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Role of Isozymes of Rabbit Microsomal Cytochrome P-450 in the Metabolism of Retinoic Acid, Retinol, and Retinal

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SUMMARY

The metabolism of retinoic acid, retinol, and retinal has been investigated with eight purified rabbit cytochrome P-450 (P-450) isozymes, including the major forms in nasal and liver microsomes. Retinoids hydroxylated at the 4-position were found to be major metabolites with each of the isozymes examined. Only two of the isozymes, polycyclic aromatic hydrocarbon-inducible P-450 1A2 and antibiotic-inducible P-450 3A6, also catalyze the oxidation of retinal to retinoic acid, a reaction not previously attributed to P-450. P-450 1A2 showed high activities in both the 4-hydroxylation and aldehyde oxidation reactions. Phenobarbital-inducible P-450 2B4 also had high activity in the 4-hydroxylation reaction of retinoids, and cytochrome b_5 was found to increase the activity of P-450 2B4 with each substrate but to increase the activity of P-450 1A2 only with retinoic acid. In microsomes, retinoic acid is converted in an NADPH-dependent manner to both 4-hydroxyretinoic acid and 4-oxoretinoic acid, but none of the isozymes investigated was found to convert the 4-hydroxy derivative to the 4-oxo derivative. Microsomes from animals treated with phenobarbital were more active than those from untreated animals in the 4-hydroxylation reaction and, consequently, showed an increase in the ratio of 4-hydroxy to 4-oxo derivatives produced. These results show that the individual forms of P-450 metabolize retinoic acid, retinol, and retinal to multiple products, and they indicate that the amounts formed may be dependent on the exposure of animals to various inducers of P-450.

Vitamin A is an essential nutrient known to be involved in the regulation of cell proliferation, vision, reproduction, and differentiation (1). All-*trans*-retinoic acid, retinol, and retinal are metabolized *in vivo* and *in vitro* to a variety of oxidized and/or conjugated metabolites (2–4). For example, cytosolic dehydrogenases have been found to oxidize retinal to retinoic acid *in vitro* (5, 6). In addition, the enzymatic activities required for the formation of one of the hydroxylated products, 4-hydroxyretinoic acid, and the metabolism of 4-oxoretinoic acid to more polar metabolites have been attributed to P-450 in the microsomal fraction obtained from various tissues (7, 8). More recently, purified rat P-450s 2B1 and 2C7 and human P-450 2C8 have been found to metabolize retinoic acid and retinol, in a reconstituted system, to more polar products, including the corresponding 4-hydroxy compounds (3, 9, 10).

The present study was undertaken to investigate the potential role of various isozymes of rabbit P-450, including the major forms present in nasal and liver microsomes, in the metabolism of retinoic acid, retinol, and retinal, in the presence or the absence of cytochrome b_5. Retinoids hydroxylated at the 4-position were found to be major metabolites with each of the isozymes examined. Two of the most active isozymes in the 4-hydroxylation were P-450 1A2 and P-450 2B4. Cytochrome b_5 was found to increase the activity of P-450 2B4 with each substrate but to increase the activity of P-450 1A2 only with retinoic acid. Furthermore, P-450s 1A2 and 3A6, in addition to catalyzing the hydroxylation at the 4-position, bring about the conversion of retinal to retinoic acid.

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Experimental Procedures

Materials. All-*trans*-retinoic acid, all-*trans*-retinol, and all-*trans*-retinal were obtained from Sigma. [11,12-^{3}H]Retinoic acid and [11,12-^{3}H]retinol were from NEN Research Products and were 98% pure when analyzed by HPLC. Standard compounds (5,6-epoxy-, 4-hydroxy-, and 4-oxoretinoic acid) were a generous gift from Dr. Peter F. Sorter, Hoffmann-La Roche, Inc. (Nutley, NJ), and the authentic 4-hydroxyretinol was kindly provided by Dr. Joseph L. Napoli, University of Buffalo. Retinol was purified by HPLC before use. The chloroform or methanol solutions of the retinoids used in the enzyme assays were flushed with nitrogen and stored at −70°. HPLC-grade acetonitrile, ethyl acetate, and methyl alcohol were from Mallinckrodt, and catalase and superoxide dismutase were from Sigma. Male New Zealand White rabbits (2.0–2.5 kg in weight) were untreated or given 0.1% (w/v) phenobarbital in place of the drinking water for 7 days. All animals were fasted for 12–14 hr before being sacrificed. Pyrophosphate-washed liver microsomes were prepared as described (11) and stored at −70° in 100 mM Tris-acetate buffer, pH 7.4, containing 0.1 mM EDTA and

ABBREVIATIONS: P-450, cytochrome P-450; HPLC, high performance liquid chromatography.

20% (v/v) glycerol. The individual P-450s were purified from either rabbit nasal (12) or liver microsomes, as summarized elsewhere (13), except that 2E1 was purified from liver microsomes of acetone-treated animals (14). NADPH-P-450 reductase (15) and cytochrome b_5 (16) were purified from rabbit liver microsomes.

Enzyme assays. All reaction mixtures had a final volume of 1.0 ml. A chloroform solution of 1,2-dilauroyl-*sn*-glycero-3-phosphorylcholine, to give a concentration of 30 μg/ml in the final reaction mixture, and a chloroform solution of the retinoid were added to glass tubes. The solvent was evaporated under nitrogen, and deionized glass-distilled water, ascorbic acid, and 1.0 M potassium phosphate buffer, pH 7.4, were added to give final concentrations of 1.0 mM and 50 mM, respectively. The contents were mixed by a Vortex instrument for 30 sec and then centrifuged to remove the reaction components from the sides of the glass. In all experiments, the P-450, reductase, and cytochrome b_5, when present, were added in a 1:2:2 molar ratio. The individual isozymes of P-450 were incubated with the reductase for about 20 min at 4° before addition to the mixture. Cytochrome b_5 was then added, and after a 3-min incubation at 37° the reactions were initiated by the addition of NADPH at a final concentration of 1.0 mM. Ethyl acetate (3.0 ml), containing 50 μg/ml butylated hydroxytoluene as an antioxidant, was used to stop the reactions and to extract the products. An additional 3.0 ml of ethyl acetate were used for a second extraction. For the analysis of retinoic acid or 4-hydroxyretinoic acid, the pH of the reaction mixtures was adjusted to 3.0 with formic acid before extraction. This was found to give a recovery of >96% of [11,12-^{3}H] retinoic acid and [11,12-^{3}H]retinol. The solvent was evaporated under nitrogen, and the residue was dissolved in methanol containing 50 μg/ml butylated hydroxytoluene. For the identification of retinoic acid derived from the oxidation of retinal, the residue was allowed to react with a diethyl ether solution of diazomethane. After 20 min, the solvent was evaporated, and the residue was dissolved in methanol as described above. All procedures were carried out under minimal light or under red light.

HPLC procedures. Quantitative analysis of the reaction products was done on a Waters Resolve C_{18} reverse phase analytical column, with an automated HPLC system consisting of a Waters model 600 solvent delivery system, a model 490 UV/visible detector set at the appropriate wavelength maximum of the product, a Waters WISP model 710 autosampler, and a Hewlett-Packard model 3600 integrator. As a modification of a previously described procedure for analysis of products from retinoic acid (3), solvent A (acetonitrile/H_2O/acetic acid, 49.75:49.75:0.5) and solvent B (acetonitrile/H_2O/acetic acid, 90:10:0.04), each containing 0.01 M ammonium acetate, were used. Isocratic solvent conditions of 20% solvent B were maintained for 10 min, followed by a linear gradient to 80% solvent B over 20 min and then to 100% solvent B in 5 min, at a flow rate of 0.5 ml/min. In the analysis of both retinoic acid and its methyl ester, solvent C (acetonitrile/H_2O/acetic acid, 95:5:0.04) and solvent D (H_2O/acetic acid, 99.5:0.5), each containing 0.01 M ammonium acetate, were used. Isocratic solvent conditions of 38% solvent D were maintained for 2 min, followed by a linear gradient over 12 min to 100% solvent D, at a flow rate of 1.0 ml/min. In the analysis of 4-hydroxyretinol, 4-hydroxyretinal, and the methyl ester of retinoic acid, a gradient system consisting of acetonitrile and water was used. In the quantitative analysis of the 4-hydroxyretinoids and the methyl ester of retinoic acid, the areas of the peaks were in a range found to be linear with respect to known amounts of the standards. The 4-hydroxyretinal used to produce a standard curve was enzymatically generated from retinal, in a complete reconstituted system containing P-450 2B4, and was subsequently purified by HPLC. The concentrations of the 4-hydroxy standards were determined using the extinction coefficients of the parent compounds (17). In-line UV/visible absorption spectra were taken after trapping of the peak of interest in the flow cell of the HPLC detector.

Additional procedures. HPLC-purified 4-hydroxyretinal was reduced in methanol with $NaBH_4$ (1 mg/ml), and the identity of the product was confirmed, by HPLC and Uv/visible spectrometry, to be 4-hydroxyretinol. The methyl ester of retinoic acid was formed by derivatization of the standard or enzymatically produced retinoic acid with diazomethane in diethyl ether. With the enzymatically generated product, the derivatization was done either on HPLC-purified retinoic acid or on the extracted and dried reaction components. After evaporation of the solvent, the products were dissolved in methanol and analyzed by HPLC. The electron impact mass spectral analysis of the enzymatically produced methyl ester of retinoic acid was done by direct probe on a VG Analytical 70S mass spectrometer, with an electron energy of 70 eV, by James Windak, Department of Chemistry, The University of Michigan.

Results

P-450 2B4-dependent metabolite formation. The major metabolites produced from the NADPH-dependent reaction of retinoic acid, retinol, and retinal in a reconstituted enzyme system containing P-450 2B4, in the presence of cytochrome b_5, are shown in the HPLC profiles given in Fig. 1. The peaks at 4.2 min in Fig. 1A and 10.1 min in Fig. 1C co-migrated with the authentic standards of 4-hydroxyretinoic acid and 4-hydroxyretinol, respectively, and had the same absorption spectra as the standards. The compound in the 8.4-min peak in Fig. 1E, produced from retinal, was spectrally similar to retinal. Identification of this product as 4-hydroxyretinal was based upon the observation that, upon reduction with $NaBH_4$, it gave a product having the HPLC retention time (Fig. 2A) and spectral properties (Fig. 2B) of the standard 4-hydroxyretinol.

The requirements for the 4-hydroxylation of retinoids in the reconstituted enzyme system containing P-450 2B4 are shown in Table 1. With all of the substrates examined, the rate of 4-hydroxylation was insignificant upon omission of NADPH, reductase, or P-450. The rate was 2–3-fold higher in the complete system than in the reactions where cytochrome b_5 was omitted. Ascorbic acid was included in all reaction mixtures, as used for another P-450 substrate (18), to prevent the formation of nonenzymatic oxygenated products of the retinoids. The addition of 0.1 or 0.3 mM desferrioxamine, as an iron chelator (19–22), to the reaction mixtures also prevented the generation of nonenzymatic products. To ensure that reduction products of oxygen that are formed by the P-450 system, such as hydrogen peroxide (23) and superoxide (24–26), were not involved in the formation of 4-hydroxyretinoic acid or 4-hydroxyretinal, the effects of catalase and superoxide dismutase on the complete system were examined. As shown in Table 2, these additions had no effect on the formation of 4-hydroxyretinal. Similar results (data not shown) were obtained for the hydroxylation of retinoic acid.

Reaction kinetics. In other experiments, for which the results are not given, the P-450 2B4-dependent formation of 4-hydroxyretinoic acid was linear for 25 min with 0.1 nmol of P-450 and linear with respect to the amount of P-450 up to 0.3 nmol. Maximal rates of hydroxylation of retinoic acid were obtained at a substrate concentration of 24 μM. The formation of 4-hydroxyretinol and 4-hydroxyretinal was linear for at least 15 min with 0.025 nmol of P-450 2B4 and linear with respect to the amount of P-450 2B4 up to 0.1 nmol. Maximal rates of hydroxylation with retinol and retinal were obtained at substrate concentrations of 15 and 18 μM, respectively. Inhibition was observed at higher substrate concentrations with all three retinoids. Fig. 3 shows typical double-reciprocal plots for retinal in the absence and presence of cytochrome b_5, from which kinetic constants were obtained. The apparent Michaelis con-

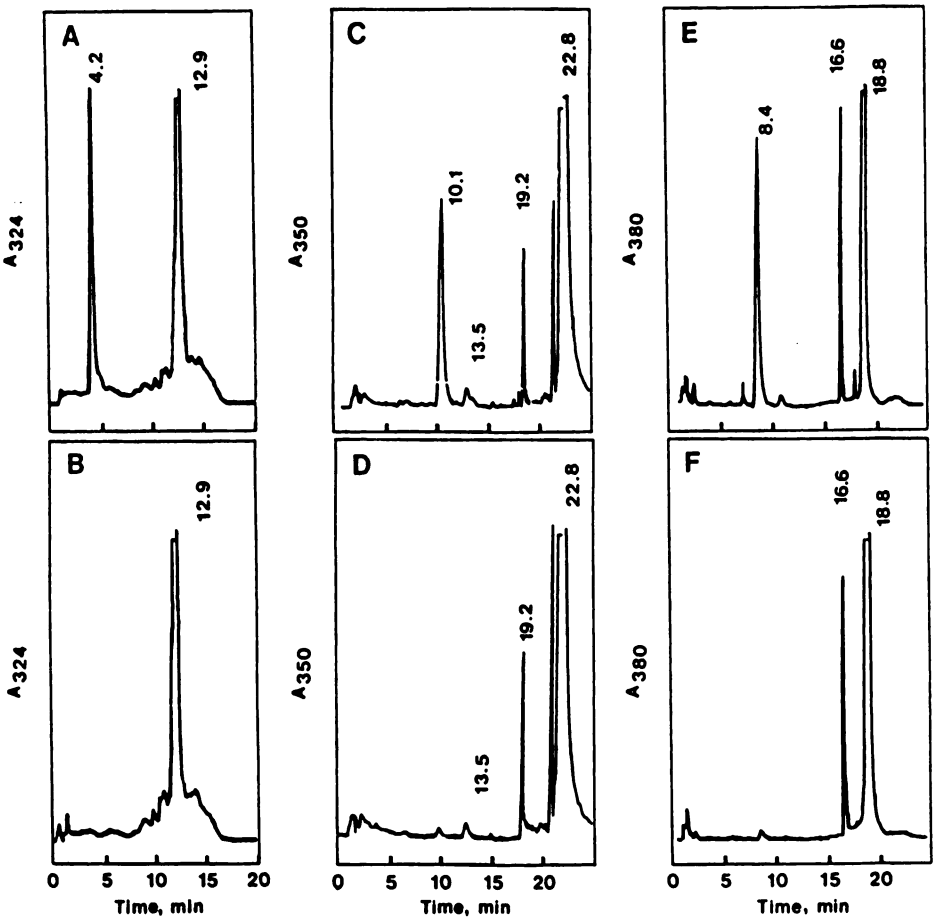

Fig. 1. HPLC profile of the major metabolites produced from retinoic acid, retinol, or retinal in a reconstituted system containing P-450 2B4. The wavelength monitored was at the absorption maximum of the substrate. Upon incubation with a complete system, consisting of P-450 2B4, reductase, cytochrome b_5, NADPH, and phospholipid, one major metabolite was found with each of the substrates, retinol (A), retinoic acid (C), or retinal (E). The HPLC profile of a boiled enzyme blank for each substrate (B, retinol; D, retinoic acid; and F, retinal) is also shown. The complete reconstituted system was as described in Experimental Procedures, with 0.1 nmol of P-450 with 24 μM retinoic acid or 0.05 nmol of P-450 with 18 μM retinal or 15 μM retinol. For heat inactivation, the enzymes were placed in a boiling water bath for 5 min. The incubation was for 10 min at 37°, and the wavelength monitored was at the absorption maximum for the substrate used. HPLC conditions are described in Experimental Procedures for analysis of retinoic acid. For retinol and retinal analysis, the isocratic solvent was acetonitrile/water (62:38 and 55:45, respectively), maintained for 8 min, followed by a linear gradient to 100% acetonitrile in 10 min.

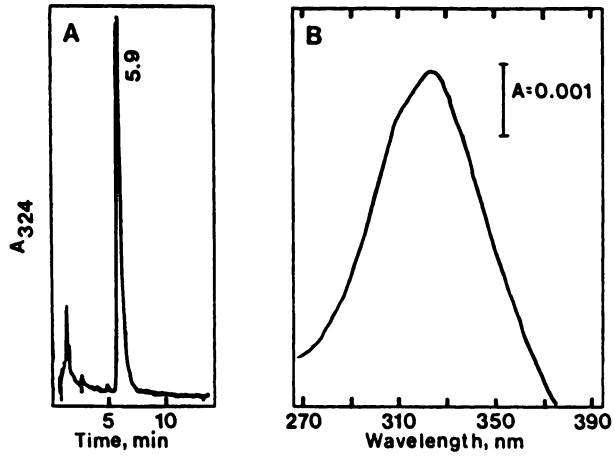

Fig. 2. Identification as 4-hydroxyretinal of the major metabolite from the incubation of P-450 2B4 and retinal. A, HPLC profile of the polar retinal metabolite after reduction with $NaBH_4$. The solvent was acetonitrile/water (55:45), and the wavelength monitored was 324 nm. B, Absorption spectrum of the 5.9-min peak shown in A.

TABLE 1

Component requirements for 4-hydroxylation of retinoic acid, retinal, and retinol by P-450 2B4

Conditions were as described in Fig. 1. The data are an average of three experiments, reported as the mean ± variance from the mean.

System	Rate of 4-hydroxylation		
	Retinoic acid	Retinal	Retinol
	nmol of product/min/nmol of P-450 2B4		
Complete	0.22 ± 0.01	1.4 ± 0.1	1.7 ± 0.2
Cytochrome b_5 omitted	0.066 ± 0.001	0.45 ± 0.03	0.86 ± 0.06
NADPH omitted	<0.005	0	0
Reductase omitted	<0.005	0	0
P-450 omitted	<0.005	0	0
P-450 and reductase omitted	<0.005	0	0
Heat-inactivated enzymes	<0.005	0	0

stants and maximum velocities were determined from the linear portion of the curve, as indicated in Fig. 3, *inset*. The correlation coefficients for the curves in the double-reciprocal plots for each substrate ranged from 0.96 to 0.99. The apparent K_m and V_{max} values for the three substrates, in the presence and absence of cytochrome b_5, are given in Table 3. The maximum velocities for the reaction of P-450 2B4 with retinol and retinal were much greater than seen with retinoic acid. In addition, with these substrates there was a marked increase in the V_{max} values in the presence of cytochrome b_5, with much less of an effect on the K_m values.

P-450 isozyme activities. The activities of eight purified forms of rabbit liver and nasal microsomal P-450 in the 4-hydroxylation of each of the substrates are shown in Table 4. Polyaromatic hydrocarbon-inducible P-450 1A2 had the highest activity with all substrates in the absence of cytochrome b_5, whereas phenobarbital-inducible P-450 2B4 was the most active in the presence of cytochrome b_5. With the other six isozymes, the rate of 4-hydroxylation of retinoic acid was much lower, and cytochrome b_5 had no effect. In the 4-hydroxylation of retinol and retinal, the alcohol-inducible form, P-450 2E1, showed a decrease in activity in the presence of cytochrome b_5.

TABLE 2

Effect of catalase and superoxide dismutase on the 4-hydroxylation of retinal by P-450 2B4

Conditions were as described in Fig. 1. The data are an average of two experiments.

System	Activity
	nmol/min/nmol of P-450
Complete	1.25 ± 0.07
Catalase added (240 units)	1.26 ± 0.06
Catalase added (960 units)	1.34 ± 0.04
Superoxide dismutase added (60 units)	1.20 ± 0.05
Superoxide dismutase added (360 units)	1.21 ± 0.09

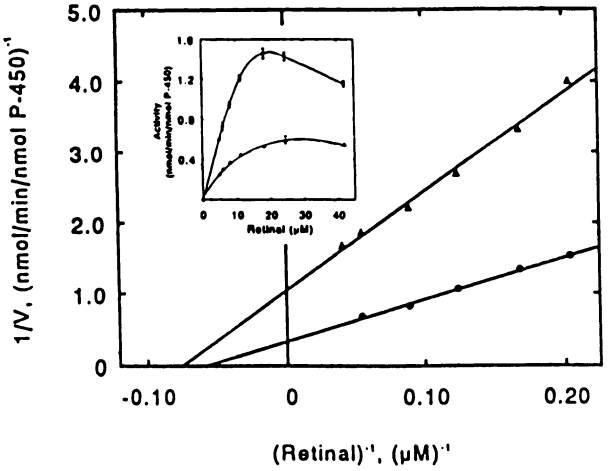

Fig. 3. Effects of cytochrome b_5 on kinetics of retinal 4-hydroxylation. Double-reciprocal plot of the reaction velocity versus substrate concentration and substrate saturation curve (*inset*) for the 4-hydroxylation of retinal in the absence (▲) or presence (●) of cytochrome b_5. Each data point is the average of three experiments, and *error bars* are not given when the deviation was smaller than the size of the *symbol*. The conditions were as described in Table 2.

TABLE 3

Effect of cytochrome b_5 on kinetic constants for retinoic 4-hydroxylation by P-450 2B4

The apparent kinetic constants were obtained from double-reciprocal plots. The amount of P-450 2B4 present in the assays with retinoic acid was 0.2 nmol and with the other substrates was 0.035 nmol. All other conditions for the complete system, with and without cytochrome b_5, were as described in Fig. 1. The values for retinal are taken from Fig. 3. The experimental procedure for obtaining the values for retinoic acid and retinol was similar to that for retinal.

Substrate	Cytochrome b_5 added	K_m	V_{max}
		μM	*nmol/min/nmol of P-450*
Retinoic acid	−	9	0.17
	+	13	0.33
Retinol	−	6	0.97
	+	13	2.50
Retinal	−	13	0.95
	+	17	2.88

Furthermore, the noninducible form of P-450, 2C3, and the antibiotic-inducible form, P-450 3A6, both showed considerable activity with retinal. In the absence of cytochrome b_5, P-450 2C3 catalyzed the 4-hydroxylation of retinal with an activity as high as seen with P-450 1A2 but showed a marked inhibition in the presence of cytochrome b_5.

Microsomal activities. When the metabolism of retinoic acid was investigated with rabbit liver microsomes, products

TABLE 4

Activity of various forms of liver and nasal microsomal P-450 in catalyzing the 4-hydroxylation of retinoids

Conditions were as in Fig. 1, with 0.15 nmol of P-450 and an incubation time of 15 min in the assays with retinoic acid and 0.075 nmol of P-450 in the assays with retinol and retinal. The data are an average of two experiments.

Form of P-450[a]	Cytochrome b_5 added	Rate of 4-hydroxylation		
		Retinoic acid	Retinol	Retinal
		pmol/min/nmol of P-450		
2B4 (LM2)	−	71 ± 10	690 ± 50	570 ± 10
	+	230 ± 13	1630 ± 90	1400 ± 20
1A2 (LM4)	−	98 ± 13	800 ± 50	790 ± 50
	+	150 ± 13	760 ± 90	670 ± 40
2E1 (LM3a)	−	20 ± 1	181 ± 1	270 ± 10
	+	16 ± 1	132 ± 1	160 ± 20
2E2 (LM3d)	−	7 ± 1	ND[b]	130 ± 20
	+	6 ± 1	ND	131 ± 2
2C3 (LM3b)	−	18 ± 1	103 ± 5	753 ± 3
	+	19 ± 1	130 ± 20	45 ± 5
(NMa)	−	36 ± 4	49 ± 5	ND
	+	43 ± 3	47 ± 4	ND
2G1 (NMb)	−	13 ± 1	<20	<20
	+	15 ± 1	50 ± 3	<20
3A6 (LM3c)	−	<5	113 ± 12	387 ± 5
	+	<5	96 ± 1	40 ± 5

[a] Trivial names are given in parentheses.
[b] ND, not determined.

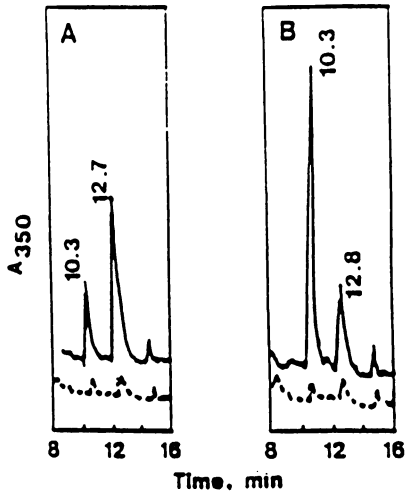

Fig. 4. HPLC profile of the products from the incubation of retinoic acid with liver microsomes from untreated (A) or phenobarbital-induced (B) rabbits. The HPLC profiles of incubations without NADPH are also given (– – –). Assay conditions were as described in Fig. 1, with 1 nmol of P-450/assay. The 10.3-min peak coeluted with 4-hydroxyretinoic acid, and the 12.7-min peak with 4-oxoretinoic acid.

more polar than the substrate were produced, as seen in Fig. 4. In the reaction with microsomes from uninduced animals, the HPLC chromatogram (Fig. 4A) showed products at 10.3 and 12.7 min that coeluted with standard 4-hydroxy- and 4-oxo-retinoic acid, respectively. In other experiments, which are not presented, none of the P-450 isozymes examined in this study were found to oxidize 4-hydroxyretinoic acid to 4-oxoretinoic acid. With microsomes from rabbits treated with phenobarbital (Fig. 4B), there was an increase in the formation of 4-hydroxy-retinoic acid. Also, the ratio of the 4-hydroxy to the 4-oxo derivative increased after phenobarbital induction. The activities in the 4-hydroxylation of retinoic acid were determined to be 5.73 ± 0.08 and 22 ± 2 pmol/min/nmol of total P-450 for

the microsomes from the uninduced and phenobarbital-treated rabbits, respectively.

Formation of retinoic acid from retinal. P-450 forms 1A2 and 3A6 were found to oxidize retinal to a polar product that was identified as retinoic acid. Fig. 5A shows a chromatogram of the authentic retinoic acid (14.5 min) and its methyl ester (18.9 min). The HPLC-purified, NADPH-dependent product from a reaction of reconstituted P-450 1A2 and retinal is shown in Fig. 5B. Under these conditions, the peak that eluted at 14.4 min co-migrated with standard retinoic acid. After treatment with diazomethane, the 14.4-min peak, shown in Fig. 5C, was almost completely replaced by the 18.9-min peak, which co-migrated with the standard methyl ester of retinoic acid. A portion of the electron impact fragmentation pattern of the enzymatically produced retinoic acid after derivatization to the methyl ester is shown in Fig. 5D. The M^+ and M^+-15 peaks are characteristic of those seen with the authentic standard (27). Under the same assay conditions used for the formation of the 4-hydroxyretinal, the relative activities of the two forms active in the oxidation of retinal to retinoic acid are given in Table 5. The product was quantitated as the methyl ester derivative, and it was concluded that P-450 1A2 is about 5-fold more active than P-450 3A6 and that cytochrome b_5 does not have an effect on the activity of either enzyme.

Discussion

It has been shown *in vivo* and *in vitro* that retinoic acid, retinol, and retinal are metabolized to a variety of oxidized and/or conjugated metabolites (2–4). Many of the metabolites identified from *in vivo* studies contain a hydroxyl or keto group at the 4-position of the cyclohexenyl ring (1). Previously, purified rat P-450s 2B1 and 2C7 and human P-450 2C8 were shown to metabolize retinoic acid and retinol, in reconstituted systems, to more polar products, including the respective 4-hydroxy derivatives (3, 9, 10). The present study demonstrates that a series of eight purified rabbit isozymes of P-450, in a reconstituted system containing NADPH, hydroxylate retinoic acid, retinol, and retinal almost exclusively at the 4-position. These reactions are believed to be involved in deactivation and subsequent elimination of the retinoids from the body (4). Recently, it was reported that 4-hydroxy- and 4-oxoretinoic acid were 70–86% as effective as retinoic acid in stimulating retinoic acid receptor-dependent reporter gene transcription *in vitro* (28). In addition, all-*trans* derivatives of retinoic acid (3,4-didehydro, 4-hydroxy, 4-oxo, and 18-hydroxy) had affinities similar to that of retinoic acid in binding to bovine adrenal cellular retinoic acid-binding proteins (29). Thus, isozymes of P-450 form a specific metabolite that represents either the deactivation or a physiological function of vitamin A.

The isozymes with the highest activities in the 4-hydroxylation of these retinoids are P-450s 2B4 and 1A2. Moreover, with retinoic acid as substrate, the activities of these isozymes are about 2 orders of magnitude greater than previously reported with the rat isozymes (9). With P-450 2B4, cytochrome b_5 increases the rate of hydroxylation of all three retinoids. With P-450 1A2, cytochrome b_5 stimulates the 4-hydroxylation of retinoic acid but has much less of an effect on the activity with retinol and retinal. Similar results on the differential effects of cytochrome b_5 on the activities of P-450 forms 2B4 and 1A2 were seen in the hydroxylation of prostaglandins, in which cytochrome b_5 is completely essential for metabolism by P-450 2B4 but plays a facilitory role in the reactions catalyzed by P-450 1A2 (30). In addition, with some of the other isozymes examined in this study, cytochrome b_5 inhibits the 4-hydroxylation of the retinoid substrates. The diverse effects that cytochrome b_5 has on retinoid metabolism are in accord with previous results in which cytochrome b_5 was shown in cultured cells to stimulate (31) and *in vitro* to stimulate, inhibit, or have no effect on various monooxygenation reactions, and these effects are P-450 isozyme specific and dependent on the substrate used, as previously reviewed (32). Most studies support the idea that, with certain isozymes of P-450 and certain substrates, cytochrome b_5 enhances electron transfer to the intermediate oxy-ferro P-450-substrate complex, thus enhancing the monooxygenation of the substrate and decreasing the amount of H_2O_2 formed (32).

With the P-450 isozymes examined, 4-hydroxyretinoic acid did not appear to be oxidized in a P-450-dependent manner to the 4-oxo derivative, as described by others (9, 10). Alcohol-inducible P-450 2E1, which exhibits high activity in alcohol oxidations (14, 33), surprisingly did not oxidize 4-hydroxy- to 4-oxoretinoic acid or retinol to retinal. The activity of P-450 2E2, a protein that differs from P-450 2E1 by only 16 amino acid residues located throughout the protein (34), is about 30% that of 2E1 with retinoic acid and about 50% that of 2E1 with retinal in the absence of cytochrome b_5 and nearly equal in the presence of cytochrome b_5.

The present study demonstrates for the first time that a

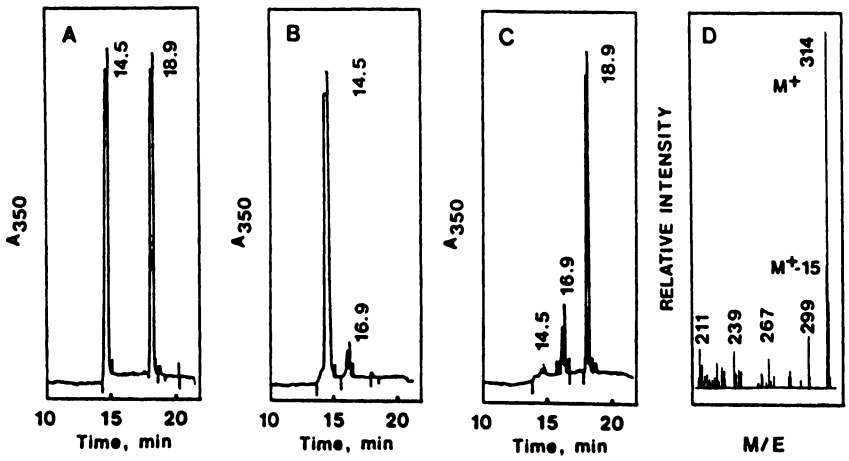

Fig. 5. P-450 1A2-dependent formation of retinoic acid from retinal. A, HPLC chromatogram of standard retinoic acid after a 10-min incubation with diazomethane; the standard and the methyl ester eluted at 14.5 min and 18.9 min, respectively. The HPLC conditions are given in Experimental Procedures. B, HPLC chromatogram of the HPLC-purified enzyme reaction product at 14.4 min, which coeluted with standard retinoic acid. C, Enzymatic reaction product after derivatization with diazomethane. The peak at 18.9 min coeluted with the methyl ester of retinoic acid. D, Electron impact fragmentation pattern for the enzymatically generated retinoic acid derivatized with diazomethane, with mass per unit charge (*M/E*) shown as a function of ion intensity. This was obtained after subtraction of the spectral contribution of dioctyl phthalate, a plasticizer that comes from Tygon tubing.

TABLE 5

Activity of two forms of liver microsomal P-450 in the formation of retinoic acid from retinal

Assay conditions were as described in Table 4. Retinoic acid was quantitated as the methyl ester after derivatization with diazomethane. For HPLC analysis of the ester, the mobile phase was acetonitrile/water (65:35), with a linear gradient to 100% acetonitrile over 15 min. The data are mean of three experiments.

Form of P-450	Cytochrome b_5 added	Rate of retinoic acid formation
		nmol/min/nmol of P-450
1A2	−	1.1 ± 0.1
	+	0.96 ± 0.03
3A6	−	0.23 ± 0.01
	+	0.17 ± 0.01

purified P-450 reconstituted with reductase and phospholipid, in the presence of NADPH, can oxidize retinal to retinoic acid. Recently, aldehydes were shown to be oxidized to carboxylic acids by microsomes (35–38) and by a P-450 purified from mouse liver microsomes (39). Retinal has been shown to undergo conversion to retinoic acid by cytosol and microsomes and may be an intermediate in the pathway from retinol to retinoic acid (5, 35, 40–44). The biological oxidation of aldehydes to acids is usually catalyzed by aldehyde dehydrogenase, which is located mainly in the mitochondria and cytosol (45). On the other hand, cytosolic dehydrogenases, distinct from the several classes of alcohol dehydrogenases, convert retinal to retinoic acid *in vitro* and may contribute significantly to this conversion *in vivo* (5, 6). Two of the isozymes examined, polycyclic aromatic hydrocarbon-inducible P-450 1A2 and antibiotic-inducible P-450 3A6, oxidize retinal to retinoic acid, with the activity of the former being about 5-fold higher than that of the latter.

Interestingly, P-450 1A2 efficiently catalyzes 4-hydroxylation on the ring and oxidation of the polar head group, sites on the retinal molecule far removed from each other. Previously, similar results were reported, in which a conformationally restricted molecule, progesterone, was metabolized by a rat P-450 to two primary hydroxylated products, with the two sites of catalysis being on opposite faces and far apart from each other (46). With retinal, the 4-hydroxylation appears to be more widely catalyzed by the series of isozymes examined, whereas the oxidation to retinoic acid is more specific to P-450 1A2. The results from retinal and P-450 1A2, in which 4-hydroxyretinal and retinoic acid but not 4-hydroxyretinoic acid were detected as products, imply that the substrate is specifically bound at the active site in one of two orientations in which the 4-position of the ring or the polar end group is oriented close to the reactive iron-oxene intermediate.

From results in the present study, under conditions that induce P-450 2B4, such as phenobarbital treatment (47), the rate of 4-hydroxylation increases over that seen with uninduced microsomes, resulting in a change of the ratio of 4-hydroxyretinoic acid to 4-oxoretinoic acid. Consequently, the metabolism of vitamin A in different tissues may be affected by exposure of an animal to various inducers of P-450. As shown in this study, vitamin A, which is known to be involved in vision, fetal development, cellular proliferation and differentiation, and the prevention of carcinogenesis (1), when metabolized by specific isozymes of P-450 is converted to various metabolites that may have physiological roles in the cell. As a result, P-450 may be an important enzyme in the well regulated transport and storage system that provides tissues with suitable amounts of retinoids in spite of normal fluctuations in daily vitamin A intake.

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