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Hepatic drug metabolism in retinol-deficient rats

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Oxidative drug and steroid metabolism by hepatic microsomes requires NADPH and molecular oxygen and utilizes cytochrome P-450 as the terminal oxidase [1]. Among the factors affecting the activity of hepatic oxidative enzymes is the nutritional status of the animal. For example, protein deficiency in growing rats has been shown to lower hepatic content of cytochrome P-450 and reduce the rates of ethylmorphine and aniline metabolism [2]. Starvation also influences hepatic drug metabolism [2–5], the specific effects deficiency in growing rats has been shown to lower hepatic animal and the hepatic substrate employed. For example, Gram *et al.* [4] found starvation of male rats to produce increases in hepatic microsomal concentration of cytochrome P-450 and in the rates of ethylmorphine demethylation and aniline hydroxylation. Little change in drug oxidation was seen in starved females. Fasted guinea pigs [5], on the other hand, metabolized aniline but not aminopyrine faster than controls, and had normal hepatic levels of cytochrome P-450.

Some vitamins also seem to have an important role in the regulation of hepatic oxidative metabolism. Patel and Pawar [6] reported that chronic riboflavin deficiency in male and female rats resulted in a decline in hepatic cytochrome P-450 concentration and the metabolism of several drug substrates. Administration of riboflavin to deficient animals reversed the effects of vitamin deficiency. Vitamin C deficiency also lowers the rate of hepatic drug oxidation [5, 7]. Hepatic microsomes obtained from guinea pigs on vitamin C-deficient diets for 21 days contained less cytochrome P-450 and NADPH cytochrome P-450 reductase activity than those obtained from controls, resulting in diminished metabolism of aniline, aminopyrine and *p*-nitroanisole. Each of these effects was reversed by ascorbic acid treatment *in vivo*. Recent studies [8] indicate that α-tocopherol also plays a role in the regulation of drug metabolism. Because of the apparent importance of some vitamins for the maintenance of normal hepatic oxidative metabolism, we have now examined the effects of retinol deficiency on these enzymes in rats.

Disease-free, weanling male albino Wistar rats were obtained from Hilltop Farms, Scottdale, Pa. Animals were caged individually under standardized conditions of light

Table 1. Effects of retinol deficiency on hepatic oxidative metabolism in rats*

Group	Body wt (g)	Liver wt (g)	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)	Drug metabolism (nmoles/min g liver)	
					Ethylmorphine	Aniline
Control	345 ± 12 (12)	11·7 ± 0·6 (12)	24·9 ± 1·1 (12)	0·98 ± 0·08 (12)	746·7 ± 75·3 (6)	43·3 ± 3·5 (6)
Retinol-deficient	247 ± 11 (12)†	7·3 ± 0·3 (12)†	19·3 ± 0·7 (12)†	0·56 ± 0·02 (12)†	346·6 ± 66·9 (8)†	25·1 ± 4·2 (8)†
Pair-fed	320 ± 10 (4)‡	10·2 ± 0·5 (4)‡	25·7 ± 1·9 (4)‡	0·91 ± 0·10 (4)‡	673·8 ± 83·2 (4)‡	39·7 ± 5·1 (4)‡
Retinol-deficient + retinol	325 ± 13 (5)†	10·8 ± 0·7 (5)†	26·3 ± 1·8 (5)†	0·90 ± 0·07 (5)†	709·3 ± 69·4 (5)†	41·3 ± 3·9 (5)†

* Values are expressed as mean ± S.E.; number of observations per group is indicated in parentheses.
† $P < 0.05$ (vs control group).
‡ $P < 0.05$ (vs retinol-deficient group).

Table 2. Effects of retinol deficiency on the spectral dissociation constants (K_s) of type I substrates in hepatic microsomes*

Group	Spectral dissociation constants (10^6) Hexobarbital	Ethylmorphine	Ethylbenzene	Naphthalene
Control	27·6 ± 1·0 (6)	22·1 ± 1·5 (4)	589·2 ± 42·2 (4)	239·1 ± 20·1 (5)
Retinol-deficient	76·8 ± 8·5 (6)†	47·2 ± 4·6 (4)†	208·3 ± 38·2 (4)†	83·2 ± 8·2 (5)†
Retinol-deficient + retinol	24·9 ± 1·2 (6)‡	20·7 ± 1·5 (4)‡	561·2 ± 62·0 (4)‡	188·3 ± 16·2 (5)‡

* Values are expressed as mean ± S.E.; number of observations per group is indicated in parentheses.
† $P < 0.05$ (vs control group).
‡ $P < 0.05$ (vs retinol-deficient group).

(06:00–18:00) and temperature (24°). Rats were made retinol deficient as previously described [9], and all animals were fasted 12 hr prior to sacrifice. Pair-fed controls were employed, since *ad lib.* food intake by retinol-deficient animals was approximately 20 per cent less than normal. Retinol-replaced rats received 6 mg retinol/kg of deficient diet for 8 weeks.

All animals were decapitated between 9:00 and 10:00 a.m., and livers were quickly removed and homogenized in cold 1·15% potassium chloride. Homogenates were centrifuged at 9000 *g* for 20 min in a Sorvall refrigerated centrifuge. Aliquots of the supernatant were removed for enzyme assays, and the rest was centrifuged at 105,000 *g* for 60 min in a Beckman preparative ultra-centrifuge. The microsomal pellet was suspended in 1·15% potassium chloride containing 0·05 M Tris-HCl buffer, pH 7·4. Substrate-induced type I difference spectra (Δ O.D.$_{385-420}$) were obtained using a Cary 17 recording spectrophotometer at room temperature. Microsomal suspensions contained 3–4 mg protein/ml. Spectral dissociation constants were calculated by the method of Schenkman *et al.* [10]. Cytochrome P-450 was measured as described by Omura and Sato [11] and microsomal protein determined by the method of Lowry *et al.* [12].

Reaction mixtures for drug metabolism assays contained either aniline (5 μmoles) or ethylmorphine (10 μmoles) and 0·5 ml liver 9000 *g* supernatant (500 mg/ml), glucose 6-phosphate (9 μmoles), $MgSO_4$ (24·2 μmoles), NADP (2·08 μmoles) and Tris-HCl buffer (0·02 M), pH 7·4, in a final volume of 3·0 ml. Semicarbazide HCl (25 μmoles) served as a trapping agent for formaldehyde produced from ethylmorphine. Incubations were carried out in a Dubnoff metabolic incubator at 37° for 15 min under air. Formaldehyde [13] and *p*-aminophenol [14] were assayed by methods previously described. All samples were read against appropriate tissue blanks.

Retinol-deficient rats weighed significantly less than controls at the end of the treatment period (Table 1). Livers were also proportionately reduced in size and contained no detectable retinol as determined by the trifluoroacetic acid reaction [15]. Rats pair-fed with retinol-deficient animals showed no significant decline in body or liver weights. Even pair-fed animals, therefore, weighed significantly more than retinol-deficient rats. Hepatic microsomal protein concentration was reduced in retinol-deficient rats but not in pair-fed controls. Cytochrome P-450 concentration in hepatic microsomes from retinol-deficient rats was also significantly lower than that in controls. Hepatic metabolism of ethylmorphine (demethylation) and aniline (hydroxylation), whether expressed per unit tissue weight (Table 1) or per mg microsomal protein, was significantly impaired in retinol-deficient animals. Pair-feeding had no effect on drug metabolism. Retinol administration (6 mg/kg of diet) for 6 weeks fully reversed the effects of retinol deficiency (Table 1). Livers from retinol-treated animals contained approximately 250 μg retinol/g of tissue.

Retinol deficiency also altered the apparent (spectral) affinity of cytochrome P-450 for several type I oxidative substrates (Table 2). Binding affinities for ethylmorphine and hexobarbital were reduced whereas those for ethylbenzene and napthalene, compounds which bind largely by hydrophobic interaction [16], increased in retinol-deficient animals. These changes were also fully reversed by administration of retinol to retinol-deficient rats.

Addition of retinol *in vitro* to hepatic microsomes from retinol-deficient rats further lowered oxidative metabolism. At a concentration of $2·8 \times 10^{-4}$ M, retinol inhibited ethylmorphine and aniline oxidation 47 and 59 per cent respectively. Inhibition of hepatic mixed function oxidase activity by retinol *in vitro* has previously been reported by Hill and Shih [17]. Spectral dissociation constants for hydrocarbon or drug substrates in hepatic microsomes were unaffected by similar concentrations of retinol *in vitro*.

Previous investigations have established the importance of various nutritional factors in determining the activity of hepatic mixed function oxidase systems. The present studies indicate that retinol is also required for the maintenance of normal hepatic cytochrome P-450 levels and oxidative metabolism. Others have shown that food deprivation can markedly influence hepatic drug oxidation [2–5], and retinol-deficient animals do indeed consume somewhat less food than normal animals. However, changes in caloric intake probably do not contribute to the effects reported here, since pair-fed animals showed none of the changes seen in retinol deficiency. Furthermore, the effects of starvation on drug oxidation, previously reported [2–5], are for the most part opposite to those resulting from retinol-deficiency.

The decline in drug metabolism in retinol-deficient rats can apparently be attributed to both an overall reduction in hepatic microsomal protein and a fall in the specific activity of oxidative enzymes. Although full delineation of the mechanisms responsible for the effects of retinol deficiency on hepatic function will require further investigation, the decline in hepatic cytochrome P-450 content in the present studies closely paralleled the changes in drug metabolism. Moreover, the changes in the rates of hydroxylation of aniline, a type II substrate, and demethylation of ethylmorphine, a type I substrate, induced by retinol deficiency were similar, perhaps suggesting a relatively non-specific decline in hepatic oxidative metabolism. Studies employing additional substrates are needed to determine the validity of this hypothesis. In addition, although oxidative enzyme activity when expressed per unit liver weight or per mg microsomal protein was substantially reduced by retinol deficiency, activity per nmole of cytochrome P-450 was unaffected, further suggesting that changes in hemoprotein content contribute significantly to the changes in drug metabolism.

Our observations also suggest a change in the nature of hepatic cytochrome P-450 in retinol-deficient animals.

The apparent affinity of cytochrome P-450 for several substrates, as determined spectrally, was significantly different in controls and retinol-deficient rats. Binding affinity for drug substrates (ethylmorphine and hexobarbital) decreased in retinol deficiency, whereas affinity for simple aromatic hydrocarbons (ethylbenzene and naphthalene) increased. Each of these effects was reversed by retinol administration to deficient animals. We have recently demonstrated [16] that hydrocarbon binding to hepatic cytochrome P-450 follows a "partition" law, the hydrocarbon behaving as if "extracted" from the aqueous medium by the enzyme (nonaqueous phase). Hydrophobic interaction was shown to be the principal determinant of hydrocarbon binding to cytochrome P-450. The present results are, therefore, consistent with an increase in the hydrophobicity of cytochrome P-450 (or decrease in the hydrophobicity of its environment) in retinol-deficient animals.

None of the changes produced by retinol deficiency could be reversed by addition of retinol to hepatic microsomes. In fact, the oxidation of both aniline and ethylmorphine was further inhibited by retinol *in vitro*. Thus, direct effects of retinol on hepatic microsomes cannot explain the changes in oxidative metabolism seen in retinol-deficient rats. Studies are now in progress to further clarify the actions of retinol on hepatic mixed function oxidase systems.

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NOTE ADDED IN PROOF

Observations made by G. C. Becking (*Can. J. Physiol. Pharmac.* **51**: **6**, 1073) which have recently come to our attention are consistent with the effects of retinol presented here.