

EFFECTS OF DIETARY MOLYBDENUM UPON RAT GROWTH, LIVER AND BLOOD  
DISTRIBUTIONS OF COPPER AND MOLYBDENUM AND UPON PHENOL TOXICITY

by

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## INTRODUCTION

Early molybdenum toxicity studies were concerned with the practical problem of molybdenum toxicity as encountered in field situations (15) (16). After it was found that copper could overcome molybdenum toxicity (9) (16), the problem became more of an academic one and more attention was directed to the effects of artificially administered Mo upon the animal body. At first these studies were concerned with gross changes such as discoloring of hair coat, diarrhea, and bone abnormalities (12) (1) (16) then the effects of Mo and sulfate upon body concentrations of Mo and Cu were ascertained. Molybdenum was found to retard growth and elevate liver levels of Mo and Cu while concomittant addition of dietary inorganic sulfate restored growth and lowered liver levels of Mo and Cu (22). More recently subcellular distributions of Mo and Cu (4) and especially enzyme systems have been studied (24) (8). Dietary Mo has been reported to increase the activity of liver alkaline phosphatase and to decrease kidney and intestinal alkaline phosphatase (33).

Since Mo toxicity resembles a Cu deficiency which can be overcome by dietary additions of Cu, much work has been directed toward an attempt to relate Mo toxicity to a Cu deficiency. These attempts at the enzyme level have often been unsuccessful (8) (31). However, the

activity of sheep skin tyrosine oxidase may be reduced with Mo feeding (29).

One recent hypothesis explaining the mechanism of Mo toxicity is that Mo inhibits the enzyme sulfide oxidase, thus allowing increased concentrations of sulfide to accumulate in the tissue. The sulfide precipitates the Cu as the insoluble  $Cu_2S$  and renders the animal Cu deficient (19). Subsequent investigation, however, indicated that the reduced enzyme level may have been due to decreased feed intake which resulted from the rat rejecting the Mo containing diet (26). Furthermore, some of the toxic effects of Mo may be a result of impaired feed absorption and/or utilization (20).

An observation which may bear a relationship to Mo caused anemia in ruminants is the report that Mo given orally to lambs caused the production of a large population of short-lived erythrocytes. However these short-lived erythrocytes may have been produced rapidly in response to anemia and were thus functionally inadequate (21).

A report that may influence future experimental procedures and interpretation of past results is the observation that Mo toxicity of rats is enhanced by housing the animals in galvanized cages rather than stainless steel cages (5).

The general topic of Mo toxicity has recently been reviewed (23) (3). Also, a review which focuses attention on the comparison between ruminants and non-ruminants has recently appeared (25).

The following experiments were performed in order to gain an insight into the mechanism of Mo toxicity and its alleviation by inorganic sulfate.

## DISTRIBUTION OF COPPER AND MOLYBDENUM IN LIVER CENTRIFUGAL FRACTIONS\*

The results reported here were obtained from studies to determine the influence of dietary molybdenum and sulfate upon distribution of copper and molybdenum in particular fractions isolated from the livers of rats and also perhaps to furnish information about the mechanism of molybdenum toxicity.

### EXPERIMENTAL

The rats used in these studies (weanling Sprague-Dawley strain males) were individually housed in stainless steel wire floored cages and were provided food and water ad libitum. The basal diet used had the following percentage composition: sucrose 81.5, vitamin-free casein (N. B. Co.) 10.0, Wesson oil 5.0, mineral mixture 2.5, vitamin mixture 0.8, and L-cystine 0.2. Vitamins were added as follows (milligrams per kilogram of ration): choline chloride, 1,000; inositol, 100; calcium pantothenate, 20; niacin, 10; menadione, 10; thiamine HCl, 5; riboflavin, 3; pyroxidine HCl, 3; folic acid, 0.2; biotin, 0.1; and vitamin B<sub>12</sub>, 0.01. Vitamins A, D and E were supplied by two drops/rat/week of 50% percomorph liver oil in cottonseed oil (containing 0.5 gm of  $\alpha$ -tocopherol acetate/10 ml). The composition of the salts used in grams

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per kilogram of ration follows:  $\text{Ca}_3(\text{PO}_4)_2$ , 12.5; NaCl, 6.5; KCl, 5.5; MgO, 0.7;  $\text{Fe}_2\text{O}_3$ , 0.15;  $\text{MnO}_2$ , 0.10; KI, 0.03;  $\text{ZnCO}_3$ , 0.02. All salts were C. P. or A. R. Copper at the level of 1.6 ppm was added as the sulfate to bring total copper, as analyzed, to approximately 2.5 ppm. Four levels of molybdenum and 3 levels of sulfate were added to the diets of the 6 treatment groups as indicated in Table I. After feeding the experimental diets for 6 weeks, the rats were sacrificed by decapitation and the livers removed. Approximately half of each liver was dried, then analyzed for copper and molybdenum. The other portion of the fresh liver was immediately placed on ice and homogenized with 9 volumes of cold 0.25 M sucrose for 2.5 minutes. The homogenate was centrifuged at 700 X g for 10 minutes, the supernatant removed, the nuclei and debris precipitate resuspended in approximately 5 ml of ice cold 0.25 M sucrose and recentrifuged at 700 X g for 10 minutes. The 2 supernatants were combined and centrifuged at 5,000 X g for 10 minutes. The supernatants were combined designated as the supernatant fraction in the case where no further fractionation was done. In a second study a microsomal fraction was prepared by centrifuging the supernatant, from which the mitochondria had been isolated, at 75,000 X g for one hour. The microsomal pellet was washed but not resuspended. A standard wet ashing method, using nitric and perchloric acids, was

used to digest the samples. Copper was determined by the carbamate method of the A.O.A.C. (2) and molybdenum by the thiocyanate method of Evans et al. (14).

## RESULTS

The feeding of up to 400 ppm of molybdenum severely limited rat growth and increased the liver levels of copper and molybdenum (Table I). Additions of inorganic sulfate improved the growth of molybdenum-fed rats and reversed the tendency toward increased concentration of liver copper and molybdenum. Distributions of copper and molybdenum in the centrifugal fractions are also given in Table I. An average of 66% of the total copper was present in the supernatant of the livers of the rats fed the control diet (-Mo) while in the molybdenum-fed rats, supernatant copper was approximately 45% of the total. This difference was statistically significant. The percentage of the total copper in the nuclei and debris and mitochondria fractions prepared from the livers of rats fed molybdenum was increased over that found in the same fractions prepared from livers of rats fed the control diet. When molybdenum was fed, it was found predominately in the supernatant. There was no significant effect of increasing dietary molybdenum above the lowest level added upon distribution of molybdenum or copper. It appeared that dietary sulfate had no significant effect on distribution of copper and molybdenum

TABLE I

DISTRIBUTION OF COPPER AND MOLYBDENUM IN THREE LIVER CENTRIFUGAL FRACTIONS (% OF TOTAL,  
FIVE RATS / TREATMENT)

Diet additions								
ppm Mo*	0	200	300	400	500	500	500	
ppm SO <sub>4</sub> **	0	0	0	0	400	750	1500	
Wt gain (g)‡	174 ± 26	97 ± 33	65 ± 23	38 ± 11	38 ± 13	72 ± 19	124 ± 29	
COPPER								
Nuclei and debris	14 ± 3.7	25 ± 4.5	23 ± 7.2	28 ± 7.4	30 ± 4.7	34 ± 8.5	24 ± 6.4	
Mitochondria	20 ± 4.7	28 ± 3.4	27 ± 2.4	28 ± 1.9	29 ± 6.2	28 ± 8.5	27 ± 4.5	
Supernatant	66 ± 5.4	46 ± 4.6	50 ± 2.5	44 ± 8.3	42 ± 9.2	38 ± 3.7	48 ± 8.3	
Total (μg)‡‡	31 ± 3.8	36 ± 6.1	27 ± 3.5	27 ± 5.3	25 ± 8.1	33 ± 2.2	34 ± 9.5	
Conc. (ppm)‡‡	10 ± 2.8	15 ± 4.4	18 ± 3.0	29 ± 11	20 ± 8.0	19 ± 4.0	14 ± 3.6	

\* Mo added to diet as Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O.

\*\* SO<sub>4</sub> added to diet as an equimolar mixture K<sub>2</sub>SO<sub>4</sub> : Na<sub>2</sub>SO<sub>4</sub>.

‡ 6-wk gain in body wt.

‡‡ Calculated from total liver wt and analysis of an aliquot.

‡‡‡ Dry wt basis.

TABLE I (CONT'D)

Diet additions

ppm Mo*	0	200	300	400	500	500	500
ppm SO <sub>4</sub> **	0	0	0	0	400	750	1500
MOLYBDENUM							
Nuclei and debris -	10 ± 2.9	8 ± 3.3	13 ± 4.3	13 ± 3.3	17 ± 3.1	10 ± 3.8	
Mitochondria -	15 ± 1.0	14 ± 4.1	16 ± 1.9	15 ± 3.5	17 ± 4.0	16 ± 5.2	
Supernatant -	76 ± 3.4	78 ± 6.7	72 ± 5.8	72 ± 6.4	66 ± 3.5	74 ± 7.5	
Total (μg)‡	2 ± .4	40 ± 13	45 ± 13	31 ± 8.6	42 ± 17	36 ± 10	43 ± 11
Conc. (ppm)‡‡	.7 ± .2	20 ± 5.5	33 ± 10	35 ± 14	35 ± 13	21 ± 3.2	18 ± 6.6

\* Mo added to diet as Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O.

\*\* SO<sub>4</sub> added to diet as an equimolar mixture K<sub>2</sub>SO<sub>4</sub> : Na<sub>2</sub>SO<sub>4</sub>.

‡ Calculated from total liver wt and analysis of an aliquot.

‡‡ Dry wt basis.

in the particulate fractions of liver.

To determine the amount of copper and molybdenum associated with the microsomes as a result of molybdenum feeding a second experiment was initiated. Six rats were fed the basal diet and 6 were fed basal plus 400 ppm molybdenum for 6 weeks. From the livers of rats in this trial, a microsomal fraction was prepared in addition to the other particulate fractions. The distribution results were qualitatively similar to those reported in Table I. In addition, the copper associated with the microsomal fraction represented about 10% of the total and did not appear to be altered when molybdenum was included in the diet. Likewise, the amount of molybdenum in the microsomal fraction was approximately 10% of the total.

#### DISCUSSION

The largest amount of copper and molybdenum on an absolute and molar basis was not found in the same liver fractions, thus it appears unlikely that molybdenum causes copper concentration to rise by simply forming a copper-molybdenum complex. The increase in copper concentration in the particulate fractions may constitute a compensatory mechanism, namely, an attempt to overcome a copper deficiency induced by a molybdenum-block of a copper dependent metabolic reaction. Perhaps the failure to demonstrate a

copper deficiency at the enzyme level is due to this extreme compensation.

The copper appeared to be associated with the particulate fractions of liver containing a high concentration of protein. The converse appears to be true for molybdenum. This suggests that the copper may be bound to protein as it is in blood.

#### SUMMARY

In the liver of control rats (-Mo) the copper appeared to be concentrated (> 55%) in the supernatant fraction (nuclei and debris < 15%; mitochondria 20%; microsomes 10%), while in the liver from molybdenum-fed rats more copper was found in the nuclei and debris (27%) and mitochondria (27%) fractions and less in the supernatant (< 37%). The percentage of total copper in the microsomes appeared to be unaltered by dietary molybdenum. The molybdenum was usually found to be concentrated (> 60%) in the supernatant (nuclei and debris < 15%; mitochondria 15%; microsomes 10%). The feeding of sulfate had little or no effect on distribution of copper or molybdenum.

## THE CORRELATION OF MOLYBDENUM TOXICITY WITH VARIOUS PARAMETERS

It has been known for some time that inorganic sulfate lowers the body stores of Mo and also limits the increase of Cu which usually occurs in Mo toxicity in rats (22). Sulfate may also alleviate the toxic action of Mo by replacing some of the body sulfate which is depleted because of the inhibiting action of Mo upon sulfide oxidase.

The following experiment was designed to test if the only beneficial effect of sulfate was to lower body stores of Mo and Cu.

### EXPERIMENTAL

Approximately one half of each liver sample obtained in the previous experiment (Distribution of Cu and Mo in Liver Centrifugal Fractions) was dried as whole liver and analyzed for Cu and Mo according to the usual procedures.

### RESULTS AND DISCUSSION

The results of this dry weight analysis are given in the following table:

LOT	TREATMENT	WEIGHT GAIN	CONC IN $\mu$ g g. DRY LIVER	
			CU	MO.
I	Control	174	10	0.7
II	C + 200 ppm Mo	97	15	20
III	C + 300 ppm Mo	65	18	33
IV	C + 400 ppm Mo	38	29	35
V	C + 500 ppm Mo + 400 ppm SO <sub>4</sub>	38	20	35
VI	C + 500 ppm Mo + 750 ppm SO <sub>4</sub>	72	19	21
VII	C + 500 ppm Mo + 1500 ppm SO <sub>4</sub>	124	14	18

These results seem to indicate that the beneficial effect of sulfate was to lower the concentration of Mo since when the addition of sulfate to the 500 ppm Mo-fed lot lowered the liver Mo concentration to the same level as the 400 ppm Mo-fed lot (35  $\mu$ g), the weight gain was the same (38 g.). Looking at the individual values, however, the same results were not evident. In fact, statistical analysis showed that weight gain (or lack of weight gain) was not even very well correlated with Mo concentration in the liver. In order to find a parameter to which weight gain was well correlated, the values were divided into two groups, set I: -SO<sub>4</sub> (Lots II, III, IV) and set II: +SO<sub>4</sub> (Lots V, VI, VII); and extensive statistical analyses were conducted. The results of some of the correlation analyses are given in Table II. Since the single parameters which best correlated with weight gain were Mo Conc/body wt

TABLE II

WEIGHT GAIN AS A MEASURE OF MOLYBDENUM TOXICITY AND ITS CORRELATION WITH VARIOUS  
PARAMETERS

Parameter	Without added dietary inorganic sulfate			Added dietary sulfate		
	Correlation Coefficient (r)	t*	Level of Significance**	Correlation Coefficient (r)	t	Level of Significance
Mo conc	0.387	1.51	-	0.518	2.18	0.05
Cu conc	0.548	2.36	0.05	0.368	1.43	-
<u>Mo conc</u> <u>body wt</u>	0.638	2.99	0.05	0.718	3.73	0.01
<u>Cu conc</u> <u>body wt</u>	0.683	3.37	0.01	0.720	3.74	0.01
(log wt gain) vs	0.640	t <sub>Mo</sub> 1.05	-	0.469	t <sub>Mo</sub> 1.60	-
Mo conc+Cu conc		t <sub>Cu</sub> 2.40	0.05		t <sub>Cu</sub> 1.11	-
Total Mo +	0.670	t <sub>Mo</sub> 1.05	-	0.655	t <sub>Mo</sub> 0.15	-
Total Cu		t <sub>Cu</sub> 1.87	-		t <sub>Cu</sub> 2.88	0.01

\* t="student's " t

\*\* (-=non-significance)

TABLE II (CONT'D)

Parameter	Without added dietary inorganic sulfate			Added dietary sulfate		
	Correlation Coefficient (r)	t*	Level of Significance**	Correlation Coefficient (r)	t	Level of Significance
Total $\frac{\text{Mo}}{\text{body wt}}$	0.624	$t_{\text{Mo}}$ 0.21	-	0.591	$t_{\text{Mo}}$ 1.48	-
+ Total $\frac{\text{Cu}}{\text{body wt}}$		$t_{\text{Cu}}$ 2.47	0.05		$t_{\text{Cu}}$ 1.00	-
(log wt gain) vs $\frac{\text{Mo conc}}{\text{body wt}}$	0.755	$t_{\text{Mo}}$ 0.83	-	0.728	$t_{\text{Mo}}$ 1.99	0.05
+ $\frac{\text{Cu conc}}{\text{body wt}}$		$t_{\text{Cu}}$ 2.10	0.05		$t_{\text{Cu}}$ 0.85	-
(log wt gain) vs Total Mo	0.714	$t_{\text{Mo}}$ 1.70	-	0.692	$t_{\text{Mo}}$ 0.14	-
+ Total Cu		$t_{\text{Cu}}$ 1.68	-		$t_{\text{Cu}}$ 3.17	0.01

\* t="student's" t

\*\* - = non-significance

( $r=0.638, 0.718$ )\* and Cu Conc/body wt ( $r=0.683, 0.720$ ), it might be profitable to look at some individual values of Cu conc/body wt and Mo conc/body wt. If the body weights of the two groups are arranged in descending order, it can be seen that often when the weight gains were about equal, the Cu conc/body wt and Mo conc/body wt were also about equal.

Wt. Gain	<u>Mo conc</u> <u>body wt</u>	<u>Cu conc</u> <u>body wt</u>	Wt. Gain	<u>Mo conc</u> <u>body wt</u>	<u>Cu conc</u> <u>body wt</u>
147	9.4	8.7	171	7.5	6.9
103	24.7	11.6	120	17.0	7.6
102	14.2	7.6	115	13.0	12.0
102	8.6	7.1	107	6.5	8.2
75	27.0	13.0	104	11.0	9.9
69	24.3	19.0	95	12.0	7.0
65	17.0	14.0	68	17.0	21.0
61	43.0	21.0	66	17.0	15.0
53	27.0	17.0	66	22.0	14.0
49	30.0	25.0	53	22.0	22.0
46	32.0	24.0	48	38.0	17.0
45	41.0	34.0	45	26.0	33.0
42	27.0	19.0	44	45.0	27.0
31	80.0	64.0	39	63.0	27.0
22	29.0	29.0	16	33.0	14.0

\*  $r$  = correlation coefficient for  $-SO_4$  and  $+SO_4$  groups, respectively

Notice for example the Mo and Cu associated with the weight gains 65 g. ( $-SO_4$ )-66 g. ( $+SO_4$ ), 53 g., 102 g.-104 g., and 49 g.-48 g. Sometimes the concentrations of the  $-SO_4$  groups were greater, notice for example weight gains 103-104 g., 69-68 g. and 45 g. Rarely are the concentrations of the  $+SO_4$  group greater at comparable weight gain values (42-44 g.). If sulfate had any outstanding beneficial effect other than lowering body Mo and Cu, it would have enabled the sulfate fed rats to have a higher weight gain while still having a high liver Mo or Cu value. This was not observed.

In order to test if there was some particular combination or ratio of Mo and Cu which was especially toxic, the study which yielded the highest r (log wt. gain vs Mo conc/body wt + Cu conc/body wt) was selected for further investigation. The studies which yielded the best prediction were:

$$\text{Set I } \log (\text{wt gain}) = 1.984 + 0.589 \frac{\text{Mo conc}}{\text{body wt}} - 1.793 \frac{\text{Cu conc}}{\text{body wt}}$$

$$\text{Set II } \log (\text{wt gain}) = 2.153 - 0.902 \frac{\text{Mo conc}}{\text{body wt}} - 0.733 \frac{\text{Cu conc}}{\text{body wt}}$$

It is doubtful whether this line of investigation was very helpful since the coefficients are quite different in the two sets.

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\* Much of the above statistical analysis was done by Dr. R. Bargmann, V.P.I. Stat Dept.

## DISTRIBUTION OF COPPER AND MOLYBDENUM IN BLOOD FRACTIONS

This experiment was initiated as a follow up to the experiment where the distribution of Cu and Mo in liver fractions was ascertained.

## EXPERIMENTAL

Approximately equal 10 to 20 ml portions of pooled whole heparinized blood from control and Mo-fed rats were used in this study. In each run, one sample each of control and Mo-fed blood was used, three such runs were made. Two of the Mo-fed samples were from 400 parts per million Mo-fed rats and one was from 300 ppm Mo-fed. Since there appeared to be little difference between the 300 ppm Mo-fed rat blood and the 400 ppm Mo-fed rat blood, the distributions were averaged together in the final analysis. The erythrocytes were separated by strong centrifugation and were not washed. The various plasma protein fractions were separated using the ammonium sulfate precipitation method of Cohn, et al (7). The gamma globulin was precipitated at 34% of saturation, the beta globulin at 40%, the alpha globulin at 50% and the albumin at 63%. The precipitated fractions were separated by centrifugation and analyzed for Cu and Mo according to the usual procedures (p. 8). The percentage distribution is given in Table III.

It can be seen that when Mo was fed, less of the

TABLE III

PERCENTAGE OF TOTAL BLOOD COPPER AND MOLYBDENUM FOUND IN VARIOUS BLOOD FRACTIONS

	CONTROL RATS		MO-FED RATS	
	COPPER		COPPER	MOLYBDENUM
Erythrocytes	27.5	± 2.3*	16.5	± 3.5
Gamma Globulin	9.1	± 1.7	10.9	± 1.7
Beta Globulin	7.3	± 0.8	8.3	± 1.8
Alpha Globulin	15.7	± 2.4	15.4	± 2.3
Albumin	23.7	± 2.3	37.9	± 7.8
Supernatant	16.5	± 2.3	11.0	± 1.0

\* ± Standard Deviation

total blood Cu (16.5% vs 27.5%) was found in the erythrocytes. Because of elevated total blood Cu, the concentration of Cu in the Mo-fed rat erythrocytes was higher, however, ( $1.75 \mu\text{g}$  Cu/ml packed R.B.C. for the Mo-fed rats vs  $0.96 \mu\text{g}$  Cu/ml packed R.B.C. for the controls). As was noticed with liver fractions, the feeding of Mo lowered the relative amount of Cu found in the supernatant fraction of blood. Also, similarly as with liver, most of the blood Mo was found in the supernatant fraction (36.9%). Total blood Mo was about  $13 \mu\text{g}/\text{ml}$  whole blood.

Interpretation of these results is difficult since the feeding of Mo to rats appears to have little consistent effect upon the blood picture.

The observation that much of the Cu is associated with the albumin was verified using 0.01 ml plasma and subjecting it to paper electrophoresis. Spraying the paper with cuprizone and ammonia showed faint bands of Cu associated with the albumin and also with the fibrinogen-gamma globulin area (origin). Perhaps a better means to determine the Cu and Mo distribution would be to inject the animals with the radioactive isotopes, subject the blood to electrophoresis and place the paper strips on photographic film or in a strip counter.

EFFECTS OF MOLYBDENUM FEEDING UPON SULFATE EXCRETION AND  
PHENOL TOXICITY

If the feeding of molybdenum lowers the activity of sulfide oxidase thus inhibiting the formation of sulfate, then the molybdenum toxic rats fed a low sulfate diet should excrete less sulfate normally and should have less sulfate available for detoxification purposes when given a toxic amount of a substance such as phenol.

The following experiment was designed to test whether the molybdenum fed rats excrete less sulfate or handle a toxic amount of phenol differently from the control rats.

EXPERIMENTAL

Weanling Sprague-Dawley strain male rats housed individually in stainless steel cages were used in this experiment. The basal diet was essentially the same as described elsewhere (p.6) and contained in per cent: sucrose 81.5, vitamin-free casein 10.0, Wesson oil 5.0, mineral mixture 2.5, and vitamin mixture 1.0. Copper at the level of 1.6 ppm was added as the sulfate to bring total copper, as analyzed, to approximately 2.5 ppm. Molybdenum at the level of 200 parts per million and 400 ppm was added as an aqueous solution of the molybdate to lots II and III. Lot I was the ad libitum control lot. The amount of feed consumed by the 400 ppm

Mo-fed lot was measured daily and the average of this lot was fed on the succeeding day to the rats in the pair-fed lot (lot IV). Originally each lot contained seven rats, however, in order to obtain the eight or nine values as given in Tables IV and VII, one or two rats were used again approximately two weeks after their first phenol administration. On the night before the phenol administration, feed was removed from all rats. One hour before phenol administration, 2.5 grams feed per 100 g. rat body weight was offered in the hope that all the rats would eat all the feed, however, only the pair-fed rats did this. Phenol was administered by polyethylene stomach tube. On any one phenol administration day, three rats of each lot were given phenol and in all subsequent analyses these 12 samples were treated as much alike as possible. The first four rats (one from each lot) were given 50 mg (526 micromoles) of phenol in one ml water per 100 g. body weight. Very soon both Mo-fed rats died. Thereafter, 40 mg (420  $\mu$ M) of phenol were administered. One 24 hour urine sample was collected immediately before and one immediately after phenol administration. The collected urine was stored at 0°-4° C until analyses were completed. Glucuronic acid was determined first since it would likely be effected most by storage. According to Schmidt (30) free and conjugated phenol values are not affected by

long periods (30 days) of cold storage.

Total and conjugated urinary glucuronic acid was determined by the method of Fishman and Green (17) using naphthoresorsinol. An important modification was initiated in the determination of total glucuronic acid, namely, the iodine solution and bisulfite solutions were mixed before addition to the reaction mixture. Free and total sulfate (before and after hydrolysis with 0.01 N HCl for one hour, respectively) was determined by the gelatin stabilized BaSO<sub>4</sub> turbidimetric method of Dodgson (13). Urine color and cloudiness were corrected for by subtracting a urine-NaCl-gelatin blank. Free urinary phenol was separated by distillation from pH 8 solution using tris-phosphate buffer. Conjugated phenol was hydrolyzed and distilled from pH 3 solution as outlined by Volterra (32). The distilled phenol was redistilled and determined by the method of Folin and Ciocalteau (18).

#### RESULTS AND DISCUSSION

As expected, the feeding of Mo limited the five week weight gain. The weight gains of the four lots were as follows:

Lot I ( <u>ad libitum</u> controls)	84 g
Lot II (C + 200 ppm Mo)	41 g
Lot III (C + 400 ppm Mo)	30 g
Lot IV (pair-fed controls)	38 g

The urinary values before phenol was administered are given in Table IV. The results when calculated as amount excreted

TABLE IV

## URINARY EXCRETION VALUES BEFORE PHENOL ADMINISTRATION

All values expressed as micromoles

Average nine values per mean

LOT	Total Glucuronic Acid		Conjugated Glucuronic Acid		Total Inorganic Sulfate	
	Per Day	Per Day Per 100 g. body wt.	Per Day	Per Day Per 100 g. body wt.	Per Day	Per Day Per 100 g. body wt.
I	17.2	10.5 $\pm$ 4.7*	13.2	8.0 $\pm$ 4.2	69.1	45.2 $\pm$ 26
II	9.6	9.0 $\pm$ 6.7	7.0	6.5 $\pm$ 5.2	39.1	37.9 $\pm$ 26
III	7.4	8.9 $\pm$ 5.2	5.2	6.2 $\pm$ 4.9	27.0	35.0 $\pm$ 13
IV	5.1	5.1 $\pm$ 2.9	4.0	4.0 $\pm$ 1.9	30.0	30.0 $\pm$ 17

LOT	Free Inorganic Sulfate		Free Phenol		Conjugated Phenol	
	Per Day	Per Day Per 100 g. body wt.	Per Day	Per Day Per 100 g. body wt.	Per Day	Per Day Per 100 g. body wt.
I	49.2	32.5 $\pm$ 15	3.7	2.4	0.9	0.5
II	35.7	33.8 $\pm$ 24	3.1	2.8	1.0	0.9
III	23.0	32.0 $\pm$ 15	1.4	2.0	0.5	0.7
IV	25.0	24.0 $\pm$ 14	2.3	2.8	0.8	0.8

\*  $\pm$  Standard Deviation

per 100 g. body weight per day, with the possible exception of glucuronic acid of lot IV, show little difference from control values. The differences are nullified by the large standard deviation values.

In order to test for the possibility that the variability was due to differences between different runs, values were calculated as percentages of the average control value within the same run. The results of these calculations are given in Table V. It can be seen that this method of calculation gives values which are hardly any different from those obtained from the absolute values, consequently, this percentage method of calculation was abandoned.

The urinary excretion values following phenol administration are given in Table VI. Again there was large variability within treatment lots with little difference between means. An indication that sulfate excretion may be more influenced by diet than by the tissue levels of sulfide oxidase is given by the high value of total sulfate excreted by the rats which consistently consumed all the allotted feed before phenol administration (lot IV). That feed consumption may be important in preventing phenol toxicity is evidenced by the low mortality of this group (Table VI ).

The relatively low amount of administered phenol which was excreted (about 18%) is, however, much more than the trace amount reported by others (10). In this respect the animal of choice for any future experiments might be the rabbit,

TABLE V

## URINARY EXCRETION VALUES BEFORE PHENOL ADMINISTRATION

## PERCENT OF AVERAGE CONTROL

LOT	Total Glucuronic Acid Per Day Per 100 g. wt.	Conjugated Glucuronic Acid Per Day Per 100 g. wt.	Total Inorganic Sulfate Per Day Per 100 g. wt.	Free Inorganic Sulfate Per Day Per 100 g. wt.
I	100 $\pm$ 46*	100 $\pm$ 55	100 $\pm$ 54	100 $\pm$ 48
II	86 $\pm$ 54	77 $\pm$ 52	82 $\pm$ 53	92 $\pm$ 53
III	89 $\pm$ 48	78 $\pm$ 58	77 $\pm$ 37	93 $\pm$ 37
IV	50 $\pm$ 26	49 $\pm$ 18	73 $\pm$ 40	77 $\pm$ 46

\*  $\pm$  Standard Deviation

TABLE VI

## URINARY EXCRETION VALUES AFTER PHENOL ADMINISTRATION

All values except percentages expressed as micromoles

Average eight values per mean

LOT	Total Glucuronic Acid		Conjugated Glucuronic Acid		Total Inorganic Sulfate	
	Per Day	Per Day Per 100 g. body wt.	Per Day	Per Day Per 100 g. body wt.	Per Day	Per Day Per 100 g. body wt.
	I	286	177 $\pm$ 97*	192.2	115 $\pm$ 91	23
II	155	152 $\pm$ 94	95	94 $\pm$ 54	27	29 $\pm$ 30
III	110	126 $\pm$ 113	71	81 $\pm$ 64	29	36 $\pm$ 46
IV	131	131 $\pm$ 93	79	79 $\pm$ 50	44	46 $\pm$ 23

LOT	Free Inorganic Sulfate		Free Phenol		Conjugated Phenol	
	Per Day	Per Day Per 100 g. body wt.	Per Day	Per Day Per 100 g. body wt.	Per Day	Per Day Per 100 g. body wt.
	I	7.5	6.4 $\pm$ 11	120	80 $\pm$ 37	19
II	6.3	7.4 $\pm$ 9.6	67.2	61 $\pm$ 51	8.1	7.9 $\pm$ 7.1
III	10.0	13.0 $\pm$ 8.4	50.5	58 $\pm$ 50	4.1	5.5 $\pm$ 3.5
IV	5.1	5.9 $\pm$ 6.2	69.3	71 $\pm$ 64	10.0	11 $\pm$ 11

\*  $\pm$  Standard Deviation

TABLE VI (CONT'D)

LOT	% Administered Phenol found in urine	% Mortality
I	21 $\pm$ 9.0	16.7
II	16 $\pm$ 12.0	25.0
III	15 $\pm$ 12.0	50.0
IV	19 $\pm$ 16.0	00.0

an animal which has been reported to excrete 77% of a half lethal dose in 24 hours, half of which is free and half conjugated (10). Others have also reported that rabbits excreted most of an ingested dose (27).

The large significant increase in glucuronic acid excretion after phenol administration would be expected as a result of the animals attempt to detoxify the phenol.

Although it is possible that differences actually existed between treatment groups but were obscured by the high variability, the results of this experiment indicate that control and Mo-fed rats handle toxic amounts of phenol in approximately the same manner. The only evidence that Mo-fed rats may be less able to meet the challenge of toxic amounts of phenol was the high mortality of the Mo-fed rats and this may have been due to the generally poor condition of these rats.

## MISCELLANEOUS OBSERVATIONS

## Erythrocyte Fragility

Subjecting red blood cells to progressively greater hypotonicity results in hemolysis. Preliminary studies indicated that erythrocytes of molybdenum-fed rats began hemolysis at a higher NaCl concentration and were also completely hemolyzed at a higher NaCl concentration than erythrocytes from control rats. Twelve hypotonic NaCl solutions were constituted and the hemolysis was ascertained visually (11). The results are given in the following table:

	Control	Mo-fed
Begin lysis	0.45 $\pm$ 0.0245	0.47 $\pm$ 0.015
Finish lysis	0.30 $\pm$ 0.0245	0.36 $\pm$ 0.015

A method used to test for erythrocyte fragility in vitamin E deficiency situations utilizes the susceptibility of erythrocytes to dialuric acid lysis (28). This method resulted in no differences between control and Mo-fed rats.

## Cytochrome-c Oxidase Activity

Earlier investigation resulted in the observation that the activity of cytochrome-c oxidase, a copper dependent enzyme, was inhibited by feeding the animal molybdenum (3). Subsequent experimentation, however,

failed to verify this observation. The results of one of the experiments are given in the following table:

M Cytochrome-c oxidized/min/10 mg fresh liver  
(6 rats per value)

Control	0.040
C + 100 ppm Mo	0.047
C + 400 ppm Mo	0.048

#### Distribution of Mo<sup>99</sup> in Blood and Liver Fractions

A 53 mg sample of molybdenum as molybdic acid in 10 ml water was irradiated with  $10^{11}$  neutrons/sec/cm<sup>2</sup> for one hour. The resulting sample had a specific activity of approximately 100 counts/min/mg Mo. The sample was neutralized with solid sodium carbonate and one ml was injected intraperitoneally into each of four rats. Two of the rats were controls and weighed about 84 g. each, the other two had been fed 400 ppm Mo for four weeks and weighed about 65 g. each. After 16 hours, blood and liver samples were removed. The liver was fractionated as described previously (p. 7) and the blood was separated into erythrocytes and plasma by centrifugation. A portion of the plasma was separated into a TCA precipitate and a TCA supernatant. The activities of all samples were counted in a well type gamma scintillation counter. The liver samples were also analyzed according to the usual colorimetric procedures (p. 8). The results of the distribution of radioactivity and of total Mo are given

in the following tables:

Percent of Mo<sup>99</sup> found in blood fractions

	Control	Mo-fed
R.B.C.	20	26
Plasma	80	74
TCA precipitate	24	26
TCA supernatant	56	49

Percent of Mo<sup>99</sup> found in liver fractions

	Control	Mo-fed
Nuclei debris	9.6	10.0
Mitochondria	8.6	9.8
Microsomes	18.5	9.4
Supernatant	63.3	70.8

Percent of total Mo found in liver fractions

	Control	Mo-fed
Nuclei debris	10.4	18.6
Mitochondria	6.7	16.8
Microsomes	14.3	8.7
Supernatant	68.6	55.9

In the case where the only liver Mo was that injected (control), the distribution of Mo<sup>99</sup> agrees quite well with the distribution of total Mo. Considering the Mo-fed rats it appeared as if the injected Mo<sup>99</sup> did not distribute itself uniformly among the already present

fed Mo but went predominately to the supernatant fraction (70.8% vs 55.9%). It is not surprising that injected Mo does not equilibrate with all stores of Mo, some of which may be tightly bound, in the short time allotted.

## THESIS SUMMARY

The feeding of 200 to 500 parts per million of molybdenum to rats resulted in a greater percentage of the total liver copper being found in the nuclei and debris and mitochondrial fractions at the expense of the supernatant fraction, while the molybdenum was found to be concentrated in the supernatant fraction. Dietary inorganic sulfate while lowering liver levels of copper and molybdenum appeared to have little or no effect upon their distribution. Dietary molybdenum also caused the relative amount of copper in the supernatant fraction and erythrocytes of blood to decrease, while the increase occurred in the albumin fraction. As with liver, the blood fraction which contained the most molybdenum was the supernatant. Statistical analysis indicated that a quantity which was relatively well correlated with molybdenum toxicity as measured by weight gain was a combination of liver molybdenum and copper concentrations divided by body weight. It appeared that the only beneficial effect of sulfate was to lower this quantity. Sulfate excretion or the manner in which rats handled a toxic amount of phenol was found to be unaffected by molybdenum feeding. Earlier reports that dietary molybdenum caused a decrease in cytochrome-c oxidase activity and an increase in erythrocyte fragility could not be substantiated. An injected dose of radioactive molybdenum was found not to be equilibrated with the already present dietary molybdenum in sixteen hours.

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## ABSTRACT

The feeding of 200 to 500 parts per million of molybdenum to rats resulted in a greater percentage of the total liver copper being found in the nuclei and debris and mitochondrial fractions at the expense of the supernatant fraction, while the molybdenum was found to be concentrated in the supernatant fraction. Dietary inorganic sulfate while lowering liver levels of copper and molybdenum appeared to have little or no effect upon their distribution. Dietary molybdenum also caused the relative amount of copper in the supernatant fraction and erythrocytes of blood to decrease, while the increase occurred in the albumin fraction. As with liver, the blood fraction which contained the most molybdenum was the supernatant. Statistical analysis indicated that a quantity which was relatively well correlated with molybdenum toxicity as measured by weight gain was a combination of liver molybdenum and copper concentrations divided by body weight. It appeared that the only beneficial effect of sulfate was to lower this quantity. Sulfate excretion or the manner in which rats handled a toxic amount of phenol was found to be unaffected by molybdenum feeding. Earlier reports that dietary molybdenum caused a decrease in cytochrome-c oxidase activity and an increase in erythrocyte fragility could not be substantiated. An injected dose of radioactive molybdenum was found not to be equilibrated with the already present dietary molybdenum in sixteen hours.