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Biotransformation of all-*trans*-Retinal, 13-*cis*-Retinal, and 9-*cis*-Retinal Catalyzed by Conceptal Cytosol and Microsomes

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ABSTRACT. Oxidative conversions of all-*trans*-retinal (t-RAL), 13-*cis*-retinal (13-cRAL), and 9-*cis*-retinal (9-cRAL) to their corresponding retinoic acids (RAs) catalyzed by rat conceptal cytosol (RCC) or microsomes (RCM) were studied. The primary product of RCC-catalyzed oxidations of both t-RAL and 13-cRAL was t-RA, with only trace amounts of 13-cRA and 9-cRA. In the RCC-catalyzed oxidation of 9-cRAL, generated t-RA, 9-cRA, and 13-cRA constituted approximately 56, 34, and 10%, respectively, of the total RAs. For all RCC-catalyzed retinal oxidations, NAD was a much more effective cofactor than NADP. And t-RAL and 13-cRAL were much better substrates than 9-cRAL. Formaldehyde, acetaldehyde, citral, and disulfiram were investigated as inhibitors, but only citral and disulfiram effectively inhibited the RCC-catalyzed conversion of t-RAL or 13-cRAL to t-RA. Methanol and ethanol failed to inhibit either reaction even at very high concentrations ($\geq$10 mM). RCM exhibited lower specific enzymatic activities than RCC in catalyzing oxidations of t-RAL, 13-cRAL, and 9-cRAL, indicating that the cytosolic fraction was dominant for converting retinals to RAs. The predominant RA produced from RCM-catalyzed oxidations of t-RAL, 13-cRAL, or 9-cRAL was t-RA for each substrate, and again NAD was a much more effective cofactor than NADP in all cases. For RCM-catalyzed oxidations of RALs, 13-cRAL was a much better substrate than t-RAL or 9-cRAL. Methanol and ethanol were not effective inhibitors for RCM-catalyzed oxidations of t-RAL or 13-cRAL. In RCM-catalyzed reactions, citral (10 mM) completely inhibited oxidation of t-RAL but showed only a minor effect on oxidation of 13-cRAL. 13-cRA was converted almost completely to t-RA after 2 hr of incubation with RCC. BIOCHEM PHARMACOL 53;6:877–885, 1997. © 1997 Elsevier Science Inc.

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The retinoids comprise a very large family of compounds that includes both natural and synthetic derivatives of vitamins A_1 (t-ROH†) and A_2 (all-*trans*-3,4-didehydroretinol) and their respective esters [1]. Retinoids have received extraordinary attention in terms of their capacities to combat a variety of pathologic skin conditions and to act as cancer chemopreventive agents. A major problem in the development of clinically useful retinoids, however, has been the capacity of many retinoids to elicit profound embryotoxic and dysmorphogenic effects [2, 3]. Current research suggests that endogenous RAs, such as t-RA and 9-cRA, are highly embryotoxic and act as proximate/ultimate teratogens in experimental animals such as rodents [4–7]. It is well documented that 13-cRA is a potent human teratogen, and both t-RA and 9-cRA (>10 μM) can produce severe malformations in cultured rat embryos during organogenesis (gestational days 9.5–11.5) [8–12].

Increasing evidence suggests that most, if not all, of retinoid-elicited embryotoxic and dysmorphogenic effects are produced via receptor-mediated activation or repression of genes [2, 3]. Currently, at least three RARs (RAR-α, RAR-β, and RAR-γ) and three RXRs (RXR-α, RXR-β, and RAR-γ) are known to regulate a variety of genes at the transcriptional level after they are activated by specific ligands. t-RA, all-*trans*-3,4-didehydro-RA, 9-*cis*-3,4-didehydro-RA, and all-*trans*-4-oxo-RA are reported to bind RARs with high affinity, whereas 9-cRA binds both RXRs and RARs with high affinity [13–17]. The nature of ligand-dependence of nuclear retinoid receptors strongly suggests that regulation of endogenous levels of RAs can be crucial for either preventing or inducing retinoid-related embryotoxicity. Clearly, biosynthesis of RAs from their precursors

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† *Abbreviations:* t-ROH, all-*trans*-retinol; RA, retinoic acid; t-RA, all-*trans*-retinoic acid; 13-cRA, 13-*cis*-retinoic acid; 9-cRA, 9-*cis*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RCC, rat conceptal cytosol; RCM, rat conceptal microsomes; RHC, rat hepatic cytosol; RHM, rat hepatic microsomes; t-RAL, all-*trans*-retinal; 13-cRAL, 13-*cis*-retinal; 9-cRAL, 9-*cis*-retinal; and ALDH, aldehyde dehydrogenase.

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is an important regulatory process for the homeostasis of endogenous retinoid receptor ligands.

In recent studies, we have demonstrated that embryonic biotransformation of t-ROH to t-RA involves two consecutive enzymatic reactions similar to those in adult hepatic tissues [18]. In the first reaction, t-ROH is oxidized to t-RAL, and an NAD/NADP-dependent retinol dehydrogenase catalyzes the reaction. In the second reaction, formed t-RAL is further oxidized to t-RA, and an NAD-dependent retinal dehydrogenase catalyzes that reaction. The oxidation of t-RAL to t-RA appears not to be the rate-limiting reaction under many ordinary conditions but can, of course, be rate limiting under certain conditions. Detailed investigations of this reaction will provide important new insights into the biosynthesis of RAs in embryos. First, t-RAL is an important intermediate in biosynthesis of retinoids. After generation from the oxidative cleavage of carotenoids such as β-carotene, t-RAL can either be oxidized to RAs or reduced to retinol, depending on the conditions [19]. Thus, biotransformation of RALs to RAs plays a role in the homeostasis of endogenous levels of retinoid receptor ligands. Second, it has been demonstrated that retinals can undergo rapid isomerizations in ocular tissues and then execute crucial physiological functions in the visual cycle [20, 21]. Similar isomerizations may also occur in embryonic tissues during organogenesis, and these reactions could be important in directing the formation of specific retinoid receptor ligands such as t-RA and 9-cRA. Third, studies have shown that citral (3,7-dimethyl-2,6-octadienal) can effectively inhibit biotransformation of t-retinal to t-RA [18, 22]. It is important to examine whether other ALDH inhibitors can also inhibit the reaction because some of the inhibitors, such as disulfiram, have clinical applications, and the usage of these inhibitors may complicate the homeostasis of endogenous retinoids.

In this study, we have investigated biotransformation of t-RAL, 13-cRAL, and 9-cRAL to their corresponding RAs as catalyzed by RCC and RCM. The objectives of the study were: (1) to examine the activities of retinal dehydrogenase(s) in different subcellular fractions (RCC vs RCM) of rat conceptuses during organogenesis; (2) to characterize the embryonic retinal dehydrogenase(s) with respect to various substrates and cofactors (e.g. NAD vs NADP) as well as to various inhibitors of ALDHs; and (3) to investigate any possible embryonic tissue-dependent interconversions among formed RAs.

MATERIALS AND METHODS

Materials

All retinoids used, except 9-cRA, were purchased from the Sigma Chemical Co. (St. Louis, MO). 9-cRA was a gift from Dr. Sorter of Hoffmann-La Roche Inc. (Nutley, NJ). NAD, NADP, citral, and disulfiram were purchased from Sigma. All other chemicals and reagents utilized were of the highest purity commercially available.

Preparation of Cytosol and Microsomes

Time-mated pregnant rats (Sprague–Dawley, Wistar-derived) were obtained from Tyler Laboratories (Bellevue, WA) on day 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 embryos proper plus the amniotic membranes and visceral yolk sacs, were removed from ether-anesthetized dams and washed with Hanks' balanced salt solution (pH 7.5). The whole conceptuses were frozen immediately 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 first centrifuged at 700 *g* for 10 min to remove large particulates. The resultant supernatant was further centrifuged at 105,000 *g* at 4° for 1.5 hr, and the supernatant was defined as RCC. The resultant particulates were washed and suspended in the same buffer. The washed particulates were recentrifuged at 105,000 *g* at 4° for another 1.5 hr. The resultant particulates were washed twice, suspended in buffer, and defined as RCM. Preparations of adult RHC and RHM followed the same procedures as described for the preparations of RCC and RCM except that freshly prepared adult rat liver homogenates were used as starting materials.

Oxidation of Retinals to RAs

Freshly prepared RCC, RCM, RHC or RHM 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. t-RAL, 13-cRAL or 9-cRAL in acetone (1 mg/mL) was added to the test tube, and the final volume was brought to 1 mL with buffer. Final concentrations (18 μM) of retinoid substrates appeared to saturate the enzyme(s) responsible for catalysis of conversion, thus permitting estimates of levels of expression of enzyme in conceptal tissues. This also permitted accurate estimates of quantities of metabolites generated. Additions of retinals were completed in a darkened room with yellow lights to prevent photoisomerization. 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 stored in a freezer (−20°) for subsequent HPLC analyses.

Inhibition of Oxidation of Retinal to RAs with Enzyme Inhibitors

The experimental procedure was the same as described above except that RCC or RCM were preincubated with enzyme inhibitors for 20 min prior to the addition of substrate. The enzyme inhibitors used in these experiments

were citral, disulfiram, formaldehyde, acetaldehyde, methanol, and ethanol.

Conversion of 13-cRA to t-RA in the Presence of RCC

The experimental procedures were the same as those described above for oxidation of retinal catalyzed by RCC except that 13-cRA was used as substrate.

Identification and Quantitation 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. Identification and quantitation of retinoids for conversions of retinals to RAs catalyzed by RCC or RCM were conducted with a Zorbax octadecylsilane (ODS) column (0.46 × 15 cm) (MacMod Anal. Inc., Chadds Ford, PA) following the procedures described by Kim *et al.* [23] with slight modifications. The analytical eluents consisted of solvent A (acetonitrile: H_2O:acetic acid, 49.75:49.75:0.5, by vol.) and solvent B (acetonitrile:H_2O:acetic acid, 90:10:0.04, by vol.), both containing 10 mM ammonium acetate. The HPLC conditions were as follows: 80% solvent A plus 20% solvent B with a flow rate of 1.5 mL/min for 15 min. A mixture of authentic retinoids was analyzed with the same procedure. 13-cRA, 9-cRA, and t-RA were mixed with t-ROH and t-RAL and then were diluted with distilled water before the HPLC injection. One hundred microliters of a mixture of standard retinoids or supernatant fraction of incubation mixture was 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. The detection limit of the HPLC system for concentrations of RAs was 1 ng/100 μL. Concentrations of RAs below 1 ng/100 μL were designated as not detectable.

Protein Determination

The method of Lowry *et al.* [24] was used to quantitatively determine the concentrations of protein in RCC, RCM, RHC, and RHM. Bovine serum albumin was used as a standard protein for the quantitation.

Statistical Analysis

All experimental data are expressed as the means of three to five experimental measurements with standard deviations. The *t*-tests were conducted with a Microexcell statistics package (Microsoft, Redmond, WA) to test for the statistical significance of differences between mean values.

RESULTS

Table 1 shows RCC-catalyzed biotransformation of t-RAL, 13-cRAL, or 9-cRAL in the presence of either NAD or NADP added to the reaction vessels. In all reactions, t-RA was the predominant RA generated. In addition, NAD appeared to be a much more effective cofactor than NADP in catalysis of the bioconversions of retinals to RAs. For the conceptal retinal dehydrogenase(s), t-RAL was the substrate exhibiting the most rapid conversion, while 9-cRAL was the substrate exhibiting the slowest conversion. Interestingly, as 13-cRAL was oxidized, neither 13-cRA nor 9-cRA was detected.

RCM-catalyzed conversions of t-RAL, 13-cRAL, or 9-cRAL are shown in Table 2. Similarly, t-RA was the predominant RA formed, and NAD was a better cofactor in all reactions. Conversely, 13-cRAL appeared to be the best substrate, while t-RAL and 9-cRAL were poor substrates for RCM dehydrogenase(s). RCM exhibited 10- and 3-fold lower specific enzymatic activities for t-RAL and 13-cRAL oxidation than those assessed in RCC. RCM preparations exhibited slightly lower specific activities for 9-cRAL than did RCC preparations.

Table 3 presents data for the RHC- or RHM-catalyzed biotransformation of t-RAL, 13-cRAL, or 9-cRAL in the presence of added NAD. Compared with RCC- and RCM-catalyzed reactions, RHC and RHM fractions expressed 2- to 10-fold higher retinal dehydrogenase activities. Similar to conceptal biotransformation of retinals to RAs, t-RA was the predominant isomer generated by adult tissues except for oxidation of 9-cRAL. RHM appeared to lack dehydrogenases capable of catalyzing the conversion of 9-cRAL to RAs.

A variety of ALDH inhibitors were investigated for their potential capacity to inhibit RCC-catalyzed biotransformation of t-RAL and 13-cRAL to t-RA. As shown in Fig. 1,

TABLE 1. Biotransformation of all-*trans*-retinal, 13-*cis*-retinal or 9-*cis*-retinal catalyzed by rat conceptal cytosol (RCC)

Substrate (18 μM)	Coenzyme (4 mM)	Retinoic acids (pmol/mg protein)		
		13-cRA	9-cRA	t-RA
t-Retinal	NAD	342 ± 35	234 ± 24	4450 ± 150
t-Retinal	NADP	ND*	ND	231 ± 79
13-*cis*-Retinal	NAD	ND	ND	3117 ± 170
13-*cis*-Retinal	NADP	ND	ND	1060 ± 43
9-*cis*-Retinal	NAD	101 ± 35	349 ± 89	589 ± 5
9-*cis*-Retinal	NADP	ND	ND	198 ± 52

RCC (0.030 mg protein) was preincubated with sodium phosphate buffer (0.1 M, pH 7.5) containing NAD or NADP (4 mM) and NaCl (40 mM) for 20 min at 37°. Reactions were then initiated by adding substrate (final concentration 18 μM) and continued for 2 hr. Incubations without additions of substrate, cytosol, or cofactors served as controls, and the values obtained were subtracted from those of test samples. Values are expressed as means of four experimental measurements with standard deviations. For further details, see Materials and Methods.

* ND indicates that values were under the detection limit (1 ng/100 μL) of the UV detector at 354 nm.

TABLE 2. Biotransformation of all-*trans*-retinal, 13-*cis*-retinal or 9-*cis*-retinal catalyzed by rat conceptal microsomes (RCM)

Substrate (18 μM)	Coenzyme (4 mM)	Retinoic acids (pmol/mg protein)		
		13-cRA	9-cRA	t-RA
t-Retinal	NAD	ND*	ND	389 ± 27
t-Retinal	NADP	ND	ND	96 ± 63
13-*cis*-Retinal	NAD	ND	ND	1621 ± 27
13-*cis*-Retinal	NADP	ND	ND	435 ± 10
9-*cis*-Retinal	NAD	ND	124 ± 6	442 ± 9
9-*cis*-Retinal	NADP	ND	ND	ND

RCM (0.032 mg protein) was preincubated with sodium phosphate buffer (0.1 M, pH 7.5) containing NAD or NADP (4 mM) and NaCl (40 mM) for 20 min at 37°. Reactions were then initiated by adding substrate (final concentration 18 μM) and continued for 2 hr. Incubations without additions of substrates, cytosol, or cofactors served as controls, and the values obtained were subtracted from those of test samples. Values are expressed as means of four experimental measurements with standard deviations. For further details, see Materials and Methods.

* ND indicates that values were under the detection limit (1 ng/100 μL) of the UV detector at 354 nm.

methanol and ethanol showed no inhibitory effects on either reaction even at a very high concentration (10 mM). Interestingly, formaldehyde and acetaldehyde (substrates for ALDHs) also did not inhibit either reaction at a relatively high concentration (0.1 mM). Addition of disulfiram (a well-known inhibitor of acetaldehyde dehydrogenase), on the other hand, reduced generation of t-RA by approximately 40% at 10 μM concentrations. At 100 μM concentrations, disulfiram completely inhibited the biotransformation of t-RAL to t-RA and reduced the conversion of 13-cRAL to t-RA by approximately 80%. Methanol and ethanol, precursors of formaldehyde and acetaldehyde, did not inhibit either reaction.

The inhibitory effects of citral on RCC biotransformation of t-RAL or 13-cRAL to t-RA were investigated. As exhibited in Fig. 2, citral reduced the generation of t-RA from t-RAL and 13-cRAL by approximately 90 and 65%, respectively, at concentrations as low as 10 μM. At the same concentration (10 μM), citral was more effective than disulfiram in inhibiting the biotransformation of either t-RAL or 13-cRAL to t-RA.

The effects of citral on RCM-catalyzed biotransformation of t-RAL and 13-cRAL to t-RA also were investigated. As shown in Fig. 3, citral inhibited both reactions but only at a high concentration (10 mM). Similar to the inhibition by citral of RCC-catalyzed biotransformations, RCM-catalyzed oxidation of t-RAL appeared to be more sensitive to citral than the oxidation of 13-cRAL.

Effects of ethanol on the biotransformation of 13-cRAL to t-RA as catalyzed by RCC or RCM are shown in Fig. 4. Within a wide range of concentrations of ethanol, no significant inhibition of generation of t-RA was observed for either reaction. On the contrary, increases in t-RA formation were observed. These increases were both consistent and statistically significant.

Generation of t-RA from RCC-catalyzed oxidation of 13-cRAL was verified by comparing the elution times of putative retinoids with those of authentic retinoid standards. Figure 5A shows the separation of RAs, t-retinol, and t-RAL under the HPLC conditions utilized. Figure 5B is a typical HPLC chromatogram after 2 hr of incubation of 13-cRAL. The retinoids produced comigrated with an authentic t-RA standard and exhibited an elution time of 11.5 min. After addition of an authentic 13-cRA standard to the incubation mixture, 13-cRA migrated with an elution time of 9.5 min and was well separated from the RA peak (Fig. 5C). Based on these observations, we concluded that t-RA was the predominant product of oxidation of 13-cRAL catalyzed by RCC.

To examine the possible mechanisms pertaining to the interconversion between 13-cRA and t-RA in rat embryonic tissues, 13-cRA was incubated with RCC in the dark and the profile of the reaction is shown in Fig. 6. 13-cRA was converted rapidly to t-RA in the presence of RCC. As indicated in Fig. 6, approximately 80% of 13-cRA was converted to t-RA after a 30-min incubation.

DISCUSSION

In this study, we demonstrated that subcellular fractions of whole rat conceptal tissue homogenates (RCC and RCM) possessed catalytic activities for the oxidative conversions of retinals to RAs during organogenesis. Combining these observations with earlier reports that t-ROH was the predominant endogenous retinoid in embryonic tissues [9], our findings strongly suggest that biotransformation of retinoids to RAs catalyzed by conceptal enzymes can play an important role in the regulation of endogenous levels of retinoid receptor ligands. That is, the levels of ligands in embryonic tissues can be regulated by both maternal circulation (ligand levels in plasma) and by *localized* biotransformation.

In a rat conceptal homogenate system, conversion of t-RAL to t-RA appeared to be catalyzed primarily by an NAD-dependent retinal dehydrogenase(s) [18]. This is in harmony with previous observations in studies with adult mammalian tissues [25–30]. For both RCC and RCM, an NAD-dependent ALDH(s) was evidently the primary enzyme(s) in catalysis of oxidative conversions of t-RAL, 13-cRAL, and 9-cRAL, and RCC appeared to be the major site of generation of corresponding RAs. Both conceptal cytosolic and microsomal retinal dehydrogenase(s) appeared to be somewhat substrate specific. For example, the RCC retinal dehydrogenase(s) exhibited the highest catalytic efficiency for t-RAL and the lowest efficiency for 9-cRAL. On the other hand, the RCM retinal dehydrogenase(s) exhibited the highest catalytic efficiency for 13-cRAL and about the same efficiencies for t-RAL and 9-cRAL. These observations indicated substrate preferences for biotransformations of retinals to RAs in embryonic tissue during organogenesis.

Various enzyme inhibitors were applied to further characterize the conceptal retinal dehydrogenase activities.

TABLE 3. Biotransformation of all-*trans*-retinal, 13-*cis*-retinal or 9-*cis*-retinal catalyzed by adult rat hepatic cytosol (RHC) or microsomes (RHM)

Substrate (18 μM)	Coenzyme (4 mM)	Enzyme source	Retinoic acids (pmol/mg protein)		
			13-cRA	9-cRA	t-RA
t-Retinal	NAD	Cytosol	ND*	ND	8155 ± 205
t-Retinal	NAD	Microsomes	ND	ND	1042 ± 29
13-*cis*-Retinal	NAD	Cytosol	154 ± 9	ND	6907 ± 288
13-*cis*-Retinal	NAD	Microsomes	ND	ND	814 ± 66
9-*cis*-Retinal	NAD	Cytosol	ND	2804 ± 529	ND
9-*cis*-Retinal	NAD	Microsomes	ND	ND	ND

RHC (0.032 mg protein) or RHM (0.036 mg protein) were preincubated with sodium phosphate buffer (0.1 M, pH 7.5) containing NAD (4 mM) and NaCl (40 mM) for 20 min at 37°. Reactions were then initiated by adding substrate (final concentration 18 μM) and continued for 2 hr. Incubations without additions of substrate, cytosol, or cofactors served as controls, and the values obtained were subtracted from those of test samples. Values are expressed as means of four experimental measurements with standard deviations. For further details, see Materials and Methods.

* ND indicates that values were under the detection limit (1 ng/100 μL) of the UV detector at 354 nm.

Chemically, t-RAL and its stereoisomers are a group of long-chain hydrophobic aldehydes, and the conceptal retinal dehydrogenase(s) is thus expected to share some common characteristics with classical ALDHs. Indeed, disulfiram, a well-known inhibitor of acetaldehyde dehydrogenase, effectively inhibited the oxidative conversions of t-RAL and 13-cRAL. On the other hand, conceptal retinal dehydrogenase(s) appeared to be functionally different from formaldehyde and acetaldehyde dehydrogenases since the additions of formaldehyde and acetaldehyde showed no competitive inhibitory effects for the same reactions. These observations suggested that retinals probably have higher affinities than the short-chain aldehydes for the conceptal NAD-dependent retinal dehydrogenase(s). Citral, a long-chain aldehyde (relative to formaldehyde and acetaldehyde), was reported as an effective blocking agent for the biosynthesis of retinoic acids both *in vitro* and *in vivo* [18, 22]. In this study, citral was more effective than disulfiram in inhibiting oxidations of t-RAL and 13-cRAL. For example, in RCC, additions of citral (10 μM) reduced the generation of RA by approximately 90 and 65% in oxidative conversions of t-RAL and 13-cRAL, respectively. At the same concentration, additions of disulfiram reduced the genera-

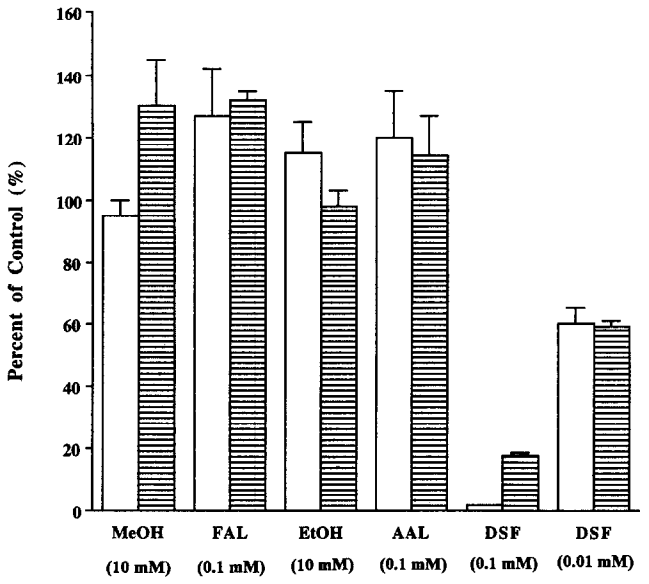

FIG. 1. Effects of enzyme inhibitors on oxidative conversions of t-RAL (□) or 13-cRAL (▤) (18 μM) to t-RA catalyzed by RCC (0.026 mg protein): methanol (MeOH), formaldehyde (FAL), ethanol (EtOH), acetaldehyde (AAL), and disulfiram (DSF). Incubations were conducted at 37° for 2 hr in the presence of NAD (4 mM) in the dark. Values are means ± SD, N = 3–5. Absolute (pmol/mg protein) for controls were 3917 ± 92 (generation of t-RA from t-RAL) and 3371 ± 15 (generation of t-RA from 13-cRAL).

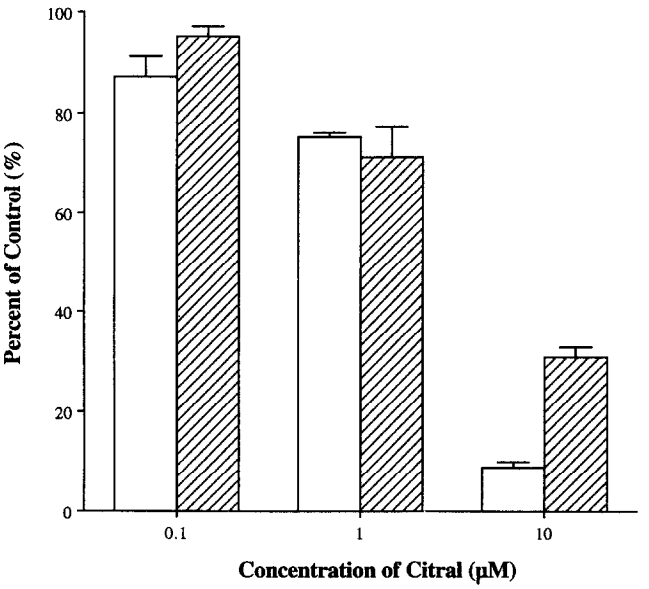

FIG. 2. Effects of various concentrations of citral on oxidative conversion of t-RAL (□) or 13-cRAL (▨) (18 μM) to t-RA catalyzed by RCC (0.026 mg protein). Incubations were conducted at 37° for 2 hr in the presence of NAD (4 mM) in the dark. Values are means ± SD, N = 3–5. Absolute values (pmol/mg protein) for controls were 3917 ± 92 (generation of t-RA from t-RAL) and 3371 ± 15 (generation of t-RA from 13-cRAL).

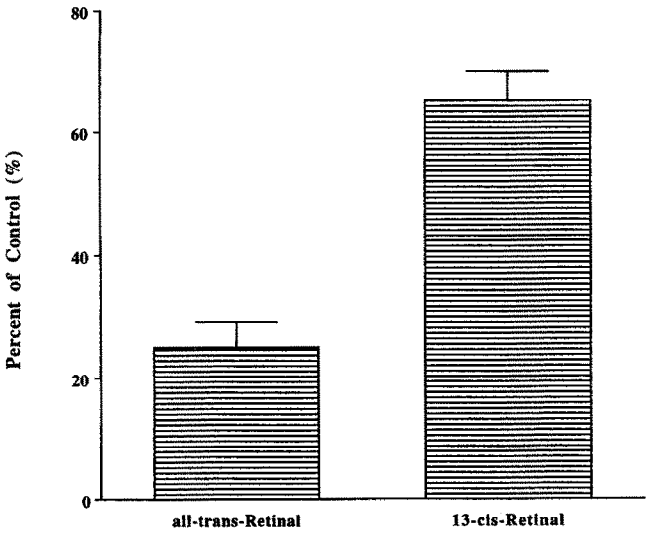

FIG. 3. Effects of citral (10 mM) on oxidative conversions of t-RAL or 13-cRAL (36 μM) to t-RA catalyzed by RCM (0.02 mg protein). Incubations were conducted at 37° for 2 hr in the presence of NAD (4 mM) in the dark. Values are means ± SD, N = 3–5. Absolute values (pmol/mg protein) for controls were 535 ± 64 (generation of t-RA from t-RAL) and 917 ± 115 (generation of t-RA from 13-cRAL).

tion of retinoic acid by approximately 40% in both reactions. RCM-catalyzed retinal oxidation appeared to be less sensitive to citral than the reactions catalyzed by RCC, suggesting that the RCM retinal dehydrogenase(s) may be structurally and enzymatically different from the RCC retinal dehydrogenase(s), at least as manifested in terms of affinity to citral. It is also interesting to observe that citral was more effective in blocking oxidation of the all-*trans* isomer than oxidation of the 13-*cis* isomer in both RCC- and RCM-catalyzed biotransformation of retinals to RAs. When considered together with the fact that RCC showed different catalytic efficiencies for t-RAL and 13-cRAL, this observation suggested that conversions of t-RAL and 13-cRAL may be catalyzed by a different conceptal dehydrogenase(s).

Ethanol is a highly important human teratogen in western countries, and mechanisms pertaining to ethanol-elicited embryotoxicity and dysmorphogenic effects have been explored widely. The possible inhibition by ethanol of the biosynthesis of RAs has been suggested as one of the causes of human fetal alcohol syndrome (FAS) [31, 32]. Even though ethanol can inhibit the biosynthesis of RAs in adult hepatic, testicular, and ocular tissues [33, 34], whether similar inhibition by ethanol occurs in embryonic tissues during organogenesis has only been investigated recently. We recently investigated the possible inhibition by ethanol of biotransformations of t-ROH and t-RAL to t-RA using RCC as an enzyme source *in vitro*. Within a wide range of concentrations, no inhibition by ethanol of either reaction was observed [35]. In the present study, effects of ethanol on oxidations of t-RAL and 13-cRAL catalyzed by RCC and RCM also were investigated. Again, we did not observe inhibition by ethanol of biotransformation of retinals to RAs over a wide range and high level of concentrations. Methanol, another short-chain alcohol and environmental hazardous chemical, likewise did not produce inhibition of either reaction. Instead, increased generation of RAs was observed after retinals were incubated with ethanol or methanol. One possible explanation for the ethanol/methanol-induced increases in retinal biotransformation is that additions of ethanol or methanol may have changed significantly the physical characteristics of the solvent system comprising the reaction mixture. The altered system may provide a better solvent for the hydrophobic substrate, resulting in greater accessibility of substrate to the active site(s) of the enzyme. This idea represents a speculation at this point. Nevertheless, this study indicated that ethanol, at very high concentrations, failed to inhibit biotransformation of retinals to RAs and further suggested that ethanol is not a factor causing inhibition of biosynthesis of RA, at least in terms of the oxidations of t-RAL and its isomers.

One interesting finding was that RCC exhibited the capacity to rapidly convert 13-cRA to t-RA. An investigation of isomerization of 13-cRA to t-RA was thus triggered by the discovery and that the predominant product of 13-cRAL oxidation was t-RA rather than 13-cRA. This unexpected observation immediately suggested that an enzymatic or nonenzymatic mechanism(s) (or both) exists either for conversion of 13-cRAL to t-RAL before the retinal oxidation or for conversion of 13-cRA to t-RA after the retinal oxidation or both. Isomerization of 13-cRAL to t-RAL was not investigated with the existing HPLC system

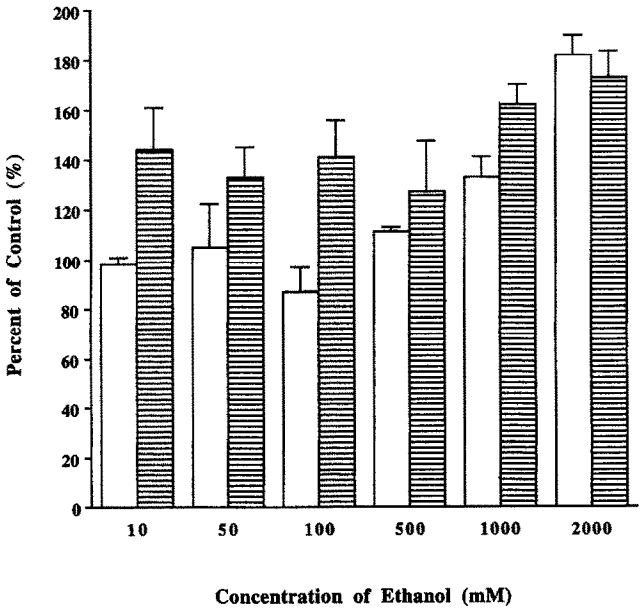

FIG. 4. Effects of various concentrations of ethanol on oxidative conversion of 13-cRAL (18 μM) to t-RA catalyzed by RCC (□) (0.026 mg protein) or RCM (▤) (0.02 mg protein). Incubations were conducted at 37° for 2 hr in the presence of NAD (4 mM) in the dark. Values are means ± SD, N = 3–5. Absolute values (pmol/mg protein) for controls were 4383 ± 600 (RCC-catalyzed reaction) and 740 ± 89 (RCM-catalyzed reaction).

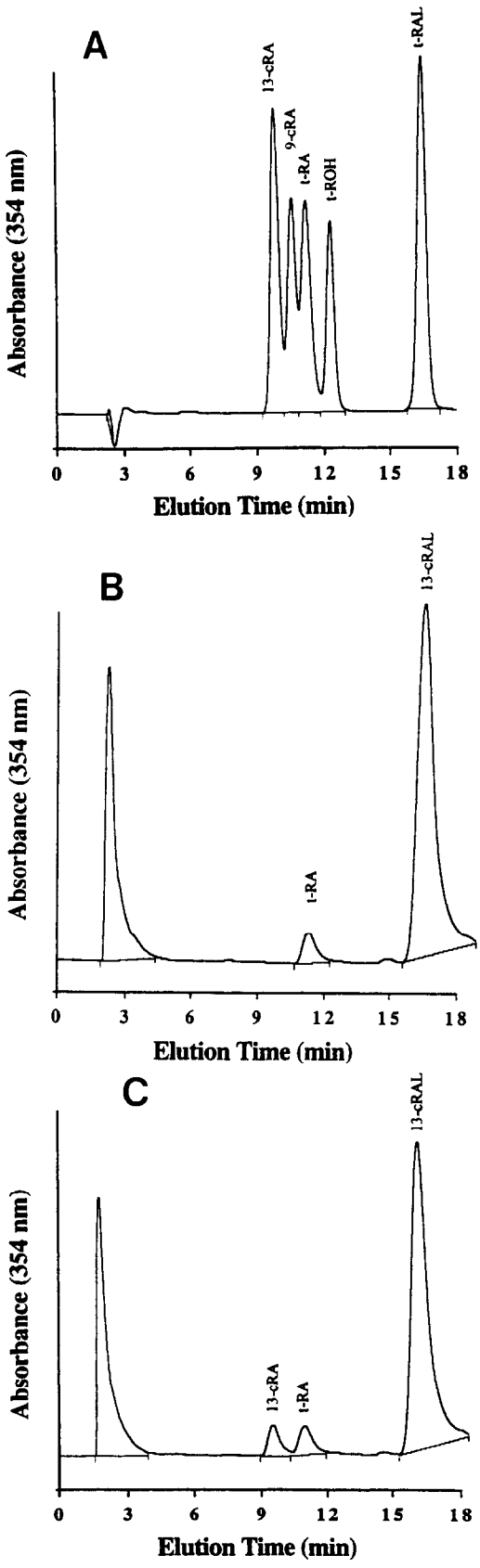

FIG. 5. Identification and verification of generation of t-RA from oxidative conversion of 13-cRAL catalyzed by RCC (0.026 mg protein) by HPLC. (A) standard retinoids; (B) mixture of incubation of 13-cRAL with RCC after 2 hr; and (C) addition of internal standard 13-cRA to the mixture in B. For further experimental details, see Materials and Methods.

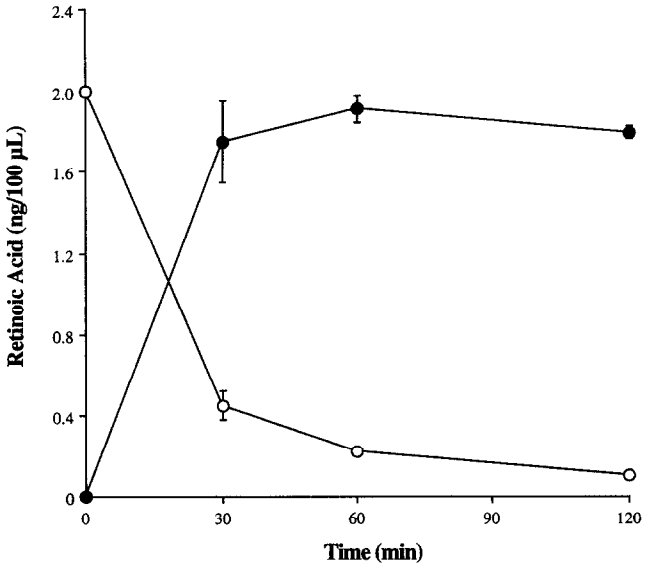

FIG. 6. Interconversion of 13-cRA (0.36 µM) to t-RA in the presence of RCC (0.053 mg protein). Key: (○) 13-cRA; and (●) t-RA. Incubations were conducted at 37° in the presence of NAD (4 mM) in the dark. No t-RA was detected in mixtures of incubations without conceptal cytosol. Values are means ± SD, N = 3–5.

because 13-cRAL was not separated from t-RAL. t-RA, however, was readily separated from 13-cRA by the HPLC system utilized. The results of the study showed that RCC, during organogenesis, can rapidly convert 13-cRA to t-RA.

Early studies indicated that all-*trans*-retinoyl β-glucuronide was a major metabolite when 13-cRA was administered to rats *in vivo* [36, 37]. Based on their observations, McCormick *et al.* [36] suggested that isomerization of 13-cRA to t-RA may (but not necessarily) occur before the conjugation reaction. Recent studies also reported that t-RA was the predominant RA in embryonic tissues after whole rat embryos were cultured with 13-cRA for various times [9]. Interestingly, 13-cRA was barely observable in those studies. The results presented here are not only in harmony with those earlier studies but also demonstrate, for the first time, that embryonic tissue cytosol is a major site for rapid isomerization of 13-cRA to t-RA. The mechanism(s) pertaining to the RCC-dependent isomerization is now being investigated vigorously in this laboratory.

The RCC-dependent, rapid conversion of 13-cRA to t-RA may have important physiological significance. It has been speculated that the biological functions of 13-cRA are probably, at least in major part, attributed to activation through an isomerization to t-RA [38]. Two lines of evidence have supported this concept. First, it is now clear that 13-cRA does not bind (or binds only with very low affinity) to RAR/RXR receptors. Even though the possibility that 13-cRA may bind to other unknown nuclear receptors cannot be ruled out at this time, the striking effects of RARs and RXRs on normal and abnormal development suggest that 13-cRA is likely to be converted to its all-*trans* isomer to execute its biological functions. Second, investigations of cellular RA binding proteins (CRABP), which

play an important regulatory and functional role in the homeostasis of retinoids, indicated that 13-cRA exhibits approximately 25-fold lower affinity to CRABP than t-RA [39, 40]. This tends to suggest that 13-cRA is not likely to participate in the CRABP-mediated biological processes unless it is first converted to isomers that have higher binding affinities with CRABP.

In summary, we have demonstrated that RCC and RCM both contain enzymes capable of catalyzing oxidations of t-RAL, 13-cRAL, and 9-cRAL to RAs and that conceptal NAD-dependent retinal dehydrogenase(s) appeared to be the primary enzyme(s) in catalysis of the reactions. RCC also exhibited the capacity to rapidly convert 13-cRA to t-RA, and detained investigations of the possible participation of conceptal cytosolic isomerase(s) in that reaction are currently underway. Also, the possibility that significant isomerization may occur at the level of the aldehyde as well as at the level of the acid would merit future investigative attention. The data of Table 1 also suggest isomeric conversion of the 9-*cis* isomer to the 13-*cis* isomer and possibly that the 9-*cis* isomer may tend to inhibit conversion of 13-*cis* isomer to the all-*trans* configuration.

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