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Reduction of Flavins by Molybdenum(V)†

G. Colovos and J. T. Spence*

ABSTRACT: The mechanism of the reduction of flavins by molybdenum(V) in tartrate buffer (pH 2.50–5.25) has been investigated as a model for molybdenum–flavin reactions in enzymes. The reaction reaches a pH-dependent equilibrium, and the rate of reduction is independent of flavin concentration and dependent on molybdenum(V) concentration to the first power. A two-electron reduction mechanism involving molybdenum(IV) as a reactive intermediate has been developed and applied to the reaction using a computer curve-fitting program to obtain rate constants. No evidence was found for the involvement of flavosemiquinone or molybdenum–flavin complexes in the reduction. A second, much slower reaction between reduced flavin and molybdenum(VI) producing a molybdenum(V)–flavosemiquinone complex was observed. The implications for the reactions of molybdenum-containing enzymes are discussed.

Molybdenum is now well established as a necessary constituent of several redox flavoenzymes (Bray *et al.*, 1967; Spence, 1969). Current evidence suggests a redox role for the metal, as in xanthine oxidase, where it appears to transfer electrons from substrate to flavin: xanthine → Mo(VI) → FAD → Fe(III) → O_2. Although it is generally thought to function between the +6 and +5 oxidation states, some recent work indicates lower states, particularly +4, may be involved (Palmer and Massey, 1969; Massey and Edmondson, 1970). Whether the reaction between molybdenum and flavin is a one- or two-electron-transfer process is unknown, either in enzymatic or model systems.

Preliminary studies of the reaction between the flavin and molybdenum(V,VI) redox systems have been reported

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(Mitchell and Williams, 1964; Hemmerich and Spence, 1966; Spence *et al.*, 1967; Spence, 1970), but no detailed kinetic investigations which would give information useful in interpreting the enzymatic reactions have appeared. This work was undertaken to obtain such data from the model molybdenum(V)–flavin system.

Experimental Section

Materials. Flavin mononucleotide (FMN), obtained from Nutritional Biochemicals Co., was found to be chromatographically pure and used without purification. Lumiflavin-3-acetic acid was kindly furnished by Dr. Peter Hemmerich, Fachbereich Biologie, University of Constance, Germany. Sodium molybdate, determined to be 99.8% pure (Knowles, 1932), was purchased from B. & A. Chemicals. Molybdenum(V) was used in the form $(NH_4)_2MoOCl_5$, prepared according to the method of Palmer (1954), and its purity checked spectrophotometrically (Frank, 1964). All buffers were prepared from reagent grade chemicals.

Methods. The reaction was followed polarographically, since flavins give a completely reversible wave over the pH range used with no interference by molybdenum(V) or -(VI). The method was calibrated by measuring the wave height at the appropriate potential of solutions of known concentrations of flavin.

For a typical run, stock solutions of flavin and $(NH_4)_2MoOCl_5$ were prepared in buffer deaerated with He (99.995%) and allowed to reach temperature equilibrium in a thermostat. Aliquots were then transferred to the proper amount of deaerated buffer in a thermostatted polarographic cell with a gas-tight syringe and mixed with the gas stream. All vessels containing flavin solutions were kept in ruby flasks or covered to prevent the known photochemical reactions of flavins.

Spectrophotometric measurements were made with a Cary 15 recording spectrophotometer and electron spin resonance (esr) data obtained on a Varian V-4500 electron spin resonance (esr) spectrometer equipped with 100-kc field modulation.

Rate constants were obtained using least-squares and curve-fitting programs on a Digital Computer Corp. PDQ-8 computer.

Results

In order to determine the possible influence of the ribityl side chain of FMN on the reaction rate, lumiflavin-3-acetic acid[1] was reduced with $Mo(V)_2$ under identical conditions as FMN. The kinetic results were essentially the same, however, so FMN was used in all runs reported.

Equilibria. When excess $(NH_4)_2MoOCl_5$ and FMN are mixed in tartrate buffer at pH 4.20 the reaction proceeds almost to completion. If Mo(VI) (as Na_2MoO_4) is added to the system, however, the reaction reaches an equilibrium. Similarly, if Mo(VI) is added to a solution of FMN reduced with $Na_2S_2O_4$ or $TiCl_3$, the reverse reaction proceeds to the same equilibrium:[2] FMN + $Mo(V)_2 \rightleftarrows FMNH_2$ + 2Mo(VI) + H^+. (Molybdenum(V) exists primarily as a dimer under these conditions and both $Mo(V)_2$ and Mo(VI) are undoubtedly present as tartrate complexes, but their structures are uncertain) (Spence and Heydanek, 1967). At pH 4.20 this equilibrium is strongly in favor of the products; it is, however, pH dependent and shifts somewhat toward the reactants at lower pH. A plot of log K_e *vs.* pH in the pH range 2.50–3.80 (Figure 1) gave a slope of 0.80 ± 0.16, indicating one proton is released in the reaction. Above pH 4.20, K_e becomes independent of pH. At pH 4.20, K_e = 0.360 ± 0.059 (12 determinations).

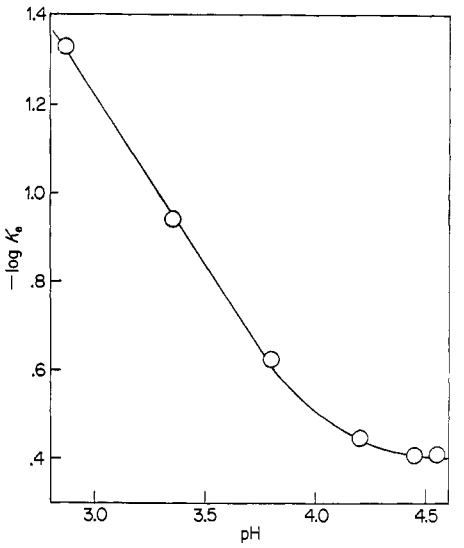


FIGURE 1: Variation of equilibrium constant with pH. −log K_e is plotted *vs.* pH. 0.769 M tartrate, 37°.

Because of the greater ease of preparation of solutions, and the value of K_e, the kinetic data were obtained from a study of the forward reaction.

Kinetics. At pH 4.20, in the absence of added Mo(VI) the reaction proceeds almost to completion. Kinetic studies indicated the rate in the absence of Mo(VI) is independent of FMN concentration and, in the presence of excess $Mo(V)_2$, follows zero-order kinetics for close to 2 half-lives (Figure 2). Because of the slowness of the reaction, most runs were made with excess $Mo(V)_2$; by varying the concentration of $Mo(V)_2$, however, the rate was found to be first order in $Mo(V)_2$

$$-\mathrm{d[FMN]}/dt = k_1[\mathrm{Mo(V)_2}] = k_1' \qquad (1)$$

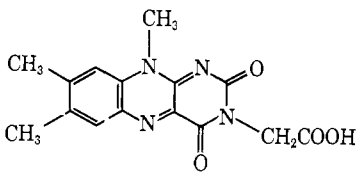
this expression was used to obtain the rate constants in Table I.

In order to explain these results, a rate controlling step involving only $Mo(V)_2$ is necessary. Previous work has shown that both $Mo(V)_2$ and Mo(VI) may react *via* an Mo(IV) intermediate (Guymon and Spence, 1967; Huang and Spence, 1968). Using this suggestion, and taking into account the reverse reaction, the following mechanism can be postulated

$$\mathrm{Mo(V)_2} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{Mo(IV)} + \mathrm{Mo(VI)}$$

$$\mathrm{Mo(IV)} + \mathrm{FMN} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{Mo(VI)} + \mathrm{FMNH_2}$$

I



lumiflavin-3-acetic acid

[2] Abbreviations used are: FMN, flavoquinone; FMNH·, flavosemiquinone; $FMNH_2$, flavohydroquinone.

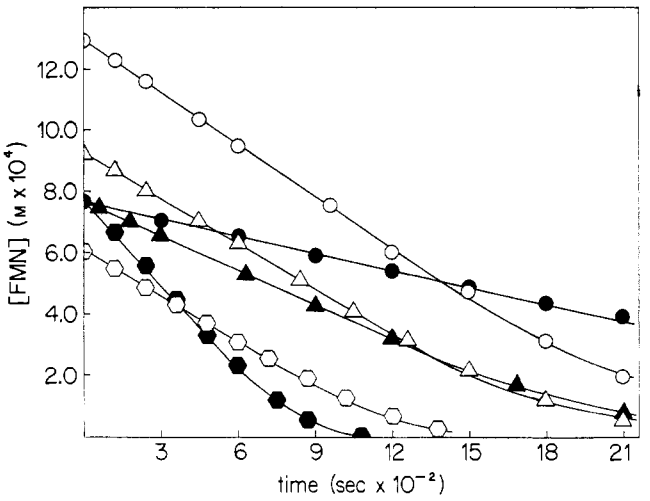


FIGURE 2: Zero-order kinetic plots for forward reaction. [FMN] is plotted *vs.* time. (○) $[FMN]_0 = 12.96 \times 10^{-4}$ M, $[Mo(V)_2] = 6.94 \times 10^{-3}$ M. (△) $[FMN]_0 = 9.26 \times 10^{-4}$ M, $[Mo(V)_2] = 6.94 \times 10^{-3}$ M. (○) $[FMN]_0 = 6.07 \times 10^{-4}$ M, $[Mo(V)_2] = 6.94 \times 10^{-3}$ M. (●) $[FMN]_0 = 7.69 \times 10^{-4}$ M, $[Mo(V)_2] = 2.62 \times 10^{-3}$ M. (▲) $[FMN]_0 = 7.69 \times 10^{-4}$ M, $[Mo(V)_2] = 4.99 \times 10^{-3}$ M. (●) $[FMN]_0 = 7.69 \times 10^{-4}$ M, $[Mo(V)_2] = 11.88 \times 10^{-3}$ M. pH 4.20, 0.769 M tartrate, 37°.

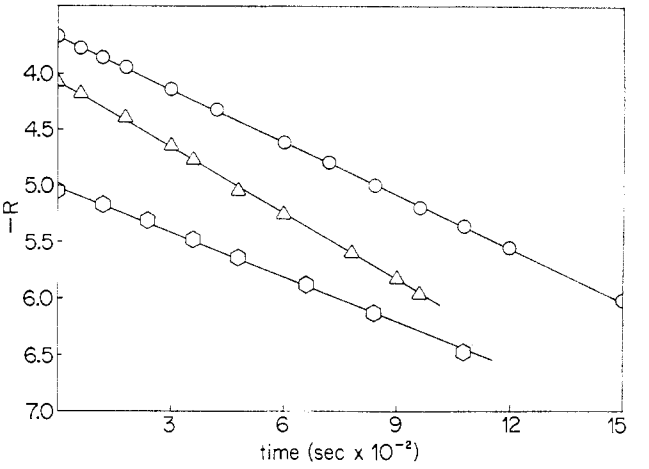


FIGURE 3: Kinetic plots for complete reaction. $-R[(\log (a + b \times [FMN])] + b[FMN]/2.303(bc - a)]$ is plotted *vs.* time (see eq 4). (○) $[FMN]_0 = 7.69 \times 10^{-4}$ M, $[Mo(V)_2] = 1.19 \times 10^{-2}$ M, [Mo(VI)] = 1.54×10^{-2} M. (△) $[FMN]_0 = 7.69 \times 10^{-4}$ M, $[Mo(V)_2] = 1.83 \times 10^{-2}$ M, [Mo(VI)] = 3.08×10^{-2} M. (○)$[FMN]_0 = 3.08 \times 10^{-4}$ M, $[Mo(V)_2] = 1.19 \times 10^{-2}$ M, [Mo(VI)] = 3.08×10^{-2} M. pH 4.20, 0.769 M tartrate, 37°.

Applying the steady-state assumption to [Mo(IV)], this mechanism gives the rate expression

$$-\mathrm{d[FMN]}/\mathrm{d}t = \frac{k_1k_2[Mo(V)_2][FMN] - k_{-1}k_{-2}[Mo(VI)]^2[FMNH_2]}{k_{-1}[Mo(VI)] + k_2[FMN]} \quad (2)$$

Since $K_e = k_1k_2/k_{-1}k_{-2}$, eq 2 can be written as

$$-\mathrm{d[FMN]}/\mathrm{d}t = \frac{k_1\left([Mo(V)_2][FMN] - \dfrac{[Mo(VI)]^2(FMNH_2)}{K_e}\right)}{\dfrac{k_{-1}}{k_2}[Mo(VI)] + [FMN]} \quad (3)$$

Since $C_0 = [FMN] + [FMNH_2]$ and both $Mo(V)_2$ and Mo(VI) concentrations are essentially constant, eq 3 upon integration may be written

$$\log (a + b[FMN]) + \frac{b[FMN]}{2.303(bc - a)} = -\frac{b^2k_1t}{2.303(bc - a)} + I \quad (4)$$

where $a = -[Mo(VI)]^2Co/K_e$, $b = [Mo(V)_2] + (Mo(VI)]^2/K_e$, $c = k_{-1}/k_2[Mo(VI)]$, and I = constant of integration. In order to apply this equation to the rate data, the ratio k_{-1}/k_2 must be known. By neglecting the second term in the numerator of eq 3, which is a good approximation in the absence of added Mo(VI), approximate limits of k_{-1}/k_2 can be estimated. The best value of k_{-1}/k_2 was then obtained by treating the data with a computer curve-fitting program starting with these limits, which gave the results in Table II and the plots in Fig-

TABLE I: Estimated Rate Constants, Forward Reaction.[a]

Run	$[FMN]_0$, M × 10^4	$[Mo(V)_2]_0$, M × 10^3	k_1[b], sec^{-1} × 10^5
1	7.69	1.97	7.02
2	7.69	2.62	6.98
3	7.69	3.68	8.16
4	7.69	4.99	7.63
5	7.69	6.04	7.85
6	7.69	6.94	7.78
7	7.69	8.16	7.66
8	7.69	10.61	7.63
9	7.69	11.88	7.88
10	6.07	6.94	7.29
11	7.41	6.94	6.63
12	9.26	6.94	7.30
13	11.10	6.94	7.20
14	12.96	6.94	8.43

[a] pH 4.20, 0.769 M tartrate, 37°. [b] Average with std dev = $(7.53 \pm 0.49) \times 10^{-5}$.

TABLE II: Rate Constants.[a]

Run	$[FMN]_0$, M × 10^4	$[Mo(V)_2]_0$, M × 10^2	$[MoVI]_0$, M × 10^2	k_{-1}/k_2[b], × 10^2	k_1[c], sec^{-1} × 10^5
1	7.69	2.06	3.08	1.62	12.0
2	7.69	1.83	3.08	1.14	9.62
3	7.69	1.46	3.08	1.22	9.28
4	7.69	1.19	3.08	2.28	12.1
5	7.69	0.852	3.08	2.28	11.2
6	6.15	1.19	3.08	0.920	7.88
7	4.62	1.19	3.08	1.10	8.10
8	3.08	1.19	3.08	0.906	7.68
9	7.69	1.19	6.15	1.62	13.1
10	7.69	1.19	1.54	1.32	7.06

[a] pH 4.20, 0.769 M tartrate, 37°. [b] Average with std dev = $(1.44 \times 0.51) \times 10^{-2}$. [c] Average with std dev = $(9.80 \times 2.14) \times 10^{-5}$ sec^{-1}.

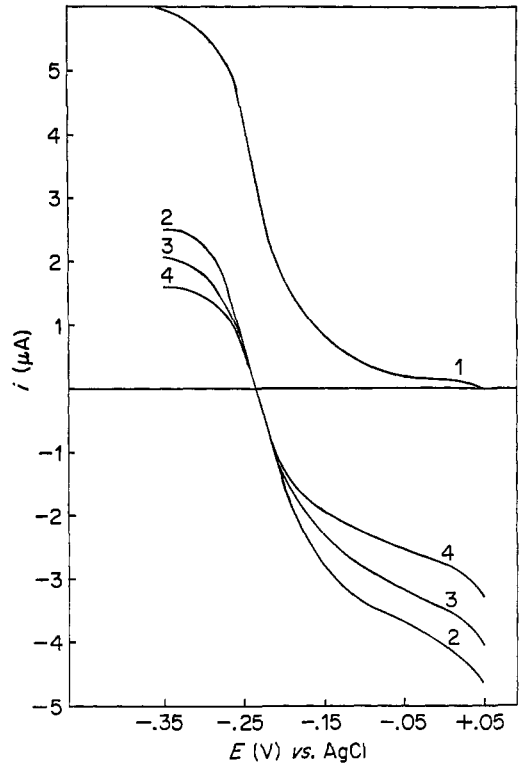


FIGURE 4: Polarographic waves during formation of red complex. Current (i) is plotted *vs.* E. 1, zero time. 2, 57 min. 3, 528 min. 4, 1185 min. $[FMN]_0 = 7.41 \times 10^{-4}$ M, $[Mo(V)_2] = 9.39 \times 10^{-3}$ M, [Mo(VI)] $= 4.44 \times 10^{-2}$ M. pH 4.20, 0.769 M tartrate, 37°.

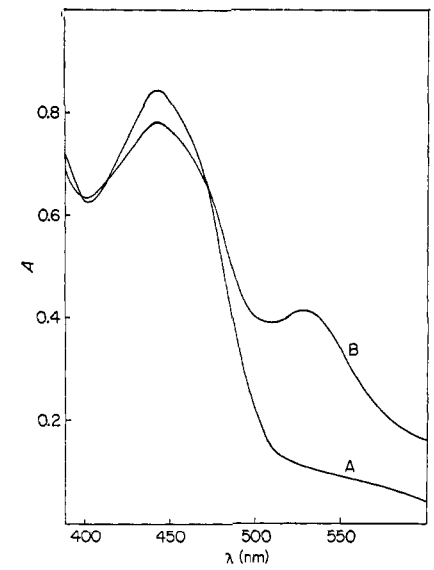


FIGURE 5: Visible absorption spectra for FMN and red complex. (A) Zero time. (B) 1185 min. $[FMN]_0 = 7.41 \times 10^{-4}$ M, $[Mo(V)_2] = 9.39 \times 10^{-3}$ M, [Mo(VI)] $= 4.44 \times 10^{-2}$ M. pH 4.20, 0.769 M tartrate, 37°. 0.1-cm cells.

ure 3. From this same program the slope was obtained, allowing k_1 to be calculated. The results (Table II) are seen to be in satisfactory agreement with the approximate value of k_1 obtained by eq 1 (Table II). It should be noted that the plots (Figure 3) give good straight lines to 90–95% reaction.

In the absence of excess Mo(VI), the second term in the numerator and the first term in the denominator of eq 3 can be neglected for about 70% reaction, giving eq 1.

Equation 3 also suggests that in the presence of a large excess of Mo(VI), the forward reaction should become first order in FMN. Consideration of the value of k_{-1}/k_2, however, indicates that in the range of useful FMN concentrations ($\geqq 10^{-4}$ M), Mo(VI) would have to be $\sim 2 \times 10^{-1}$ M in order to achieve this. At such high concentrations, the reaction would only proceed to a small extent before equilibrium would be reached, and the evaluation of rate constants would be difficult if not impossible.

The kinetic results, therefore, are in agreement with the assumed mechanism and indicate the reduction proceeds *via* a two-electron step.

Using the values of K_e, k_1, and k_{-1}/k_2, k_{-2} can then be calculated: $k_{-2} = k_1/(k_{-1}/k_2 K_e) = 1.89 \times 10^{-2}$ sec^{-1} M^{-1}.

If the reaction mixture containing a large excess of Mo(VI) was allowed to stand for considerable time after reaching equilibrium, a second, very slow change was observed. The solution gradually became a deep wine red, and the total polarographic wave height (cathodic plus anodic waves) decreased (Figure 4). The same result could also be obtained by adding an excess of Mo(VI) to a solution of $FMNH_2$ under similar conditions. This same phenomenom has been observed before in phosphate buffer, pH ~8, where it was attributed to the formation of a Mo(V)·flavosemiquinone complex (Hemmerich and Spence, 1966; Spence *et al.*, 1967): Mo(VI) + $FMNH_2 \rightleftarrows$ Mo(V)·FMNH·. The Mo(V)·FMNH· complex does not give a polarographic wave in this potential region; hence, the total wave height due to FMN and $FMNH_2$ decreases as the equilibrium shifts with complex formation. The same reaction must occur in this case, since the spectrum of the final solution (Figure 5) is the same as reported above (Hemmerich and Spence, 1966).

In the above work in phosphate buffer (Hemmerich and Spence, 1966; Spence *et al.*, 1967) this complex was assumed to be an intermediate in the reverse reaction between Mo(VI) and $FMNH_2$; in the present work, however, this cannot be the correct interpretation, since the complex does not form until long after the faster reaction between $Mo(V)_2$ and FMN has reached equilibrium. The complex formation must, therefore, be regarded as competing with the reverse reaction. Apparently, this complex formation does not compete favorably with the reverse two electron redox reaction in tartrate buffer, probably due to complexing of Mo(VI) with buffer. Because of the low rate of complex formation, no further rate studies were made at this time. In the previous work in phosphate buffer, (Spence *et al.*, 1967) some evidence was obtained for the intermediate formation of an esr active, Mo(VI)·FMNH· complex. In the present work an esr analysis of the reaction mixture gave no evidence for such a complex. No increase in flavosemiquinone in solutions containing $Mo(V)_2$ and Mo(VI) above that obtained from solutions containing the same concentrations of FMN and $FMNH_2$ was found. Some flavosemiquinone is always present in such solutions, due to the well-known disproportionation equilibrium (Hemmerich *et al.*, 1963): FMN + $FMNH_2 \rightleftarrows$ 2FMNH . Again, this difference in behavior of the two systems can most likely be attributed to the presence of $Mo(V)_2$ and Mo(VI) tartrate complexes.

Discussion

The results indicate the reaction between the flavin and Mo(V,VI) redox systems goes by a two electron transfer, with Mo(IV) as a reactive intermediate. This lends some support

to the recent suggestion that Mo(IV) may be involved in the catalytic cycle of the enzyme xanthine oxidase (Palmer and Massey, 1969; Massey and Edmondson, 1970). In water, Mo(IV) is unstable, reacting immediately with flavin or Mo(VI). In the enzyme, however, this oxidation state may be stabilized by the various amino acid side chains of the protein. In such a case, the presence of an esr signal during substrate oxidation might be attributed to Mo(V) in equilibrium with Mo(IV) and Mo(VI), or, less likely, to an Mo(IV) species in a reasonably symmetrical field.[3]

The second, much slower reaction of Mo(VI) with $FMNH_2$ to produce the red complex indicates another possible pathway for electron transfer, in this case a one-electron step

$$Mo(VI) + FMNH_2 \rightleftharpoons Mo(V)\cdot FMNH\cdot \text{ (red complex)}$$

$$Mo(V)\cdot FMNH\cdot \rightleftharpoons Mo(V) + FMNH\cdot$$

$$2Mo(V) \rightleftharpoons Mo(V)_2$$

$$2FMNH\cdot \rightleftharpoons FMN + FMNH_2$$

Since this reaction seems to compete favorably with the two-electron step in phosphate buffer, but not in tartrate, it appears that the ligand environment of the molybdenum is important in deciding which path is preferred, again suggesting a critical role for the binding groups present at the active site of the enzyme. Work concerning the influence of various ligands on the relative rates of these two reactions is underway in this laboratory.

[3] In a symmetric octahedral field, Mo(IV) should have two unpaired electrons. No esr signals for Mo(IV) species have been reported in solution, however. This is most likely due to severe line broadening caused by an efficient spin–spin interaction relaxation process. Such appears to be the case for Mo(III) complexes, which also give no esr signals in solution.

Acknowledgment

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