p-**Phenoxypropiophenone.** This ketone was made by the procedure of Bun-Hoi, *et al.*,[74] mp (CH_3OH) 35.5–36.0°, lit. 36°.

p-**Phenylthiopropiophenone.** This compound was synthesized by the Friedel–Crafts reaction of diphenyl sulfide and propionyl chloride using $AlCl_3$ as catalyst. The crude product contained some disubstituted material. The desired compound was separated by fractional crystallization and consisted of white crystals, mp 59–61°. The nmr spectrum showed an A_2B_2-type multiplet splitting from the disubstituted benzene ring with peaks at 7.81, 7.74, 7.21, and 7.13 ppm, a broad resonance from the monosubstituted benzene ring at 7.37 ppm, a 1:3:3:1 quartet from the methylene protons centered at 2.86 ppm, and 1:2:1 triplet at 1.20 ppm.

p-**Phenylselenopropiophenone.** This compound was synthesized in the same manner as the corresponding sulfur derivative. Faint yellow crystals were obtained, mp 71–73°. The methylene and methyl resonances were centered at 2.850 and 1.17 ppm, respectively. The B-type lines of the A_2B_2 multiplet were obscured by the resonance from the monosubstituted benzene ring.

B. Procedures. Oxidations were carried out in the apparatus described earlier.[75] The concentrations of base and ketone were 0.1 and 0.05 *M*, respectively. Esr spectra were obtained with a Varian V-4500-10 esr spectrometer equipped with a 12-in. magnet and 100-kc/sec field modulation. Sweep rates were calibrated from the spectrum of *p*-benzosemiquinone in 1-butanol.[76]

Acknowledgments. We are grateful to Mr. Robert Petkun for the synthesis of *p*-*t*-butylpropiophenone, *m*-methoxypropiophenone, and *m*,*p*-dimethylpropiophenone. We thank Mr. Leland Nelson for running nmr spectra of the new compounds, and Drs. B. S. Snowden, Jr., and G. H. Meyer for developing the iterative program to determine optimum McLachlan molecular orbital parameters. We wish to thank Mobil Research and Development Corporation for permission to publish this work.

(73) G. K. Hughes and E. O. P. Thompson, *J. Proc. Roy. Soc. N. S. W.*, 82, 262 (1948).
(74) Ng. Ph. Bun-Hoi, Mg. D. Xuong, and D. Lavit, *J. Chem. Soc.*, 1034 (1954).
(75) G. A. Russell, E. G. Janzen, and E. T. Strom, *J. Amer. Chem. Soc.*, 86, 1807 (1964).
(76) B. G. Segal, M. Kaplan, and G. K. Fraenkel, *J. Chem. Phys.*, 43, 4191 (1965).

A Paramagnetic Monomeric Molybdenum(V)–Cysteine Complex as a Model for Molybdenum–Enzyme Interaction

T. J. Huang and G. P. Haight, Jr.

Contribution from the Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois 61801. *Received November 5, 1969*

Abstract: A paramagnetic Mo(V)–cysteine complex has been studied anaerobically in 0.2 *M* phosphate buffer over a pH range from 6 to 10. Mo(V) and cysteine form a diamagnetic dimer at low pH which then dissociates slowly into a paramagnetic monomer on addition of base. Epr measurements of the isotopically enriched ^{95}Mo(V)–cysteine complex give the following parameters: $g = 1.975$, $g_{zz} = 2.029$, $g_{yy} = 1.972$, $g_{xx} = 1.931$; and $a = 0.0032$ (35 G), $A_{zz} = 0.0051$ (54 G), $A_{yy} = 0.0022$ (24 G), $A_{xx} = 0.0031$ cm^{-1} (34 G). The g and a values, calculated on the basis of $g = \frac{1}{3}(g_{xx} + g_{yy} + g_{zz})$ and $a = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$, are in good agreement with those corresponding values measured at room temperature, suggesting that the paramagnetic complex possesses the same structure in both frozen and liquid solutions. A six-line hyperfine splitting follows the monomeric pattern. A line broadening which depends on the nuclear spin orientation was observed in solution at room temperature. The temperature dependence of the epr signals suggests that an equilibrium between the dimer and the monomer exists in solution. Results show the dioxo bridge of the dimer can be broken either by the attack of OH^- in solution or by heat in solid state. A broad weak band, tentatively assigned as a d–d transition, centered at 580 nm was observed for the paramagnetic monomer in solution. A possible structure for the paramagnetic monomer and the biological implications for molybdenum–enzyme interaction, especially in xanthine oxidase, are discussed.

An electron paramagnetic resonance (epr) signal, attributed to the d^1 system of monomeric molybdenum(V), has been found in a number of sulfhydryl enzymes such as xanthine oxidase,[1] aldehyde oxidase,[2] nitrate reductase,[3] and nitrogenase.[4] Molybdenum–sulfur binding at the active site due to the cysteine residue has been proposed.[1,2] This fact led us to study the paramagnetic monomeric Mo(V)–cysteine complex as a possible model for molybdenum–enzyme interaction. Although a few papers dealing with the Mo(V)–cysteine complex have recently appeared in the literature,[5–8] no stable epr signal which includes measurement of anisotropic parameters has been reported in this system. The study of molybdenum thiol complexes by Meriwether, *et al.*,[9] revealed that neither cysteine nor glutathione produces an epr signal in solution with Mo(V). Following the earlier work of Spence and Chang,[5] Kay and Mitchell[6] and Melby[8] independently isolated a diamagnetic Mo(V)–cysteine complex which contains the dimeric grouping $Mo_2O_4^{2+}$, with ligands coordinated to each Mo atom. The structure of this compound was determined by Knox and Prout,[7] and X-ray data suggest that the diamagnetism is due to the formation of a direct Mo–Mo bond. Recently, Mar-

(1) R. C. Bray and L. S. Meriwether, *Nature*, 212, 467 (1966).
(2) P. Handler and K. V. Rajagoplarr, *J. Biol. Chem.*, 239, 2027 (1964).
(3) C. A. Fewson and D. J. D. Nicholas, *Biochim. Biophys. Acta*, 49, 335 (1961).
(4) D. J. D. Nicholas, P. W. Wilson, W. Heinen, G. Palmer, and H. Beinert, *Nature*, 196, 433 (1962).
(5) J. T. Spence and H. H. Y. Chang, *Inorg. Chem.*, 2, 319 (1963).
(6) A. Kay and P. C. H. Mitchell, *Nature*, 219, 267 (1968).
(7) J. R. Knox and C. K. Prout, *Chem. Commun.*, 1127 (1968).
(8) L. R. Melby, *Inorg. Chem.*, 8, 349 (1969).
(9) L. S. Meriwether, W. F. Marzluff, and W. G. Hodgson, *Nature*, 212, 456 (1966).

tin and Spence[10] reported time-dependent (both g value and line shape are time dependent) epr signals from 10^{-2} M Mo(V) and excess cysteine in 1 M phosphate buffer at pH 6; however, these unstable signals rapidly disappear and precipitation occurs.[11] Using lower concentrations ($\sim 10^{-3}$ M Mo(V)), we have observed a stable, well-defined, and time-independent epr signal from a Mo(V)–cysteine complex. Neither rapid disappearance of the epr signal nor precipitation was observed. Moreover, isotopically enriched ^{95}Mo was used to cause hyperfine splitting in order to obtain anisotropic epr parameters which give information about the electronic environment of this complex. In order to make comparisons with biological systems, this complex was studied anaerobically in 0.2 M phosphate buffer over the pH range 6–10. In conjunction with the study of this model system for molybdenum-containing enzymatic material, epr signals of Mo(V) complexes with cysteine-related compounds, namely, ethanedithiol, aminoethanethiol, β-mercaptopropionic acid, and glutathione (a cysteine-containing peptide), are also reported.

Experimental Section

The solutions of the complexes were prepared in the following manner. The 0.2 M phosphate buffer at desired pH was deaerated with argon (99.996% purity) and a specific amount of ligand was dissolved in the buffer. A deaerated solution of Mo(V) in 3 N HCl (with an equal volume of deaerated 3 N NaOH for neutralization) was then added to start the reaction. Samples were removed periodically with a gas-tight syringe through a rubber diaphragm and frozen in liquid nitrogen in 3-mm i.d. bore quartz tubes under argon for epr measurements. In all cases, excess ligand was used in order to overcome difficulties caused by low solubility of Mo(V) uncoordinated to cysteine in basic solution. The concentrations of Mo(V) and ligand were of the order of 10^{-3} and 10^{-2} M, respectively. The low concentration of Mo(V) was employed in order to slow down the rate of the reduction of Mo(V) by the ligands within the time of study and to avoid any possible dipolar broadening of the epr signals.

Epr Measurements. All epr spectra were recorded with a Varian V-4502 X-band spectrometer equipped with an automatic temperature controller, using 100-kc modulation. The g values were determined by comparison with the signal of powdered DPPH radical (diphenylpicrylhydrazyl radical), which has an isotropic g value of 2.0036.[12] For the study of reversible temperature dependence of the epr signal of the Mo(V)–cysteine complex in solution, a glass capillary, formed from the thin section of a disposable pipet, was used for the epr measurements at temperatures greater than the room temperature. Difficulty was encountered in tuning the instrument because of the high dielectric constant of the system when a 3-mm i.d. bore quartz tube was used. All epr measurements on frozen samples were made at 77°K.

Quantitative Epr Measurements. A solution of $K_3Mo(CN)_8$, prepared by oxidation of $K_4Mo(CN)_8$ with Ce(IV) in 1 M H_2SO_4,[13] was used as a standard for determining the Mo(V) monomer concentration from epr data. $K_4Mo(CN)_8$ was prepared by the method of Furman and Miller.[13] For quantitative epr measurements, the derivative signal obtained from the $K_3Mo(CN)_8$ was doubly integrated and compared with a double-integrated signal for Mo(V) at each set of conditions. (After integration, the epr base line was arbitrarily adjusted to ensure that the absorption curve goes to zero at both ends of the spectrum.)

Electronic Spectra. Electronic spectra were obtained at ambient temperature with a Cary 14 spectrophotometer, using cells of appropriate path length. Since Mo(V) can be oxidized by atmospheric oxygen, a specially designed reaction vessel was constructed, to which a silica absorption cell which could be evacuated was joined. After mixing of the reactants, a sample was drawn off into the evacuated cell by means of a stopcock and the closed cell removed from the vessel and placed in the spectrophotometer for absorption measurements.

Thermogravimetric Analysis. A thermogram was recorded under a stream of nitrogen gas with a homemade thermogravimetric analyzer which has a temperature range of 25–360°, especially designed for the study of thermodecomposition of coordination compounds.

Materials. Stock solutions of $[Mo(V)]_2$ were prepared by reduction of Na_2MoO_4 by shaking over Hg in 3 N HCl,[14] and standardized spectrophotometrically at 298 nm. Since molybdenum pentachloride gives the same result as Mo(V) in 3 N HCl does, isotopically enriched $^{95}MoCl_5$ and $^{98}MoCl_5$ were used to prepare the corresponding complexes. Micro-scale syntheses of $^{95}MoCl_5$ and $^{98}MoCl_5$ were made by the modified method of Porterfield and Tyree;[15] 150 mg of $^{95}MoO_3$ was added to a 5-ml flask containing 2 ml of hexachloropropene. By means of an oxygen torch, the reaction mixture was brought to a boil and refluxed until reaction was complete (about 20 min) as evidenced by the change of the solid to a clear solution. Then the flask was allowed to cool to room temperature and evacuated by a vacuum pump until crystalline $MoCl_5$ was obtained. It was washed with a small quantity of cold dry reagent-grade CCl_4, and the evacuation process was repeated. The resulting dark-colored crystals were stored *in vacuo* over KOH.

Isotopically enriched $^{95}MoO_3$ (isotopic purity 96.8%) and $^{98}MoO_3$ (98.28%) were obtained from Oak Ridge National Laboratory. Hexachloropropene was obtained from Matheson Coleman and Bell.

L-Cysteine hydrochloride monohydrate, L-cystine, glutathione, EDTA, L-alanine, L-histidine hydrochloride monohydrate, β-mercaptopropionic acid, 1-propanethiol, 2-aminoethanethiol hydrochloride, and 1,2-ethanedithiol are all commercially available and reagent grade. All compounds were used without further purification.

Results

Electron Paramagnetic Resonance. Monomeric Mo(V) has one unpaired electron in the 4d orbital and is paramagnetic. It undergoes dimerization very rapidly in solution at pH >6 with consequent loss of its epr signal. However, in the presence of cysteine, Mo(V) monomers can be stabilized and an epr signal can be obtained.

Upon mixing of Mo(V) with cysteine, a light orange color develops immediately, followed by a further slower change of color to light greenish brown. Appearance of an epr signal is observed during the second color change. This indicates that a species, probably diamagnetic, is formed initially which then dissociates into a paramagnetic monomer. The higher the pH, the faster the rate of the formation of the paramagnetic species. The ultimate intensity of the epr signal also depends on pH. At pH 6, only a weak, poorly defined signal was observed. In the pH range 7–10, a time-independent, well-defined signal developed within a couple of hours at room temperature, and reached its maximum several hours later. No study was made after 24 hr because of the occurrence of the reduction of Mo(V) by cysteine ligand. Strengths of signals increase with increasing pH, reach a plateau at pH 9–10, and then fall off and disappear as pH is further increased to 12. Quantitative epr analyses revealed that the content of paramagnetic monomers never exceeds 2% of total molybdenum.

The solution spectrum of isotopically enriched ^{95}Mo(V)–cysteine complexes is shown in Figure 1. The

(10) J. M. Martin and J. T. Spence in "Progress in Coordination Chemistry," M. Caes, Ed., Elsevier Publishing Co., Amsterdam, 1968, p 492.
(11) J. T. Spence, private communication, May 24, 1969.
(12) A. N. Holden, C. Kittel, F. R. Merritt, and W. A. Yager, *Phys. Rev.*, 77, 147 (1950).
(13) N. H. Furman and C. O. Miller, *Inorg. Syn.*, 3, 160 (1950).
(14) N. F. Howell and W. M. Murray, *J. Amer. Chem. Soc.*, 58, 1689 (1936).
(15) W. W. Porterfield and S. Y. Tyree, Jr., *Inorg. Syn.*, 9, 133 (1967).

Table I. Electron Spin Resonance Parameters of Paramagnetic Mo(V) Complexes with Cysteine and Cysteine-Related Ligands[a]

Ligand	pH	g	a, cm^{-1} (gauss)	g_{zz}	g_{yy}	g_{xx}	A_{zz}, cm^{-1} (gauss)	A_{yy}, cm^{-1} (gauss)	A_{xx}, cm^{-1} (gauss)	Ref
Cysteine	9	1.975 (1.977)[c]	0.0032 (35)[d]	2.029	1.972	1.931	0.0051 (54)[d]	0.0022 (24)[d]	0.0031 (34)[d]	b
Ethanedithiol	6	2.002 (2.000)[c]	(32)	2.058	1.993	1.947				b
1:1 EtOH-H_2O		2.002	(30)	2.050	1.978	1.978				9
β-Mercapto-propionic acid	10	1.969 (1.967)[c]		1.992	1.954	1.954				b
Glutathione	8	1.951	(32)		(See Figure 4)					b
2-Aminoethane-thiol	12				(See Figure 4)					b
Xanthine oxidase										
γ,δ form	10	1.977	(34)	2.025	1.956	1.951	(41)	(24)	(37)	1
α,β form	10	1.977	(41)	1.990	1.971	1.971	(67)	(28)	(28)	1
"slowly developing signal"	8.2	1.967		1.975	1.970	1.957	(70)			16
Nitrate reductase		1.970								3
Aldehyde oxidase		1.97								2
Nitrogenase	6.5	1.97								4

[a] $[Mo(V)]_t = 10^{-3}$ [ligand] $= 10^{-2}$ M. [b] This work. [c] Calculated on the basis of $g = {}^1/_3(g_{zz} + g_{yy} + g_{xx})$. [d] Experimentally observed separation of hyperfine components (in the unit of gauss).

six hyperfine lines due to ^{95}Mo with nuclear spin of $^5/_2$ indicate that the paramagnetic species is a monomer and that there is only one molybdenum nucleus per molecule. Therefore, the possibility of the existence

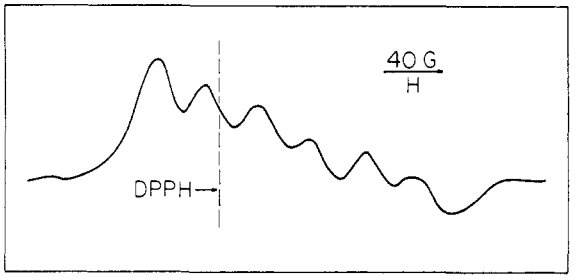


Figure 1. First-derivative epr spectrum of isotopically enriched $^{95}Mo(V)$–cysteine complexes at room temperature; $[Mo(V)]_t \approx 10^{-3}$ M and [cysteine] $\approx 10^{-2}$ M.

of a paramagnetic dimer appears to be ruled out. When the complex is prepared from naturally occurring Mo(V), the six satellites due to $^{95,97}Mo$ (nuclear spin (I) $= {}^5/_2$, natural abundance 25%) are too weak to be observed. Presumably they are covered by the broad intense line due to $^{94,96,98}Mo$ ($I = 0$, natural abundance 75%). Introduction of isotopically enriched $^{95}Mo(V)$ seems necessary in this case. The epr spectra of frozen solutions of $^{95}Mo(V)$–cysteine and $^{98}Mo(V)$–cysteine at 77°K are shown in Figure 2. The anisotropies of the g tensor and the hyperfine tensor are clearly demonstrated. The hyperfine splitting constants are expressed in both the proper unit of cm^{-1} and the conventional unit of G.

In frozen solution at 77°K the epr spectrum of $^{95}Mo(V)$–cysteine complexes may be described by the following spin Hamiltonian

$$\mathcal{H} = \beta H \cdot g \cdot S + I \cdot A \cdot S$$

where $S = {}^1/_2$, $I = {}^5/_2$. At room temperature in liquid solution, the anisotropies are averaged to zero, and the Hamiltonian becomes

$$\mathcal{H} = g\beta H \cdot S + aI \cdot S$$

The average g and hyperfine splitting constant, a, are determined from the solution spectrum in Figure 1. Since the spacings between each two neighboring peaks are approximately the same, the second-order effect is presumably negligible and therefore no correction was

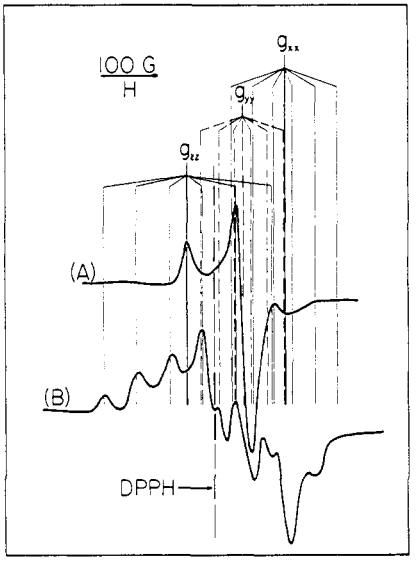


Figure 2. First-derivative epr spectra of Mo(V)–cysteine complexes at 77°K, $[Mo(V)]_t \approx 10^{-3}$ M and [cysteine] $\approx 10^{-2}$ M: (A) with 98.28% isotopically enriched ^{98}Mo, (B) with 96.80% isotopically enriched ^{95}Mo.

made to obtain g and a. Although the g and A tensors need not have coincident axes, the analysis proceeded with the assumption of coincidence. The g tensor is determined from the frozen solution spectrum of $^{98}Mo(V)$–cysteine complexes in Figure 2 where no hyperfine interaction exists, and the hyperfine tensor is determined from that of $^{95}Mo(V)$–cysteine complexes in Figure 2. The epr parameters are listed in Table I. The examination of A_{xx} must be considered to be only tentative because one expected line at the extreme right is not observed. However, according to the assignment in Figure 2, the isotropic hyperfine splitting con-

stant, 0.0035 cm^{-1}, calculated on the basis of $a = {}^1/_3(A_{xx} + A_{yy} + A_{zz})$, is in good agreement with 0.0032 cm^{-1}, measured from the room-temperature spectrum. The failure to observe the last expected hyperfine component may arise from the inadequacy of the assumption of coincidence of g and A tensors or from the inadequacy of the Hamiltonian employed. Furthermore, difficulty in determining A_{xx} was also encountered in the analysis of epr spectra of the Mo(V)-containing enzyme.[16] From the expression $g = {}^1/_3(g_{xx} + g_{yy} + g_{zz})$, a value of 1.977 is obtained, again in good agreement with 1.975, measured from the room-temperature spectrum. This result suggests that the complex possesses the same structure in both liquid and frozen solutions.

The anisotropies could result from rhombic distortions of an octahedral complex. If such is the case, the electronic ground-state orbital, d_{yz} (or d_{xz}), should not interact with the lone-pair 2p orbital of the nitrogen nucleus and consequently, no matter where nitrogen is coordinated to Mo(V), no superhyperfine splitting due to ^{14}N ($I = 1$) would be observed. The absence of ^{14}N splitting in Figures 1 and 2 supports this point.

A line broadening is observed in the solution spectrum (Figure 1) at room temperature which is dependent on the nuclear spin orientation, m_I. This effect could be interpreted in terms of two anisotropic magnetic interactions which govern the spin relaxation.[17] One comes from anisotropy of the g tensor, the other from dipolar hyperfine coupling with magnetic nuclei. Both interactions fluctuate in a tumbling molecule in solution and produce broadening. The same broadening has also been found in the vanadyl ion VO^{2+} [18] and sodium vanadyl glycolate.[19] Figure 3 shows the pH-dependent line broadening of the epr spectra of isotopically enriched $^{95}Mo(V)$–cysteine complexes at 77°K. This may be attributed to a spin–lattice relaxation process. At lower pH, the crystal lattice facilitates energy transfer from the electron spin to other degrees of freedom, including the vibrations of the lattice, and shortens the spin-lattice relaxation time. Therefore, it produces more broadening at lower pH than at higher pH, as demonstrated in Figure 3. Under such dilute concentration conditions, line broadening from dipole–dipole interaction seems very unlikely. Moreover, the pH dependence of the line broadening cannot be explained in terms of dipole–dipole interaction. However, we cannot exclude the possibility that the line broadening may also arise from a mixture of similar chemical species. When naturally occurring molybdenum was used, a signal with three g components as shown in Figure 2A was observed at pH 7–10, and the pH-dependent line broadening at 77°K was diminished to such an extent that only broadening of the g_{xx} component could be seen clearly. The reason that the presence of nuclear spins accelerates the spin–lattice relaxation and consequently causes more pH-dependent line broadening at 77°K remains to be explained.

Integrated epr signals were proportional to the square root of the $[Mo(V)]_2$ concentration and independent of excess ligand. Signal intensities increase reversibly with increasing temperature indicating that the dissociation of the dimers is endothermic. The temperature dependence at pH 9.0 is shown in Table II.

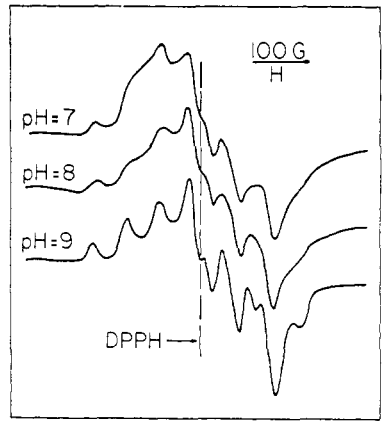


Figure 3. First-derivative epr spectra of $^{95}Mo(V)$–cysteine complexes at 77°K, at various pH's; $[Mo(V)]_t \approx 10^{-3}$ M and [cysteine] $\approx 10^{-2}$ M.

Table II

Temp, °C	Signal height, arbitrary units	Temp, °C	Signal height, arbitrary units
25	1.5	75	3.3
35	2.1	55	2.9
55	2.8	35	2.0
75	3.2	25	1.5
90	4.0	$\Delta H \sim 3$ kcal	

Owing to the possible reduction of Mo(V) by cysteine ligand, the possible formation of paramagnetic species from Mo(III) and Mo(V) with cystine and cysteine was also investigated. Mo(III) forms complexes with cystine and cysteine, and gives epr signals which look completely different from those reported here. Mo(V) does not give any epr signal with cystine in solution. Therefore, we conclude that there is no reduction of Mo(V) by cysteine within the time of study under the conditions employed and the epr signal must definitely be attributed to the paramagnetic monomeric Mo(V)–cysteine complex. Because Meriwether found no epr signal for Mo(V) with cysteine in solution[9] we speculated that the attack of phosphate species might cause the dissociation of the dimers into monomers. However, the results of our investigation show that the Mo(V)–cysteine complex prepared in water (no phosphate species) at pH 8.0 (pH was adjusted with NaOH) gives the same epr result as that in phosphate buffer at the same pH. This indicates that the dissociation of the dimers is in no way dependent on the various species of phosphate present.

It must be noted that no epr signal was observed with L-alanine, L-histidine, L-cystine, L-propanethiol, or EDTA. Stable epr signals were also observed with β-mercaptopropionic acid, 1,2-ethanedithiol, 2-aminoethanethiol, and glutathione. Their epr spectra are shown in Figure 4 and their epr parameters are compiled in Table I. The results demonstrate that Mo(V)

(16) R. C. Bray, P. F. Knowles, F. M. Pick, and T. Vanngard, *Biochem. J.*, 107, 601 (1968).
(17) A. Carrington and A. D. McLachlan, "Introduction to Magnetic Resonance," Harper and Row Publishers, Inc., New York, N. Y., 1967, p 197.
(18) R. N. Rogers and G. E. Pake, *J. Chem. Phys.*, 33, 1107 (1960).
(19) R. L. Belford, N. D. Chasteen, H. So, and R. E. Tapscott, *J. Amer. Chem. Soc.*, 91, 4675 (1969).

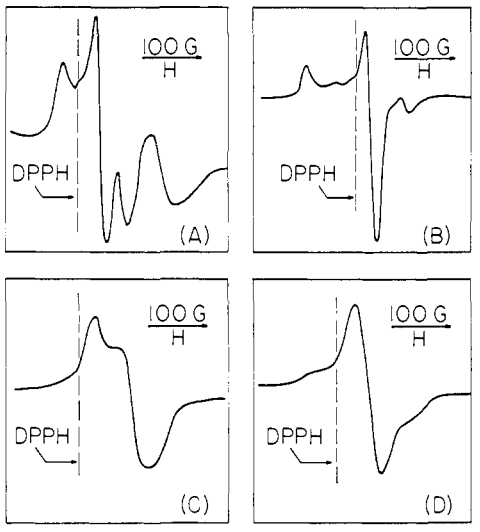


Figure 4. First-derivative epr spectra of Mo(V) complexes with cysteine-related thiol compounds at 77°K, $[Mo(V)]_t = 10^{-3}$ *M*, and [ligand] = 10^{-2} *M*. Naturally occurring molybdenum compounds were used in all cases. (A) Mo(V)–glutathione complexes at pH 8, (B) Mo(V)–ethanedithiol complexes at pH 6, (C) Mo(V)–β-mercaptopropionic acid complexes at pH 10, (D) Mo(V)–aminoethanethiol complexes at pH 12.

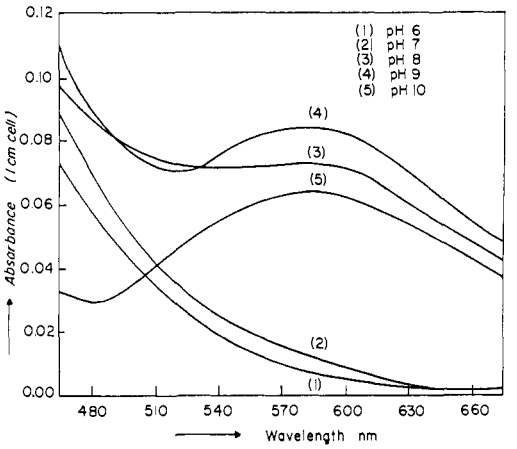


Figure 5. Visible spectra of Mo(V)–cysteine complexes at various pH's. In all cases, $[Mo(V)]_t = 10^{-3}$ *M* and [cysteine] = 10^{-2} *M*.

monomer can be stabilized by polydentate ligands containing at least one sulfhydryl group.

Electronic Absorption Spectra. Solutions of free Mo(V) at the pH range of interest are light yellow-orange in color due to an absorption maximum at 300 nm with a long shoulder sloping into the visible. The spectra of Mo(V)–cysteine complexes at this pH range in the ultraviolet region are similar in line shape to that of free Mo(V); however, the intensities are different, and a shift of 6 nm of the maximum absorption peak toward higher wavelength is observed at all pH's studied. In the visible region (Figure 5), a broad, weak absorption centered at 580 nm was observed. The extinction coefficient at 580 nm is about 160 on the basis of the dimer concentration while it is about 4000 on the basis of the monomer concentration which was quantitatively measured by epr. In comparison with the strong absorption ($\epsilon \sim 12{,}000$ at 306 nm) in the ultraviolet region, this band is tentatively assigned to a d–d transition. The pH dependence of the intensity of this

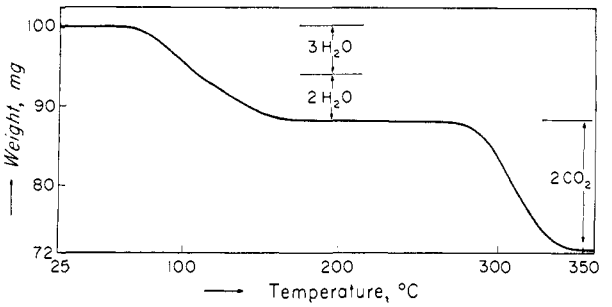


Figure 6. Thermogravimetric analysis of $Na_2Mo_2O_4(cyst)_2 \cdot 5H_2O$ (I). The thermogram was recorded under a stream of nitrogen gas (flow rate 120 ml/min) at a heating rate of 2°/min. The weights are the actual weights of the sample.

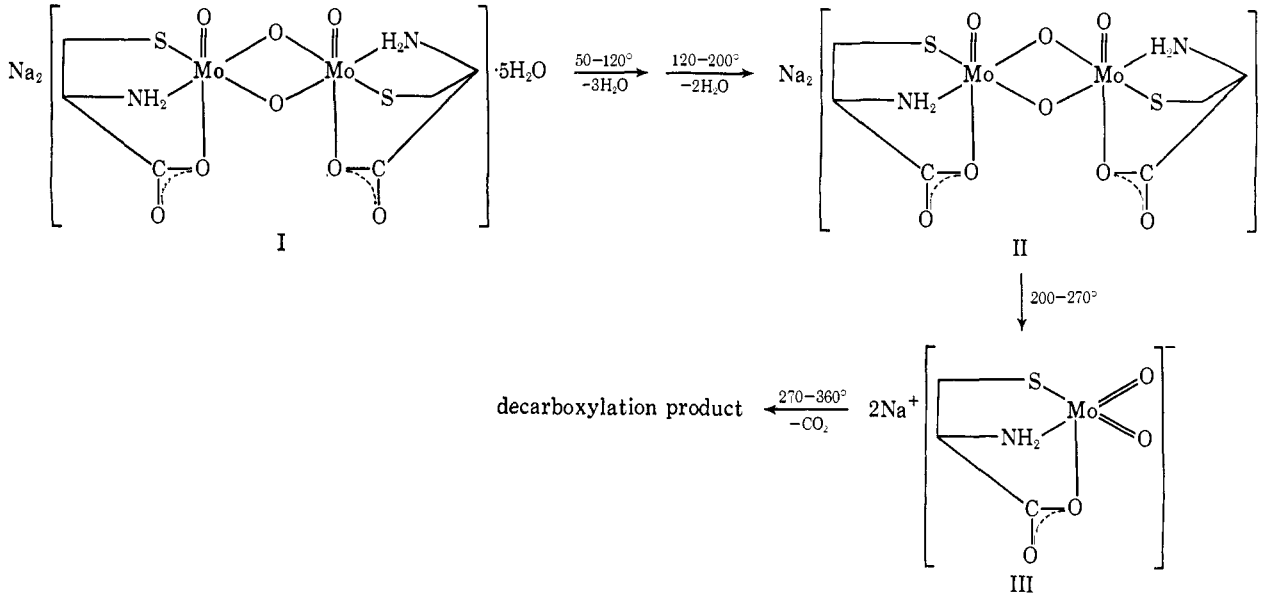
580-nm transition follows the same pattern as the pH dependence of the intensity of the epr signal of the paramagnetic monomer. Furthermore, this 580-nm transition is only observed after the second color change where the solution gives an epr signal, but is not observed during the first color change where only dimers exist in solution. Therefore, it is suspected that the 580-nm transition arises from the paramagnetic monomer. However, the results of quantitative epr analysis and spectrophotometric measurement do not confirm or deny this possibility because of the small amount of paramagnetic species and the low absorbance of this 580-nm transition. A similar 580-nm transition is observed in xanthine oxidase and was tentatively assigned to molybdenum.[20] Like Mitchell, *et al.*,[6] we were unable to detect d–d transitions in the spectrum of the cysteine complex in aqueous solution at pH <8. The above transition (580 nm) was observed only in the Mo(V)–cysteine and Mo(V)–glutathione complexes, but not observed in the other complexes studied in this work.

The spectrophotometric investigation (Job's plot) shows a metal-to-ligand ratio of 1:1 in the complex of Mo(V) with cysteine, which is in good agreement with the result of Mitchell, *et al.*,[6] and Melby.[8]

Thermodecomposition Pattern of the Diamagnetic Dimer in Solid State. The result of epr study shows that the dissociation of dimers into monomers in solution is endothermic. This suggests that the dioxo bridge of the dimer may be broken by heat. Therefore a thermogravimetric analysis with an epr study was made to confirm this possibility. The thermogram of the binuclear complex (I) is shown in Figure 6. There are two different types of water possible, lattice ($3H_2O$) and coordinated ($2H_2O$) water. The latter are probably hydrogen-bonded with the carboxylate oxygen or the terminal oxygen in the proximity of molybdenum nuclei. The presence of two types of water is in good agreement with Mitchell's ir result.[6] That no weight loss was observed at 200–270° reflects two possibilities, first, that the dimer retains its form (II); second, that the dimer (II) decomposes into monomer (III) without weight loss. If the first is the case, either no epr signal or a reversible epr signal from the triplet state of the binuclear complex due to thermal excitation of electrons should be observed. However, no such phenomena were found. The second possibility is

(20) K. Garbett, R. D. Gillard, P. F. Knowles, and J. E. Stangroom, *Nature*, 215, 824 (1967).

Scheme I



supported by the evidence that an irreversible epr signal with anisotropy was observed in this temperature range. The signal finally becomes symmetric and narrower as temperature is increased to over 270°. This second epr signal probably arises from the decarboxylation product of the paramagnetic monomer (III). Scheme I is proposed for the thermodecomposition of the Mo(V)–cysteine dimer (I).

The result gives support for the existence of a paramagnetic monomeric Mo(V)–cysteine complex.

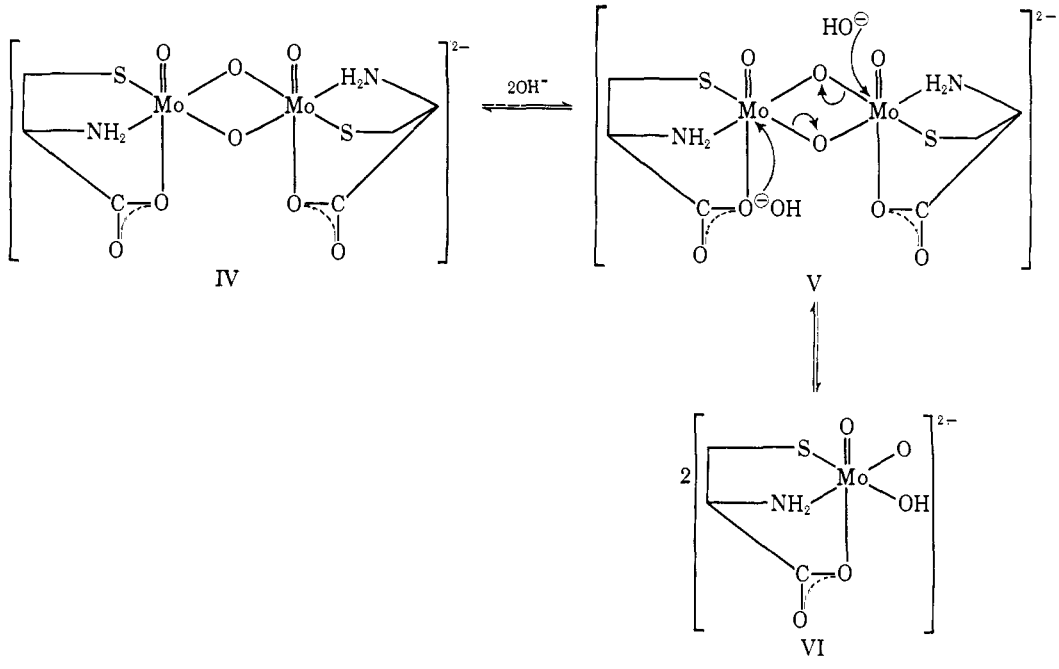
Discussion

The epr results of six hyperfine lines, the low content of paramagnetic monomers, and the reversible temperature dependence of the epr signals, strongly suggest that an equilibrium between dimers and monomers exists in solution for Mo(V)–cysteine complexes. The fact that intensities of epr signals are proportional to the square root of $[Mo(V)]_2$ concentration and independent of excess cysteine is consistent with the following equilibrium

$$M_2L_2 \rightleftarrows 2ML$$

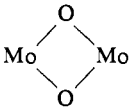
where M is Mo(V) and L is the ligand, cysteine. The X-ray structure (I)[7] of Mitchell's Mo(V)–cysteine dimer enables us to speculate on the structure of paramagnetic monomers in solution for the cysteine complex (IV–VI).

Since Mo(V) with cysteine gives essentially the same epr results in phosphate-free water solution at pH 8.0 as in phosphate-buffered solution at the same pH, the following three possible mechanisms are proposed for the breaking of the dimer in solution: (1) through a rapid equilibrium established by the attack of H^+ on the oxygen bridges followed by a slower attack of OH^- to break the bridges; (2) through the attack of water molecules to form an unstable seven-coordinated binuclear complex, followed by a rapid breaking of the bridges to form a monomer; and (3) through the attack



of OH^- ions to break the oxygen bridges to form the paramagnetic monomer (VI). Since dissolution of the dimer (I) in very dilute NaOH solution (pH 9) shows a decrease of pH, the first mechanism is rejected. In view of the pH dependences of the rate, the extent of dissociation of the dimer, and the intensities of the epr signals, the second mechanism should also be eliminated. The third mechanism as shown above seems more plausible. This mechanism is further supported by the fact that the solution behavior (uv, visible, and epr) of the cysteine complex is the same whether formed directly in solution from Mo(V) and cysteine or from crystals of $Na_2Mo_2O_4(cyst)_2 \cdot 5H_2O$ (I), prepared in England and sent to us by P. C. H. Mitchell.

The lability of the dioxo bridge



in the physiological pH region is created by the sulfhydryl group of the polydentate ligands, as evidenced by the fact that no epr signal was obtained with monodentate ligands containing the sulfhydryl group or with polydentate ligands containing no sulfhydryl group, and epr signals were observed with polydentate ligands containing at least one sulfhydryl group. The lability is enhanced by increasing the chain length of the ligand, as evidenced by the fact that stronger epr signals were obtained with glutathione and apoenzyme of putidaredoxin, both containing cysteine residues.

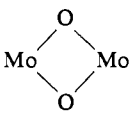
Mo(V), with all of the polydentate ligands containing only one sulfhydryl group, shows a g value and a hyperfine splitting constant which are very close to those of xanthine oxidase,[1,16] as well as to those of other enzymes[2-4] (see Table I), indicating that the ligands coordinated to Mo(V) in the enzymes do so through the cysteine residue of the apoenzyme.

Since xanthine oxidase has been extensively studied,[1,16,20-23] the following striking comparisons are made between the Mo(V)–cysteine complex and xanthine oxidase. (1) It forms a binuclear complex, which is in equilibrium with a paramagnetic monomer. This meets the requirements of having two molybdenum(V) coordinated by a biologically important sulfur-donor ligand and of having a paramagnetic monomer, established for xanthine oxidase by epr measurements.[1,9,23] (2) In xanthine oxidase, an absorption maximum at 580 nm is tentatively attributed to the molybdenum chromophore.[20] A weak, broad d–d transition with center at 580 nm of the Mo(V)–cysteine complex could well correspond to this feature of xanthine oxidase. (3) No ^{14}N–ligand superhyperfine splitting was observed in either case.[1] (4) From the anisotropies of the epr spectra, both xanthine oxidase and the Mo(V)–cysteine complex are shown to be highly distorted (possibly an octahedral complex with rhombic distortion[1]). (5) The similarity of the epr spectral line shapes[1,16] and the near equal values of the epr parameters (see Table I) suggest that Mo(V) may exist in the same electronic environment in both cases. The above features lead us to the conclusion that the Mo(V)–cysteine complex may serve as a model for the molybdenum–enzyme interaction in the biological system.

(21) R. C. Bray and P. F. Knowles, *Proc. Roy. Soc., Ser. A*, **302**, 351 (1968).
(22) G. Palmer, R. C. Bray, and H. Beinert, *J. Biol. Chem.*, **239**, 2657 (1964).
(23) R. C. Bray, P. F. Knowles, and L. S. Meriwether, Second International Conference on Magnetic Resonance in Biological Systems, Stockholm, 1968.

Summary

The existence of Mo(VI)–cysteine complexes has been demonstrated by Spence, *et al.*,[5] and Melby.[8] This, combined with the present result, suggests that the active site in the oxidized form of molybdenum-containing enzymes involves Mo(VI) coordinated to cysteine residues. After Mo(VI) is reduced to Mo(V) in the electron transfer sequence, Mo(V) with cysteine residues forms a diamagnetic binuclear Mo(V)–cysteine complex which then is in equilibrium with a paramagnetic monomeric Mo(V)–cysteine complex through the rupture of the dioxo bridge



The epr signal in the reduced form of molybdenum-containing enzymes may arise from this paramagnetic unit. That the equilibrium generally lies in the direction of the dimer could account for the low content of total molybdenum detectable as Mo(V) by epr measurement for reduced molybdenum enzymes.[23]

Acknowledgment. The authors wish to thank Mrs. Susan Bekiares for her technical assistance, Dr. E. Hertzenberg for running the thermogram, Dr. R. L. Belford for helpful discussion and valuable comments on the original manuscript, and Professor P. C. H. Mitchell for his generous supply of a sample of Mo(V)–cysteine dimers. This work is supported by a grant (No. 14348) from the National Institutes of Health.