Q_b/Q_f is close to one; at longer times a limiting value is reached.

Electrolysis at -1.3 V probably yields reduced forms which rapidly undergo irreversible reactions with the solvent to produce non-electroactive species. This type of behavior is observed in the reduction of aromatic hydrocarbons[7], where formation of the dianion is followed by a rapid protonation reaction. The cyclic voltammetry experiments in the presence of a proton donor (HQ) indicate that the radical anions can also undergo a protonation reaction when excess acid is present.

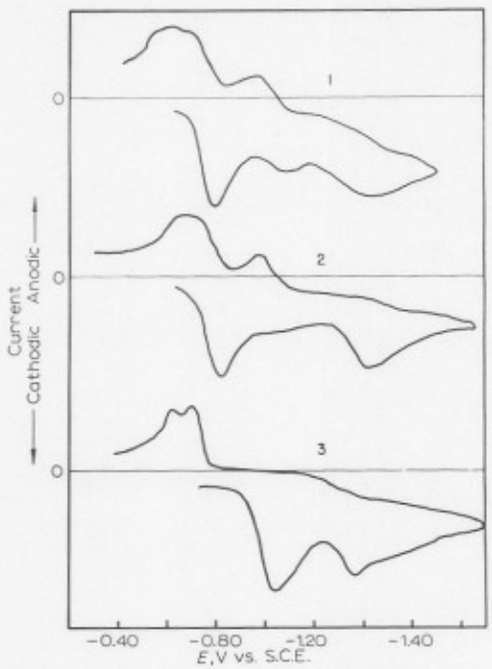


Fig. 6. Cyclic voltammograms of: (1), riboflavin; (2), lumiflavin; (3), lumichrome in DMSO.

In an attempt to identify the species A and B, a brief study of compounds related to riboflavin was undertaken. Lumiflavin (riboflavin with the ribityl at the 9-position replaced by a methyl group), and lumichrome (complete dealkylation at the 9-position, with the molecule in the alloxazine form), known as photochemical decomposition products of riboflavin, were investigated. Typical cyclic voltammo-

grams of these species and riboflavin are shown in Fig. 6. As expected, the electrochemical behavior of lumiflavin is very similar to that of riboflavin. The coulometric reduction of lumiflavin at potentials of the first reduction wave consumes one faraday per mole and produces a solution which shows a strong ESR signal similar to that of riboflavin, but with somewhat better resolution. Lumichrome shows reduction peaks at about -1.1 V and -1.4 V *vs.* SCE. Another possibility for a product of the reaction of riboflavin anion radical is a dimer of riboflavin. MICHAELIS AND SCHWARZENBACH[10] have reported dimerization reactions in aqueous solutions and the apparent concentration-dependence of the second cathodic wave is suggestive of this kind of reaction. To eliminate the electrode material, supporting electrolyte or solvent as major reactants in the chemical reactions following electron transfer, some experiments were performed using a platinum rather than a mercury electrode, using tetra-*n*-butylammonium iodide or perchlorate rather than $NaClO_4$ as supporting electrolyte, or using N,N-dimethylformamide rather than DMSO as solvent. The general electrochemical behavior of riboflavin was the same in these experiments. A definite assignment of the products and mechanisms of the following chemical reactions must await better product analysis and further electrochemical studies.

EXPERIMENTAL

Riboflavin was obtained from Eastman Organic Chemicals and used without further purification. Its purity was checked by melting point, and coulometry in aqueous solution. Lumiflavin was obtained from Professor DAVID METZLER and Professor GORDON TOLLIN. Lumichrome was obtained from Aldrich Chemical Company. Sodium perchlorate was G. Frederick Smith reagent-grade, and was dried at 120° before use. Dimethylsulfoxide was obtained from Matheson, Coleman and Bell and was purified by treating with anhydrous alumina overnight and then distilling under vacuum. Solutions of riboflavin were protected from light by covering volumetric ware and electrochemical cells with black vinyl tape. Photochemical reactions of riboflavin in DMSO were shown to occur when the solutions were exposed to sunlight or strong ultraviolet light.

The procedures and apparatus for the electrochemical studies were generally as described previously[7]. In some experiments, the solutions were de-aerated with purified nitrogen, and in others vacuum line techniques[7] were employed with no difference in electrochemical behavior. Spectrophotometry was carried out by pumping solution from the electrolysis cell through Tygon tubing to a quartz flow cell contained in a Cary Model 14 spectrophotometer. A Varian V-4502-14 spectrometer was employed for ESR studies.

ACKNOWLEDGEMENTS

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SUMMARY

The electroreduction of riboflavin in dimethylsulfoxide solutions at a mercury electrode was studied by polarographic, cyclic voltammetric and coulometric techniques. The solutions produced upon reduction were examined by electron spin resonance spectroscopy and spectrophotometry. A mechanism for the reaction, based on an initial one-electron transfer to form the riboflavin radical anion which decomposes by two parallel reactions, is proposed.

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