pulsion between the chloride ion and the electron pair of the ammonia. The ammonia adds predominantly between the ethylenediamine molecules, which results in a *trans* configuration. The D*-*cis* product occurs only when ammonia adds adjacent to the chloride of the trigonal bipyramid or when a tetragonal pyramid is the intermediate.

It is interesting to note that a tetragonal pyramid is the most logical intermediate for the second step of the reaction because of its almost complete retention of configuration but rather unlikely for the first step at low temperatures.

A comparison of the analogous reactions in water are interesting. The aquation (acid hydrolysis) of *trans*-$[Co(en)_2(NH_3)Cl]^{+2}$ occurs with retention of configuration[18] (as is also noted for the corresponding ammonation) and is believed to involve largely *cis*-displacement. On the other hand, aquation of *cis*-$[Co(en)_2Cl_2]^+$ is thought to take place predominantly *via* an S_N1 mechanism,[9b,17] which does not allow a D*⟷L* inversion as has been noted for the analogous ammonation. The greater polarizability and greater base strength of ammonia may account for this difference.

A kinetic study in liquid ammonia is now in progress in an attempt to determine the thermodynamic constants of possible competing mechanisms.

Experimental

Synthesis of Dichlorobis-(ethylenediamine)-cobalt(III) Chlorides.—The *trans*-dichlorobis-(ethylenediamine)-cobalt(III) chloride was prepared as described by Bailar[19] with a slight modification—oxygen was bubbled into the cobalt(II) solution through a sintered glass gas bubbler for 1.5 to 2 hr. (longer bubbling for up to 4 hr. does not appreciably alter the yield) instead of bubbling air through the solution for 10 to 12 hr. The *cis*- and D*-*cis*-dichlorobis-(ethylenediamine)-cobalt(III) chlorides were also prepared by the method described by Bailar[19] with comparable yields.

Anal. (resolved *cis* complex) Calcd. for $CoC_4H_{16}N_4Cl_3$: C, 16.83; H, 5.65. Found: C, 16.80; H, 5.66; $[M]_D = 2380 \pm 75°$.

Synthesis of Chloroamminebis-(ethylenediamine)-cobalt(III) Chlorides.—The *cis*- and the D*-*cis*-chloroamminebis-(ethylenediamine)-cobalt(III) chlorides were prepared by the directions of Work[20] with similar yields.

Anal. (resolved *cis*-chloroammine complex) Calcd. for $CoC_4H_{19}N_5Cl_3$: C, 15.88; H, 6.33; N, 23.15. Found: C, 15.92; H, 6.25; N, 22.95; $[M]_D = 580 \pm 17°$.

Apparatus.—Rotatory dispersion measurements were made on instruments reading directly to 0.001°. Both a Schmidt and Haensch polarimeter with a monochromator and a Rudolph Photoelectric Spectro-Polarimeter were used. Jacketed cells with path lengths of one and two decimeters were used. All rotations are reported as molecular rotations, $[M]$.

The absorption measurements were taken on a Cary Recording Spectrophotometer, Model 14, with one cm. matched quartz cells.

Constant low temperatures were maintained with a small heater, a Fenwal thermoregulator, Dry Ice cold fingers and a methanol bath in a large Dewar flask.

Ammonation of Optically Active Complexes.—Refrigeration grade ammonia, distilled from a vessel containing sodium, was used in this study. Moisture was excluded by the use of potassium hydroxide or mercury traps during all experiments and by flashing the reaction vessels with gaseous ammonia prior to their cooling for an experiment. Wherever appropriate, the complex was dissolved in liquid ammonia just above the melting point of ammonia (−77°) and warmed to the reaction temperature in the constant temperature bath. At the end of the reaction period, the excess liquid ammonia was flashed off; the precipitate was dried at room temperature *in vacuo* for two days and then dissolved in water to make the appropriate measurements. The ammonia concentrations in methanol were determined by titration with standard hydrochloric acid solution.

Ammonation of Racemic *cis*-Chloroamminebis-(ethylenediamine)-cobalt(III) Chloride.—Three grams (3 g., 0.01 mole) of racemic *cis*-$[Co(en)_2(NH_3)Cl]Cl_2$ was allowed to react with 500 cc. of liquid ammonia for two weeks. One-half gram (0.5 g.) of the resulting yellow-orange precipitate was dissolved in 100 cc. of water and 50 cc. of a saturated solution of sodium dithionate (saturated in 5% acetic acid) was added. Only a few fine crystals (less than a mg.) of the very slightly soluble *trans*-$[Co(en)_2(NH_3)_2]_2(S_2O_6)_3$ were isolated, even with seeding. However, 0.4 g. of the *cis*-$[Co(en)_2(NH_3)_2](I_x)_3$ complex was isolated by the addition of an excess of potassium periodide solution to the filtrate.

Acknowledgment.—We are indebted to Socony Mobil Co. and Texaco Inc. for providing an assistantship and a fellowship, respectively, for one of the authors (R.D.A.) during the early part of the study, to J. Nemeth and his staff for the analyses and to the staff at the California Institute of Technology for the use of their Rudolph spectropolarimeter.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF UTAH STATE UNIVERSITY, LOGAN, UTAH]

The Interaction of Molybdenum with Riboflavin and Flavin Mononucleotide

BY J. T. SPENCE AND JACQUES TOCATLIAN[1]

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An investigation of the complexing properties of molybdenum has led to a study of the interaction of molybdenum (V) and (VI) with FMN, riboflavin and mannitol. Polarimetric studies have indicated a strong complex is formed in acidic solution between molybdenum (VI) and FMN. The complex was found to contain two molybdenums per FMN. Riboflavin probably forms a similar complex. Mannitol was found to complex with molybdenum (VI) in the same ratio as FMN and an apparent stability constant was calculated. No evidence was found for a complex of molybdenum (V) with FMN or mannitol. Kinetic studies indicated FMN acts as a catalyst for the oxidation of Mo (V) to Mo (VI) by oxygen. Results are interpreted as indicating the ribitol side chain of FMN is involved in complex formation with Mo (VI). The biological implications with regard to molybdenum containing enzymes are discussed.

An investigation in this Laboratory of the interaction of molybdenum with compounds of biochemical interest has led to a study of complexes of molybdenum with FMN (flavine mononucleotide) and riboflavin. Molybdenum has been reported to be present in four enzymes,[2-5] all of which contain

(1) Abstracted in part from a thesis submitted by J. T. in partial fulfillment of the requirements for the M.S. degree at Utah State University, 1960.

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FAD (flavin-adenine dinucleotide) as coenzymes. In view of the known ability of riboflavin to complex other metals,[6,7] it seemed of interest to investigate the interaction of molybdenum with FMN and riboflavin as a possible model system for the enzymes in which these compounds occur (as part of the FAD molecule). Since recent work[8] has indicated that the molybdenum in xanthine oxidase undergoes a change in oxidation state from (VI) to (V) in the presence of the substrate, both oxidation states of molybdenum have been investigated.

Although a number of complexes of molybdenum with compounds of biochemical interest have been reported,[9-12] little quantitative work has been done, and no reports of complexes between molybdenum and riboflavin or FMN have appeared.

Experimental

Polarimetric data were obtained with two instruments. The variation of specific rotation with *p*H for the complexes and the molar ratio data for the mannitol complex were determined with a Schmidt and Haensch polarimeter. The molar ratio data for the FMN complex was obtained with a Rudolph high precision polarimeter. Due to the color of FMN solutions, it was found best to use a light source of 5780 Å. (yellow line of mercury) to obtain reasonable optical rotations along with good contrast. The sodium lamp was used for the mannitol complex. Temperature was 25.2 ± 0.1°. The precision of the optical rotation data was ±0.01 degrees of arc with the Rudolph instrument and ±0.02 degrees of arc with the Schmidt and Haensch instrument.

Kinetic data were obtained by pipetting aliquots of stock solutions of molybdenum (V) (in 3 *N* HCl) into flasks containing the proper quantity of sodium hydroxide solution immersed in a thermostat at 25.2 ± 0.1° and withdrawing samples at selected time intervals, diluting and measuring the absorbance at 298 mμ *vs.* a blank of FMN or water. The *p*H of the mixtures was determined on an aliquot immediately after mixing.

Spectrophotometric data were obtained with a Perkin-Elmer Model 4000 A recording spectrophotometer and a Beckman Model DU spectrophotometer. Specially constructed pressure cells 1 cm. thick, made by the American Instruments Corporation, were used for spectrophotometric measurements in the absence of air.

Molybdenum (VI) solutions were prepared from B & A reagent grade sodium molybdate dried for 24 hr. at 110° and stored in a desiccator. This reagent was analyzed by the α-benzoinoxime method[13] and found to contain 99.7% $Na_2MoO_4 \cdot 2H_2O$. Molybdenum (V) solutions were prepared by reduction of molybdenum (VI) solutions by shaking vigorously in a mechanical shaker with mercury in 3 *N* hydrochloric acid and standardized against cerric sulfate.[14] It was found that these solutions could be kept for relatively long periods (a week or more) by storing over mercury.

Eastman Kodak Co. riboflavin was used without further purification since chromatographic analysis[15] did not reveal the presence of any impurities. FMN was purchased from Nutritional Biochemicals Company and was used without further purification since chromatographic analysis showed no impurities. Solutions of riboflavin and FMN were prepared fresh and discarded after 12 hr. Every effort was made to protect them from light and keep them at the proper *p*H to minimize decomposition.[16]

Ribitol was obtained from Nutritional Biochemicals Company and used without further treatment. Eastman Kodak Co. mannitol was used without further purification. All other materials were reagent grade.

Results

Polarimetric Studies of Molybdenum (VI).—Figure 1 shows the dependence of specific rotation on *p*H for solutions of FMN and complexes of FMN, riboflavin and mannitol. The optical rotation of mannitol is too small to be measured and the

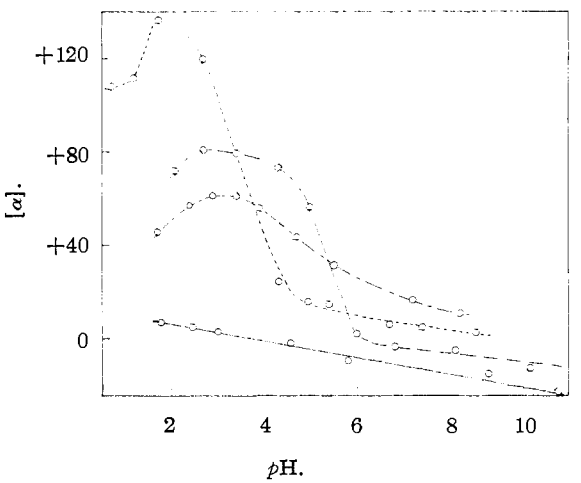

Fig. 1.—Variation of specific rotation ([α]) with *p*H: —, FMN, 1.0 × 10^{-2} *M*· — — —, FMN, 1.0 × 10^{-2} *M*, + Mo(VI), 2.0 × 10^{-2} *M*; — — — —, riboflavin, 1.0 × 10^{-2} *M* + Mo(VI) 2.0 × 10^{-2} *M*; - - - -, mannitol, 5.0 × 10^{-2} *M* + Mo(VI) 0.10 *M*. FMN solutions measured at 5780 Å., mannitol measured at 5890 Å.

low solubility of riboflavin in acidic solutions prevents measurement of its optical rotation. The addition of molybdenum (VI) to solutions of FMN and mannitol enhances the specific rotation, with the maximum increase occurring at a *p*H between 2 and 3. This increase in optical rotation has been used in analytical determinations of mannitol and other sugar alcohols.[17] The addition of molybdenum (VI) to riboflavin increases the solubility of the latter, giving a specific rotation-*p*H curve similar to that for FMN although somewhat lower in magnitude. This increase of solubility is good evidence for complex formation with riboflavin.

Figure 2 is a plot of molar ratio of molybdenum (VI) *vs.* optical rotation for FMN–molybdenum (VI) solutions. The data show a sharp break at a ratio of 2 Mo per FMN. Furthermore, the sharp-

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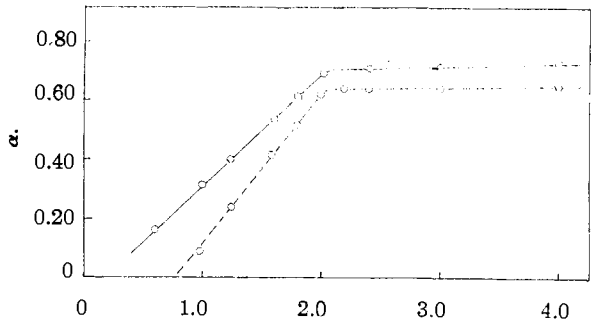

Fig. 2.—Variations of optical rotation (α) with molar ratio Mo(VI)/FMN. The optical rotations of a series of solutions containing a FMN concentration of 1.0×10^{-2} M and increasing Mo concentrations are plotted *vs.* molar ratio Mo/FMN. —, pH = 2.50, chloroacetate buffer; - - - -, pH = 4.68, acetic acid buffer.

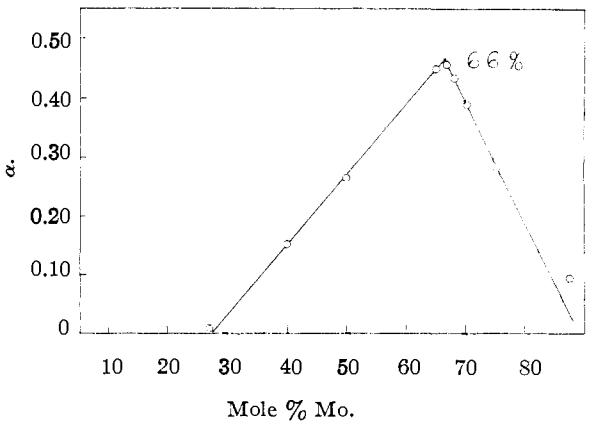

Fig. 3.—Job's method of continuous variations for FMN in the presence of Mo(VI). The sum of the FMN and the Mo(VI) concentrations is equal to 2.0×10^{-2} M. Optical rotation (α) is plotted *vs.* mole % Mo (VI) at pH 2.50 in chloroacetate buffer.

ness of the break indicates a very strong complex is formed; in fact, the complex is so strong that no estimate of the stability constant could be made from the graph. Increasing the pH decreases the total rotation of the complex but affects the sharpness of the break only slightly. No reliable measurements above pH 5 could be made due to the small rotation of the complex. The ratio of 2 Mo per FMN is confirmed by Fig. 3, which represents a plot of mole fraction of molybdenum (VI) *vs.* optical rotation according to Job's method of continuous variations adapted to polarimetry.

In an effort to find out what part of the FMN molecule is involved in complex formation, ribitol and mannitol were treated in the same way. The observed rotations with ribitol were too small to be useful, but in the case of mannitol a complex involving a ratio of 2 Mo per mannitol was found, as seen in Fig. 4. The break in the curve is not as sharp as in the case of FMN and an estimate of the apparent formation constant gave a value of 2×10^4 (mole/l.)$^{-2}$.

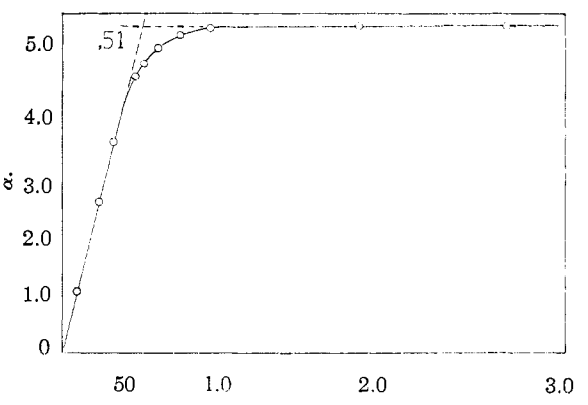

Fig. 4.—Variation of optical rotation (α) with molar ratio mannitol/Mo(VI). The optical rotations of a series of solutions containing a Mo(VI) concentration of 0.10 M and increasing mannitol concentrations are plotted *vs.* molar ratio mannitol/Mo(VI) at pH = 2.80 in chloroacetate buffer.

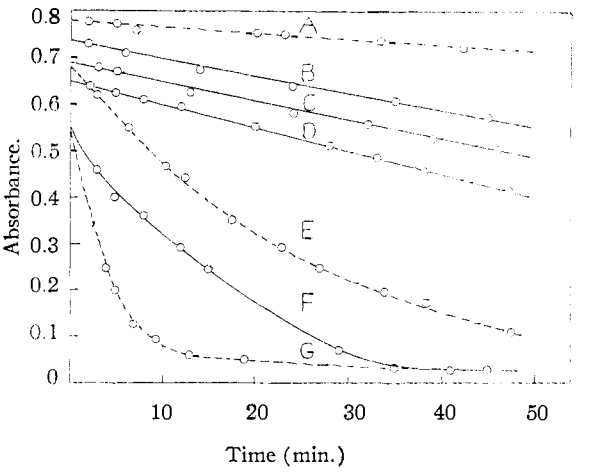

Fig. 5.—Effect of FMN on the rate of oxidation of Mo(V). Absorbances at 298 mμ are plotted *vs.* time for a series of solutions containing an initial concentration of Mo(V) of 5.39×10^{-4} M (solid curves) or an initial concentration of Mo(V) of 5.39×10^{-4} M plus a concentration of FMN of 5.39×10^{-4} M (dashed curves) at the pH values: A, 2.45; B, 2.70; C, 5.15; D, 5.95; E, 5.50; F, 6.90; G, 6.70.

Because of its low solubility, no significant results with riboflavin could be obtained.

Solutions of molybdenum (V) are so intensely colored in the wave length range of interest that no reliable polarimetric measurements were possible.

Spectrophotometric and Kinetic Studies of Molybdenum (V).—Molybdenum (V) has an absorption maximum near 298 mμ which can be used to identify it. This band rapidly disappears in the absence of a complexing agent, due to oxidation to molybdenum (VI) by atmospheric oxygen. Molybdenum (V) can be stabilized against oxidation by the presence of a suitable chelating agent, such as EDTA.[18] Studies of solutions of molybdenum (V)

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and FMN showed that FMN did not stabilize molybdenum (V) toward oxidation at any *p*H, suggesting that no complex is formed. In fact, the rate of oxidation of molybdenum was actually enhanced by addition of FMN, particularly at *p*H values above 5. Figure 5 shows the oxidation of molybdenum (V) to molybdenum (VI) as measured by the disappearance of the absorption band at 298 mμ. Addition of FMN catalyzes the oxidation at *p*H values above 5. No attempt was made to study the catalysis above *p*H 7 because of the speed of the reaction.

In an attempt to understand this catalysis it was postulated that Mo(V) might be reducing FMN, which in turn would be oxidized very rapidly by oxygen. This was tested by mixing deaerated solutions of Mo(V) and FMN under nitrogen at various *p*H values and recording the spectra in special pressure cells which had been evacuated and flushed with nitrogen. No change in the spectrum of the solutions was observed up to 24 hr. after mixing. Since the spectrum of reduced FMN is quite different[19] from oxidized FMN, it was concluded that no reduction of FMN by molybdenum (V) occurs in the absence of oxygen.

Addition of molybdenum (VI) did not alter the spectrum of FMN in any way.

Discussion

All of the data obtained indicate a very strong complex is formed between the molybdenum(VI) and FMN in the *p*H range studied, with a maximum stability near *p*H 3, as indicated by the variation of specific rotation with *p*H. Furthermore, the results of the variation of specific rotation with *p*H and the increased solubility in the presence of molybdate make it probable that a similar complex is formed with riboflavin, although its stability may not be as great.

Although it is as yet tentative, the following considerations support the conclusion that the molybdenum is complexed with the ribitol side chain of FMN or riboflavin. First, no change was observed in the visible or ultraviolet spectrum of FMN upon addition of molybdenum (VI). Such a change might be expected if the molybdenum disturbed the resonance system of the isoalloxazine nucleus by complexing between the number 10 nitrogen and the hydroxy group *peri* to it. Second, a rather large change in the optical rotation of FMN occurs upon the addition of molybdenum (VI), and the optical rotation of FMN is due to the ribitol side chain. Third, a similar complex of the same ratio is formed between mannitol and molybdenum, although it appears to be of a lower stability. The difference in optical rotation between the FMN–Mo complex and the riboflavin–Mo complex may be due to the involvement of the phosphate group of the former. The apparent increase of stability of the FMN-complex over the mannitol complex might be ascribed to coordination of the molybdenum with one of the nitrogen atoms of the isoalloxazine nucleus. Work to confirm these conclusions is currently being pursued in this Laboratory.

The catalysis of the oxidation of Mo(V) to Mo(VI) by FMN may be similar to that reported for the photo-oxidation of Fe(II) to Fe(III).[20] Further studies of this catalysis are planned.

The results reported in this paper support the idea that Mo(VI) may be complexed with FAD in the enzyme systems in which it is found. Furthermore, the catalysis of the oxidation of Mo(V) by FMN may have implications for those oxidation–reduction enzyme systems in which molybdenum is known to undergo a change in oxidation state.[8,21]

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