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THE RELATIONSHIP OF MOLYBDENUM TO IRON STATUS IN PREGNANCY AND ANEMIA IN RATS AND HUMANS

by

Jo Ann Mortensen

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

IN

Nutrition and Food Sciences

UTAH STATE UNIVERSITYM
Logan, Utah

1977

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JoAnn Mortensen

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ABSTRACT

The Relationship of Molybdenum to Iron Status in Pregnancy

and Anemia in Rats and Humans

by

Jo Ann Mortensen, Master of Science

Utah State University, 1977

Major Professor: Dr. Deloy G. Hendricks
Department: Nutrition and Food Science

Weanling male rats were made anemic and fed diets supplemented with 20 ppm iron and/or 2 ppm molybdenum. A decrease in serum iron was observed in the rats supplemented only with iron and a significant decrease in hemoglobin was observed in rats given no supplementation.

In a second experiment, pregnant female rats were also fed diets supplemented with 20 ppm iron and/or 2 ppm molybdenum. An inverse relationship was apparent between iron and copper in both the serum and the liver of the female rats. The livers of their pups displayed an inverse relationship between molybdenum and copper. Hemoglobin in both dams and pups tended to decrease when: (1) supplemental molybdenum was absent but supplemental iron was present; (2) supplemental iron was absent but supplemental molybdenum was present; and (3) no supplementation was given at all. While there appeared to be little placental transfer of molybdenum, iron and copper seemed to be transferred from the dams' liver.

In a third experiment, serum was collected from pregnant women in first, second, and third trimester, and at postpartum. Both serum iron and serum molybdenum decreased significantly at postpartum. Inverse relationships were apparent between (1) serum iron and serum copper, and (2) serum molybdenum and serum copper.

(68 pages)

INTRODUCTION

We hear a lot about "iron-poor blood" or iron deficiency anemia, and solutions for it, such as iron tablets in many forms and iron fortification of various foods. There is, however, evidence that the whole solution to "iron-poor blood" is not just iron supplementation, and that the trace mineral molybdenum may play an important part in solving this problem.

Studies undertaken in the late 1940s on anemic pregnant women provided evidence that the molybdenum and ferrous sulfate together enhanced hemoglobin synthesis more so than ferrous sulfate alone. Subsequent studies with rats supported the hypothesis that molybdenum via xanthine oxidase aided in iron mobilization from body stores to be used for hemoglobin synthesis.

This study was undertaken to gather further evidence for the role of molybdenum in iron mobilization. While the literature did support such a role, no one included the direct analysis of molybdenum in experimental procedures. This study was designed to include molybdenum analysis as well as analyses for iron, copper. hemoglobin, and xanthine oxidase.

REVIEW OF LITERATURE

Therapeutic Use of Molybdenized Ferrous Sulfate

One of the first studies (Neary, 1946) to implicate the therapeutic use of molybdenum in iron deficiency anemia came from the Medical Department of White Laboratories, Inc., Newark, New Jersey, when they produced a molybdenized ferrous sulfate tablet that other research laboratories consequently used in similar studies.

Since this product was used in all the related studies which follow, a description of the tablet itself is appropriate at this point. Molybdenized ferrous sulfate was prepared by a special process, involving the co-precipitation of molybdenum sesquioxide (Mo_2O_3) and ferrous sulfate, with the production of a complex in which elemental molybdenum and ferrous iron are present in an approximate ratio of 1 to 15 parts, respectively. It was made available for clinical study in the form of tablets, each of which contained 3 mg of molybdenum sesquioxide (approximately 2.5 mg of elemental molybdenum) and 195 mg of ferrous sulfate (approximately 50 mg of ferrous iron). These tablets were analyzed for copper by spectrographic and colorimetric (dithiazone) methods. The results showed that copper was present to an extent not greater than 5 ppm (Neary, 1946).

Since iron-deficiency anemia is common among pregnant women, it is not surprising that Neary (1946) chose twenty-two anemic pregnant women as subjects for his clinical study. An initial hematological study of

each patient was made during a pre-medication period of at least 7 days. The twenty-two patients were divided into two numerically equal groups: Group 1 received a daily dose of approximately 250 mg ferrous sulfate, while Group 2 received approximately the same daily dose of ferrous iron plus 15 mg molybdenum in the form of molybdenized ferrous sulfate tablets.

Patients treated with molybdenized ferrous sulfate displayed a greater hemoglobin response than did those on ferrous sulfate alone. The rate of hemoglobin formation in Group 2 patients seemed to follow a distinctive pattern in which increments in hemoglobin concentration occurred with uniform rapidity until normal levels were attained. Conversely, patients in Group 1 received the same dosage of iron (but it was in the form of ferrous sulfate) as those in Group 2. They exhibited an initially satisfactory therapeutic response followed by a progressively slower rate of hemoglobin synthesis, until hemoglobin values, still subnormal, were unaffected despite continued treatment with the same dose of ferrous sulfate.

When the curve for therapeutic response in Group 1 had definitely assumed its plateau, molybdenized ferrous sulfate was substituted for ferrous sulfate in Group 1 patients. The rate of renewed hemoglobin formation was uniformly rapid until normal values were virtually reached.

In view of these results, it was suspected that the superior therapeutic effect of molybdenized ferrous sulfate might be due to increased absorption of iron; however, in four apparently normal adults, the researchers were unable to show any significant difference in degree of

iron absorption following oral administration of ferrous sulfate and molybdenized ferrous sulfate.

About the same time, Healy (1946) from Boston published a similar study. Instead of using pregnant anemic women, Healy treated patients with hypochromic anemia. Forty-nine patients were treated with the molybdenum-iron complex (Group I), while twenty-one patients were treated with ferrous sulfate tablets (Group II). The average daily intake of elemental iron in Group I was approximately 230 mg, while in Group II it was approximately 380 mg. The total therapeutic iron intake averaged 3.53 gm for Group I and 7.87 gm for Group II. Group I, with half as much supplemental iron and about half as much time as Group II, increased total hemoglobin twice as much as Group II.

In another study done by Chesley and Annito (1948), two comparable groups of anemic, pregnant women were also given supplements of ferrous sulfate with and without molybdenum. Those with the molybdenized ferrous sulfate showed a significantly better hemoglobin response than did those on ferrous sulfate alone.

Prompted by the results of Neary (1946), Healy (1946), and Chesley and Annito (1948), Dieckmann and Priddle (1949) supplemented 49 pregnant anemic patients in their third trimester with molybdenized ferrous sulfate, having already obtained negative results from therapy with standard iron salts (Talso and Dieckmann, 1948). Untreated anemic patients were used as the control group. In their results Dieckmann and Priddle not only compared the molybdenized treated group and the control (untreated) group, but also the Talso-Dieckmann group that was treated with

standard iron salts (ferrous sulfate--1 gm/day, ferric ammonium citrate--5 gm/day, or ferrous carbonate--5 gm/day). The molybdenum-iron complex was comparatively more effective in causing significant increases in the hemoglobin concentration of most patients with anemia of pregnancy. Dieckmann and Priddle thereafter decided that if any patient's hemoglobin did not show a significant increase with molybdenized iron during a three-week period, further hematological studies were to be done to determine a more complicated cause of the anemia.

Since the molybdenized iron had been so efficacious in treating anemia of pregnancy during the last six weeks of pregnancy and also postpartum, Dieckmann, et al. (1950) decided to test the prophylactic effects of the same complex. Sixty-seven patients comprised the treated group and fifty-six comprised the control group; the treated group was given a prophylactic dose of three tablets of the molybdenum-iron complex, which is half the therapeutic dose beginning about the middle of pregnancy and continuing to term. The molybdenized iron, even in the absence of anemia, resulted in a more optimum hemoglobin level than was observed for the control.

The results from all the preceding studies prompted Gullberg and Vahlquist (1950) to perform a series of iron absorption tests in healthy female adults, using "Mol-Iron"--as the molybdenized-iron preparation came to be called. The control preparation was of the same composition except for the absence of molybdenum.

These authors concluded that: (1) the serum iron response was almost exactly the same whether or not molybdenum was present in the

preparation, and the slight difference in the rise after four hours (11γ percent) was well below any statistical significance; and (2) molybdenum did not improve absorption of iron, and if others observed better effects with molybdenum in clinical trials, it must be explained by other mechanisms.

With new pharmaceutical research developments, the 1960s brought on a new form of Mol-Iron--a sustained-release form called Mol-Iron Chronosule (also supplied by White Laboratories). To test the efficiency and gastrointestinal tolerance of this new product, Mouratoff and Batterman (1961) performed iron absorption tests in normal, healthy adult volunteers; one group was given the sustained-release molybdenized ferrous sulfate and the control group was given another currently available product--a sustained release capsule of exsiccated ferrous sulfate called Feosol Spanule. Total dosage of the ferrous sulfate was equivalent to 135 mg of elemental iron, and that of the molybdenized ferrous sulfate was equivalent to 120 mg of elemental iron and 6 mg of molybdenum oxide. (The original Mol-Iron contained approximately 40 mg elemental iron and 5 mg elemental molybdenum.) A significant increase of serum iron above the fasting level was observed with the sustained release molybdenized iron preparation.

The results were quite different from those of Gullberg and Vahlquist (1950), who found no significant differences in non-anemic adults who took either ferrous sulfate or molybdenized ferrous sulfate tablets. Besides producing sustained elevations of the serum iron levels, the sustain-released molybdenized ferrous sulfate was also

very well tolerated by the gastrointestinal tract. Since, primarily, the only difference between these two studies was a sustained-release factor, the difference in results was attributed to the same factor.

On first thought, this result implies that the mechanism behind the improved performance of the molybdenized iron is the more efficient absorption; however, there are still a few questions that need to be answered if this is the case. First, it is well-known that iron absorption is generally dependent upon the body's need for iron--normal, healthy individuals will not absorb _extra_ iron, even if it is available. Serum iron of normal healthy subjects given molybdenized ferrous sulfate in the previous experiment increased much greater after fasting than the control subjects on ferrous sulfate alone. This observation indicates a mechanism other than increased absorption of iron alone, but leaves the possibility that molybdenum is rapidly absorbed and initiates iron mobilization from body stores. This mobilization may or may not stimulate erythropoiesis; if it does, the body's need of extra iron for erythropoiesis may consequently lead to increased absorption of iron as well. Second, there is conflicting evidence that questions the validity of improved performance as a result of the sustained-release factor. Norrby and Sölvell (1971) performed a study involving 20 male blood donors, who were given either regular ferrous sulfate tablets or the sustained-release form; there was a 29 percent increase in iron absorption with the sustained-release preparation. Nielsen, et al. (1973) performed a comparable study (also using 20 male blood donors and same method of analysis), but found no difference between the regular and

the sustained-release ferrous sulfate. Obviously, more studies comparing regular and time-released preparations are necessary to help clear the question.

Regardless of the mechanism behind Mouratoff and Batterman's study, Stevenson (1962), noting significant increases in serum iron of nonanemic subjects with use of Mol-Iron Chronosule, decided to evaluate the therapeutic activity of the same capsule. Seventeen female outpatients with uncomplicated and moderately severe iron deficiency anemia were selected for the study. The dosage prescribed was one capsule (containing approximately 80 mg of elemental iron) twice a day. The sustained-release preparation of molybdenized ferrous sulfate was capable of promptly correcting iron deficiency anemia. This result is expected; since iron-deficient bodies need more iron, they will absorb more.

Posner and Wilson (1963) compared the effectiveness of Mol-Iron Chronosule with a conventional capsule of ferrous sulfate in the treatment of iron deficiency anemia in 205 pregnant patients. Hematocrit, hemoglobin level, and red blood cell count were measured biweekly until term and again at six weeks postpartum. The results indicated that the molybdenized ferrous sulfate gave a greater and a more uniform hematologic response than the conventional ferrous sulfate. The greatest increase in hematologic response was observed after term.

Iron Mobilization via Xanthine Oxidase

To understand a possible mechanism behind these types of observations, molybdenum should be looked at as part of the xanthine oxidase molecule, recognizing that it is essential for optimal xanthine oxidase activity. Lehninger (1962) described the xanthine oxidase molecule as follows:

Molecular weight	300,000
Prosthetic group	2 FAD
Metal	8 Fe, 2 Mo

Seelig (1972), reviewing the role of molybdenum as a component of xanthine oxidase, described several experiments in which molybdenum supplements increased xanthine oxidase levels in liver and/or intestinal mucosa of weanling and/or molybdenum-deficient rats. These experiments, along with several others, led to the verification of molybdenum as a component of the xanthine oxidase molecule.

The specific function of molybdenum as part of the xanthine oxidase molecule seems to be one of internal electron transport. Palmer et al. (1963), from their kinetic study, suggested that flavin, molybdenum, and iron participate in oxidation-reduction, with transport of electrons in the order: $e^- \rightarrow Mo \rightarrow FAD \rightarrow Fe$. Handler et al. (1964) also suggested a similar sequence of electron transfer. However, Bray and Swann (1972), with improved methods of EPR (electron paramagnetic resonance) made the following revision of the above proposed sequence (Figure 1):

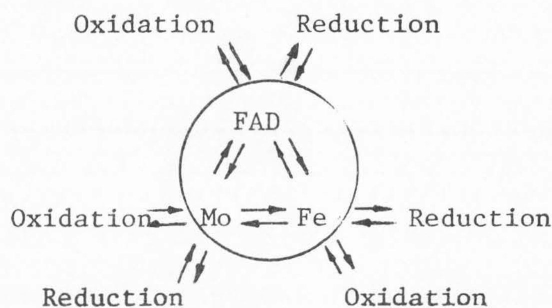


Figure 1. Possible oxidation-reduction reactions between reducing and oxidizing substrate molecules and the molybdenum, flavin and iron of xanthine oxidase. The enzyme molecule is represented by the circle and arrows indicate transfer of reducing equivalents (adapted from Bray and Swann, 1972).

Concerning xanthine oxidase substrates, xanthine oxidase is necessary for the oxidation of xanthine to uric acid. However, evidence is being presented that it also plays a part in the reduction of ferric-ferritin to ferrous-ferritin (Figure 2).

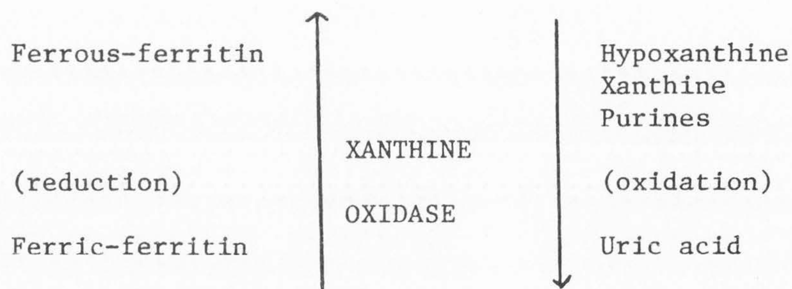


Figure 2. Reduction of ferric-ferritin to ferrous-ferritin (Fridovich and Handler, 1958).

Reduction of storage iron is necessary before the iron can be mobilized for use in hemoglobin synthesis.

Mazur and Green (1957) did an *in vitro* study with rat liver slices which demonstrated this role of xanthine oxidase in reducing ferric-ferritin to the ferrous form. Since ferritin iron is mainly in the ferric state, they measured the amount of ferrous iron formed per millimole ferritin total iron in a xanthine oxidase system over a 60 minute period, and found appreciable reduction of ferritin iron as opposed to no reduction found when an inhibitor was added to the system.

In a subsequent *in vivo* study, Mazur et al. (1958) confirmed the participation of xanthine oxidase in liberating tissue iron to the circulation. Upon the basis that xanthine oxidase acts upon hypoxanthine, xanthine, and purines to produce uric acid, several animal experiments were performed. In all the experiments, conditions which would normally stimulate xanthine oxidase activity were induced, such as hemorrhagic or traumatic shock (which normally results in an accumulation of products of nucleotide catabolism), or elevating the level of xanthine oxidase substrates in the tissues. The first experiment showed that rats subjected to hemorrhagic or traumatic shock had plasma uric acid levels two to three times higher than normal or nephrectomized rats. The second experiment showed that injections of large amounts of purine or hypoxanthine in guinea pigs and dogs caused increases in plasma iron. The increased level of iron was maintained for longer than 30 minutes in the guinea pig and for 2 hours in the dog. In the dog, elevated values of plasma uric acid accompanied a rise in plasma iron. In a third

experiment with rabbits, large doses of purine and hypoxanthine also elevated levels of plasma iron that required 1 to 2 hours to return to normal levels. In another experiment, rabbits were administered doses of xanthine, inosine or adenylic acid (purines). This treatment resulted not only in increased plasma iron but also increased plasma uric acid levels. On the other hand, when uric acid was injected into the rabbits in amounts that provided levels as high as those achieved with purine or hypoxanthine, there was no increase in plasma iron. Neither was hyperferremia produced by saline, glycine, or sodium butyrate; in fact, a fall in plasma iron was observed, undoubtedly due to the repeated removal of plasma with each blood sample, and according to Mazur et al. (1958), this serves to make the hyperferremia in previous experiments more significant. Finally, a man who was given a 1 gm intravenous injection of inosine showed a rise in plasma iron from a control value of 72 μ g/1oo ml plasma to 103 μ g/1oo ml after 5.5 hours and returned to preinjection values by 9.5 hours (C. A. Finch in a personal communication--see Mazur et al., 1958). Thus, plasma iron increased as plasma uric acid, an end-product of xanthine oxidase activity, increased.

Several years after the Mazur associates study, Ayvazian (1964) observed a clinical case of xanthinuria and hemochromatosis simultaneously in a 47 year old man. He offered as one explanation, referring to Mazur et al. (1957 and 1958), a close relation between purine and iron metabolism, which is mediated through liver xanthine oxidase. Ayvazian's article resulted in several letters to the editor plus an editorial in the New England Journal of Medicine (1964). R. A. MacDonald (1964) stated to the editor that approximately 1400 cases of

hemochromatosis had been reported to that date and that Ayvazian was the first to associate it with abnormal xanthine metabolism.

In the unsigned editorial (1964), certain inconsistencies were pointed out in the Mazur et al. concept as related to Ayvazian's observations. The writer stated that xanthine oxidase is normally found in intestinal mucosa, liver and bone marrow but not in other tissues; since enzyme deficiencies in most inborn errors of metabolism are not organ specific, one would expect diminished xanthine oxidase activity and failure of ferritin mobilization not only in the liver but also in the intestinal mucosa. The writer further reasoned that under these circumstances, excessive quantities of iron might not accumulate in the patient.

On the other hand, Crosby (1964) hypothesized that ferritin is present in the intestinal epithelium to prevent absorption of excess iron. Crosby stated that in his working hypothesis, anything that interferes with the ferritin apparatus--either its formation or its function--would permit the absorption of available excess dietary iron and might, therefore, result in hemochromatosis. The exact mechanism of iron absorption is presently unknown.

Mazur and Carleton (1965) reported an inverse relationship between ferritin iron content and xanthine oxidase activity in the liver of the developing rat; hence, the more xanthine oxidase present the less iron remained in the liver as stores.

Powell and Emmerson (1966) found that hepatic iron concentration increased at a significantly more rapid rate in rats fed diets containing allopurinol, a xanthine oxidase inhibitor, than those without the inhibitor. This implies a role of xanthine oxidase in taking iron out of the stores of the liver. The rats fed the diets including supplemental iron plus allopurinol eventually developed hemosiderosis, while those receiving only laboratory meal plus allopurinol did not.

To follow up on Powell and Emmerson's association of hemosiderosis with xanthine oxidase inhibition, Mazur and Sackler (1967) analyzed 18 human liver samples for xanthine oxidase activity. Liver samples came from three groups of patients: control liver samples obtained during surgery or necropsy from patients without liver disease, biopsy samples from patients with cirrhosis, and a third group from patients with idiopathic hemochromatosis. The xanthine oxidase activity in the liver samples from patients with cirrhosis or hemochromatosis were all below those in the control group, and in a number of instances there was no evidence for any xanthine oxidase activity.

In a study on the biological effects of dietary molybdenum on chicks, molybdenum was added to a diet adequate in all known nutrients including 100 ppm Fe and 100 ppm Cu (Anders and Hill, 1970). Highly significant increases in chick growth resulted when 5 ppm molybdenum was added to the basal diet. A three-fold increase of liver xanthine oxidase activity accompanied this addition. According to these workers, hemoglobin concentration can decrease by one-third during the first week of life of the chick. This decrease of hemoglobin was unaffected

when the iron content of the diet was increased to 150 ppm; however, when 5 ppm molybdenum was added to the diet containing 150 ppm iron, the early depression of hemoglobin was essentially prevented. Moreover, Anders and Hill commented that intestinal xanthine oxidase was extremely low in day-old chicks (2.9 Δ A/min/gr. protein); however, this enzyme increased rapidly with age and reached a value of 9.6 Δ A/min/gr. protein at 10 days. The addition of 1.2 ppm molybdenum to the diet resulted in a further increase to 14.0 Δ A/min/gr. protein at this age.

EXPERIMENTAL METHODS

Experimental Objective

The purpose of this study was to gather further evidence for the role of molybdenum in iron mobilization under two stressful conditions--pregnancy and anemia. Three different experiments were performed in order to evaluate the relationships between molybdenum, iron, copper, and hemoglobin: (1) normal male rats made anemic were given iron supplements with and without molybdenum; (2) pregnant rats were fed diets with and without iron supplements, with and without molybdenum; and (3) serum was collected from pregnant human subjects.

Experimental Design

Male rat experiment

Thirty-six male Sprague Dawly rats (average weight 86 gm) were housed in individual stainless steel cages with wire bottoms and kept at a controlled room temperature (25° C). They were given feed and distilled water ad libitum. While feeding on rat chow, they were bled twice (0.7 ml each bleeding) the first week to help make them anemic. At the beginning of the second week all rats were placed on a diet without supplemental iron or supplemental molybdenum (see Appendix Table 4 for diet compositions), and were bled twice to compound the anemia. At the beginning of the third week, the rats were assigned to

one of three groups such that the mean initial weights and hemoglobins for each group were almost identical (weights: 86.5, 85.4, and 86.4 gm; hemoglobins: 4.72, 4.71, and 4.71 gm/dl). The three groups were then fed one of the following diets: (1) iron with molybdenum ($Fe_+ Mo_+$); (2) iron without molybdenum ($Fe_+ Mo_-$); or (3) no supplementation of either iron or molybdenum ($Fe_- Mo_-$). The quantity of iron supplemented was 20 ppm; analysis of the diet indicated that the mean iron content in the supplemented diets was 25 ppm and the mean iron content in the unsupplemented diets was 10 ppm. After three weeks on the appointed diet, all rats were decapitated and blood and tissue samples were collected. Blood samples were analyzed for hemoglobin, iron, and copper while livers for molybdenum, iron and copper content.

Female rats and pups experiment

Thirty-six female adult rats (average weight 275.4 gm) were housed in individual stainless steel cages with wire bottoms. Feed and distilled water were given ad libitum. After eating regular rat chow for one week, they were divided into four groups according to one of the following diet supplements: (1) iron with molybdenum ($Fe_+ Mo_+$); (2) iron without molybdenum ($Fe_+ Mo_-$); (3) molybdenum without iron ($Fe_- Mo_+$) or (4) neither iron nor molybdenum ($Fe_- Mo_-$). After one week on their respective diets they were mated. Date of conception was recorded after sperm were obtained from a vaginal swab. As pregnancy was detected, they were placed in separate stainless steel cages with a solid bottom covered with sawdust. They maintained their diet until delivery. On the day of delivery, both the mothers and the pups were

decapitated. Blood samples and livers were collected. Blood samples were analyzed for hemoglobin, iron, and copper. The livers were assayed for molybdenum, iron, and copper content.

Pregnant women experiment

Blood samples were collected from 121 pregnant women: 56 in the first trimester; 29 in the second trimester; 21 in the third trimester; and 15 postpartum. Two blood samples were taken from each woman: one heparinized and the other non-heparinized. The non-heparinized samples were centrifuged, and the serum was saved for molybdenum, iron, and copper analyses. The heparinized blood was analyzed for xanthine oxidase and hemoglobin content. Records were kept of vitamin and mineral supplements being taken at the time of collection.

Analytical Methods

To aid in analyses of metals, the samples were ashed for concentration purposes and to eliminate protein. All rat livers were ashed at 600° C for 48 hours, then dissolved with 6N nitric acid, and brought to a 25 ml volume with deionized water.

All serum to be analyzed for iron and copper was mixed with trichloroacetic acid (TCA) in a ratio of 2:1 (TCA:serum) to precipitate the protein, thus eliminating possible difficulties with matrix interferences. The mixture was then centrifuged and the supernatant was used for the analyses.

All serum analyzed for molybdenum was mixed with 0.1 percent Triton-X-100 (Culver, 1975) in deionized water in a 1:1 ratio to hydrolyze the protein.

Iron and copper were analyzed by conventional flame methods on a Varian Model AA1200 Atomic Absorption Spectrophotometer. The parameters used for each element were according to the instrument manufacturer's recommendations.

Molybdenum was analyzed by means of a Carbon Rod Atomizer (CRA). The instruments used were the Varian Model AA6 Spectrophotometer and the Varian Model 90 Carbon Rod Atomizer. The limited sample volume and low levels of molybdenum made conventional flame analysis impractical. The CRA is capable of measuring levels as low as 0.6 ppb in aqueous media. Since total volume placed in the CRA is analyzed, a volume of five microliters was sufficient for analysis. Although larger volumes would increase the detected signal, more difficulties are encountered with molecular background interferences. The following parameters were determined either empirically or from recommended procedures of the manufacturer.

Table 1. Instrument and carbon rod atomizer conditions

Instrument conditions		Carbon rod atomizer conditions	
Wave length	313.3 nm	Dry	100° C/25 sec
Spectral band width	0.5 nm	Ash	800° C/15 sec
Lamp current	8 na	Atomize	2500° C/4 sec/ 500° C sec^{-1}
Slit height	4 mm		
		Sheathing gas	Argon (20 psi)
		Tube	threaded
		Sample volume	5 nl

Background correction was required to reduce molecular absorption and scattering, which would interfere with the analysis. Argon was used instead of less expensive nitrogen to aid in prolonging the useable life of the graphite in the CRA. Internally threaded graphite tubes provided more uniform drying characteristics during the initial heating step due to the increased surface area over the standard tube.

Calibration was accomplished with spiked standards in matching matrices. An aqueous sample was used intermittently to verify the condition of the CRA graphite.

Xanthine oxidase was analyzed according to the method of Hashimoto (1972). The standard contained: 1 ml (0.2 moles) xanthine, 1 ml (0.3 moles) potassium oxonate, 1 ml (150 moles) phosphate buffer, and 25 λ xanthine oxidase. The blank was the same, excluding the xanthine oxidase. Each sample contained 0.2 ml whole blood, 1 ml (0.2 moles) xanthine, 1 ml (0.3 moles) potassium oxonate, and 1 ml (150 moles) phosphate buffer. The samples were incubated for 30 minutes in a water bath at 30° C. The reaction was then stopped by adding 0.1 ml of (W/V) 100 percent TCA. After thorough mixing, samples were centrifuged for 15 minutes. The resultant clear supernatant was read at 292 nm on a Beckman spectrophotometer against a reagent blank.

Statistical Methods

Means, standard deviations, F-tests, and least significant differences were calcualted by standard methods (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Male Rat Experiment

No statistical differences of liver iron, liver molybdenum, or liver copper were observed among any of the treatments. While liver iron was almost identical for all three treatments, liver molybdenum and liver copper tended to have higher values in rats fed the Fe_- Mo_- diet; but because of the wide numerical range of individual observations, no statistical significance was observed (Figures 3, 4 and 5). The liver iron stores were low for rats on all three treatments, which was to be expected since all rats were anemic (according to their hemoglobin values--Table 2) beginning the feeding of each diet.

Table 2. Male rat weight and hemoglobin

	Initial		Sub-final		Final	
Diet	Wt (gm)	Hb(gm/dl)	Wt (gm)	Hb(gm/dl)	Wt (gm)	Hb(gm/dl)
Fe_+ Mo_+	86.4	4.72	111.1	6.44	116.4	7.13
Fe_+ Mo_-	85.4	4.71	114.8	6.72	111.3	7.58
Fe_- Mo_-	86.4	4.71	103.2	4.52	107.3	4.30

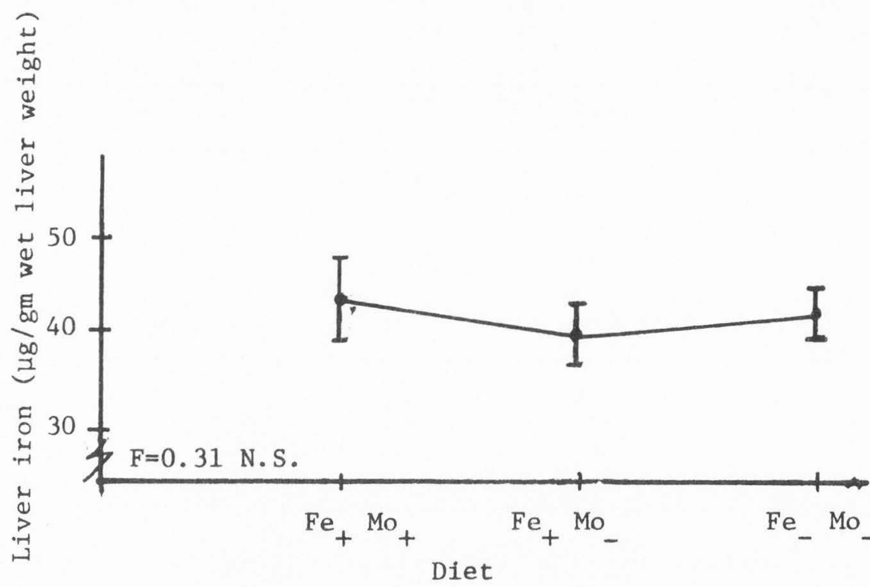


Figure 3. Influence of dietary iron and molybdenum on male rat liver iron.

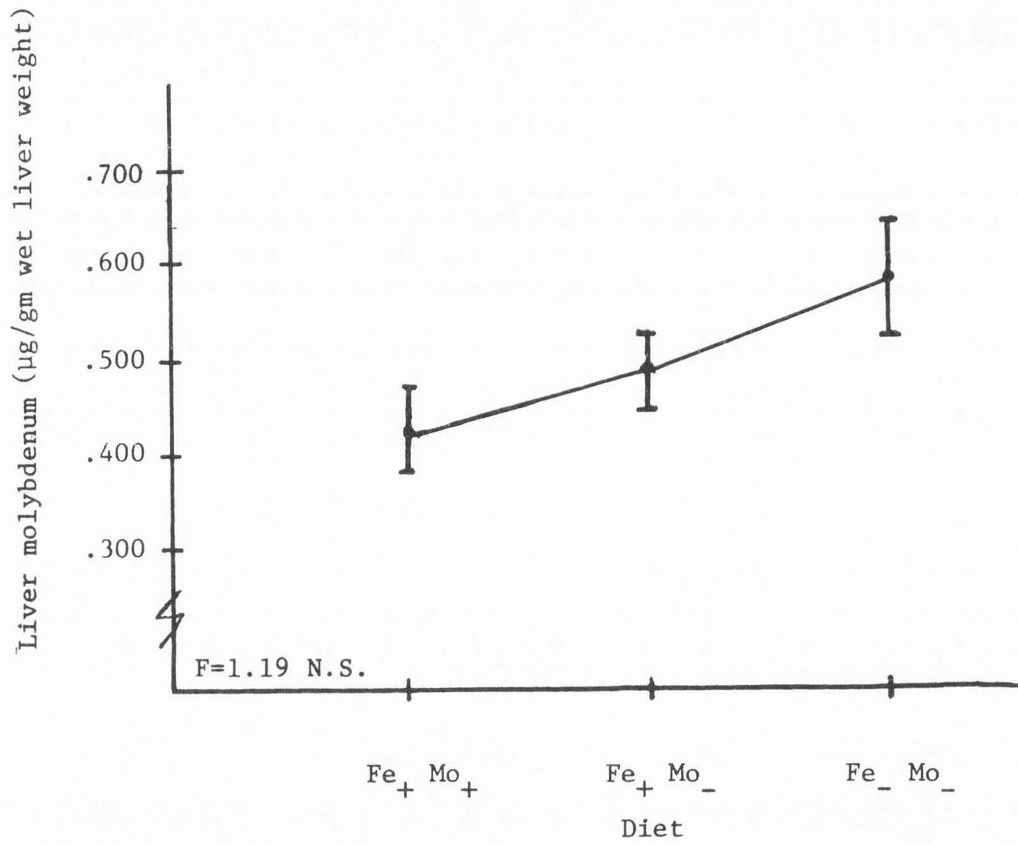


Figure 4. Influence of dietary iron and molybdenum on male rat liver molybdenum.

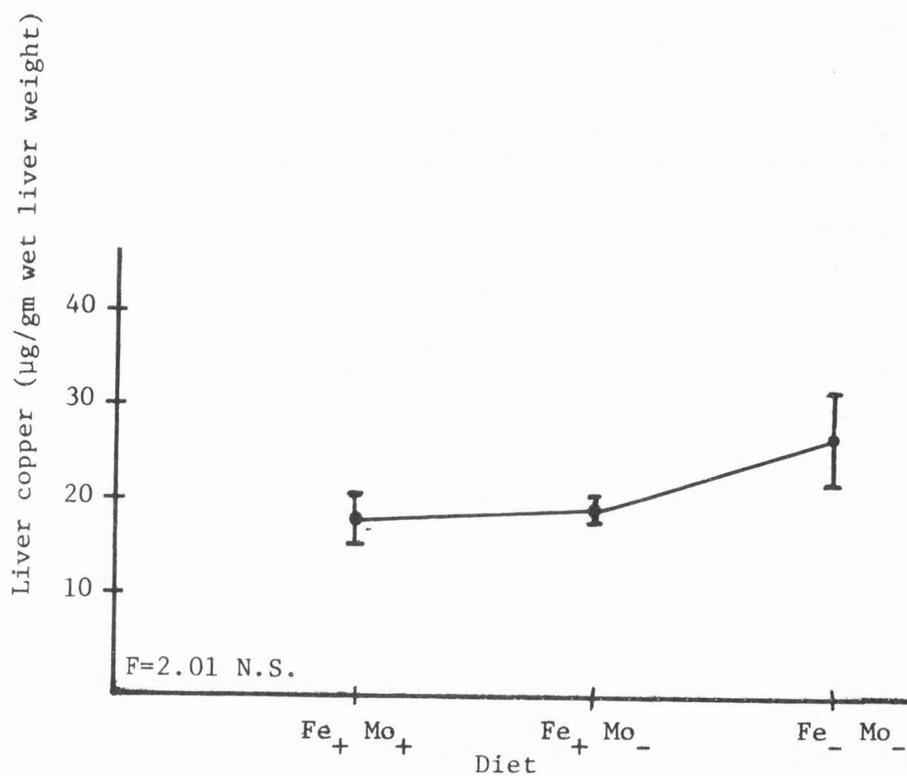


Figure 5. Influence of dietary iron and molybdenum on male rat liver copper

Figures 6, 7 and 8 indicate the status of serum iron, serum copper and hemoglobin at the time of sacrifice. Serum copper was almost identical for all three treatments, but significant differences were shown in serum iron and hemoglobin. Surprisingly, serum iron for the Fe_+ Mo_+ treatment was almost identical to that of the Fe_- Mo_- treatments; however, there was a significantly lower serum iron for the Fe_+ Mo_- treatment. Another interesting result--a lower hemoglobin for the Fe_+ Mo_- treatment did not accompany the significantly lower serum iron for the same group. The significant difference in hemoglobin occurred with the Fe_- Mo_- treatments, as was expected. Note the high F value (43.71) for that significant decrease in hemoglobin.

The confusion of these results starts with the fact that while the rats on the Fe_- Mo_- diet remained anemic, according to hemoglobin values, the final liver and serum iron were comparable to the relative "non-anemic" rats; liver copper and liver molybdenum however, tended to increase in the anemic rats; serum copper of the anemic rats was comparable to the relative "non-anemic" groups. Initially these rats were all hemorrhagically anemic; those who maintained the lowest level of anemia did so because of a nutritional deficiency.

Female Rat and Pups Experiment

No significant differences were found with liver iron and liver copper among any of the female groups, as shown in Figures 9 and 10. Nor were there significant differences in serum iron and serum copper among any of the groups (Figures 11 and 12); however, an inverse relationship appeared to be apparent between iron and copper in both the serum and the liver.

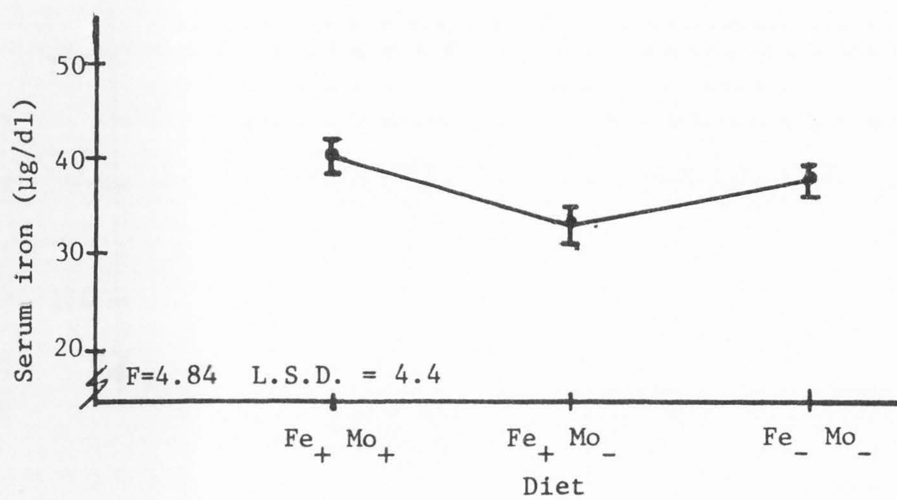


Figure 6. Influence of dietary iron and molybdenum on male rat serum iron.

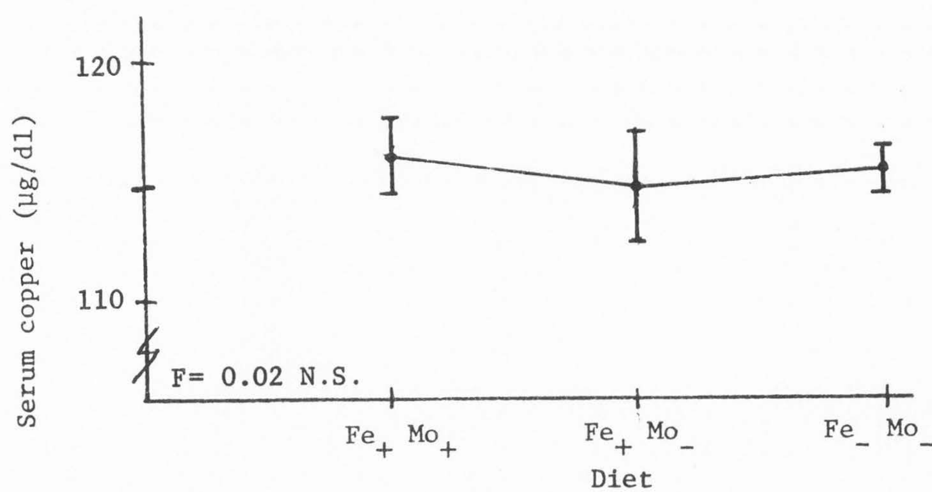


Figure 7. Influence of dietary iron and molybdenum on male rat serum copper.

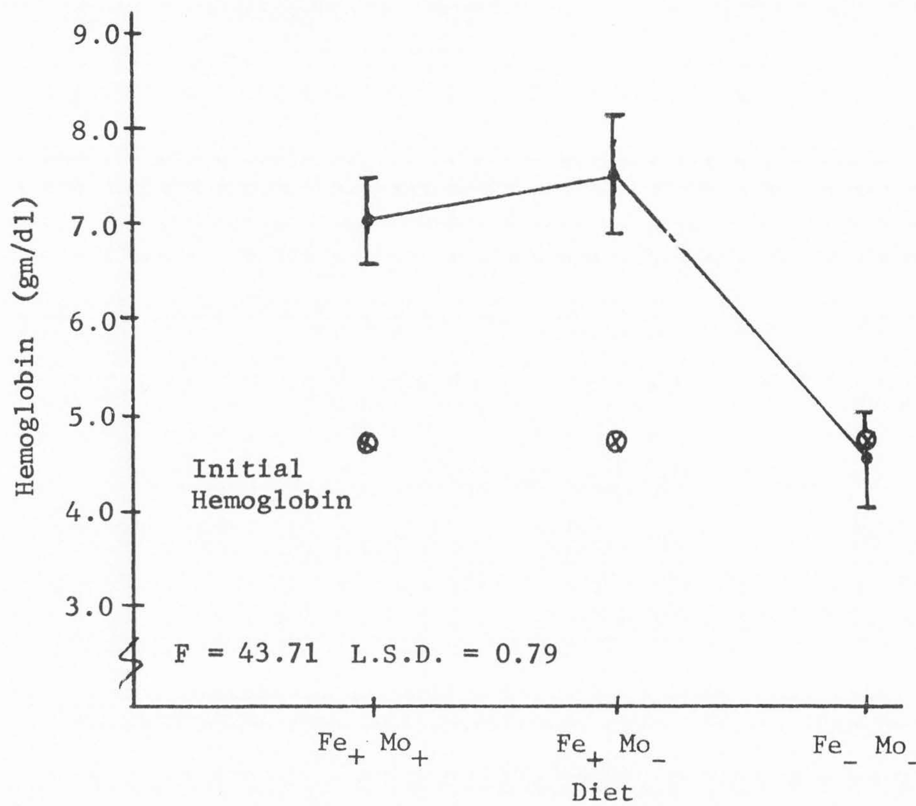


Figure 8. Influence of dietary iron and molybdenum on male rat hemoglobin.

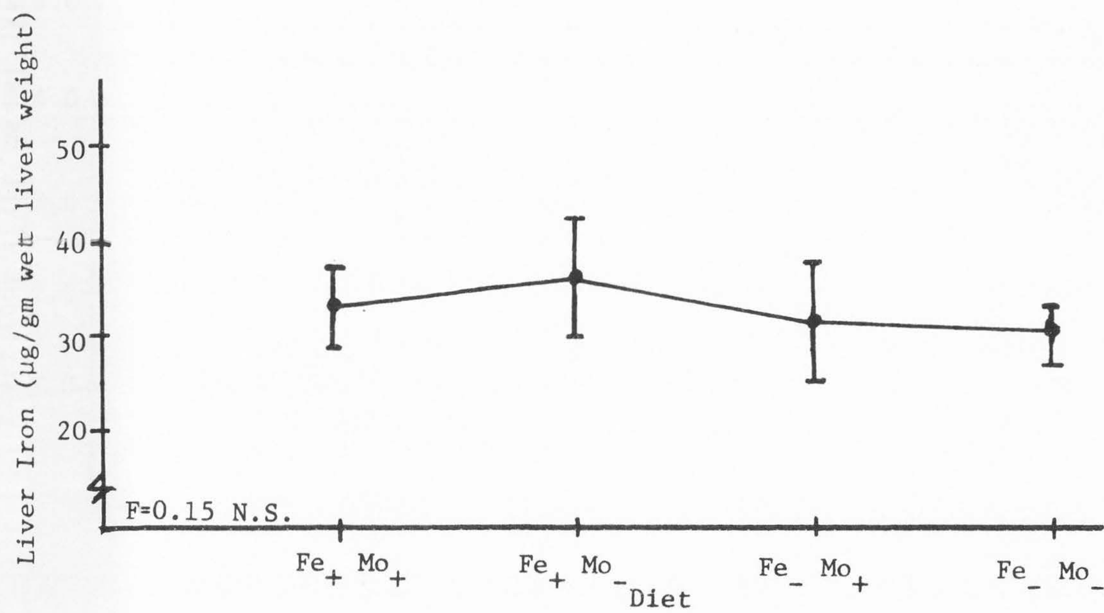


Figure 9. Influence of dietary iron and molybdenum on female rat liver iron.

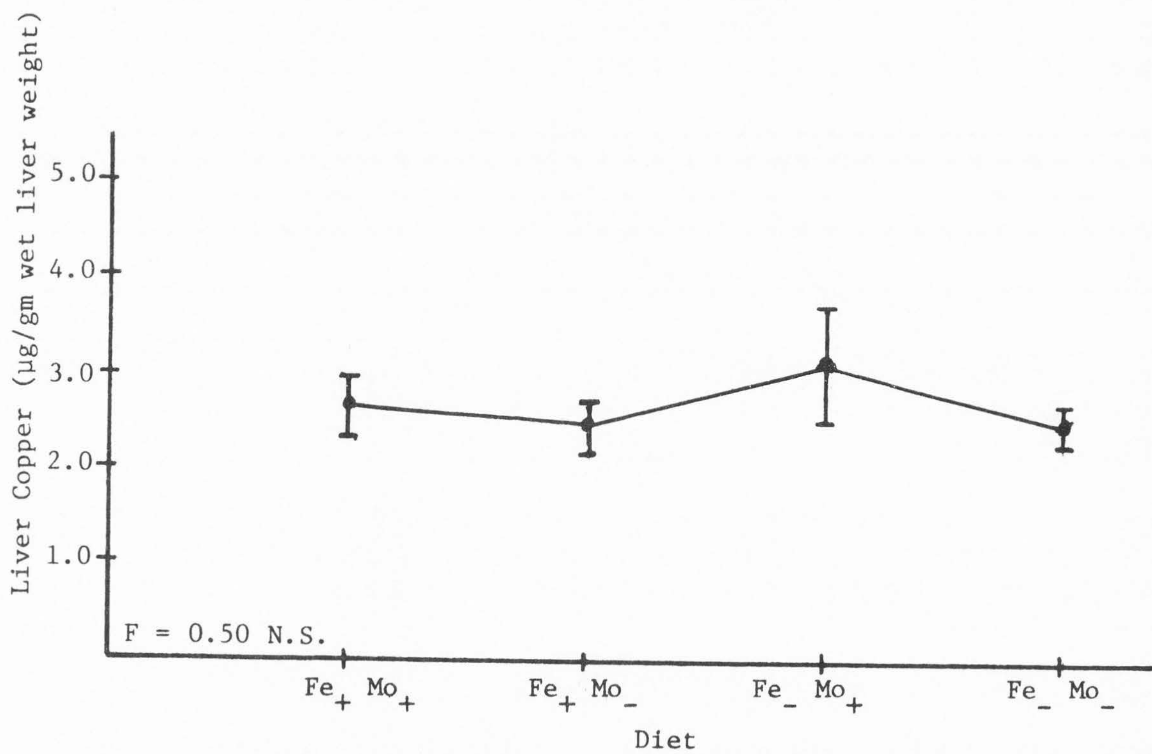


Figure 10. Influence of dietary iron and molybdenum on female rat liver copper.

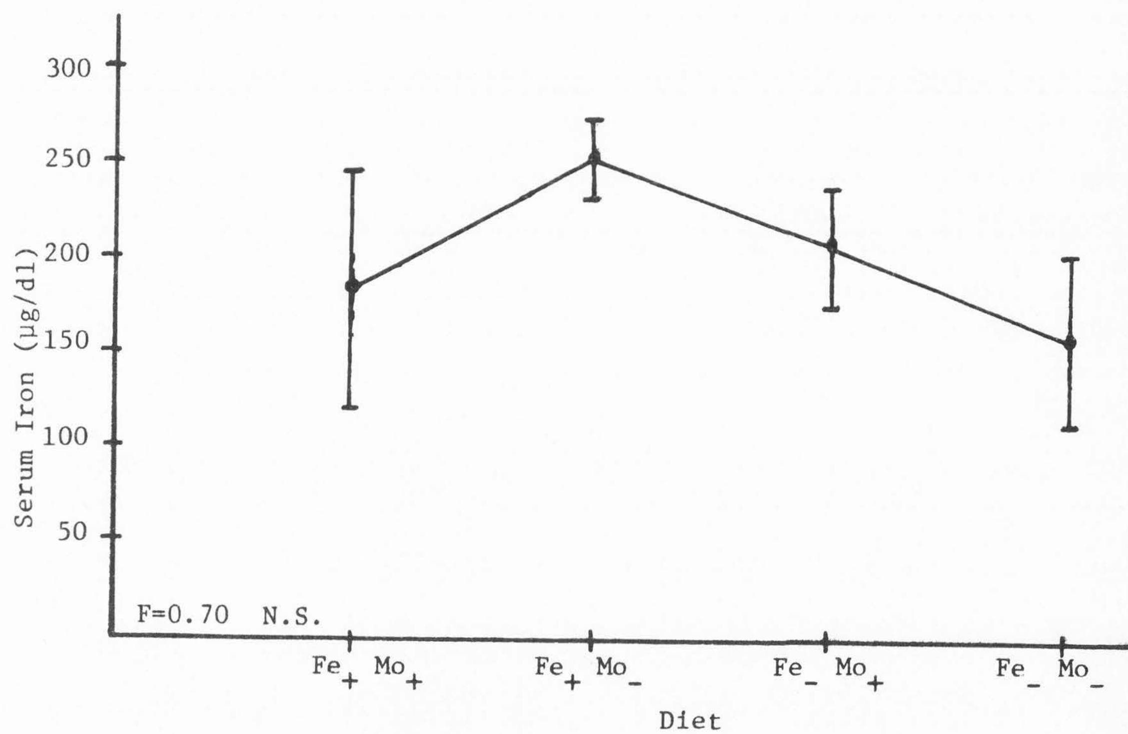


Figure 11. Influence of dietary iron and molybdenum on female rat serum iron.

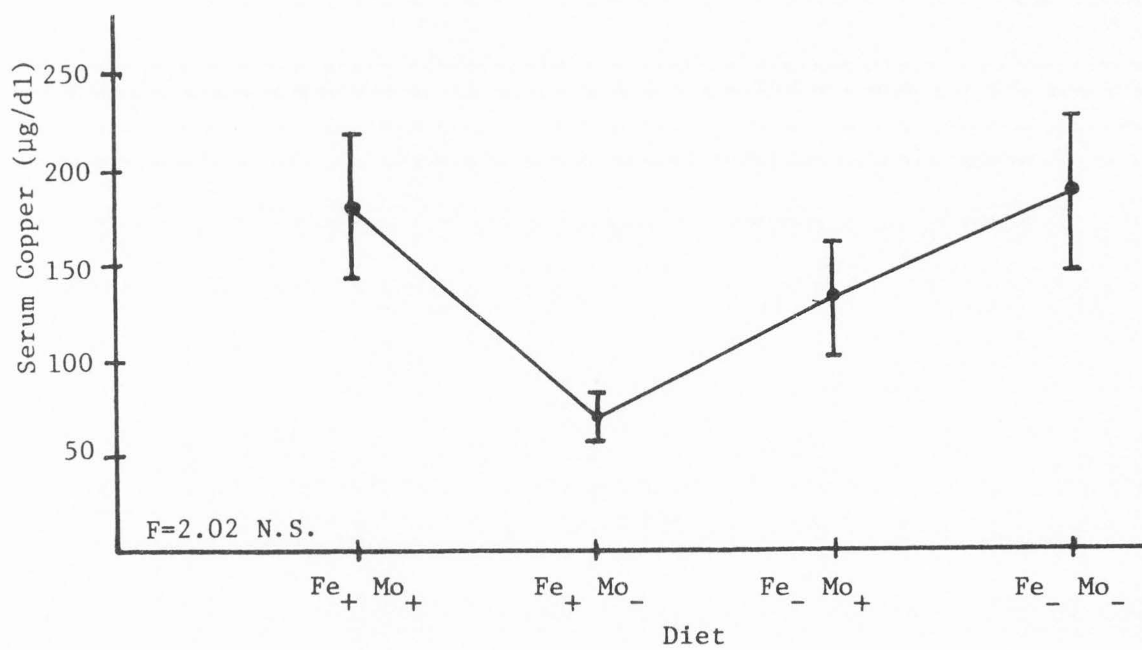


Figure 12. Influence of dietary iron and molybdenum on female rat serum copper.

Although the differences in liver molybdenum among the groups were not quite significant, Figure 13 indicated that rats fed diets without supplemental molybdenum had the lowest liver molybdenum. Hemoglobin was significantly lower in rats fed diets without supplemental iron ($Fe_- Mo_+$ and $Fe_- Mo_-$) than in rats fed diets supplemented with both iron and molybdenum ($Fe_+ Mo_+$); however, these same differences were not seen when comparisons were made with the rats on the $Fe_+ Mo_-$ diet (Figure 14). Hemoglobin tended to decrease as supplemental iron and/or molybdenum was absent.

In reviewing the results of the pregnant female rats, it needs to be kept in mind that the numbers and graphs represent only the post-partum phase of their "stress" condition. Also, in relation to the male rat experiment, the hemoglobins of the females were all "normal" or "borderline" cases, whereas the male rats were all definitely anemic, the differences among the male rats only showing one group significantly more anemic than the other two groups.

Differences found in the dams' hemoglobin were reflected in the pups' liver iron. Liver iron was significantly lower in rats fed diets without supplemental iron ($Fe_- Mo_+$ and $Fe_- Mo_-$) than in rats fed diets supplemented with both iron and molybdenum ($Fe_+ Mo_+$); again, significant differences were not seen when comparisons were made with rats on the $Fe_+ Mo_-$ diet (Figure 15). Liver iron in the pups tended to decrease when supplemental iron and/or molybdenum was absent in the dams, indicating that the diets had the most effect on placental transfer rather than on the dams themselves.

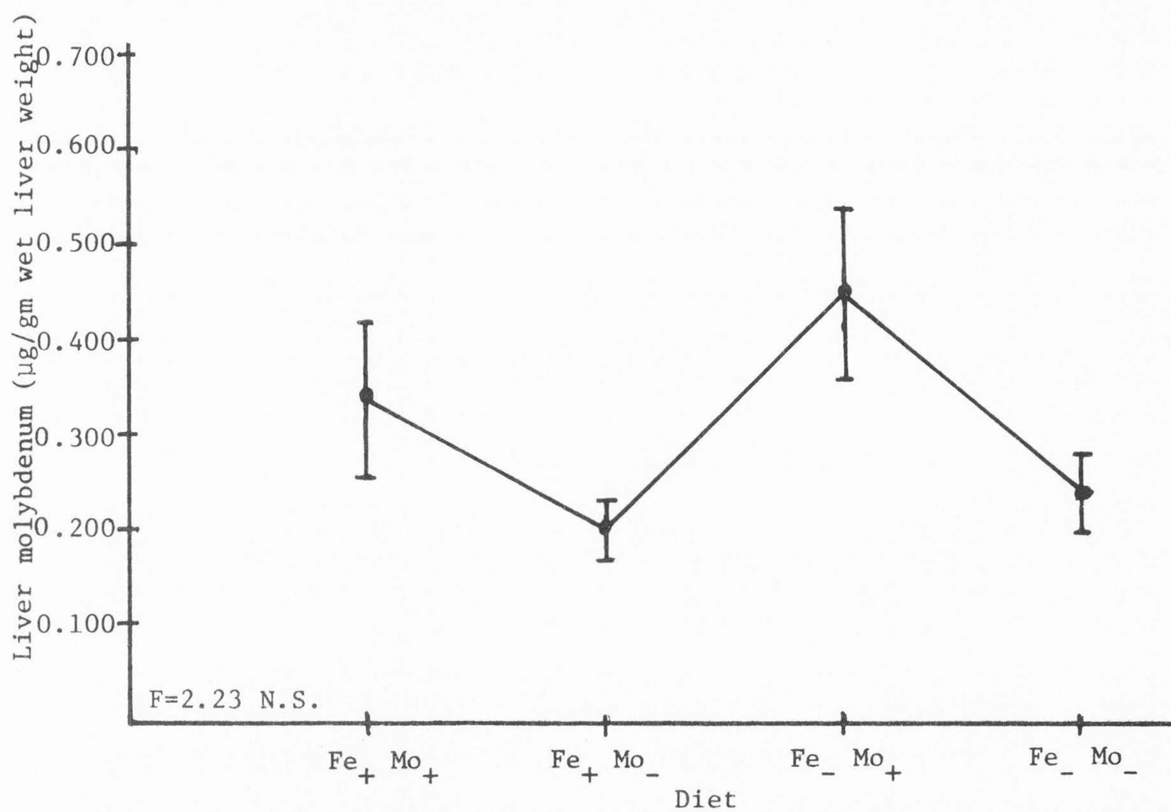


Figure 13. Influence of dietary iron and molybdenum on female rat liver molybdenum.

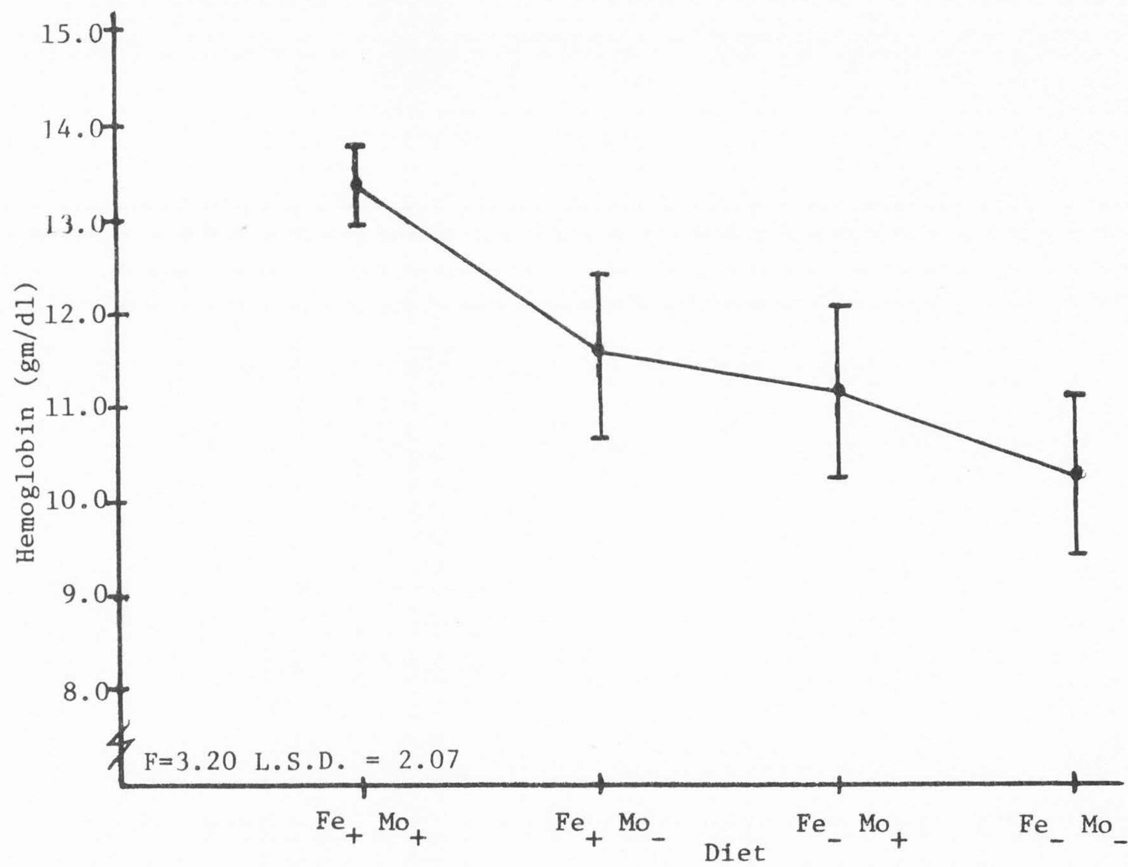


Figure 14. Influence of dietary iron and molybdenum on female rat hemoglobin.

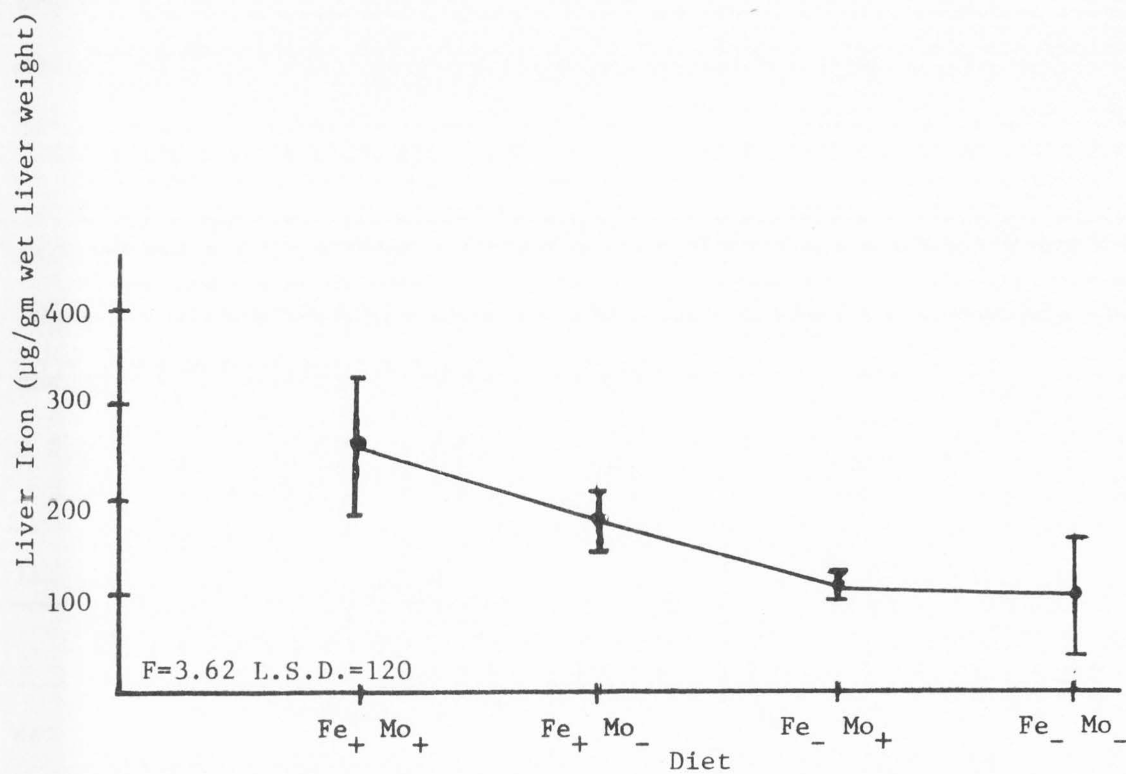


Figure 15. Influence of dietary iron and molybdenum in rat pup liver iron.

The pup's hemoglobin decreased as supplemental iron was taken out of the diet and then as supplemental molybdenum was taken out as well (Figure 16).

Although no significant differences were detected among the groups concerning the pups' liver molybdenum and liver copper, liver molybdenum and liver copper apparently had an inverse relationship (Figures 17 and 18).

Relationships were noticed between the dams and the pups. As seen in Table 3, liver values for iron and copper were higher in the pups than in the dams. The opposite was true for molybdenum, in which the highest values remained in the dams. These observations indicated a placental transfer of iron and copper, but not of molybdenum.

Table 3. Dam and pup liver relationships

	Fe_+ Mo_+	Fe_+ Mo_-	Fe_- Mo_+	Fe_- Mo_-	L.S.D. (.05)
Wet liver iron, µg/gm					
Dams	34.1	37.4	33.5	32.2	N.S.
Pups	266.7	185.7	110.6	106.2	120
Wet liver copper, µg/gm					
Dams	2.7	2.5	3.2	2.6	N.S.
Pups	26.7	17.1	18.7	22.9	N.S.
Wet liver molybdenum, µg/gm					
Dams	0.35	0.21	0.45	0.25	N.S.
Pups	0.12	0.18	0.15	0.12	N.S.

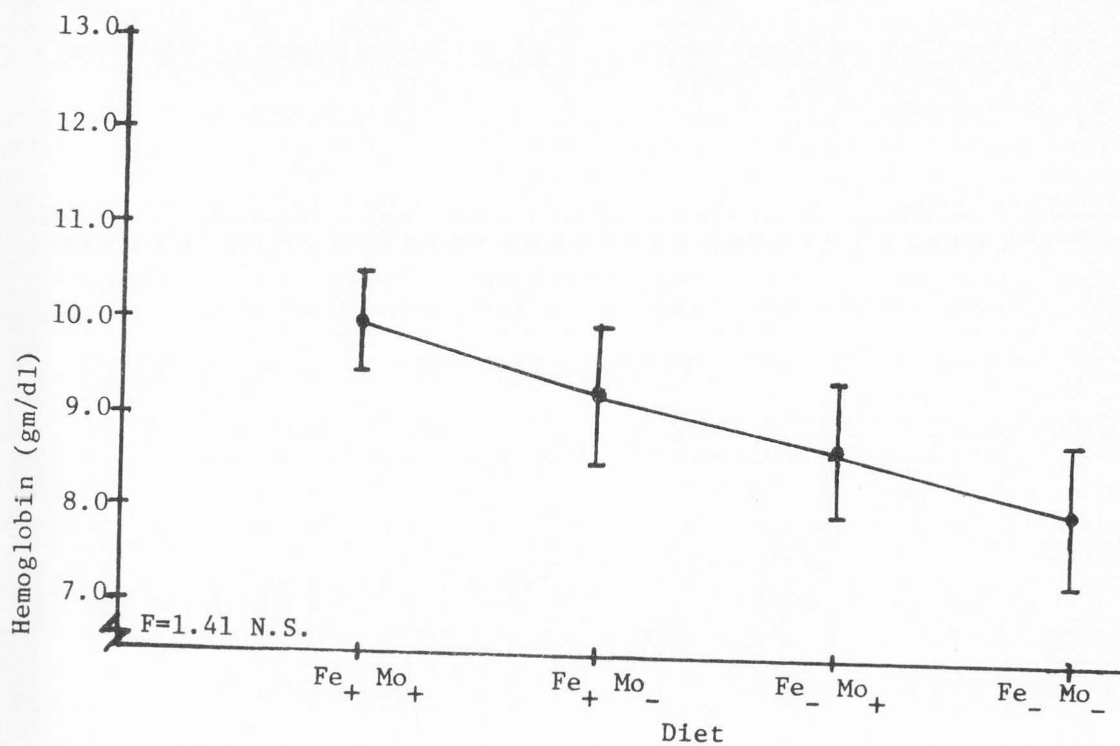


Figure 16. Influence of dietary iron and molybdenum in rat pup hemoglobin.

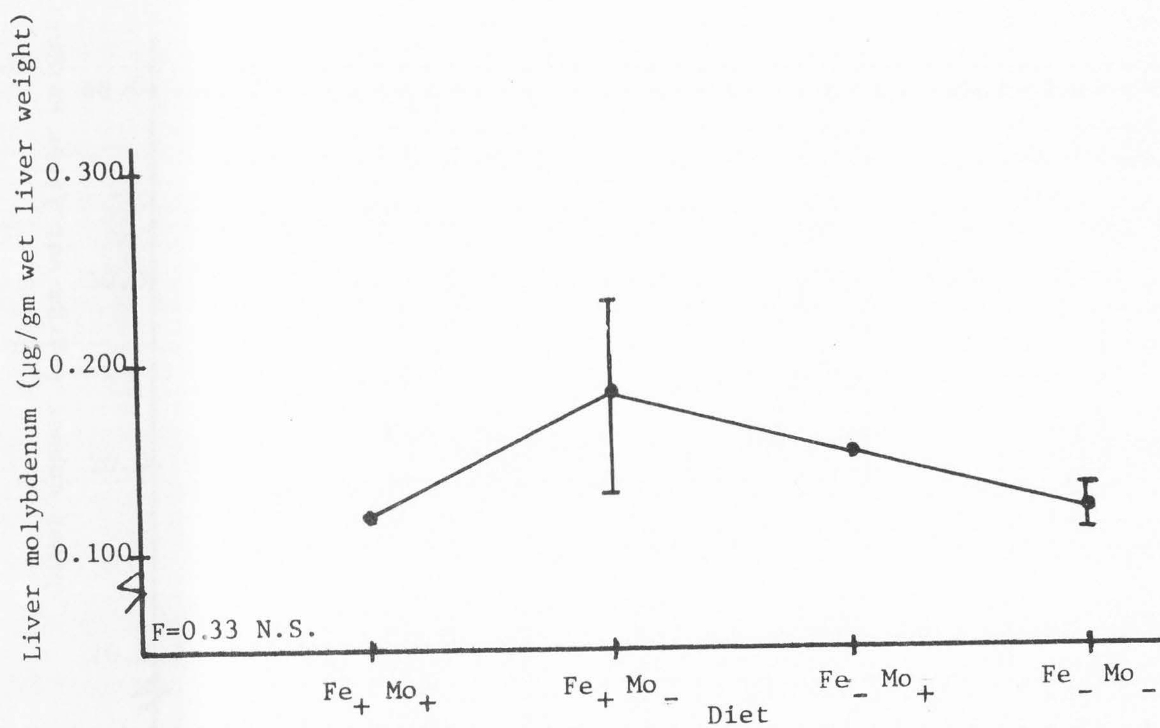


Figure 17. Influence of dietary iron and molybdenum on rat pup liver molybdenum.

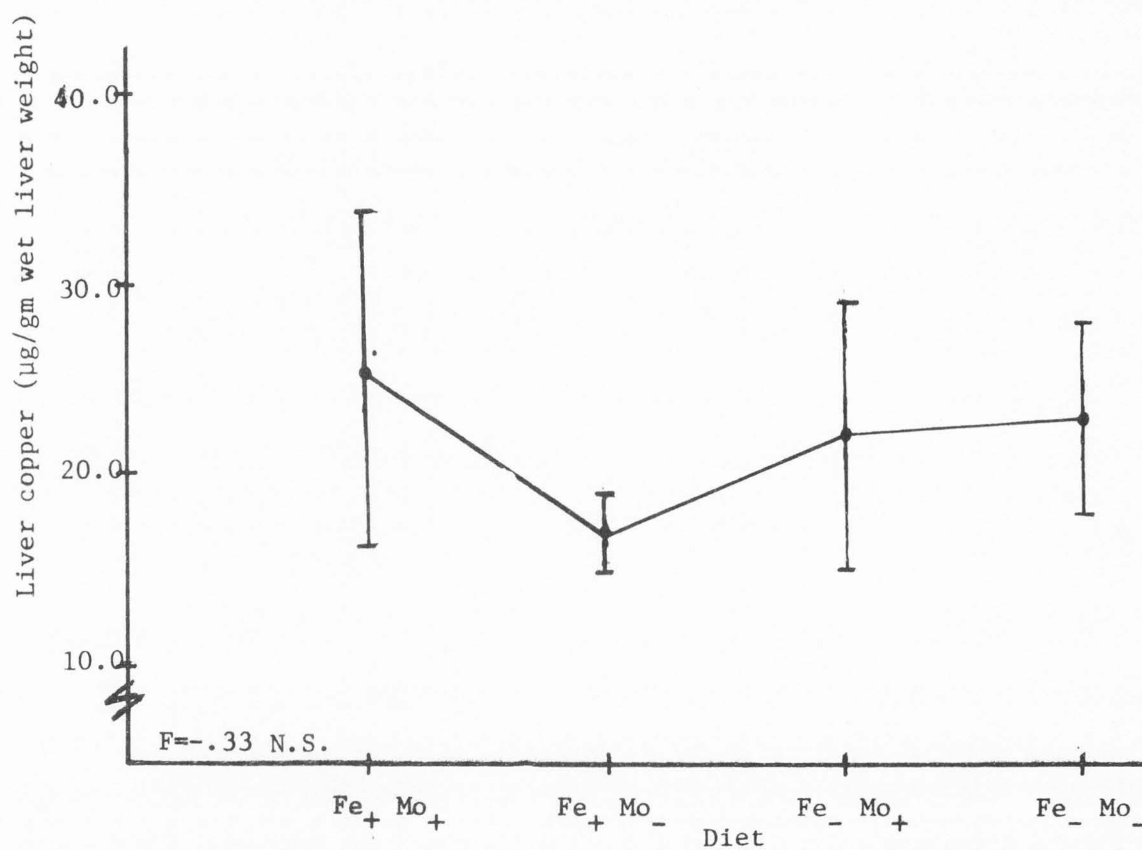


Figure 18. Influence of dietary iron and molybdenum on rat pup liver copper.

Pregnant Women Experiment

There were significant differences among the groups in relation to serum copper, serum iron and serum molybdenum; however, there were none in relation to hemoglobin and xanthine oxidase.

Serum copper during the first trimester was significantly lower than all the other groups (Figure 10). The tendency for serum copper to rise during pregnancy was also seen in women using oral contraceptives (Horwitt, et al., 1975). Serum iron did not differ significantly until postpartum, when there was a notable decrease below normal serum iron levels (Figure 20). The same observation held true with serum molybdenum--no differences before postpartum and then a significant decrease at postpartum (Figure 21). An inverse relationship was observed between serum copper and both serum iron and molybdenum.

The quantity of molybdenum in human serum as determined by the CRA method was comparable to that found in other methods. The lowest detectable quantity found by CRA was 0.52 ppb molybdenum. The highest quantity in serum was 5.91 ppb molybdenum. If the value of "0" ppb was arbitrarily assigned to the non-detectable values, the overall mean was 0.83 ppb molybdenum. If the nondetectable values were disregarded, the overall mean was 1.57 ppb molybdenum. Of the 122 samples, 58 were nondetectable.

Baert, et al. (1976), using neutron activation, determined that in human serum the molybdenum content was less than 1.1 ppb. Brune et al. (1966), also using neutron activation, reported 3.3 ppb molybdenum in whole blood. Bala and Lifshits (1966) used a "fractional-spectrographic"

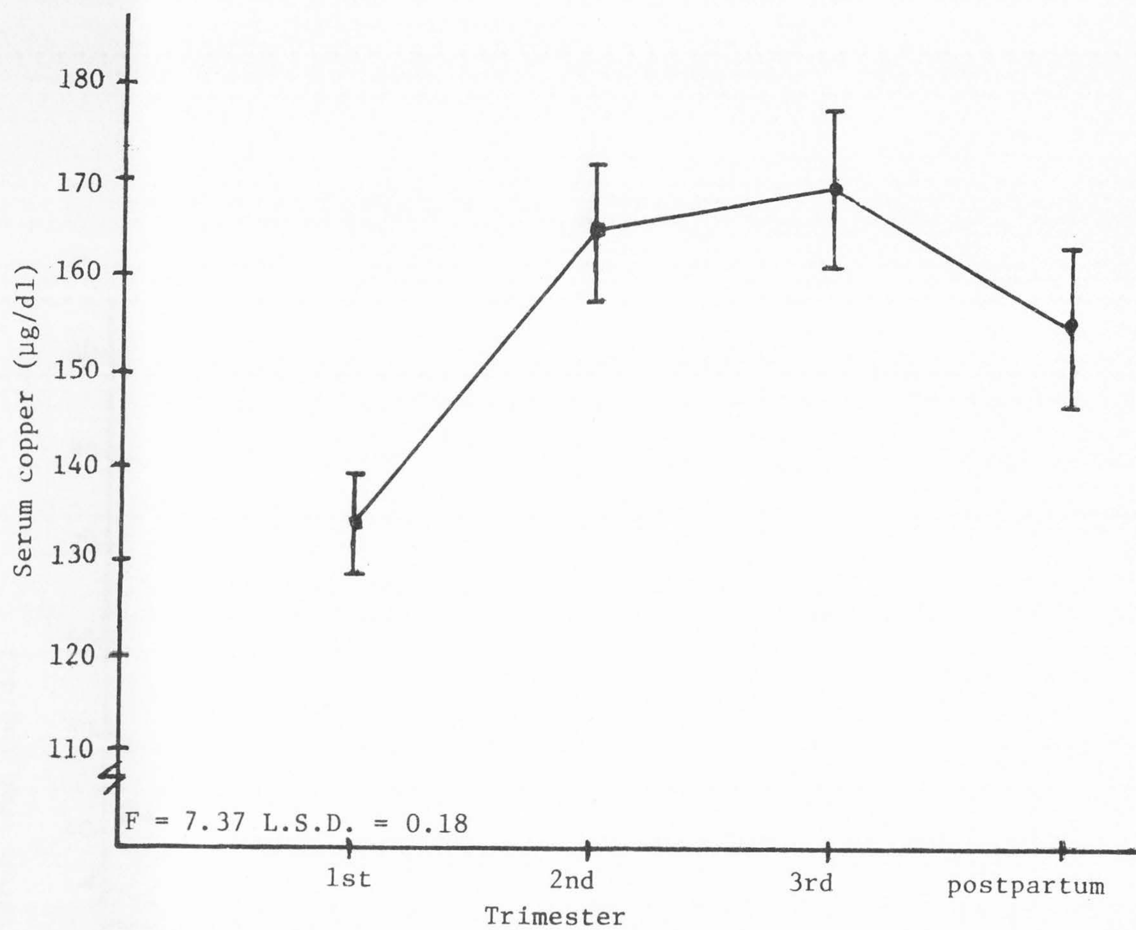


Figure 19. Influence of gestation time on serum copper in women.

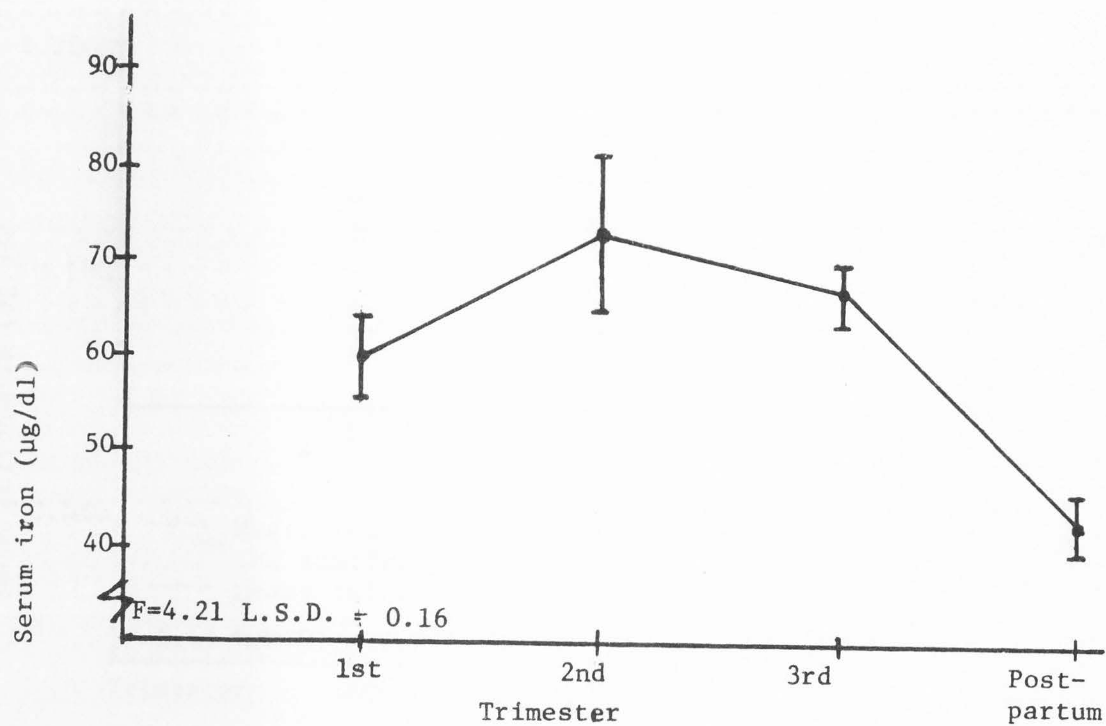


Figure 20. Influence of gestation time on serum iron in women.

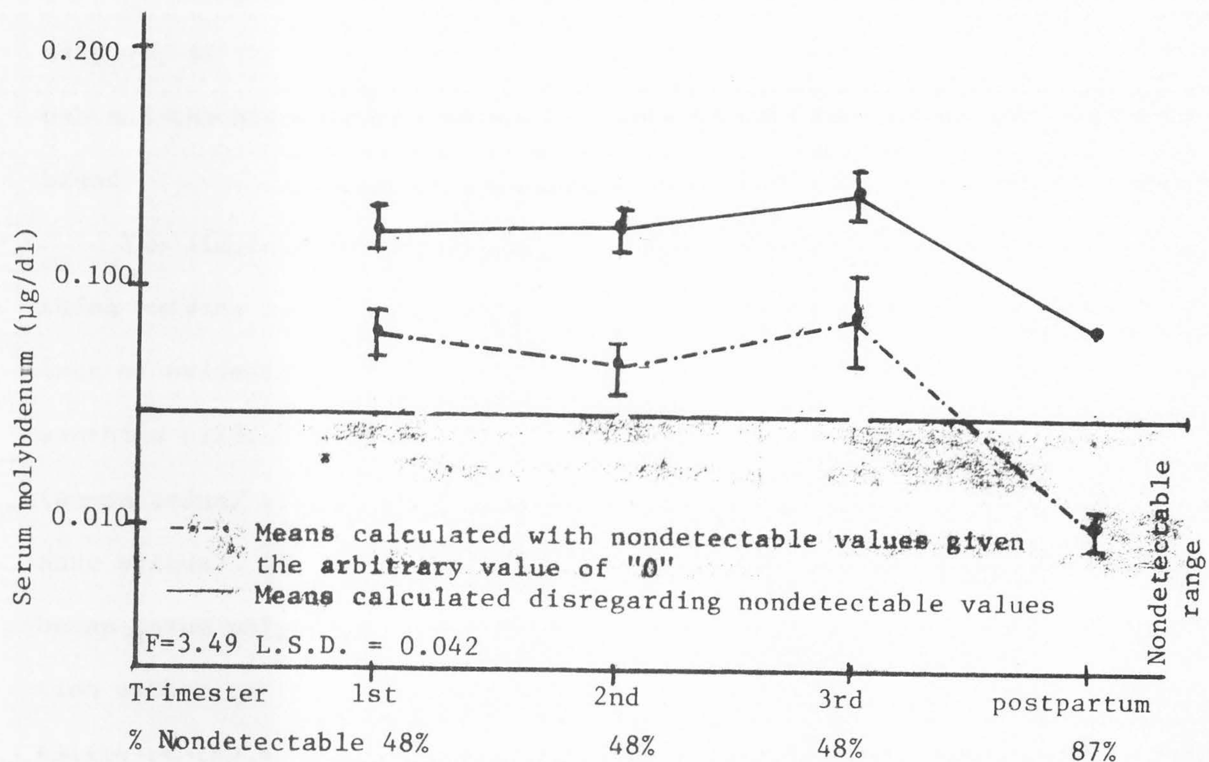


Figure 21. Influence of gestation time on serum molybdenum in women.

method, reporting 7.2 ppb molybdenum in plasma. Two other studies in the literature have reported molybdenum in human blood. Butt, et al. (1964) used emission spectroscopy, reporting ranges of 12 ppb to 34 ppb molybdenum in human serum (mean of 22 ppb molybdenum) which is slightly higher than the previous methods reported. Their study also reported nondectable levels of molybdenum. Allaway, et al. (1968) measured molybdenum colorimetrically by the thiocyanate method in 229 samples; of the 48 that were detectable, the lowest quantity was 5.0 ppb and the highest was 157 ppb, averaging 30.9 ppb molybdenum in whole blood.

The limited results of this study did not indicate a role of xanthine oxidase in iron mobilization (Figure 22). One reason for this lack of evidence was that in only one of the three experiments was xanthine oxidase able to be measured, and even then from a source (human serum) that may not even be a good indicator of xanthine oxidase status. Also, the fact that there was not any correlation between human serum molybdenum and human serum xanthine oxidase leaves a question either to the validity of the actual enzyme assay or to the possibility of the observed serum molybdenum levels reflecting the total molybdenum in the serum and not just the amount in serum xanthine oxidase. Supporting the latter possibility is the report of Bala and Lifshits (1966) that molybdenum was observed in three parts of the plasma: the globulins, the albumins, and the mineral part.

Williams, et al. (1974) also refuted the role of xanthine oxidase in iron mobilization. It has been hypothesized that in order to

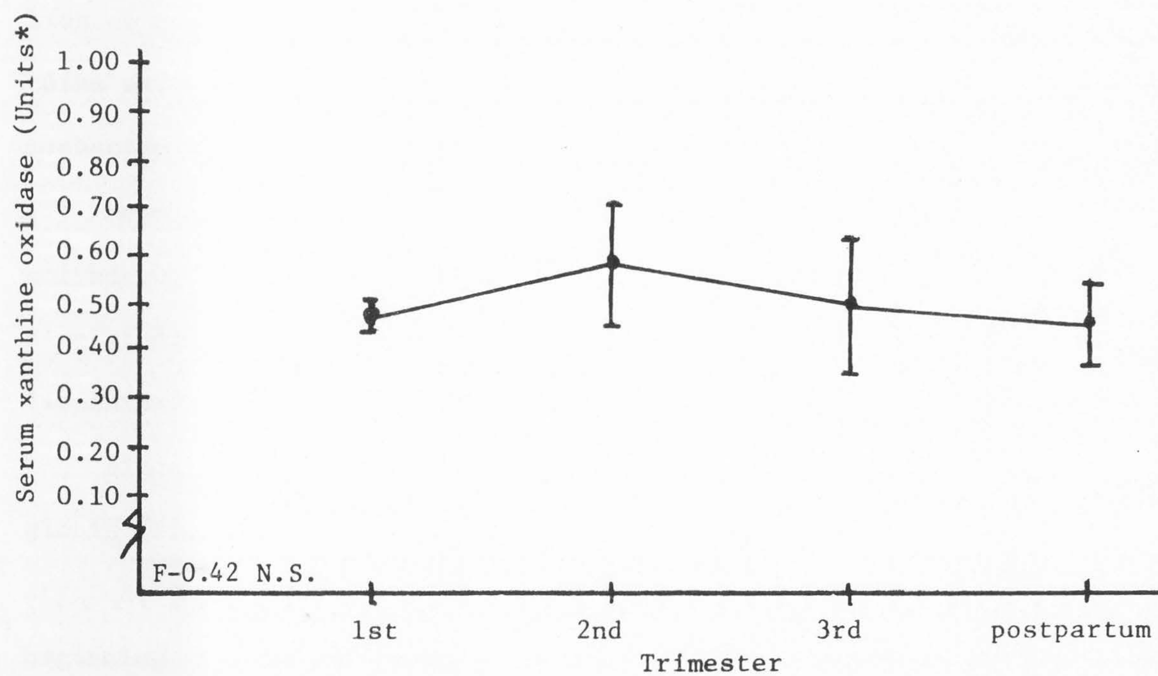
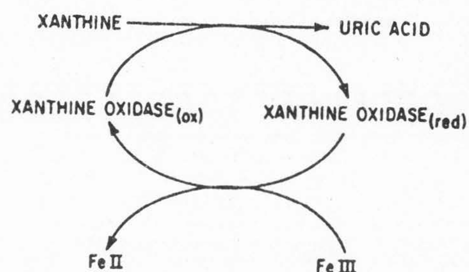


Figure 22. Influence of gestation time on serum xanthine oxidase in women. (*μM xanthine to uric acid/min at 7.5 pH.)

release iron from ferritin, iron must be reduced, accepting an electron from xanthine oxidase directly (Figure 23A). According to Williams et al., however, further studies of the enzymatic properties of xanthine oxidase established that many of its reactions depend upon the generation of the superoxide anion radical ($O_2^-\cdot$). Their *in vitro* study showed inhibition by superoxide dismutase of the xanthine oxidase--mediated reduction of iron in ferric chloride, FeADP, and ferritin, indicating that the superoxide anion radical is an intermediate in the reaction (Figure 23B). They also pointed out that hepatic concentration of superoxide dismutase is very high, and concluded that the xanthine oxidase reaction was unlikely to be an important physiological mechanism for mobilization of iron from hepatic stores.

More studies concerning xanthine oxidase and iron need to include molybdenum analysis as part of the experiment, as well as more extensive studies on xanthine oxidase, superoxide dismutase and iron relationships.

Since there were no significant differences in relation to hemoglobin (Figure 24), a molybdenum-hemoglobin relationship was not demonstrated. Most of these women were taking supplemental iron from the beginning of pregnancy, and had little chance to become anemic, even in their stress condition.



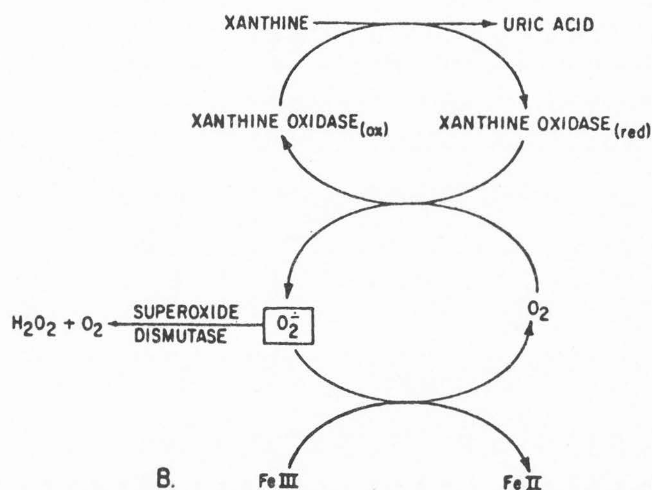


Figure 23. Alternative hypotheses for the reduction of iron by xanthine oxidase. A. Iron accepts an electron directly from xanthine oxidase. B. The superoxide anion radical serves as an intermediate electron carrier. Superoxide dismutase would be expected to inhibit the latter reaction but not the former (Williams, et al., 1974).

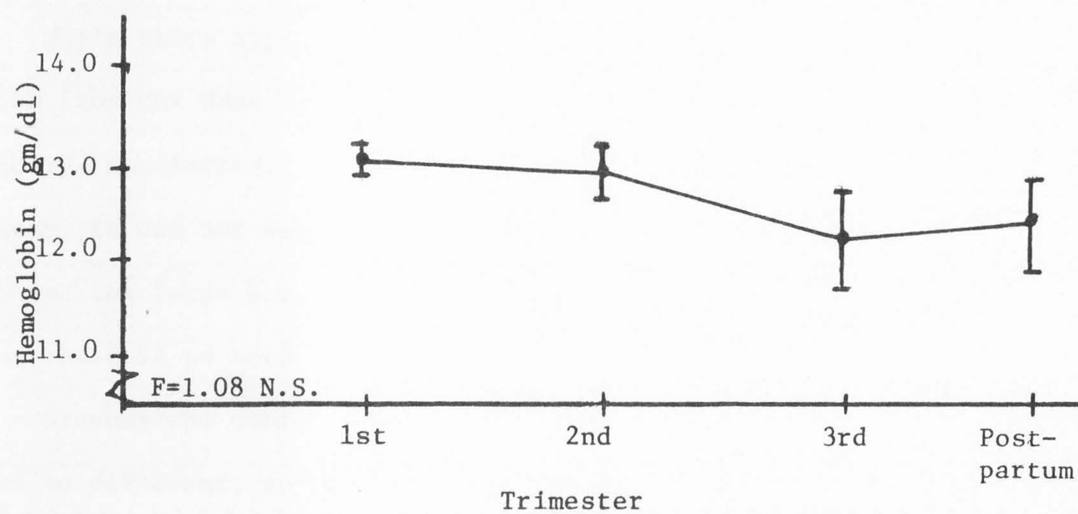


Figure 24. Influence of gestation time on hemoglobin in women.

SUMMARY

The purpose of this study was to gather further evidence for the role of molybdenum in iron mobilization during pregnancy and anemia.

Evidence for a molybdenum-iron relationship was seen in the pregnant women data. It was plainly seen that both serum iron and serum molybdenum decreased significantly at postpartum, while serum copper and hemoglobin remained relatively the same. The evidence was not as clear cut in the rat experiments.

While there appeared to be little placental transfer of molybdenum from the dams to their pups, iron and copper appeared to be readily transferred. While molybdenum absorbed well from the digestive system, it did not seem to accumulate across a placental membrane. Perhaps the fetus has little requirement for molybdenum at first, and thus there is no need for more placental transfer.

Because the control and stress factors of the three experiments were so different, no general correlations can be made among the experiments. The only correlation that can be made between the two rat experiments was that regardless of hemoglobin status, liver iron appeared to remain unaffected; neither the severity of anemia nor a normal henoglobin appeared to make any difference in liver iron stores. A longer duration of the rat experiments may have told a different story.

The only indication of a molybdenum-hemoglobin relationship occurred in the female rat experiment, where hemoglobin in both dams and pups tended to decrease as supplemental molybdenum and/or supplemental iron was absent.

Inverse relationships between molybdenum and copper were observed in both the pups' livers and the pregnant women's serum; as copper levels increased, molybdenum levels decreased. Inverse relationships between iron and copper were apparent in the pregnant women's serum and the pregnant female rats' serum and liver; as copper levels increased, iron levels decreased.

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APPENDIX

Table 4. Rat diet composition

Ingredients	gm/kg	*Mineral mix	gm/kg
Glucose	615	$CuSO_4$	0.290
Casein	200	KCl	200.00
(EDTA washed)		$Na_2HPO_4 \cdot H_2O$	360.00
Fiber	25	$MgCO_3$	37.60
Corn oil	100	$CaCO_3$	395.00
Vitamin mix	20	KI	0.79
Mineral mix*	40	$MnSO_4 \cdot H_2O$	3.78
Total	1000 gm/kg	$ZnSO_4 \cdot 7\ H_2O$	0.288
		$CaCl_2 \cdot 6\ H_2O$	0.023
		Supplements:	
		$FeSO_4 \cdot 7\ H_2O$	0.100
		$Na_2MoO_4 \cdot 2\ H_2O$	0.005

Table 5. Male rat liver mineral content

	Diet		
	Fe_+ Mo_+	Fe_+ Mo_-	Fe_- Mo_-
Wet liver iron (μg/gm liver)	$\bar{x}$ = 44 s.d. = 16 n = 12	$\bar{x}$ = 40 s.d. = 12 n = 11	$\bar{x}$ = 43 s.d. = 8 n = 12
Wet liver molybdenum (μg/gm liver)	$\bar{x}$ = 0.43 s.d. = 0.17 n = 11	$\bar{x}$ = 0.50 s.d. = 0.14 n = 11	$\bar{x}$ = 0.58 s.d. = 0.22 n = 10
Wet liver copper (μg/gm liver)	$\bar{x}$ = 18 s.d. = 9.9 n = 12	$\bar{x}$ = 19 s.d. = 4.9 n = 11	$\bar{x}$ = 27 s.d. = 17 n = 12

Table 6. Male rat serum mineral content and hemoglobin

	Diet		
	Fe_+ Mo_+	Fe_+ Mo_-	Fe_- Mo_-
Serum iron (μg/dl)	$\bar{x}$ = 41 s.d. = 4.9 n = 10	$\bar{x}$ = 34 s.d. = 5.9 n = 12	$\bar{x}$ = 39 s.d. = 4.0 n = 11
Serum copper (μg/dl)	$\bar{x}$ = 116 s.d. = 5.4 n = 8	$\bar{x}$ = 115 s.d. = 8.0 n = 11	$\bar{x}$ = 116 s.d. = 3.4 n = 12
Hemoglobin (gm/dl)	$\bar{x}$ = 7.1 s.d. = 0.8 n = 12	$\bar{x}$ = 7.6 s.d. = 1.1 n = 12	$\bar{x}$ = 4.3 s.d. = 0.9 n = 12

Table 7. Female rat liver mineral content

	Diet			
	Fe_+ Mo_+	Fe_+ Mo_-	Fe_- Mo_+	Fe_- Mo_-
Wet liver iron (µg/gm liver)	$\bar{x}$ = 34 s.d.=10 n = 6	$\bar{x}$ = 37 s.d.=16 n = 6	$\bar{x}$ = 33 s.d.=17 n = 8	$\bar{x}$ = 32 s.d.=8.4 n = 6
Wet liver molybdenum (µg/gm liver)	$\bar{x}$ = 0.35 s.d.=0.19 n = 6	$\bar{x}$ = 0.21 s.d.=0.07 n = 6	$\bar{x}$ = 0.45 s.d.=0.27 n = 8	$\bar{x}$ = 0.25 s.d.=0.09 n = 5
Wet liver copper (µg/gm liver)	$\bar{x}$ = 2.7 s.d.=0.7 n = 6	$\bar{x}$ = 2.5 s.d.=0.6 n = 6	$\bar{x}$ = 3.2 s.d.=1.7 n = 8	$\bar{x}$ = 2.6 s.d.=0.5 n = 5

Table 8. Female rat serum mineral content and hemoglobin

	Diet			
	Fe_+ Mo_+	Fe_+ Mo_-	Fe_- Mo_+	Fe_- Mo_-
Serum iron (µg/dl)	$\bar{x}$ = 181 s.d.=108 n = 3	$\bar{x}$ = 253 s.d.=30 n = 2	$\bar{x}$ = 212 s.d.=59 n = 4	$\bar{x}$ = 159 s.d.=89 n = 4
Serum copper (µg/dl)	$\bar{x}$ = 178 s.d.=97 n = 7	$\bar{x}$ = 72 s.d.=22 n = 4	$\bar{x}$ = 135 s.d.=65 n = 5	$\bar{x}$ = 185 s.d.=71 n = 3
Hemoglobin (gm/dl)	$\bar{x}$ = 13.4 s.d.=1.0 n = 6	$\bar{x}$ = 11.5 s.d.=2.1 n = 6	$\bar{x}$ = 11.2 s.d.=1.8 n = 4	$\bar{x}$ = 10.3 s.d.=1.7 n = 4

Table 9. Rat pup liver mineral content and hemoglobin

	Diet			
	Fe_+ Mo_+	Fe_+ Mo_-	Fe_- Mo_+	Fe_- Mo_-
Wet liver iron (µg/gm liver)	$\bar{x}$ = 266 s.d.=156 n = 5	$\bar{x}$ = 186 s.d.=87 n = 6	$\bar{x}$ = 111 s.d.=36 n = 8	$\bar{x}$ = 106 s.d.=69 n = 5
Wet liver molybdenum (µg/gm liver)	$\bar{x}$ = 0.12 s.d.=0.00 n = 3	$\bar{x}$ = 0.18 s.d.=0.09 n = 4	$\bar{x}$ = 0.16 s.d.=0.06 n = 8	$\bar{x}$ = 0.12 s.d.=0.02 n = 3
Wet liver copper (µg/gm liver)	$\bar{x}$ = 27 s.d.=18 n = 4	$\bar{x}$ = 17 s.d.=4.4 n = 5	$\bar{x}$ = 22 s.d.=19 n = 7	$\bar{x}$ = 23 s.d.=11 n = 5
Hemoglobin (gm/dl)	$\bar{x}$ = 10.0 s.d.=1.3 n = 6	$\bar{x}$ = 9.4 s.d.=1.6 n = 5	$\bar{x}$ = 8.8 s.d.=1.6 n = 5	$\bar{x}$ = 8.1 s.d.=1.49 n = 4

Table 10. Pregnant women serum mineral content, hemoglobin and serum xanthine oxidase

	Trimester			
	1st	2nd	3rd	Postpartum
Serum iron (µg/dl)	$\bar{x}$ = 60 s.d.=24 n = 36	$\bar{s}$ = 73 s.d.=37 n = 20	$\bar{x}$ = 67 s.d.=10 n = 9	$\bar{x}$ = 42 s.d.=10 n = 14
Serum molybdenum (µg/dl) (nondetectable assigned "0")	$\bar{x}$ = 0.79 s.d.=0.71 n = 56	$\bar{x}$ = 0.69 s.d.=0.72 n = 29	$\bar{x}$ = 0.83 s.d.=1.24 n = 22	$\bar{x}$ = 0.11 s.d.=0.30 n = 15
Serum copper (µg/dl)	$\bar{x}$ = 134 s.d.=20 n = 39	$\bar{x}$ = 166 s.d.=32 n = 32	$\bar{x}$ = 169 s.d.=29 n = 13	$\bar{x}$ = 156 s.d.=32 n = 15
Hemoglobin (gm/dl)	$\bar{x}$ = 13.0 s.d.=1.2 n = 44	$\bar{x}$ = 13.0 s.d.=1.6 n = 23	$\bar{x}$ = 12.3 s.d.=2.3 n = 18	$\bar{x}$ = 12.4 s.d.=1.8 n = 13
Serum xanthine oxidase (µM xanthine to uric acid/min at 7.5 pH)	$\bar{x}$ = 0.48 s.d.=0.23 n = 32	$\bar{x}$ = 0.59 s.d.=0.41 n = 12	$\bar{x}$ = 0.50 s.d.=0.51 n = 12	$\bar{x}$ = 0.48 s.d.=0.34 n = 16

VITA

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