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HEPATIC DRUG METABOLISM IN ZINC-DEFICIENT RATS

G. C. BECKING and A. B. MORRISON

Research Laboratories, Food and Drug Directorate, Ottawa, Canada

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Abstract—The rates of metabolism *in vitro* of pentobarbital, aminopyrine and *p*-nitrobenzoic acid were significantly reduced and the duration of action of pentobarbital *in vivo* was prolonged in zinc-deficient rats. Zinc deficiency did not result in an alteration in the rate of aromatic ring hydroxylation *in vitro*, with either aniline or zoxazolamine as substrate. Microsomal protein and RNA levels were maintained during zinc depletion, but a significant decrease in the cytochrome P-450 content of the microsomes was noted in zinc-deficient rats at approximately the same time as the lower rate in metabolism of pentobarbital was found. Biochemical lesions and gross signs of zinc deficiency were completely ameliorated by refeeding zinc-deficient rats a zinc-containing diet for 14 days.

IT HAS become increasingly evident during the last few years that any alteration in microsomal drug-metabolizing enzyme systems may lead to a change in the rate of biotransformation of drugs and to altered duration of drug activity or toxicity or both.[1] The nutritional status of an animal can markedly alter the rate at which it can metabolize drugs, as shown by studies on the effects of starvation,[2] dietary protein content[3, 4] and calcium deficiency.[5]

Zinc is required for a variety of biochemical reactions[6, 7] in mammalian metabolism. Many enzymes important in both protein and energy metabolism are dependent on zinc ions for maximal activity. A zinc deficiency syndrome has recently been described in man.[8] The studies described herein were conducted to determine whether zinc plays a role in the metabolism of drugs by hepatic microsomes.

MATERIALS AND METHODS

Immature male rats of the Wistar strain, weighing approximately 75 g, were divided into three groups. Group 1 (*ad lib.* control) received *ad lib.* a diet containing in per cent: egg white, 15; corn starch, 65; corn oil, 10; cellulose, 5; minerals,[9] 4; and vitamins,* 1. Group 2 (zinc deficient) received *ad lib.* a similar diet, except that the zinc was omitted from the mineral mixture. Group 3 (isocaloric control) received the zinc-containing diet in an amount equal to that consumed by the rats in group 2. Food was supplied in aluminum and stainless steel cans; the animals were housed individually in stainless steel cages and received glass-distilled water *ad lib.*

Enzyme preparation. Rats were killed by cervical dislocation; the livers were removed and immediately chilled in crushed ice. All subsequent preparative steps were carried

* Composition similar to that used by D. G. Chapman, R. Castillo and J. A. Campbell, *Can. J. Biochem. Physiol.* 37, 679 (1959), except that the vitamin A content was increased to 5000 I.U./kg of diet.

out at 0–4°. The livers were homogenized in a Teflon-glass homogenizer with 3 vol. of isotonic KCl containing 1×10^{-4} M EDTA and the homogenate was centrifuged at 17,500 *g* for 20 min. The supernatants were used for all drug metabolism studies *in vitro*. Where microsomes were required, the appropriate high-speed supernatants were centrifuged at 140,000 *g* (av.) for 1 hr. Microsomal pellets were suspended in isotonic KCl, recentrifuged for 1 hr and suspended finally in 0·1 M sodium phosphate buffer (pH 7·4) at a concentration such that 1·0 ml suspension was equivalent to 200–300 mg liver. To avoid differences in drug metabolism caused by daily rhythmic variation,[10] all animals were killed between 8:00 and 9:00 a.m. throughout these studies.

Enzyme assays. Drug metabolism *in vitro* was studied by adding 1·0 ml of the 17,500 *g* supernatant to incubation mixtures containing sodium phosphate buffer (300 μmoles, pH 7·4), glucose 6-phosphate (50 μmoles), nicotinamide (100 μmoles), magnesium chloride (25 μmoles), NADP (3 μmoles) and various substrates (2 μmoles pentobarbital, 2·4 μmoles zoxazolamine, 10 μmoles aminopyrine aniline or *p*-nitrobenzoic acid) in a final volume of 5·0 ml.

The mixtures were incubated for 1 hr in a Dubnoff metabolic shaker at 37° under air, except for the reduction of *p*-nitrobenzoic acid, which was carried out in an atmosphere of nitrogen. Under these conditions, essentially linear rates of metabolism were obtained for all drug substrates.

Metabolism of pentobarbital was determined essentially as outlined by Brodie *et al.*[11] Enzyme activity was terminated by the addition of 5·0 ml of 1 N HCl and the barbiturate was extracted with 40 ml of a petroleum ether–isoamyl alcohol mixture. Excess nicotinamide was removed by washing the petroleum ether phase with 5 ml of 1·5 M sodium dihydrogen phosphate and shaking vigorously for 3 min. The reduction of *p*-nitrobenzoic acid and the demethylation of aminopyrine were determined as previously described.[12] Hydroxylation of zoxazolamine was determined by measuring the disappearance of substrate as outlined by Conney *et al.*[13] and aniline hydroxylation was determined as outlined by Schenkman *et al.*[14]

Glucose 6-phosphate dehydrogenase activity was measured at room temperature by the method of Balinsky and Bernstein.[15] Glucose 6-phosphatase assays were carried out by the procedure of Appelmans *et al.*[16] and inorganic phosphorus was measured by the method of Hurst and Becking.[17]

Chemical analyses. Protein was measured by the biuret procedure.[18] Prior to biuret analysis, 10% sodium deoxycholate was added to all samples to eliminate turbidity.

Microsomal RNA was determined as outlined by Hayden and Becking[19] without removal of lipid-soluble material prior to alkaline hydrolysis.

In studies on urinary ascorbic acid excretion, urine was collected in 10% oxalic acid and the ascorbic acid was assayed the same day by the 2,6-dichlorophenolindophenol method.

Cytochrome P-450 was assayed essentially by the method of Omura and Sato.[20] Duplicate aliquots of microsomal suspensions (containing 2–3 mg protein/ml in sodium phosphate buffer, pH 7·4) were placed in glass-stoppered tubes. Oxygen-free carbon monoxide was slowly bubbled through one tube for 1 min and oxygen-free nitrogen through the second sample. A few milligrams of solid sodium dithionite were added to each tube and the carbon monoxide-treated sample was regassed for 30 sec. Using the nitrogen-treated sample as a reference, the quantity of P-450 was calculated

from the difference of the optical densities between 450 and 490 mμ. The levels of P-450 were expressed as millimicromoles per gram of liver (wet weight) using the extinction coefficient reported by Omura and Sato.[21]

Sleeping time. This measured the time which elapsed between the intraperitoneal injection of sodium pentobarbital (35 mg/kg) and the reappearance of the righting reflex. Each group contained eight animals.

Statistical evaluation of all data was made by the rank sum method of Wilcoxon.[22]

RESULTS

Curves of weight gain (Fig. 1) for the three groups of animals showed the marked retardation of growth commonly found during zinc depletion and indicated the effect of adding zinc to the diet of deficient animals. After 14 days on a zinc-containing

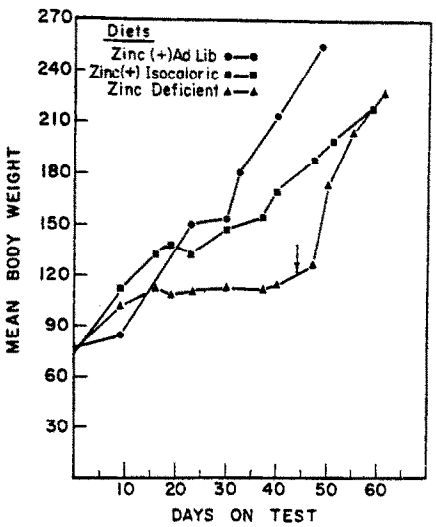

FIG. 1. Growth of control and zinc-deficient rats. The arrow indicates the time when zinc-deficient animals were fed the zinc-containing control diet.

diet, formerly deficient (40–44 days on test) animals appeared normal and their food consumption returned to that of the control animals. A marked anorexia, typical of zinc deficiency, was noted approximately 15 days after administration of the zinc-deficient diet was begun. Signs of severe zinc deficiency recently reported[23] did not appear in our animals until they had received the zinc-deficient diet for more than 50 days.

A significant decrease in the urinary excretion of ascorbic acid was noted after 24 days on the zinc-deficient diet (Table 1). The reduced rate of ascorbic acid excretion was maintained until the animals were given a diet containing zinc. After 14 days of recovery on a zinc-containing diet, urinary ascorbic acid levels were essentially equal to those of control rats.

The prolonged pentobarbital sleeping time shown in Table 2 was the first indication that zinc deficiency altered the rate of drug metabolism. This effect was noted prior to the onset of severe zinc deficiency symptoms other than growth retardation and

TABLE 1. EFFECT OF ZINC DEPLETION ON URINARY EXCRETION OF ASCORBIC ACID*

Diet	Days on test	Ascorbic acid excretion (mg/24 hr) 24	56	42 + 14
Ad lib. control		0·33 ± 0·02	0·30 ± 0·01	
Isocaloric control		0·29 ± 0·02	0·32 ± 0·02	
Zinc deficient		0·17 ± 0·01†	0·12 ± 0·01†	
Zinc deficient + zinc				0·28 ± 0·03

* Results are expressed as the mean values obtained with five rats ± S. E. M.
† Significantly different from isocaloric control values ($P < 0·01$).

TABLE 2. EFFECTS OF ZINC DEPLETION ON PENTOBARBITAL* SLEEPING TIMES

Diet	Days on test	Sleeping time (min)
Ad lib. control		49 ± 4
Isocaloric control	38	47 ± 3
Zinc deficient		70 ± 5†
Isocaloric control	55	41 ± 4
Zinc deficient + zinc	40 + 15	46 ± 5

* Administered as an i.p. dose of 35 mg/kg to groups of eight rats. Results are expressed as the mean value obtained with eight rats ± S. E. M.
† Significantly different from control values ($P < 0·01$).

TABLE 3. EFFECT OF ZINC DEPLETION ON THE METABOLISM *IN VITRO* OF PENTOBARBITAL*

Diet	Days on test	Pentobarbital metabolized (mμmoles/mg protein/hr) 37	58	44 + 14
Ad lib. control		9·90 ± 1·77	11·40 ± 0·38	
Isocaloric control		6·51 ± 0·80	11·34 ± 0·56	
Zinc deficient		4·29 ± 0·29†	4·21 ± 0·59‡	
Zinc deficient + zinc				10·37 ± 0·64

* Results are expressed as the mean value obtained with five rats ± S.E.M.
† Significantly different from isocaloric control values ($P = 0·05$).
‡ Significantly different from isocaloric control values ($P < 0·01$).

changes in hair texture. The return to control values after 14 days of zinc repletion indicated that the alteration in pentobarbital metabolism *in vivo* was a direct result of the lack of zinc in the diet.

No significant alteration in the rate of drug metabolism *in vitro* was found prior to 37 days on the zinc-deficient diet. The only alteration found at 37 days was a marked lowering of pentobarbital oxidation (Table 3). This lower rate of hepatic metabolism

of pentobarbital probably accounted for the prolonged sleeping times found in zinc-deficient rats (Table 2). It is apparent from Table 3 that after 14 days of feeding a diet containing zinc to formerly deficient animals pentobarbital metabolism was essentially the same as that of the isocaloric control rats.

Other hepatic drug-metabolizing systems studied were not found to be as sensitive to zinc depletion as was pentobarbital oxidation. A gradual decrease in the rate of metabolism of aminopyrine (Table 4) was found, but no significant alteration in the *N*-demethylation of aminopyrine was found until 44–46 days on the zinc-deficient diet. As with pentobarbital, repletion of zinc-deficient animals with zinc for 14 days resulted in a return to normal of the rate of aminopyrine metabolism.

TABLE 4. EFFECT OF ZINC DEPLETION ON THE METABOLISM *IN VITRO* OF AMINOPYRINE*

		4-Aminoantipyrine (mμmoles/mg protein/hr)		
Diet	Days on test	46	58	44 + 14
Ad lib. control			6·42 ± 0·85	
Isocaloric control		4·75 ± 0·30	5·09 ± 0·47	
Zinc deficient		2·99 ± 0·41†	2·41 ± 0·40‡	
Zinc deficient + zinc				4·26 ± 0·39

* Results are expressed as the mean value obtained with five rats ± S. E. M.
† Significantly different from isocaloric control values ($P = 0·05$).
‡ Significantly different from isocaloric control values ($P < 0·01$).

TABLE 5. EFFECT OF ZINC DEPLETION ON THE METABOLISM *IN VITRO* OF *p*-NITROBENZOIC ACID*

		p-Aminobenzoic acid (mμmoles/mg protein/hr)		
Diet	Days on test	46	58	44 + 14
Ad lib. control			2·22 ± 0·28	
Isocaloric control		1·11 ± 0·20	2·41 ± 0·25	
Zinc deficient		0·69 ± 0·05†	1·09 ± 0·07‡	
Zinc deficient + zinc				1·99 ± 0·23

* Results are expressed as the mean value obtained with five rats ± S. E. M.
† Significantly different from control values ($P = 0·05$).
‡ Significantly different from control values ($P < 0·01$).

Nitroreductase activity during zinc depletion (Table 5) was significantly ($P < 0·01$) reduced after 58 days of feeding the zinc-deficient diet.

No alteration in the rate of ring hydroxylation *in vitro* was found (Table 6) even when rats were fed the zinc-deficient diet for 58 days. It is evident that similar results were obtained when aniline or zoxazolamine was used as the substrate to measure aromatic ring hydroxylation.

Protein, RNA and cytochrome P-450 levels in rat liver microsomes from zinc-deficient and control animals are summarized in Table 7. The RNA and protein content of liver microsomes from zinc-deficient rats was essentially the same as in control animals. After 42 days on the zinc-deficient diet, the cytochrome P-450 content of microsomes from the deficient animals was significantly lower than that of controls ($P < 0.05$). Since repletion of zinc-deficient animals with zinc increased the P-450

TABLE 6. RING HYDROXYLATION *IN VITRO* OF ZOXAZOLAMINE AND ANILINE DURING ZINC DEPLETION*

		Zoxazolamine metabolized		*p*-Aminophenol formed
		(mμmoles/mg protein/hr)		
Diet	Days on test	37	58	58
Ad lib. control		5·82 ± 0·79	4·19 ± 0·43	
Isocaloric control		4·73 ± 0·54	3·74 ± 0·64	2·99 ± 0·28
Zinc deficient		3·33 ± 0·74	3·46 ± 0·60	3·44 ± 0·44

* Results are expressed as the mean value obtained with five rats ± S. E. M.

TABLE 7. MICROSOMAL PROTEIN, RNA AND CYTOCHROME P-450 LEVELS IN ZINC-DEFICIENT RATS*

		Protein	RNA	
Diet	Days on test	(mg/g wet liver)		Cytochrome P-450 (mμmoles/g wet liver)
Isocaloric control	35	24·7 ± 1·5	40·8 ± 0·33	20·9 ± 0·6
Zinc deficient		26·0 ± 1·1	4·66 ± 0·29	18·3 ± 0·7†
Isocaloric control	42	27·3 ± 1·0	5·25 ± 0·24	21·8 ± 0·8
Zinc deficient		25·1 ± 1·2	4·67 ± 0·36	17·7 ± 1·0‡
Isocaloric control	56	26·2 ± 0·9	4·79 ± 0·20	22·2 ± 0·6
Zinc deficient + zinc	42 + 14	24·2 ± 1·3	4·96 ± 0·17	21·1 ± 0·8

* Results are expressed as the mean value obtained with five rats ± S. E. M.
† Significantly different from control values (P approaches 0·05).
‡ Significantly different from control values ($P < 0.05$).

level to that found in controls, the lower level of P-450 found during zinc depletion was a result of the low level of dietary zinc and was not due to some other dietary stress.

No evidence was obtained during our studies that liver homogenates of zinc-deficient rats had altered glucose 6-phosphate dehydrogenase or glucose 6-phosphatase activities.

DISCUSSION

These studies show the effect of zinc deficiency on the ability of rats to metabolize several classes of drugs. Since the addition of zinc to the deficient diet eliminated the growth retardation and lowered drug metabolism, the observed alterations were clearly the result of zinc deficiency and cannot be attributed to other causes. Use of an

isocaloric control eliminated, in part, the possibility that the reduced food consumption by zinc-deficient animals could have produced the effects observed.

It is well known that the biosynthesis of ascorbic acid by the glucuronic acid pathway occurs in rat liver microsomes.[24] The marked decrease in ascorbic acid excretion in zinc-deficient rats could have resulted, in part, from a decrease in the specific activity of the enzymes of the glucuronic acid pathway or from a decrease in the protein content of the microsomes. Since no alteration was noted in the total protein content of microsomes during zinc depletion, it seems possible that the specific activities of both the glucuronic acid pathway and most drug-metabolizing enzymes were lowered at varying rates during zinc deficiency. Of the microsomal enzyme systems studied, ascorbic acid biosynthesis would appear to be the most sensitive to zinc deficiency, while the ring hydroxylation system and glucose 6-phosphatase activity of the rat liver microsomes were unaffected by this dietary stress.

Decreased drug metabolism in zinc-deficient rats cannot be explained by decreased availability of the usual NADPH regenerating system, since no change in glucose 6-phosphate dehydrogenase activity was found. A more probable explanation for the decreased metabolic ability would seem to be the lower levels of cytochrome P-450 found in zinc-deficient rats. This could lead to a decrease in the activity of the electron transport system required for drug metabolism,[25] thus altering the biotransformation of the drugs studied.

It is difficult to explain why the activity of ring hydroxylation, as measured by zoxazolamine and aniline metabolism, was unaffected by zinc deficiency. It is tempting to speculate that zinc may play some role in the binding of drugs (other than zoxazolamine and aniline) to cytochrome P-450 or other components of the microsomal electron transport system.

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