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EFFECT OF EXCESS AND DEFICIENT COPPER INTAKE ON RAT LIVER MICROSOMAL ENZYME ACTIVITY*

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Abstract—The effect of copper loading and copper deficiency on rat liver microsomal enzyme activity was studied. A significant reduction of liver aniline hydroxylase activity was produced by the administration of 450 ppm copper in the drinking water to rats for 15 and 30 days. Lower levels of copper (50 and 150 ppm) administered to rats for the same time had no significant effect on enzyme activity. Copper loading did not alter the activities of liver glucose 6-phosphatase or benzpyrene hydroxylase. Aniline hydroxylase and hexobarbital oxidase activities were significantly reduced, and hexobarbital sleeping time was prolonged in copper-deficient rats. Liver enzyme activities and hexobarbital sleeping times were restored to control levels by feeding copper-deficient rats a copper-containing diet for 14 days. The addition of divalent copper *in vitro* to 10,000 *g* supernatants from liver homogenates from copper-deficient rats did not restore enzyme activity to control levels. Instead, high levels of added copper produced a decreased rate of aniline hydroxylation *in vitro*. Copper deficiency did not prevent the induction of liver microsomal enzyme activity by phenobarbital. Phenobarbital administration significantly increased microsomal copper in rats receiving a normal copper intake and increased both whole liver and microsomal copper concentration in copper-deficient rats. The normal developmental increase in liver aniline hydroxylase and hexobarbital oxidase activities with age was delayed in the offspring of female rats maintained on a copper-deficient diet.

MOFFITT *et al.*[1] recently reported that administration of phenobarbital produced marked increases in rat liver microsomal copper, manganese and zinc concentrations. In contrast, the acute administration of low doses of carbon tetrachloride (CCl_4) produced a reduction in the liver microsomal concentration of these elements. The changes in metal concentrations observed in that study were accompanied by changes in liver microsomal enzyme activity. Hilderbrand *et al.*[2] demonstrated that the chronic administration of phenobarbital to rats increased whole liver copper content, but did not alter the liver content of other essential trace elements. Fahim *et al.*[3] produced the same effect by the chronic administration of 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT). Lal and Sourkes[4] showed that the hepatic copper concentration of rats treated for 1 to 8 weeks with CCl_4 was significantly reduced. Since these compounds are known to be potent inducers or inhibitors of liver microsomal enzyme activity,[5] it appears that changes in microsomal enzyme

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activity may be linked to alterations in mineral balance in general, and specifically to liver copper concentration. A possible role of essential trace metals in the maintenance of normal levels of microsomal enzyme activity is also suggested by recent reports that hepatic drug metabolism is significantly reduced during zinc,[6] magnesium[7] and calcium[8] deficiencies in rats. In addition, the metabolism of various substrates by microsomal enzyme systems in rat liver[9] and lung[10] *in vitro* is highly responsive to changes in metal ion concentrations in the assay system.

Copper is an essential trace element[11] and is a critical constituent of a number of enzymes involved in both protein and energy metabolism. Recently, human copper deficiency has been identified in both premature[12] and malnourished[13] infants. It has been suggested that high dietary copper intake by man might result from the use of copper supplements as growth stimulants in livestock.[14] Although the principal effects of copper deficiency and copper toxicity in animals are well known,[14] more subtle effects, such as possible alterations of the metabolism of drugs and other foreign compounds by the hepatic microsomal enzyme system, have not been investigated.

The present study was conducted to determine whether dietary factors which might alter hepatic copper content, i.e. copper loading and copper deficiency, would also influence basal levels of hepatic microsomal enzyme activities or would alter the induction of these enzyme systems by phenobarbital. The effect of copper deficiency on the normal developmental increase in hepatic microsomal enzyme activity in neonatal rats was also investigated. The enzyme systems selected for these studies were aniline hydroxylase, hexobarbital oxidase and benzpyrene hydroxylase, since it has been reported that they differ in response to inducers and inhibitors.[5,15] Liver glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) activity was also studied to provide a nonoxidative microsomal enzyme, which has been shown to be altered by agents which induce liver injury.[16]

MATERIALS AND METHODS

Animals and treatments. Adult male Holtzmann rats (175–250 g) were used in most experiments. Females and mixed sexes of young rats were used for studies of the development of liver microsomal enzyme activity with age. All animals were housed in plastic cages with stainless steel covers in an air-conditioned room. Food was provided in glass jars. In the copper-loading study, rats were maintained on a standard Purina rat chow diet (containing 13·9–15·9 mg copper/kg of diet). Additional copper was provided in drinking water solutions of 50, 150 and 450 ppm copper, prepared with reagent grade cupric sulfate ($CuSO_4$–$5H_2O$). Drinking water solutions were made fresh every 1 or 2 days, and the amount of water consumed was measured. Control animals received distilled water *ad lib.*

For the copper-deficiency studies, rats were maintained on a commercial copper-deficient diet* (Nutritional Biochemicals Co., Cleveland, Ohio), which contained 1–2 mg copper/kg of diet by atomic absorption analysis. This diet contained normal levels of other essential elements and vitamins. Control animals were maintained on the same diet supplemented with cupric sulfate (15 mg copper/kg of diet). All animals were provided with distilled water *ad lib.*

* Composition similar to that used by C. F. Mills and G. R. Murray, *J. Sci. Fd Agric.* **11**, 547 (1960).

Saline (0·9%) solutions of phenobarbital sodium (75 mg/kg, i.p.) were administered daily to groups of copper-deficient and control animals for 3 days prior to sacrifice. Saline was administered to groups of both copper-deficient and control animals as a control procedure. All animals were fasted overnight and sacrificed 24 hr after the last injection.

In order to study the development of liver microsomal enzyme activity in copper-deficient newborn and young rats, females (175–200 g) were maintained on a copper-deficient or control diet from 1 week prior to mating until 21 days after the birth of their litters. Litters were separated from the mothers at 21 days after birth and were maintained on a copper-deficient or control diet until the day of sacrifice. Animals from each dietary group were sacrificed at 5, 10, 20, 30 and 60 days after birth. Sexes were combined and livers were pooled for the determination of liver enzyme activities and copper content in the 5-, 10- and 20-day-old rats. Because of the low liver enzyme activity in older female rats, only male 30- and 60-day-old rats were used.

Tissue preparation and enzyme assays. At appropriate time intervals, rats were sacrificed by cervical section and exsanguinated. The livers were removed and immediately chilled in crushed ice. All subsequent steps were carried out at 0–4°. The livers were homogenized in a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, N.Y.) with 2 vol. of isotonic KCl (1·15%). Aliquots of the whole homogenate were used for the determination of glucose 6-phosphatase activity and liver metal concentrations. The remaining homogenate was centrifuged at 10,000 *g* for 15 min in a Beckman model L ultracentrifuge (rotor 40). The resulting supernatants, containing microsomes and soluble fraction, were used for all measurements of aniline hydroxylase, hexobarbital oxidase and benzpyrene hydroxylase activities. The activity of the 10,000 *g* supernatant is referred to as "microsomal enzyme activity" in this report, unless otherwise indicated. For measurements of microsomal protein and copper contents, the 10,000 *g* supernatants were centrifuged at 105,000 *g* for 1 hr. The supernatant phase was discarded and the microsomal pellet was then resuspended in sufficient KCl to give the volume of the original homogenate. Aliquots of this preparation were used for the assay of microsomal protein and copper concentrations.

Aniline hydroxylation was determined as described by Dixon *et al.*,[17] and hydroxylation of benzpyrene was determined by the method of Sunderman.[18] The metabolism of hexobarbital *in vitro* was determined by adding 1·5 ml of the 10,000 *g* supernatant to incubation mixtures containing glucose 6-phosphate (40 μmoles), NADP (3 μmoles), nicotinamide (30 μmoles), magnesium chloride (40 μmoles) and hexobarbital (3 μmoles). The incubation mixture was adjusted to a final volume of 7·5 ml with 0·1 M Tris–KCl buffer (pH 7·4). The disappearance of hexobarbital was determined as described by Cooper and Brodie.[19] All reaction mixtures were buffered at pH 7·4 and were incubated with shaking (140 cycles/min) in a Dubnoff metabolic shaker at 37° under air. Enzyme activity was expressed as micromoles of product formed or micromoles of substrate lost per g of liver per incubation time.

Liver glucose 6-phosphatase activity was determined by measuring the liberation of inorganic phosphate by the method of Cori and Cori,[20] as modified by Murphy.[21] Protein was measured by the biuret procedure.[22] All assays were performed in duplicate.

For the determination of the effect of added copper *in vitro* on microsomal enzyme

activity, no other divalent metal ions were added to the reaction mixture. The 10,000 *g* supernatants from two to three animals were pooled and increasing amounts (0·1 to 50·0 μg) of divalent copper were added as a constant volume (0·5 ml) to aliquots of the pooled supernatants. These experiments were repeated on a second pool of 10,000 *g* supernatants from different animals.

Metal analyses. Whole liver copper, manganese and zinc concentrations, and microsomal copper concentration were determined by atomic absorption spectrophotometry as previously described.[1]

Sleeping time. Sleeping times after intraperitoneal administration of hexobarbital sodium (100 mg/kg) served as an index of the rate of drug metabolism *in vivo*. The sleeping time was considered to be the time during which the righting reflex was absent.

Statistical significance of differences between means was determined with *t*-tests.

RESULTS

Effect of copper loading. Groups of rats were given drinking water containing 50, 150 or 450 ppm copper for 15 and 30 days. Whole liver copper concentration and liver enzyme activities were determined at both time intervals (Table 1). Liver copper concentration increased with increasing concentrations of copper in the drinking

TABLE 1. EFFECT OF EXCESS COPPER INTAKE ON HEPATIC ENZYME ACTIVITIES AND COPPER CONCENTRATION*

Treatment	*N*	Per cent control activity†			Liver copper (μg/g wet wt)†
		Glucose 6-phosphatase	Aniline hydroxylase	Benzpyrene hydroxylase	
Control, pooled	10	100·0 ± 10·8	100·0 ± 6·9	100·0 ± 13·9	4·7 ± 0·8
Copper, 50 ppm, 15 days	5	112·2 ± 6·6	110·1 ± 11·7	106·1 ± 16·5	7·4 ± 0·6‡
Copper, 50 ppm, 30 days	5	78·5 ± 10·6	85·6 ± 4·0	97·3 ± 8·4	8·6 ± 0·4§
Copper, 150 ppm, 15 days	5	103·1 ± 3·2	78·3 ± 8·0	92·1 ± 3·4	9·4 ± 0·8§
Copper, 150 ppm, 30 days	5	99·7 ± 1·1	80·3 ± 8·7	98·8 ± 7·6	14·6 ± 1·3§
Copper, 450 ppm, 15 days	5	103·3 ± 6·9	25·9 ± 2·2§	120·3 ± 15·9	36·1 ± 3·5§
Copper, 450 ppm, 30 days	5	104·5 ± 2·9	13·5 ± 4·0§	81·2 ± 4·7	46·1 ± 4·1§

* Copper administered via drinking water as $CuSO_4$-$5H_2O$. Purina rat chow fed *ad lib.* during the study. Control animals received distilled water.

† Values are means ± S.E. of *N* animals per test group. Enzyme activities are expressed as per cents of control activities. Respective control activities in this study were: 32·8 ± 3·6 μg Pi/mg/hr (glucose 6-phosphatase); 0·59 ± 0·04 μmoles *p*-aminophenol/g/30 min (aniline hydroxylase); and 0·94 ± 0·13 pmoles 3-hydroxybenzpyrene/g/20 min (benzpyrene hydroxylase).

‡ Significantly different from control values ($P < 0·05$).

§ Significantly different from control values ($P < 0·01$).

water. Liver glucose 6-phosphatase and benzpyrene hydroxylase activities were not significantly changed by dietary copper loading. In contrast, liver aniline hydroxylase activity was reduced to approximately 20 per cent of control levels by 450 ppm copper at both 15 and 30 days. Body weights and water consumption were recorded during the course of this study, but no significant differences from controls were detected in any of the experimental groups.

Effect of copper deficiency on liver microsomal enzyme activity. Animals that were maintained on a copper-deficient diet for 21 and 42 days showed the retardation of growth commonly found during copper deficiency[23] (Table 2); however, liver/body

TABLE 2. BODY WEIGHT GAIN, LIVER/BODY WEIGHT RATIOS AND LIVER PROTEIN IN COPPER-DEFICIENT AND CONTROL RATS*

Dietary group	Days on diet	Body wt gain (g)†	Liver (g/100 g body wt)	Liver protein (mg/g liver)
Copper-deficient	21	32·5 ± 6·1‡	3·34 ± 0·23	225·5 ± 10·8
Control	21	56·1 ± 3·2	3·82 ± 0·09	249·5 ± 14·5
Copper-deficient	42	84·7 ± 3·2‡	2·84 ± 0·30	282·0 ± 11·5
Control	42	133·0 ± 5·8	2·90 ± 0·05	301·0 ± 9·0

* Values represent mean ± S.E. of at least four animals per experimental group.
† Represents gain in weight during the indicated times on the respective diets.
‡ Significantly different from respective control values ($P < 0·05$).

weight ratios and liver protein levels were not significantly changed. Daily food consumption was recorded, but no differences from controls were detected.

Copper deficiency produced the expected reduction in whole liver copper (Table 3). The whole liver concentrations of two other essential elements, zinc and manganese, were not affected. When the deficient animals were returned to a copper-containing

TABLE 3. WHOLE LIVER METAL CONCENTRATIONS IN COPPER-DEFICIENT AND CONTROL RATS

Days on Cu-deficient diet	Metal (μg/g tissue wet wt)*		
	Cu	Zn	Mn
0(14)	5·04 ± 0·23	34·92 ± 4·03	3·64 ± 0·53
21(6)	3·79 ± 0·37†	31·19 ± 2·61	4·20 ± 0·40
42(6)	3·81 ± 0·40†	39·93 ± 1·61	3·61 ± 0·20
42 + Cu(4)‡	5·58 ± 0·21	36·84 ± 2·97	3·70 ± 0·32

* Values represent mean ± S.E. of (N) animals per test group.
† Significantly different from control values ($P < 0·05$).
‡ Copper-deficient rats were returned to a copper-containing diet (15 mg Cu/kg diet) for 14 days.

diet (15 mg/kg diet), whole liver copper concentrations returned to control values.

The effects of copper deficiency on liver microsomal enzyme activities are summarized in Table 4. A significant ($P < 0·01$) decrease in liver aniline hydroxylase activity was observed at 21 days on a copper-deficient diet. This reduced rate of aniline hydroxylation was maintained throughout the 42-day test period with deficient diet. When the deficient diet was replaced with a copper-containing diet for 14 days, aniline hydroxylase activity returned to control levels. The metabolism of hexobarbital *in vitro* was reduced to 29 per cent of control values after 42 days of feeding

TABLE 4. EFFECT OF COPPER DEFICIENCY ON HEPATIC MICROSOMAL ENZYME ACTIVITY

Days on Cu-deficient diet	N	Per cent control activity*			Hexobarbital† sleep time (min)
		Aniline hydroxylase	Benzpyrene hydroxylase	Hexobarbital oxidase	
0	14	100·0 ± 6·4	100·0 ± 4·4	100·0 ± 7·1	13·0 ± 1·5
21	6	47·4 ± 4·6‡	124·7 ± 7·7	86·4 ± 9·5	24·1 ± 3·0‡
42	6	38·8 ± 6·2‡	131·9 ± 7·2§	29·0 ± 7·3‡	32·3 ± 1·5‡
42 + Cu (14 days)	4	109·7 ± 8·9	108·2 ± 9·5	107·4 ± 5·4	15·0 ± 2·7

* Values represent mean ± S.E. of N animals per group. Respective control enzyme activities are: 0·85 ± 0·05 μmoles *p*-aminophenol/g/30 min (aniline hydroxylase); 1·20 ± 0·05 pmoles 3-hydroxybenzpyrene/g/20 min (benzpyrene hydroxylase); and 3·51 ± 0·33 μmoles hexobarbital disappeared/g/30 min (hexobarbital oxidase).

† Hexobarbital, 100 mg/kg, i.p.

‡ Significantly different from pooled control values ($P < 0·01$).

§ Significantly different from pooled control values ($P < 0·05$).

the copper-deficient diet, and hexobarbital sleeping time was prolonged at both 21 and 42 days. Both of these parameters of hexobarbital metabolism returned to control values after 14 days of copper repletion. In contrast to effects on aniline hydroxylase and hexobarbital oxidase, liver benzpyrene hydroxylase activity was slightly increased at both time periods on the copper-deficient diet. Liver glucose 6-phosphatase activity was also measured, but no differences from control activity were observed during copper deficiency.

Since the reduction in liver microsomal enzyme activity produced by copper deficiency was completely reversed by restoring copper to the diet, it seemed possible that the addition of copper *in vitro* to liver enzyme preparations from copper-deficient rats might restore activity. To test this possibility, various amounts of divalent copper were added to 10,000 *g* supernatants from control and copper-deficient rats, and the metabolism of aniline and hexobarbital *in vitro* was measured (Table 5). Low levels of copper (0·1 to 2·5 μg) produced little or no effect on aniline hydroxylation, but higher concentrations of copper were inhibitory in liver homogenates from both control and deficient rats. The metabolism of hexobarbital *in vitro* was not significantly altered by added copper at any concentration studied.

In the copper deficiency experiments, the effects noted were apparently due to an alteration of the microsomal fraction and not an effect of copper deficiency on the NADPH generating system of the soluble fraction. The metabolism of aniline and hexobarbital *in vitro* was significantly inhibited, when either the 10,000 *g* supernatant, fortified with NADP and glucose 6-phosphate, or isolated microsomes and NADPH were tested (Table 6). Furthermore, addition of the soluble fraction (105,000 *g* supernatant) from control liver to microsomes from deficient animals did not increase activity, and the activity of microsomes from control animals was not altered by addition of soluble fraction from deficient animals (Table 7).

Effect of copper deficiency on response to phenobarbital. In a previous study,[1] a marked increase in liver microsomal copper concentration occurred in rats on a normal copper-containing diet following three daily doses of phenobarbital (75 mg/kg, i.p.). This suggested that increased microsomal copper concentration might be

TABLE 5. EFFECT OF COPPER ON THE METABOLISM *in vitro* OF ANILINE AND HEXOBARBITAL IN COPPER-DEFICIENT AND CONTROL RAT LIVER 10,000 *g* SUPERNATANTS

Diet	Substrate	Copper concn (μg/incubation volume)*							
		0	0·1	0·5	1·0	2·5	5·0	10·0	50·0
Copper-deficient†	Aniline‡	0·44 ±0·02	0·47 ±0·02	0·47 ±0·02	0·50 ±0·03	0·35 ±0·01	0·33§ ±0·03	0·18§ ±0·02	0·00§ ±0·00
Control	Aniline	0·61 ±0·05	0·62 ±0·07	0·66 ±0·03	0·70 ±0·04	0·73 ±0·02	0·51 ±0·03	0·49§ ±0·03	0·24§ ±0·01
Copper-deficient†	Hexobarbital‡	3·13 ±0·29	3·15 ±0·40	3·10 ±0·33	3·63 ±0·20		4·15 ±0·025	3·05 ±0·18	2·01 ±0·35
Control	Hexobarbital	4·03 ±0·39	4·37 ±0·44	4·23 ±0·29	4·20 ±0·50		4·89 ±0·50	3·86 ±0·71	3·53 ±0·20

* Respective incubation volumes were 5·5 ml for aniline hydroxylase and 7·5 ml for hexobarbital oxidase.

† Copper-deficient animals were maintained on deficient diet for 21 days.

‡ Values are micromoles of product formed (aniline) or micromoles of substrate disappeared (hexobarbital)/g liver/30 min. Mean ± S.E. of two experiments on pooled 10,000 *g* supernatants from control or copper-deficient rat livers.

§ Significantly different from 0 μg metal ($P < 0·05$).

TABLE 6. METABOLISM *in vitro* OF ANILINE AND HEXOBARBITAL BY 10,000 *g* SUPERNATANTS AND WASHED MICROSOMES FROM COPPER-DEFICIENT AND CONTROL RAT LIVERS

Enzyme system	Dietary group*	Substrate†	
		Aniline	Hexobarbital
10,000 *g* Supernatant, NADP and glucose 6-phosphate	Control	0·85 ± 0·11	4·16 ± 0·30
	Copper-deficient	0·39 ± 0·07‡	2·30 ± 0·11‡
Microsomes and NADPH	Control	0·73 ± 0·15	1·76 ± 0·09
	Copper-deficient	0·34 ± 0·06‡	0·61 ± 0·05‡

* Groups of three rats were maintained on either a copper-deficient or the control diet for 6 weeks. Pooled livers were homogenized and centrifuged at 10,000 *g* for 15 min. The 10,000 *g* supernatant fractions were subsequently recentrifuged at 105,000 *g* for 60 min to obtain the microsomes. Aliquots of 10,000 *g* supernatants and resuspended microsomes (from 0·33 g liver) were used for enzyme activity determinations.

† Expressed as micromoles product formed (aniline) or substrate lost (hexobarbital)/g liver/30 min. Values represent mean ± S.E. of two experiments.

‡ Significantly different from control value ($P < 0·01$).

TABLE 7. INTRACELLULAR LOCALIZATION OF EFFECT OF COPPER DEFICIENCY ON LIVER MICROSOMAL ENZYME ACTIVITY

Cell fraction*		Enzyme activity†	
Soluble	Microsomes	Aniline hydroxylase	Hexobarbital oxidase
Control	Control	1·12 ± 0·21	3·44 ± 0·22
Control	Copper-deficient	0·58 ± 0·10	2·02 ± 0·13
Copper-deficient	Control	1·23 ± 0·12	3·46 ± 0·18
Copper-deficient	Copper-deficient	0·56 ± 0·09	2·14 ± 0·20

* Groups of three rats were maintained on either a copper-deficient or control diet for 6 weeks. After sacrifice, pooled livers were fractionated into microsomes and soluble fraction. Various combinations of microsomes and soluble fractions were made as indicated. Each assay was performed in duplicate, and contained microsomes and supernatant from 0·33 g liver, with NADP and glucose 6-phosphate added as cofactors.

† Expressed as micromoles product formed or substrate lost/g liver/30 min. Values represent mean ± S.E. of two experiments.

required for microsomal enzyme induction. If this were true, the reduction in whole liver and subcellular copper produced by copper deficiency[24] might diminish the induction of liver microsomal enzyme activity by phenobarbital.

To test this possibility, phenobarbital (75 mg/kg/day, i.p., for 3 days) was administered to groups of copper-deficient and control rats, and liver aniline hydroxylase and hexobarbital oxidase activities were determined. In both control rats and rats on copper-deficient diet, phenobarbital administration produced a significant increase in liver aniline hydroxylase activity (Table 8). Since copper deficiency alone produced a marked reduction in aniline hydroxylase activity, the appropriate control here should be the deficient animals. On this basis, phenobarbital treatment produced a greater relative increase in aniline hydroxylase activity in copper-deficient rats than in controls. Liver hexobarbital oxidase activity was also significantly increased by

TABLE 8. EFFECT OF PHENOBARBITAL ON LIVER MICROSOMAL ENZYME ACTIVITY IN COPPER-DEFICIENT AND CONTROL RATS

Weeks on diet	Dietary group	Enzyme activity (μmoles/g/30 min)*			
		Aniline hydroxylase	% of control	Hexobarbital oxidase	% of control
3	Control	0·85 ± 0·05		3·51 ± 0·33	
3	Control and PB†	2·60 ± 0·15‡	306·0	6·44 ± 0·25‡	183·4
3	Copper-deficient	0·40 ± 0·04		3·03 ± 0·08	
3	Copper-deficient and PB	2·18 ± 0·13‡	545·0	5·11 ± 0·46‡	168·6
6	Control	0·94 ± 0·07		3·35 ± 0·30	
6	Control and PB	2·30 ± 0·03‡	266·0	5·71 ± 0·24‡	170·5
6	Copper-deficient	0·38 ± 0·08		1·50 ± 0·31	
6	Copper-deficient and PB	1·47 ± 0·25‡	386·8	4·60 ± 0·19‡	306·1

* Values represent mean ±S.E. of at least four animals per group.

† Phenobarbital sodium (PB) was administered i.p., 75 mg/kg/day, on days 18–20 (3-wk study) or on days 39–41 (6-wk study). Control animals received saline. All animals were sacrificed 24 hr after the last dose of phenobarbital.

‡ Significantly different from respective saline-treated control or copper-deficient animals ($P < 0·01$).

phenobarbital in both dietary groups at both time periods. At 6 weeks, phenobarbital administration produced a greater relative increase in hexobarbital oxidase activity in copper-deficient rats than in controls.

In another experiment, the time response of changes in liver microsomal enzyme activity, protein and copper concentration after the administration of phenobarbital for 1, 2 or 3 consecutive days was determined in rats on the control diet and on a copper-deficient diet for 6 weeks (Tables 9 and 10). Although feeding the copper-deficient diet reduced the activity of both liver aniline hydroxylase and hexobarbital oxidase by more than 50 per cent, it did not alter microsomal protein concentration in rats not treated with phenobarbital (Table 9). Aniline hydroxylase and hexobarbital oxidase activities were significantly increased after the first dose of phenobarbital in both dietary groups. Microsomal protein was not significantly increased until after the second dose. Although copper deficiency decreased whole liver copper levels by approximately 35 per cent in this study, microsomal copper was not significantly reduced. Whole liver copper concentrations in copper-deficient rats were significantly increased by phenobarbital at all time periods studied, while control values remained unchanged. On the second and third day of phenobarbital injections, microsomal copper levels in control and copper-deficient rats were significantly increased.

Effect of copper deficiency on development of liver microsomal enzyme activity. In man and many animal species, the copper concentration in neonatal liver is greater than that in adult liver due to a decreased capacity for hepatic copper removal in the neonate.[25] Age also influences the subcellular distribution of copper. Microsomal copper is approximately five times greater in 10-day-old rats than in adults.[25] Since liver microsomal enzyme activity also follows a normal developmental pattern of increasing activity with increasing age,[5] it seemed possible that the high liver copper concentration in the neonate may play some role in the development of microsomal enzyme activity. The relationship between these two phenomena was studied by

Table 9. Time response of phenobarbital effect on liver microsomal enzyme activity and microsomal protein in copper-deficient (6 weeks) rats

No. of phenobarbital injections*	Control†			Copper-deficient†		
	Aniline hydroxylase	Hexobarbital oxidase	Microsomal protein	Aniline hydroxylase	Hexobarbital oxidase	Microsomal protein
0	0·94 ± 0·07	3·35 ± 0·30	22·8 ± 2·3	0·38 ± 0·08	1·50 ± 0·31	24·0 ± 2·5
1	1·41 ± 0·06‡	4·62 ± 0·33§	26·3 ± 1·4	0·65 ± 0·09§	3·20 ± 0·18‡	27·7 ± 2·1
2	2·05 ± 0·13‡	4·91 ± 0·14‡	35·3 ± 2·3§	1·03 ± 0·05‡	4·03 ± 0·17‡	33·1 ± 1·8§
3	2·30 ± 0·03‡	5·71 ± 0·24‡	35·1 ± 2·7§	1·47 ± 0·25‡	4·60 ± 0·19‡	34·9 ± 2·7§

* Phenobarbital was administered i.p., 75 mg/kg/day, for the indicated number of consecutive days. Control animals received saline. Animals were sacrificed 24 hr after the last injection.

† Enzyme activity is expressed as micromoles product formed or substrate lost/g/30 min. Microsomal protein is expressed as milligrams protein/g wet liver. Each value represents the mean ±S.E. of at least four animals per group. The control group (0 phenobarbital injections) represents seven animals accumulated over all time periods.

‡ Significantly different from saline-treated animals of the respective dietary group ($P < 0.01$).

§ Significant difference ($P < 0.05$).

TABLE 10. EFFECT OF PHENOBARBITAL ON WHOLE LIVER AND MICROSOMAL COPPER CONCENTRATIONS IN COPPER-DEFICIENT (6 weeks) AND CONTROL RATS

No. of phenobarbital injections*	Copper concn (μg/g liver wet wt.)†			
	Control		Copper-deficient	
	Whole liver	Microsomal	Whole liver	Microsomal
0	6·06 ± 0·56	0·94 ± 0·09	3·94 ± 0·20	0·78 ± 0·10
1	6·23 ± 0·12	0·98 ± 0·15	4·82 ± 0·31‡	0·85 ± 0·29
2	6·09 ± 0·44	1·59 ± 0·11§	5·38 ± 0·41§	1·44 ± 0·18§
3	6·40 ± 0·60	2·10 ± 0·16§	6·38 ± 0·33§	1·74 ± 0·15§

* Phenobarbital was administered i.p., 75 mg/kg/day, for the indicated number of consecutive days. Control animals received saline.

† Each value represents the mean ±S.E. of at least four animals per group.

‡ Significant difference ($P < 0·05$).

§ Significantly different from saline-treated animals of respective dietary group ($P < 0·01$).

determining the effect of copper deficiency on microsomal enzyme activity in the developing rat (Fig. 1).

Female rats were maintained on a copper-deficient or control diet from 1 week prior to mating until 21 days after the birth of their litters. Copper deficiency did not

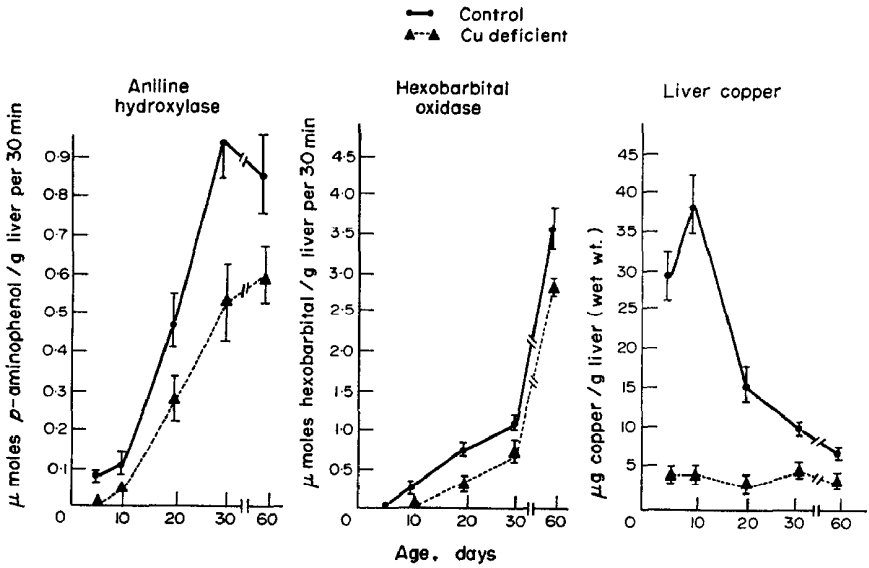

FIG. 1. Effect of copper deficiency on the development of liver enzyme activity and copper concentration with age. The offspring of copper-deficient and control female rats were maintained on the respective diets until sacrifice at the indicated days after birth. Values for 5-, 10- and 20-day-old rats represent the mean ± S.E. of four groups of three pooled livers, sexes combined. Values for 30- and 60-day-old rats represent the mean ± S.E. of five to eight male rats. Each value for copper-deficient rats (▲ - - - ▲) differs significantly from the corresponding control (● —— ●) value ($P < 0·05$).

affect the course of pregnancy, but litter size was significantly reduced (control, 12·5 ± 1·7; copper-deficient, 8·1 ± 1·2, $P < 0·05$). The offspring of the deficient and control mothers were maintained on the respective diets after separation from their mothers at 21 days. Animals were sacrificed at various ages, and liver microsomal enzyme activities and copper concentrations were determined. Copper deficiency produced an apparent delay in onset of the developmental increase in hepatic aniline hydroxylase and hexobarbital oxidase activities; however, the rate of increase in activity with age appears to be the same in both dietary groups, with copper-deficient rats exhibiting lower activity at each age studied. Copper deficiency also prevented the marked increase in liver copper concentration observed in control animals at 5 and 10 days after birth. Whole liver copper levels were significantly reduced in copper-deficient rats at all ages studied.

DISCUSSION

The results of the present investigation indicate that an optimum level of dietary copper is essential for the metabolism of certain substrates by rat liver microsomal enzymes, and that both excesses and deficiencies of copper result in diminished enzyme activity.

Yamane *et al.*[26] have reported that the chronic administration of 0·5% basic cupric acetate in a maize diet to rats produced a significant enhancement of liver microsomal azo-reductase activity and a depression of microsomal oxidative *N*-demethylation. In our studies, the administration of 50, 150 and 450 ppm copper (as cupric sulfate) in the drinking water for 15 and 30 days had no apparent effect on liver benzpyrene hydroxylase or glucose 6-phosphatase activities. However, liver aniline hydroxylase activity was significantly reduced by the highest level of copper (450 ppm). This result correlates with the depression of aniline hydroxylation produced by high levels of divalent copper *in vitro*, reported by Peters and Fouts[9] and confirmed in this study. Therefore, it appears that the effect of high dietary copper intake on liver microsomal enzyme activity may depend upon the particular substrate (drug) studied.

The reduction of the metabolism of aniline *in vitro* and the metabolism of hexobarbital both *in vitro* and *in vivo* in copper-deficient rats was reversed by the restoration of copper to the diets. Therefore, it is probable that the observed alteration of liver microsomal enzyme activity was a direct result of a lack of copper in the diet. Although a reduced growth rate was observed in our rats fed copper-deficient diets, it is unlikely that the reduction of microsomal enzyme activity was an effect of starvation,[27] because copper-deficient rats did not have reduced food consumption. Weight loss has been described as characteristic of copper deficiency, and is not due to anorexia.[23] The effects of copper deficiency on hepatic microsomal enzyme activity cannot be explained by a decreased availability of the NADPH generating system of the soluble fraction, since the source of soluble fraction (control or deficient animals) had no effect on enzyme activity (Table 7).

Although copper deficiency decreased the metabolism of hexobarbital and aniline *in vitro*, representative type I and type II substrates of liver microsomal enzymes[28] respectively, the hydroxylation of benzpyrene, another type I substrate, was not depressed during copper deficiency. A possible explanation of this disagreement may be that copper is essential for the binding of certain chemical classes of substrates

(e.g. nitrogen-containing compounds) to microsomal lipoprotein membranes or to cytochrome P-450. Such a mechanism has been postulated for the role of zinc in hepatic drug metabolism.[6] Copper does not appear to be essential to the catalytic activity of the liver microsomal enzyme system, since the addition of cupric ion to crude liver enzyme preparations from either copper-deficient or control rats either inhibited or did not alter the metabolism of aniline or hexobarbital. One possible explanation of the effects of copper loading or high levels of cupric ion *in vitro* on microsomal enzyme activity is that excess copper may displace or block the site of action of other divalent metals in the microsomes.

Copper deficiency did not prevent the induction of liver microsomal enzyme activity or microsomal protein by phenobarbital. In agreement with a previous study,[1] repeated phenobarbital administration significantly increased liver microsomal copper concentration. In the present study, a single dose of phenobarbital significantly increased liver microsomal enzyme activity in control and copper-deficient rats, but did not elevate microsomal copper. This suggests that the induction of microsomal enzymes by phenobarbital is not dependent upon increased microsomal copper. This is further supported by the observation that developmental increases in microsomal enzyme activities occurred, though delayed in onset, in copper-deficient neonates. The increased microsomal copper levels that were observed after two or three doses of phenobarbital may be related to an increased binding of cytoplasmic copper by the induced levels of microsomal protein. The chronic administration of phenobarbital has been shown to alter copper metabolism markedly, producing dramatic decreases in muscle copper and increases in serum and liver copper.[2] The increase in whole liver copper content in copper-deficient rats after phenobarbital may possibly be explained by the effect of this compound on whole body copper metabolism. In addition, Owen and Hazelrig[29] reported that copper-deficient liver has a higher affinity for copper than control liver, as demonstrated by ^{64}Cu perfusion studies.

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