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# INTERACTION OF DRUGS AND RETINOL

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**Abstract**—In liver microsomes of ethanol-fed rats, retinol competitively inhibited the hydroxylation of aniline, the demethylation of dimethylnitrosamine, and the oxidation of ethanol to acetaldehyde, whereas the inhibition of benzphetamine demethylation was of the mixed type in microsomes of phenobarbital-treated, ethanol-treated or control rats. Conversely, benzphetamine exerted a striking inhibition of the 4-hydroxylation of retinol in microsomes of phenobarbital-treated rats. At the concentration used, ethanol (100 mM) and dimethylnitrosamine (10 mM) had no such effect. *In vivo* administration of phenobarbital resulted in a 9-fold increase in the $V_{max}$ of the microsomal retinol 4-hydroxylase activity, with a 3-fold increase of the $K_m$, whereas ethanol feeding resulted in a doubling of the $V_{max}$ with no significant change in the $K_m$. The induction of this microsomal retinol-metabolizing system may contribute to the hepatic vitamin A depletion that has been reported previously after either ethanol or drug administration. Conversely, the observed inhibition, by retinol, of microsomal drug metabolism, including the demethylation of dimethylnitrosamine, may be of significance with regards to the interaction of retinol with carcinogenesis.

In both humans [1] and animals [2], ethanol consumption as well as experimental drug administration [3] result in the depletion of hepatic vitamin A. Furthermore, the combination of ethanol with drugs as well as food additives results in a striking potentiation of the depletion [4], but the mechanism of this effect is unknown. Since xenobiotics are metabolized by microsomal systems inducible by drug administration *in vivo* and since a similar cytochrome P-450-dependent retinol-metabolizing system was described recently [5], the question was raised whether ethanol and other drugs interact with retinol metabolism at the microsomal level and, conversely, whether retinol affects ethanol and drug metabolism at this site. Furthermore, it is known that microsomal cytochrome P-450 consists of a family of isoenzymes with selective affinities for various drugs; therefore, we also wished to assess in the present study to what extent the selective induction of some of these systems by drug administration results in differential effects on retinol metabolism.

## MATERIALS AND METHODS

*Animals.* Weanling male Sprague–Dawley rat littermates (Crl:CD$^R$ (SD) BR strain, Charles River Breeding Laboratories, Wilmington, MA) were fed, for 2–3 weeks, our regular control liquid diet [6] containing, per ml, 1 kcal of total energy, 6 units of vitamin A (retinyl acetate) with the addition of 0.3 mg phenobarbital, which resulted in a daily intake of about 130 mg/kg. Littermates were pair-fed daily the same diet without the phenobarbital. Similarly, other rat littermates were pair-fed the ethanol-containing (36% of total energy) or the isocaloric control diet for 24 days.

*Methods.* After a 16-hr fast, rodents were decapitated, liver microsomes were prepared [7], and the concentration of cytochrome P-450 was determined [8]. Measurements were carried out of the activities of microsomal ethanol oxidizing system [7], microsomal aniline hydroxylase [9], and benzphetamine and dimethylnitrosamine (DMN) demethylases [10], except that the incubation conditions were as described below for microsomal retinol metabolism, with determination of formaldehyde according to Werringloer [11]. Microsomes (0.5 to 1 mg protein/ml) were incubated with [11, 12-$^3$H]-all-*trans*-retinol, specific activity 40–60 Ci/mmole (Amersham Corp, Arlington Heights, IL), dissolved in ethanol–water [9:1 (v/v) + 1 mg/ml butylated hydroxytoluene (BHT)], and 97.1 ± 0.41% pure, as verified by high performance liquid chromatography (HPLC). The substrate was stored up to 2 months under Argon at −80°, in separately sealed small aliquots. An aliquot of this labeled retinol together with another aliquot of unlabeled all-*trans*-retinol (provided by Hoffmann-LaRoche, Nutley, NJ) were evaporated to near dryness, and bovine serum albumin (fraction V), 3.2 mg/ml of water, was added to the substrate in a ratio of 6.5 μg of albumin/nmole of retinol. After vigorous mixing, the substrate was evaporated for 15 min and then sonicated for 10 min at 200 W in cold water with a Braun-Sonic 1510 sonicator. Except when otherwise indicated, the concentration of retinol varied between 0.1 and 0.15 mM with or without DMN, ethanol or benzphetamine. The incubation was carried out at 37°. The reaction mixture contained 0.1 M Tris–HCl, 0.15 M KCl buffer, pH 7.4 (at 37°), with 5 mM $MgCl_2$, 1 mM EDTA, with or without a 1 mM of concentration NADPH, NADP or NAD. After a 3-min preincubation, the reaction

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was started with the addition of the cofactor. After a 5- to 10-min incubation, the reaction was stopped by adding a mixture of L-ascorbic acid and EDTA (0.25 mg each) and rapid freezing in dry ice and acetone. The samples were then lyophilized and extracted overnight with 1 ml methanol (containing 1 mg BHT) as previously described [5].

All HPLC analyses were carried out with a 1084B liquid chromatograph equipped with a variable wavelength detector and a fraction collector or an HP-1090 liquid chromatograph equipped with a photo diode-array spectrophotometric detector (Hewlett–Packard, Palo Alto, CA). The principal HPLC system used was as follows: a Zorbax ODS column (0.46 × 15 cm) (DuPont, Wilmington, DE) 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 contained 0.01 M ammonium acetate. The final solvent conditions were kept for 8 min. Thereafter, the column was equilibrated with the initial solvent mixture for 10 min before the next sample injection [12]. Detection was carried out at 325 nm. With this system, the retention time for 4-hydroxyretinol was 5.01 ± 0.01 min with the HPLC 1084B and 3.83 ± 0.006 with the HPLC 1090. Recovery of 4-hydroxyretinol added to microsomes was 91.6 ± 2.2%. All these analyses were done at 30°. Fractions (0.25 and 0.5 min) were collected and counted in a Beckman LS 7800 liquid scintillation counter (Beckman Scientific Instruments, Irvine, CA) after the addition of 5 ml Aquasol (New England Nuclear, Boston, MA).

*Chemicals.* Ammonium acetate, acetic acid, and all HPLC solvents were obtained from the J. T. Baker Chemical Co. and were filtered on 0.5 μm Fluoropore filters (Millipore, Bedford, MA). Butylated hydroxytoluene, NADPH, NADP, NAD, bovine serum albumin (fraction V), L-ascorbic acid, DMN and aniline hydrochloride were purchased from the Sigma Chemical Co. (St. Louis, MO); benzphetamine hydrochloride was from the Upjohn Co. (Kalamazoo, MI).

*Statistics.* Results are expressed as mean ±S.E., and the significance of the difference was calculated by Student's *t*-test [13]. Kinetics of retinol and drug metabolism were determined from double-reciprocal plots of velocity versus substrate concentrations and calculations of the best fitted lines with the Stat-Pro statistical package for the IBM microcomputer.

## RESULTS

*Effect of retinol on microsomal drug metabolism.* The effect of retinol on microsomal aniline hydroxylation was studied in preparations obtained from ethanol-fed rats (Fig. 1) and controls (not shown). In both preparations, retinol exerted a competitive-type inhibition. A similar inhibition was also observed in terms of the microsomal ethanol-oxidizing system (Fig. 2) and the DMN demethylase activity (Fig. 3). It should be noted that some of the

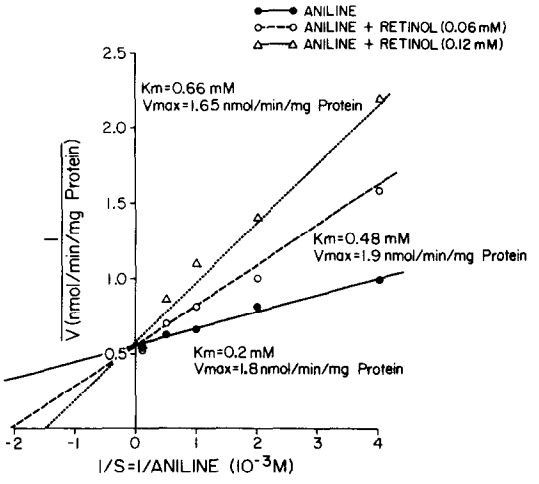


Fig. 1. Effect of retinol on hepatic microsomal aniline hydroxylation (Lineweaver–Burk plot). Liver microsomes of rats fed ethanol (as described in Materials and Methods) were incubated with various concentrations of aniline, with or without retinol.

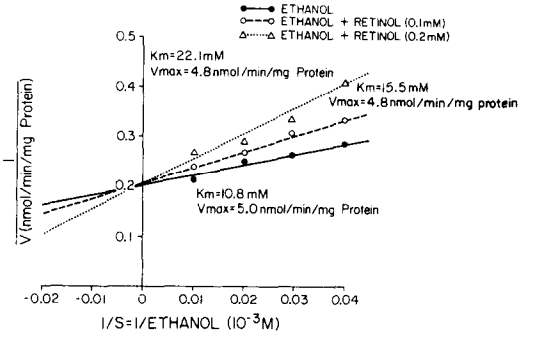


Fig. 2. Effect of retinol on hepatic microsomal ethanol oxidation. Liver microsomes of rats fed ethanol (as described in Materials and Methods) were incubated with various concentrations of ethanol, with or without retinol.

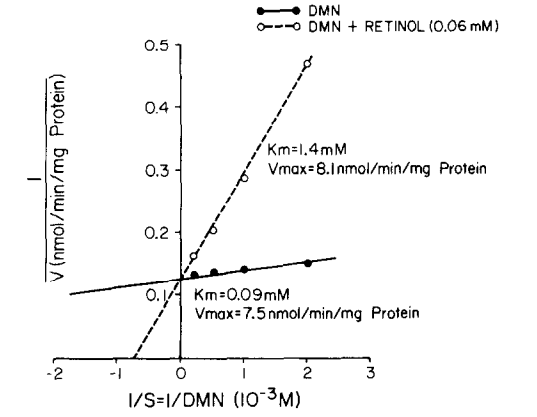


Fig. 3. Effect of retinol on hepatic microsomal DMN demethylation. Liver microsomes of rats fed ethanol (as described in Materials and Methods) were incubated with various concentrations of DMN, with or without retinol.

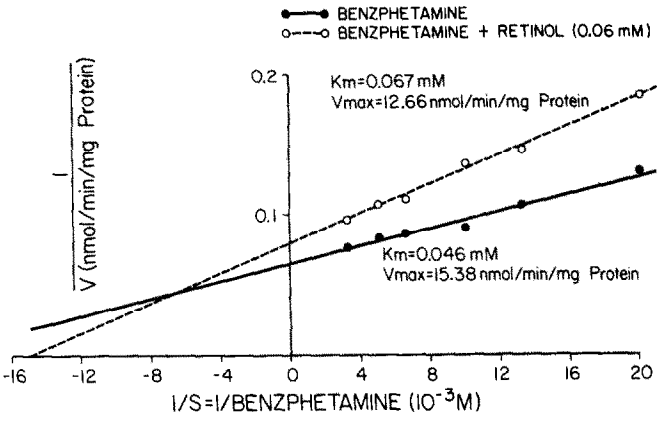


Fig. 4. Effect of retinol on hepatic microsomal benzphetamine demethylation in phenobarbital-treated rats. Liver microsomes of phenobarbital-treated rats (as described in Materials and Methods) were incubated with various concentrations of benzphetamine, with or without retinol, as indicated.

points did not coincide with the best calculated single line. Given the plurality of cytochrome P-450 isozymes, curvilinear Lineweaver–Burk plots are not unexpected. However, with the number of points available, linear regression appeared to be the most suitable method of analysis.

A different type of inhibition was observed when benzphetamine was used as substrate: in microsomes of phenobarbital-treated (Fig. 4), ethanol-treated (Fig. 5) or control (not shown) rats, mixed inhibition was found.

*Effects of various drugs on microsomal 4-hydroxylation of retinol.* At the concentrations used, ethanol (100 mM) and DMN (10 mM) had no significant effect on microsomal retinol 4-hydroxylation in microsomes of either phenobarbital- or ethanol-treated rats. By contrast, benzphetamine exerted a striking competitive inhibition of the 4-hydroxylation of retinol in microsomes of phenobarbital-treated rats (Fig. 6).

*Effects of ethanol consumption or drug administration on the activity of the microsomal retinol-oxidizing system.* Administration of either phenobarbital or ethanol resulted in significant increases in the activity ($V_{max}$) of the microsomal retinol 4-hydroxylase system. This induction was demonstrated in two groups of rats: one shown in Table 1, and an additional group of seven ethanol-treated rats ($0.21 \pm 0.03$ vs $0.09 \pm 0.01$ nmoles/min/mg protein in controls; $P < 0.005$). The $K_m$ of the induced form(s) differed as shown for an illustrative case in Fig. 7. After ethanol, the $K_m$ of the system was not significantly different from that observed in controls, whereas after phenobarbital a significantly higher $K_m$ was measured (Table 1).

## DISCUSSION

This study reveals that, *in vitro*, retinol competitively inhibits the microsomal metabolism of

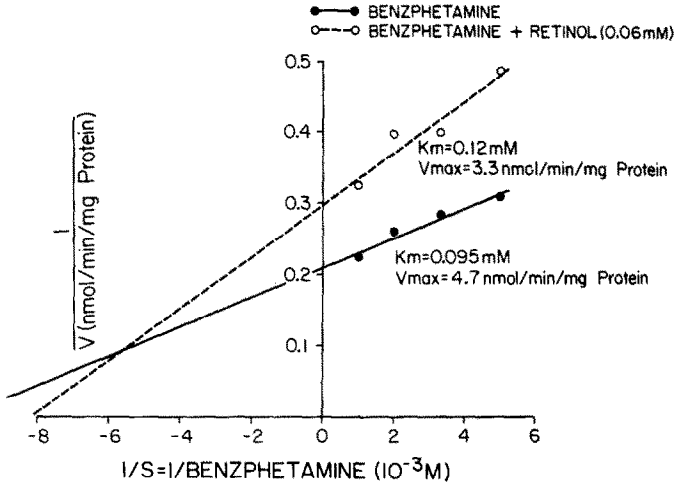


Fig. 5. Effect of retinol on hepatic microsomal benzphetamine demethylation in ethanol-treated rats. Liver microsomes of rats fed ethanol (as described Materials and Methods) were incubated with various concentrations of benzphetamine, with or without retinol.

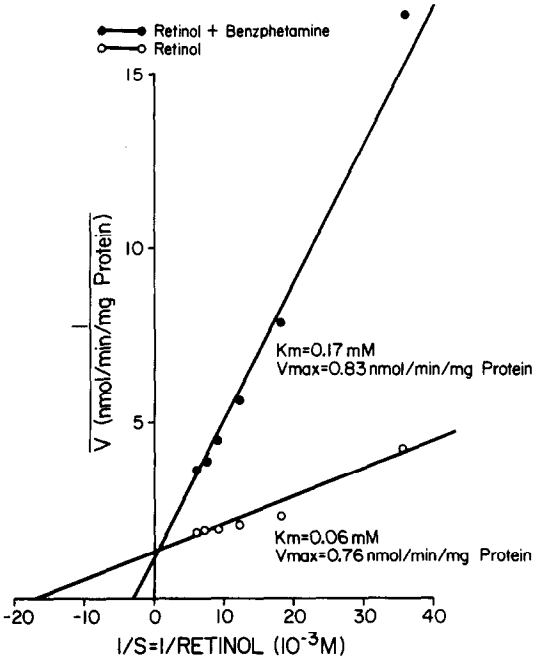


Fig. 6. Effect of benzphetamine on the 4-hydroxylation of retinol. Liver microsomes of phenobarbital-treated rats were incubated with 0.5 mM benzphetamine, as described under Materials and Methods.

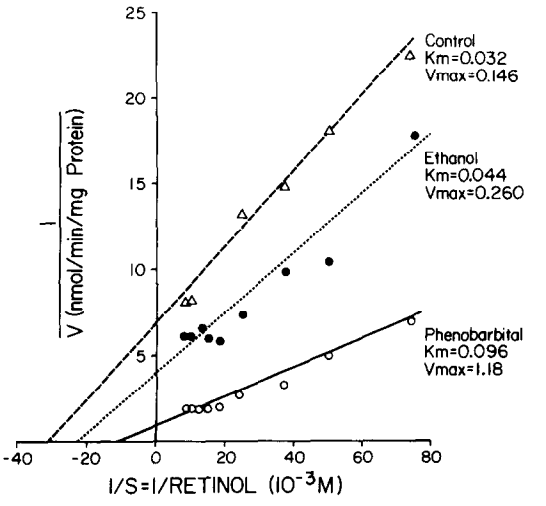


Fig. 7. Representative Lineweaver–Burk plot of the activity of the microsomal retinol 4-hydroxylase in liver preparations from control, ethanol- and phenobarbital-treated rats. The means of all results obtained are given in Table 1. Values for $K_m$ are in mM, and those for $V_{max}$ are in nmoles/min/mg protein.

ethanol, aniline and DMN; mixed inhibition of benzphetamine hydroxylation was also observed. Conversely, benzphetamine inhibited the 4-hydroxylation of retinol in microsomes, as previously reported [5]. *In vivo*, administration of either ethanol or phenobarbital resulted in a striking induction of the activity of the microsomal retinol-metabolizing system.

Our recent observation of an active microsomal cytochrome P-450-dependent retinol-oxidizing system [5] raised the possibility that retinol might interact with the metabolism of other drugs at this site. Hepatic interactions between drugs and retinol had been suspected previously on the basis of indirect evidence, such as the hepatic vitamin A depletion after drug administration [3]. Such interaction has now been documented more directly in the present study. These results support the importance of the cytochrome P-450-dependent system in retinol oxidation. In addition to these pharmacologic implications, these observations may also be of significance with regard to the interaction of retinol with carcinogenesis. Epidemiological studies in the U.K. [14–16] and the U.S. [17] have revealed an increased risk of bronchogenic carcinoma in association with low serum vitamin A levels. Surveys of dietary habits in Norway [18, 19], Singapore [20], Japan [21] and the U.S. [22] also revealed a negative relation between human lung cancer and various indices of vitamin A consumption. Moreover, deficiency of vitamin A in experimental animals leads to the development of squamous metaplasia in the tracheobronchial epithelium as well as in other epithelial organs [23–26]. The metaplastic changes of the respiratory epithelium are frequently found to precede, or be associated with, tumour development [27–37]. It was found that concomitant ethanol consumption and vitamin A deficiency resulted in an increased incidence of squamous metaplasia of the trachea [38]. It is conceivable that this potentiation may predispose the tracheal epithelium to develop neoplasia in the presence of a carcinogenic insult. Indeed, it has been shown in animal studies that vitamin A deficiency increases the susceptibility to neoplasia and enhances carcinogenesis in the respiratory tract following the administration of car-

Table 1. Effect of ethanol or phenobarbital treatment on the 4-hydroxylation of [$^3$H] retinol by rat liver microsomes

| | N | $K_m$ (mM) | $V_{max}$ (nmoles/min/mg protein) |
|---|---|---|---|
| Control | 4 | 0.028 ± 0.006 | 0.11 ± 0.02 |
| Ethanol | 5 | 0.040 ± 0.009 | 0.23 ± 0.04* |
| Phenobarbital | 4 | 0.080 ± 0.005† | 0.98 ± 0.01‡ |

Each value is the mean ±S.E.M.
*–‡ Significantly different compared to control: * $P < 0.05$, † $P < 0.01$, and ‡ $P < 0.001$.

cinogenic polycyclic hydrocarbons [39, 40]. Conversely, treatment of animals with vitamin A or its derivatives in high doses protects against the induction of tumors of the respiratory tract [41, 42]. These animal studies are consistent with a growing body of epidemiological data that human lung cancer risks are inversely correlated with blood retinol levels and dietary vitamin A intake. Obviously multiple mechanisms are involved. In addition to those reviewed by Orr [43], one now must also consider the possibility that retinol may be beneficial through direct interference with the activation of chemical carcinogens, as illustrated by the inhibition of DMN metabolism by retinol (Fig. 3).

In addition to the interaction of retinol with ethanol and other drugs *in vitro* in liver microsomes, we also found that, *in vivo*, the administration of either ethanol or phenobarbital resulted in a striking induction of the activity of the hepatic microsomal 4-hydroxylase of retinol (Table 1). The $V_{max}$ of the system was much more increased after phenobarbital than after ethanol but there was also an associated increase of the $K_m$. The intracellular retinol concentrations at the site of retinol metabolism are not known. It is conceivable, however, that at "physiological" intracellular retinol concentration the lower $K_m$ for retinol of the microsomal 4-retinol hydroxylase in the ethanol-treated rats (as compared to the phenobarbital-treated ones) may offset, in part, the differences in the magnitude of the overall induction. In any event, the induction of this system might contribute to enhanced retinol degradation in the liver and hence to the hepatic vitamin A depletion which has been described both in humans and experimental animals after either ethanol or drug administration [1–3].

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