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Electrochemical Aspects of a NADH/FAD Model System

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A model system of the NADH/FAD redox couple has been studied by the use of electrochemical techniques. The system studied was composed of 1-benzyl-1,4-dihydronicotinamide (BNAH) and riboflavin (RF), which were used in place of NADH and FAD respectively, dissolved in a DMSO solution. The results obtained were as follows. RF is reduced either by BNAH or sodium borohydride, obeying a second-order rate law, presumably by the mechanism of hydride-ion transfer. The resultant product is inactive to the electrochemical reduction at a mercury-pool electrode. Entirely different products were produced when RF was reduced electrochemically. RF was reduced at a rate of 1 *F*/mol at the initial stage of controlled potential electrolysis to form, probably, a dimeric compound, but the latter product underwent further reduction and the total quantity of electricity necessary for the complete reduction exceeded 10 *F*/mol. Interestingly, the electrolytic reduction gives the same product as that obtained by BNAH when electrolysis is performed in the presence of a sufficient amount of BNAH, indicating that the initial product of electrochemical reduction receives the hydride ion rather than further electrons.

$$2RF + 2e \rightleftarrows (RF)_2^{2-} \xrightarrow{+H^-,\ +H^+} 2RFH^-$$

The Zn^{2+} added to the BNAH/RF system was found to act as the oxidizing agent of RFH^- and regenerated the neutral RF. This is markedly different from the case of BNAH/organic carbonyl compounds, where the metal ions behave as catalysts to promote the reaction.

In a series of papers,[1–4] we have reported on our electrochemical studies of the reaction of 1-benzyl-1,4-dihydro-nicotinamide (BNAH) with several aromatic compounds. The results may be outlined as follows:

1) Metal cations, which catalyze the redox reactions between BNAH and organic substrates, act as bridging agents in a ternary Michaelis complex composed of BNAH, a substrate, and a metal catalyst.

2) The force sustaining the ternary complex is mainly due to ion-pairing stabilization (Coulomic force between the organic anion and the metal ion).

3) Zinc exhibits the highest catalytic activity.

In the present paper, we should like to report the somewhat different behavior of metal ions observed in the redox reaction between BNAH and riboflavin (Vitamin B_2).

Riboflavin, which will hereafter be abbreviated as RF, is known as a constituent of flavocoenzymes. An excellent review article on the nature and reactions of flavins and flavocoenzymes is available.[5] Electrochemical studies of the same subject were reviewed extensively by Elving.[6]

Studies hitherto reported were done in aqueous media, and only a little is known about nonaqueous systems. By means of cyclic voltammetry, Sawyer[7] studied the qualitative behavior, including the metal-ion interactions, of several flavin compounds in DMF, acetonitrile, and DMSO.

Yoshinaga[8] also studied the electrochemistry of RF in DMF solutions using cyclic voltammetry and a.c. and d.c. polarography. According to these works, RF is reduced exhibiting two one-electron waves the first electron transfer being quasi-reversible, and the second, irreversible.

Experimental

Reagents. The solvent used in this experiment was dimethyl sulfoxide (DMSO). In some preliminary experiments, *N,N*-dimethylformamide (DMF) was also tested. Although the basic behavior of RF was found to be more or less similar in the two solvents, a slight advantage was found with DMSO in the solubility of RF. Commercial DMSO was first treated with Molecular Sieve 3A overnight and then distilled under reduced pressure in a nitrogen atmosphere. The supporting electrolyte was tetraethyl ammonium perchlorate (TEAPC). The RF of Katayama Chemicals was used as purchased. BNAH and its oxidized form BNA^+ were supplied by Dr. Ohnishi of the Sagami Chemical Research Center. The zinc salt was in the form of perchlorate.

Equipment and Procedures. The polarograph used was a Yanagimoto P-8 type, and the basic conditions of measurements were as follows:

Height of mercury column: 63.5 cm
Drop rate: 1.84 mg s^{-1} (at −1.0 V in DMSO)
Drop life: 3.81 s (at −1.0 V in DMSO)
Scan rate: 0.2 V s^{-1}.

The reference electrode was a SCE in a DMSO solution.

The homogeneous reaction of BNAH with RF was studied in a 20-ml polarographic cell in which 10 ml of a test solution had been placed. The test solution, initially composed of 0.1 M (1M=1 mol dm^{-3}) TEAPC and 1 mM RF, was first deoxygenated with bubbling nitrogen, and then BNAH equivalent to 30 mM was dissolved in it. The progress of the reaction was monitored periodically by means of d.c. polarography.

Electrolysis was carried out in a two-compartment cell separated by a glass frit. The working electrode was a mercury pool, and the counter electrode, a platinum foil. In the mercury-pool compartment, a dropping mercury electrode was also inserted to monitor the advance of the electrolysis.

Results

Electrochemical Behavior of RF in DMSO. The electrochemical behavior of RF has been studied by means of a.c. and d.c. polarography as well as by means of cyclic voltammetry.[8] Here, we shall cite only a part of our results.

The d. c. polarogram of 1 mM of RF in DMSO is

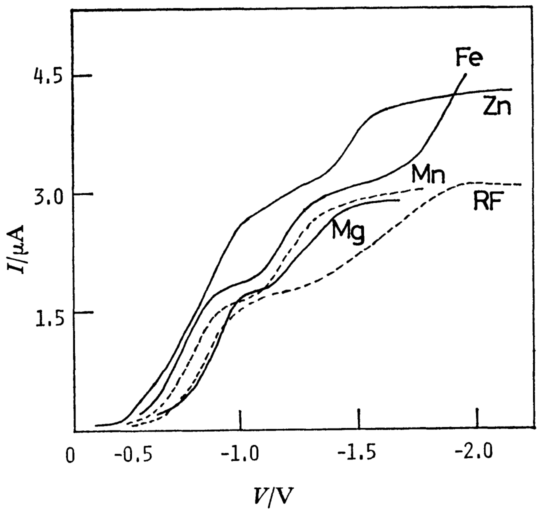


Fig. 1. D. c. polarograms of riboflavin (1 mM) in the presence of equimolar amount of several metal cations.

shown by the dotted curve in Fig. 1. As may be seen, the first one-electron wave appears at $E_{1/2}=-0.90$, and the second, at -1.56 V. Both of the waves are diffusion-limiting, the first one being quasi-reversible, and the second, totally irreversible.

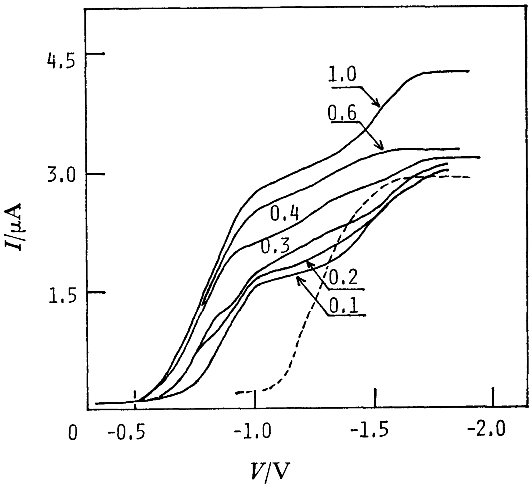


Fig. 2. Effect of zinc ion concentration on the polarogram of riboflavin (1 mM). Numbers indicate Zn^{2+} concentration in mM. Broken curve stands for 1 mM Zn^{2+}.

When equimolar amount of various metal salts are added, the wave shifts in a more positive direction, indicating the existence of ion-pair formation (Fig. 1). Figure 2 shows the effect of the zinc ion at a variety of concentrations. When the concentration of the zinc ion exceeds 0.6 mM, the wave corresponding to Zn^{2+} itself appears and the change in the wave form of RF almost ceases. This may be an indication that, in the ion-pair formed, two molecules of reduced RF are fixed per Zn^{2+} ion, on the average.

Reduction of RF by BNAH in Homogeneous DMSO Solutions. When RF is reacted with a large excess of BNAH in DMSO solutions containing 0.1 M TEAPC, the following reaction proceeds:

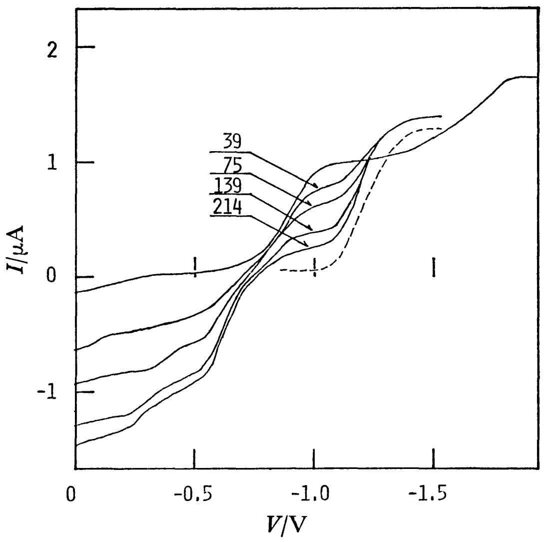


Fig. 3. Change in polarograms with the progress of reaction between BNAH and RF. Numbers indicate reaction time in minutes. The uppermost curve stands for RF before adding BNAH, and broken curve for BNA^+ (1 mM). RF: 1 mM, BNAH: 30 mM.

$$\mathrm{RF} + \mathrm{BNAH} \xrightarrow{k_{11}} \mathrm{RFH}^- + \mathrm{BNA}^+. \qquad (1)$$

The progress of the reaction was monitored periodically by the aid of polarography with the reacting solution. Figure 3 shows a part of the results, where the initial concentrations of the reactants were 1 mM for RF and 30 mM for BNAH respectively. When the cathodic wave height at -1.1 V, as a measure of the amount of RF remaining unreacted, and the anodic wave height at -0.2 V, for the amount of reduced product, are plotted against each other, Curve A in Fig. 4 is obtained. The slope of the curve is exactly 2, indicating that the reduction occurring is equivalent to a two-electron transfer per molecule of RF. The reaction as a whole can be expressed by Eq. 1. The apparent rate constant was determined to be $(3.7\pm0.2)\times10^{-3}\ \mathrm{M^{-1}\ s^{-1}}$.

Westheimer[9] showed that the reaction between NAD^+ and certain organic substrates occurs *via* a hydride-ion mechanism. We are inclined to conclude

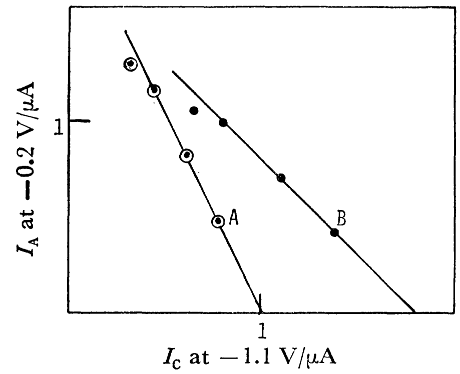


Fig. 4. Equivalency between RF and its reduced product. The abscissa stands for the concentration of RF remained unreacted and the ordinate for reduced RF. A: Without additives, B: with 0.7 mM Zn^{2+}.

that the present reaction, as expressed by Eq. 1, proceeds by the same mechanism, *i.e.*, hydride-ion transfer.[10]

The product, $\mathrm{BNA^+}$, is polarographically reactive and gives a cathodic wave with the half-wave potential at −1.5 V. The curve of the broken line in Fig. 3 stands for the solution of 1 mM $\mathrm{BNA^+}$ observed separately. The curves in Fig. 3 clearly indicate that the wave corresponding to the $\mathrm{BNA^+}$ reduction increases progressively with the reaction time. In this figure, there are two facts worthy of note. The first is that the limiting current at the $\mathrm{BNA^+}$ wave remains constant, regardless of the reaction time, and the second, that the second wave of RF itself disappears completely.

The reason why the second wave disappears can be explained by assuming that the following sequence of reactions is taking place at the dme surface:

$$\mathrm{RF} + \mathrm{e} \rightleftharpoons \mathrm{RF}^{\cdot-} \qquad (2)$$

$$2\mathrm{RF}^{\cdot-} \longrightarrow (\mathrm{RF})_2{}^{2-} \qquad (3)$$

$$(\mathrm{RF})_2{}^{2-} + \mathrm{BNAH} + \mathrm{H^+} \longrightarrow \mathrm{BNA^+} + 2\,\mathrm{RFH^-}. \qquad (4)$$ [11]

If both the reactions, (3) and (4), are assumed to occur in the closest vicinity of dropping mercury, and if they are assumed to proceed sufficiently rapidly, there may be no $\mathrm{RF}^{\cdot-}$ which is responsible for the second wave.

The constancy of the wave height at the plateau of the $\mathrm{BNA^+}$ wave also supports the occurrence of Reaction 4. The species responsible for the wave height concerned should be both the RF remaining unreacted and the $\mathrm{BNA^+}$ formed by the homogeneous reaction (1). If the RF remaining unreacted undergoes a series of reactions, (2) to (4), rapidly and quantitatively, the concentration of $\mathrm{BNA^+}$ at the electrode surface will be equal to the sum of those due to the homogeneous reaction (Eq. 1) and produced by the follow-up chemical reaction (Eq. 4). Accordingly, the concentration of $\mathrm{BNA^+}$ should always be equal to the initial concentration of RF itself, 1 mM in this case.

It is worthy of note that RF gives the same product, $\mathrm{RFH^-}$, even when the reaction is initiated by electronation (Eq. 2) as long as a sufficient amount of BNAH is present in the system. In other words, $(\mathrm{RF})_2{}^{2-}$ reacts more easily with BNAH than it receives further electrons. Very similar behavior was observed when sodium borohydride was used in place of BNAH. This suggests that the role of BNAH is as a hydride-ion source rather than as an electron donor.[10]

Behavior of $\mathrm{Zn^{2+}}$ in the Redox Reaction between BNAH and RF. As has been reported previously, BNAH reduces various aromatic carbonyl compounds, and several metal ions have been found to accelerate the rate of the reaction. In contrast to this, the existence of some metal ions apparently retards the reduction of RF with BNAH.

Figure 5 shows the results of a measurement similar to that of Fig. 3. The only difference was that the starting solution contained 0.7 mM $\mathrm{Zn^{2+}}$ besides 1 mM of RF and 30 mM of BNAH. Because of the ion-pairing phenomena, the polarogram of RF changed to one two-electron wave. As had been done before the cathodic wave height was plotted against the anodic one to get Curve B in Fig. 4. The slope is exactly unity.

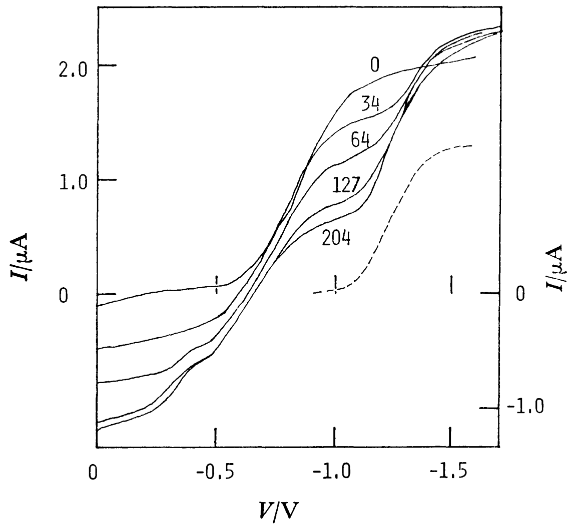


Fig. 5. Change in polarograms with progress of reaction between BNAH and RF in the presence of 0.7 mM $\mathrm{Zn^{2+}}$. RF: 1 mM, BNAH: 30 mM.

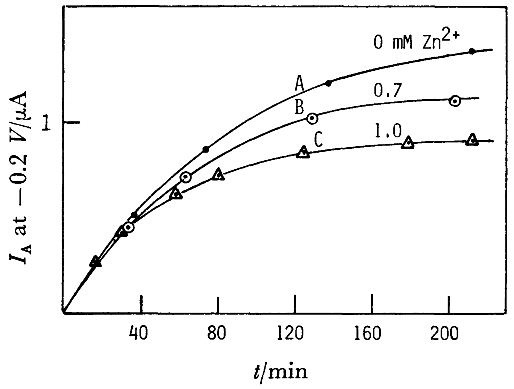


Fig. 6. Accumlation of reduced RF and the effect of $\mathrm{Zn^{2+}}$ ion on it.

Since the cathodic wave was two-electron, the anodic wave corresponds to a two-electron reaction. The shape of the anodic wave is indistinguishable from that in Fig. 3. Accordingly, one may conclude that the presence of $\mathrm{Zn^{2+}}$ does not affect the production of $\mathrm{RFH^-}$.

The anodic wave appearing in the polarograms of Figs. 3 and 5 is due to the reoxidation of the reduction product, $\mathrm{RFH^-}$. Therefore, its wave height can be used as a measure of the concentration of $\mathrm{RFH^-}$. This is examined in Fig. 6, where Curve A stands for the measurement in the absence of $\mathrm{Zn^{2+}}$, and B and C for those in the presence of 0.7 mM and 1.0 mM $\mathrm{Zn^{2+}}$ respectively. It is clearly indicated that the accumulation of $\mathrm{RFH^-}$ is retarded by the presence of $\mathrm{Zn^{2+}}$.

In Fig. 5, the limiting current at the second plateau, where $\mathrm{BNA^+}$ is reduced, is roughly constant, irrespective to the reaction time. However, the value is much greater than that observed in Fig. 3. This requires an additional explanation. The species responsible for the total wave height of interest are, of course, the $\mathrm{BNA^+}$ produced homogeneously and the RF remaining unreacted, the latter gives a two-electron wave in this case[12] owing to the presence of the $\mathrm{Zn^{2+}}$ ion. Accordingly, the difference in the heights of the first and second

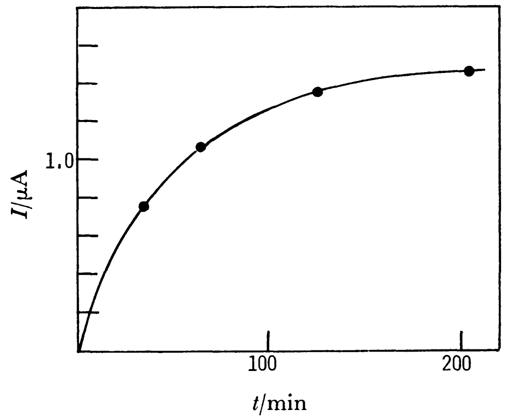


Fig. 7. Increase of BNA$^+$ concentration with reaction time observed by polarography.
1.5 μA corresponds to 1.2 mM roughly.

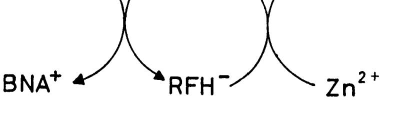
waves must correspond to the concentration of BNA$^+$ produced by the homogeneous reaction. This is plotted in Fig. 7 against the reaction time. It should be noted that the final value is approximately 1.5 μA, which corresponds to a BNA$^+$ concentration of 1.2 mM or more. Undoubtedly, this value exceeds the initial concentration of RF. This is a result of the following regeneration reaction:

$$RFH^- + Zn^{2+} \xrightarrow{k_{reg}} RF + H^+ + Zn^0. \qquad (6)$$

In order to confirm the occurrence of Reaction 6, we carried out the analysis of the zinc ion in the solution phase by means of atomic-absorption spectrometry. If Reaction 6 actually takes place, the Zn^{2+} ion will disappear from the solution. The first measurement was made with 1.0 mM of Zn^{2+} added to a solution of DMSO containing 1 mM of RF and 30 mM of BNAH, and the second with 1.5 mM of Zn^{2+}. The reaction mixture was stirred by means of a magnetic stirrer and kept for several hours at room temperature (*ca.* 22 °C). When the resultant solution was subjected to atomic-absorption spectrometry without any additional treatment, 75 to 80% of the Zn^{2+} was recovered, indicating that the major part of the metallic zinc was still in the solution. Therefore, the solution was brought into contact with mercury and the whole system was vigorously agitated. By this treatment, the metallic zinc can be transferred into the mercury phase.

For the two solutions tested, the initial zinc concentrations of which were 1.0 and 1.5 mM, the values were reduced to 0.05 mM and 0.1 mM respectively. This means, on the average, that, during a period of homogeneous reaction of several hours, each molecule of reduced riboflavin had a chance to return to the oxidized form (RF).

The above findings indicate the following combination of redox cycles:



This is a fact unexpected at the beginning of the present study, some comments will be made below.

Electrochemical Reduction of RF. The controlled potential electrolysis of RF was also studied with a mercury-pool electrode. The change in solution composition was studied also by means of polarography.

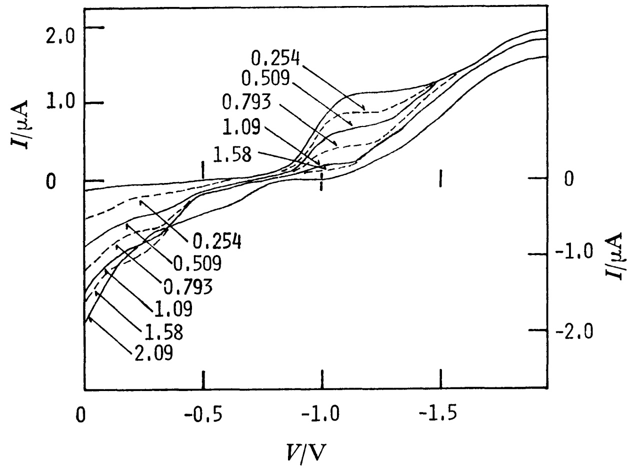


Fig. 8. Progress of controlled potential electrolysis of RF as observed by polarography. Electrolysis was made at −1.90 V. Numerical figures indicate *F*/mol.

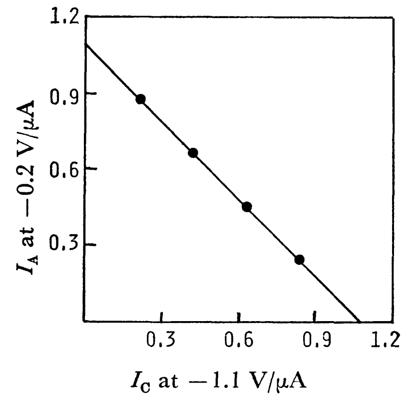


Fig. 9. Coulometric correlation in the electrolytic reduction of RF. The ordinate stands for the amount of reduced product and the abscissa for RF remained.

As is shown in Fig. 8, the first wave of RF reduction decreases with the progress of electrolysis and disappears after passing the electricity of *ca.* 1 *F*/mol. The decreasing cathodic wave and the increasing anodic one are compared in Fig. 9. The slope of this curve is unity, in contrast to Curve A in Fig. 4, where the slope was 2. Since the cathodic wave under consideration is of one-electron, the product which is responsible for the anodic wave seems to be a dimeric compound. The reaction taking place at the cathode electrode may thus be written as:

$$RF + e \rightleftharpoons RF^{\cdot -} \qquad (7)$$

$$2RF^{\cdot -} \longrightarrow (RF)_2^{2-}. \qquad (8)$$

Unexpectedly, the wave height at the potential region where RF gives the second wave does not show a marked decrease.

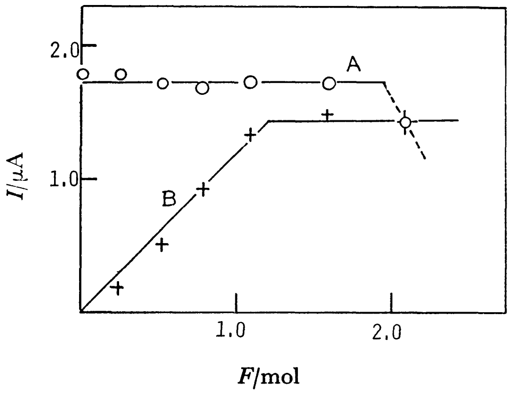


Fig. 10. Analysis of polarograms during the controlled potential electrolysis. Curve A is the wave height at −1.9 V, where not only neutral RF but also several reduced products may contribute to the wave height. Curve B may be considered as the concentration of dimeric product, $(RF)_2^{2-}$.

When the change in wave height measured at −1.9 V is plotted against the quantity of electricity consumed, Curve A of Fig. 10 is obtained. It may be seen that the decrease begins after 2 F/mol or so of electricity is consumed. This suggests that the dimer anion $(RF)_2^{2-}$ produced by Eq. 8 is further reduced to contribute to the wave height. The rest of the wave height is, of course, due to the RF remaining. Since the contribution of the latter is simply twice the height of the first wave, the difference will give the concentration of the dimer anion. Curve B in Fig. 10 shows this. The linear increase ceases at a point corresponding to 1 F/mol, supporting the foregoing estimation that the initial electrode product is a dimeric compound.[13]

It should be noted that the constant potential coulometry made at −1.9 V indicated that the number of electrons involved exceeds 10. This indicates that, upon electrochemical reduction, RF undergoes a complete reduction for almost all of the reducible groups.

Anodic Oxidation of Reduced Riboflavin (Preliminary Report). From the foregoing descriptions, it is clear that there are at least two types of reduction products. One is the BNAH-product, which is assumed to be the so-called leucoflavin,[14] while the other consists of electrode products. The latter is, in general, a mixture of compounds differing in their stages of reduction. In order to avoid confusion, we will hereafter confine the meaning of "electrode product" to the "dimer compound" which is obtained within the limit of an applied charge of 1 F/mol.

The BNAH-product gives a two-stage anodic wave in polarograms, with half-wave potentials at −0.65 (Wave A1) and −0.35 V (A2). The former is much higher than the latter amounting to 85% of the total height. The reoxidation of the electrode product also exhibits a two-stage wave, but the half-wave potentials appear at −0.35 V (A2) and 0 V (A3).

The BNAH-product is sensitive to oxygen, and, if a sufficient amount of oxygen is introduced, the polarogram of the original RF recovers quantitatively. Similarly, RF is regenerated, at least polarographically, when the BNAH-product is subjected to the anodic oxidation at the mercury-pool electrode at −0.5 V.

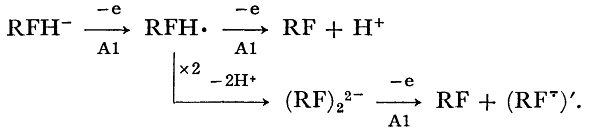
On the other hand, the electrode product behaves somewhat complicatedly in response to the anodic oxidation. When the electrode product is reoxidized at −0.2 V, Wave A2 disappears after passing a charge of 0.5 F/mol, but at this same stage Wave A3 remains unchanged. The disappearance of Wave A2 gives rise to the regeneration of the reduction wave of RF. However, the recovery is, atmost, 60% of the original RF wave.

A tentative explanation for these findings is that the electrode product, which is assumed to be a dimer anion, undergoes one-electron oxidation to give half the original RF:

$$(RF)_2^{2-} + e \xrightarrow{A1} RF + (RF^{\bar{\cdot}})'.$$

One of the products $(RF^{\bar{\cdot}})'$ is solely an assumption in order to explain both the one-electron behavior of the A2 wave and the 50% recovery of the RF wave. When the electrode product is anodically oxidized at +0.2 V, all of the anodic wave disappears, but the reaction is complicated.

The A2 wave appeared in the reoxidation of both the BNAH and electrode products. If the active species responsible for this wave is the same, it should be the dimer anion. As a possibility, the following reaction seems to operate:



$$\begin{tikzcd} RFH^- \arrow[r, "-e", "A1"'] & RFH\cdot \arrow[r, "-e", "A1"'] \arrow[d, "\times 2", "-2H^+"'] & RF + H^+ \\ & (RF)_2^{2-} \arrow[r, "-e", "A1"'] & RF + (RF^{\bar{\cdot}})'. \end{tikzcd}$$

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10) The electrochemical behavior of BNAH is totally irreversible. Its anodic oxidation exhibits a two-electron wave at +0.6 V and produces BNA^+, while the reduction of BNA^+ takes place at about −1.0 V. This means that the electron-transfer reaction of BNA^+/BNAH couple is much hindered. Taking into account the result of Westheimer,[9] who indisputablly verified the hydride-ion mechanism for the NAD^+/NADH couple, the homogeneous reaction between BNAH and RF seems to proceed by means of a hydride mechanism. We have found that sodium borohydride, known as a typical hydride-ion source, also reduces RF.

11) There is another way to describe the reaction:

$$2RF^{\overline{\cdot}} + BNAH + H^+ \longrightarrow BNA^+ + 2RFH^-. \quad (5)$$

This insists that BNAH attacks the $RF^{\overline{\cdot}}$ radical anion, but not the dimer anion $(RF)_2{}^{2-}$. We, however, prefer the mechanism of Eq. 4.

12) The latter species might produce BNA^+ as a result of a follow-up chemical reaction, as in the manner of Eq. 4. However, this does not affect the total wave height.

13) After passing 1 *F*/mol, the current appears to be constant until *ca.* 2 *F*/mol. This constancy is, however, meaningless for discussion, since the species responsible for this current can not be uniquely specified.

14) Spectrometric identification has not yet been attempted, because the absorption of BNAH masks the flavin spectra.