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RIBOFLAVIN AND DRUG METABOLISM IN ADULT MALE AND FEMALE RATS*

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Abstract—Oxidative metabolism of drugs *in vitro* in liver 9000 *g* supernatant fraction from riboflavin-deficient adult male and female rats was investigated. A significant decrease in overall oxidation of aminopyrine, ethylmorphine, *N*-methylaniline, aniline and acetanilide was observed. Studies at two deficiency levels indicate that a marked decrease in specific activities of *N*-demethylation of aminopyrine, ethylmorphine, *N*-methylaniline and hydroxylation of aniline and acetanilide occurs in both male and female rats. The levels of drug-metabolizing enzymes were further lowered with increase in deficiency. The levels of flavin, NADPH cytochrome c reductase, cytochrome P-450 and cytochrome b_5 were reduced in riboflavin-deficient (7 weeks) male rats as compared to normal rats. The activity of drug enzymes from riboflavin-deficient rats was stimulated by pretreatment with phenobarbital. A significant reversal of drug enzyme activities was noted when riboflavin was administered to deficient animals.

HEPATIC preparations from various species can metabolize foreign lipid-soluble substances.[1,2] Cytochrome P-450 is considered to be an important component of the mixed-function oxidase involved in the hydroxylation of steroids and many foreign compounds.[3,4] It has been reported that the hepatic drug-metabolizing enzyme is influenced by several factors such as starvation,[5] sex and species.[6–8] Studies regarding nutritional status of animals have indicated the role of vitamin C,[9] lipids, proteins,[10,11] iron and iodine[12] in drug metabolism. Catz *et al.*[12] have observed a marked increase in the levels of cytochrome P-450 content in weanling and old mice fed on a riboflavin-deficient diet. However, oxidative metabolism *in vitro* was augmented only slightly. Our preliminary studies[13] using riboflavin-deficient young rats indicated a significant decrease in the metabolism of aminopyrine, ethylmorphine and aniline.

Very little information is available regarding chronic riboflavin deficiency and drug metabolism. The present paper reports drug oxidation reactions *in vitro* using two different types of substrate, namely type I and type II, at different deficiency levels. The study also reports the liver protein content and activities of drug enzymes when riboflavin was administered for up to 15 days to animals with a 7 week riboflavin deficiency. Furthermore, it describes the effect of phenobarbital pretreatment on various drug enzymes in riboflavin-deficient animals.

MATERIALS AND METHODS

Adult albino male and female rats of the Haffkine strain, weighing 130–140 g, were obtained from Haffkine Institute, Bombay. The rats were housed in individual cages at room temperature and kept away from exposure to pharmacologically

* Dedicated to Prof. N. G. Magar, Professor and Head of Biochemistry Department, Institute of Science, Bombay, with a deep sense of gratitude on the occasion of his 60th birthday.

active compounds. All animals were fed a synthetic diet for 1 week prior to initiation of the experiments. The rats were then divided into two groups: control and riboflavin-deficient. Pair feedings were done up to 7 weeks. The contents of the two diets are listed in Table 1.

Refeeding. Riboflavin was administered to 7-week riboflavin-deficient male and female rats. The effect of riboflavin administration on growth, liver weight and activities of various drug enzymes was investigated after 5, 7, 10 and 15 days.

Phenobarbital treatment: At the end of 4 and 7 weeks, as well as during refeeding, the rats were injected intraperitoneally with phenobarbital sodium (50 mg/kg body wt) for 5 successive days.

TABLE 1. COMPOSITION OF THE CONTROL AND RIBOFLAVIN-DEFICIENT DIETS

Ingredients	Control diet	Riboflavin-deficient diet
Casein	13	
Vitamin-free casein		13
Corn starch	45	45
Sucrose	31	31
Groundnut oil	6	6
Salt mixture*	4	4
Vitamin mixture†	1	1‡

* Salt mixture of Hegsted *et al.*[14] with the following modifications: $CuSO_4$-5 H_2O, 3·266 g; $COCl_2$-6 H_2O, 2·16 g; NaF, 0·432 g.

† Vitamin mixture of Schultze[15] supplying 125 μg riboflavin/day/rat; 100 I.U. vitamin A, 20 I.U. vitamin D and 0·5 mg vitamin E/day/rat were also added to diet along with groundnut oil.

‡ Riboflavin-free vitamin mixture.

Tissue preparation. The animals were fasted for the last 12–14 hr and sacrificed 24 hr after the last injection by decapitation. The livers were excised, weighed, washed in 0·9% ice-cold saline and minced. The tissues were homogenized (1 : 10, w/v) in 0·25 M ice-cold sucrose with a glass homogenizer fitted with a motor-driven Teflon pestle. All tissue preparations were performed at 0–2°. The homogenates were centrifuged at 9000 *g* for 20 min at −2° and the drug enzyme activities of the supernatant fraction were studied. The microsomes were prepared by centrifuging the 9000 *g* fraction for 60 min at 105,000 *g* in a preparative Spinco model L2 ultracentrifuge. The pellets were washed with 0·15 M KCl and centrifuged again at 105,000 *g* for 30 min. The resulting microsomal pellets were resuspended in 0·15 M KCl containing 50 mM Tris-HCl buffer, pH 7·4, and the levels of the microsomal electron transport components were studied.

Liver 9000 *g* supernatant fraction protein and microsomal protein were measured according to the biuret method.[16]

Drug enzyme activities. Drug enzyme activities were determined in a typical incubation mixture consisting of 50 mM Tris-HCl buffer, pH 7·4, 5 mM $MgCl_2$, 50 μl NADPH (50 mg/ml), 2 mg/ml of protein and various substrates, 8 mM each, in a final volume of 15 ml.

The following substrates were used: (type I) aminopyrine and ethylmorphine, and (type II) aniline, acetanilide and *N*-methylaniline.

The mixtures were incubated in a Dubnoff metabolic shaker under air at 37°, 10 min for *N*-demethylation and 15 min for hydroxylation. The product formed was estimated as described by Schenkman *et al.*[17] Aminopyrine, ethylmorphine and *N*-methylaniline demethylations were determined by measuring the formaldehyde produced according to the procedure of Nash.[18] Aniline and acetanilide hydroxylations were measured by following the formation of *p*-aminophenol[19] and *p*-hydroxyacetanilide[20] respectively. Drug enzyme activities were expressed as nmoles of product formed/min/mg of protein.

Assays of microsomal electron transport components. Microsomal NADPH cytochrome c reductase activity was assayed at room temperature according to the procedure of Masters *et al.*[21] Cytochrome b_5 content was determined from the increment of absorbancy between 424 and 409 nm for NADH reduced and air-saturated samples. Cytochrome P-450 content was measured by the procedure of Omura and Sato.[22] Flavins were determined according to the procedure of Kings *et al.*[23]

RESULTS

Effect of riboflavin deficiency on body weight, liver weight and liver protein content. The effect of riboflavin deficiency after maintaining the rats on a deficient diet for 4 and 7 weeks was studied. Adult male and female rats were started on the riboflavin-deficient diet when they weighed 130 ± 10·0 g. Seven weeks after initiation of the diet, deficient male and female rats weighed 146·0 ± 3·0 g and 138·0 ± 3·0 g respectively, while corresponding control male and female rats weighed 253·0 ± 11·0 g and 180·0 ± 7·0 g respectively (Table 2). Liver weights of the control and riboflavin-deficient male rats were 6·8 ± 0·3 g and 4·8 ± 0·3 g respectively; those of control and deficient female rats were 4·6 ± 0·1 g and 3·7 ± 0·2 g respectively. Three to 4 weeks after initiation of the riboflavin-deficient diet, some animals showed loss of hair. Also in some animals the hair appeared to have less luster and smoothness than that of the normal rats. With increased deficiency of riboflavin, physical lesions characteristic of riboflavin deficiency were seen around the ears, mouth, shoulders and the back of almost all the animals. A significant increase in liver weight in all groups was observed due to phenobarbital pretreatment.

Liver 9000 *g* fraction protein concentrations were markedly increased in riboflavin-deficient animals as compared to normal rats. Phenobarbital pretreatment had a stimulatory effect on protein concentration in all animals.

Drug-metabolizing enzymes. Activities of drug-metabolizing enzymes at two different deficiency levels are included in Table 3. The activities of drug-metabolizing enzymes using type I and type II substrates were found to be reduced in riboflavin-deficient animals even at the end of the fourth week of deficiency and continued to decrease further till the end of of the experimental period. During the chronic deficiency stage (7 weeks), various oxidative enzyme activities were reduced 40–67 per cent in male and 40–71 per cent in female rats as compared to their respective normals.

The magnitude of the decrease was less with *N*-demethylation of type I substrates (aminopyrine and ethylmorphine) as compared to hydroxylation of type II compounds (e.g. aniline and acetanilide). The *N*-methylaniline *N*-demethylase activity was maximum as compared to that of other drug enzymes.

TABLE 2. EFFECT OF RIBOFLAVIN DEFICIENCY AND PHENOBARBITAL PRETREATMENT ON BODY WEIGHT, LIVER WEIGHT AND LIVER PROTEIN (9000 *g*) CONTENT IN ADULT MALE AND FEMALE RATS*

Rats	Feeding period (weeks)	Sex	Body wt (g)		Liver wt (g)		Liver protein content (mg/g liver)	
			Control	Phenobarbital-treated	Control	Phenobarbital-treated	Control	Phenobarbital-treated
Control	4	Male	223 ± 9·0	226 ± 7·0	7·2 ± 0·2	10·2 ± 0·7	227·0 ± 7·0	261·0 ± 5·0
	7	Male	253 ± 11·0	260 ± 9·0	6·8 ± 0·3	11·2 ± 0·5	250·0 ± 9·0	285·0 ± 5·0
Riboflavin-deficient	4	Male	189 ± 4·0	188 ± 4·0	5·7 ± 0·1	11·1 ± 0·6	232·0 ± 10·0	270·0 ± 9·0
	7	Male	146 ± 3·0	150 ± 7·0	4·8 ± 0·3	7·7 ± 0·4	290·0 ± 7·0	312·0 ± 5·0
Control	4	Female	186 ± 5·0	188 ± 6·0	4·1 ± 0·2	7·3 ± 0·3	205·0 ± 7·0	233·0 ± 3·0
	7	Female	180 ± 7·0	183 ± 4·0	4·6 ± 0·1	6·2 ± 0·4	217·0 ± 4·0	247·0 ± 7·0
Riboflavin-deficient	4	Female	160 ± 5·0	160 ± 4·0	4·5 ± 0·4	7·5 ± 0·3	220·0 ± 6·0	260·0 ± 8·0
	7	Female	138 ± 3·0	142 ± 6·0	3·7 ± 0·2	7·6 ± 0·3	231·0 ± 7·0	269·0 ± 5·0

* Values are expressed as mean ± S.E. (five rats in each group).

TABLE 3. EFFECT OF RIBOFLAVIN DEFICIENCY AND PHENOBARBITAL PRETREATMENT ON OXIDATIVE DRUG METABOLISM IN ADULT MALE AND FEMALE RATS*

		Normal rats				Riboflavin-deficient rats			
		Feeding period				Feeding period			
		4 weeks		7 weeks		4 weeks		7 weeks	
Sex		Without PB	With PB	Without PB	With PB	Without PB	With PB	Without PB	With PB
	N-demethylation of:								
Male	Aminopyrine†	4·3 ± 0·1†	12·5 ± 0·5	5·0 ± 0·2	13·2 ± 0·2	3·0 ± 0·2	7·3 ± 0·4	2·6 ± 0·1	7·0 ± 0·3
Female		3·3 ± 0·1	6·3 ± 0·4	4·0 ± 0·1	8·8 ± 0·2	2·6 ± 0·1	4·8 ± 0·2	2·0 ± 0·3	4·1 ± 0·3
Male	Ethylmorphine†	3·9 ± 0·2	10·8 ± 0·4	4·9 ± 0·2	11·6 ± 0·3	2·6 ± 0·2	5·5 ± 0·3	1·6 ± 0·1	4·2 ± 0·2
Female		2·6 ± 0·1	5·9 ± 0·2	3·3 ± 0·2	8·2 ± 0·3	1·7 ± 0·1	4·0 ± 0·1	1·3 ± 0·1	2·2 ± 0·1
Male	*N*-methylaniline†	7·2 ± 0·2	13·0 ± 0·2	6·5 ± 0·1	14·2 ± 0·1	5·0 ± 0·2	8·3 ± 0·3	3·7 ± 0·3	7·0 ± 0·1
Female		6·8 ± 0·2	12·2 ± 0·3	5·2 ± 0·1	10·7 ± 0·4	5·2 ± 0·2	8·2 ± 0·4	3·0 ± 0·1	6·5 ± 0·2
	Hydroxylation of:								
Male	Aniline†	3·8 ± 0·1	8·2 ± 0·4	5·3 ± 0·2	10·8 ± 0·5	2·4 ± 0·2	5·0 ± 0·3	2·0 ± 0·1	4·2 ± 0·2
Female		2·6 ± 0·1	4·9 ± 0·2	5·0 ± 0·1	8·7 ± 0·3	2·0 ± 0·2	3·5 ± 0·1	1·4 ± 0·1	2·7 ± 0·1
Male	Acetanilide†	3·4 ± 0·4	8·7 ± 0·2	5·5 ± 0·1	4·9 ± 0·2	2·1 ± 0·1	5·1 ± 0·3	2·0 ± 0·2	4·0 ± 0·1
Female		2·9 ± 0·1	5·1 ± 0·1	4·9 ± 0·2	9·0 ± 0·3	1·8 ± 0·1	3·4 ± 0·2	1·5 ± 0·1	2·7 ± 0·1

* Values are expressed as mean ± S.E. (five rats in each group). PB = phenobarbital treatment.

† Units of activity are given in Materials and Methods. All results between phenobarbital-treated and untreated animals indicate a significant difference: $P < 0.001$.

The activities of all drug-metabolizing enzymes increased linearly in normal rats with the increases in feeding period. However, a very small sex variation was observed for *N*-demethylation of type I compounds. The effect of riboflavin deficiency on the levels of electron transport components in 7-week riboflavin-deficient adult male rats was examined. It was noted that the levels of NADPH cytochrome c reductase, cytochrome b_5, cytochrome P-450 and flavin were significantly reduced in deficient male rats as compared to normal rats (Tables 4 and 5).

TABLE 4. EFFECT OF 7 WEEKS OF RIBOFLAVIN DEFICIENCY ON THE LEVELS OF ELECTRON TRANSPORT COMPONENTS IN ADULT MALE RATS*

Rats	NADPH cytochrome c reductase ΔO.D. 550–539 assuming ϵ = 21 nmoles cytochrome c reduced/mg/min)	Cytochrome b_5 (nmoles/mg protein)	Cytochrome P-450 (nmoles/mg protein)
Normal	160	0·77	1·46
Riboflavin-deficient	75†	0·52†	1·16†

* These determinations were carried out at the Institute of Science, Bombay. Values are the average of three determinations of pooled livers of three rats with two different preparations.

† $P < 0.001$.

TABLE 5. EFFECT OF 7 WEEKS OF RIBOFLAVIN DEFICIENCY ON FLAVIN CONTENT OF ADULT MALE RATS*

Rats	FMN (nmoles/mg protein)	FAD (nmoles/mg protein)	Total flavin (nmoles/mg protein)
Normal	0·17	0·58	0·75
Riboflavin-deficient	0·10	0·33†	0·43†

* Values are the average of three determinations of pooled livers of three rats with two different preparations.

† $P < 0.001$.

Induction of drug-metabolizing enzymes by phenobarbital pretreatment. The effects of phenobarbital pretreatment on drug-metabolizing enzymes in both normal and riboflavin-deficient animals are summarized in Table 3. It was found that the deficient animals have decreased drug oxidation even at the end of 4 weeks and it continued to decrease with the progressive increase in the deficiency state. It was noted that drug metabolism was enhanced by phenobarbital pretreatment in deficient animals even at an advanced state of deficiency. It was interesting to note that the magnitude of the stimulation was of the same order as that in their respective normals.

Effect of riboflavin administration on body weight, liver weight and liver protein content. Both male and female rats on the riboflavin-deficient diet for 7 weeks were given riboflavin for 5, 7, 10 and 15 days. Marked stimulation in growth was noted after administration of riboflavin to the deficient rats. At the end of day 15, their physical appearance was exactly the same as that of normal animals. There was an average gain in body weight of 42 g in male and 15 g in female rats after administration of riboflavin to the deficient rats for 15 days (Fig. 1). The response in riboflavin-deficient female rats was less. The change in liver protein content at the end of various refeeding times is presented in Fig. 2. A decrease in liver protein content after 7 days of

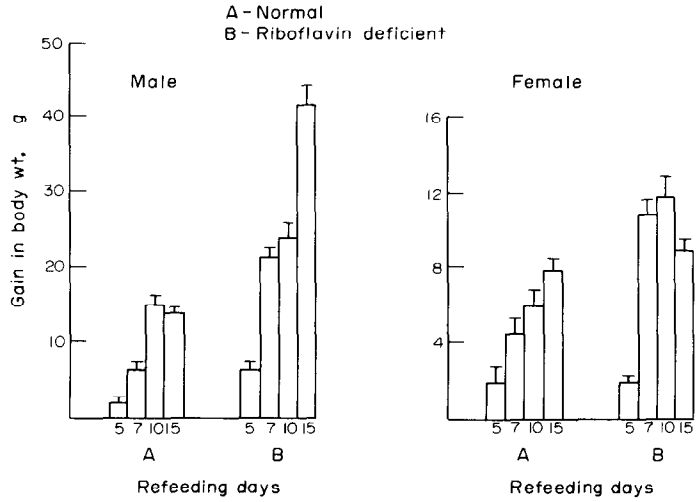

FIG. 1. Change in body weight after 15 days of refeeding in normal and 7-week riboflavin-deficient male and female rats.

refeeding was observed in both male and female rats; a very slight increase was noted at the end of second week.

Effect of riboflavin administration on drug metabolism. The activities of all drug-metabolizing enzymes were lowered at the end of 7 weeks of chronic deficiency. There was a reversal in the activity of various drug-metabolizing enzymes after administration of riboflavin, the reversal in both male and female rats was linear during refeeding (Figs. 3–7). The increase in the activities of the drug enzymes was faster

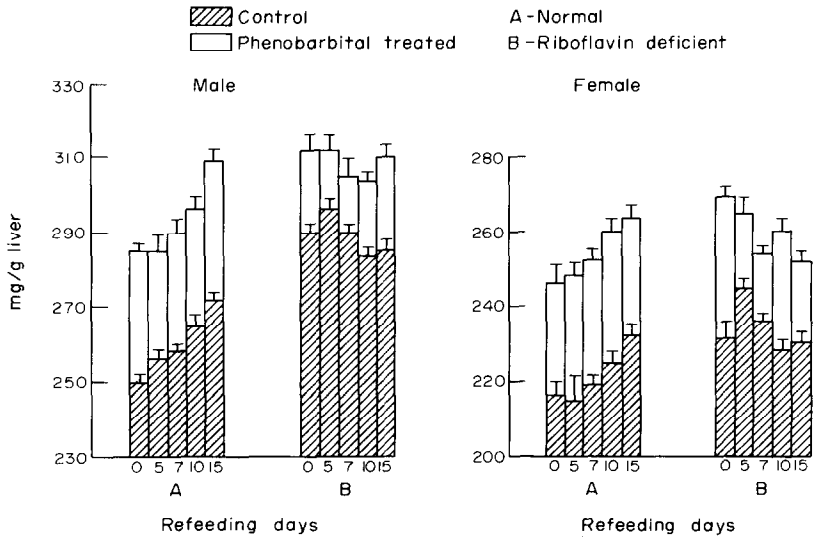

FIG. 2. Effect of refeeding and phenobarbital treatment on liver protein content in normal and 7-week riboflavin-deficient male and female rats.

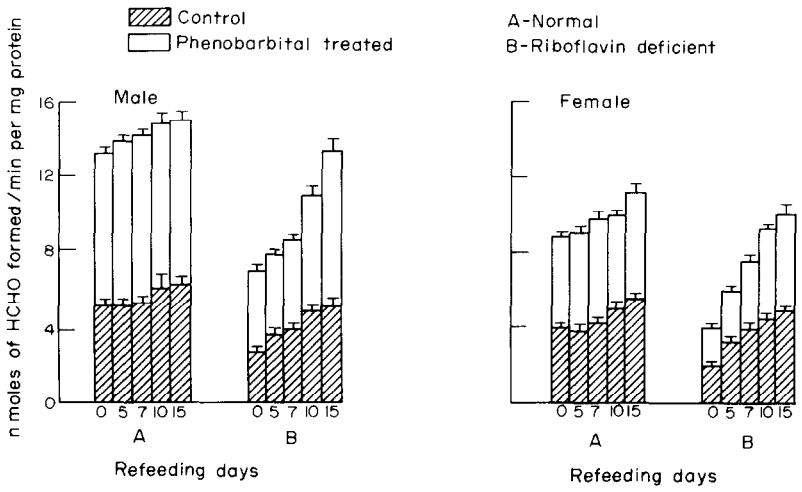

FIG. 3. Effect of refeeding on *N*-demethylation of aminopyrine in normal and 7-week riboflavin-deficient adult male and female rats.

in the deprived animals during refeeding periods than that in normal animals. Riboflavin administration produced an almost 50–60 per cent reversal of drug oxidative enzyme activities in both male and female deficient rats as compared to their respective levels at the end of the 7-week deficiency stage.

DISCUSSION

Earlier we have reported[13] the role of riboflavin in drug metabolism in male rats weighing 40–50 g. The present study reports riboflavin and drug metabolism in adult male and female rats at two deficiency levels. The results indicate that the activity

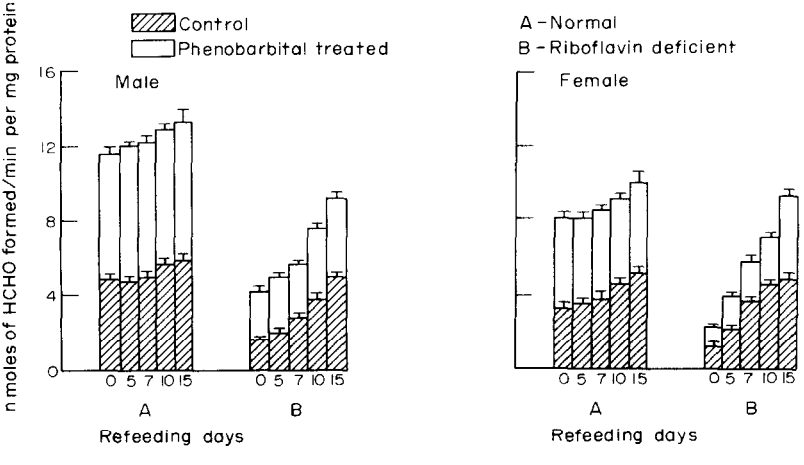

FIG. 4. Effect of refeeding on *N*-demethylation of ethylmorphine in normal and 7-week riboflavin-deficient adult male and female rats.

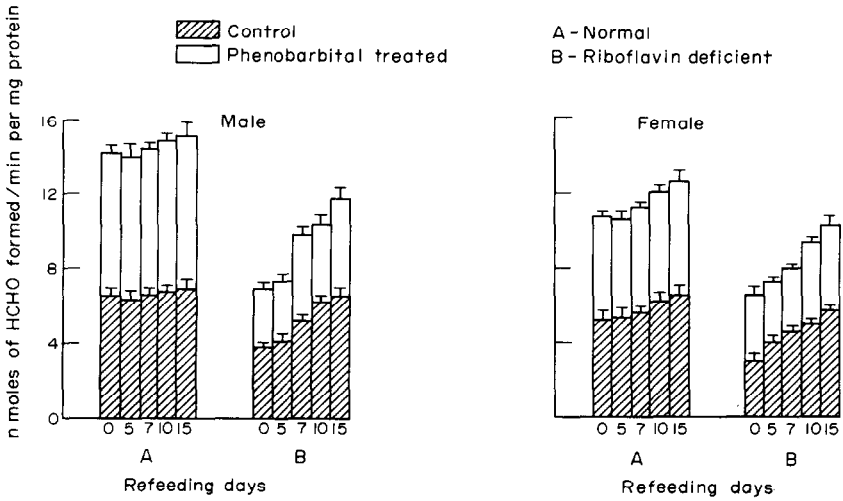

FIG. 5. Effect of refeeding on *N*-demethylation of *N*-methylaniline in normal and 7-week riboflavin-deficient adult male and female rats.

of hepatic drug-metabolizing enzymes is markedly reduced in rats fed a riboflavin-deficient diet. We also found that the liver had a decreased capacity for drug oxidation.[13] The levels of cytochrome P-450 and cytochrome b_5 were slightly increased and the total flavin and cytochrome c reductase were slightly lowered. In the present study in 7-week riboflavin-deficient adult male rats the levels of NADPH cytochrome c reductase, cytochrome P-450, cytochrome b_5 and flavin were markedly reduced. Catz *et al.*[12] have reported an increase in cytochrome P-450 content in both weanling and old mice fed a riboflavin-deficient diet *ad lib.* However, they found a slight increase in *N*-demethylation of aminopyrine and hydroxylation of aniline, and a significant decrease in hydroxylation of benzpyrene. They have no explanation for these

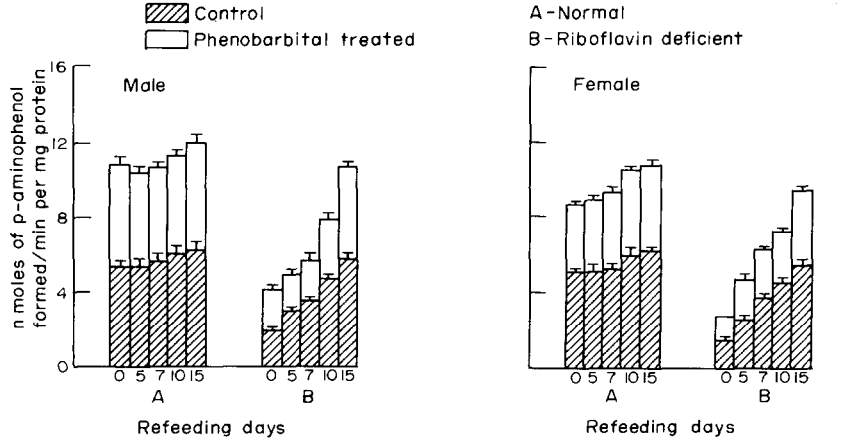

FIG. 6. Effect of refeeding on hydroxylation on aniline in normal and 7-week riboflavin-deficient adult male and female rats.

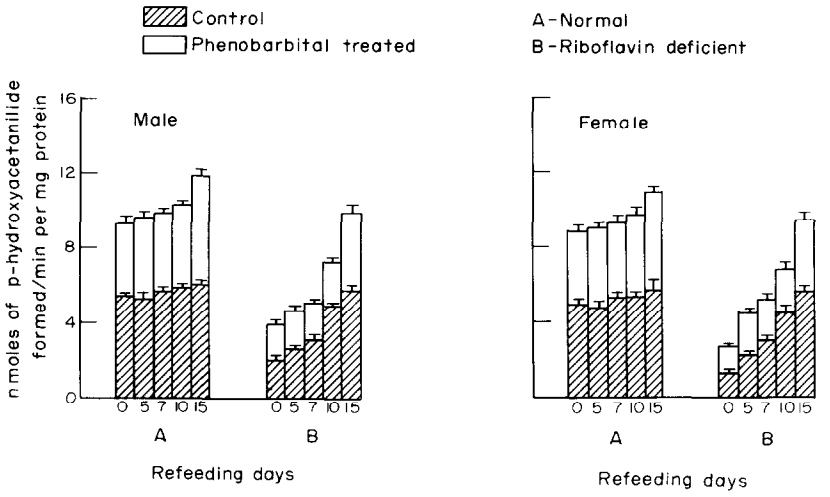

FIG. 7. Effect of refeeding on hydroxylation of acetanilide in normal and 7-week riboflavin-deficient adult male and female rats.

observations. Earlier one of us observed* the reduced levels of cytochrome P-450, cytochrome c reductase and flavin with concurrent lowering of *N*-demethylation of aminopyrine and ethylmorphine in adult flavin-deficient male rats when fed a flavin-deficient or a flavin and galactoflavin-deficient diet for 3–4 weeks. The present results confirm these unpublished observations. Recently we have noted slightly higher levels of cytochrome P-450 and cytochrome b_5 in riboflavin-deficient young (40–50 g) growing male rats as compared to normal rats.[13] However, in adult male rats the levels of all electron transport components were found to be reduced by a riboflavin-deficient diet. The reason for this discrepancy remains to be determined.

Tandler *et al.* studied the ultrastructural changes in hepatocytes of riboflavin-deficient mice and noted that the changes in endoplasmic reticulum (ER) were evident. The orderly complex of ER was replaced by randomly distributed rough-surfaced dilated vacuoles with clear contents. Fragmentation of the ER was also evident in electron micrographs.

In the present study, the observed low levels of drug enzymes resulting from the reduced ability of liver to oxidize drugs are possibly due to the low levels of cytochrome P-450, NADPH cytochrome c reductase, cytochrome b_5 and flavin. The loss of structural integrity of the endoplasmic reticulum could also be an additional factor in lowering the drug enzyme activities. A 2-to-2-fold difference due to sex variation for *N*-demethylation of type I compounds has been reported by Schenkman *et al.*[25] and Gigon *et al.*[26] The very small sex difference observed in the present study must be due to the different strain of rats.

Induction due to phenobarbital pretreatment in rats fed a 0·5% lactalbumin,[27] low casein content,[10,11] and vitamin C-deficient diet[9] has been reported. In the present study, the activities of drug enzymes from riboflavin-deficient rats were induced to the same level as that of controls. Thus, riboflavin deficiency does not inhibit induction of drug-metabolizing enzymes by phenobarbital.

* S. S. Pawar, unpublished observations from R. W. Estabrook's laboratory.

Reversal of inhibited microsomal drug-metabolizing enzyme activity in riboflavin-deficient animals by administration of riboflavin has indicated that the levels of the components of the electron transport system could be restored and/or the integrity of the system could be partially repaired. It was noted that the levels of drug-metabolizing enzymes during refeeding were higher in male rats than in female rats. The activities were linearly induced by phenobarbital pretreatment during the entire refeeding period in both sexes.

The results suggest that the female rats required more time to restore the drug enzyme levels than did male rats, possibly due to the sex difference or different physiological response to the riboflavin administration. There was a reversal of drug metabolism with changes in parameters such as body weight, liver membrane and liver protein due to the administration of riboflavin. Hence, there might be restoration of integrity of microsomal phospholipids with the cytochrome P-450. Similar observations have been reported in vitamin C-deficient guinea-pigs.[9]

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