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BIOCHEMICAL ASPECTS OF MOLYBDENUM COORDINATION CHEMISTRY

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CONTENTS

A. INTRODUCTION

It is now well established that molybdenum is an important trace element[1], participating in a number of biochemical redox reactions, among which are the oxidation of xanthine and purines and the reduction of nitrate and molecular nitrogen. The importance of nitrate reduction and nitrogen fixation cannot be overemphasized, since they are the major routes of nitrogen incorporation for plants and, therefore, animals In each of these systems the molybdenum is bound to an enzyme and, in at least two, associated with a coenzyme, flavin adeninedinucleotide (FAD). It thus seems probable that the metal ion is coordinated with certain amino acid side chains of the protein, and possibly with the coenzyme as well.

This article will be limited to the aspects of molybdenum chemistry that are relevant to its biochemical function. The literature concerning the general coordination chemistry of molybdenum has recently been reviewed[2,3] and the bio-

logical work on the various enzymes has been covered in a number of monographs and symposia[4–7].

B. BIOCHEMISTRY

(i) *Occurrence of molybdenum enzymes*

Molybdenum is known to be a necessary metal for four enzymes: xanthine oxidase[8], aldehyde oxidase[7], nitrate reductase[4] and nitrogenase (molybdoferredoxin)[9]. In the first two, iron and FAD are also cofactors, while the evidence regarding nitrate reductase indicates molybdenum is the only metal present and the FAD generally found with the enzyme may not be an integral part of the system[10]. Nitrogenase appears to contain non-heme iron also[9], but no coenzyme has yet been identified. This is summarized in table 1.

TABLE I

MOLYBDENUM ENZYMES[a]

Enzyme	*Coenzyme*	*Metals*	*Substrate*
Xanthine Oxidase	FAD(2)	Mo(2), Fe(8)	Xanthine, Purines
Aldehyde Oxidase	FAD(2)	Mo(2), Fe(8)	Aldehydes, Purines
Nitrate Reductase	FAD(?)	Mo(?)	Nitrate
Nitrogenase (molybdoferredoxin)		Mo(?), Fe(?)	Nitrogen

[a] Numbers in parentheses indicate number of coenzyme molecules or metal atoms per enzyme molecule.

Xanthine oxidase is found in milk and the livers of various animal species Aldehyde oxidase is present mainly in liver, while nitrate reductase is widely distributed in plants and microorganisms Nitrogenase was originally found in free living bacteria (*Azotobacter vinelandii* and *Clostridium pasteurianum*) but it also appears likely to be present in bacteria involved in symbiotic nitrogen fixation in certain plants.

(ii) *Oxidation state of molybdenum*

The oxidation states of molybdenum to be expected in an aqueous or semi-aqueous environment are 6+, 5+ and 3+. Some relatively stable complexes of the 4+ state are known ($Mo(CN)_8^{4-}$, *e.g.*) but these are unlikely to be present in biological systems. Both 5+ and 3+ states, particularly the latter, are rather easily oxidized by oxygen except when complexed with certain ligands.

At present it seems to be generally accepted that molybdenum is present in the 6+ and 5+ states in the enzymes, probably alternating between the two during active electron transport. The evidence for this comes from three sources. First, both 5+ and 6+ states were identified by complexing with 8-hydroxyquinoline

in the case of nitrate reductase[11,12]. Second, ESR signals characteristic of Mo^{V} have been obtained from all the molybdenum enzymes in the presence of substrate[7,8,13,14]. Third, a consideration of reduction potentials suggests the 3+ state is not likely to be found in biological systems[15]. This evidence, however, is not conclusive and the presence of the 3+ state (and possibly 4+) must not be ruled out. In the case of nitrate reductase, the method of formation of the 8-hydroxyquinoline complexes would most likely not have prevented oxidation of any 3+ species present, because the 3+ species itself appears to form a complex with this ligand only slowly[16]. Although the ESR signals detected during enzymatic reaction are most likely due to Mo^{V}, in no case has more than ~37% of the total Mo been accounted for, even under conditions that should result in total reduction[7] of Mo^{VI}. Furthermore, since Mo^{III} is a d^3 ion, the three electrons would be unpaired in an octahedral ligand field, resulting in severe line broadening and probable loss of the ESR signal. In fact, no ESR spectra for the Mo^{III} species in solution have been reported in the literature. Thus, any Mo^{III} present would probably not be observed by ESR. Finally, the argument concerning reduction potentials is not conclusive since these depend to a great extent on the ligand. Williams estimated the reduction potential of the Mo^{V}/Mo^{III} couple to be about -0.6 to -1.0 volt at neutral pH, thus putting it outside the potential of the biological system, particularly if it must react with flavins ($E_0' \cong -0.25$ volts for flavins)[15]. Recent work has demonstrated, however, that in the presence of excess thioglycollic acid (mercaptoacetic acid), which appears to complex both 5+ and 3+ states, Mo^{V} is reduced to the 3+ state:

$$2RSH + Mo^{V} \rightarrow RSSR + Mo^{III}$$

Since the reduction potential of this thiol is about -0.30 volts[17] at pH 7, the potential for the Mo^{V}/Mo^{III} couple must be shifted considerably more positive by complexing.

It seems clear then that at present, although the 6+ and 5+ states are most probable for enzyme bound Mo, the 3+ state cannot be completely overlooked. The most recent work on xanthine oxidase has, in fact, postulated that Mo^{VI} is reduced to Mo^{III} and possibly even Mo^{II} in order to explain the uptake of electrons by the enzyme[18]. More work on the reduction potentials of the enzymes and of model complexes must be done before unequivocal answers can be given

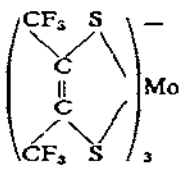
(iii) *Binding sites*

Molybdenum in the 6+ and 5+ states exhibits its greatest affinity for oxygen ligands, although many complexes with nitrogen and sulfur species are known. It might therefore be expected that it will be bound through the free carboxy group of acidic amino acids or the hydroxyl groups of tyrosine or serine side chains. Work involving inhibition studies with sulfhydryl binding agents, however, has implicated cysteine (or cystine) as the binding site of the metal ion. Furthermore,

ESR studies of the Mo^V signal in the enzymes and of those obtained from certain complexes support this conclusion Ligands that are easily polarized, such as CN^- and RS^-, give rise to high g values and small A values (hyperfine splitting due to the 5/2 spin of the ^{95}Mo and ^{97}Mo nuclei), as is the case with xanthine oxidase, and the other molybdenum enzymes This is summarized in Table 2.

TABLE II

ESR PARAMETERS FOR MOLYBDENUM ENZYMES AND REPRESENTATIVE COMPLEXES

Species	*g*	*A (Gauss)*
Xanthine Oxidase	1 977	37–41
Aldehyde Oxidase	1 97	
Nitrate Reductase	1 97	
Nitrogenase (molybdoferredoxin)	1 97	
$Mo(CN)_8^{3-}$	1.99	35
Mo^V–TGA	1 978–2 006	34–41
Mo^V–BAL	2 002	
Mo^V–TA	1 94	
$MoOCl_5^{2-}$	1 947	50
Mo^V–EDTA	1 936	56
	2 0097	12 2

TGA = thioglycollic acid (mercaptoacetic acid) BAL = 2,3-dimercapto-1-propanol TA = tartaric acid. EDTA = ethylenediamine-tetraacetic acid

Many Mo^V complexes exhibit charge transfer spectra in the region[19] of 350–500 nm. Unfortunately, absorption due to flavins and flavin radicals and possibly non-heme iron also occurs in this region, which severely limits the usefulness of these measurements in obtaining information about the binding site of the molybdenum in the enzymes. Iron-free xanthine oxidase has been reported[20,21], but its spectrum appears to be essentially identical with that of the native enzyme, suggesting the absorption is due to flavin or molybdenum (or both). Flavin free nitrate reductase, which also contains no iron, shows no absorption bands at wavelengths greater[22] than about 280 nm In this case, however, the Mo is most likely in the 6+ state, for which visible absorption is not so likely. No spectra of the flavin-free enzyme in a reduced state, when appreciable amounts of Mo^V might be present, have been published.

Recently, evidence has been obtained that Mo^V in xanthine oxidase interacts with the flavosemiquinone radical. It was demonstrated by Beinert and Hemmerich[23] that the ESR relaxation behavior of flavosemiquinone is considerably altered by the presence of metal ions. This was ascertained by measuring the microwave power necessary to saturate the radical. It was found that much greater power

levels were required for saturation when metal ions are present, presumably due to an additional mechanism for relaxation *via* the metal ion. Furthermore, similar behavior was found when metal-free flavoenzymes were compared with metal containing flavoenzymes. Knowles has found that a very strong effect on relaxation of the flavosemiquinone ESR signal is produced when Mo^V is added to solutions of glucose oxidase[24]. Beinert has also shown that behavior of the Mo^V species in aldehyde oxidase is altered when signals due to flavosemiquinone and iron are formed in the presence of the substrate[7,25,26]. Although magnetic dipolar interactions may be the cause of this behavior, chelation of the Mo^V with the flavin radical might also be involved

Finally, it appears from recent studies with xanthine oxidase and aldehyde oxidase that certain inhibitors (methylalcohol, cyanide, arsenite) and the substrate are also bound to the Mo of the enzyme, no doubt by coordination[27–29].

Clearly the coordination chemistry of the enzymatic Mo is rather complicated, with hydroxy and carboxy amino acids, cysteine, flavin coenzymes and substrates serving as possible ligands during the redox process.

(iv) *Function*

All of the molybdenum containing enzymes are redox enzymes, involved in the oxidation (xanthine oxidase, aldehyde oxidase) or reduction (nitrate reductase, molybdoferredoxin) of substrates. It seems most likely that the function of the metal ion is to transfer electrons between substrate and coenzyme, or substrate and iron. It has been found that the ESR signals for Mo^V in xanthine oxidase and aldehyde oxidase appear in the presence of substrate and disappear again as the substrate is oxidized. Although the data are not good enough to be certain of the exact sequence, the electron transfer path appears to be[7,18,25,26].

$$\text{substrate} \rightarrow Mo^{VI}/Mo^{V} \rightarrow \text{FAD/FADH}\cdot \rightarrow Fe^{3+}/Fe^{2+} \rightarrow O_2$$

FADH = flavosemiquinone

In the case of xanthine oxidase, Bray indicates an alternative path, depending on substrate and pH, may be[8].

$$\text{substrate} \rightarrow \text{FAD/FADH}\cdot \rightarrow Mo^{VI}/Mo^{V} \rightarrow Fe^{III}/Fe^{II} \rightarrow O_2$$

It was found by Palmer, Bray and Beinert that the Mo^V of xanthine oxidase is bound in two different states, each giving rise to a characteristic ESR signal. Recent kinetic studies by Palmer and Massey[18] indicate that only one of the ESR signals (α,β, fast) is probably involved in the oxidation of substrate by the enzyme Further ESR work by Bray *et al.*[8,30], has shown that the Mo^V is interacting with a proton, causing each line of the ESR signal to be split into a doublet (Fig. 1a and b). Moreover, Bray and Knowles[29] have shown that the proton interacting with the Mo^V is initially derived from the C-8 position of the substrate (xanthine, purines), and when CH_3OH is used to inactivate the enzyme, interaction between

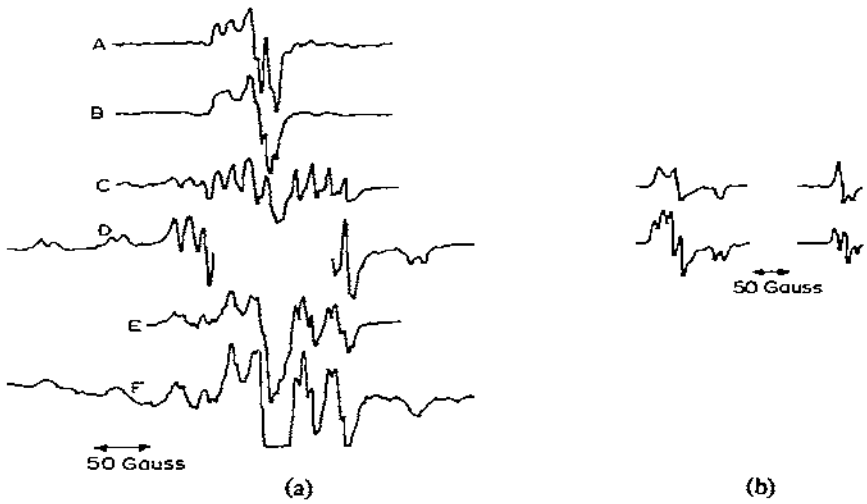


Fig 1a α, β ESR signal from xanthine oxidase in H_2O and D_2O Enzyme reduced with purine for 1 min , pH 10 A, Natural enzyme in H_2O, B, Natural enzyme in D_2O; C, Enzyme enriched with ^{95}Mo in H_2O, D, Enzyme enriched with ^{95}Mo in D_2O, higher modulation, center of trace omitted; E, Enzyme enriched with ^{95}Mo in D_2O, F, ^{95}Mo enriched enzyme in D_2O, higher modulation. −150 °C Redrawn from ref 8

Fig 1b Slowly appearing ESR signal from xanthine oxidase reduced with sodium dithionite Bottom, H_2O; top, D_2O Spectra on left, 35 GHz; on right, x-band pH 8 2, −150° Redrawn from ref 30

a proton from the methyl group and Mo^V occurs[30]. Clearly, this work indicates that the initial binding of the substrate, at least with xanthine and purines, is at the molybdenum site and that electron transfer probably occurs as in the first scheme above.

It should be pointed out that the ESR data may also be interpreted, somewhat less probably, in terms of a change of ligand of the Mo^V not necessarily involving any electron transfer[8]. In this regard, it has been shown with aldehyde oxidase that a small Mo^V signal is obtained from the resting enzyme[7]. Since it is well known that Mo^V has a great tendency to dimerize, forming ESR inactive oxygen (or sulfur) bridged species in which the Mo^V spins are paired[3], the possibility exists that such a dimer is partially converted to monomer in the presence of substrate due to ligand substitution by the substrate, accounting for the increase in ESR signal[26].

In the case of nitrate reductase, again an ESR signal attributed to Mo^V has been observed during nitrate reduction. No ESR kinetic studies have been reported, however, probably because of the difficulty of obtaining a pure enzyme preparation. On the basis of limited evidence, Nicholas has proposed the electron transfer sequence to be[11]:

$$NADH^{\dagger} \rightarrow FAD \rightarrow Mo^{VI} \rightarrow NO_3^-$$

† NADH = Nicotine adenine dinucleotide, reduced

This sequence is supported by model studies (see below). Nicholas also indicates, at least with certain nitrate reductases, that the enzyme is flavin-free, with the flavin component belonging to another enzyme system coupled to the nitrate reductase.

At present, very little is known about the function of the molybdenum in molybdoferredoxin[31]. One possibihty is tbat it supplies electrons to a bound N_2 molecule (bound to azoferredoxin, the non-heme iron protein also necessary for N_2 reduction), enabling its subsequent reduction to NH_3. This might be effected directly, or by reduction of the iron species to which the N_2 is presumably bound.

C. MODEL STUDIES

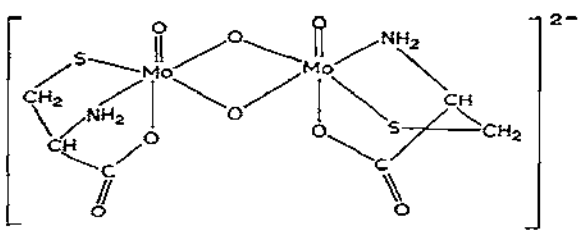
(1) *Complexes*

Although a rather large number of complexes of molybdenum in various oxidation states is known[2,3], not many of biochemical interest have been investigated.

In view of the evidence concerning cysteine as the probable binding site of the metal in the enzymes, complexes with thiols would appear to be the best models. A number of such complexes have been used for analytical purposes, but their structures have not been established[32]. More recently, work has been reported on complexes with cysteine, thioglycollic acid, β-mercaptopropionic acid, β-mercaptoethylamine, and related compounds in which more definite conclusions concerning structure have been reached.

Spence and Chang first reported that both Mo^V and Mo^{VI} complex with cysteine in dilute aqueous solution, with the Mo^{VI}: cysteine ratio varying from 1:1 to 1:3 depending on concentration, while Mo^V forms a 1:1 complex[33]. The Mo^{VI} complex involved the mercapto group and either the carboxy or amino group. Similar complexes of Mo^{VI} were demonstrated for cysteine ethyl ester and β-mercaptopropionic acid, while β-mercaptoethylamine forms a 1:1 complex.

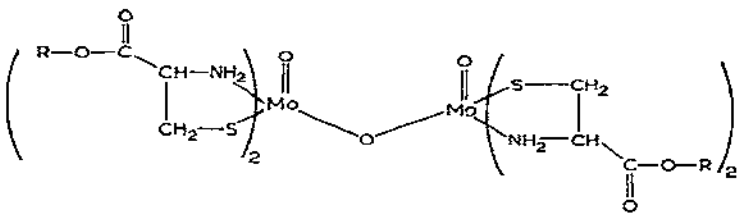
More recently, a solid Mo^V cysteine complex has been prepared and its structure determined by X-ray crystallography[34]. This complex is a 1:1 binuclear dioxobridged species in which the cysteine is tridentate:



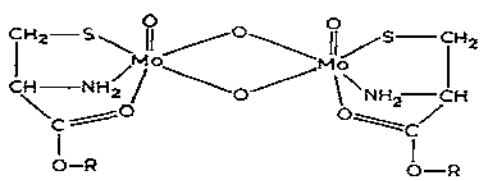
The complex is diamagnetic, as with most Mo^V complexes, possibly due to spin

pairing *via* a direct Mo–Mo bond (2 569 Å). Mitchell[64] has discussed this complex as a model for xanthine oxidase and reports that while it reacts with oxygen to produce the Mo^{VI} species, it does not catalyze the oxidation of xanthine by oxygen. It has an absorption maximum in water at 306 nm, $\varepsilon = 12{,}800$.

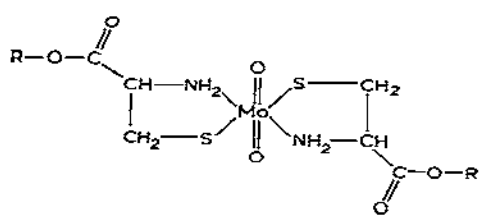
Melby has found that Mo^{V} forms a 1:2 binuclear oxobridged complex with methyl or ethyl esters of cysteine[35]. Based on analysis and infrared spectra of the solid, he formulated its structure as:



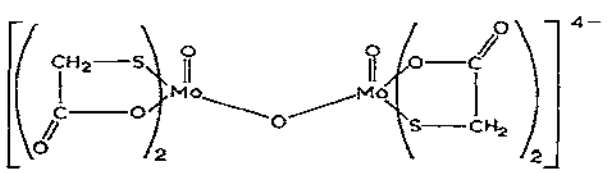
This complex, when dissolved in organic solvents, gave a 1:1 complex which appears to have a dioxobridge structure, having lost two ligands:



Although this is formulated as tridentate, no evidence for coordination of the $>C=O$ was given. In addition, he obtained a 1:2, mononuclear complex from Mo^{VI} and cysteine esters:



Thioglycollic acid forms a 1:2 complex with Mo^{V}, which by analogy with other complexes is probably a binuclear bridged species, since it also is mainly ESR inactive[36]:



This complex undergoes reduction in the presence of excess ligand to give a new complex, the structure of which has not yet been determined[37].

All of these complexes are essentially diamagnetic in the solid state. As a result they are not comparable to the enzymatic Mo^V, which gives an ESR signal accounting for a maximum of ~37% of the total Mo. Careful examination of the aqueous solutions of many thiol complexes, however, has revealed a small amount of ESR active species present, most likely a monomer, in equilibrium with the dimer. Meriwether *et al.* first reported ESR signals in solutions[38] of thioglycollic acid or BAL† and Mo^V. The signal with thioglycollic acid was found to change g value with time, possibly indicating some change in symmetry of the complex. In nonaqueous solvents, 100% of the Mo^V is present as monomer, while in water, a maximum of ~10% was present under the most favorable conditions, while the usual signal level was much lower (<0.4%). As seen in Table 2, the g values and hyperfine splitting constants (A) are comparable to those found in xanthine oxidase and have been interpreted as indicating a high degree of delocalization of the electron onto the sulfur ligands.

This group of workers also reported that no signals were obtained with cysteine and glutathione, but subsequent work has shown signals indeed are present for both complexes[36]. In the case of cysteine, the g values and signal shape changes with time as it does with thioglycollic acid (Fig. 2)

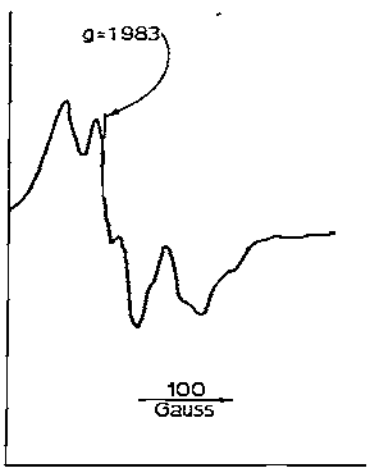


Fig. 2 ESR spectrum of Mo^V+cysteine, pH 5.00, −195°; 2 minutes after mixing

It therefore seems that the thiol complexes are, with regard to ESR spectra, qualitatively similar to the enzymes. The main difference appears to be in the fraction of the Mo^V present in a monomeric state. It seems quite reasonable that in the enzyme the higher levels of monomer might be achieved by bonding to the protein, the coenzyme or the substrate. Whether the molybdenum in the resting enzyme is essentially all dimeric with a shift of the dimer–monomer equilibrium

† BAL = 2,3-dimercapto-1-propanol

in favor of monomer occurring in the presence of substrate, and therefore not necessarily involving any electron transfer, remains to be seen.

Another possibility for the appearance of ESR active Mo^V species, both in the enzymes and the complexes, is a thermal equilibrium between ESR active and inactive dimers:

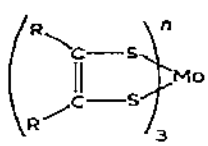
$$\underset{\text{ESR inactive (singlet)}}{\text{dimer}} \rightleftharpoons \underset{\text{ESR active (triplet)}}{\text{dimer}}$$

In such a case, the ESR active dimer would be a triplet having two unpaired electrons. Such a transformation might occur by a twisting about the Mo–O–Mo bridge, which would result in uncoupling of spins, as suggested by Cotton with regards to a binuclear bridged Mo^V–xanthate complex[39] Such an explanation seems quite likely in the case of $MoOCl_5^{2-}$ in HCl. Here, Bernal and Gray[74] found that this compound is 100% monomer in > 10M HCl. As the HCl concentration is lowered, however, both the intensity of the ESR signal and the paramagnetism of the solution decrease, but not the same amount. This was explained by postulating the existence of both paramagnetic and diamagnetic dimers, with the paramagnetic dimer contributing to the total paramagnetism but not to the ESR signal:

$$\underset{\substack{\text{monomer}\\\text{(ESR active)}}}{2\,Mo^V} \rightleftharpoons \underset{\substack{\text{paramagnetic dimer}\\\text{(ESR inactive)}}}{Mo^V{}_2} \rightleftharpoons \underset{\substack{\text{diamagnetic}\\\text{dimer}}}{Mo^V{}_2}$$

The paramagnetic dimer, as determined by the difference in ESR signal and the total paramagnetism, has a maximum concentration at ~6M HCl All paramagnetism disappeared below 2M HCl. The reason that the paramagnetic dimer is not ESR active is presumably due to the strong interaction of two unpaired electrons in solution, resulting in extensive line broadening, as with Mo^{III} species If this is the case, then such an equilibrium would not explain the ESR signals found with the enzymes and the complexes. Furthermore, the g values and line shapes in such cases are similar to those observed for monomeric Mo^V species. Work on the magnetic susceptibility of the enzymes and complexes in solution might reveal an appreciable amount of paramagnetic dimers, but such data have not been reported.

Another class of sulfur containing complexes of Mo^V, the dithiolates, are of interest because of the strong delocalization of the unpaired electron over the ligand, again resulting in high g values and small hyperfine splittings[40].

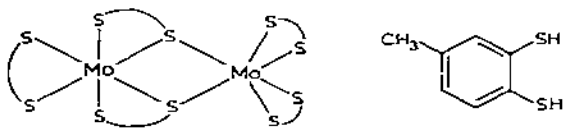


R = CF_3 , CN
n = 0, −1 , −2

A series of these complexes has been prepared with charges of 0, −1, and −2, in which the formal oxidation state of the metal is 6+, 5+, and 4+. Analysis of

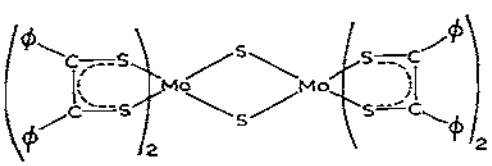
the ESR spectra for the 1− species (one unpaired electron) indicates, however, that the unpaired electron resides largely in a ligand orbital, giving rise to the high g value and small hyperfine splittings (Table 2). These complexes are water insoluble, but their high degree of electron delocalization and their absorption spectra (λ_{max} 670, 500 nm in CH_2Cl_2) makes them of interest as models for the enzyme bound Mo.

Another complex of this type is the binuclear Mo^V–toluene-3,4-dithiol species[41]. This is of considerable interest since it appears to involve a disulfur bridge between the Mo^V ions, having a ligand· Mo^V ratio of 5:2



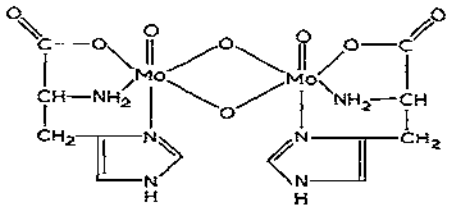
This complex is essentially diamagnetic, but a solution in CH_2Cl_2 gives a weak ESR signal with $g = 1.999$ and $A = 32$ Gauss. The absorption spectrum shows a charge transfer band at 515 nm with a shoulder at 610 nm. Molybdenum(VI) also forms a complex with this ligand of the formula $Mo(L)_3$.

A similar complex involving a disulfur bridge with dithiodiketone has been reported[42]. In this case, the formal oxidation state of the Mo is 2+, but this is without much significance because of extensive delocalization in the ground state:



These complexes suggest the possibility of the existence of sulfur bridged species in the molybdenum enzymes.

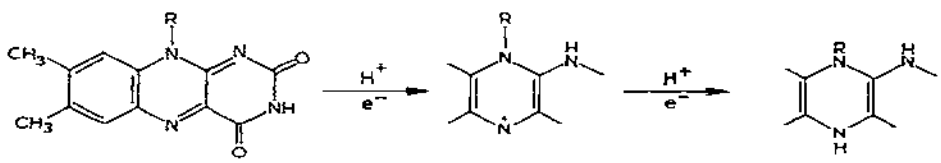
Because of its acid–base properties, the amino acid histidine is often regarded as a possible binding site for metal ions in enzymes, and many studies of its complexes with divalent and trivalent metal ions have been made. Spence and Lee reported that Mo^{VI} forms a 1:1 complex with histidine at pH 6.00 and, based on NMR investigations and studies of the methyl ester, concluded that binding to the metal ion occurred through the α-amino group and the 1-nitrogen of the imidazole ring[43]. No evidence for complex formation was obtained below pH 4. In the case of Mo^V evidence was obtained for a weak 1:1 complex at pH 4.60 in solution. Recently, Melby has isolated a solid 1:1, Mo^V histidine complex which appears to have a structure similar to the cysteine complex, the ligand being tridentate and the two molybdenum atoms joined through a dioxo bridge[44]:



This complex was found to be ESR inactive and to dissociate in dilute acid, in agreement with the solution work of Spence and Lee[43].

Since at least two of the molybdenum enzymes contain flavin (FAD) as coenzyme, and the ESR work of Beinert[25,26] indicates an interaction between these components, complexes of molybdenum with flavins are of considerable interest

Work by Hemmerich *et al.*[45] has shown that flavins, in spite of their structural similarity to 8-hydroxyquinoline, have little affinity for metal ions in either their oxidized state (flavoquinones) or completely reduced state (flavohydroqui-

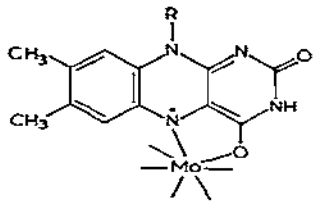


nones). The flavosemiquinone state, however, forms relatively stable complexes with a number of metal ions in aqueous solution[45].

Spence and Tocahan found that Mo^{VI} forms a strong complex with flavin mononucleotide (FMN) at pH near 2. The site of the complexing, as shown by work with polyols and the absence of an effect on the flavin absorption spectrum upon complexing, is undoubtedly the ribityl side chain[46]. The complex appears to have a Mo^{VI} : flavin ratio of 2:1, and, as the pH is raised above 2, dissociates. A number of similar complexes with sugars and polyols have been reported in the literature, but their biological significance is doubtful[47].

In preliminary studies of the reaction between reduced flavins and Mo^{VI} (or oxidized flavins and Mo^{V} since the system is a pH dependent equilibrium) Mitchell and Williams found spectral evidence of enhanced concentrations of the flavosemiquinone, which they suggested might be due to complex formation[48] with Mo^{V} or Mo^{VI}.

Studies by Spence *et al.* of this same reaction have shown that when Mo^{VI} is added to a solution of reduced flavin at pH 6.0, a red, ESR inactive intermediate is formed which is most likely an Mo^{V}–flavosemiquinone complex[49]. This complex appears to be thermodynamically unstable, but kinetically rather slow to react, thus allowing its detection.



The absence of an ESR signal for this complex may be attributed to spin pairing between the semiquinone and Mo^V or severe line broadening due to the presence of 2 spins in the system. Hemmerich has found that when Mo^V is added to a solution of flavin in CH_3CN, a spectrum characteristic of flavosemiquinone–metal complexes is obtained, probably due to a charge transfer complex in which Mo^V has transferred an electron to the flavin, thus forming an Mo^{VI} flavosemiquinone species[50]. Studies by Spence *et al.* have shown that in water at pH > 8, Mo^V reacts with flavin giving Mo^{VI} and reduced flavin[49].

$$Mo^V{}_2 + Fl \rightleftharpoons 2Mo^{VI} + FlH_2$$

By means of ESR it was demonstrated that the flavosemiquinone concentration was greatly enhanced above that normally present in a metal free solution containing both oxidized and reduced flavin and preliminary power saturation studies indicated considerable interaction between the flavosemiquinone and metal ion. These results most probably are due to the presence of a charge transfer complex similar to that reported by Hemmerich[50] in CH_3CN.

Most Mo^V complexes in water are ESR inactive, due to spin pairing in the dimers[19]. Because of the presence of ESR active Mo^V in the enzymes, any complex that gives detectable ESR signals in water is of interest as a model Recent work has shown that a number of ligands besides thiols give rise to a monomer–dimer equilibrium (strongly in favor of the dimer) with Mo^V. The most important group of these ligands are α-hydroxy or polyhydroxy acids—tartaric, gluconic and citric acids all give a small amount of monomer at or near biological pH. Unlike the thiol ligands, these complexes have low g values, reflecting a much smaller degree of electron delocalization onto the ligand. Tartaric acid forms a complex at pH 4.72 that appears to have a 3:1 ligand: Mo^V ratio in the monomer[51]. This species is in equilibrium with a 2:1 dimeric complex:

$$(L)_2Mo(O)—O—Mo(O)(L)_2 + 2L \rightleftarrows 2Mo(L)_3$$

L = tartaric acid, $(CHOHCOOH)_2$

The constant for the monomer–dimer equilibrium was estimated to be 3.1×10^6 at pH 4.72 and 25°. Citric acid forms a complex with Mo^V with a maximum concentration of monomer[52] at pH ≅ 3.0. Under these conditions, the equilibrium constant for monomer–dimer formation was estimated to be: 2.5×10^5 at

25°. Because of the relatively small amount of monomer present, no further information concerning the structure of these complexes was obtained.

Although the affinity of Mo for nitrogen ligands is somewhat lower than for oxygen species, a small equilibrium concentration of monomer is present even with EDTA† at biological pH in the presence of excess ligand[53]. The ESR signal of this system was observed to be a maximum at pH near 3.4, decreasing to zero at pH 10. When the EDTA: Mo^V is <1, no signal is observed, due to formation of 2: $1Mo^V$: EDTA complex that has been studied by a number of workers[54]. The monomer–dimer equilibrium constant for this complex at pH 3.4 was found to be 7.2×10^4 at 25°. Again, the g-value is low, as would be expected for this ligand.

It thus appears that a number of ligands can stabilize the monomer to varying degrees under biological conditions, giving rise to ESR active species.

(ii) *Reactions*

Little is known concerning the redox reactions of molybdenum species in solution. Of those reactions which might be considered as models for enzymatic processes, systems related to nitrate reduction have received the most attention.

Haight studied the Mo^{VI} catalyzed reduction of nitrate at the dropping mercury electrode and concluded that the active species involved in the reduction of the nitrate ion is Mo^{IV}, formed from Mo^{VI} at the electrode[55]. In other studies of the Mo^{VI} catalyzed reduction of nitrate by Sn^{II} in acid solution he again proposed Mo^{IV} as the intermediate and observed that NH_2OH and NH_3 are the products in HCl, while N_2O is produced[56] in H_2SO_4. The conclusion concerning Mo^{IV} was questioned by Kolthoff who also studied the catalyzed polarographic reduction[57]. He interpreted his data in terms of an Mo^V intermediate, indicating that the Mo^V formed in the reaction may not be the same species obtained when Mo^V is deliberately added to test its effect, as Haight did. Kolthoff's work was substantiated by Guymon and Spence[58], who investigated the reduction of nitrate by Mo^V in various buffers. They found that no reduction occurred in phosphate, acetate, bicarbonate or borate buffers, but when tartrate was used, nitrate was reduced to NO. Furthermore, a study of the kinetics of the reduction indicated it was first order in nitrate and half-order in total Mo^V. A mechanism involving Mo^V monomer as the active intermediate was proposed to explain the kinetics:

$$Mo_2^V \rightleftarrows 2Mo^V \tag{1}$$

$$Mo^V + NO_3^- \rightarrow Mo^{VI} + NO_2 \tag{2}$$

$$2NO_2 \rightarrow NO^+ + NO_3^- \tag{3}$$

$$Mo^V + NO^+ \rightarrow Mo^{VI} + NO \tag{4}$$

Reaction (2) is the rate controlling step in this scheme. Evidence in support of this

† EDTA = ethylendiamine–tetraacetic acid

mechanism was obtained by the finding of an ESR signal for Mo^V in the tartrate buffer. No signal was observed in acetate, bicarbonate or borate buffers while only a weak signal was detected in phosphate. This was confirmed by further studies of Mo^V in tartrate (see above) indicating a 3:1 tartrate–Mo^V monomeric complex is present in equilibrium with a 2:1 dimer. These results explain the observation of Haight in the catalyzed reduction of nitrate that the addition of Mo^V (as $MoOCl_5^{2-}$) had no effect on the reactions[56]. In this case, the Mo^V immediately dimerized, and the dimer is probably unreactive. However, when Mo^V is formed *in situ* from Mo^{VI} it may react quickly with nitrate before dimerization occurs. Since the catalyzed reactions proved much faster in HCl than H_2SO_4, Cl^- may also stabilize the monomer to some extent.

Contrary to the results with nitrate, the oxidation of $Mo^V{}_2$ by I_2 and O_2 was found to proceed at a rate independent of the concentration of oxidant and dependent on the concentration of $Mo^V{}_2$ to the first power in phosphate buffer[59], pH 1.65–7.20. Furthermore, ESR studies of the rate of monomer formation under these conditions ruled out Mo^V monomer as the reacting species, since it is formed too slowly to account for the observed kinetics. A mechanism involving the formation of Mo^{IV} as the rate controlling step was proposed, but no direct evidence was obtained to substantiate it:

$$Mo^V{}_2 \rightleftarrows Mo^{IV} + Mo^{VI}$$

$$Mo^{IV} + I_3^- \rightarrow 3I^- + Mo^{VI}$$

If this mechanism is correct, it appears $Mo^V{}_2$ can react in either one (Mo^V monomer) or two (Mo^{IV}) electron steps, depending on the oxidant. Possibly, with oxidants like I_2, which probably favor two electron transfers, the Mo^{IV} route is energetically more favorable, while with nitrate, which can add one electron to produce NO_2, the single transfer is easier. In any case, the possibility of different reaction mechanisms may account for the involvement of molybdenum in the rather diverse enzymatic processes in which it is found.

The results of Guymon and Spence suggest that the enzymatic reduction of nitrate also proceeds *via* monomeric Mo^V, since an ESR signal is observed in the presence of substrate. The function of the protein may be to provide ligands to stabilize the monomer as does tartrate in the model reaction. The kinetic results of the model reaction clearly indicate that the higher the concentration of monomer (which is present at less than 0.1% in tartrate) the faster nitrate reduction occurs.

A nitrate reducing system that is closer to the enzymatic process has been reported by Spence and Frank[60]. They found that when solutions of nitrate, FMN and Mo^{VI} were irradiated with light of wavelength greater than 3750 Å traces of nitrite and NO gas were detected. These results were interpreted as a photochemical reduction of FMN proceeding by an abstraction of hydrogen from its ribityl side chain, followed by reduction of Mo^{VI} to Mo^V by the photoreduced flavin and subsequent reduction of nitrate by Mo^V:

$$FMN \xrightarrow{h\nu} FMNH_2^*$$
$$FMNH_2^* + 2Mo^{VI} \rightarrow FMN^* + 2Mo^{V}$$
$$Mo^{V} + NO_3^- \rightarrow NO_2 + Mo^{VI}$$
$$2NO_2 \rightarrow NO_3^- + NO^+$$
$$Mo^{V} + NO^+ \rightarrow NO + Mo^{VI}$$

In this reaction, the reoxidized flavin, FMN*, is mainly lumichrome, arising from the splitting off of the ribityl side chain after hydrogen abstraction. Subsequent study of these reactions has confirmed the proposed mechanism, although only small amounts of nitrate are reduced in phosphate buffer[61,62]. Recently, a model system which quantitatively reduces nitrate has been developed. When solutions of reduced FMN (reduced by Ti^{3+} or $S_2O_4^{2-}$) are mixed with nitrate in tartrate or citrate buffer at pH 2–4, and a small amount of Mo^{VI} (or Mo^{V}_2) is added, the flavin is oxidized and nitrate is reduced[63]:

$$3FMNH_2 + 2NO_3^- \xrightarrow{Mo^{VI}} 3FMN + 2NO$$

No reduction occurs in the absence of Mo. An investigation of the kinetics has shown the rate of disappearance of $FMNH_2$ to be independent of reduced flavin concentration and dependent on nitrate concentration to the first power and Mo^{VI} concentration to the one-half power. Furthermore, a short initial fast period of $FMNH_2$ disappearance was observed corresponding to the reduction of Mo^{VI} to Mo^{V}, before the zero order reaction began. Clearly, this reaction is proceeding by the same mechanism as the reduction of nitrate by Mo^{V} discussed above, involving the formation of Mo^{V} monomer, stabilized by the citrate buffer, as the reactive species of the metal ion:

$$2Mo^{VI} + FMNH_2 \rightleftarrows FMN + 2Mo^{V} \quad (5)$$
$$2Mo^{V} \rightleftarrows Mo^{V}_2 \quad (6)$$
$$Mo^{V} + NO_3^- \rightarrow Mo^{VI} + NO_2 \quad (7)$$
$$2NO_2 \rightarrow NO^+ + NO_3^- \quad (8)$$
$$Mo^{V} + NO^+ \rightarrow Mo^{VI} + NO \quad (9)$$

Reaction (5) is much faster than reaction (7) which is rate determining. The initial fast disappearance of $FMNH_2$ corresponds to reaction (5), confirmed by adding Mo^{V}_2 instead of Mo^{VI}, in which case zero order kinetics are observed from the beginning Since the Mo^{V} is quantitatively reduced to Mo^{V}_2 by $FMNH_2$ in reaction (5), its concentration remains essentially constant throughout the major part of the reaction, and, in the presence of excess nitrate, overall zero kinetics are observed:

$$\frac{-d\,[FMNH_2]}{dt} = k\,[Mo^{V}]\,[NO_3^-] = k'\,[Mo^{V}_2]^{\frac{1}{2}}\,[NO_3^-] = k''$$

Again it was found that no reduction of nitrate occurs in phosphate or acetate buffers, which do not stabilize the monomer to any appreciable extent. This system, then, behaves in a manner very similar to the enzymatic system and supports the electron transfer scheme proposed originally by Nicholas for nitrate reductase[11]:

$$NADH \rightarrow FAD \rightarrow Mo^{VI} \rightarrow NO_3^-$$

Although other complexes of Mo^V which give ESR signals (thiols, EDTA) have not been investigated in nitrate reduction, it seems clear that the key to the reaction is the presence of at least a small concentration of the monomer. In this regard, Mitchell and Williams reported the Mo^V 8-hydroxyquinoline complex, which is ESR inactive, does not reduce nitrate[19]. Complexes which give enhanced monomer concentration may prove much more effective in this model reaction.

No model reactions in which xanthine or purines are oxidized by molybdenum complexes have been reported. In fact, it has been found that Mo^V complexes of cysteine[64] and oxalate[65] do not catalyze the oxidation of xanthine by oxygen.

In the study of Mo^{VI} complexes with sugars it was found that at low pH solutions of the sugars and Mo^{VI} gradually turned blue, due to the formation of molybdenum blue, a mixed valence Mo^V–Mo^{VI} species[66]. Thus, oxidation of the sugar occurred, and since sugars may be regarded as reactive aldehydes, this reaction might be considered a model for the enzyme aldehyde oxidase. Recently, it has been found that a Mo-phthalocyanine complex, when impregnated on a carbon electrode, catalyzes the electrochemical oxidation of glucose[67]. No investigation of aldehyde oxidations beyond these observations have been reported, however.

Although there are now a number of complexes known to bind molecular nitrogen ($Ru(NH_3)_5N_2^{2+}$, e g) no such species containing molybdenum has been reported Two reactions involving possible intermediates in nitrogen reduction have been studied, however. Haight and Swift[68] have investigated the reduction of hydroxylamine to ammonia by Mo^V in acid. They found the rate to be:

$$\frac{-d[Mo^V]}{dt} = [Mo^V]\,[NH_2OH]\,[H^+]^2$$

They proposed a mechanism in which NH_2OH first complexes with $Mo^V{}_2$, followed by decomposition of the complex into NH_3 and Mo^{VI} in the presence of acid:

$$Mo^V{}_2\text{–}OH + NH_3OH^+ \rightleftarrows Mo^V{}_2\text{–}O\text{–}NH_3 + H_2O$$

$$2H^+ + Mo^V{}_2\text{–}O\text{–}NH_3 \rightleftarrows Mo^{VI}{}_2\text{–}OH_2 + NH_3$$

They also observed that the absorption due to Mo^V is enhanced by NH_2OH, lending support to their proposed NH_3OH^+–$Mo^V{}_2$ complex.

The oxidation of hydrazine by Mo^{VI} in phosphate buffer, pH 1.2–3.2, has been studied by Huang and Spence[69]. They found the reaction to produce N_2 exclusively. Based on this and trapping experiments which detected diimide as an

intermediate, they proposed Mo^{VI} is a two electron oxidant, being reduced to Mo^{IV}, which subsequently reacts with Mo^{VI} to give Mo^{V}_2:

$$Mo^{VI}+N_2H_5^+ \rightarrow Mo^{IV}+N_2H_2+3H^+$$

$$H^+ + 2N_2H_2 \rightarrow N_2 + N_2H_5^+$$

$$Mo^{IV}+Mo^{VI} \rightarrow 2Mo^{V}$$

$$2Mo^{V} \rightleftarrows Mo^{V}_2$$

These authors also studied the Mo^{VI} catalyzed oxidation of hydrazine by methylene blue[69] They found the rate controlling step in this case to be the same as for the uncatalyzed reaction, with the Mo^{IV} and Mo^{V}_2 being reoxidized in fast steps to Mo^{VI}:

$$Mo^{VI}+N_2H_5^+ \rightarrow Mo^{IV}+N_2H_2$$

$$H^+ + 2N_2H_2 \rightarrow N_2 + N_2H_5^+$$

$$Mo^{IV}+MB \rightarrow Mo^{VI}+MBH$$

$$Mo^{IV}+Mo^{VI} \rightarrow 2Mo^{V}$$

$$Mo^{V}+MB \rightarrow MBH\cdot + Mo^{VI}$$

$$2MBH\cdot \rightleftarrows MB+MBH+H^+$$

MB = methylene blue (oxidized)

MBH · = methylene blue radical

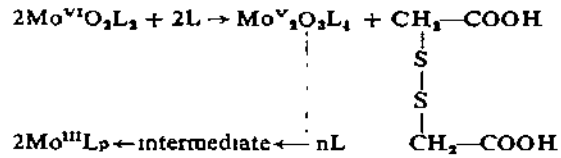
MBH = methylene blue (reduced)

Evidence in support of this mechanism was obtained from ESR studies and kinetic investigations of the reaction of Mo^{V}_2 with methylene blue in solution.

As discussed above, the molybdenum of the enzymes appears to be bound to the sulfhydryl group of cysteine. Since the cysteine–cystine system is redox active, although no evidence implicating it in the enzymatic electron transfer processes has been reported, it is possible the Mo^{VI}/Mo^{V} couple might interact with it.

Spence and Chang first reported that at pH <5, Mo^{VI} is reduced by cysteine, as indicated by the formation of molybdenum blue[33]. Many other thiols are known to reduce Mo^{VI} to Mo^{V} and they have been used as analytical reagents for this purpose[32]. In a study of the reactions of Mo^{VI} with thioglycollic acid at pH 6.0 in phosphate buffer it was found that Mo^{VI} is first reduced quantitatively to Mo^{V} by excess ligand. The initial product is a 2 : 1 binuclear, ESR inactive complex, in equilibrium with a small amount of monomeric species. In the presence of excess ligand, this initial complex slowly disappears, as evidenced by the disappearance of its polarographic wave and the absorption due to the Mo^{V}–8-hydroxyquinoline complex when the reagent is added to a sample of the solution in ethanol, and the

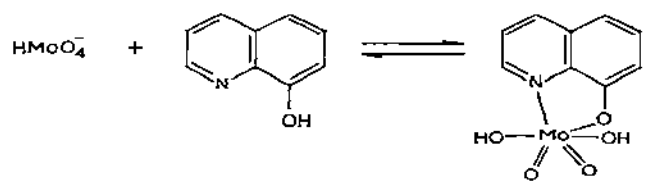
appearance of a new and more intense peak in the solution at 365 nm. After considerable time (~48 hours) the absorption of the solution changes to that obtained when K_3MoCl_6 is added to a solution of the ligand (λ_{max} = 420 nm), indicating the Mo^V complex is reduced to an Mo^{III} species[66]. The appearance of the Mo^{III} absorption, however, does not correspond in time to the disappearance of the original Mo^V complex, suggesting an intermediate species (the 365 nm absorption) is formed. The nature of this intermediate, which is an Mo^{IV} species, as well as the nature of the Mo^{III} complex have not yet been determined:



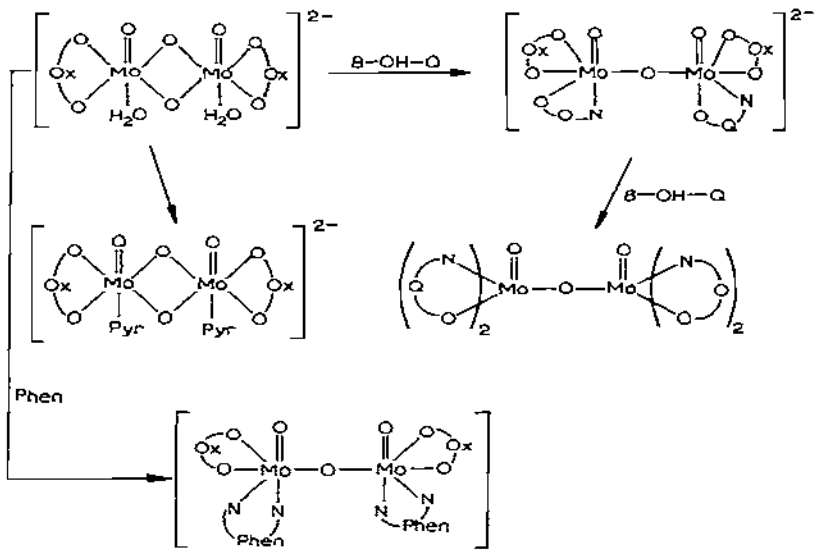
The intermediate and the Mo^{III} complex are surprisingly stable in solution to oxidation, even upon standing in air, and are essentially inert to substitution by 8-hydroxyquinoline in ethanol. The results, although preliminary, indicate the possibility of redox reactions between the molybdenum and cysteine of the enzymes, and suggest that Mo^{III} (and possibly Mo^{IV}) states cannot be ruled out.

Regardless of whether the molybdenum is involved in the electron transfer sequence of the enzymes or not, there is evidence (see above) that it is involved in binding the substrate, at least in xanthine oxidase and aldehyde oxidase. Furthermore, it is likely that during the course of reaction the ligand environment about the metal ion changes. Therefore, ligand substitution reactions of Mo–complexes are of considerable interest as models

Knowles has studied the formation of the Mo^{VI}–8-hydroxyquinoline complex by fast reaction techniques as a model for substrate binding in xanthine oxidase[70]. He found that if Mo^{VI} exists as a protonated species in the reaction, a rate constant approaching that of the enzyme for substrate was obtained:



More recently, a study of the ligand substitution reactions of the μ-dioxo-bis [oxo-oxalato-aquomolybdate(V)] ion has been reported by Mitchell[65]. He found that 8-hydroxyquinoline, pyridine-2-carboxylic acid, 1,10-phenanthroline, pyridine, and EDTA will replace H_2O, a bridging oxygen, or oxalate, giving a variety of products



He obtained solids with all ligands except EDTA and the structures are based on analysis, infra red and electronic spectra, and magnetic measurements. All complexes were essentially diamagnetic (0.4–0.5 BM as compared with 1.73 BM expected for Mo^{V}), although no ESR spectra of solutions were reported. He observed that the ease of displacement is: H_2O > one bridging O > oxalate ≫ terminal O, while the ease of substitution is oxine > oxine-5-sulfonic acid ≫ EDTA ≫ pic > phen > pyr. If the oxalate complex can be considered as a model for the enzyme (xanthine oxidase), then it appears there is a number of ways by which substrate might be bound, producing a variety of products. It is of interest to note, however, that no reaction of the complex was found with xanthine.

Beyond this work, no other detailed investigations of substitution reactions of Mo species in aqueous solution have been reported.

D. CONCLUSIONS

The biochemical uniqueness of molybdenum appears to be due to its ability to coordinate a variety of different ligands, to exist in at least two (and possibly more) oxidation states, and to participate in both redox and ligand exchange reactions *via* a number of different mechanisms. These at least are the conclusions of model studies and seem to be relevant to the enzymatic systems.

Clearly, however, a number of fundamental questions remain to be answered. First, the oxidation state of the metal, both in the resting and the working enzymes needs to be established. Although the evidence favors 6+ and 5+ states, in no case has more than 37% of the molybdenum been accounted for as ESR

active Mo^V. This question might be pursued by studying the redox behavior of the enzyme, although the interpretation of titration curves is difficult with such large molecules (MW of ~300,000 for xanthine oxidase), particularly when a number of other redox active species are present (thiols, iron, flavin). Recently, the preparation of flavin-free xanthine oxidase has been reported[71]. A study of its electronic spectrum, particularly in the working state, as well as that of flavin-free nitrate reductase, might reveal what absorption (if any) is due to molybdenum. This could then be compared with model complexes, which might indicate both the oxidation state and something about the metal ion environment. In this regard, a suggestion of Dennard and Williams might prove most interesting[72]. By replacing the Mo of the enzyme with W, which should exhibit charge transfer spectra at shorter wavelengths, perhaps allowing its distinction from flavin absorption, the enzymatic chromophore of Mo might be identified.

A second problem is to determine whether the Mo participates directly in the electron transfer process, or whether it simply undergoes ligand substitution reactions. Following a suggestion of Cotton[39], who proposed that in binuclear, bridged Mo^V complexes, spin pairing might be overcome if sufficient twisting about the Mo–O–Mo (or Mo–S–Mo) bridge bond occurred. It should then be possible to prepare Mo^V complexes with large ligands which, because of steric factors, might adopt such a configuration, giving rise to paramagnetic species. The work of Mitchell on substitution reactions of the Mo^V–oxalate complex indicates substitution can occur readily with a variety of ligands[65].

Finally, questions of the nature of the binding site and the mechanism of enzymatic redox reactions can be approached by a systematic study of the structures, electronic and ESR spectra, reduction potentials and reaction mechanisms of Mo complexes in 6+, 5+, 4+ and 3+ states Complexes with sulfur ligands, with peptides and with flavin-peptide species containing both the sulfur and flavin moieties in the same molecule seem particularly promising[73]. The correlation of such work with biochemical investigations of the enzymes, perhaps including degradation studies to isolate active fragments and, ultimately, X-ray crystallographic analysis should give a deeper understanding of the biological function of this fascinating transition metal.

E NOTES ADDED IN PROOF

The absorption spectrum of deflavo-xanthine oxidase, both in the oxidized and reduced forms has recently been published by Komai *et al.* [H. Komai, V. Massey and G. Palmer, *J. Biol. Chem.*, 244 (1969) 1692]. In both cases, the spectrum is essentially similar to that of other iron–sulfur enzymes and indicates the characteristic absorption of the native enzyme is due mainly to flavin with little contribution from Mo.

A Mo–nitrogen complex, prepared from Mo^{III}–acetylacetonate and triisobutyl aluminum in toluene has now been reported [M. Hidai, K. Tominari and Y. Uchida, *Chem. Commun*, (1969) 814].

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