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Metabolism of All-*trans*, 9-*cis*, and 13-*cis* Isomers of Retinal by Purified Isozymes of Microsomal Cytochrome P450 and Mechanism-based Inhibition of Retinoid Oxidation by Citral

GREGORY M. RANER, ALFIN D. N. VAZ, and MINOR J. COON

Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, Michigan 48109-0606

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SUMMARY

The involvement of a series of microsomal cytochrome P450 (P450) isozymes in all-*trans*-retinoid metabolism, including the conversion of all-*trans*-retinal to all-*trans*-retinoic acid, was previously described. In the current study, we examined the role of seven liver microsomal P450 isozymes in the oxidation of three isomers of retinal. P450 1A1, which was not tested previously, is by far the most active in the conversion of all-*trans*-, 9-*cis*-, and 13-*cis*-retinal to the corresponding acids, as well as in the 4-hydroxylation of all-*trans*- and 13-*cis* retinal. In contrast, P450s 2B4 and 2C3 are the most active in the 4-hydroxylation of 9-*cis*-retinal, with turnover numbers ~7 times as great as that of P450 1A1. The inclusion of cytochrome b_5 in the reconstituted enzyme system is without effect or inhibitory in most cases but stimulates the 4-hydroxylation of 9-*cis*-retinal by P450 2B4, giving a turnover of 3.7 nmol of product/min/nmol of this isozyme, the highest for any of the retinoid conversions we have studied. Evidence was obtained for two additional catalytic reactions not previously attributed to P450 oxygenases: the oxidation of all-*trans*- and 9-*cis*-retinal to the corresponding 4-oxo derivatives by isoform 1A2, and the oxidative cleavage of the acetyl ester of vitamin A (retinyl acetate) to all-*trans*-retinal, also by isoform 1A2. The physiological significance of the latter reaction, with a K_m for the ester of 32 μM and a V_{max} of 18 pmol/min/nmol of P450, remains to be established. We also examined the effect on P450 of citral, a terpenoid α,β-unsaturated aldehyde and a known inhibitor of cytosolic retinoid dehydrogenases. Evidence was obtained that citral is an effective mechanism-based inactivator of isozyme 2B4, with a K_I of 44 μM as determined by the oxidation of 1-phenylethanol to acetophenone, and by isozyme 1A2 in the oxidation of all-*trans*-retinal to the corresponding acid and by isozyme 2B4 in the 4-hydroxylation of all-*trans*-retinol and retinoic acid. Thus, citral is not suitable for use in attempts to distinguish between retinoid conversions catalyzed by dehydrogenases in the cytoplasm and by P450 cytochromes in the endoplasmic reticulum.

Intense interest in vitamin A metabolism has been generated by the growing number of important biological processes whose regulation has been attributed to the retinoid metabolites. These products are known to play important roles in cell growth and differentiation (1–5) as well as in limb (6), nervous system (7), urogenital (8), and cranial development (8) and have also been implicated in the prevention and treatment of certain types of cancer (9, 10). Current understanding of the biological activities is incomplete, but it is well established that at least two retinoids, all-*trans*-retinoic acid and 9-*cis*-retinoic acid, use specific receptors, RAR and RXR, respectively, in exerting their effects at the transcriptional level (11–13). An additional retinoid metabolite, 14-hydroxy-4,14 retroretinol, has been reported to have a role in the activation and proliferation of B and T cells in human lymphocytes and lymphoblastoid cell lines (14–16), but the cellular mechanism by which this activation occurs is currently unknown. All-*trans*-retinoic acid has no effect on these cells. It has also been suggested that retinoylation (covalent modification of proteins by retinoic acid) may be an alternative means for modulation of a cell phenotype (17–19). In general, the biological activity of retinoids seems to parallel their ability to interact with the two known classes of retinoid receptors, which suggests that transcriptional regulation is probably central to the response. For example, 4-oxo-all-*trans*-retinoic acid is approximately half as active as all-*trans*-retinoic acid in promoting cell differentiation in F9 embryonal carcinoma cells (20) and in producing dysmorphogenic effects in rat embryonal tissue (21). Likewise, 4-oxo-all-*trans*-retinoic acid binds to RAR with slightly lower affinity than does all-*trans*-retinoic acid (22).

Two dominant pathways are involved in the metabolism of retinoids. First, the activation of retinol is associated with its conversion to retinoic acid, which involves the stepwise de-

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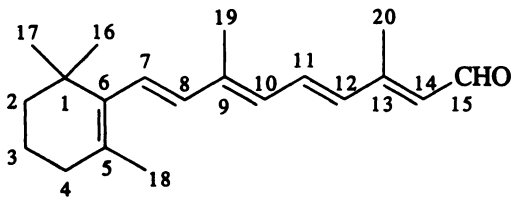
ABBREVIATIONS: P450, cytochrome P450; HPLC, high performance liquid chromatography; DLPC, 1,2-dilauroyl-*sn*-glycerol-3-phosphorylcholine; RAR, retinoic acid receptor; RXR, retinoic X receptor; TFA, trifluoroacetic acid.

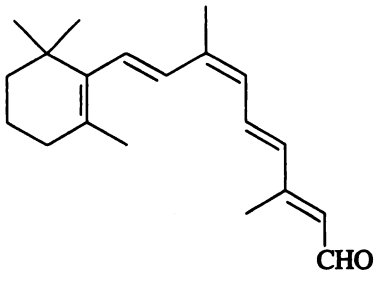
hydrogenation or oxygenation of the alcohol function. Cytosolic alcohol and aldehyde dehydrogenases are known to catalyze the conversion of retinol to retinoic acid via the retinaldehyde intermediate (23–25). In addition, several purified rabbit liver P450 isoforms have recently been shown to catalyze the oxidation of all-*trans*-retinal to all-*trans*-retinoic acid (26), and the reaction has also been shown to occur in microsomal membranes (27). A second metabolic pathway, the hydroxylation of the various retinoid compounds at the 4 position of the β-ionone ring, is apparently catalyzed exclusively by P450 enzymes, as shown by studies with CO and other inhibitors (28). This pathway is thought to represent the inactivation of the retinoids, but, as with 4-oxo-all-*trans*-retinoic acid, some of these metabolites may possess some biological activity.

The goals of the current study were to determine the role of various purified rabbit liver P450s in the metabolism of 9-*cis*- and 13-*cis*-retinals and to examine the metabolism of the all-*trans* compound with purified P450 1A1, which was previously untested. The structures of the retinoid substrates are shown in Fig. 1. P450 1A1 has proved to be the most active isoform in the conversion of all three retinals to the corresponding retinoic acids. In addition, 1A1 is highly effective in hydroxylating both all-*trans*- and 13-*cis*-retinal, indicating that this isoform may be responsible for much of P450-mediated retinoid metabolism. In addition, P450 1A2 was shown to catalyze the oxidation of 9-*cis*- and all-*trans*-retinal to the 4-oxo compounds, as well as the oxidative ester cleavage of retinyl acetate to form retinal. These two reactions had not previously been attributed to the P450 monooxygenase system. We also examined the effects of citral (3,7-dimethyl-2,6-octadienal), a monoterpene natural product composed of the *cis* (neral) and *trans* (geranial) isomers, on P450-catalyzed reactions, including the oxidation of 1-phenylethanol and of retinoids. Citral has been used in various laboratories as an inhibitor of retinoid dehydrogenases (29, 30), but our results demonstrate that it is also an effective mechanism-based inhibitor of P450 cytochromes.

All-*trans* -Retinal



9-*cis* -Retinal



13-*cis* -Retinal

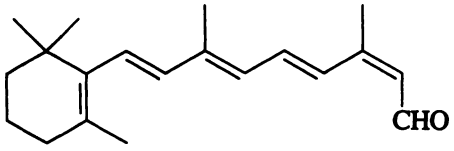


Fig. 1. Chemical structure and numbering system of the three retinal isomers used as substrates.

Experimental Procedures

Materials. Citral, NADPH, and DLPC were obtained from Sigma Chemical Co. (St. Louis, MO) along with all of the retinoids that were used as substrates. The standard 4-oxoretinoic acid was a generous gift from Dr. Jerry Sepinwall, Hoffmann-La Roche (Nutley, NJ). All retinoid solutions were made fresh each day in chloroform and stored on ice. HPLC-grade solvents (acetonitrile, methyl alcohol, chloroform, and ethyl acetate) were obtained from Mallinckrodt (Paris, KY), and TFA was obtained from Sigma. The citral isomers neral and geranial were separated and purified by HPLC with the use of a Waters Nova-pak C-18 column (3.9 × 300 mm) with the following system. A 10-min linear gradient starting with 40% acetonitrile and 60% solvent A (99.5% H_2O, 0.5% TFA, and 0.01 M ammonium acetate) and ending with 45% acetonitrile and 55% solvent A was followed by a 25-min isocratic flow at the final solvent conditions. The flow rate was 1.0 ml/min, and detection was at 240 nm. Under these conditions, the peaks were well resolved, and the isomers were collected in 1.0-ml fractions and pooled according to content. The purification of the various rabbit liver P450 isoforms, NADPH-cytochrome P450 reductase, and cytochrome b_5 has been described previously (26, 31).

Retinoid metabolism. All experiments involving retinoids as substrates were carried out as described by Roberts *et al.* (26), with several minor variations. The appropriate amount of a stock retinoid solution, in chloroform, was added to a glass test tube (to give a final retinoid concentration of 2–100 μM) along with 30 μg of DLPC, also in chloroform, and the solvent was evaporated. To each tube, 10 μl of a 0.1 M stock solution of L-ascorbic acid was added with 100 μl of 1.0 M potassium phosphate buffer, pH 7.4, and deionized water in an amount to bring the final volume to 1.0 ml, and the solution was mixed with a vortex instrument for 30 sec. In a separate tube, P450 and reductase were combined in a 1:2 molar ratio and stored on ice for 20 min before the addition of an aliquot to the retinoid solution to give a final concentration of 0.10 μM P450 and 0.20 μM reductase (and 0.2 μM cytochrome b_5 where indicated). All mixtures were preincubated at 30° for 1.0 min before the reaction was initiated with 1.0 mM NADPH (final concentration). Reactions were carried out for 15 min at the same temperature and then quenched with 2.0 ml of ethyl acetate containing 50 μg/ml butylated hydroxytoluene. The ethyl acetate extract was removed, and the remaining aqueous solution was acidified with 10 μl of 88% formic acid and extracted with an additional 2.0 ml of ethyl acetate. The solvent was evaporated from the combined extracts, and the residue was dissolved in 0.20 ml of methanol for analysis by HPLC. All reactions and other procedures were carried out in the absence of overhead light.

Inhibition by citral. P450 2B4 (1.0 μM) was incubated at 30° in the presence of reductase (2.0 μM), phosphate buffer, pH 7.4 (100 mM), DLPC (30 μg/ml), and varying amounts of citral, with NADPH (1.0 mM) as the final addition. For the assay of remaining P450

activity, the mixtures were then diluted 1:20 into a reaction mixture (final volume, 1.0 ml) containing 10 mM 1-phenylethanol, 30 μg/ml DLPC, 100 mM phosphate buffer, pH 7.4, and 1.0 mM NADPH, to give final concentrations of 0.05 and 0.1 μM for P450 and the reductase, respectively. The reaction was allowed to proceed for 20 min at 30°, at which point it was quenched with 0.40 ml of 6% perchloric acid. After storage for 15 min on ice, the mixtures were centrifuged for 15 min at 3000 × *g* to remove the protein and analyzed by HPLC for the reaction product, acetophenone. This assay has been described in detail previously (32). Inhibition by citral of the P450 1A2-catalyzed oxidation of retinal was examined in a similar manner except that after the first incubation, the mixture was diluted 1:20 into a solution containing, instead of 1-phenylethanol, 40 μM all-*trans*-retinal and 1 mM L-ascorbic acid. The reaction was allowed to proceed for 15 min at 30° and quenched as described above under retinoid metabolism.

HPLC methods. A Waters Nova-pak C18 column (3.9 × 300 mm) was used for all assays in conjunction with a Waters HPLC system consisting of a model 600E controlling unit, a model 610 solvent delivery system, a model 700 satellite WISP, a model 490E UV/vis detector, and the Millennium 2010 software package. For the analysis of metabolites of 9-*cis* and all-*trans* retinoids, a single solvent system was used that consisted of solvent A (100% acetonitrile) and solvent B (99.9% H_2O and 0.1% TFA). An 8-min linear gradient from 45% A/55% B to 55% A/45% B was followed by a 22-min linear gradient to 100% A, which was maintained for an additional 5 min (flow rate, 1 ml/min). For experiments with retinal, products were detected at 350, 360, 370, and 380 nm, whereas the products from retinoic acid metabolism were detected at 310, 330, 350, and 380 nm. Under the conditions used, 13-*cis*-retinal and 13-*cis*-retinoic acid were coeluted, so a different solvent system was used. With solvent B containing 0.01 M sodium bicarbonate, pH 7.0, and the same gradient system described above, very good separation was achieved between the aldehyde and the acid. Standard curves for all three retinal isomers, the corresponding alcohols, and 13-*cis*- and all-*trans*-retinoic acid were generated using the gradients described above. Quantification of the 4-hydroxyretinals was done using the standard curves for the parent compounds with the assumption that the extinction coefficient of the corresponding product was not significantly different. Analysis of the product of 1-phenylethanol oxidation involved the use of an isocratic system consisting of 25% acetonitrile and 75% H_2O containing 0.1% TFA at a flow rate of 1.0 ml/min. The product, acetophenone, was eluted between 7.5 and 8 min under the conditions used.

Other analytical methods. A Hewlett Packard model 8452A diode array spectrophotometer was used to obtain visible absorption spectra of the products of retinoid metabolism after purification by HPLC. Gas chromatography-mass spectrometry analysis was used to look for citral metabolites in a reaction mixture containing 2 μM P450 2B4, 2 μM reductase, 1 mM citral, 50 μg/ml DLPC, 100 mM phosphate buffer, pH 7.4, and 1.0 mM NADPH. The reaction was allowed to proceed for 1 hr at 30° in a closed vessel. The mixture was then heated at 60° for 20 min, and 1.0 ml of headspace gas was removed and injected onto a Supelcowax-10 capillary column (0.3 mm i.d., 0.25-μm film thickness, 30-m length) over a period of 1–2 min. A Hewlett Packard gas chromatography-mass spectrometry instrument consisting of a model 5890 Series II gas chromatograph and a model 5970 detector was used for the analysis. The column was maintained at 35° for 5 min, and the temperature was then increased to 100° at a rate of 5°/min. The reaction mixture was extracted with methylene chloride, the solvent was evaporated from the extract, and the residue was dissolved in 10 μl of methylene chloride and injected onto a DB-5 fused silica capillary column attached to a Finigan gas chromatograph, which was connected to a Finigan 4021 mass spectrometer operating at electron impact conditions of 70 eV. The injector temperature was maintained at 200° and the column temperature was kept at 50° for 2 min after injection of the sample, followed by an increase to 275° at a rate of 20°/min, and held at that level for an additional 30 min. Data were processed with INCOS software.

Results

Oxidation of all-*trans*, 9-*cis*, and 13-*cis* isomers of retinal by P450 isozymes. The reaction of various isoforms of P450 in a reconstituted enzyme system with the three isomeric retinal compounds resulted in several products, as shown by HPLC analysis. The chromatogram displayed in Fig. 2 shows products from the P450 1A2-catalyzed oxidation of 9-*cis*-retinal carried out at 30° for 15 min. In control experiments, P450 was omitted or the enzymes were heat-denatured before their addition. Peak E represents the parent 9-*cis*-retinal on the basis of its absorption spectrum and coelution with the authentic standard. The product in peak D was spectroscopically identical to 9-*cis*-retinoic acid and had the corresponding molecular mass of 300 Da as determined by mass spectrometry analysis. Furthermore, when column pH, ordinarily 2.0, was increased to 7.2, a striking decrease in retention time resulted, providing further evidence for the 9-*cis* acid. Peak A was characterized by the absorbance maximum at 374 nm and molecular mass of 300 Da, but no pH-dependent shift in retention on the column was observed, thus indicating the presence of 4-hydroxy-9-*cis*-retinal. Peak B was observed only in those reaction mixtures that contained P450 1A2 and was nearly identical to peak A spectroscopically but had a molecular mass of 298 Da, thus providing identification of 4-oxo-9-*cis*-retinal. Finally, peak C, an autoxidation product that was present even when the enzymes had been heat-denatured, was identified as 5,8-epoxy-9-*cis*-retinal on the basis of its spectral properties and molecular mass. In similar experiments, the major oxidation products formed from 13-*cis*-retinal by P450 1A2 were characterized as 13-*cis*-retinoic acid based on comparison with an authentic sample and as 4-hydroxy-13-*cis*-retinal based on mass spectral and spectroscopic analysis. The products of all-*trans*-retinal metabolism by P450 have been characterized previously and include the 4-hydroxy derivative of the aldehyde and all-*trans*-retinoic acid (26). The formation of the 4-hydroxy derivatives, the 4-oxo product, and the retinoic acids as determined in this study was linear with time in all

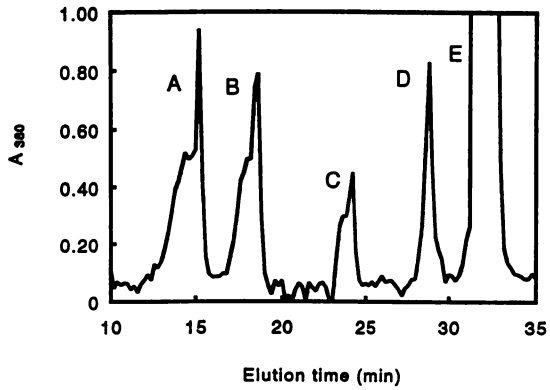


Fig. 2. HPLC profile of products from the reaction of P450 1A2 with 9-*cis*-retinal in a reconstituted enzyme system containing NADPH and NADPH-cytochrome P450 reductase. Conditions for the reaction and the analytical procedures are described in Experimental Procedures. The identity of the peaks was determined by a combination of electronic absorption spectroscopy, HPLC, and mass spectrometry analysis.

cases for at least 15 min at 30° and also increased linearly with increasing P450 concentration under the conditions used (results not shown). The substrate concentration used in further experiments (40 μM) was saturating as judged by experiments in which the level of all-*trans*-, 9-*cis*-, and 13-*cis*-retinals varied with P450 1A1 as the catalyst.

Each of the three retinal isomers was used as a substrate for seven different purified rabbit liver P450 isozymes, including 1A1, 1A2, 2B4, 2E1, 2E2, 3A6, and 2C3, in the reconstituted system, and the rates of 4-hydroxylation and of retinoic acid formation were determined. The results are presented in Fig. 3, except that the data with 2E2 are omitted because it exhibited no detectable activity in any of the reactions. Although some rates of all-*trans*-retinal metabolism were reported previously (26), this retinoid was also included in the current study because the experimental conditions were different and because P450 1A1, which gave particularly important results, was not examined previously. The general finding was that for each reaction and for each isomer of retinal, multiple cytochromes have significant catalytic activity. However, the rates vary from some that are barely measurable to others that are sufficiently high to contribute in a major way to retinoid metabolism, and no single isozyme can be assigned the role of "retinoid oxygenase." As shown in the Fig. 3, for the 4-hydroxylation reaction, P450 1A1 is the most active with the all-*trans*- and 13-*cis*-retinals, with turnover numbers of 1.1 and 1.5 nmol/min/nmol P450, respectively. In contrast, for the 4-hydroxylation of 9-*cis*-retinal, P450s 2C3 and 2B4 are the most active, with values of 2.6 and 2.8 nmol/min/nmol P450, which are among the highest determined in this study, and 1A1 is relatively ineffective. For oxidation of the isomeric aldehydes to the retinoic acids, however, P450 1A1 is the most effective for the all-*trans*, 9-*cis*, and 13-*cis* forms, with turnover numbers of 0.72, 2.1, and 1.3 nmol/min/nmol P450, respectively, and the only other isoform with significant activity is 1A2. The effect of including cytochrome b_5 in the reconstituted system at a 1:2 P450/cytochrome b_5 molar ratio was determined with all of the P450s with the three retinals in 4-hydroxylation and retinoic acid formation. No effect or a slight inhibition was observed in most cases. For example, 2E2 had no detectable activity with any of the retinals, with or without cytochrome b_5. The results with the three cytochromes that are the most active in retinal metabolism are given in Table 1. Cytochrome b_5 was generally inhibitory, and only in the case of 9-*cis*-retinal 4-hydroxylation was a stimulation seen, with an increase in the turnover number from 2.8 to 3.7. The latter value, representing an increase of >30%, was the highest determined in the current study.

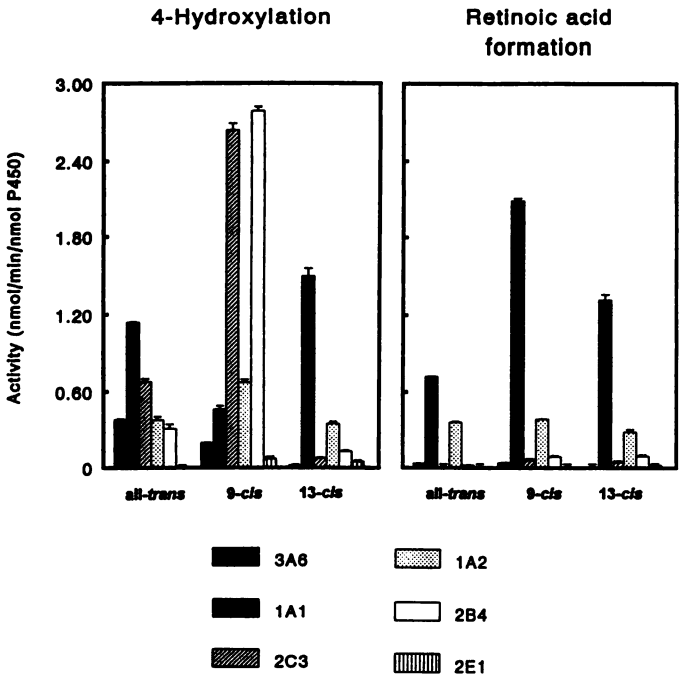


Fig. 3. Rates of 4-hydroxylation and aldehyde oxidation of three retinal isomers by various isoforms of P450 in the absence of cytochrome b_5. Activities were measured at 30° with 40 μM retinoids under conditions described in Experimental Procedures.

Steady state reaction parameters. P450 1A1 was selected for further kinetic analysis because of its primary importance in retinoid metabolism. Each of the retinal isomers was examined in detail with respect to the kinetic parameters, k_{cat} and K_m, and partitioning between the two oxidative reactions. Fig. 4 is a representative Lineweaver-Burk plot for the oxidation of 9-*cis*-retinal to 9-*cis*-retinoic acid by this cytochrome. The k_{cat} for this reaction is 3.4 nmol/min/nmol P450, with a K_m of 18 μM, whereas the corresponding 4-hydroxylation reaction was characterized as having a k_{cat} of 0.5 nmol/min/nmol and a K_m of 40 μM (results not shown). The kinetic constants for all of the 1A1-catalyzed reactions are summarized in Table 2. For all of the retinals, concentrations of >50 μM had an inhibitory effect. As a result, in some cases it was not possible to obtain meaningful reaction rates at substrate concentrations that were twice the K_m values, and the range of useful substrate concentrations was therefore limited. The addition of catalase or superoxide dismutase had no effect on the results. Because the two metabolic processes, aldehyde oxidation and 4-hydroxylation, apparently represent the activation and inactivation of retinals, respectively, the partitioning ratio represents an important biological property of the enzyme. The ratio of the catalytic efficiencies (k_{cat}/K_m) for the two competing reactions with P450 1A1 was calculated for each of the retinal isomers (Table 2). The highest value for this ratio, 15, is seen with 9-*cis* retinal, whereas the ratios for the all-*trans* and 13-*cis* isomers are both in the range of 1.

Data presented above (Fig. 3) indicate that P450 1A2 is slightly more active than 1A1 in catalyzing the 4-hydroxylation of 9-*cis*-retinal and much less active in forming the retinoic acid, but the former cytochrome has the interesting property of producing 4-oxo-9-*cis*-retinal. P450 1A2 was also shown to convert all-*trans*-retinal to the 4-oxo compound

TABLE 1
Effect of cytochrome b_5 on P450-catalyzed retinal oxidation

Form of P450	Retinal isomer	Product	Change in rate
			%
1A1	*trans*	4-Hydroxy	−39
1A1	*trans*	Acid	0
1A1	9-*cis*	4-Hydroxy	−24
1A1	9-*cis*	Acid	−32
2C3	9-*cis*	4-Hydroxy	−61
2B4	9-*cis*	4-Hydroxy	+33
1A1	13-*cis*	4-Hydroxy	−46
1A1	13-*cis*	Acid	−100

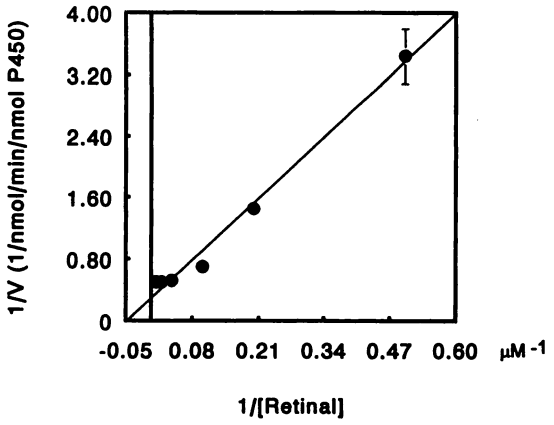


Fig. 4. Lineweaver-Burk analysis of the oxidation of 9-*cis*-retinal to 9-*cis*-retinoic acid by P450 1A1. The retinal concentration was varied from 2 to 50 μM in reaction mixtures incubated for 15 min at 30° with 0.1 μM P450 and 0.2 μM reductase present. Data represent an average of results from duplicate experiments.

(results not shown). Under the conditions used, the 13-*cis* isomer did not undergo this reaction, and the other P450 isozymes were apparently ineffective in forming the 4-oxo compound. The V_{max} value for formation of the 4-oxo compound is higher than that for 4-hydroxylation (Table 3). The results do not distinguish between free and bound 4-hydroxyretinal as the intermediate for further oxidation at this position. In addition, we have found that P450 1A2 is active in the oxidative ester cleavage of retinyl acetate to give retinal as the product. The cleavage of a variety of xenobiotic esters by P450 isozymes has been described (33–35), but this is the first example of the oxidative cleavage of an ester of a biologically occurring compound. A kinetic analysis of the formation of retinal from the retinyl ester by P450 1A2 is given in Fig. 5 as a Lineweaver-Burk plot, and the kinetic constants are reported in Table 3. The other P450 isozymes examined had no significant activity in the cleavage of the acetyl ester, and in additional experiments retinyl palmitate did not yield detectable levels of retinal with P450s 1A1, 1A2, and 2B4, even with cytochrome b_5 present. The ester cleavage reaction is relatively slow, and its biological significance remains to be determined.

Inhibition by citral. The effect of a monoterpene natural product, citral, on the activity of P450 2B4 was determine (Table 4). Inhibition by citral or its individual isomers was extensive and in each case required the presence of NADPH, suggesting mechanism-based formation of the actual inhibitor. The effect of citral was unchanged with either catalase or rabbit liver epoxide hydrolase present, indicating that H_2O_2 generation was not the cause of the inhibition and that a reactive epoxide intermediate was probably not formed. Furthermore, the target of the inhibition was not the reductase, since the addition of fresh reductase at the start of the assay for 1-phenylethanol oxidation did not change the results. The time course and concentration dependence for the inhibition of cytochrome P450 2B4 by citral, as judged by the effect on the rate of oxidation of 1-phenylethanol, are shown in Fig. 6. The resulting K_I was determined to be 44 μM, and the maximum rate of inactivation to be 0.078/min. The only product identified in the reaction of P450 2B4 with citral was the corresponding acid, but its formation was very slow. Furthermore, small amounts of the corresponding alcohol were also present in the commercial compound. The inhibition by citral was found not to be attributable to the corresponding alcohol or acid (data not shown). Under the same conditions where citral gave ~60% inhibition, similar results were obtained with the *cis* and *trans* compounds (Table 4). However, the acid and alcohol corresponding to citral gave only 10% and 20% inhibition, respectively (data not presented). Citral also extensively inhibits P450 1A2, as determined by the oxidation of retinal to retinoic acid, as well as P450 2B4, as determined by the 4-hydroxylation of both retinol and retinoic acid (Table 5). However, the results were somewhat different from those obtained when phenylethanol oxidation was used as the assay. In the three retinoid reactions, the average inhibition was 72% with NADPH and 34% without. Thus, approximately half of the inactivation by citral can be attributed to a mechanism-based effect and the other half to the use of retinoids as substrates, possibly indicating competition with citral because of its similar structure. Accordingly, citral could be particularly effective in blocking retinoid metabolism, and we have found that 200 μM citral added to the reconstituted isozyme system with all-*trans*-retinal completely prevents 4-hydroxylation and retinoic acid formation (data not shown).

Discussion

The involvement of cytochrome P450 in retinoid metabolism has been well established, as reviewed previously (26). In our earlier study, the roles of individual purified isoforms of rabbit liver P450 in all-*trans* retinoid metabolism were examined, and it was shown that although P450s 2B4, 1A2, and 2C3 are all active in 4-hydroxylation, only 1A2 and, to a much lesser extent, 3A6 oxidize all-*trans*-retinal to all-*trans*-retinoic acid. This P450-linked aldehyde-oxidizing activity has also been demonstrated in liver microsomes (27). A particularly interesting finding in the current study was the major role of P450 1A1 in retinoid metabolism, including relatively high activity in the conversion of all-*trans*-retinal to the 4-hydroxy compound and to retinoic acid. 4-Hydroxylation is generally accepted as representing the initial step in the elimination of retinoids from tissues, whereas all-*trans*-retinoic acid is the specific ligand for the RAR, which has been implicated in the regulation of a wide range of cellular functions (11). Therefore, the partitioning between these two metabolic fates should reflect the physiological role of a particular P450 in retinoid metabolism. In the case of all-*trans*-retinal, the k_{cat}/K_m value for 4-hydroxylation by P450 1A1 is identical to that for acid formation, and the partition ratio of 1 suggests a dual role for this cytochrome in the oxidation of the all-*trans* aldehyde.

Recently, 9-*cis*-retinoic acid has been found to be a high affinity ligand for the RXR, which is known to form heterodimers with other hormone receptors and thus influence the level of transcription of target genes (12). Furthermore, the 9-*cis* acid binds to RAR with the same affinity as all-*trans*-retinoic acid (11) and can influence gene transcription in this manner as well. The formation of 9-*cis*-retinoic acid *in vivo* therefore represents an important biological process and one likely to be regulated at multiple levels. Isomerase activities have been reported in rat (36) and bovine liver microsomes that convert all-*trans*-retinoic acid to the 9-*cis* acid in what seems to be a thiol-mediated process (37). Alterna-

TABLE 2

Kinetic constants for P450 1A1-catalyzed oxidation of retinal isomers

Retinal substrate	4-Hydroxylation			Retinoic acid formation			Partition ratio[a]
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	
	nmol/min/nmol	*μM*		*nmol/min/nmol*	*μM*		
All-*trans*	1.7	20	0.085	1.2	14	0.085	1.0
9-*cis*	0.5	40	0.013	3.4	18	0.19	15.0
13-*cis*	1.8	60	0.031	1.3	29	0.045	1.5

[a] Catalytic efficiency for retinoic acid formation divided by that for 4-hydroxylation.

TABLE 3

Kinetic parameters for various P450 1A2-catalyzed reactions in the oxidation of retinoids

The usual reconstituted enzyme system was used, and the incubation period was 20 min.

Substrate	Product	V_{max}	Apparent K_m
		nmol/min/nmol	*μM*
All-*trans* retinyl acetate	Retinal	0.018 ± 0.002	32 ± 2
9-*cis*-Retinal	4-Oxo-9-*cis*-retinal	1.3 ± 0.1	60 ± 5
9-*cis*-Retinal	4-Hydroxy-9-*cis*-retinal	0.72 ± 0.06	22 ± 2
9-*cis*-Retinal	9-*cis*-Retinoic acid	0.40 ± 0.02	16 ± 1

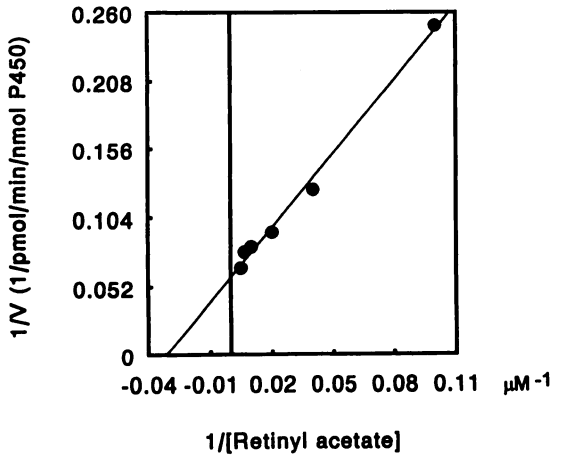


Fig. 5. Lineweaver-Burk plot for the oxidative ester cleavage of retinyl acetate by P450 1A2. Substrate concentrations were in the range of 10–200 μM; P450 was 0.2 μM; and reductase and cytochrome b_5 were each 0.4 μM. The reactions were carried out for 1 hr at 30°.

TABLE 4

Inhibition by citral of P450 2B4 as determined by 1-phenylethanol oxidation

The conditions were as described in Experimental Procedures. To the complete system containing P450 2B4, the reductase, phosphatidylcholine, NADPH, and the inhibitor (200 μM), were added where indicated. After 15 min at 30°, the activity of P450 was determined in the oxidation of 1-phenylethanol to acetophenone.

System	Inhibitor	Activity
		%
Complete	None	100
Complete	Citral	42 ± 6
NADPH omitted	Citral	103 ± 3
Catalase added	Citral	48 ± 3
Epoxide hydrolase added	Citral	51 ± 2
Complete; additional reductase added in the assay of 1-phenylethanol oxidation	Citral	47 ± 3
Complete	Geranial	45 ± 3
NADPH omitted	Geranial	100
Complete	Neral	42 ± 4
NADPH omitted	Neral	100

tively, the formation of 9-*cis*-retinoic acid from 9-*cis*-β-carotene is known to occur (38, 39). Invoking the "central cleavage" mechanism discussed by Wang *et al.* (37) would necessitate the intermediate formation of 9-*cis*-retinal, which could then be converted to 9-*cis*-retinoic acid via the action of aldehyde dehydrogenases or cytochrome P450. The oxidation of 9-*cis*-retinal was examined in the current study with purified P450 isoforms in a reconstituted system, and, as with the all-*trans* isomer, 1A1 was found to be the most active isoform in the generation of retinoic acid, with a k_{cat} of ~3.0 nmol/min/nmol, nearly 6 times greater than the next highest value with 1A2. The 4-hydroxylation reaction, on the other hand, is catalyzed much more efficiently by P450s 2C3 and 2B4, particularly in the presence of cytochrome b_5, whereas 1A1 is only 10% as active. The ratio of the catalytic efficiencies of the competing reactions for 1A1 is 15:1 in favor of the conversion to retinoic acid. The 2B4 and 2C3 isoforms have almost no detectable activity in 9-*cis*-retinoic acid formation but very high activities in 4-hydroxylation, indicating that their primary role may be in the deactivation of retinoids, a conclusion that is consistent with the findings of Westin *et al.* (40), which show the induction of human 2C7 on treatment with vitamin A.

Our results show that the metabolism of 13-*cis*-retinal is catalyzed predominantly by the P450 1A1 isoform, which is approximately equally active in 4-hydroxylation and in the formation of 13-*cis*-retinoic acid. The significance of this reaction is unclear, however, since a unique physiological role for the 13-*cis*-acid remains to be established. The teratogenic effects associated with all-*trans*-retinoic acid occur at 10-fold lower concentrations than with the 13-*cis* form (21), presumably because of the lower affinity of the latter compound for RAR or RXR (41). Interestingly, pretreatment of animals with 13-*cis*-retinoic acid stimulates retinoic acid metabolism in the intestinal mucosa of vitamin A-deficient rats to a greater extent than does treatment with all-*trans*-retinoic acid (42), which suggests a possible regulatory function for the 13-*cis* compound. If this were the case, the formation of the acid could represent an important step in the regulation process.

The natural product citral has been shown to inhibit the formation of retinoic acid from retinol or retinal in a number of different tissues (29, 30) and is generally believed to block cytosolic alcohol and aldehyde dehydrogenases, but its ability to inhibit P450 has not been examined previously. We have shown, however, that citral acts primarily as a mechanism-based inactivator of cytochrome P450 and thus is not a useful agent to distinguish between the P450-mediated and dehydrogenase-mediated reactions. The K_I for citral in the 2B4-

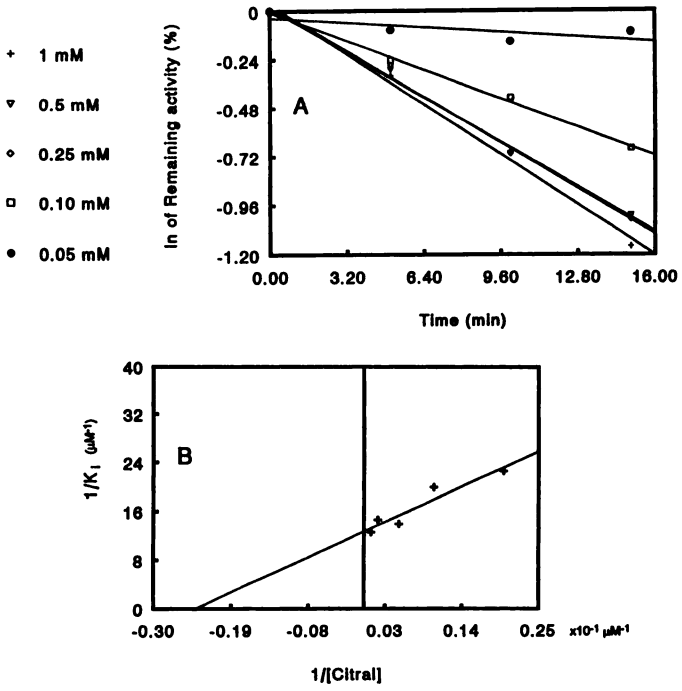


Fig. 6. Analysis of the inhibition of P450 2B4 by citral. A, Time course for the inactivation at various inhibitor concentrations. B, Double reciprocal plot of the K_I values (obtained in A) versus inhibitor concentration. The K_I for citral was found to be 44 μM, with a maximum rate of inactivation of 0.078/min.

TABLE 5

Inhibition of P450 by citral as determined by rates of retinoid metabolism

P450 2B4-catalyzed 4-hydroxylation of all-trans-retinol and retinoic acid and of P450 1A2-catalyzed oxidation of all-trans-retinal to the acid were examined. The system was otherwise as in Table 4.

System	Inhibitor added	Activity with retinoid substrate		
		Retinol	Retinal	Retinoic acid
			%	
P450 1A2				
Complete	None		100	
Complete	Citral		31 ± 3	
NADPH omitted	Citral		68 ± 2	
P450 2B4				
Complete	None	100	100	
Complete	Citral	25 ± 5		27 ± 4
NADPH omitted	Citral	60 ± 2		70 ± 3

catalyzed oxidation of 1-phenylethanol was found to be 44 μM, with a maximum rate of inactivation of 0.078/min. This value is well below the concentrations generally used to inhibit retinoic acid formation. Citral was also shown to inhibit both 1A2 and 1A1 in the oxidation of all-*trans*-retinal to both all-*trans*-retinoic acid and 4-hydroxy-all-*trans*-retinal. The mechanism of inactivation is not yet known, but formation of the inhibitor is clearly P450 and NADPH dependent, unaffected by catalase or epoxide hydrolase, and irreversible as the activity of the enzyme is not recovered on subsequent dialysis.

4-Oxo-all-*trans*-retinoic acid is active in promoting cell growth and differentiation, as well as causing teratogenic effects in rats at concentrations that are only approximately twice as high as those that bring about similar effects with all-*trans*-retinoic acid (20, 21, 43). Thus, the formation of the oxo compound from all-*trans*-retinoic acid, a process that was thought to represent deactivation, actually gives rise to a product with only marginally lower activity. This reaction has been demonstrated in hamster liver microsomes (41), with an absolute requirement for NAD, but NADPH is ineffective, a finding that is apparently inconsistent with the involvement of P450. In addition, previous findings by Roberts *et al.* (26) with rabbit liver microsomes also suggest that P450 does not have a role in forming the 4-oxo-all-*trans*-retinoic acid. In the current study, we have shown that formation of the 4-oxo product of all-*trans*- and 9-*cis*-retinal is catalyzed by P450 1A2 but not by any other isoform tested. The 4-oxo compound was not observed in the current study when all-*trans*-retinoic acid was used as a substrate, which is consistent with the previous reports. The question then remains of whether 4-oxo-retinal is a precursor of 4-oxo-retinoic acid. In reactions with P450 1A1 carried out for longer times, as the concentration of the retinal substrates became subsaturating during the course of the reaction, secondary products began to accumulate, including 4-hydroxy retinoic acid, presumably due to the further oxidation of the 4-hydroxy retinal. Therefore, the generation of 4-oxo-retinoic acid from 4-oxo-retinal would require only that aldehyde oxidation be favored over alternative metabolic processes, such as hydroxylation at the 2 or 3 position of the β-ionone ring (44).

Finally, the finding that P450s can catalyze the oxidative cleavage of a variety of esters to carbonyl products (33–35) prompted an examination of the ability of the purified isoforms to bring about the formation of all-*trans*-retinal from retinyl acetate and retinyl palmitate. The acetate ester, a major source of vitamin A in multivitamin food supplements, is metabolized primarily in the intestinal tract, where it is rapidly hydrolyzed to retinol and subsequently esterified to long-chain fatty acid esters (45), including retinyl palmitate, before transport to the liver. Kolars *et al.* (46, 47) have shown that in humans, a P450 enzyme, 3A4, is present in small bowel enterocytes and gut epithelium, which are the primary sites of retinyl acetate metabolism. In addition, Peters and Kremers (48) detected two P450s in the intestinal mucosa of humans, thus justifying the proposed role of P450 in retinyl ester cleavage. Our study indicates that 1A2 catalyzes the oxidative cleavage of retinyl acetate with a V_{max} of 18 pmol/min/nmol P450 and a K_m of 40 μM. The V_{max} is quite low, but it may still be of physiological importance. In this connection, retinal formation from β-carotene in human intestinal homogenates has been reported to occur at rates as low as 2.3 pmol/hr/mg homogenate (30). Retinyl palmitate was not cleaved to retinal at a significant rate by any of the rabbit liver isoforms that we examined in this study.

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Send reprint requests to: Dr. Minor J. Coon, Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, MI 48109-0606. E-mail: mjcoon@umich.edu