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INCREASED HEPATIC RETINAL DEHYDROGENASE ACTIVITY AFTER PHENOBARBITAL AND ETHANOL ADMINISTRATION

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Abstract—Cytosolic retinal dehydrogenase (EC 1.2.1.36, retinal: NAD^+ oxidoreductase) activity was measured by assessing the conversion of retinal to retinoic acid by HPLC. *In vitro*, acetaldehyde, chloral hydrate and disulfiram were found to be inhibitors, whereas 95% of the activity remained in the presence of cyanide or in the absence of oxygen. In rats, retinal dehydrogenase activity prevailed over that of retinal oxidase. By contrast, in deermice, 80% of retinal oxidation was due to the oxidase rather than the retinal dehydrogenase activity in a normal strain (ADH^+) as well as in one lacking alcohol dehydrogenase (ADH^-). In ADH^- deermice, retinal oxidase activity was greater than in ADH^+ animals. *In vivo*, in the rat, chronic ethanol administration resulted in a significant increase of the dehydrogenase activity in the liver, but not in other tissues. After phenobarbital administration, hepatic retinal dehydrogenase activity was increased 8-fold, but no extrahepatic induction was observed. Conversely, feeding rats with a diet devoid of the precursor for the substrate (retinal) by replacing retinyl acetate with an equivalent amount of retinoic acid resulted in decreased retinal dehydrogenase activity. Under conditions in which retinal dehydrogenase activity is rate-limiting for the metabolism of retinol to retinoic acid, its induction after phenobarbital or ethanol administration may contribute to hepatic vitamin A depletion.

The observations that phenobarbital administration [1, 2] and chronic ethanol consumption [3] are associated with significant depletion of hepatic vitamin A in animals and that a correspondent reduction was found in humans [1, 4] raised the question of the possible mechanisms involved. Some results suggested that malabsorption was not the main mechanism since ethanol was shown to promote hepatic vitamin A depletion even in the absence of dietary vitamin A [3]. Thus, enhanced mobilization of vitamin A from the liver as well as enhanced degradation had to be considered as putative mechanisms. Subsequently, evidence was gathered in favor of increased mobilization after acute [5] and chronic [2, 6] ethanol administration as well as enhanced degradation. Indeed, phenobarbital administration and chronic ethanol consumption were found to increase retinol breakdown via a cytochrome P-450 dependent microsomal system [7, 8]. There was also increased activity of a newly described microsomal retinol dehydrogenase but the latter effect was relatively small [9] for ethanol, and non-existent for phenobarbital. Classically, the main pathway for retinol disposition in the liver proceeds via a cytosolic NAD^+-dependent retinol dehydrogenase, the activity of which is also increased, but only modestly after chronic ethanol consumption, and not at all after phenobarbital [9]. The product of both cytosolic and microsomal retinol dehydrogenases, namely retinal, is metabolized further in various tissues by a cytosolic NAD^+-dependent, cyanide-insensitive retinal dehydrogenase (EC 1.2.1.36, retinal:NAD^+ oxidoreductase) or an oxygen-requiring, cyanide-sensitive retinal oxidase [10–15]. The present study was undertaken to assess the possible effects of phenobarbital administration and chronic ethanol consumption on retinal oxidation.

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MATERIALS AND METHODS

Chemicals. Ammonium acetate, acetic acid, and all HPLC solvents were obtained from the J. T. Baker Chemical Co. and filtered through 0.5 μm Fluoropore filters (Millipore, Bedford, MA). Butylated hydroxytoluene (BHT), NAD, bovine serum albumin (BSA, fraction V), L-ascorbic acid, EDTA, *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), dilsulfiram, dithiothreitol (DTT), Me_2SO, potassium cyanide, retinoic acid and retinal were purchased from Sigma (St. Louis, MO). Retinol RO 1-4955 was donated by Hoffman-La Roche (Nutley, NJ). 4-Methylpyrazole was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Chloral hydrate was provided by the V.A. Medical Center Pharmacy.

Animals. Weanling male and female Sprague–Dawley rats [Crl:CD] BR strain, were purchased from the Charles River Breeding Laboratories (Wilmington, MA) and fed Purina Laboratory Chow. Unless stated otherwise, the results were obtained in male animals. Some of these rats, when they reached a weight of 120–150 g, were given for 4 weeks our regular liquid diet [16], with or without ethanol (isocalorically substituted for carbohydrates

providing 36% of total energy), containing 1 kcal of energy and 6 units of vitamin A (retinyl acetate) per ml. Dietary ingredients were purchased from Dyets, Inc. (Bethlehem, PA). Over the 4 weeks of pair feeding, the ethanol-fed rats (N = 12) and their respective control littermates (N = 12) consumed comparable amounts of diet (1765 ± 71 vs 1762 ± 69 ml) and reached body weights of 240 ± 8 and 261 ± 6 g respectively. Another group of weanling rats was given the liquid diet described above except that retinyl acetate was replaced by an equimolar concentration of retinoic acid. After 8 weeks, the animals reached body weights of 296 ± 7 and 310 ± 9 g (N = 6) for the ethanol-fed and the control group respectively. Six additional animals (90–100 g body weight) were fed for 2 weeks the control liquid diet with the addition of 0.3 mg phenobarbital/ml, which resulted in a daily intake of 21 ± 0.3 mg. Littermates were pair-fed daily the same diet without phenobarbital. Over the 2 weeks, the animals treated with phenobarbital had an intake of 974 ± 16 vs 992 ± 37 ml in pair-fed controls and reached a comparable body weight (175 ± 2 vs 182 ± 4 g, N = 6). Deermice originally obtained from Dr. M. Felder (Columbia, SC) were bred in our laboratory and fed a mouse diet (Wayne Rodent Blox, 8604-00, Continental Grain Co., Chicago, IL).

Procedures. The rats were killed by decapitation, and the deermice by cervical dislocation. The animals were sacrificed in the fed state, except for the rats fed the liquid diets [16], which were killed after a 16-hr fast. Liver, kidney, testes and eyes were rapidly removed, rinsed in ice-cold KCl (1.15%), and 20%, 30%, 30%, and 50% homogenates were made with the same solution respectively. Microsomes and fat-free cytosol were obtained from liver, kidney and testes as previously reported for rats [17] and deermice [18] with the following modifications: the fat-free cytosol was centrifuged for an additional 30 min at 100,000 *g* to avoid microsomal contamination. Mitochondria were prepared from another aliquot of the liver as previously described [19]. Proteins were measured according to Lowry *et al.* [20]. The substrate retinal was dissolved in Me_2SO with BHT (1 mg/ml) at a concentration of about 50 mg/ml; an aliquot was then mixed with BSA (3.2 mg/ml) at the desired concentration.

Cytosol (1 mg protein/ml) was incubated at 37° under air and dim light with retinal (0.15 mM, except for the phenobarbital-treated rats, their controls, and the deermice, for which 0.2 mM was used). Higher concentrations were avoided, because of substrate inhibition. The reaction was performed in triplicate in disposable borosilicate glass culture tubes (13 × 100 mm). The reaction mixture contained 50 mM Hepes, 0.15 mM KCl buffer, pH 8.5, with 1 mM EDTA, 50 μM DTT, 200 μM 4-methylpyrazole, 0.075% Me_2SO and 0.8 mg/ml BSA with or without 3 mM NAD^+. The pH 8.5 was selected on the basis of preliminary experiments indicating optimal activity. A similar pH was also used by others [11, 21]. The reaction was started with the simultaneous addition of the cofactor and the substrate. When indicated, ethanol, chloral hydrate (aqueous solutions) or disulfiram (in 40 mM ethanol) was added to the mixture (at the required concentration) prior to addition of the cytosol. When acetaldehyde was used, the incubation was carried out in air-tight conditions to avoid evaporation of the compound. With boiled cytosol or homogenate, no significant reaction was detected. In the absence of cofactor, in the rats, 5–10% of the activity remained (retinal oxidase). The addition of potassium cyanide (0.1 mM) completely inhibited this residual reaction, without affecting the NAD^+-dependent activity. The retinal dehydrogenase activity was calculated as the difference of the activities between the presence and the absence of NAD^+. This was equivalent to the cyanide-insensitive part of the reaction. Microsomal and mitochondrial fractions (1 mg protein/ml) were incubated in the same mixtures as the one used for the cytosol, with the omission of 4-methylpyrazole and the addition of 1 μM rotenone.

After 10 min of incubation, the reaction was stopped by adding a mixture of L-ascorbic acid and EDTA (0.25 mg each), and 0.5 ml of cold absolute ethanol followed by rapid freezing in dry ice–acetone. Samples were extracted twice with 2 ml hexane containing BHT (1 mg/ml) and centrifuged for 10 min at 2000 *g*. After freezing in dry ice–acetone, the supernatant fraction was decanted rapidly into a second set of disposable tubes, and the combined supernatant was evaporated completely under nitrogen and resuspended in 2-propanol/water (9:1) for HPLC analysis.

HPLC procedures. All HPLC analyses were carried out with an HP-1090 Chemstation or with an HP-1090L, equipped with a diode-array detector, an HP-3392A Integrator, a Think-jet printer and an HP-7440A Plotter (Hewlett–Packard, Avondale, PA). The HPLC system used was as follows: a Zorbax ODS column (0.46 × 15 cm) (Mac-Mod Anal. Inc., Chadds Ford, PA) for which the mobile phase was programmed with a gradient from 25% mobile phase B (75% A) to 90% mobile phase B (10% A) in 10 min at a flow rate of 1.5 ml/min. The mobile phase A was acetonitrile/H_2O/acetic acid (49.75/49.75/0.5; by vol.) and the mobile phase B was acetonitrile/H_2O/acetic acid (90/10/0.04; by vol.), both containing 0.01 mM ammonium acetate as previously reported [7, 22]. Simultaneous multiwavelength detection at 325, 350 and 365 nm was obtained for all samples.

Statistics. Results are expressed as mean ± SEM, and the significance of the differences was calculated by Student's paired *t*-test or group *t*-test. Data among 4 groups were analyzed by one-way ANOVA and Newman–Keuls post-hoc tests. [23]. Enzyme kinetics were determined from weighted double-reciprocal plots of velocity versus substrate concentrations [24] with an IBM PC computer.

RESULTS

In rat liver cytosol retinal dehydrogenase activity (tested at retinal concentrations of 0.008 to 0.15 mM) was found to have a V_{max} of 1.58 ± 0.15 nmol/min/mg protein and an apparent K_m of 0.066 ± 0.002 mM in rats fed Purina Chow, and weighing 197 ± 7.8 g (N = 5). Fasting was found to decrease the activity by as much as 50%: in three fasted rats weighing

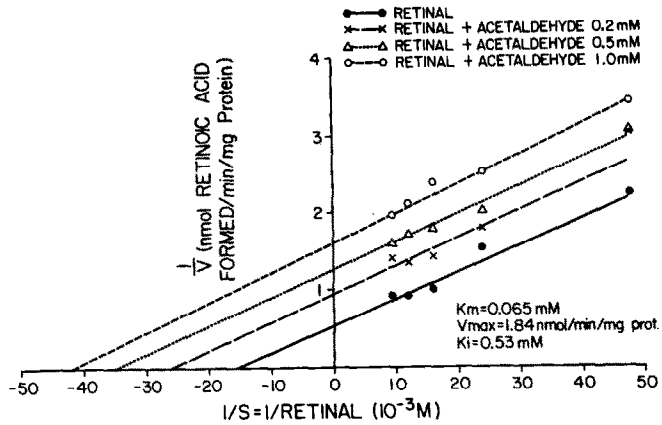

Fig. 1. Effect of substrate concentration on the rate of retinal oxidation and uncompetitive inhibition of retinal dehydrogenase activity by acetaldehyde. (Lineweaver–Burk double-reciprocal plot.)

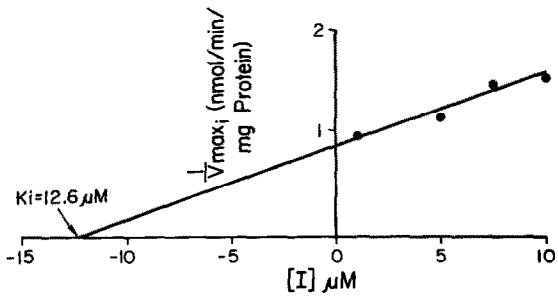

Fig. 2. Non-competitive inhibition of retinal dehydrogenase activity by chloral hydrate. $1/V_{max_i}$ (obtained from reciprocal plots) is replotted here versus the chloral hydrate inhibitor concentrations [*I*]. The non-competitive inhibition was linear and had a K_i of 12.6 ± 0.2 μM. Concentrations of retinal used were 40, 60, 80, 100 and 120 μM.

172 ± 2.6 g, the $K_m$was 0.030 ± 0.006 mM and the V_{max} 0.65 ± 0.14 nmol/min/mg protein. In addition to the retinoic acid formed, a small peak of retinol was also detected, probably reflecting reduction of retinal to retinol by the back reaction of the cytosolic retinol dehydrogenase. Quantitatively, this represented 3 ± 0.4% (N = 6) of the forward reaction. Appearance of the retinol peak could be avoided by the addition of 4-methylpyrazole (200 μM), a well-known inhibitor of the cytosolic retinol dehydrogenase. Therefore, 4-methylpyrazole (200 μM) was incorporated in our assay procedure. *In vitro*, addition of ethanol (50 and 100 mM) had no significant effect on retinal dehydrogenase activity. Acetaldehyde (tested at 0.2, 0.5, 1.0 mM) showed uncompetitive hyperbolic inhibition, with a K_i of 0.53 ± 0.01 mM (Fig. 1). Chloral hydrate, a known aldehyde dehydrogenase inhibitor, was found to be a potent linear non-competitive inhibitor of retinal dehydrogenase activity (Fig. 2) with a K_i of 12.6 ± 0.2 μM. Disulfiram (20 and 50 mM) was also found to inhibit the enzyme activity.

As previously reported, after chronic ethanol consumption, liver vitamin A was decreased significantly in ethanol-fed rats compared to controls (563 ± 53 vs 1268 ± 75 nmol/g; $P < 0.001$ and 1980 ± 204 vs 2221 ± 153 nmol/total liver/100 g body weight; $P < 0.001$, N = 6). Concomitantly, there was a striking increase in retinal dehydrogenase activity in the liver cytosol (Fig. 3) with a corresponding increase in the homogenate. In the liver homogenate of ethanol-treated and pair-fed rats the enzyme activities were found to be 0.16 ± 0.02 vs 0.10 ± 0.01 nmol/min/mg protein, 41.0 ± 4.1 vs 22.5 ± 1.9 nmol/min/g liver and 143.7 ± 14.2 vs 60.0 ± 6.5 nmol/min/total liver/100 g body weight (N = 6). In absolute values these activities were 20–30% lower than those determined in the cytosol (Fig. 3), probably because of the presence of some interfering substances. The K_m of retinal dehydrogenase activity in the cytosol (0.52 ± 0.05 mM; N = 4) was not affected significantly by ethanol feeding. Retinal oxidase activity was too low to be measured accurately. No significant retinal oxidation was detected in the microsomes of all groups. The activity in the mitochondrial fraction could be ascribed to cytosol contamination, as assessed by enzyme markers.

Contrasting with the induction of cytosolic retinal dehydrogenase activity in the liver, there was no corresponding increase in other tissues: there was no significant change in kidney cytosol of the ethanol-fed rats compared to controls (56.4 ± 2.0 vs 60.2 ± 3.6 nmol/min/g; N = 6) nor was there a change in the eye homogenate (4.2 ± 0.5 vs 4.0 ± 0.6 nmol/min/g; N = 6). In the testes there was even a modest but significant decrease in ethanol-fed rats when the results were expressed per gram of tissue (21.8 ± 1.1 vs 26.8 ± 1.5; $P < 0.05$) but not per total tissue per 100 g body weight (24.6 ± 0.9 vs 27.5 ± 1.2; N = 6).

After phenobarbital treatment, the liver vitamin A was reduced significantly when compared to the pair-fed control (405 ± 29 vs 761 ± 28 nmol/g, $P < 0.001$ or 2099 ± 135 vs 2518 ± 115 nmol/total liver/100 g body weight, $P < 0.05$, N = 6), and the retinal dehydrogenase activity was increased 8-fold in the liver cytosol (Fig. 4). In phenobarbital-treated rats, the apparent K_m and V_{max} were

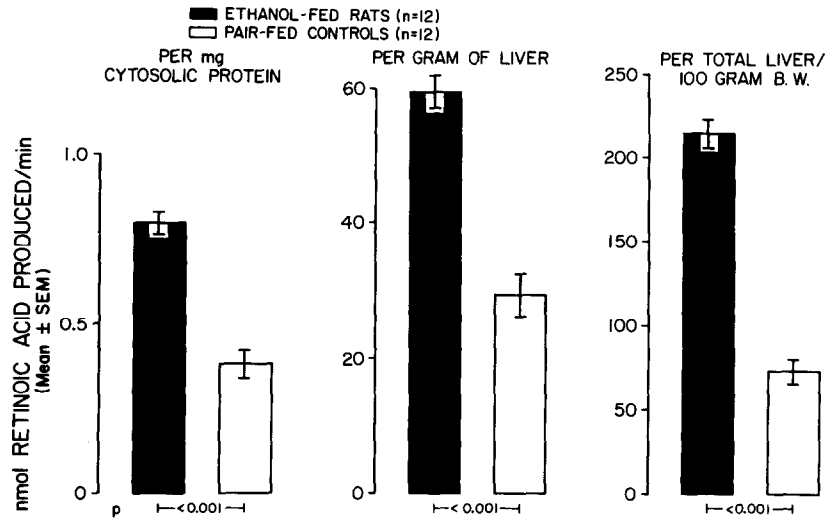

Fig. 3. Effect of chronic ethanol consumption on retinal dehydrogenase activity of rat liver cytosol.

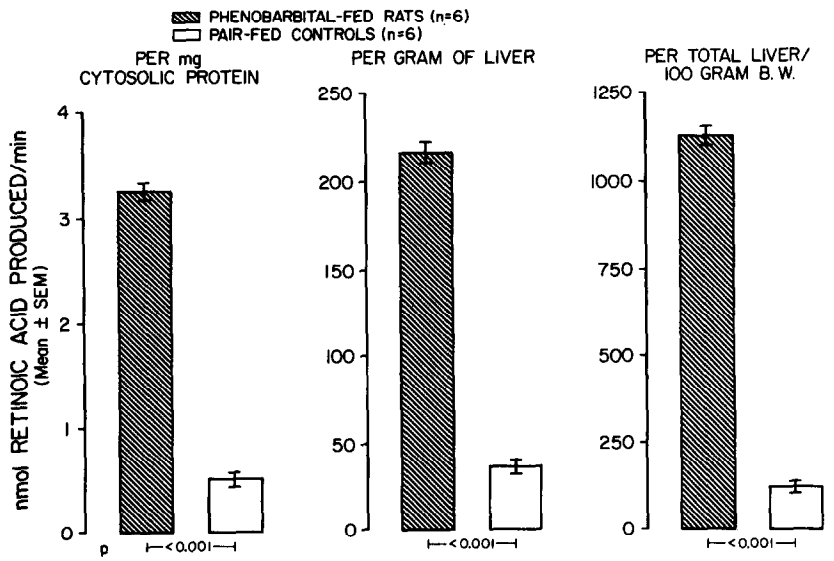

Fig. 4. Effect of phenobarbital administration on retinal dehydrogenase activity of rat liver cytosol.

significantly different from those of the pair-fed controls: 0.37 ± 0.07 vs 0.022 ± 0.003 mM ($P < 0.005$; $N = 6$) and 9.3 ± 1.7 vs 0.68 ± 0.10 nmol/min/mg protein ($P < 0.001$; $N = 6$) respectively. No difference in activity was detected in extrahepatic tissues of phenobarbital-treated rats compared to controls: in the kidneys the activity was 61.4 ± 1.8 vs 62.3 ± 5.1 nmol/min/g ($N = 6$) and, in the testes, it was 19.1 ± 1.0 vs 18.6 ± 1.2 nmol/min/g ($N = 6$).

In the animals fed the retinoic acid-rich diet, which is devoid of any precursor of the substrate (retinal) for retinal dehydrogenase, there was a significant decrease in the activity of this enzyme, both in ethanol-fed and control rats (Table 1, to be compared with Fig. 3). The difference was significant ($P < 0.001$ for ethanol-fed and $P < 0.01$ for controls), with the results expressed either as nmol/min/per mg protein, per g liver or per total liver per 100 g body weight.

In deermice, $81.8 \pm 1.4\%$ and $76.7 \pm 2.9\%$ of the hepatic retinal oxidation was due to the oxidase activity in the ADH^- and ADH^+ strains respectively (Table 2). In the liver, the activity was significantly higher in the cytosol of the ADH^- than in the ADH^+ animals. As for the rat, the enzyme activity in the homogenate was lower than in the cytosol: $82.3 \pm 2.7\%$ and $86.0 \pm 2.5\%$ ($N =$

Table 1. Retinal dehydrogenase activities in liver cytosol of retinoic acid-fed rats

	Retinal dehydrogenase activity (nmol/min)		
	Ethanol	Control	P*
Per mg protein	0.34 ± 0.06†	0.17 ± 0.03‡	<0.02
Per g liver	24.0 ± 4.7†	13.5 ± 2.7‡	<0.02
Per total liver/100 g body wt	84.0 ± 12.3†	37.3 ± 6.2§	<0.01

Values are means ± SEM (N = 6).

* Significance of difference between ethanol and control groups was analyzed by Student's paired *t*-test.

†–§ Values were significantly lower (†$P < 0.001$, ‡$P < 0.01$ or §$P < 0.025$) than the corresponding results obtained in animals fed the regular (retinyl-acetate-containing) diets (Fig. 3); significance of differences between groups was obtained by one-way ANOVA and Newman–Keuls post-hoc tests.

Table 2. Retinal oxidase and dehydrogenase activities in liver cytosol of ADH^- and ADH^+ deermice

	ADH^-		ADH^+	
	Oxidase	Dehydrogenase (nmol/min)	Oxidase	Dehydrogenase (nmol/min)
Per mg protein	3.25 ± 0.25*	0.70 ± 0.04	1.76 ± 0.20	0.58 ± 0.11
Per g liver	244 ± 37†	51.8 ± 6.5	121 ± 9	38.8 ± 6.0
Per total liver/100 g body wt	979 ± 131†	210 ± 25	492 ± 35	157 ± 23

Liver cytosol was prepared as described under Materials and Methods. The enzyme activity was measured with 0.8 mg protein/ml, 0.2 mM retinal and 3 mM NAD (retinal dehydrogenase) or without the cofactor (retinal oxidase).

Values are means ± SEM (N = 6).

*† Significantly different compared to ADH^+: * $P < 0.001$, and † $P < 0.01$.

6) of the cytosolic activity respectively. It is noteworthy that, even without any treatment, the retinal oxidase activity of the deermice was relatively high and much more elevated than in the rat, particularly in the ADH^- animals. These ADH^- deermice were devoid of cytosolic retinol dehydrogenase activity, confirming our previous finding [25].

DISCUSSION

This study revealed that chronic ethanol consumption and phenobarbital administration are associated with a significant increase in activity of cytosolic retinal dehydrogenase in the liver. No such increase was found in eyes, kidneys and testes. After chronic ethanol consumption, there was even a slightly decreased activity in the testes. Acetaldehyde was found to be an uncompetitive inhibitor of the dehydrogenase but the concentrations required were well above those found *in vivo* [26–28]. Thus, although acetaldehyde levels are increased after chronic ethanol consumption [28, 29], they may not reach concentrations which affect the enzyme activity to a meaningful extent. Cloral hydrate was found to be a non-competitive inhibitor. Ethanol (50–100 mM) and 4-methylpyrazole (200 μM) were not inhibitory, but disulfiram was a non-competitive inhibitor. Similar results have been reported in a pig kidney epithelial cell line with ethanol, 4-methylpyrazole and disulfiram [21]. The physiological substrate for the enzyme could be the retinal derived from β-carotene, as postulated by Napoli [21], or formed from retinol by the cytosolic or microsomal retinol dehydrogenases [9]. Consistent with this view is the finding that the retinal dehydrogenase activity was decreased in animals fed a diet in which retinyl acetate had been replaced by retinoic acid and thus was devoid of substrate for this enzyme (Table 1). It is of interest that in ADH^- deermice, which lack the cytosolic retinol dehydrogenase [25], retinal oxidation was nevertheless rather high (Table 2), when compared to the rat (Fig. 3). In these animals, retinal is produced by the recently discovered microsomal retinol dehydrogenase [9].

The increased retinal oxidation described in this paper may be involved in the regulation of the level of hepatic vitamin A. Multiple factors determine the amount of retinol (as retinyl esters) in the liver. Dietary vitamin A is one of the most obvious factors but, since our animals were strictly pair-fed, their intakes were comparable. Differences in hepatic vitamin A between ethanol-fed and control animals were found previously to occur

even in the absence of dietary vitamin A, indicating that malabsorption is not a major factor under those conditions [3]. Other factors such as the induction of microsomal cytochrome P-450 dependent retinol degradation to polar metabolites [7] may be contributory to the hepatic vitamin A depletion. Furthermore, both acute [5] and chronic [6] ethanol administration were found to increase mobilization of retinol from the liver to peripheral tissues and this mechanism obviously may contribute to hepatic vitamin A depletion after ethanol [3], but not after phenobarbital, which did not provoke such mobilization [2]. The cytosolic retinol dehydrogenase has been considered as rate-limiting in the metabolism of retinol to retinoic acid, as shown most recently for mammalian pig kidney cells [21]. However, the results of the present study reveal an activity for the retinal dehydrogenase lower than that previously found for the cytosolic retinol dehydrogenase [9]. Furthermore, a new microsomal retinol dehydrogenase has been described [9] which may also provide some additional substrate for the retinal dehydrogenase. Therefore, retinal oxidation could become rate-limiting. In view of its increased activity after drug induction, the retinal dehydrogenase could sustain a significant rate of metabolism, and, to the extent that it remains rate-limiting, it may contribute to vitamin A depletion. However, the intracellular concentrations of retinol and retinal at the enzyme sites are not known, and the activity may not be saturated, particularly after phenobarbital which induces a form of the enzyme which appears to have a significantly increased K_m for retinal. Contrasting with the decrease in affinity, there was after phenobarbital a striking 8-fold increase in retinal dehydrogenase activity (Fig. 4), to a level which exceeded that of the two retinol dehydrogenases [9]. Thus, under these conditions, retinal dehydrogenase activity might not necessarily be rate-limiting in the conversion of retinol to retinoic acid in the liver. This effect of phenobarbital is comparable to its induction of cytosolic aldehyde dehydrogenase described in some rat strains [30] and, in fact, the same isozyme may be involved. By contrast, the induction effect of ethanol on retinal dehydrogenase activity described in this paper may differ from the action of ethanol on acetaldehyde dehydrogenase; the latter was found in many studies to be decreased, but the results are controversial, with some increases also reported [31].

Retinal oxidation was also found in deermice, in both the ADH^+ and ADH^- strain. Deermice lacking ADH also lack retinol dehydrogenase [25]. Nevertheless, these rodents were found in the present study to have retinal oxidase activity. In fact, the latter activity was greater in the ADH^- than in the ADH^+ animals. Therefore, this raises the question of the origin of retinal, the substrate for this retinal oxidation in ADH^- and, thus, retinol dehydrogenase negative animals. One possibility is β-carotene; indeed, it has been suggested that retinal directly derived from β-carotene may serve as a retinoic acid precursor [21]. Of interest is the observation that, in ADH^- deermice, retinal oxidase activity exceeded that previously reported for the microsomal retinol dehydrogenase [9], which could therefore become rate-limiting in the metabolism of retinol to retinoic acid in that species. Under other conditions, however, retinal oxidation may be rate-limiting and participate in the regulation of the magnitude of hepatic retinol metabolism.

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