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Female Rats Are Susceptible to Cardiac Hypertrophy Induced by Copper Deficiency: The Lack of Influence of Estrogen and Testosterone (42736)

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Abstract. In contrast to a previous report (Fields M, Lewis C, Scholfield DJ, Powell AS, Rose AJ, Reiser S, Smith, JC. Proc Soc Exp Biol Med **183**:145–149, 1986), female rats were shown to be susceptible to copper (Cu) deficiency giving rise to restriction of growth, cardiac hypertrophy, and anemia. The severity of these effects was, however, found to be less marked than in the male rats which had similar liver Cu levels. Castration or ovariectomy of Cu-deficient rats had little effect on CH or the other parameters associated with Cu deficiency, and supplementation of the neutered animals with estrogen or testosterone was similarly without effect. The ultrastructural appearance of the hypertrophied Cu-deficient female heart was similar to that previously found in males and was characterized by a large increase in mitochondrial area with disrupted cristae. The results also indicated that in contrast to Cu-deficient males iron (Fe) was not accumulated in the liver of the Cu-deficient female rats. It may be concluded that the limited protection of female rats to the effects of Cu deficiency observed in this study were unconnected with the sex steroids.

It is well established that dietary copper (Cu) deficiency can cause a number of cardiac abnormalities in several animal species. Some of these abnormalities include cardiac hypertrophy (CH) (1), necrotic and hemorrhaging hearts (2), fragmented elastic fibers of the aorta (3), and altered mechanical properties of the heart (4). Due to the similarity of these lesions produced in the cardiovascular system of Cu-deficient animals to those found in humans with coronary heart disease (CHD), Klevay (5) has proposed that Cu deficiency may play a role in the etiology of CHD in man. Other evidence linking Cu deficiency with CHD was the association of the former with CHD risk factors such as hypercholesterolemia (6), abnormal electrophysiology (7), hyperuricemia (8), and glucose intolerance (9).

Although the Cu intake in humans is thought to be less than the recommended 2–3 mg/day (10), there are few studies of Cu deficiency in humans. Nevertheless subjects fed a diet low in Cu developed abnormal electrocardiograms (11), hypercholesterolemia (12), and diminished glucose tolerance (13).

Most investigations on Cu deficiency have used male animals but the recent observation of Fields *et al.* (14) that Cu-deficient female rats were protected from CH and its sequelae suggested that there was a clear sex difference. In humans, a sex difference is firmly established in relation to CHD, where males are 10 times more at risk than premenopausal females; this protection of females is thought to be partly due to the production of ovarian hormones (15). These findings suggested that the cardiac abnormalities in Cu deficiency are under hormonal control with the female rats being protected by the production of estrogen. Estrogen has been shown to alter the subcellular distribution of liver Cu (16, 17) and raise ceruloplasmin and consequently Cu levels in the plasma (18). It has also been shown to increase lysyl oxidase activity (19), collagen maturation (20), and the amounts of mature collagen crosslinks (21). Endogenous estrogen could thus offer protection to the Cu-deficient female by increasing the activity of some of the Cu-dependent enzymes.

The present work was designed to assess in detail the influence of sex hormones on the development of CH in the Cu-deficient rat.

Materials and Methods. *Materials.* Testosterone propionate, β-estradiol-3-benzoate, vitamins, choline·HCl, and osmium tetroxide were obtained from Sigma (Poole, Dorset, UK). [2,4,6,7-3H]-Estradiol and [1,2,6,7-3H]-testosterone were purchased from Amersham International (Bucks, UK)

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and 17β-estradiol-carboxymethyloxime-ovalbumin was from Guildhay antisera (Department of Biochemistry, University of Surrey, Guildford, Surrey, UK). Dietary amino acids were purchased from Forum chemical (Reigate, Surrey, UK). Sucrose was obtained from Tate and Lyle (Bromley, Kent, UK) and hydroxocobalamin from Glaxo Laboratories (Glaxovet, Harefield, Uxbridge, UK). Arachis oil was obtained from A & J Beveridge (Edinburgh, UK). Glutaraldehyde and Araldite were obtained from Agar Aids (Stansted, Essex, UK). Stock diet was Labshure (CRM irradiated), which was obtained from Labshure Ltd. (Cambs, UK). All other materials were obtained from BDH (Poole, Dorset, UK).

Animals and diets. Thirty male and thirty female rats of the Rowett Hooded Lister strain were obtained from dams who had been receiving a Cu-deficient diet from 12 days postpartum. On the day of weaning (at 19 days old) bilateral ovariectomies were performed on 18 of the female rats using a dorsal approach (22) and 18 of the male rats were castrated. The remaining 12 female and 12 male rats were given sham-ovariectomy and -castration operations, respectively.

The rats were housed in groups of six in plastic cages with stainless-steel grid tops and floors and were given either control or Cu-deficient diet. The groups were the following:

Group 1. Cu-deficient castrated males.

Group 2. Cu-deficient sham castrated males.

Group 3. Cu-deficient castrated males + estrogen.

Group 4. Cu-deficient ovariectomized females.

Group 5. Cu-deficient sham-ovariectomized females.

Group 6. Cu-deficient ovariectomized females + testosterone.

Group 7. Cu-supplemented castrated males.

Group 8. Cu-supplemented sham-castrated males.

Group 9. Cu-supplemented ovariectomized females.

Group 10. Cu-supplemented sham-ovariectomized females.

The diet used was based on purified amino acids (Table I) and was similar to that described by Allen *et al.* (23). This basal diet having a Cu content of under 0.2 μg Cu/g of diet was fed to groups 1–6. The basal diet was supplemented with Cu sulfate to provide 10 μg Cu/g diet and was given to groups 7–10. Food and distilled water were available *ad libitum.* In an attempt to reduce spillage of the diet the rats were fed twice daily at 09.30 and 17.00 h. Their food intake (as a group) was recorded daily and the rats were weighed twice weekly.

For electron microscopic studies, two additional groups of female rats fed either Cu-deficient or supplemented diets were raised exactly as described previously but without surgical intervention.

Hormone supplements. Before the administration of hormones the rats were allowed to recover from their surgical procedures for 1 week. β-Estradiol-3-benzoate (20 μg/rat) and testosterone propanoate (2.5 mg/rat) were injected subcutaneously twice weekly in the back of the neck of the rats in group 3 and 6, respectively. Each hormone dose was dissolved in 250 μl of absolute alcohol and made up to 1 ml with arachis oil.

Experimental protocol. After 7 weeks on the diet the rats were weighed and anaesthetized with ether. Blood was obtained by cardiac puncture and was collected in heparinized tubes. The hearts, livers, and kidneys were weighed and stored at −20°C.

Analysis of blood. Hematocrits were determined in duplicate on blood which had been spun in microhematocrit tubes on a Beckman microcentrifuge. Hemoglobins were de-

TABLE I. COMPOSITION OF SYNTHETIC RAT DIET

Constituent	g/100 g diet
Sucrose	71.8
Amino acids[a]	18
Arachis oil	5
Mineral mix[b]	4.98
Vitamin mix[b]	0.12
Choline-HCl	0.1

[a] Amino acid mix used was as Allen *et al.* (23).

[b] Mineral mix and vitamins used was as Abdel Rahim *et al.* (24) except that $CuSO_4 \cdot 5H_2O$ was omitted and Na_2SeO_3 was added to give a final selenium concentration of 0.1 mg/kg.

termined by the cyano-methemoglobin technique.

Mineral analysis. Duplicate analyses of the diets for Cu and of liver samples for both Cu and iron (Fe) were performed with approximately 1 g of sample using atomic absorption spectrophotometry (25). Plasma Cu concentrations were determined similarly after precipitation of protein with 10% tricholoroacetic acid.

Estradiol and testosterone assay. Plasma samples from the appropriate groups of animals (see Table II) were assayed for either testosterone or 17β-estradiol by radioimmunoassay. The measured extraction efficiencies were 94% and 89% respectively, and the assay results were corrected accordingly. The assay for 17β-estradiol used [2,4,6,7-3H]-estradiol as tracer and an antiserum raised in sheep against 17β-estradiol-carboxymethyloxime-ovalbumin. The principal cross-reactions were with estrone (11%) and estriol (0.5%). All samples were assayed within a single batch, the within-assay variation being determined as 7.7% and the sensitivity (concentration at 2 SD of zero) being 2 pg/ml using a 0.4-ml sample size. The assay for testosterone used [1,2,6,7-3H]-testosterone as tracer and an antiserum described by Rowe *et al.* (26). The principal cross-reactions were with 5α-dihydrotestosterone (74%), androsterone (0.1%), and androstenedione (1.6%). Within-assay variation was 5.6% and sensitivity was 0.05 ng/ml using a 0.1-ml sample size.

Electron microscopy. Pieces of left ventricular tissue from the free wall close to the apex of the heart were fixed for 4 hr with 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.3. The tissue was washed with buffer and postfixed in 0.2% osmium tetroxide in 0.1 *M* sodium cacodylate buffer, pH 7.3, for 18 hr. All specimens were stained en-bloc with 5% uranyl acetate, dehydrated in a graded series of alcohols, and embeded in Araldite. Ultrathin sections were stained with lead citrate and examined on an Associated Electronics Industries (AEI) 801 electron microscope.

Statistical analysis. As the treatments applied to male and female animals were fundamentally different and there was no reason to expect that the variances for the two sexes

TABLE II. ESTRADIOL AND TESTOSTERONE LEVELS IN MALE AND FEMALE NEUTERED OR SHAM NEUTERED RATS FED A SUPPLEMENTED OR CU-DEFICIENT DIET[a]

	−Cu castrated	−Cu sham-castrated	−Cu castrated + estrogen	−Cu ovariectomized	−Cu sham-ovariectomized	−Cu ovariectomized + testosterone	+Cu castrated	+Cu sham-castrated	+Cu ovariectomized	+Cu sham-ovariectomized	Approximate coefficient of variation[b]	Significance of treatment[c]		
												Cu	Neutering	Interaction
Estradiol pg/ml	ND	ND	44.56 (5)	8.85	11.63	12.24	ND	ND	11.41	23.03	0.148	*	*	NS
Testosterone ng/ml	0.145	0.725 (4)	0.148 (5)	ND[d]	ND	1.86	0.155	2.54	ND	ND	0.170	***	***	***

[a] Each value represents the mean of six rats unless otherwise stated in parenthesis.
[b] To obtain approximate standard errors multiply coefficient of variation by the mean.
[c] Analysis of variance NS not significant; *$P < 0.05$; ***$P < 0.001$.
[d] ND not determined.

would be the same, the males and females were subjected to separate analysis of variance. The five treatments formed a 2 × 2 factorial (Copper × Neutering) plus one extra treatment. The analysis extracted the main effects and interactions for the factorial treatments. Where appropriate, the significance of difference between mean values were determined using Student's *t* test with a pooled estimate of error (27). Owing to the large differences in group variance a logarithmic transformation was applied to the results of the hormone assay prior to analysis.

Results. One rat from the −Cu (castrated + estrogen) group failed to gain weight and was removed from the experiment after 3 weeks. At the end of the sixth week on the diet two rats from the −Cu (sham-castrated) group died. There was no evidence to suggest that they had died from cardiac rupture as no hemothorax was noticeable. Both rats had however grossly enlarged kidneys and their bladders were engorged with blood suggesting that they had died from kidney failure.

All Cu-deficient rats displayed loss of hair pigment. The growth rate of the rats of the same sex were independent of the Cu status for approximately the first 25 days of the experiment but thereafter growth rates of all −Cu rats declined in comparison to the controls (Fig. 1). At the end of the experiment the body weight of the three −Cu female groups were very similar but the +Cu (ovariectomized) rats were heavier than the +Cu sham-controls (Fig. 1a). The two +Cu male groups had similar final body weights as did the unsupplemented −Cu male groups. The −Cu (castrated + estrogen) rats showed the most marked restriction of growth (Fig. 1b).

The groups of neutered +Cu male and female rats had, as expected, significantly lower levels of circulating testosterone (94%) and estradiol (50.5%), respectively, compared with controls (Table II). In Cu-deficient animals, however, there were also significantly lower levels of testosterone (71.5%) in males and of estradiol (49.5%) in female rats compared with their Cu-repleat counterparts.

Rats without hormone treatment. Body and individual organ weights are presented in Table III. Heart enlargement expressed as a percentage of body weight was evident in both male and female −Cu rats, an effect that was independent of ovariectomy or castration. The CH was, however, significantly greater in males than in the females with average increases of 103 and 51%, respectively.

The liver weight/body weight and the kidney weight/body weight ratios were significantly higher in the −Cu rats of both sexes. This hypertrophy was significantly lower in the ovariectomized and castrated groups (Table III). The cause of the liver and kidney hypertrophy in the −Cu rats appeared to be

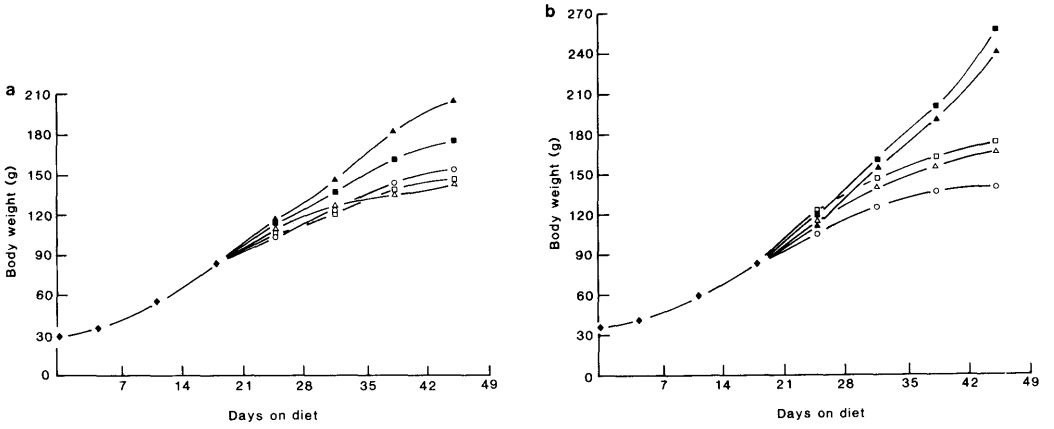

FIG. 1. Growth of (a) female and (b) male rats. The growth rates were similar for both groups up to Day 25 (♦), +Cu neutered (▲), +Cu sham-neutered (■), −Cu neutered (△), −Cu sham-neutered (□), −Cu neutered + hormone (○).

TABLE III. BODY, HEART, LIVER, AND KIDNEY WEIGHTS IN MALE OR FEMALE NEUTERED OR SHAM-NEUTERED RATS FED A SUPPLEMENTED OR CU-DEFICIENT DIET[a]

	Sex	−Cu neutered	−Cu sham-neutered	+Cu neutered	+Cu sham-neutered	−Cu hormone supplemented	Significance of treatment effects[b]			
							SED	Cu	Neutering	Interaction
Body weight (g)	M	170.3	172.1	246.7	257.4	139.5 (5)	15.34	***	NS	NS
(BW)	F	144.9	143.6	228.5	181.9	156.9	7.43	***	***	***
Heart weight (g)	M	1.19	1.22	0.91	0.91	0.85 (5)	0.10	***	NS	NS
(HW)	F	0.85	0.85	0.78	0.71	0.88	0.05	*	NS	NS
HW/100 g BW	M	0.70	0.71	0.37	0.35	0.62 (5)	0.03	***	NS	NS
	F	0.59††	0.59††	0.34	0.39	0.57	0.03	***	NS	NS
Liver weight (g)	M	8.25	10.03	9.02	10.04	8.81 (5)	0.73	NS	*	NS
(LW)	F	6.29	7.08	8.50	6.68	7.64	0.33	***	*	***
LW/100 g BW	M	4.85	5.83	3.66	3.90	6.38 (5)	0.32	***	*	NS
	F	4.37NS	4.95††	3.72	3.70	4.90	0.20	***	NS	*
Kidney weight (g)	M	0.99	1.19	1.00	1.14	1.11 (5)	0.09	NS	*	NS
(KW)	F	0.88	0.94	0.93	1.12	1.01	0.05	*	***	NS
KW/100 g BW	M	0.56	0.70	0.41	0.44	0.80 (5)	0.04	***	*	NS
	F	0.61NS	0.66NS	0.41	0.56	0.65	0.04	***	***	*

[a] Each value represents the mean of six rats unless otherwise stated in parenthesis; Significance of difference to corresponding group of male rats determined by Student's *t* test with a pooled estimate of error; NS not significant; ††$P < 0.01$.
[b] Analysis of variance; NS not significant; *$P < 0.05$, ***$P < 0.001$.

distinct from that of CH as the heart weight was significantly elevated in all the −Cu rats whereas the liver and kidney weights were not altered significantly by Cu deficiency in the males and actually were lower in the −Cu females. Thus the liver kidney "hypertrophy" appears to be associated with the lower body weight of the −Cu rats.

Indicators of Cu status are shown in Table IV. Plasma Cu (not shown) was below detectable levels in all of the −Cu animals while hemoglobin concentration, hematocrit, and liver Cu were all lowered significantly by Cu deficiency irrespective of sex or neutering. Although anemia was observed in both male and female −Cu rats it was not so marked in

TABLE IV. HEMATOLOGICAL PARAMETERS, PLASMA AND LIVER TRACE ELEMENT CONCENTRATIONS IN MALE OR FEMALE NEUTERED OR SHAM-NEUTERED RATS FED A SUPPLEMENTED OR CU-DEFICIENT DIET[a]

	Sex	−Cu neutered	−Cu sham-neutered	+Cu neutered	+Cu sham-neutered	−Cu hormone supplemented	Significance of treatment effects[b]			
							SED	Cu	Neutering	Interaction
Hemoglobin concentration (g/dl)	M	7.71	6.94 (4)	12.93	12.52	10.17 (5)	0.91	***	NS	NS
	F	9.04NS	10.16††	11.90	13.48	13.25	1.12	***	NS	NS
Hematocrit (%)	M	26.04	24.38 (4)	40.21	44.29	31.95 (5)	2.11	***	NS	NS
	F	31.83†	31.00††	38.92	39.58	41.46	2.51	***	NS	NS
Liver Cu (ng/g)	M	0.73	0.64	4.15	4.95	0.74 (5)	0.51	***	NS	NS
	F	0.82NS	0.70NS	5.52	5.38	1.17	0.31	***	NS	NS
Liver Fe (ng/g)	M	121.4	134.4	97.6	85.9	172.1 (5)	11.69	**	NS	NS
	F	144.4NS	145.0NS	171.9	189.0†††	94.0†††	25.51	NS	NS	NS

[a] Each value represents the mean of six rats unless otherwise stated in parenthesis; Significance of difference to corresponding group of male rats determined by Student's t test with a pooled estimate of error; NS not significant; †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$.
[b] Analysis of variance; NS not significant; **$P < 0.01$; ***$P < 0.001$.

the female animals. The more severe state of anemia found in the −Cu males was not associated with any differences in the liver Cu reserves as these values for both male and female Cu-deficient rats were below 1 μg/g wet weight (Table IV).

In comparison with their respective controls, the mean body weights, hemoglobin concentrations, and hematocrits of the intact −Cu female rats were 21, 25, and 22% lower, respectively, and CH was 51% higher. In the intact −Cu male rats the corresponding parameters were changed by 33, 45, 45, and 103%, respectively. This difference between the sexes was not evident in the plasma and liver Cu reserves which were similar for intact male and female rats.

Liver Fe concentrations were also unaffected by neutering but there was a significant sex difference; the +Cu female rats had significantly higher levels of Fe than the +Cu male rats. However, whereas Cu deficiency caused significantly higher liver Fe concentrations in the male rats, no difference was seen in liver Fe levels of the −Cu female rats; consequently, no significant difference in liver Fe levels of male and female Cu deficient rats.

Hormone supplemental rats. Compared with unsupplemented −Cu neutered male and female rats, estrogen-and testosterone-treated groups had higher kidney and liver weights expressed relative to body weight (Table III). The opposite effect was seen with respect to the CH. Although both −Cu neutered groups displayed CH, its magnitude was less in both hormone supplemented groups. Estrogen and testosterone supplementation apparently resulted in higher hemoglobin concentrations and hematocrits for both male and female −Cu rats. The −Cu (ovariectomized + testosterone) rats displayed no signs of anemia while the −Cu (castrated + estrogen) rats although still anemic had higher values for both parameters. The plasma and liver Cu levels were, however, unaffected by hormone treatment.

Electron microscopy. To investigate the ultrastructural pathology of the hypertrophied Cu-deficient heart in female animals, a separate group of surgically untreated rats, all showing the signs of Cu deficiency noted previously, were examined by electron microscopy. In comparison to the control tissue (Fig. 2a) the myocardium of the Cu-deficient rat was characterized by an increase in mitochondrial area and markedly enlarged individual mitochondria. The inner mitochondrial matrix was severely disrupted and vacuolated with the cristae completely absent in places (Fig. 2b).

Discussion. All of the intact (non-neutered) Cu-deficient male rats displayed several characteristic signs of Cu deficiency such as impaired growth, lower plasma and liver Cu levels, CH, anemia and higher liver Fe stores. However, in contrast to the results of Fields *et al.* (14), the female rats in this study also displayed CH, anemia, and growth inhibition. The severity of the Cu deficiency in the intact female rats, although substantial as judged by CH, anemia, and growth rate, was certainly less than that found in the corresponding −Cu male rats.

In considering possible explanations for the discrepancies between the present results and those of Fields *et al.* (14), it is likely that the severity of Cu deficiency induced in the present study was a major factor. The dietary carbohydrate source has been shown to influence greatly the severity of the final Cu deficiency (28). Fructose has been shown to produce the most severe deficiency and starch the least with glucose producing an intermediate effect (29). However, as sucrose used in this present study has been shown to produce a similar severity of Cu deficiency in rats as the fructose diet (30) used by Fields *et al.* (14), the carbohydrate source is unlikely to provide an explanation for the greater severity of the Cu deficiency induced in this work. Our experimental animals were weaned from dams receiving a Cu-deficient diet, which possibly reduced the milk Cu concentration (31) but, more importantly, the pups had no access to stock diet in the days prior to weaning.

The Cu content of the diet has been shown to be critical to the severity of the ensuing deficiency (23). Rats fed a diet containing 0.36 μg/g Cu developed more severe signs of Cu deficiency and had lower Cu reserves than rats fed a diet containing 0.7 μg/g Cu (32). It has also been shown that different dietary Cu levels can drastically alter the response of rats subjected to acute experimen-

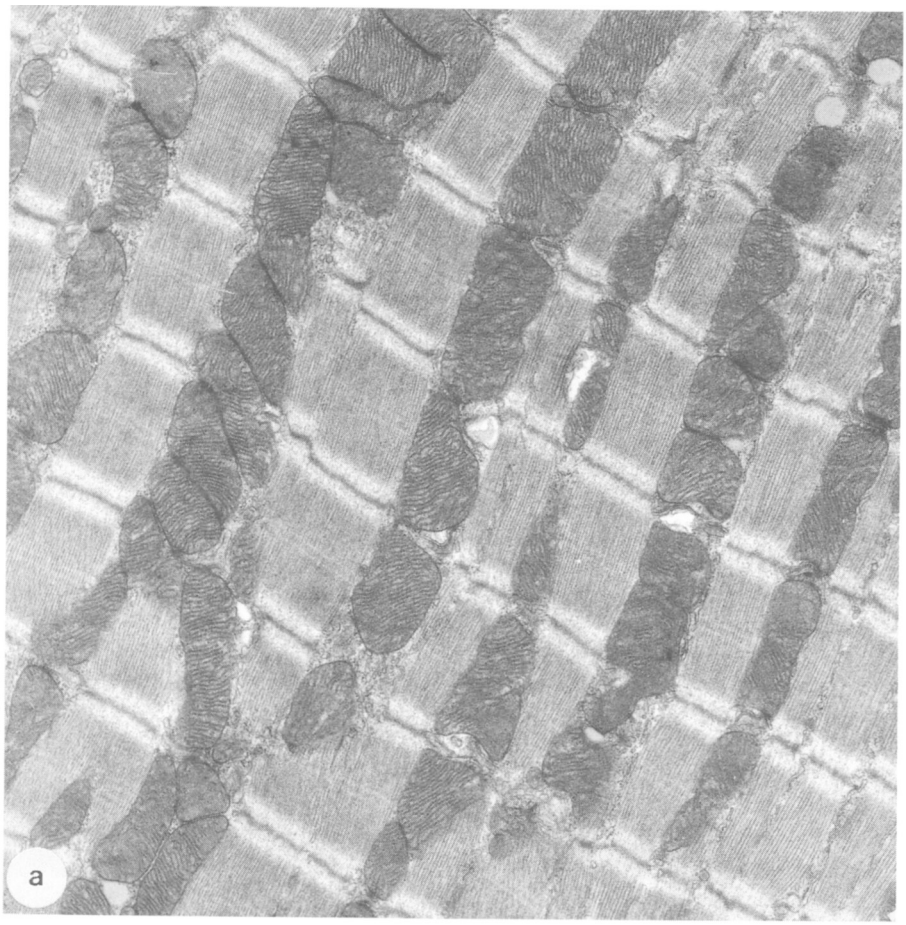

FIG. 2. Electron micrographs of myocardium from (a) control and (b) Cu-deficient rats. The mitochondria are markedly enlarged and severely disrupted in the Cu-deficient rats. Both micrographs at same magnification, ×12,680.

tal inflammation. A diet containing 0.6–0.8 μg/g Cu was found to be more protective than a diet containing 0.2 μg/g Cu (33). The lower Cu content of the diet in this study produced rats with lower liver Cu levels after a similar period on the diet than those of Fields *et al.* (14). Thus it is possible that some of the parameters of Cu deficiency such as CH and anemia are not manifest in −Cu female rats until the Cu status is extremely low. The different strain of rat could also be a contributory factor. This is however unlikely as the rats used in this study seem less susceptible to the most dramatic sign of Cu deficiency, namely cardiac rupture. In this study no aneurisms or deaths from cardiac rupture were seen, but there was a 40% mortality rate among the male rats in the study of Fields *et al.* (14). Using the same strain of rat as in the present study, Fell *et al.* (25) observed no deaths but found kidney lesions in the Cu-deficient rats. These kidney abnormalities probably contributed to the apparent kidney failure seen in this study.

The fact that the sex of the animal does not protect it from the deleterious effects of Cu deficiency is at least in part substantiated by the fact that castration or ovariectomy of the Cu-deficient rats had no dramatic effect on any of the parameters associated with Cu deficiency. The supplementation with testosterone or estrogen also did not alter greatly the severity of Cu deficiency. It is however difficult to explain why anemia in the Cu-de-

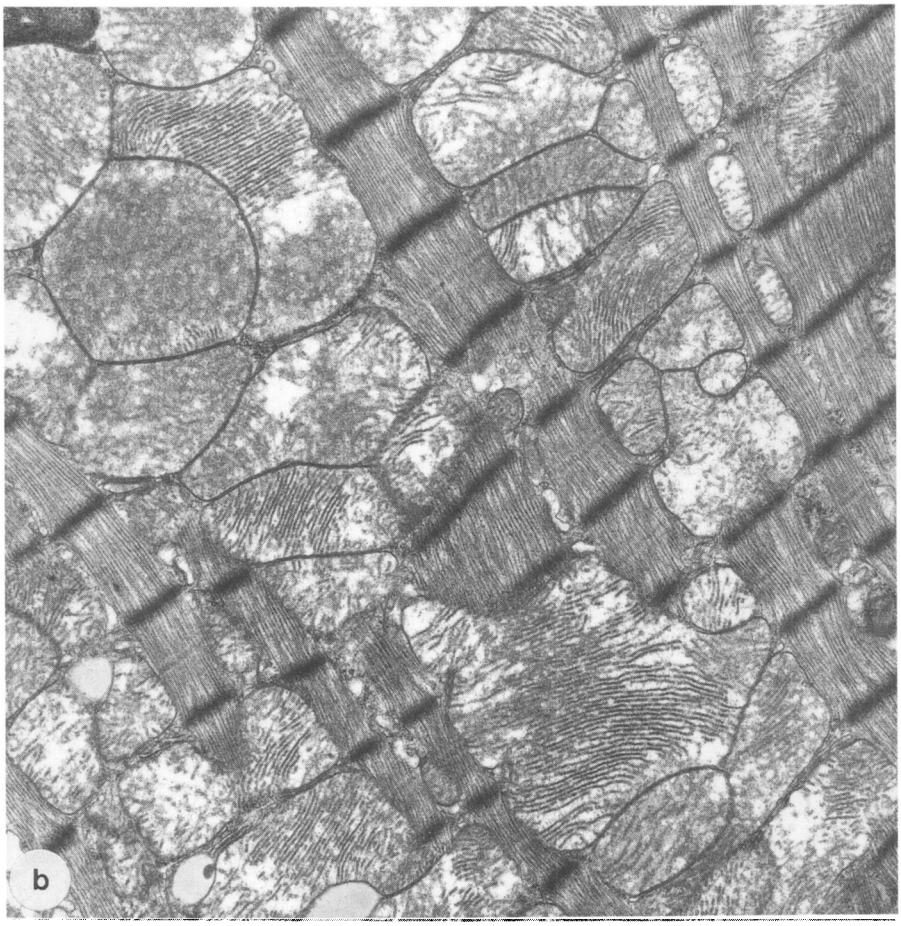

FIG. 2—*Continued.*

ficient animals was less severe in the hormone supplemented group.

The observation that female rats are susceptible to CH induced by Cu deficiency was extended by demonstrating that the ultrastructural appearance of the hypertrophied female heart was similar to that of hearts from Cu-deficient male rats (1). Disruption of the mitochondrial fine structure similar to that observed (Fig. 2b) has been demonstrated in Cu-deficient male rats (34).

As reported previously (4), the liver Fe levels in all groups of −Cu male rats were significantly higher than the +Cu controls. Higher levels of liver Fe were also found in the +Cu females in agreement with the results of others (35, 36). The −Cu female rats, however, showed no sign of Fe accumulation (Table IV), confirming the results of Cohen *et al.* (31).

Radioimmunoassays of terminal testosterone and estradiol concentrations in serum indicated that Cu deficiency resulted in significantly lower amounts compared with those of the control animals. However, variations in estradiol levels between 17 and 88 pg/ml have been noted during the 4-day estrous cycle of the rat (37) and it is impossible to conclude that the seemingly low levels of estradiol measured in the −Cu sham-ovariectomized rats were due to Cu deficiency per se. Interference of steroid metabolism could possibly result from changes in the sulfonation or liver hydroxylation of steroids (38). Further work is in progress to clarify this point.

There are conflicting accounts in the literature concerning the effects of Cu deficiency in female rodents. Sudden death and CH were reported in female mice fed a low Cu diet (39), but the CH was associated with an atypical increase in atria size. Cu deficiency induced *in utero* caused sudden death, impaired growth and CH in the resulting female offspring (31) but not when induced in nonweanling rats (40, 41). This apparent anomaly probably results from the time at which the Cu-deficient diet is introduced (31). Strause *et al.* (42), however, reported no effect on mortality rate, body weight, or hemoglobin concentration in weanling female rats fed a Cu-deficient diet containing 0.38–0.62 ppm Cu for 12 months.

The present data demonstrate that female rats are susceptible to CH, anemia, and decreased body weight induced by Cu deficiency. It seems likely that the protection noted previously in Cu-deficient female rats (14) is not maintained to the same degree when challenged by very severe Cu deficiency. The sex steroids estrogen and testosterone do not influence the susceptibility of male and female rats to Cu deficiency and are thus probably not involved with the limited protection seen in the females in this study.

During the preparation of this manuscript, Fields *et al.* (43) reported that castration of −Cu male rats ameliorated the anemia and delayed mortality whereas female rats either intact or ovariectomized were unaffected by Cu deficiency. The fact that castration delayed the mortality of −Cu male rats could not be tested in our study as no deaths from cardiac rupture were seen. Although our results are in agreement with Fields *et al.* (43) in that castration had no effect on CH further studies are necessary to resolve contradictory evidence with respect to susceptibility of female rats to Cu deficiency.

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