THE INVESTIGATION OF A COPPER-MOLYBDENUM COMPLEX

by

JOUNG-YEI FRANK CHEN

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Approved by:

Clifton E. Melton
Major Professor

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Introduction

Copper and molybdenum are essential elements for plants and animals. Copper occurs in biological systems as a copper-protein complex and is contained in some enzymes such as cytochrome oxidase and tyrosinase. Some enzymes contain molybdenum such as xanthine oxidase, sulphite oxidase, nitrate reductase, NADH dehydrogenase and nitrogenase (1).

The metabolism of copper, molybdenum and inorganic sulfate is very complex and interrelated. An interrelationship between copper and molybdenum was discovered for the first time in England by Ferguson, et al (2) at 1943. Copper poisoning in both man and animals is known, as is molybdenum poisoning. The interesting fact is that copper poisoning can be cured in animals by feeding them molybdenum, and molybdenum poisoning can be cured by feeding copper. However, sulfate $SO_4^{=}$, in small amounts, must also be present. There must then be a Cu-Mo-$SO_4^{=}$ interaction. It is suspected that the same is true with humans but few tests have been made that we are aware of because molybdenum is at present not permitted to be added to food since no need has ever been demonstrated for it. This presents a real problem in that 15% of Negroes, Indians and Asians have a chemical deficiency that makes them highly susceptible to copper poisoning.

In normal people, glutathione is present in large enough quantities in a reduced state to keep red blood cells firm and tough. The enzyme, glutathione reductase, keeps a good supply of reduced glutathione available. Copper is needed for normal production of red blood cells but copper in high doses apparently does two things; one, it complexes two molecules of glutathione thus reducing the glutathione concentrations, and two, it reduced the activity of the glutathione reductase, which further lowers the glutathione present. When glutathione reaches a low level, the red blood cells become fragile and break causing critical anemia and death.

The Negroes, Indians and Asians that have the problem have a low glutathione reductase concentration, therefore, they have a low concentration of reduced glutathione to begin with, so even a little copper is quite harmful. In India, a cheap suicide is a dose of $CuSO_4$.

From Underwood,(11) chronic copper poisoning-molybdenum deficiency syndromes include acute hemolytic crisis, hemoglobinuria, icterus anarexia, weakness and rapid death accompanied by degenerative changes in the liver, kidney, spleen and other organs.

Molybdenum poisoning is not as well worked out. It is associated with activation xanthine oxidase which converts xanthine to uric acid. Uric acid in high concentration is associated with endemic gout. Disorders resulting from copper deficiency, or chronic molybdenum poisoning include anemia,

depigmentation of hair and wool, gastrointestinal disturbances, depressed growth, neonatal ataxia, bone disorders, impaired reproduction and cardiovascular problems.

Animals are fed rations that contain Cu because Cu in small amounts is necessary for red blood cell synthesis, Cu is quite toxic to many bacteria and is fed in place of antibiotics. A deficiency in Cu causes scours. Mo is essential in animals to activate xanthine oxidase, but too much produces scours.

In animals, a ratio of Cu-Mo of 6:1 in their feed is found to be most effective,however, a 4:3 ratio compound can be made synthetically, but no one has found a way to dissolve it without destroying it.

The antagonistic effect between copper and molybdenum has been well-documented (3,4,5,10,15,16). Dowdy (5) presents an argument for the formation of a synthetic copper-molybdenum complex in vivo to explain the metabolic interaction between copper and molybdenum. The basic assumption is that copper and molybdenum bound together are biologically inactive, and the complex is absorbed, transported, and excreted as a unit. He also showed that copper and molybdenum form a complex in vitro having a molar ratio of 4:3.

Dick (6) and Wynme, et al (7) showed that added dietary inorganic sulfate could decrease copper storage. Dick (9) observed that the copper-molybdenum interaction in some cases was dependent on sulfate. He suggested a copper-molybdenum-sulfate interaction.

Huisingh, et al (8) proposed that the antagonistic interreaction between copper and sulfate was due to the formation of

cupric sulfide. The interaction of sulfate and molybdenum may occur at several sites with different effects. Since molybdate can inhibit sulfate reduction it may decrease the amount of sulfide formed from sulfate in the rumen and therefore increase the amount of copper available to the animal. They also suggest that sulfate and molybdate interact antagonistically at the membrane transport level.

Since the effect of any one of these elements(copper, molybdenum, or sulfate) is dependent on both previous and present dietary levels of the other two elements, it has been difficult to predict the effect of dietary changes. No one has been able to separate and identify the suspected complex nor have they been able to show it actually exists in either the liver or the urine.

The purpose of this investigation was to see if it was possible to dissolve the complex without destroying it, then, to develop analytical methods to determine the complex, and if possoble, to do some animal studies to see if the 4:3 complex really exists in the liver and urine.

Copper-Molybdenum Complex Structure

A comparison of X-ray diffraction powder patterns from the natural rare mineral, lindgrenite, $2CuMoO_4 \cdot Cu(OH)_2$, with a synthetic copper-molybdenum complex was made by Dowdy, et al (13). The data show that the copper-molybdenum complex prepared in the laboratory was positively identified as a synthetic form of lindgrenite. Britton, et al (12) showed that copper and molybdenum

formed a rather poorly characterized insoluble precipitate. In a study of the precipitation of copper and molybdenum it was found that in the potentiometric titration of copper sulfate with sodium molybdate a precipitate began to form at pH 5.28. The composition of the copper-molybdenum precipitate was found by Dowdy, et al (13) to be 33.4% copper and 32.3% molybdenum. The results of the continuous variation method of Job (18) indicate that the molar ratio of copper to molybdenum is approximately 4:3.

The copper-molybdenum complex was shown to be chemically homologous by varying the molar concentrations and the molar ratios of the solutions used to prepare the complex. No evidence was shown that other ionic species such as sulfate, chloride, nitrate and acetate in solution were combined into the complex. This work duplicated here and found to be correct.

X-ray diffraction results of lindgrenite obtained by Calvert, et al (14) showed an infinite three-dimensional network of $CuO_4(OH)_2$ octahedra sharing edges to form endless chains which are cross linked through $MoO_4^{=}$ tetrahedra sharing corners with adjacent octahedra as shown in Figure 1.

Figure 1.

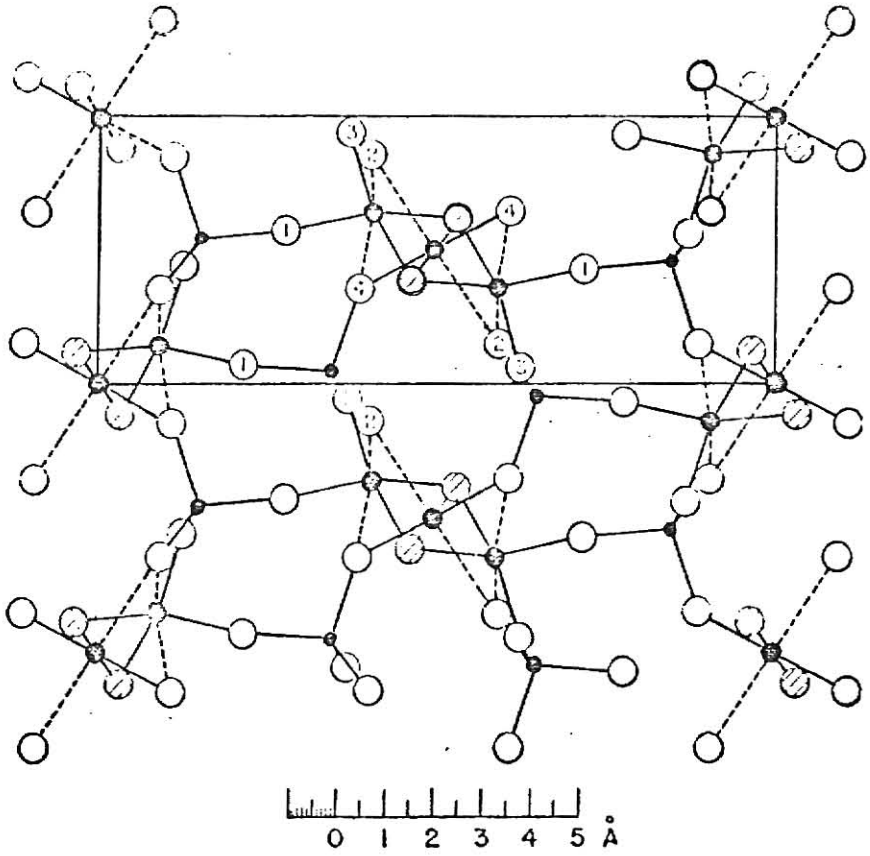
Projection of the structure of lindgrenite

Cu: large solid circle

Mo: small solid circle

O : open numbered circle

OH: hatched circle


0 1 2 3 4 5 Å

Determination of the antidotal dose of copper in the treatment of molybdenum poisoning of rats

A study of the dose of copper necessary to counteract the effect of molybdenum was made to provide information of the antidotal action and the suitable dose to be provided in the treatment of molybdenum poisoning. The experiment was designed to determine the dose of copper required to prevent the harmful effects of molybdenum.

Experiment

White in-bred rats were divided into groups of the same sex, age and similiar average weight. Those rats were fed an experimental diet*, and mineral additives were added to the feed. The mixture of diet and additives were compressed in the form of pellet under high pressure, and was easily eaten by the animals. Copper was given as $CuCl\ 2H_2O$ in order to avoid the interference of the sulphate ion as mentioned by several authors (4,28-38). for molybdenum Na MoO 2h O was used.

Following a feeding period of three weeks the animals were killed after prolonged anesthetization with ether. The liver samples were wet-ashed and dissolved in hydrochloric acid. The atomic absorption method was used for dertermination

* Purina Laboratory Chow, Rolston Purina Company, St. Louis.

of copper.

Ten male rats with an average weight of 175 g were divided into five groups. The control group A was fed the experimental diet, Group B, C, D and E received in addition, 15 mg Mo, 15 mg Mo + 5 mg Cu, 15 mg Mo + 15 mg Cu, 15 mg Mo + 50 mg Cu/rat/day, respectively, during a three weeks period.

Results and Discussion

Results of experiment are shown in Table 1.

Table 1.

Antidotal effect of Cu on body weight and Cu content of the liver

	Avg. values for the various groups				
	A	B	C	D	E
Initial wt. (g)	176	174	175	176	175
Wt. at termination of experiment (g)	234	194	231	200	136
Avg. wt. increase (g)	58	20	56	24	-39
Cu content of liver (µg Cu/g)	5.34	18.05	11.15	24.10	589.50

Daily supplemental treatment per rat:
Group A = control
Group B = 15 mg Mo
Group C = 15 mg Mo + 5 mg Cu
Group D = 15 mg Mo + 15 mg Cu
Group E = 15 mg Mo + 50 mg Cu

From Table 1. 5 mg copper per day (250 mg/kg feed) was the optimal dose as far as a growth was concerned. Higher doses than 5 mg Cu delayed growth and caused loss of weight from copper poisoning. Also, molybdenum-induced copper accumulation in liver was lowered by a daily supplement of 5 mg Cu, but higher amounts increase copper storage in liver.

This 5 mg dose was consider to be the suitable quantity for treatment, resulting in a recovery of the lose of weight induced by Mo poison.

The liver samples of these doses of copper and molybdenum are useful for further study such as trying to separate copper-molybdenum complex from liver extraction.

Synthesis Copper-Molybdenum Complex

Because the 4:3 complex was speculated on in so many papers, we tried to synthesize it as well as other complex ratios. The copper-molybdenum complex was prepared according to Dowdy, et al (13) by mixing aqueous solutions of a cupric salt (chloride, nitrate, sulfate, and acetate) and sodium molybdate at the pH range near neutrality. Reagent grade chemicals and distilled-deionized water were used for all preparations. The suspended precipitate was centrifuged for five minutes at 1000 G, and the supernatant solution discarded, the precipitate was resuspended with deionized water. This washing procedure was repeated four times. The final precipitate was collected on filter paper then dried in an oven overnight at 90 °C. The dried powder was used for our further investigations. Regardless of what mole ratios, pH or anions were used, by using the continuous variation method of Job (18) as the weight of precipitate formed the same method used by Dowdy, et al (10) indicated that we always obtained the 4:3 complex and no amount of effort was able to incorporate sulphate ion into the complex. If sulphate ion actually does effect the Cu-Mo ratio then it does so in a manner not associated with it being a part of the actual complex.

Infared Spectrum of the Copper-Molybdenum Complex

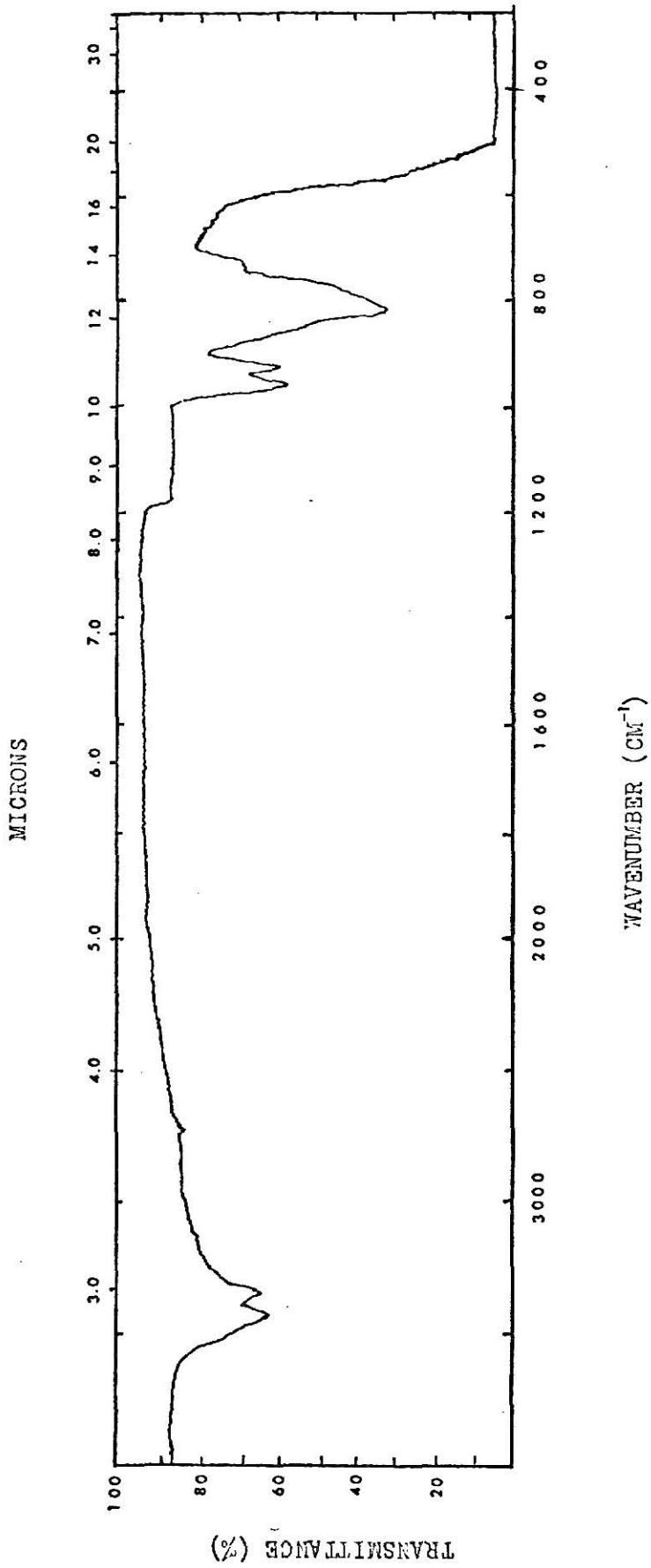
Instrumentation and methods

A Perkin Elmer 457 Grating Infared Spectrometer was used

to obtain all infared spectra. A small amount of the solid copper-molybdenum complex was ground to about 200 mesh, a drop or two of Nujor was added and the mixture reground. The slurry was transferred to a sodium chloride cell. The infared spectra of the copper-molybdenum complex obtained is shown in Figure 2.

Figure 2.

Infared spectrum of the synthetic copper-molybdenum complex


MICRONS
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
12
14
16
20
30
3000
2000
1600
1200
800
400
WAVENUMBER (CM⁻¹)
100
80
60
40
20
TRANSMITTANCE (%)

Discussion

The infared spectrum of the copper-molybdenum complex showed four charactristic peaks at 830 cm^{-1}, 920 cm^{-1}, 970 cm^{-1} and 3340 cm^{-1}, respectively. According to Ginestra, et al (25), the 830 cm^{-1}, 920 cm^{-1} and 970 cm^{-1} peaks are due to different molybdenum-oxygen vibration.

The infared spectrum was found to provide a good way to check the purity of the synthetic copper-molybdenum complex. If the precipitated complex is not washed throughly, the spectrum will show additional peaks from either copper sulfate or sodium molybdate. From Becher, et al (26), the molybdate has a peak at 1092 cm^{-1} and sulfate have peaks at 913 cm^{-1} and 907 cm^{-1}. It was found that even after three washings with water, the complex still showed a trace of copper sulfate and sodium molybdate in the infared spectrum.

By using the continuous variation method of Job (18) the same way as done by Dowdy, et al (10), we always got the copper to molybdenum 4:3 ratio no matter what kind of copper salt was used. This is a strong evidence for obtaining the same Cu-Mo complex.

Attempts to run laser Raman spectra on the copper-molybdenum complex in water failed, probably due to the low solubility of the complex in water.

Determination of Molecular Weight of The Copper-Molybdenum Complex by Gel Filtration

Whitaker J.R. (19) used gel filtration on Sephadex to determine the molecular weights of proteins. He found an excellent linear correlation between the logarithm of molecular weight of a protein and the ratio of its elution volume, V, to the void volume V_o of the column for Sephadex G-100 and G-75.

Since Sephadex is now routinely used in many laboratories, it was of interest to determine if these gels could also be used to give a molecular weight estimation of the copper-molybdenum complex.

Experimental

Reagents

Blue dextran 2000	: average molecular weight of 2,000,000
Serum albumin	: molecular weight of 70,000
Yellow dextran 20	: average molecular weight of 20,000
Cytochrome c	: molecular weight of 13,000
Vitamin B_{12}	: molecular weight of 1355.42
Sephadex G-100	: Lot. no. 9058, particle size 40-120 μ water regain 10±1g/g, obtained from Pharmacia, Uppsala Sweden.
10% $SnCl_2$	: dissolved in 1.0M HCl
10% NH_4SCN	: dissolved in water
0.1M NaCl	: dissolved in water, μ=0.194

Column

K15/90 (1.5x90 cm) borosilicate glass column obtained from Pharmacia, Uppsala, Sweden, with capillary tubing 1.0 mm i.d., 1.8 mm o.d.

Procedure

The Sephadex was suspended in enough 0.1M NaCl solution so that when it was stirred, trapped air bubbles could escape rapidly to the surface. It was then allowed to swell for two days at room temperature; otherwise it will give a column which has a very slow flow rate. The column was then half filled with NaCl solution and the gel suspension carefully added to bring the liquid level to the top of the column. A reservoir of suspended gel was placed on top of the column so that the column was poured as a continuous unit. After a 5 to 6 cm layer of Sephadex had settled to the bottom, the outlet was opened and the settling continued. After the column was poured, a filter paper disk was placed on top of the gel to prevent its disturbance when the sample was added. The column was then washed with NaCl at least overnight for the column to become completely equilibrated.

The samples were added to the top of the gel bed in 1 ml of NaCl solution. Under flow the samples were washed into the gel with additional NaCl, and NaCl was then added above the gel. Fractions of 1 ml were collected with a fraction collector with a drop counting device.

Serum albumin and Cytochrome c were determined at 280 mμ, Blue dextran 2000, Yellow dextran 20, and Vitamin B_{12} are colored compounds, Copper-molybdenum complex produces a red color by

adding a 1:1 (v/v) solution of 10% $SnCl_2$ in 1.0M HCl and 10% NH_4SCN. The void volume V_o was determined each day by using Blue dextran 2000.

Results and discussion

Amount of sample used:

Blue dextran 2000	: 0.8 mg
Serum albumin	: 8.0 mg
Yellow dextran 20	: 0.8 mg
Cytochrome c	: 8.0 mg
Vitamin B_{12}	: 0.4 mg
Cu-Mo complex	: saturated water solution
Column dxh cm	: 1.50x48 cm
Flow rate	: 0.25 ml.$\cdot$minute$^{-1}\cdot$cm^{-1}
Void volume V_o	: 18.5 ml

	elution volumn V^* (ml)	V/V_o
Serum albumin	19	1.03
Yellow dextran 20	35	1.89
Cytochrome c	40	2.16
Vitamin B_{12}	69	3.73
Cu-Mo complex	64	3.46

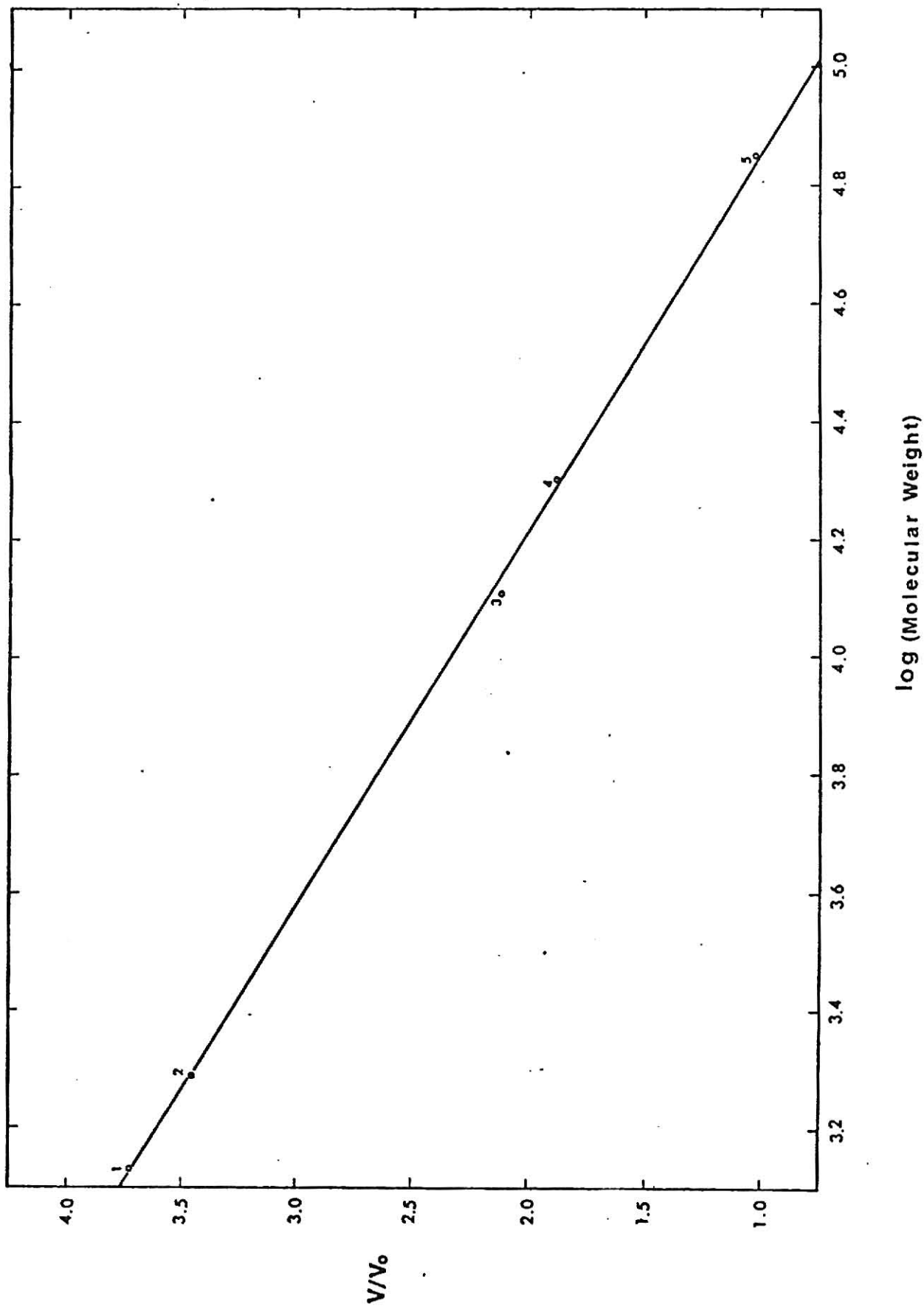
V^* = average values for three runs

Figure 3. shows the logarithm of molecular weight vs the ratio of its elution volume V to its void volume V_o.

Figure 3.

The logarithms of molecular weight vs the ratio of its elution volume V to its void volume V

1. Vitamin B_{12}
2. Cu-Mo complex
3. Cytochrome c
4. Yellow dextran 20
5. Serum albumin


4.0
3.5
3.0
2.5
2.0
1.5
1.0
V/Vo
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
log (Molecular Weight)
1
2
3
4
5

From Figure 3. it is shown that there is a linear correlation between the logarithm of molecular weight of samples and the ratio of its elution volume V to its void volume V_o. Also, from Figure 3, the elution volume of copper-molybdenum complex is 64 ml which corresponds to a molecular weight of 1950 ± 100.

Attempts to run a sample obtained from the liver of rats which were fed the copper-molybdenum complex and collecting the same elution volume (64 ml) fraction was not successful. This is explained by either three ways : (1) Due to the low concentration we were to detect it. (2) We were unable to extract the Cu-Mo complex out of rat liver, or (3) It does not exist in liver as this complex.

Dissolving the Copper-Molybdenum Complex

Because the 4:3 complex is so readily prepared in the laboratory, it is natural to assume it might well be the detoxification compound formed within the body, however, it is so insoluble that very little analytical work has been done.

The copper-molybdenum complex will dissolve in mineral acids such as hydrochloric acid, sulfuric acid, acetic acid, and nitric acid but the complex destroyed in the process. The complex is sparingly soluble in water, we found however that it was readily soluble if some chelating compound such as ethylenediaminetetraacetic disodium salt (EDTA), citric acid, tartaric acid, tiron (4,5-dihydroxybenzene m-disulfonic sodium salt) or nitrilotriacetic acid (NTA) were present. The complex was insoluble in all other organic solvents we tried.

Much to our disappointment we found that while chelating agents would dissolve the complex, they also destroyed the original compound. The various methods used to show this is as follow.

Thin Layer Chromatography

Apparatus and Reagents

TLC plates

Eastman polyester backed precoated silica gel plates were used for all chromatograms.

Buffer solutions

(1) Phosphate buffer solution

50 ml 0.1M potassium phosphate monobasic 32.1 ml 0.1M NaOH, pH= 7.10

(2) Phthalate buffer solution

50 ml 0.1M potassium hydrogen phthalate 0.1 ml 0.1M NaOH pH= 4.00

Spray reagents

(1) 10% (w/v) stannous chloride

Dissolve $SnCl_2$ in 1.0M HCl, filter before use. Make fresh every week.

(2) 10% (w/v) ammonium thiocyanate

Dissolve NH_4SCN in water.

Mix (1) and (2) in the ratio of 1:1 (v/v). the molybdate will give an intense red color after spraying. The detection limit is about 0.05 μg.

(3) 1% (w/v) sodium-diethyldithiocarbamate $(C_2H_5)_2N \cdot CS_2Na$

Dissolved in water, filter before use, store in brown bottle

make fresh every week. Copper produces an intense yellow-brown color after spraying with this solution. The detection limit is about 0.05 µg. The yellow-brown color of copper will fade when sprayed with 10% stanneous chloride solution, so, the 1% sodium-diethyldithiocarbamate must be sprayed first.

TLC plate development

The TLC plates are dried before use by heating in an oven for 30 minutes at 100 °C. After spotting, the TLC plates were allowed to equilibrate with the solvent and were then developed in a developing tank. The chromatographic tank containing 100 ml of solvent was provided with a filter paper on the wall for homogeneous chamber saturation. After an equilibration time of 15 hours, a few microliters of sample solution were applied to the chromatographic plate. It took about two to six hours to develop depending on the solvent used. The choice and composition of the solvent together with the corresponding R_f values are given separately.

Copper-molybdenum complex dissolved in chelating agents

It was found that the copper-molybdenum complex, copper sulfate and sodium molybdate all dissolved in different chelating agents such as EDTA, tiron, citric acid in a buffer solution of pH 7.10. The corresponding R_f values of each sample in the different solvent systems used are shown on Table 2.

Table 2.

TLC R_f values of samples dissolved in chelating reagents

Solvent A:
H_2O : glycolmethlethylether : ethylmethylketone : ammonia (D=0.91)
20 : 20 : 20 : 20 : 0.15

Solvent B:
H_2O : glycolmethylethylether : ethylmethyl ketone : acetone : HCl (0.1 M)
20 : 20 : 20 : 20 : 10

TLC R_f values

Sample	Solvent B	Solvent A	Methanol:NH_4NO_3:HCl(1M) 14 : 10 : 0.1	Methanol:NH_4NO_3 15 : 14
Cu-Mo complex-EDTA	0.79	[a] 0.80, 0.00	[a] 0.72, 0.00	0.80
Cu(II)-EDTA	0.79	0.80	0.72	0.80
Mo(VI)-EDTA	0.79	0.00	0.00	0.80
Cu-Mo complex-Tiron	0.81	-	-	-
Cu(II)-Tiron	0.81	-	-	-
Mo(VI)-Tiron	0.81	-	-	-
Cu-Mo complex-Citrate	0.75	[b] 0.00, 0.72	-	[b] 0.72, 0.00
Cu(II)-Citrate	0.75	0.72	-	0.72
Mo(VI)-Citrate	0.75	0.00	-	0.00
Cu-Mo complex-Tartrate	0.75	-	0.83	-
Cu(II)-Tartrate	0.75	-	0.83	-
Mo(VI)-Tartrate	0.75	-	0.83	-
Cu-Mo complex-NTA	0.88	-	-	-
Cu(II)-NTA	0.88	-	-	-
Mo(VI)-NTA	0.88	-	-	-

a Have tailing effect and is not useful for qualitative analysis
b Dissociate into two components

Results and Discussion

Despite the variation in the composition of the solvent the sequence of the R_f values show the relative migration rates of the chelated complexes to be almost constant. This is explained as being due to the possibility that the complex and the ions when chelated have about the same configuration which suppresses their specific characteristics and have close R_f values. When acid was present the molybdenum system would not move at all.

From the TLC results the composition and the pH of the solvent should be chosen very carefully. The pH of the solvent should be within very narrow limits because one has to take into account that the R_f value of the copper-molybdenum chelated complex varies with pH. Also, the stability of the chelated complex varies with pH. This could be the reason for the observed tailing effect when acid was present in the developing solvent.

Copper-Molybdenum complex in water

The spotting solution was prepared as following: 3 g of copper-molybdenum complex was washed with 30 ml of deionized-distilled water. This was repeated for 20 times, obtaining about 600 ml of wash solution. The solution was concentrated by evaporation to about 5 ml and 5 µl used for TLC analysis. The different solvent systems with their corresponding R_f values for each sample are given in Table 3.

Table 3.

TLC R_f values of samples dissolved in water

Solvent Systems	TLC R_f values		
	Cu-Mo complex in H_2O	Cu(II) in H_2O	$MoO_4^{=}$ in H_2O
NH_4NO_3 (1M) : HCl (1M) 20 ml : 5 drops	[a] 0,80, 0.60	0.80	0.60
NH_4NO_3 (1M) : Acetone : NH_3 (D=0.91) 100 ml : 100 ml : 3 drops	[a] 0.85, 0.50	0.50	0.85
H_2O : Glycomethylethylether : Ethyl-methylketone : Acetone : NH_3 20 : 20 : 20 : 20 : 0.15	[a] 0.55, 0.00	0.00	0.55

a. Dissociated into two components

Results and Discussion

The TLC values of the complex dissolved in water show that when the solvent systems contain either acid or base the copper-molybdenum complex appears to break up into two species which have the same R_f values as Cu^{++} ion and $MoO_4^{=}$ ion. With solvent systems whose pH range is near neutrality, neither the copper-molybdenum complex, the Cu^{++} nor $MoO_4^{=}$ move. So, it is hard to say from TLC data alone whether the copper-molybdenum complex in water will break up at the pH of the body.

The copper-molybdenum complex chelated by diethyldithiocarbamate

From Mitsno (17) diethylthiocarbamate will chelate Cu and Mo. These chelated complexes can be extracted from a buffer solution of pH 4.0 into chloroform. The spotting solution was prepared as follows:

To 5 ml of the sample solution in water add 10 ml of buffer pH 4.0, 5 ml 1% diethyldithiocarbamate and 5 ml of chloroform. Mix throughly for five minutes. Apply several microliters of the chloroform layer to the TLC plate.

The pH value of the buffer solution must be below 4.5 otherwise the molybdenum will not extract into chloroform. The R_f values corresponding to different solvent systems are shown in Table 4.

Table 4.

TLC R_f values of samples chelated by diethyldithiocarbamate solution

TLC R_f values

Sample	Benzene:$CHCl_3$ 1 : 1	Benzene	$CHCl_3$
Cu-Mo complex-DTTC	[a]0.56, 0.45	[a]0.85, 0.30	[a]0.75, 0.70
Mo(VI)-DTTC	0.45	0.30	0.70
Cu(II)-DTTC	0.56	0.85	0.75

DTTC = 1% Na-diethyldithiocarbamate solution in H_2O
a = Dissociate into two components

Results and Discussion

The R_f values on TLC of the copper-molybdenum complex indicated that when diethyldithiocarbamate is present the copper-molybdenum complex will break up into two components having the same R_f values as copper ion and molybdate ion. The same thing occurred again when some other chelate agents such as EDTA, citrate, tartrate and ethylenediamine are present. The copper-molybdenum complex will break up into two components having the same R_f values as copper ion and molybdate ion. This is evidence to show that the bonds between the copper part and the molybdenum part in the copper-molybdenum complex are not very strong.

The overall TLC results show that the copper-molybdenum complex is not stable when some chelate agents are present. Also, it showed that the stability of the complex varies with pH. In acid or basic developing solvent the copper-molybdenum complex will break up into two components.

Attempts to find a suitable developing solvent with pH close to neutrality failed.

Polarographic determination of the dissociation constant of copper-molybdenum complex

Polarography is a very prominent tool in the investigation of complex metal ions. The purpose of this experiment is to determine the dissociation constant of the copper-molybdenum complex.

Apparatus

Sargent recording polarography Model XXI (E.H. Sargent & Co.)
Digitec digital voltmeter (United systems Corp.)
Polarography cell with nitrogen deaerating system.

Solution

$CuSO_4$	0.025 M
Na_2MoO_4	0.025 M
KCl	1.0 M
Triton X-100	

Theory

The relationship between the dissociation constant and the number of ligands on a complex is shown by equation (A):

$$E_{1/2c} - E_{1/2s} = \frac{0.059}{n} \log K - P\frac{0.059}{n} \log C_x \qquad (A)$$

Where $E_{1/2C}$ = half wave potential of the complex ion

$E_{1/2S}$ = half wave potential of the sample metal ion

K = dissociation constant of the complex

P = number of ions involved in the complex

C_x = molar concentration of the complex ion

In practice one obtains the $E_{1/2}$ for the simple metal ion, the half-wave potentials for a series of solutions containing a given concentration of metal ion at various concentrations of the complexing agents, and plots the $E_{1/2}$ against $\log C_x$. A straight line should be obtained whose slope equals -0.059P/n, allowing calculation of P. Once P is determined, one can apply equation (A) and evaluate the dissociation constant K for the complex.

Experimental

From Meites (20) and Meloan, et al (21) the procedure is as follows: Pipet 2.0 ml of 0.025 M $CuSO_4$ solution into each of three 100 ml volumetric flasks, then add,

Flask	Substance
1	20 ml 1.0 M KCl
2	10 ml 0.025 M Na_2MoO_4 and 20 ml 1.0 M KCl
3	20 ml 0.025 M Na_2MoO_4 and 20 ml 1.0 M KCl

Add 4 drops of Triton X-100 to each flask, dilute to the mark and swirl vigorously. Bubble puried nitrogen through each solution in the polarographic cell for 15 minutes, then record the polarogram of the solution from 0.000 volt to -0.700 volt, record the voltage across the polarographic cell manually with a voltmeter to the nearest millivolts during the experiment.

Calculation and Results

Data of polarogram listed on Table 5.

Steps of calculation and results are shown as follows.

Step 1.

Use equation (B) to determine the half-wave potential $E_{1/2}$ and number of electrons involved, n.

$$E_{d.e.} = E_{1/2} - \frac{0.059}{n} \log\left(\frac{i}{i_d - i}\right) \qquad (B)$$

Where

$E_{d.e.}$ = potential at the dropping mercury electrode in volts

$E_{1/2}$ = the half wave potential in volts

n = number of electrons involved with the reducible ion

i = the current at any point on the wave in μA

i_d = limiting diffusion current in μA

Values of i and (i_d - i) have been determined from the polarogram shown in Figure 4-6, those values are then converted to workable data as shown in Tables 6-8.

Table 5.

Data of polarogram

	solution 1	solution 2	solution 3
Cu^{++}	5×10^{-4} M	5×10^{-4} M	5×10^{-4} M
$MoO_4^{=}$	0.0	2.5×10^{-3} M	5×10^{-3} M
current sensitivity (uA/mm)	0.02	0.02	0.02
EMF start (volt)	0.000	0.000	0.000
EMF end (volt)	-0.700	-0.700	-0.700
damping setting	1	1	1
DC EMF span (volt)	1.5	1.5	1.5

Figure 4.

Polarogram of solution 1

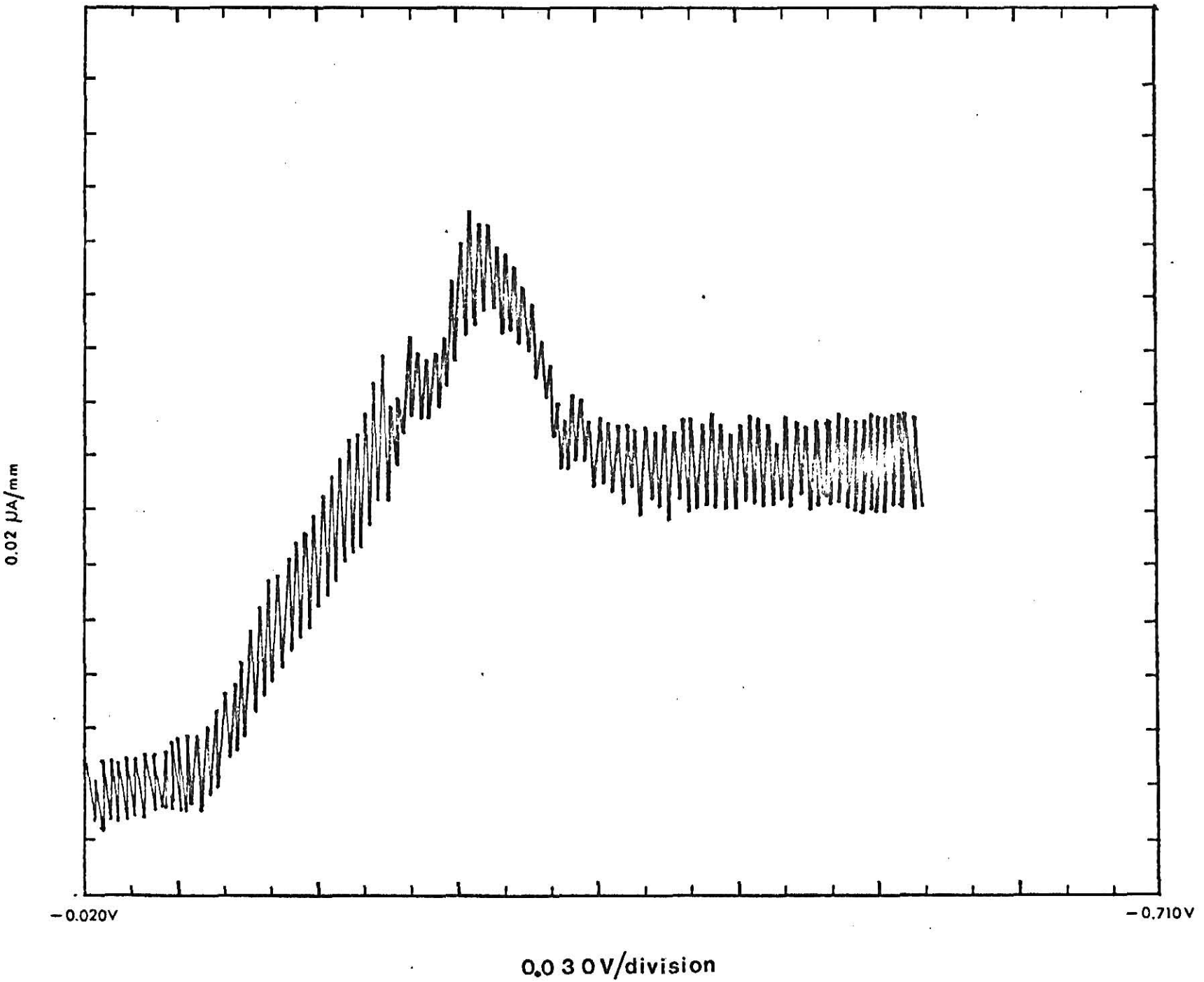

0.02 μA/mm
−0.020V
−0.710V
0.030V/division

Figure 5.

Polarogram of solution 2

0.02μA/mm
−0.004V
−0.700V
0.037V/division

Figure 6.

Polarogram of solution 3

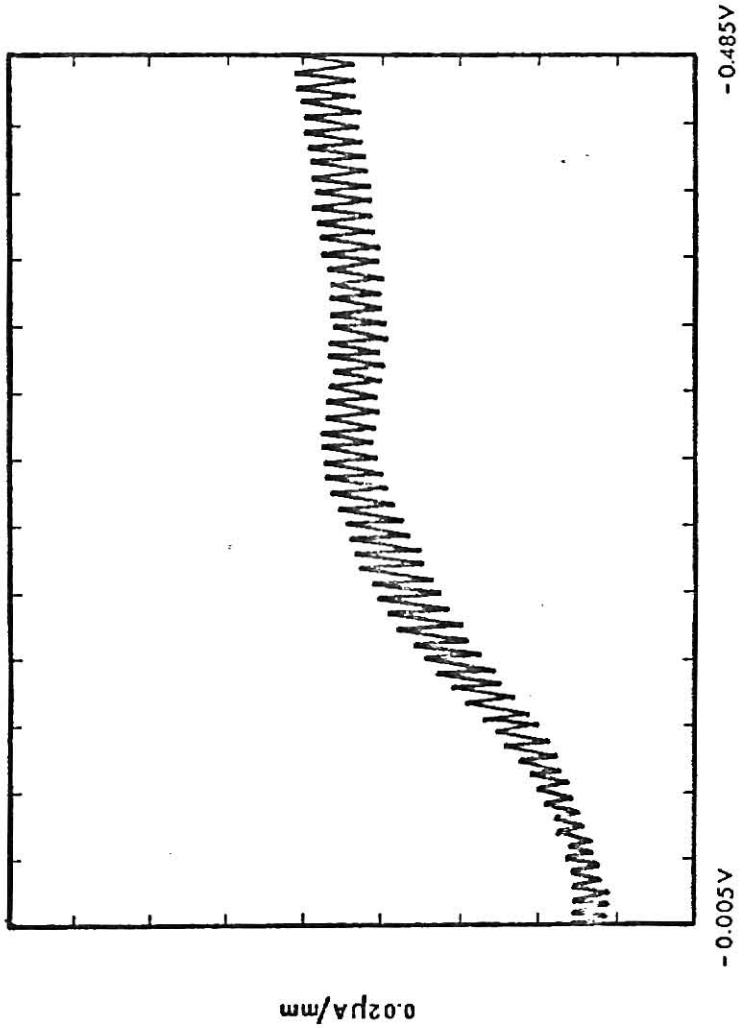

-0.485V
-0.005V
0.037V/division
0.02μA/mm

Table 6.

Workable data of polarogram of solution 1

$E_{d.e.}$	$\frac{i}{i_d - i}$	$\log(\frac{i}{i_d - i})$
-0.109	$\frac{3}{49}$	-1.213
-0.139	$\frac{15}{33}$	-0.342
-0.154	$\frac{21}{26}$	-0.093
-0.169	$\frac{28}{17}$	0.217
-0.198	$\frac{40}{1}$	1.602

Table 7.

Workable data of polarogram of solution 2

Ed.e.	$\frac{i}{i_d - i}$	$\log(\frac{i}{i_d - i})$
-0.127	$\frac{6}{51}$	-0.929
-0.146	$\frac{14}{41}$	-0.4667
-0.164	$\frac{22}{30.5}$	-0.1419
-0.182	$\frac{32}{18}$	0.2499
-0.200	$\frac{42}{5.5}$	0.8829

Table 8.

Workable data of polarogram of solution 3

$E_{d.e.}$	$\frac{i}{i_d - i}$	$\log(\frac{i}{i_d - i})$
-0.115	$\frac{2}{18}$	-0.954
-0.155	$\frac{5}{11.8}$	-0.362
-0.192	$\frac{7}{5.5}$	0.105
-0.228	$\frac{7}{2}$	0.544

A plot of $\log(\frac{i}{i_d - i})$ vs $E_{d.e.}$ is shown in Figure 7-9.

$$\text{The slope} = \frac{\log(\frac{i}{i_d - i})}{E_{d.e.}} = \frac{n}{0.059}$$

If i equals $(i_d - i)$ then E will equal the half wave potential $E_{1/2}$. Values of n and $E_{1/2}$ of solutions 1-3 are shown in Table 9.

Figure 7.

$\log(\frac{i}{i_d - i})$ vs $E_{d.e.}$ of solution 1

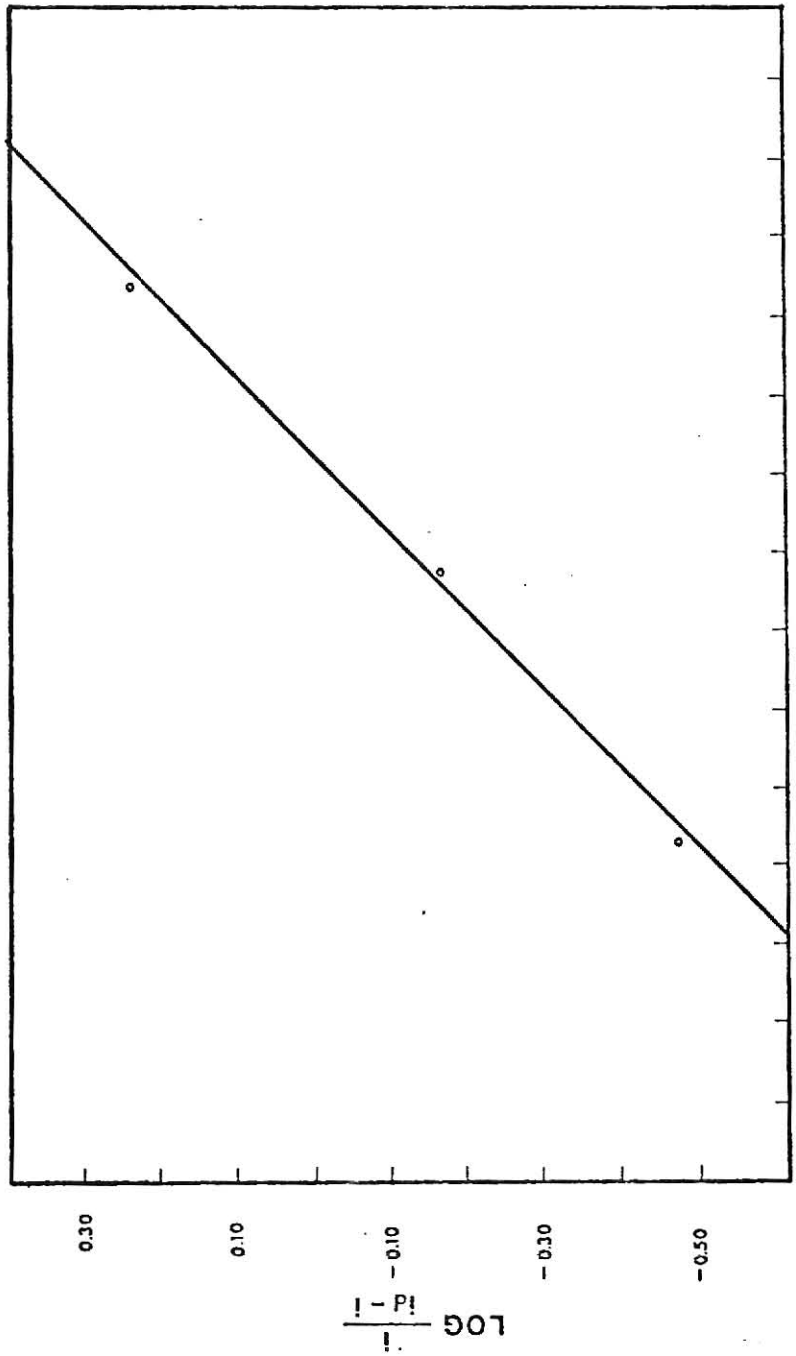

0.30
0.10
-0.10
-0.30
-0.50
LOG $\frac{i}{id - i}$
-0.13
-0.14
-0.15
-0.16
-0.17
-0.18
-0.19
$E_{d.e.}$

Figure 8.

$\text{Log}\left(\frac{i}{i_d - i}\right)$ vs $E_{d.e.}$ of solution 2

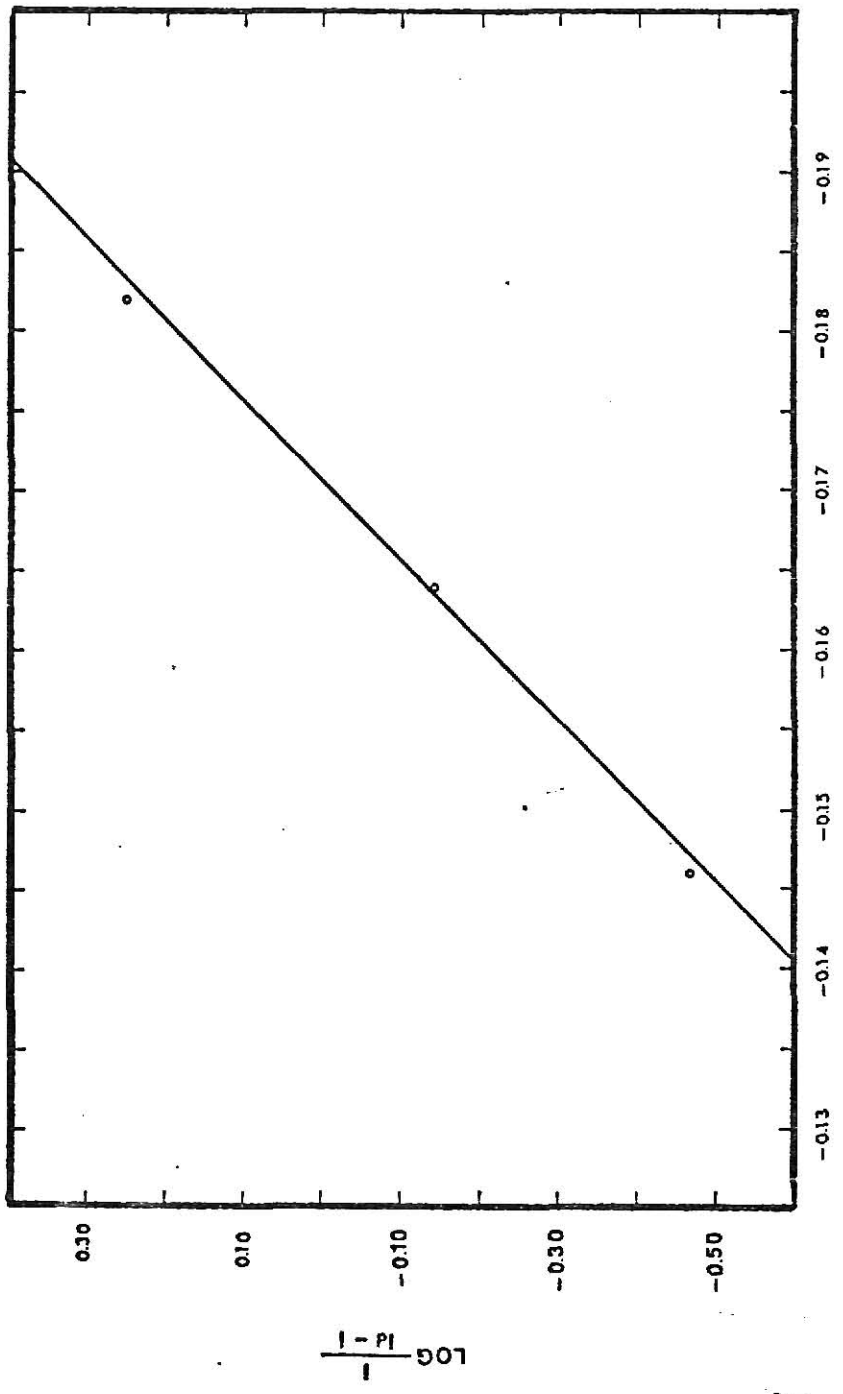

0.30
0.10
-0.10
-0.30
-0.50
-0.13
-0.14
-0.15
-0.16
-0.17
-0.18
-0.19
Ed.e.
$\log \frac{1}{id - i}$

Figure 9.

$\text{Log}\left(\frac{i}{i_d - i}\right)$ vs $E_{d.e.}$ of solution 3

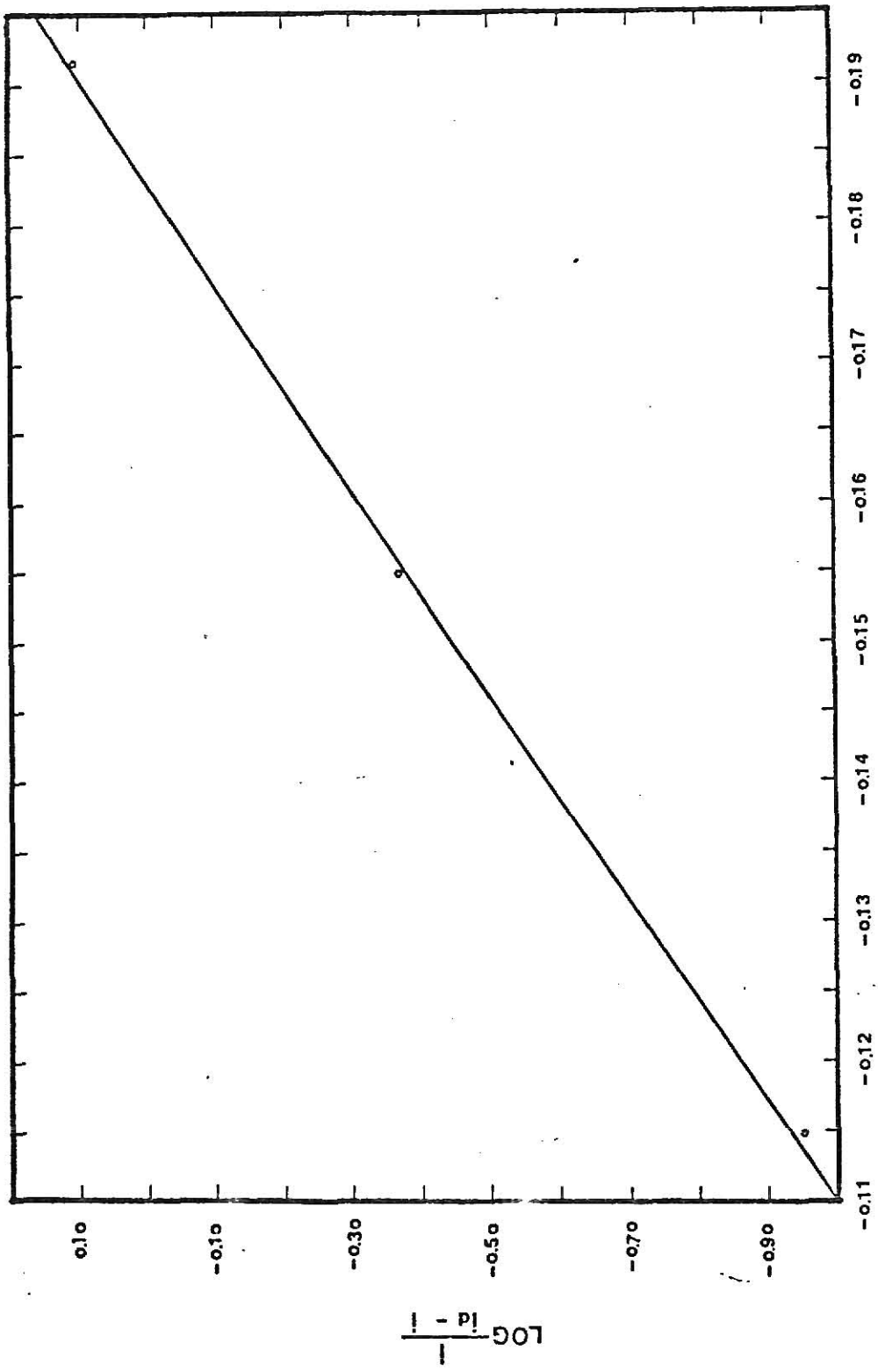

-0.11
-0.12
-0.13
-0.14
-0.15
-0.16
-0.17
-0.18
-0.19
0.10
-0.10
-0.30
-0.50
-0.70
-0.90
$\log \frac{i}{i_d - i}$
E d.e.

Table 9.

Values of n and $E_{1/2}$ of solution 1, 2,3

	n	$E_{1/2}$
solution 1	-	-0.161 volt
solution 2	1.171	-0.171 volt
solution 3	0.800	-0.178 volt

The n values of solution 2 and 3 are reasonably close to 1 since the polarogram was not corrected for iR drop.

Step 2.

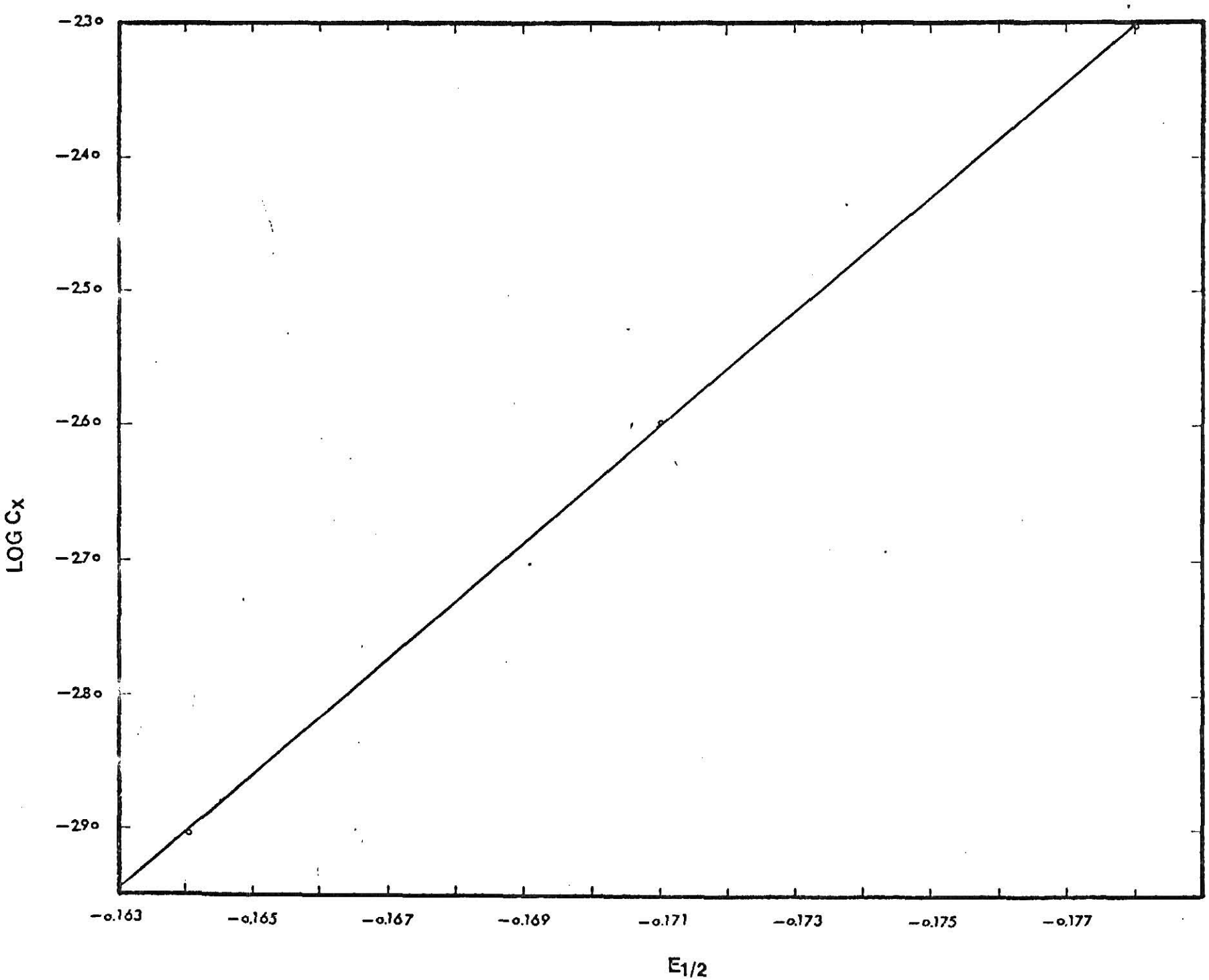
Plot $E_{1/2}$ for the complexes vs logCx to obtain P from the slope, shown in Figure 10.

$$\text{slope} = \frac{\log Cx}{E_{1/2}} = \frac{n}{P(0.059)}$$

$$\frac{0.60}{0.014} = \frac{1}{P(0.059)} \qquad \text{thus,} \quad P = 0.39$$

Figure 10.

Plot $E_{1/2}$ vs logCx of solution 1, 2, 3


−23o
−24o
−25o
−26o
−27o
−28o
−29o
LOG Cx
−o.163
−o.165
−o.167
−o.169
−o.171
−o.173
−o.175
−o.177
E1/2

Step 3.

By knowing n, P, logCx and ($E_{1/2C}$-$E_{1/2S}$) the dissociation constant K can be calculated from equation (A)

$$(-0.178)-(-0.161) = \frac{0.059}{1} \log K - (0.39)\frac{0.059}{1} \log 90.60)$$

and thus $K = 4.22 \times 10^{-1}$

The stability constant corresponding to this value is equal to 2.37 which means the copper-molybdenum complex is not a very stable complex in queous solution by comparing to the data of stability constants of copper and molybdenum with several chelating compounds shown in Table 10. (23,24).

Table 10.

Stability constants of copper and molybdenum with different chelating reagents

Ligand	Metal	Log of equilibrium constants, remarks
EDTA (H_4L)	Cu^{2+}	$K(Cu^{2+} + HL^{3-} \rightleftharpoons CuHL^{-})$ 11.54
	Mo^{6+}	$K(MoO_4^{2-} + HL^{3-} + H^{+} \rightleftharpoons MoO_3L^{4-})$ 8.8±0.3
NTA (H_3L)	Cu^{2+}	$K(CuL^{-} + A^{2-} \rightleftharpoons CuLA^{3-})$ 5.32
	Mo^{6+}	$K(MoO_4^{2-} + WO_3L^{3-} \rightleftharpoons MoO_3L^{3-} + WO_4^{2-})$ 0.15
Citrate (H_3L)	Cu^{2+}	$K(2Cu^{2+} + 2L^{3-} \rightleftharpoons Cu_2L_2^{2-})$ 13.2 ± 0.1
	Mo^{6+}	-
Tartrate (H_2L)	Cu^{2+}	$K(Cu^{2-} + 2OH^{-} + 2L^{2-} \rightleftharpoons Cu(OH)_2L_2^{4-})$ 9.85
	Mo^{6+}	$K(H_2MoO_4 + 2L^{2-} \rightleftharpoons MoO_2(H_{-1}L)_2^{4-})$ 7.66
Tiron (H_4L)	Cu^{2+}	$K(Cu^{2+} + HL^{3-} \rightleftharpoons CuHL^{-})$ 5.14
	Mo^{6+}	-
Diethyldithiocarbamate (HL)	Cu^{2+}	$K(Cu(HA)_2 + 2HL \rightleftharpoons CuL_2 + 2H_2A)$ 4.6 ± 0.2
	Mo^{6+}	-

Disc Electrophoresis

The method of disc electrophoresis has yielded excellent resolution in the separation of simple and complex protein mixtures by using polyacrylamide gels as a medium. This is polymerized with ultraviolet light without heating and is compatable with buffer solutions of any pH. This makes it a suitable method to study the copper-molybdenum complex in biological systems.

Strip electrophoresis with Eastman precoated silica gel TLC plates used as the medium was also tried, but we found that one cannot get better resolution than disc electrophoresis. However, both methods preduced the same results. The disc techniques will be the only one discussed in detail.

Apparatus

A Thomas Gel Electrophoresis unit with a Thomas power supply for electrophoresis and 8 mm × 3 inch disc electrophoresis glass tubes.

Color reagents

(1) 1% Na-diethyldithiocarbamate in water:

Filter before use. Make fresh every week, will produce an intense yellow-brown color for copper ion.

(2) 10% (w/v) $SnCl_2$ in 1.0 M HCl

Filter before use, make fresh every week.

(3) 10% (w/v) NH_4SCN in water

Molybdate ion will produce an intense red color in the mixture solution of 10% $SnCl_2$ and 10% NH_4SCN in the ratio of 1:1 (v/v).

Solutions used for disc electrophoresis:

Solution A (store in brown bottle in a refrigerator)

HCl 1.0 N	48 ml
TRIS	tris(hydroxymethyl)amino methane 36.6 g
TEMED	N,N,N,N-tetramethylethyl ethylenediamine 0.23 ml
water	to 100 ml

Solution B (store in a brown bottle in a refrigerator)

HCl 1.0 N	48 ml
TRIS	5.98 g
TEMED	0.46 ml Adjust pH to 6.7 with 1.0 N HCl
water	to 100 ml

Solution C (store in a brown bottle in a refrigerator)

Acrylamide	28 g
BIS	N,N-methylene bis acrylamide 0.735 g
water	to 100 ml

Solution D (store in a brown bottle in a refrigerator)

Acrylamide	10.0 g
BIS	2.5 g
water	to 100 ml

Solution E (store in a brown bottle in a refrigerator)

Riboflavin	4.0 mg
water	to 100 ml

Solution F (store in a brown bottle in a refrigerator)

Sucrose	40 g
water	to 100 ml

Solution I, small pore gel

1 part A

2 part C pH 8.8 to 9.0

1 part water

Solution II, small pore gel

Ammonium persulfate	0.14 g
water	to 100 ml

make fresh every week and store in a refrigerator

Large pore gel solution

1 part B

2 part D pH 6.6 to 6.8

1 part E

4 part F

Buffer solution (low buffer tank)

TRIS	6.0 g
Glycine	28.8 g
water	to 1000 ml

Buffer solution (upper buffer tank)

same as above but with 1 ml of 0.001% bromphenol blue

Experimental

Prepare the small pore gel by adding 1 ml of Solution A, 2 ml of solution C and 1 ml of water to a glass stoppered 10 ml graduated cylinder. This is Solution I. Add 4 ml of the ammonium persulfate solution or the small pore Solution II to the 4 ml of small pore Solution I that is already in the graduated cylinder and throughly mix. Fill each tube to about 3/4 inch from the top with small pore gel. Put a drop of water on top of the gel to get a smooth and flat surface at the interface. Polymerize the gel under an ultraviolet lamp for about 15 minutes. Remove the water from the top of the gel with a paper towel wick.

Prepare the large pore spacer gel by adding 1 ml of Solution B, 2 ml of Solution D, 1 ml of Solution E and 4 ml of Solution F and mix throughly. Using a syringe add about 1/4 to 3/8 inch of large pore gel on top of the small pore gel. Again, add a drop of water on top of the gel and photopolymerize under ultraviolet lamp till set. It took about ten minutes. Add about 40 µl of sample to 100 µl of large pore gel and place this on top of the large pore gel and photopolymerize. Place the tubes in the rubber holders in the bottom of the upper tank. Connect the electrodes, making the bottom tank positive. Fill the tanks with the proper buffer and run until the blue disc of bromphenol blue just gets to the small pore gel, then increase the current from 1 mA/tube to 2 mA/tube and run until the blue marker disc gets at least 30 mm into the small pore gel. Remove the gel by placing ice water on the tubes and reaming them out with the

needle end of a hypodermic syringe. Transfer the gel to a small test tube and add 1% Na-diethyldithiocarbamate solution. After the yellow color of copper appears, decant the solution, wash the gel several times with distelled - deionized water then add a mixing solution of 10% $SnCl_2$ in 1.0 M HCl and 10% NH_4SCN.

Results of Disc Electrophoresis

The results obtained from each sample are shown in Figures 11 to 19. Cu(II) represents copper from copper sulfate, Mo(VI) represents molybdate ion from sodium molybdate, Cu-Mo complex represents the synthetic copper-molybdenum complex.

Figure 11.

The actual size of small pore gel and large pore gel

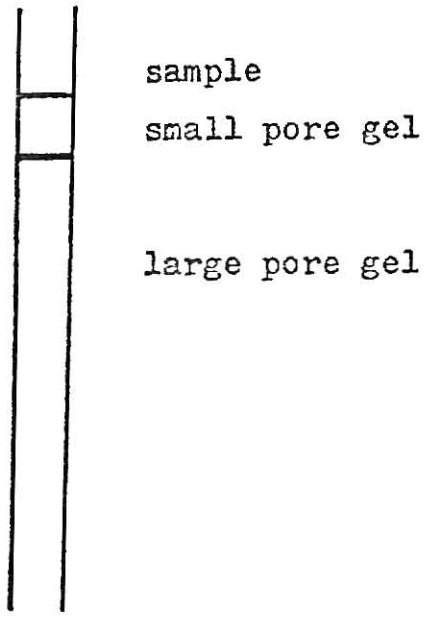

sample
small pore gel
large pore gel

Figure 12.
Samples dissolved in EDTA 1% (w/v) solution
(a) Cu-Mo complex in EDTA
(b) Cu(II)-EDTA
(c) Mo(VI)-EDTA

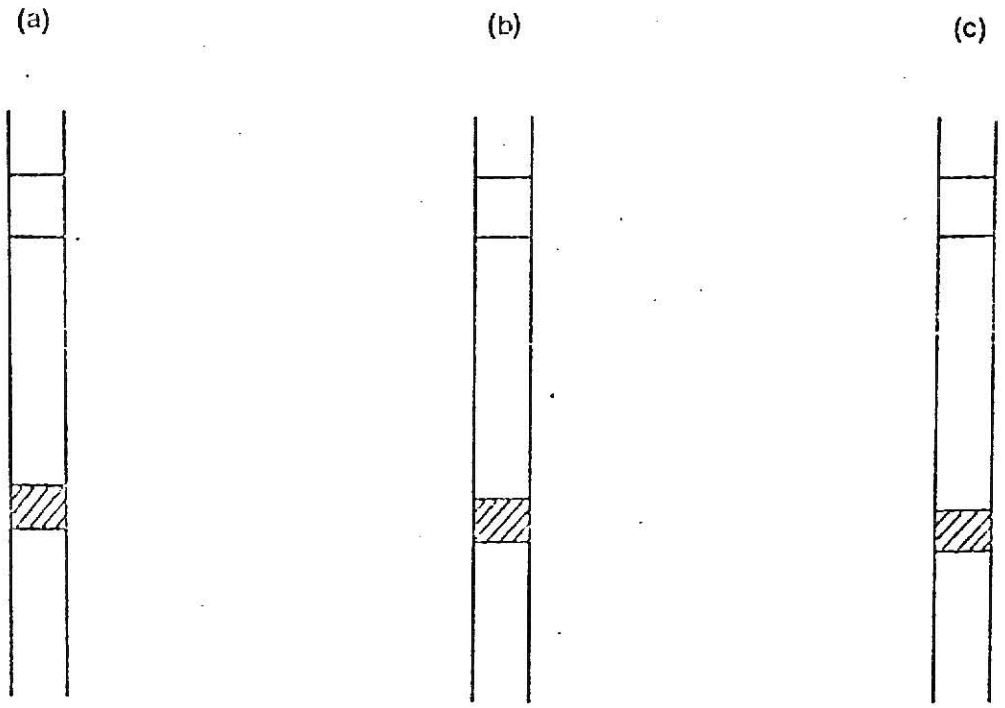

(a)
(b)
(c)

Figure 13.

Samples dissolved in citrate solution

(a) Cu-Mo complex in citrate

(b) Cu(II)-citrate

(c) Mo(VI)-citrate

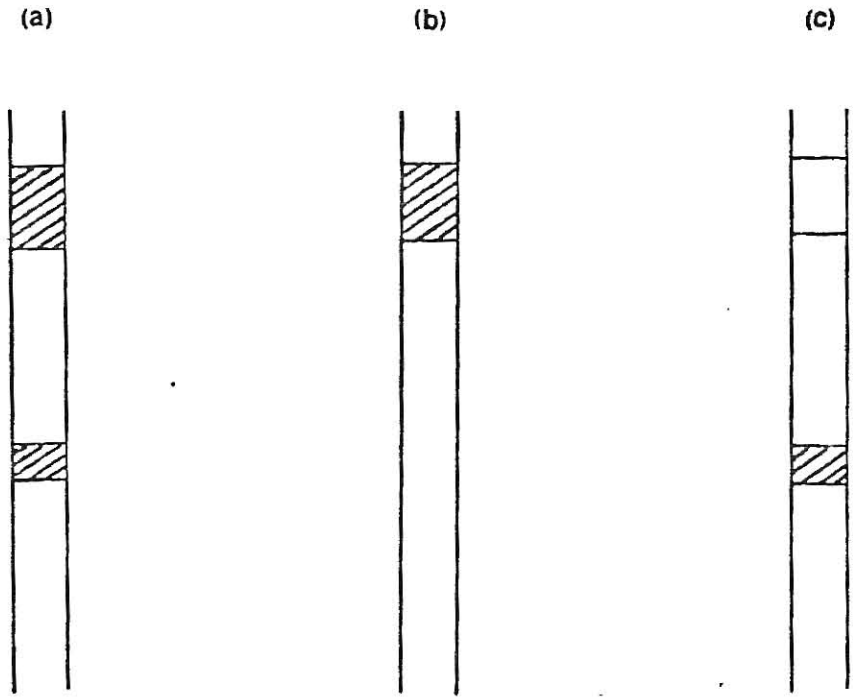

(a)
(b)
(c)

Figure 14.

Samples dissolved in tartrate solution

(a) Cu-Mo complex in tartrate

(b) Cu(II)-tartrate

(c) Mo(VI)-tartrate

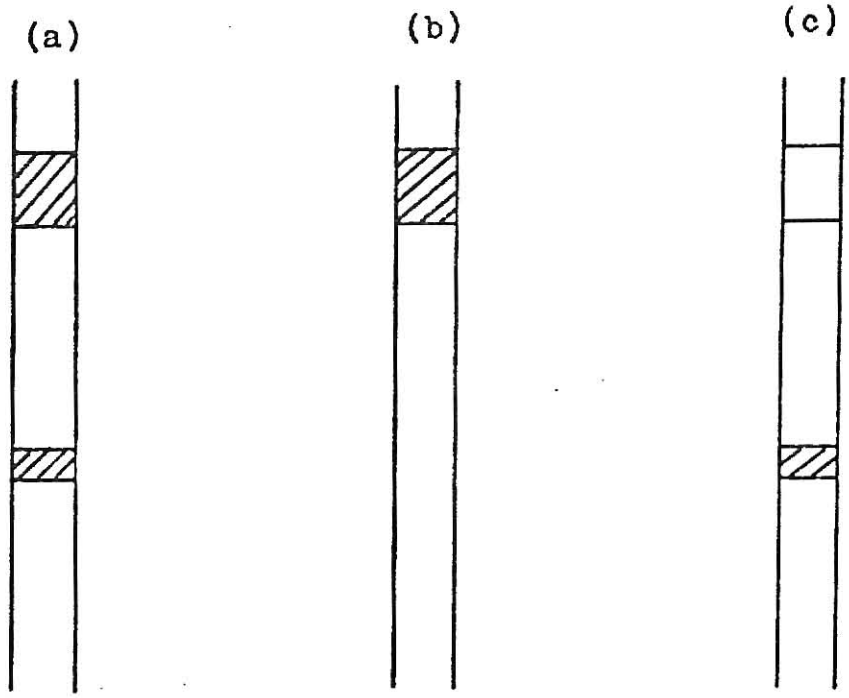

(a)
(b)
(c)

Figure 15.

Samples dissolved in tiron solution

(a) Cu-Mo complex in tiron

(b) Cu(II)-tiron

(c) Mo(VI)-tiron

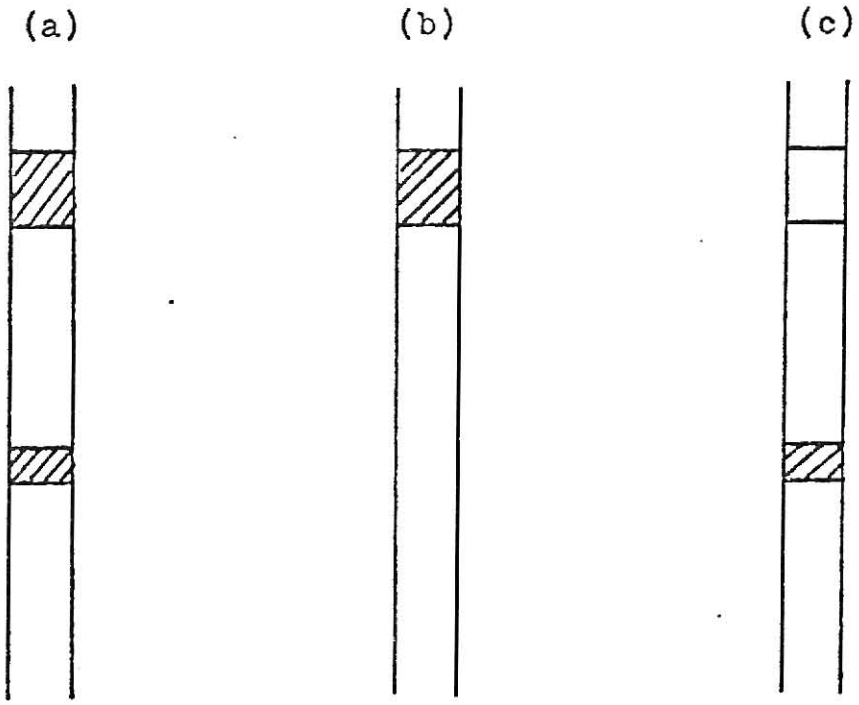

(a)
(b)
(c)

Figure 16.

Samples dissolved in NTA solution

(a) Cu-Mo complex in NTA

(b) Cu(II)-NTA

(c) Mo(VI)-NTA

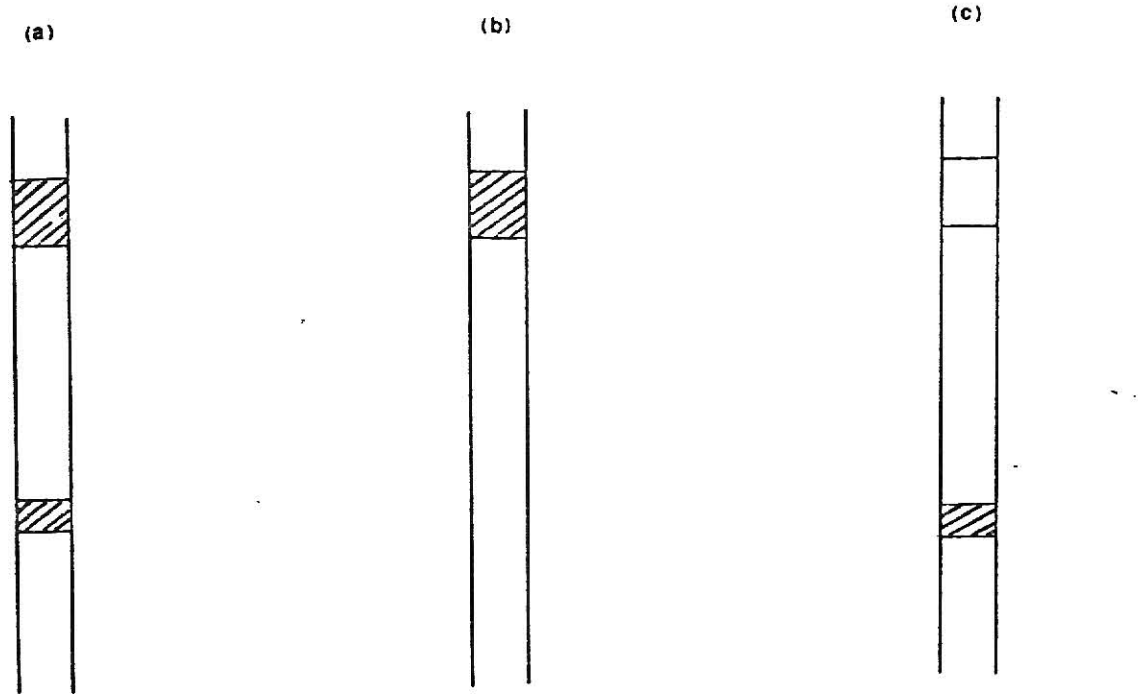

(a)
(b)
(c)

Figure 17.

Samples dissolved in HCl

(a) Cu-Mo complex in HCl

(b) Cu(II)-HCl

(c) Mo(VI)-HCl

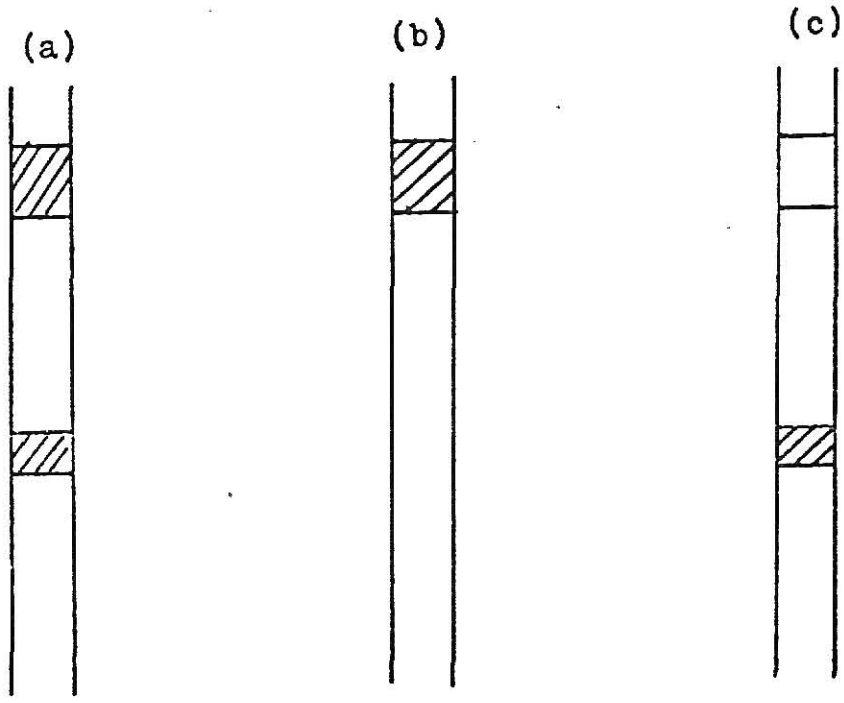

(a)
(b)
(c)

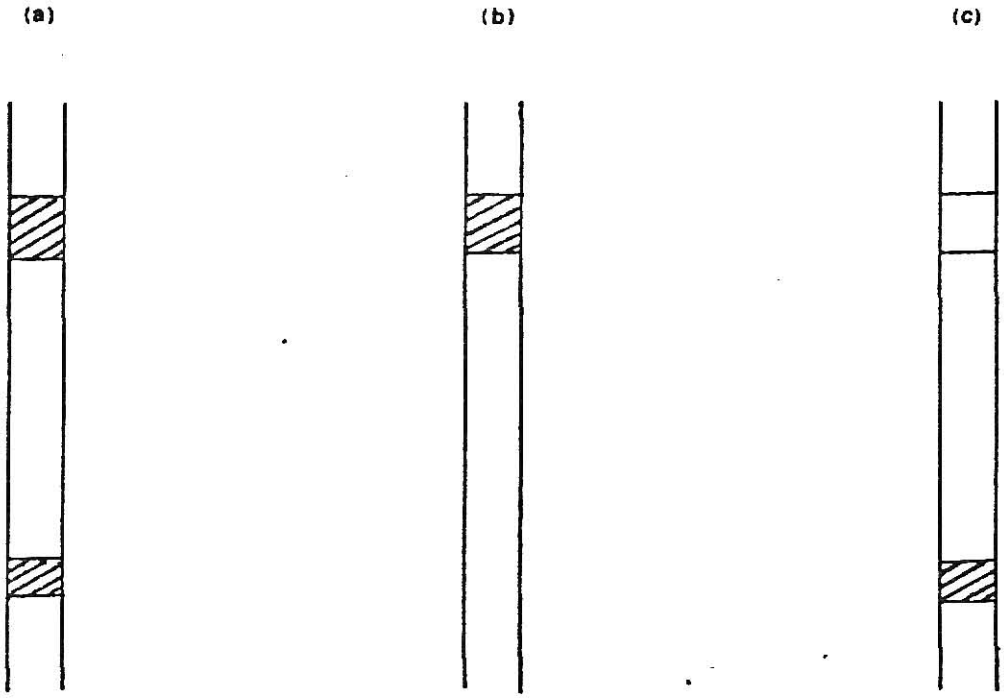
Figure 18.

Samples dissolved in water

(a) Cu-Mo complex in water

(b) Cu(II)-water

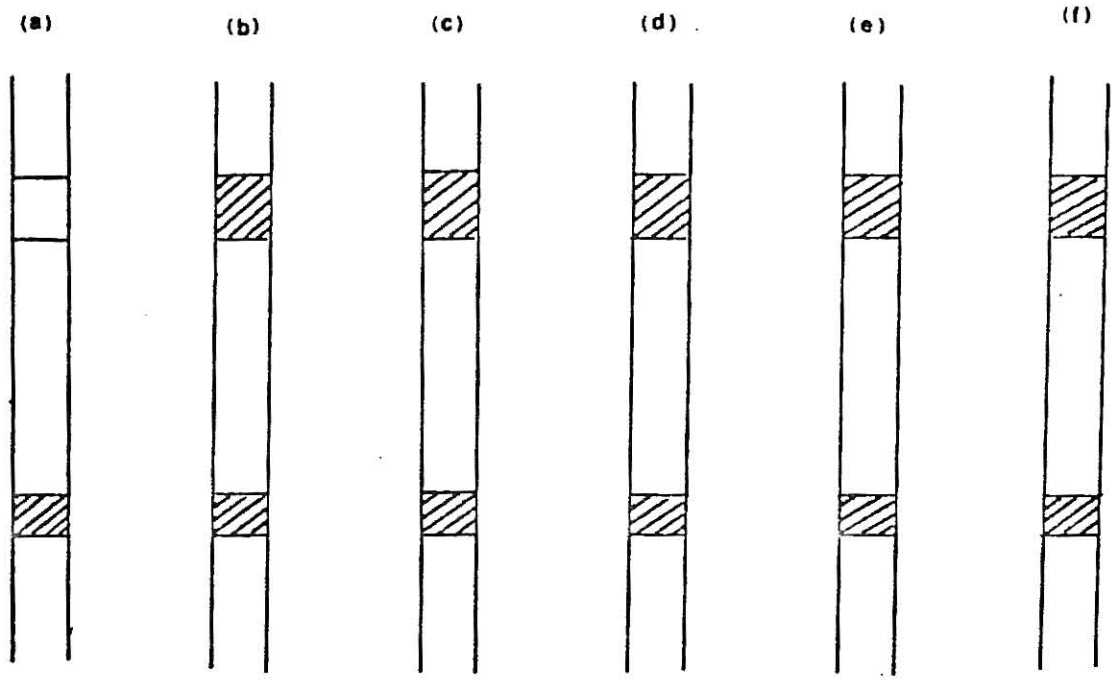
(c) Mo(VI)-water

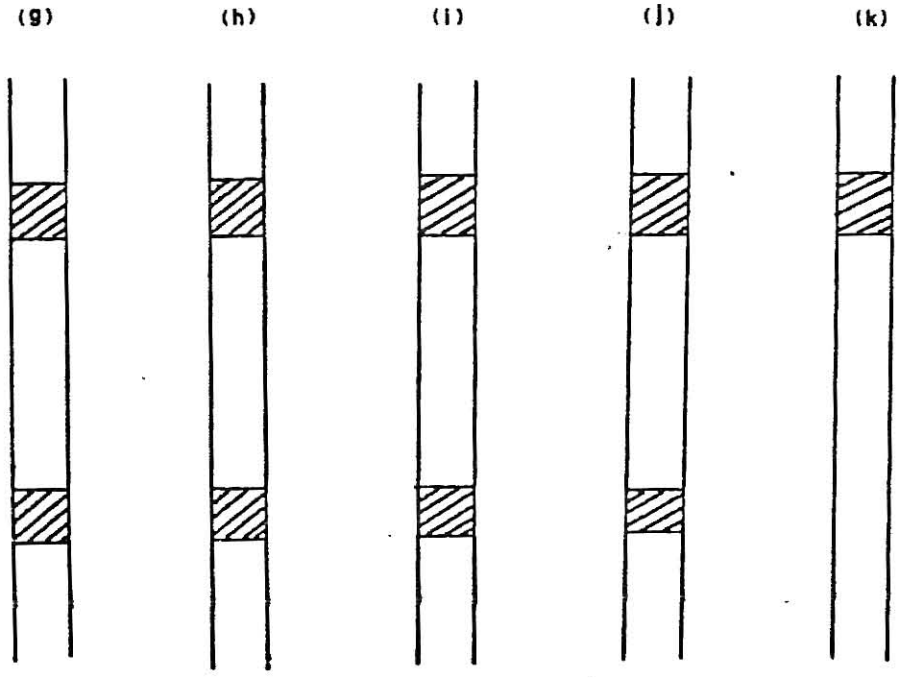

(a)
(b)
(c)

Continuous variation method

Dowdy, et al (10) used the continuous variation method of Job (18) as the measuring criteria to indicate that the molar ratio of Cu to Mo of the complex was approximate 4 to 3. In this method solutions of copper(II) and molybdate(VI) are mixed in varying mole ratios, but in such a way that the total number of moles of copper and molybdate in each mixture remains constant. The supernatant of each mixture is determined with disc electrophoresis. The results in Figure 19. show that the Cu-Mo complex dissociate into two components.

Figure 19.
Disc electrophoresis data of the supernatant of a mixture of copper sulfate and sodium molybdate solution in water according to the continuous variations method.
(a) Mo 10, Cu 0
(b) Mo 9, Cu 1
(c) Mo 8, Cu 2
(d) Mo 7, Cu 3
(e) Mo 6, Cu 4
(f) Mo 5, Cu 5
(g) Mo 4, Cu 6
(h) Mo 3, Cu 7
(i) Mo 2, Cu 8
(j) Mo 1, Cu 9
(k) Mo 0, Cu 10
values for molarity shown in the figures have been derived by multiplying the actual values by $\times 10^2$


(a)
(b)
(c)
(d)
(e)
(f)


(g)
(h)
(i)
(j)
(k)

Results and Discussion of Disc Electrophoresis

Figure 13 shows that the Cu-Mo complex in EDTA moved the same distance as Mo(VI)-EDTA and Cu(II)-EDTA. This is explained as being due to the possibility that the EDTA suppress the Cu-Mo complex specific characteristic. Figures 13-16 show that the copper-molybdenum complex will break into two components behaving like copper ion and molybdate ion with chelate compounds such as citrate, tartrate, tiron and NTA. Figure 17 shows that the copper-molybdenum complex will dissociate in HCl, and Figures 18-19 show that the copper-molybdenum complex dissociates even in water to some extent.

Conclusions

The 4:3 ratio copper-molybdenum complex was synthesized and identified as the same compound which was synthesized by Dowdy, et al (13).

The molecular weight of this copper-molybdenum complex was found to be about 1950 ±100 by using gel filtration on Sephadex G-100.

The antidotal dose of copper in the treatment of molybdenum poison of rats was determined. It was found that 5 mg Cu/rat/day was suitable quantity in a recovery of the lose of weight induced by molybdenum poison.

The copper-molybdenum complex will be destroyed when dissolved in acid such as HCl, sulfuric acid, nitric acid and acetic acid when pH value below 3.

From the results of thin layer chromatography and disc electrophoresis show that while chelating agents such as citrate, tartrate, EDTA, tiron, NTA, and diethyldithiocarbamate would dissolve the copper-molybdenum complex, they also destroyed the original compound. Also, from the evidence of disc electrophoresis the copper-molybdenum will dissociate into two components in water to some extent.

The result of polarographic determination of the dissociation constant of copper-molybdenum complex is 4.22×10^{-1}, which indicated that this complex is not a very stable compound in aqueous solution.

From all these results suggest that the copper-molybdenum complex forms in vivo probably in a different form than the synthetic copper-molybdenum complex or lindgrenite. The hypothesis that lindgrenite may exist in biological system remains in doubt.

However, the answer of this question will have to wait untill we have a suitable and sensitive method to assay the lindgrenite in biological system.

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THE INVESTIGATION OF A COPPER-MOLYBDENUM COMPLEX

BY

Joung-Yei Frank Chen

AN ABSTRACT OF A MASTER'S THESIS

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Department of Chemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

ABSTRACT

The metabolism of copper in animals is dependent on both sulfate and molybdate. The direct antagonistic effect between copper and molybdenum is that the molybdenum can initiate the copper deficiency and the copper can alleviate molybdenum toxicity. A hypothesis was developed to explain that the interaction between copper and molybdenum appears to be due to the formation of copper-molybdenum complex and that copper and molybdenum bound in this complex are biologically inactive.

The proposed copper-molybdenum complex was identified as having the same structure as mineral lindgrenite $2CuMoO_4 \cdot Cu(OH)_2$ The molecular weight of the copper-molybdenum complex was estimated by using gel filtration on Sephadex G-100.

Thin layer chromatography and disc electrophoresis experiments were conducted in an attempt to test the hypothesis that the formation of the copper-molybdenum in vitro may be the cause of the interaction between the two metals.

Water and several chelating reagents were used to dissolve the synthetic copper-molybdenum complex. The results of TLC and disc electrophoresis showed that the synthetic copper-molybdenum complex dissociates into two components in chelating agents and even in water to some extent.

The dissociation constant of the copper-molybdenum complex was determined by using a polarography method. The results show that the complex is not very stable in aqueous solution. All the results indicated that the copper-molybdenum complex formed in vivo probably is in a different form than the proposed synthetic copper-molybdenum complex.