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INFLUENCE OF DIETARY IRON LEVELS ON HEPATIC DRUG METABOLISM *IN VIVO* AND *IN VITRO* IN THE RAT

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Abstract—Rats fed an iron-deficient diet for 18 days showed a marked increase in the metabolism *in vitro* of aniline and after 25 days both the metabolism *in vitro* and *in vivo* of aniline was increased. Aminopyrine metabolism *in vivo* and *in vitro* was also markedly stimulated in iron-deficient rats but only after hemoglobin levels had decreased to approximately 50 per cent of control. Pentobarbital metabolism *in vivo* (plasma half-life) and *in vitro* was not altered in iron deficiency, although deficient animals did exhibit longer sleeping times than control animals. Microsomal cytochrome *c* reductase activity was increased during iron deficiency but only after a large decrease in hemoglobin concentration had occurred. No alteration in the microsomal cytochromes b_5 or P-450 was noted. All hematological and biochemical lesions were completely ameliorated by refeeding iron-deficient rats the iron-containing control diet. No apparent deleterious effect on drug metabolism was noted after feeding a diet containing 1·5 times the normal iron content.

RECENT reports[1–3] have shown that large segments of the population show signs of iron deficiency. An extensive compendium of nutritional status studies by Kelsay[4] show that iron, an important element for hematopoiesis, is one of the nutrients most often found deficient in the diets of both children and adults.

Studies on nutritional deficiencies such as protein,[5] calcium,[6] vitamin C,[7] zinc[8] and magnesium,[9] have indicated that the nutritional state of an animal markedly alters the rate of hepatic drug metabolism.

While the present investigation was concluding, Catz *et al.*[10] presented evidence that iron deficiency increased some hepatic drug metabolic pathways in mice. It was shown that the metabolism *in vitro* of only those substrates showing a type I spectrum when bound to cytochrome P-450 (e.g. aminopyrine and hexobarbital) was increased in iron-deficient mice. Other than sleeping time determinations, no attempt was made to determine the effect of iron deficiency on the rate of drug metabolism *in vivo* in mice. Actually no correlation between the rate of hexobarbital metabolism *in vitro* and hexobarbital sleeping time was reported by Catz *et al.*[10]

The results of studies in rats, reported in this paper, also show that iron deficiency increases the rate of hepatic drug metabolism both *in vitro* and *in vivo*. In addition, the present results in rats will be shown to differ in several respects from previous studies in iron-deficient mice.

MATERIALS AND METHODS

Animals. Male rats of the Sprague–Dawley strain weighing between 140 and 170 g were used in all experiments. Animals were randomly divided into three groups.

Group 1 (iron-deficient) received *ad lib.* the basic diet which is slightly modified from the iron-deficient diet of McCall *et al.*[11] The basic diet contained in per cent: spray-died skim milk, 65; sucrose, 21; corn oil, 10; iron free salts,[11] 3; and vitamins,[8] 1. Group 2 (isocaloric control) received the basic diet, supplemented with 220 ppm iron as ferrous sulphate, in an amount needed to maintain a growth rate essentially equal to Group 1. Group 3 (high iron) received *ad lib.* the basic diet supplemented with 330 ppm iron as ferrous sulphate. Animals were housed individually in stainless steel cages and received glass-distilled water *ad lib.*

Enzyme preparations and assays. Microsomes and crude enzyme preparations (17,500 *g* supernatants) for drug metabolism assays *in vitro* were prepared as previously described[8] after an initial homogenization in 2·5 vol. of isotonic KCl-0·0001 M EDTA. Livers were not perfused prior to homogenization. Microsomes were finally suspended in isotonic KCl–0·02 M sodium phosphate (pH 7·4) at a concentration such that 1·0 ml of suspension was equal to 600–700 mg of liver.

Drug metabolism *in vitro* was studied by adding 1·0 ml of the 17,500 *g* supernatants to each incubation mixture. Assay conditions for the determination of the metabolism *in vitro* of *p*-nitrobenzoic acid, aniline, aminopyrine and pentobarbital were those described by Becking and Morrison[8] with minor modifications. Nicotinamide was omitted from all assays and incubations were carried out for 30 min. Drug metabolism *in vitro* is reported as nanomoles metabolized per milligram of microsomal protein per hour. Under these conditions, essentially linear rates of metabolism were obtained for all drug substrates.

NADPH-cytochrome *c* reductase was determined by the method of Williams and Kamin.[12]

Isocitrate and glucose 6-phosphate dehydrogenase activities were measured as outlined previously.[9]

Drug metabolism in vivo. The plasma half-life of pentobarbital was determined after the injection of sodium pentobarbital in normal saline (35 mg/kg, i.p.). Sleeping times following this dose were determined as another indication of drug metabolism *in vivo*. Pentobarbital in plasma was determined by the extraction procedure of Brodie *et al.*,[13] after acidifying plasma samples with HCl to a pH of approximately 2.

For the determination of their respective plasma half-lives, aminopyrine (80 mg/kg) and aniline (50 mg/kg) were dissolved in normal saline and administered intraperitoneally. All plasma half-lives were calculated by the direct graphical method.[14] Individual linear regression lines were drawn for three groups of five animals, and the mean $\pm$ S. E. M., was calculated from the individual half-lives. Plasma levels of aminopyrine and total 4-aminoantipyrine were determined as described by Brodie and Axelrod.[15] Aniline and total *p*-aminophenol were extracted from plasma samples by the procedures described by Brodie and Axelrod.[16] Aniline was determined by diazotization as described for *p*-aminobenzoic acid[17] except that spectrophotometric readings were made at 560 nm after 1-hr color development in the dark. The total *p*-aminophenol in plasma was assayed, after extraction and hydrolysis, using the color reaction described by Schenkman *et al.*[18]

Chemical analyses. Protein was measured directly on aliquots of homogenates or microsomal suspensions, clarified by the addition of 10% sodium deoxycholate, utilizing the copper biuret reagent of Gornall *et al.*[19]

Microsomal cytochrome P-450 was determined as follows: microsomal suspensions

were diluted to a protein content of about 2 mg/ml with 0·25 M sucrose–0·1 M sodium phosphate buffer (pH 7·4). Duplicate aliquots were reduced with sodium dithionite, and 1 aliquot was gassed with oxygen-free carbon monoxide for 30 sec. The quantity of P-450 was expressed as nanomoles per milligram of microsomal protein and was calculated from the differences in optical density between 450 and 490 nm, using the molar extinction coefficient 91,000.[20]

The cytochrome b_5 content of diluted microsomal suspensions was determined from the difference spectrum between the oxidized and NADH-reduced samples, utilizing the molar extinction coefficient 185,000 for the difference in absorbance between 425 and 408 nm.[20]

Iron-deficiency anemia was monitored by periodic measurement of hemoglobin and hematocrits. Hemoglobin was determined by the cyanmethemoglobin method, and the hematocrit values that were reported were obtained using the microhematocrit technique.

Statistical analysis. Statistical evaluation was made by the Student's *t*-test. Results were considered significant only if $P < 0{\cdot}05$.

RESULTS

Young male rats (45–50 days old) when placed on the iron-deficient diet developed iron-deficiency anemia as early as 10 days on test (Table 1). The return of hemoglobin and hematocrit values to those of control rats after repletion of the deficient diet with iron confirms that the dietary regimen utilized results in only an iron-deficiency anemia. Growth rates were essentially equal in control and iron-deficient animals, whereas a slightly decreased rate of growth was noted in those rats fed the high iron diet (Table 1).

From the data shown in Tables 2 and 3, it is apparent that iron deficiency in rats increases the rate of both aniline and aminopyrine metabolism. This increase in metabolic rate is found both *in vitro* and *in vivo*. In iron-deficient animals aniline

TABLE 1. EFFECT OF DIETARY IRON LEVELS ON HEMOGLOBIN, HEMATOCRIT AND BODY WEIGHTS*

Diet	Days on test	Hemoglobin (g/100 ml)	Hematocrit (%)	Body weight (g)
High iron	0	14·6 ± 0·4 (6)†	42·6 ± 0·8 (6)	148 ± 6 (20)
Iron-deficient		14·4 ± 0·4 (6)	39·9 ± 0·8 (6)	150 ± 4 (20)
Control		15·0 ± 0·3 (6)	41·0 ± 0·6 (6)	146 ± 7 (20)
Iron-deficient	20	9·0 ± 0·5 (4)‡	26·1 ± 1·4 (4)‡	235 ± 7 (20)
Control		14·7 ± 0·4 (4)	39·1 ± 0·9 (4)	230 ± 5 (20)
High iron	40	15·4 ± 0·6 (4)	43·0 ± 1·1 (4)	272 ± 8 (15)‡
Iron-deficient		7·7 ± 0·4 (4)‡	21·4 ± 0·7 (4)‡	281 ± 9 (15)
Control		14·8 ± 0·4 (4)	41·6 ± 0·5 (4)	309 ± 6 (15)
Iron-deficient + iron	40 + 7	14·3 ± 0·3 (6)	41·4 ± 0·7 (6)	
Control	47	15·0 ± 0·4 (6)	42·7 ± 0·9 (6)	

* Results are expressed as the mean value ± S. E. M.
† Number of animals used for each determination.
‡ Significantly different from control values ($P < 0{\cdot}05$).

TABLE 2. METABOLISM *in vivo* AND *in vitro* OF ANILINE BY RATS FED VARYING LEVELS OF IRON

Diet	Days on test	*In vitro** *p*-Aminophenol (nmoles/mg protein/hr)	*In vivo* Plasma Half-life (min)	*In vivo* Plasma *p*-Aminophenol† (μg/ml)
Iron-deficient	18	29·2 ± 2·0		
Control		21·1 ± 2·4		
High iron	25	27·3 ± 1·9		
Iron-deficient		43·3 ± 2·5‡	58 ± 4‡	14·7 ± 2·0‡
Control		25·8 ± 2·3	89 ± 6	6·9 ± 2·2
High iron	35	21·3 ± 1·9		
Iron-deficient		39·1 ± 1·8‡		
Control		20·4 ± 1·3		
Iron-deficient + iron	40 + 7	30·2 ± 3·1	69 ± 4	13·9 ± 3·1
Control	47	26·5 ± 1·6	79 ± 7	10·9 ± 2·1

* Results are expressed as the mean value obtained with four rats ± S. E. M. Drug metabolism *in vitro* in rat liver 17,500 *g* supernatants was reported as nanomoles of *p*-aminophenol formed per milligram of microsomal protein per hour.

† Total *p*-aminophenol in plasma was determined 1·5 hr after drug administration.

‡ Significantly different from control values ($P < 0.05$).

hydroxylation *in vitro* increases significantly over that found in control rats as early as 18 days on test. After 25 days on the deficient diet, the rate of metabolism *in vitro* of aniline was a good indication of the rate of metabolism *in vivo*, as shown by changes in the plasma half-life of aniline and the different levels of total *p*-aminophenol in the plasma of deficient and control animals 1·5 hr after drug administration. No changes in hepatic drug metabolism were noted prior to the time when hemoglobin levels had decreased to approximately 65 per cent of control values. The return to control values after repletion of formerly deficient animals with ferrous sulphate for 7 days indicated that the significant increase in the metabolism in *vitro* and *in vivo* of aniline was a direct result of the low level of iron in the diet. No deleterious effect on aniline metabolism *in vitro* was noted after feeding the high iron diet for 35 days (Table 2).

Aminopyrine *N*-demethylation does not appear to be as sensitive to iron deficiency as was noted for aniline hydroxylation (Table 3). Before a significant increase in the rate of aminopyrine metabolism was noted (35–40 days on test), hemoglobin levels decreased to approximately 50 per cent of control. As shown in Table 3, rates *in vitro* gave a good indication of the rate of metabolism *in vivo* of aminopyrine. Both the decreased plasma half-life and the increased amount of total 4-aminoantipyrine found in plasma 2 hr after drug administration indicate a marked increase in the metabolism *in vivo* of aminopyrine during iron deficiency. The increased metabolism *in vitro* and *in vivo* of aminopyrine in deficient animals returned to that found in control animals after 7 days repletion with iron. Aminopyrine *N*-demethylation was not altered by feeding rats a diet high in iron for 40 days.

The metabolism *in vitro* of pentobarbital does not seem to correspond to the rate of metabolism *in vivo* when sleeping times are used as the criteria of biotransformation

TABLE 3. METABOLISM *in vivo* AND *in vitro* OF AMINOPYRINE BY RATS FED VARYING LEVELS OF IRON

Diet	Days on test	*In vitro** 4-Aminoantipyrine (nmoles/mg protein/hr)	*In vivo*† Plasma Half-life (min)	*In vivo*† Plasma 4-Aminoantipyrine (μg/ml)
Iron-deficient	25	52·3 ± 6·4		
Control		43·8 ± 3·2		
High iron	35	44·4 ± 3·1		
Iron-deficient		58·8 ± 4·6‡		
Control		37·9 ± 3·5		
High iron	40	37·7 ± 3·3		
Iron-deficient		60·8 ± 2·7‡	107 ± 8‡	15·9 ± 1·9‡
Control		40·1 ± 3·2	138 ± 6	9·8 ± 0·9
Iron-deficient + iron	40 + 7	36·9 ± 4·7	126 ± 5	9·6 ± 1·1
Control	47	38·2 ± 5·4	119 ± 6	10·4 ± 1·1

*Results are expressed as the mean value obtained with four rats ± S. E. M. Drug metabolism *in vitro* in rat liver 17,500 *g* supernatants was reported as nonamoles 4-aminoantipyrine formed per milligram of microsomal protein per hour.

† Total 4-aminoantipyrine in plasma was determined 2 hr after drug administration.

‡ Significantly different from control values ($P < 0.05$).

in vivo (Table 4). If one uses plasma half-life of the drug to monitor metabolism *in vivo*, metabolic rates *in vitro* and *in vivo* of pentobarbital reflect the same trend, that is, iron-deficient and control rats metabolize pentobarbital at essentially equal rates both *in vitro* and *in vivo*. It is apparent that a dietary overload of iron for 35 days did not alter the rate of pentobarbital metabolism *in vitro* (Table 4).

TABLE 4. EFFECTS OF VARYING DIETARY IRON LEVELS ON THE METABOLISM *in vivo* AND *in vitro* OF PENTOBARBITAL

Diet	Days on test	*In vitro** Pentobarbital metabolized (nmoles/mg protein/hr)	*In vivo* Sleeping† time (min)	*In vivo* Plasma half-life (min)
High iron	25	104·1 ± 7·0		
Iron-deficient		141·9 ± 16·7		
Control		119·4 ± 7·4		
High iron	35	96·0 ± 9·4		
Iron-deficient		107·6 ± 10·2	86 ± 8‡	78 ± 3
Control		88·9 ± 7·3	63 ± 7	70 ± 2

* Results are expressed as the mean value obtained with four rats ± S. E. M. Drug metabolism *in vitro* in rat liver 17,500 *g* supernatants was reported as nanomoles of pentobarbital metabolized per milligram of microsomal protein per hr.

† Sleeping times are expressed as the mean value obtained with ten rats ± S. E. M.

‡ Significantly different from control values ($P < 0.05$).

TABLE 5. REDUCTION *in vitro* OF *p*-NITROBENZOIC ACID BY RATS FED VARYING LEVELS OF IRON*

Diet	*p*-Aminobenzoic acid (nmoles/mg protein/hr)	
	Days on test (18)	Days on test (35)
High iron		22·9 ± 1·7
Iron-deficient	19·4 ± 1·5	24·2 ± 2·0
Control	20·2 ± 0·9	22·3 ± 0·7

* Results are expressed as the mean value obtained with four rats ± S. E. M. Drug metabolism *in vitro* in rat liver 17,500 *g* supernatants was reported as nanomoles of *p*-aminobenzoic acid formed per milligram of microsomal protein per hour.

No alteration in the rate *in vitro* of reduction of *p*-nitrobenzoic acid was noted after 35 days on either a high iron or iron-deficient diet (Table 5).

The increased rate of aniline and aminopyrine metabolism noted in iron-deficient rats cannot be explained by a lowered rate of NADPH production (Table 6). Glucose 6-phosphate or isocitrate dehydrogenase activities were not altered by feeding rats the iron-deficient or high iron-containing diets.

Concentrations of the microsomal cytochromes b_5 and P-450 and microsomal NADPH cytochrome *c* reductase activity are summarized in Table 7. It is apparent that, of the microsomal electron transport components measured, only cytochrome *c* reductase activity was found to be significantly increased in iron-deficient animals. Since it required more than 25 days on the deficient diet to significantly increase the cytochrome *c* reductase activity, this cannot account for the large increase in aniline metabolism found after 18 days on the low iron diet (Table 2).

TABLE 6. EFFECT OF VARYING DIETARY IRON LEVELS ON THE REDUCTION OF NADP *in vitro* BY RAT LIVER HOMOGENATES*

Diet	Days on test	Dehydrogenase	
		Glucose 6-phosphate	Isocitrate
Iron-deficient	18	21·2 ± 0·6	176·1 ± 9·3
Control		25·9 ± 2·3	167·7 ± 7·6
High iron	35	18·7 ± 2·1	160·0 ± 6·5
Iron-deficient		26·0 ± 1·7	134·8 ± 11·0
Control		24·9 ± 2·6	145·2 ± 8·7

* Results are expressed as the mean value obtained with four rats ± S. E. M. The values represent nanomoles of NADPH per milligram of protein per minute.

TABLE 7. EFFECTS OF DIETARY IRON LEVELS ON MICROSOMAL CYTOCHROME CONTENT AND CYTOCHROME *c* REDUCTASE ACTIVITY IN RAT LIVER*

Diet	Days on test	Microsomal cytochrome		
		b_5 (nmoles/mg protein)	P-450 (nmoles/mg protein)	*c* reductase (nmoles cyt. *c* reduced/mg protein/min)
High iron	25	0·48 ± 0·06	1·02 ± 0·11	49·2 ± 4·2
Iron-deficient		0·47 ± 0·04	0·83 ± 0·06	58·8 ± 5·8
Control		0·40 ± 0·04	0·88 ± 0·05	53·4 ± 2·9
High iron	35	0·39 ± 0·04	0·89 ± 0·05	55·1 ± 5·0
Iron-deficient		0·34 ± 0·06	0·98 ± 0·08	78·1 ± 3·8†
Control		0·38 ± 0·03	0·94 ± 0·04	60·2 ± 3·6
Iron-deficient + iron	40 + 7			60·0 ± 5·2
Control	47			58·4 ± 6·3

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

DISCUSSION

The data presented in this paper are in general agreement with the studies of Catz *et al.*,[10] that is, drug metabolic pathways which are altered in iron-deficient rats show increased activity similar to the findings in mice. Several aspects of the studies in rats reported here point out differences in the effect of iron deficiency on drug metabolism which are possibly due to species differences between mice and rats. Additional data are given to indicate, at least in the rat, that drug metabolism is increased *in vivo*, as well as *in vitro*. As in previous studies on the effects of dietary deficiencies on hepatic drug metabolism,[8,9] rates of drug metabolism *in vitro* gave good indication of alterations in drug metabolism *in vivo*. It is apparent from these studies in rats and the studies of Catz *et al.*[10] in mice, that any direct correlation between drug metabolism *in vitro* in iron-deficient animals and activity *in vivo*, as measured by barbiturate sleeping times, is extremely unlikely. Sleeping times are not only a measure of metabolic activity *in vivo* but are probably more related to changes in the central nervous system, resulting in altered sensitivity to the barbiturate as postulated by Dairman and Balazs.[21] The need for determining plasma half-life and/or metabolite levels is apparent.

From the data in this report, it is possible to show variable sensitivity of drug metabolic reactions to iron-deficient diets. The finding that aniline hydroxylation is the most sensitive, of the drug metabolic reactions studied, to decreases in the dietary iron levels is contrary to the findings of Catz *et al.*[10] during their studies in mice. These authors found no increase in the aniline hydroxylation activity even when hemoglobin levels had decreased to approximately 50 per cent of normal. It is evident from results in rats that aromatic ring hydroxylation is much more sensitive to iron deficiency than *N*-demethylation of aminopyrine. It is tempting to speculate that one reason for these differences may be due to the fact that rats have an androgen-dependent regulatory mechanism of drug metabolism which is genetically lacking in

other species.[22] It has been shown by Symes *et al.*[23] that iron deficiency in rats results in: (1) a decrease in hepatic iron levels, (2) a decrease in liver monoamine oxidase activity, and (3) an increase in the hepatic copper content. Whether the lower iron level and/or the increased copper content of the liver alters the mechanisms by which drug metabolism is controlled in the rat liver remains a matter for speculation until further work has been carried out in the area of metal deficiencies and drug metabolism.

Although the concentration is not lowered by iron deficiency, it is possible that cytochrome P-450 may show altered properties with regard to substrate binding and/or the rate of reduction of the P-450 substrate complex when hepatic iron levels decrease. This could lead to altered oxidative drug metabolism, since the rate-limiting steps in drug metabolism are those related to the capacity of the P-450 to bind to the substrate[22] and, even more important, the resulting reduction of the P-450 substrate complex.[24] Since it has been shown by Gillette *et al.*[25] that nitroreductase activity is largely dependent on the level of cytochrome P-450, the lack of alteration in nitroreductase activity during iron deficiency is understandable.

One requirement for completing any study relating nutritional deficiences to alterations in drug metabolism is proof that the results obtained are due only to the specific dietary constituent under study. This necessitates the repletion of the deficient diet with the appropriate nutrient and the return to normal of those parameters measured. It is apparent that the marked alterations in drug metabolism during the feeding of iron-deficient diets to rats are due wholly to iron deficiency, since the increased rate of aniline and aminopyrine metabolism (both *in vitro* and *in vivo*) returned to normal levels after feeding deficient animals the iron-containing diet for 7 days. Hemoglobin and hematocrit values also returned to normal during these iron-refeeding studies. Although it is likely that the same is true in mice,[10] iron repletion studies should be carried out to verify this fact.

A note of caution should be made concerning our studies on the effects of high dietary intake of iron. It has not been shown that our regimen would lead to increased iron stores and/or liver damage, nor was the study carried out for a long enough period of time to conclude positively that 1·5 times the normal intake of iron is harmless to young adult rats. Since a significant decrease in growth rate over control was noted, one must consider the inherent dangers in the consumption of high levels of iron until more clinical work has been carried out in this area.[26]

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