Pergamon

Biochemical Pharmacology, Vol. 50, No. 8, pp. 1257–1264, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02005-W

BIOTRANSFORMATION OF ALL-*TRANS*-RETINOL AND ALL-*TRANS*-RETINAL TO ALL-*TRANS*-RETINOIC ACID IN RAT CONCEPTAL HOMOGENATES

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(*Received* 6 *January* 1995; *accepted* 24 *April* 1995)

Abstract—Catalysis of the oxidation of all-*trans*-retinol (vitamin A_1) or of all-*trans*-retinal to all-*trans*-retinoic acid (all-*trans*-RA) by rat conceptal enzymes was investigated during organogenesis. Products of the reaction were identified and quantified with HPLC by comparing their elution times with those of authentic standard retinoids. Under the incubation and assay conditions utilized, all-*trans*-retinol and all-*trans*-retinal were converted to readily detectable quantities of all-*trans*-RA. Rat conceptal homogenates from gestational days 10.5, 11.5 and 12.5 each exhibited enzymatic activity for oxidation of all-*trans*-retinol and all-*trans*-retinal to all-*trans*-RA. Enzymatic catalysis was verified by showing that: (1) both reactions were coenzyme dependent; (2) the rates of reactions increased as concentrations of conceptal protein increased; (3) both reactions were abolished by heating the tissue homogenates (100°, 5 min); and (4) both reactions exhibited substrate saturation. Under the same experimental conditions, formation of all-*trans*-RA from all-*trans*-retinol was much slower than from all-*trans*-retinal, suggesting that oxidation of all-*trans*-retinol to all-*trans*-retinal was the rate-limiting step for biotransformation of all-*trans*-retinol to all-*trans*-RA in embryonic tissues. When NAD or NADP were replaced by NADH or NADPH, the rate of oxidation of all-*trans*-retinol was reduced markedly, indicating that the reaction was catalyzed primarily by an NAD/NADP-dependent dehydrogenase(s). Carbon monoxide ($CO:O_2$ = 90:10) did not inhibit the reaction. NAD appeared to be a more effective cofactor than NADP in catalyzing oxidation of all-*trans*-retinal to all-*trans*-RA. When NAD was omitted, formation of all-*trans*-RA from all-*trans*-retinal was reduced by approximately 55%. Replacing NAD by NADH or NADPH also reduced the conversion of all-*trans*-retinal to all-*trans*-RA by about 60%. These observations suggested at least two pathways for the generation of all-*trans*-RA from all-*trans*-retinal in embryos: oxidation catalyzed by an NAD/NADP-dependent dehydrogenase(s) and oxidation catalyzed by an oxidase(s) that did not require NAD, NADH, NADP or NADPH. Conversion of all-*trans*-retinol to all-*trans*-RA was inhibited strongly by low concentrations of citral, but not by high concentrations of sodium azide, 4-methylpyrazole, or metyrapone. Similarly, oxidation of all-*trans*-retinal was inhibited strongly by citral but not by metyrapone. Our studies suggested that: (1) biotransformation of all-*trans*-retinol to all-*trans*-RA in embryos was catalyzed by an NAD/NADP-dependent retinol dehydrogenase(s); (2) biotransformation of all-*trans*-retinal to all-*trans*-RA in embryos was catalyzed by an NAD/NADP-dependent retinal dehydrogenase(s) and a retinal oxidase(s); and (3) oxidation of all-*trans*-retinol to all-*trans*-retinal was the rate-limiting step in biotransformation of all-*trans*-retinol to all-*trans*-RA.

Key words: retinol; retinal; retinoic acid; retinoid receptor ligands; rat; conceptal tissues; embryo; organogenesis; dysmorphogenesis; retinol dehydrogenase; retinal dehydrogenase; retinal oxidase

In contrast to the traditional idea that vitamin A_1 (all-*trans*-retinol) plays a major role in embryonic dysmorphogenesis, evidence has suggested that the teratogenicity of a single high dose of retinol may be due, at least in part, to the metabolic conversion of retinol to the known teratogen, all-*trans*-RA† [1, 2]. Increasing evidence has supported this concept. First, several members of a family of retinoid receptors (RARs and RXRs), which are activated by retinoid receptor ligands such as all-*trans*-RA, 9-*cis*-RA and other retinoic acids, have been discovered and are known to control transcription in vertebrates [3, 4]. However, no retinol receptor has yet been found. Second, recent studies have shown that direct addition of all-*trans*-RA or 9-*cis*-RA (both are retinoid receptor ligands) to cultured embryos causes malformations of cultured embryos similar to those produced by retinol but at much lower concentrations. For example, approximately 16-fold higher concentrations of all-*trans*-retinol are required to produce the same incidence/severity of dysmorphogenesis as that produced by all-*trans*-RA in cultured whole rat embryos. All-*trans*-RA appears to be the ultimate dysmorphogen in the embryonic dysmorphogenesis produced by all-*trans*-retinol [5]. Three additional retinoic acids, namely all-*trans*-3,4-*didehydro*-RA, all-*trans*-4-*oxo*-RA and 9-*cis*-3,4-*didehydro*-RA, are now recognized as retinoid receptor ligands [6–8]. If retinoid receptor ligands play a key role in retinol-elicited embryonic dysmorphogenesis as indicated by those and other studies, the enzymatic modulation of the conversion of retinol to retinoid receptor ligands becomes crucial to both embryonic organogenesis and retinol-elicited dysmorphogenesis.

To fully understand the mechanisms of vitamin A teratogenicity, information on the embryonic biotransformation of retinol to retinoic acids seems essential. The biosynthesis of retinoic acids from retinol has been well studied in adult mammalian tissues particularly in liver.

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† Abbreviations: all-*trans*-RA, all-*trans*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; NADH, reduced nicotinamide adenine dinucleotide; and 4-MP, 4-methylpyrazole.

For those tissues, the currently recognized mechanism involves two consecutive enzymatic reactions. Retinol is first oxidized to retinal (retinaldehyde), and then retinal is further oxidized to retinoic acids. Biosynthesis of retinoic acids can be catalyzed by a wide variety of enzymes such as dehydrogenases [9–12], oxidases [13, 14], and microsomal cytochrome P450 monooxygenases [15, 16]. At present, however, whether embryos express such enzyme activities during organogenesis has not been established.

The importance of biotransformation of retinoids in relation to embryotoxicity has led us to study the enzymatic conversion of all-*trans*-retinol and all-*trans*-retinal to all-*trans*-RA in rat conceptal tissues during gestational days 10.5 to 12.5, a crucial period for organogenesis. This study was initiated by investigating enzymatic activities in a whole conceptal homogenate system. The goal was to provide a body of essential information in the area of enzymatic control of the biosynthesis of all-*trans*-RA in embryonic tissues during organogenesis. The objectives of this study, therefore, are to determine: (1) is there any conceptal enzyme(s) that catalyzes conversion of all-*trans*-retinol and/or all-*trans*-retinal to all-*trans*-RA in embryonic tissues; and (2) what specific conceptal enzyme(s) participates in such reactions if enzymatic activity is verified.

MATERIALS AND METHODS

Materials

All-*trans*-retinol and metyrapone were purchased from the Aldrich Chemical Co., Inc. (Milwaukee, WI). 13-*cis*-RA, all-*trans*-RA, all-*trans*-retinal, NAD, NADH, NADP, and NADPH were purchased from the Sigma Chemical Co. (St. Louis, MO). 9-*cis*-RA was a gift from Dr. Sorter of Hoffmann–La Roche Inc. (Nutley, NJ). Citral (3,7-dimethyl-2,6-octadienal), 4-MP, and sodium azide also were purchased from Sigma. All other chemicals and reagents utilized were of the highest purity commercially available.

Preparation of rat conceptal homogenates

Time-mated pregnant rats (Sprague–Dawley, Wistar-derived) were obtained from Tyler Laboratories (Bellevue, WA) on days 10.5, 11.5, and 12.5 of gestation. All animals were allowed free access to food and distilled water and were housed in polycarbonate cages with crushed corncob material for bedding. The morning after copulation was designated as day 0 of gestation. The conceptuses, defined as the embryo proper plus the amniotic membrane, the visceral yolk sac, and the ectoplacental cone (components of the whole embryo culture system), were removed from ether-anesthetized dams and washed with Hanks' balanced salt solution (pH 7.5). The whole conceptuses were immediately frozen at −70°. Thirty conceptuses were transferred to a Duall Tissue Grinder containing 3 mL of 0.1 M sodium phosphate buffer (pH 7.5) and homogenized by hand on ice. The homogenates were centrifuged at 600 *g* for 5 min to remove large particulates, and the supernatant fraction was used as a source of conceptal enzymes.

*Conversion of all-*trans*-retinol or of all-*trans*-retinal to all-*trans*-RA*

All-*trans*-retinol or all-*trans*-retinal were dissolved in acetone (1 mg/mL). Freshly prepared conceptal homogenates were mixed well with 0.1 M sodium phosphate buffer (pH 7.5) containing NAD (4 mM), NaCl (40 mM), and Tween-80 (0.02%) in a 5-mL glass tube. Varying amounts of all-*trans*-retinol or all-*trans*-retinal were added to the test tube, and the final volume was brought to 1 mL with buffer. The addition of retinoids was completed in a darkened room with yellow lights to prevent photoisomerization. The reactants were incubated at 37° in a water bath with continuous shaking, and light was excluded. At the end of the incubation, an equal volume of ice-cold isopropanol was added to the incubation mixture, which was then vortexed for 1 min and centrifuged for 30 min at 16,000 *g* at 4°. The supernatant was saved at −20° for HPLC analysis. Homogenates from gestational day 12.5 were used for experiments unless otherwise indicated.

*Inhibition of conversion of all-*trans*-retinol or all-*trans*-retinal to all-*trans*-RA with enzyme inhibitors*

The experimental procedure was the same as described above except that homogenates were preincubated with enzyme inhibitors for 20 min prior to the addition of substrate. The enzyme inhibitors used in these experiments were sodium azide (inhibitor of catalase), 4-MP (inhibitor of alcohol dehydrogenases), metyrapone (inhibitor of cytochrome P450-dependent monooxygenases), carbon monoxide ($CO:O_2$ = 90:10) (specific inhibitor of cytochrome P450-dependent monooxygenases), and citral (inhibitor of alcohol and aldehyde dehydrogenases).

Identification and quantification of retinoids by HPLC

The solvent delivery system for HPLC consisted of two model 100 A dual piston Beckman pumps linked together for activation of a binary gradient. The system was interfaced with a Shimadzu SPD-10A UV-VIS detector (set at 354 nm) and a Shimadzu C-R5A Chromatopac data processor. The HPLC system was equipped with a Beckman mixing chamber and manual injector. The analytical column (120 × 4.6 mm) was slurry packed with Spherisorb 3 ODS II (3 μm, phase separation). A cartridge (20 × 4.6 mm) packed with Lichrosorb RP 18 (10 μm) was used as a precolumn. The analytical eluents consisted of solvent A (methanol) and solvent B [ammonium acetate (40 mM, pH 7.4), 50% v/v, and methanol, 50% v/v]. The gradient was a slight modification of that reported previously [5], with a flow rate of 1.1 mL/min. The calibrations followed the procedures described previously [5]. Authentic retinoids were dissolved in absolute ethanol (1 mg/mL). 13-*cis*-RA, 9-*cis*-RA, and all-*trans*-RA were mixed with all-*trans*-retinol and all-*trans*-retinal and then diluted with distilled water before the HPLC injection. One hundred microliters of a mixture of standard retinoids or supernatant fraction of incubation mixture were loaded onto the HPLC column, and the elution time of each individual standard retinoid was used to identify the peaks eluting from the HPLC column.

Protein determination

The method of Lowry *et al.* [17] was used to quantitatively determine the concentration of protein in rat conceptal homogenates. BSA was used as a standard protein for the quantitation.

Statistical analysis

All experimental data are expressed as the means ± SD of three or four experimental measurements. T-tests were conducted with a Microexcell statistics package (Microsoft, Redmond, WA) to test for the statistical significance of difference between mean values.

RESULTS

Figure 1 shows HPLC separations of standard retinoic acids (13-*cis*-RA, 9-*cis*-RA, all-*trans*-RA), mixed with all-*trans*-retinol and all-*trans*-retinal. Individual retinoids were distinguishable by their elution times. The HPLC assay was capable of specifically quantifying individual retinoids, thus providing a legitimate approach for the study of bioconversion of all-*trans*-retinol and all-*trans*-retinal to all-*trans*-RA in rat conceptal tissues.

In initial experiments, all-*trans*-retinol was incubated with rat conceptal homogenates and NAD (see Materials and Methods) for periods of 1 and 2 hr. Generation of all-*trans*-RA under the described incubation conditions is illustrated in Fig. 2. Rat conceptal homogenates from 10.5, 11.5, and 12.5 gestational days each exhibited activities for the oxidation of all-*trans*-retinol. Homogenates from 12.5 gestational days possessed the highest activity.

Figure 3 illustrates the time course of the generation of all-*trans*-RA from all-*trans*-retinal with conceptal homogenates obtained from gestational day 12.5 as the enzyme source. The formation of all-*trans*-RA from all-*trans*-retinal occurred at much faster rates than from all-*trans*-retinol. Incubation of all-*trans*-retinol for 60 min resulted in the generation of a similar quantity of all-*trans*-RA as that produced by incubations of all-*trans*-retinal for 30 min under the same reaction conditions.

Table 1 exhibits the characterization of conversions of all-*trans*-retinol and of all-*trans*-retinal to all-*trans*-RA. Neither reaction proceeded measurably without the addition of fresh conceptal homogenates. Oxidation of all-*trans*-retinol appeared to be virtually totally dependent upon the addition of NAD as a cofactor. Even though NAD was not required for oxidative conversion of all-*trans*-retinal to all-*trans*-RA, participation of NAD greatly facilitated the reaction.

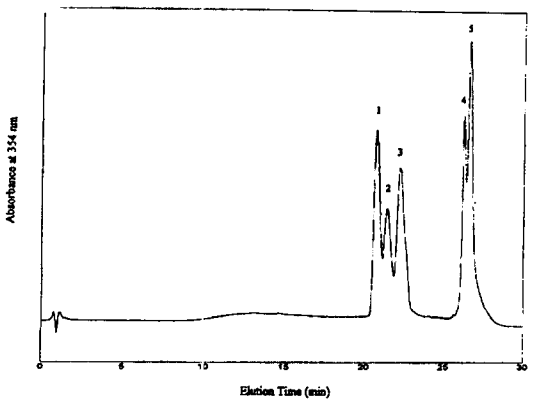

Fig. 1. Typical HPLC chromatogram for separation of standard retinoids: 1, 13-*cis*-RA; 2, 9-*cis*-RA; 3, all-*trans*-RA; 4, all-*trans*-retinol; and 5, all-*trans*-retinal. For experimental details, see Materials and Methods.

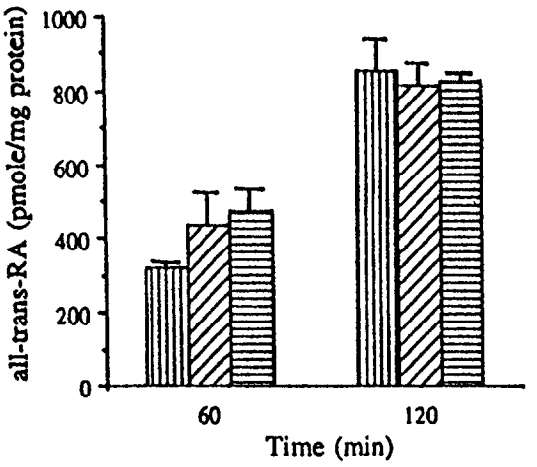

Fig. 2. Catalysis of the biotransformation of all-*trans*-retinol to all-*trans*-RA with rat conceptal homogenates obtained from 10.5 (▥), 11.5 (▨), and 12.5 (▤) gestational days. All-*trans*-retinol (36 μM) was incubated with NAD (4 mM) and with conceptal homogenates at 37° in the dark. Values are means ± SD from three experiments.

Figure 4 presents the effects of variations in substrate concentrations of all-*trans*-retinol and all-*trans*-retinal on conceptal-catalyzed formation of all-*trans*-RA. Saturation was achieved with all-*trans*-retinol concentrations of approximately 9 μM and with all-*trans*-retinal concentrations of 36 μM. At saturating substrate concentrations, the rates of generation of all-*trans*-RA from all-*trans*-retinal were approximately 3- to 4-fold greater than from all-*trans*-retinol.

Figure 5 illustrates the effects of various concentrations of conceptal protein on oxidations of all-*trans*-retinol and all-*trans*-retinal for both reactions. Formation of all-*trans*-RA increased in an approximately linear fashion as concentrations of protein increased from 0.025 to 1.0 mg/mL. Figure 5 once again demonstrated that generation of all-*trans*-RA from all-*trans*-retinal proceeded much more rapidly from all-*trans*-retinol under the same experimental conditions.

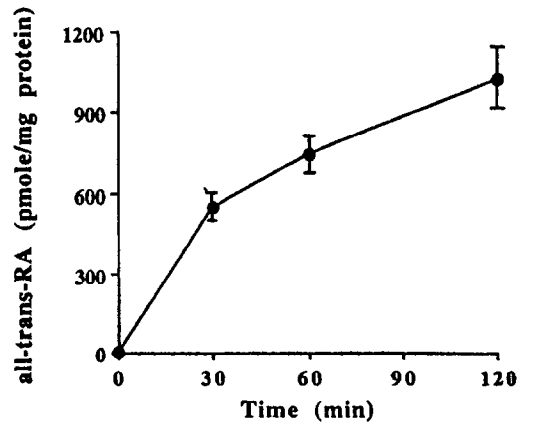

Fig. 3. Catalysis of biotransformation of all-*trans*-retinal to all-*trans*-RA with conceptal homogenates obtained from gestational day 12.5 as the enzyme source. All-*trans*-retinal (36 μM) was incubated with NAD (4 mM) and conceptal homogenates at 37° in the dark. Values are means ± SD from three experiments.

Table 1. Characterization of catalysis of the biotransformation of all-*trans*-retinol and all-*trans*-retinal to all-*trans*-RA by rat conceptal homogenates*

Homogenate	Substrate (36 μM)	Coenzyme (4 mM)	All-*trans*-RA (pmol/mg protein/hr)
Fresh	All-*trans*-retinol	NAD	233 ± 26
Fresh	All-*trans*-retinol	None	ND†
Fresh	All-*trans*-retinal	NAD	763 ± 37
Fresh	All-*trans*-retinal	None	305 ± 63
Boiled	All-*trans*-retinol or all-*trans*-retinal	NAD	ND
None	All-*trans*-retinol or all-*trans*-retinal	NAD	ND
Fresh	None	NAD	ND

* Rat conceptal homogenates of day 12.5 gestation (fresh or boiled at 100° for 5 min) were incubated with substrate and NAD (4 mM) in sodium phosphate buffer (pH 7.5) at 37° in the dark for 2 hr. All-*trans*-RA was quantitatively determined by HPLC. Values are means ± SD from three experiments.

† ND indicates that all-*trans*-RA was not detectable.

Figure 6 presents the effects of substitution of various cofactors for NAD on the generation of all-*trans*-RA from all-*trans*-retinol and from all-*trans*-retinal. Formation of all-*trans*-RA with the participation of NAD as a cofactor in both reactions was used as a control (100%) for comparisons with the participation of other cofactors. Oxidation of all-*trans*-retinol to all-*trans*-RA was clearly NAD/NADP-dependent, and there was no statistically significant difference between participations of NAD vs NADP. When both NAD and NADP were omitted from the reaction vessels, reaction rates were very close to the limits of detection. When NAD or NADP was replaced by NADH or NADPH, respectively, the formation of all-*trans*-RA was reduced markedly. To determine whether the formation of small amounts of all-*trans*-RA from retinol in the reaction with NADPH was due to catalysis by a cytochrome P450-dependent monooxygenase(s), carbon monoxide ($CO:O_2 = 90:10$) was used as a specific inhibitor of cytochrome P450. After incubation at 37° for 2 hr, no statistically significant reduction of all-*trans*-RA was observed. In addition, when NADPH was incubated in buffer for 2 hr with 100 μL of rat conceptal homogenate, the absorption of NADPH at 340 nm was reduced only by approximately 30% (data not shown). Together, these observations strongly suggested that with NADPH as cofactor, the formation of all-*trans*-RA was likely due to a low rate of oxidation of NADPH to NADP, which acted as a highly effective cofactor, and was not due to the participation of cytochrome P450-dependent monooxygenases. For oxidation of all-*trans*-retinal to all-*trans*-RA, NAD appeared to be a more effective cofactor than NADP. In contrast to the oxidation of all-*trans*-retinol, omission of NAD or NADP resulted in only an approximately 55% reduction in all-*trans*-RA formation from all-*trans*-retinal. Interestingly, for all-*trans*-retinal oxidation, replacement of NAD with NADH or NADPH resulted in decreases similar to those observed with omission of NAD or NADP, probably due to reversibility of all-*trans*-retinal oxidation.

Figure 7 shows the effects of various enzyme inhibitors on the conceptal-catalyzed conversion of all-*trans*-retinol to all-*trans*-RA. With up to 10 mM concentrations, neither sodium azide nor 4-MP produced statistically significant inhibitory effects on the oxidation of all-*trans*-retinol to all-*trans*-RA. With the same concen-

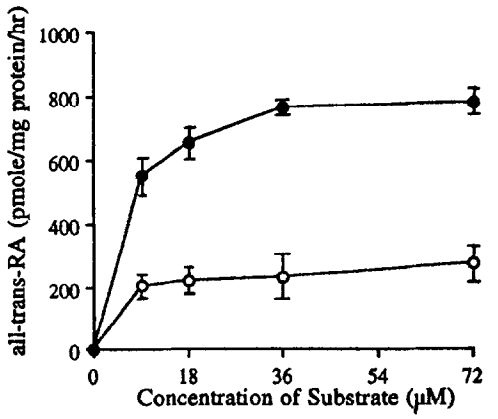

Fig. 4. Effects of various concentrations of all-*trans*-retinol (○) and all-*trans*-retinal (●) on the formation of all-*trans*-RA. Retinol or retinal was incubated with NAD (4 mM) and with conceptal homogenates at 37° in the dark for 2 hr. Concentrations of conceptal proteins in incubations of retinol and retinal were 0.86 and 0.75 mg/mL, respectively. Values are means ± SD from three experiments.

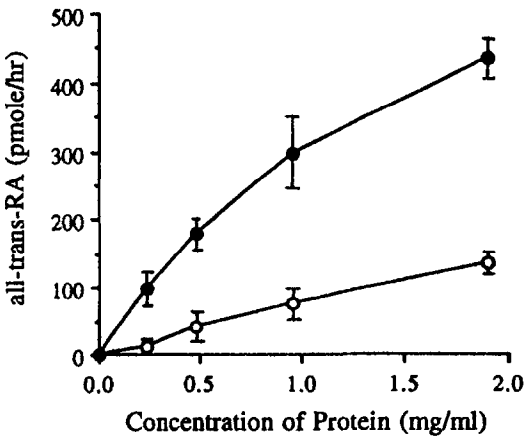

Fig. 5. Effect of various concentrations of conceptal protein on biotransformation of all-*trans*-retinol (○) and all-*trans*-retinal (●) to all-*trans*-RA. Retinol or retinal (18 μM) was incubated with NAD (4 mM) and with conceptal homogenates at 37° in the dark for 2 hr. Values are means ± SD from three experiments.

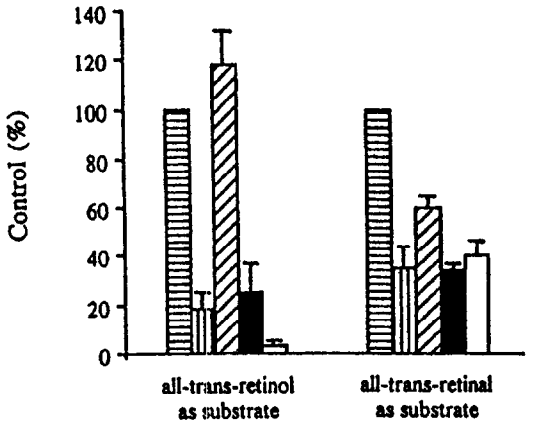

Fig. 6. Effects of coenzymes on formation of all-*trans*-RA from all-*trans*-retinol and all-*trans*-retinal: NAD (▤), NADH (▥), NADP (▨), NADPH (■), and no coenzyme (□). Substrate (36 μM) was incubated with coenzymes (4 mM) and with conceptal homogenates (0.11 mg protein/mL) at 37° for 2 hr in the dark. The effects of NADH, NADP, NADPH, and no addition of coenzyme were compared with the effect of NAD (control). Absolute values for generation of all-*trans*-RA from all-*trans*-retinol (nmol/mg protein) were: control, 0.93 ± 0.1; NADH, 0.20 ± 0.1; NADP, 1.1 ± 0.5; NADPH, 0.3 ± 0.1; and no coenzyme, 0.1 ± 0.01. Absolute values for generation of all-*trans*-RA from all-*trans*-retinal (nmol/mg protein) were: control, 9.6 ± 0.6; NADH, 3.4 ± 0.1; NADP, 5.7 ± 0.6; NADPH, 3.3 ± 0.1; and no coenzyme, 3.9 ± 1.0. Values are means ± SD from three experiments. In the reaction with NADPH, no statistically significant reduction of all-*trans*-RA was observed when carbon monoxide ($CO:O_2 = 90:10$) was used as a specific cytochrome P450 inhibitor.

trations, metyrapone reduced formation of all-*trans*-RA by approximately 35%, whereas citral completely blocked the reaction.

To further investigate the inhibitory effects of metyrapone and citral on the conceptal-catalyzed oxidation of all-*trans*-retinol, much lower concentrations of both enzyme inhibitors were added to reaction vessels. As shown in Table 2, metyrapone did not show statistically significant inhibitory effects at a 1 mM concentration, whereas citral effectively inhibited the reaction at concentrations as low as 10 μM.

Citral and metyrapone were also investigated as inhibitors of the conceptal-catalyzed conversion of all-*trans*-retinal to all-*trans*-RA. As shown in Fig. 8, metyrapone did not inhibit the reaction effectively even at an extremely high concentration (50 mM). On the other hand, additions of citral (0.1 mM) caused approximately 60% reduction of all-*trans*-RA formation. This reduction in reaction rate was roughly equivalent to the reduction observed with omission of cofactors.

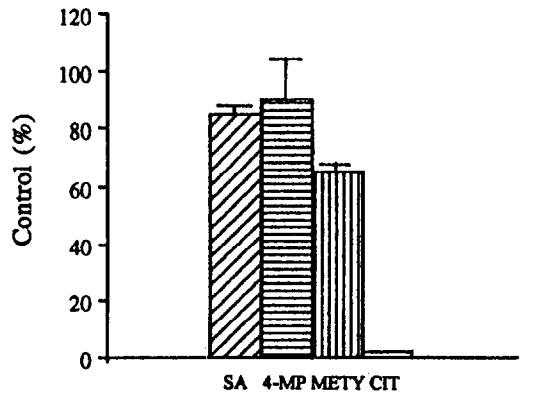

Fig. 7. Effects of enzyme inhibitors on biotransformation of all-*trans*-retinol to all-*trans*-RA: sodium azide (SA), 4-methylpyrazole (4-MP), metyrapone (METY), and citral (CIT). Enzyme inhibitors (10 mM) were preincubated with conceptal homogenates (0.33 mg/protein/mL) for 20 min prior to the addition of retinol. After addition of substrate, incubations continued for 1 hr at 37° in the dark. Absolute values for the generation of all-*trans*-RA (pmol/mg protein) were: control, 571 ± 113; SA, 485 ± 23; 4-MP, 560 ± 65; METY, 374 ± 14; and CIT, not detectable. Values are means ± SD from three experiments.

DISCUSSION

In this study, use of an HPLC assay capable of specifically quantifying all-*trans*-RA at picomolar concentrations, has enabled a demonstration that both all-*trans*-retinol and all-*trans*-retinal were enzymatically converted to all-*trans*-RA in homogenates of rat conceptal tissues at gestational days of 10.5, 11.5, and 12.5. The concept of catalysis of both all-*trans*-retinol and all-*trans*-retinal to all-*trans*-RA by conceptal enzyme(s) was supported by several pieces of experimental evidence. First, the conversion of all-*trans*-retinol to all-*trans*-RA was virtually entirely NAD/NADP dependent, and adding NAD greatly facilitated the conversion of all-*trans*-retinal to all-*trans*-RA. Second, catalyzed conversions of both all-*trans*-retinol and all-*trans*-retinal to all-*trans*-RA were abolished by heat denaturation. Third, rates of conversions of both all-*trans*-retinol and all-*trans*-retinal to all-*trans*-RA increased as concentrations of conceptal protein increased. Finally, rates of conversions of both all-*trans*-retinol and all-*trans*-retinal to all-*trans*-RA exhibited substrate saturation.

In adult tissues, it is generally agreed that the oxidation of all-*trans*-retinol to all-*trans*-retinal is the rate-limiting step in the biosynthesis of all-*trans*-RA from all-*trans*-retinol [11]. In adult rat liver and kidney, for example, the rates of retinoic acid synthesis from retinal are 4- and 7-fold faster, respectively, than from retinol

Table 2. Inhibition by metyrapone and citral of biotransformation of all-*trans*-retinol to all-*trans*-RA catalyzed by rat conceptal homogenates*

Enzyme inhibitor	Concentration (mM)	All-*trans*-RA (pmol/mg protein)	All-*trans*-RA (% of control)
None	0	926 ± 56	100
Metyrapone	5	500 ± 139	54 ± 15
	1	760 ± 287	82 ± 31
Citral	0.05	28 ± 9	3 ± 1
	0.01	111 ± 37	12 ± 4

* All-*trans*-retinol (36 μM), NAD (4 mM), and freshly prepared rat conceptal homogenates were incubated at 37° for 20 min prior to the addition of metyrapone or citral. After addition of metyrapone or citral, incubation continued for 2 hr. Controls were conducted in the same procedure except for the addition of metyrapone or citral. Values are means ± SD from four experiments.

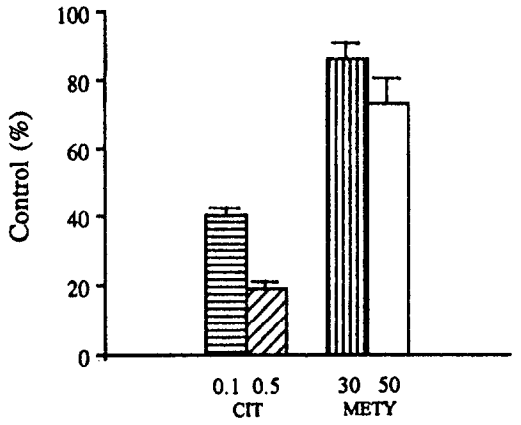

Fig. 8. Effects of citral (CIT) and metyrapone (METY) on biotransformation of all-*trans*-retinal to all-*trans*-RA. Enzyme inhibitors (shown in millimolar concentrations) were preincubated with rat conceptal homogenates (0.33 mg protein/mL) for 20 min prior to the addition of retinal. After addition of substrate, incubations continued for 2 hr at 37° in the dark. Absolute values for the generation of all-*trans*-RA (pmol/mg protein) were: control, 1055 ± 44; CIT (0.1 mM), 417 ± 28; CIT (0.5 mM), 196 ± 16; METY (30 mM), 897 ± 53; and METY (50 mM), 770 ± 106. Values are means ± SD from three experiments.

[10]. Our experimental results with conceptal tissues exhibited similar differences in reaction rates. Conversion of all-*trans*-retinal to all-*trans*-RA was invariably more rapid than that of all-*trans*-retinol to all-*trans*-RA indicating that, in conceptal tissues, oxidation of all-*trans*-retinol to all-*trans*-retinal is the rate-limiting step for the biotransformation of all-*trans*-retinol to all-*trans*-RA. In addition, with all-*trans*-retinol as substrate, no all-*trans*-retinal was detected in the chromatograms, further supporting this concept.

In adult rat liver and kidney, the biosynthesis of retinoic acids from retinol is catalyzed by a cytosolic retinol dehydrogenase that requires NAD, but not NADP as a coenzyme. NADH inhibited that reaction by 70% in liver and by 50% in kidney, probably as the results of enhancement of the conversion of retinal to retinol [10]. It was reported that a microsomal NAD-dependent retinol dehydrogenase, which was distinct from the microsomal cytochrome P450 system and cytosolic NAD-dependent retinol dehydrogenase, is capable of catalyzing the conversion of all-*trans*-retinol to all-*trans*-retinal in rat liver *in vitro* [18]. It was also reported that an NADPH-dependent retinol oxidase catalyzes oxidation of retinol to retinal in rat liver microsomes *in vitro* [13]. Furthermore, rodent class I and II alcohol dehydrogenases can also participate in the oxidation of retinol [12–21]. In rat conceptal homogenates, conversion of all-*trans*-retinol to all-*trans*-RA appeared to be catalyzed by a conceptal retinol dehydrogenase(s) but not by an NADPH-dependent retinol oxidase or by a cytochrome P450-dependent system. The conceptal retinol dehydrogenase(s), unlike that in adult rat liver and kidney, was less cofactor specific since the reaction was well supported by both NAD and NADP. Indeed, an NAD-dependent retinol dehydrogenase from bovine retinol rod outer segments has been purified recently [22].

The coenzyme participation in the formation of all-*trans*-RA from all-*trans*-retinal was more complex than from all-*trans*-retinol. In contrast to observations in experiments assessing the oxidation of all-*trans*-retinol to all-*trans*-RA, replacement of NAD with NADP reduced the formation of all-*trans*-RA from all-*trans*-retinal by approximately 40%. When NAD was not added to the incubation vessels, formation of all-*trans*-RA was reduced by approximately 55%, suggesting that: (1) an NAD-dependent conceptal dehydrogenase(s) played a major role in the reaction; and (2) an enzyme(s) that did not require NAD as a coenzyme also participated in the reaction. In experiments with adult rat liver and kidney, omission of NAD caused a reduction of biosynthesis of RA from retinal by about 25 and 30%, respectively [10]. It appears, therefore, that conversion of all-*trans*-retinal to all-*trans*-RA is regulated by multiple enzymes in both adult and conceptal tissues. Additions of NADH or NADPH caused approximately 60% reduction in all-*trans*-RA formation from all-*trans*-retinal. This was probably due to facilitated reduction of all-*trans*-retinal to all-*trans*-retinol.

Recently a cytosolic retinal oxidase (retinoic acid synthase) has been characterized in rabbit liver [14]. Activity of the retinal oxidase was not affected by any coenzymes such as NAD, NADH, NADP, or NADPH in the oxidation of all-*trans*-retinal to all-*trans*-RA. It was also reported that a xanthine dehydrogenase was able to catalyze oxidation of retinal to retinoic acid [23]. Whether rat conceptal tissues may express a similar retinal oxidase and xanthine dehydrogenase activities is not clear at present. Nevertheless, our experimental data suggested that a similar retinal oxidase could be present in rat conceptal tissues and might contribute to oxidation of all-*trans*-retinal to all-*trans*-RA.

Currently, catalase has been suggested to play a major role in ethanol oxidation *in vivo* in deer mice [24]. Whether catalase plays any role in the oxidation of longer chain alcohols, such as all-*trans*-retinol, appears not to have been reported. In this study, sodium azide was incubated with all-*trans*-retinol, and on significant inhibitory effect on the formation of all-*trans*-RA was found even though sodium azide was present in an extremely high concentration (10 mM). It would seem that catalase does not play a significant role in the conversion of all-*trans*-retinol to all-*trans*-RA in rat conceptal tissues.

Rodent class I and II alcohol dehydrogenases (ADH) are capable of catalyzing the biosynthesis of retinoic acid from retinol [25]. However, 4-MP, the widely used selective inhibitor of ADH, did not show significant inhibitory effects on the oxidation of all-*trans*-retinol to all-*trans*-RA, even when the final concentration of 4-MP was 10 mM. This observation suggested two possibilities. First, the conceptal dehydrogenase(s) that catalyzed the formation of all-*trans*-RA from all-*trans*-retinol differs from the 4-MP-inhibitable (class I) alcohol dehydrogenases, at least in terms of sensitivity to 4-MP. Second, a class II alcohol dehydrogenase may be the primary conceptal dehydrogenase that catalyzes the observed reaction since class II alcohol dehydrogenase is far less sensitive to 4-MP inhibition than class I alcohol dehydrogenase [26].

Studies have shown that cytochrome P450-dependent monooxygenases can also participate in the oxidation of retinoids in adult mammalian tissues. It was reported that a reconstituted cytochrome P450-dependent monooxygenase system catalyzes oxidation of retinol to 4-hy-

droxyretinol in human liver *in vitro* [27]. Whether the same enzyme activity is expressed in rat conceptuses or whether certain conceptal cytochrome P450-dependent monooxygenases may participate in catalysis of the oxidation of all-*trans*-retinol to all-*trans*-RA in conceptal tissues is not clear at present. Experimental evidence obtained from this study, however, indicated that cytochrome P450-dependent monooxygenases were unlikely to play a significant role in the oxidation of all-*trans*-retinol to all-*trans*-RA in rat conceptal tissues. First, the reaction observed appeared to be strictly NAD/NADP dependent, indicating that dehydrogenase(s), rather than monooxygenases, was involved in the reaction. Second, no statistically significant reduction of all-*trans*-RA was observed when carbon monoxide ($CO:O_2$ = 90:10) was used as a specific inhibitor of cytochrome P450-dependent monooxygenases with NADPH as a cofactor. Third, metyrapone inhibited the reaction only at extremely high concentrations (5 mM) and, compared with citral, was a very ineffective inhibitor. Finally, there is no evidence suggesting that constitutive cytochrome P450-dependent monooxygenase activity is expressed in rat conceptuses during organogenesis [28].

Citral was proposed as a vitamin A antagonist [29] and inhibited oxidation of all-*trans*-retinol to all-*trans*-RA in mouse epidermis both *in vitro* and *in vivo* [20] and also inhibited oxidation of all-*trans*-retinol to all-*trans*-retinal in rat liver microsomes *in vitro* [13]. In this study, the rat conceptal dehydrogenase(s) that catalyzed oxidation of all-*trans*-retinol to all-*trans*-RA was highly sensitive to inhibition by citral. At concentrations as low as 10 μM, conversion of all-*trans*-retinol to all-*trans*-RA was blocked almost entirely.

It has been reported that a polycyclic aromatic hydrocarbon-inducible P4501A2 and an antibiotic-inducible P4503A6 catalyze oxidation of all-*trans*-retinal to all-*trans*-RA in rabbit liver microsomes *in vitro* [15]. In our studies, the possible participation of cytochrome P450-dependent monooxygenases in the oxidation of all-*trans*-retinal to all-*trans*-RA was investigated. Even at an extremely high concentration (50 mM), metyrapone did not inhibit conversion of all-*trans*-retinal to all-*trans*-RA effectively, suggesting that cytochrome P450 was unlikely to be significantly involved. In addition, the fact that NADPH did not promote oxidation of all-*trans*-retinal to all-*trans*-RA also suggested that the reaction was not catalyzed by a cytochrome P450-dependent monooxygenase. Total resolution of this issue will require further study.

In contrast to results obtained with metyrapone, formation of all-*trans*-RA from all-*trans*-retinal was inhibited markedly by citral. For instance, addition of citral (10 μM) reduced the formation of all-*trans*-RA from all-*trans*-retinol by more than 90%. Ten-fold higher concentrations of citral (100 μM) reduced the formation of all-*trans*-RA from all-*trans*-retinal by only approximately 60% but by the same magnitude as omission of NAD, suggesting that citral may be less effective in inhibition of oxidation of all-*trans*-retinal through a non-dehydrogenation pathway(s).

In summary, we have demonstrated with rat conceptal tissues as enzyme source that all-*trans*-retinol and all-*trans*-retinal can be converted to all-*trans*-RA *in vitro*. Both reactions were catalyzed by conceptal enzymes. Oxidation of all-*trans*-retinol appeared to be catalyzed solely by an NAD/NADP-dependent dehydrogenase(s) that was sensitive to inhibition by low concentrations of citral. An NAD-dependent dehydrogenase(s) and an oxidase(s) that did not require NAD, NADH, NADP or NADPH appeared to be the primary enzymes catalyzing oxidation of all-*trans*-retinal to all-*trans*-RA. Catalase and cytochrome P450 appeared unlikely to play significant roles in the catalysis of conceptal biotransformation of all-*trans*-retinol to all-*trans*-RA.

Acknowledgements—We thank Dr. J. Creech Kraft and Trina Irico for their assistance in this study. We also thank Drs. A. Levin and P. Sorter from Hoffmann-La Roche for their contribution of the retinoids. This work was supported by NIEHS grants (ES-04041 and ES-05861), and H.C. was supported by an NIEHS training grant (ES-07032).

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