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Activation of retinoic acid by coenzyme A for the formation of ethyl retinoate

(vitamin A/retinoids/metabolism)

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ABSTRACT **All-*trans*-retinoic acid is metabolized to a less polar metabolite in rat liver microsomes. This metabolite was proven to be ethyl retinoate by cochromatography on high-performance liquid chromatography, base hydrolysis to all-*trans*-retinoic acid, and gas chromatography/mass spectral analysis. The formation of ethyl retinoate is a specific enzymatic process; the apparent K_m for all-*trans*-retinoic acid is 9.8 μM. The production of ethyl retinoate is greatly stimulated by the addition of coenzyme A, suggesting the formation of a retinoic acid-coenzyme A intermediate (retinoyl-coenzyme A).**

Vitamin A (retinol) is required for several biological activities: vision, reproduction, growth, and cellular differentiation. The acidic form of the vitamin, all-*trans*-retinoic acid, is made by way of an irreversible oxidation of vitamin A aldehyde (1). All-*trans*-retinoic acid supports only cellular differentiation and growth functions of vitamin A. There is considerable interest in the metabolism of all-*trans*-retinoic acid (2, 3), since it may be converted to an active form of the vitamin required for growth and differentiation.

Stephens-Jarnagin *et al.* (2) recently examined the metabolism of all-*trans*-retinoic acid in vitamin A-deficient rats following administration of a pharmacological dose. They concluded that in the intestine only three retinoids exhibited biological activity in the rat vaginal smear assay: all-*trans*-retinoic acid, 13-*cis*-retinoic acid, and retinoyl β-glucuronide. Silva and DeLuca (3) studied the metabolism of all-*trans*-retinoic acid *in vivo* in several target tissues following administration of a physiological dose. They found several metabolites more polar than retinoic acid and few less polar. Conclusions drawn from both studies are that *in vivo* the metabolism of all-*trans*-retinoic acid is extensive and that only one identified metabolite (retinoyl β-glucuronide) other than the 13-*cis* isomer of all-*trans*-retinoic acid possesses biological activity equivalent to that of all-*trans*-retinoic acid. During the course of studying the *in vitro* biosynthesis of retinoyl β-glucuronide in rat liver microsomes (9), we noted the concomitant production of a nonpolar metabolite. A similar nonpolar metabolite of retinoic acid has been detected in F9 teratocarcinoma stem cells (unpublished results). We have identified this metabolite as ethyl retinoate. The activity of the system that produces ethyl retinoate is greatly increased in the presence of coenzyme A.

MATERIALS AND METHODS

Materials. All-*trans*-[11-^{3}H]retinoic acid (1.15 Ci/mmol; 1 Ci = 37 GBq) was generously supplied by the National Institutes of Health (Bethesda, MD). Unlabeled all-*trans*-retinoic acid was obtained from Eastman. 5,6-Epoxyretinoic acid was generously supplied by Hoffmann–La Roche. Esters of all-*trans*-retinoic acid were synthesized by derivitizing retinoic acid with either diazomethane, generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, or diazoethane, generated from *N*-ethyl-*N'*-nitro-*N*-nitroguanidine (Aldrich), to obtain methyl retinoate and ethyl retinoate, respectively. All other chemicals were obtained through commercial suppliers and were of analytical grade. The solvents used for HPLC were of HPLC grade (EM Science, Gibbstown, NJ).

Animals. Weanling male rats were obtained from Holtzmann (Madison, WI) and were maintained on a purified vitamin A-deficient diet (4). The diet did not contain the fat-soluble vitamins; these were added just prior to feeding [50 μg of β-carotene per week, 5.85 μg of calciferol (vitamin D_2) per week, 0.6 mg of α-tocopherol per week, and 75 μg of mendione per week]. Animals were allowed free access to food and water.

Isolation and Storage of Microsomal Membrane Preparations. Rats were killed ≈3 weeks after weaning, and livers were quickly removed and rinsed in ice-cold 0.25 M sucrose. The hepatic tissue was then homogenized in 0.25 M sucrose with a Brinkmann Polytron (Westbury, NY), setting no. 5, for 10- to 15-sec intervals until a homogenous mixture was produced. The liver homogenate was centrifuged for 15 min at 9000 × *g* (P_1). The supernatant was then centrifuged at 80,000 × *g* for 90 min to produce a microsomal membrane pellet (P_2). The supernatant (S_2) was discarded and the microsomal pellet was resuspended in 50 mM Tris buffer (pH 7.4). The suspension was assayed immediately for protein content by the method of Bradford (5). The microsomal preparation either was used immediately or was stored at −70°C for no longer than 8 months before use. No difference in activity was observed between freshly prepared and frozen microsomes or between microsomes stored 1 week or 8 months in these studies.

Enzyme Assays. Each reaction mixture contained 100 μM all-*trans*-retinoic acid (specific activity = 1 × 10^6 dpm/200 nmol), 1.7 M ethanol, 10 mM UDP-glucuronic acid, 2 mg of microsomal protein, 5 mM saccharic acid 1,4-lactone, and 0.04% Tween 40 added to 50 mM Tris buffer (pH 7.4, 37°C) in a final volume of 2 ml. The enzymatic reactions were initiated with the addition of the microsomes. To ensure continuous mixing, each tube contained a magnetic stirring bar and was placed over a magnetic stirrer. Incubation mixtures that were used to examine the effect of coenzyme A contained an ATP-regenerating system (1 mM ATP/20 mM phospho*enol*pyruvate/2 units of pyruvate kinase). The reactions were terminated after 60 min with the addition of 10 ml of ethanol and were placed in a dry ice/ethanol bath until analyzed.

Metabolite Preparation and HPLC. Reaction mixtures were warmed to room temperature and concentrated to dryness under vacuum. The residue was dissolved in ethanol containing 50 μg of butylated hydroxytoluene and *n*-propyl

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gallate per ml to prevent nonspecific oxidation. The alcohol-soluble material was brought to dryness under a stream of nitrogen, dissolved in 200 μl of ethanol containing antioxidants, and subjected to HPLC analysis.

Chromatography was performed with a Beckman model 420 liquid chromatograph (Beckman Instruments, Arlington Heights, IL). This chromatography system utilizes a Waters U6K injector (Waters Associates) and a Beckman 160 fixed-wavelength UV detector. A Zorbax-ODS reverse-phase column (4.6 mm × 25 cm, DuPont) was used to separate retinoic acid and its metabolites. The column was equilibrated with a 35% water in methanol mixture containing 10 mM ammonium acetate. After loading the sample onto the column, a linear gradient of water in methanol, 35–0%, was begun. The solvent gradient was run over 35 min. Known retinoid standards were monitored with this HPLC system by measurement of absorbance at 340 nm. Fractions (1.1 ml) were collected for measurement of metabolites by scintillation spectroscopy.

Scintillation Spectroscopy. To each 1.1-ml HPLC fraction, 3 ml of 3a70B scintillation fluid (Research Products International, Mount Prospect, IL) was added. ^3H content of each fraction was determined by a Packard Prias model 400 CL/D liquid scintillation counter (Packard Instruments, Downers Grove, IL).

Changes in Atmospheric Conditions. The incubation mixtures containing all substrates except the microsomal preparation were first mixed in culture tubes capped with serum stoppers. Each incubation tube was flushed continuously with a single gaseous mixture. Ten minutes after the atmospheric conditions of the incubation vessel were altered, the microsomal preparation was added by syringe.

Other Instrumentation. Mass spectral analyses were performed on a Kratos MS-25/DS-55 gas chromatograph/mass spectrometer (Kratos Analytical Instruments, Ramsey, NJ). Conditions for gas chromatography were splitless injection, a 10-m RSL-150 (methyl silicone) column by Alltech (Deerfield, IL) with a head pressure of 1 kg/cm^2, and a hyperbolic heat curve of 40–250°C. The source temperature of the mass spectrometer was 150°C above ambient and electron impact voltage was 70 eV.

RESULTS

Formation of a Nonpolar Metabolite of All-*trans*-Retinoic Acid. The formation *in vitro* of a metabolite less polar than all-*trans*-retinoic acid by a microsomal preparation is shown in Fig. 1*A*. This metabolite was formed whether UDP-glucuronic acid was present (Fig. 1*A*) or not (Fig. 1*B*). The formation of this material was not dependent on the biosynthesis of retinoyl β-glucuronide since no glucuronide was formed in the absence of UDP-glucuronic acid. Therefore, transesterification is not likely to be the process by which this metabolite is formed *in vitro*, as suggested (6, 7).

Identification of the Nonpolar Metabolite of All-*trans*-Retinoic Acid. The nonpolar metabolite of retinoic acid comigrated with synthetic ethyl retinoate on reverse-phase HPLC (Fig. 2*A*). The metabolite was isolated following reverse-phase HPLC separation and subjected to base hydrolysis in aqueous methanol. The hydrolyzed product was then rechromatographed (Fig. 2*B*). Hydrolysis of the metabolite yielded a product that comigrated with all-*trans*-retinoic acid.

The purified metabolite was subjected to gas chromatography/mass spectrometry; the mass spectrum is shown in Fig. 3. The mass spectral fragmentation pattern for the *in vitro* biosynthesized ethyl retinoate (Fig. 3*B*) was identical with that of synthetically derived ethyl retinoate (Fig. 3*A*). Therefore, this less polar metabolite of retinoic acid is ethyl retinoate.

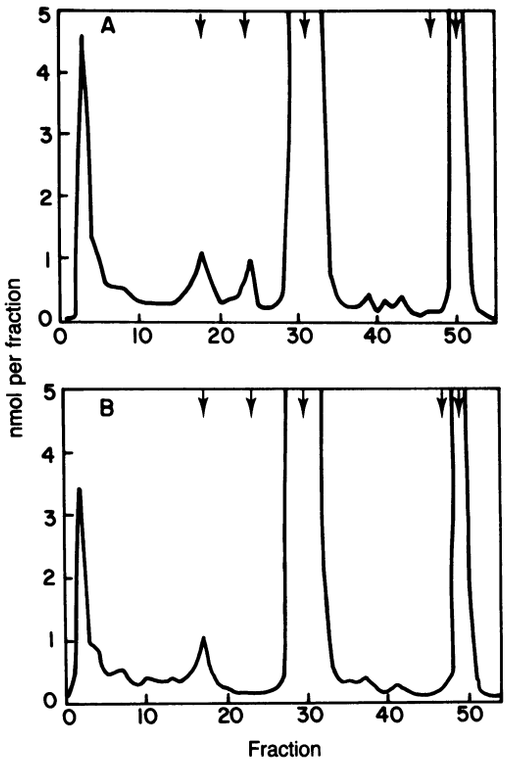

FIG. 1. HPLC profile of rat liver microsomal metabolism of all-*trans*-retinoic acid. (*A*) Standard reaction mixture with UDP-glucuronic acid added. (*B*) Standard reaction mixture with no UDP-glucuronic acid added. Arrows represent elution positions of internal standards (from left to right): 5,6-epoxyretinoic acid, retinoyl β-glucuronide, all-*trans*-retinoic acid, methyl retinoate, and ethyl retinoate.

Apparent K_m and V_{max} for the Reaction. The apparent K_m for retinoic acid is 9.8 μM and the V_{max} is 5 pmol/mg per min,

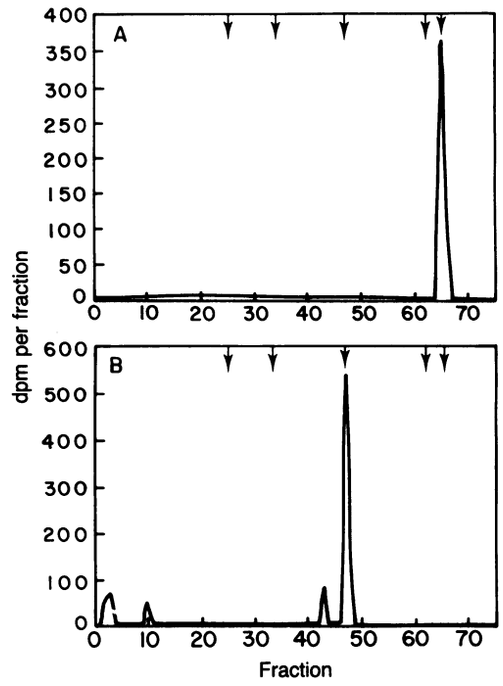

FIG. 2. Hydrolysis of the nonpolar metabolite of all-*trans*-retinoic acid. (*A*) HPLC profile of the metabolite. (*B*) HPLC profile of the metabolite after base hydrolysis. Arrows represent elution positions of internal standards (see legend to Fig. 1).

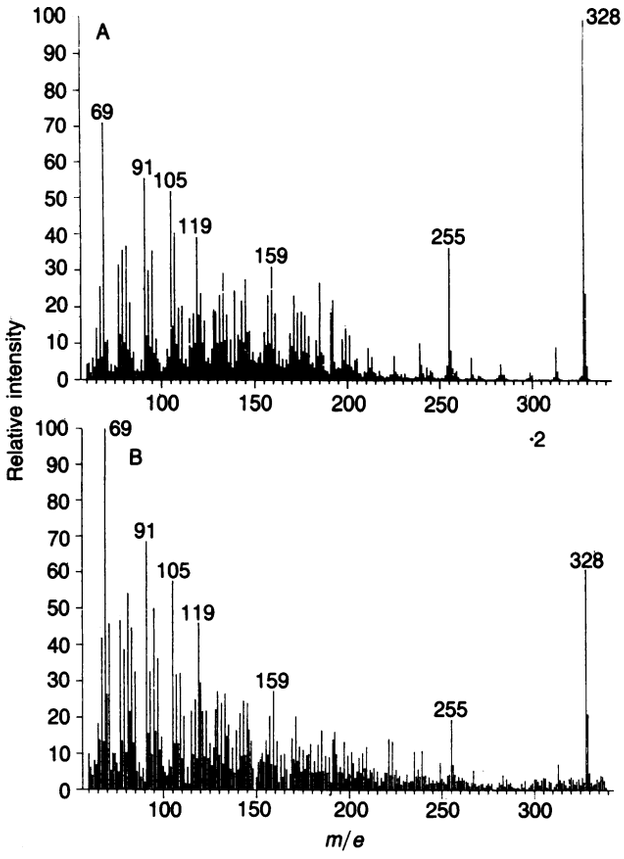

FIG. 3. Gas chromatography/mass spectrometry of synthetic and biosynthetic retinoids. (*A*) Synthetic ethyl retinoate. (*B*) Less polar metabolite of all-*trans*-retinoic acid.

as determined from Lineweaver–Burk analysis. The data analysis typified a double-reciprocal plot (data not shown).

Effect of Ethanol, Time, and Microsomal Protein Concentration on Ethyl Retinoate Formation. No ethyl retinoate was detected when ethanol was omitted from the incubation mixture (Table 1). Increasing the ethanol concentration in the reaction mixture markedly increased the production of ethyl retinoate (Table 1). These results illustrate that the ethyl portion of the metabolite originates from the ethanol used in the incubation and not from the ethanol used in the extraction procedure. Also, no ethyl retinoate was formed without incubation despite the presence of ethanol in the reaction mixture and extraction procedure. The time course study showed a linear relationship for up to 90 min at a microsomal protein concentration of 5 mg/ml (data not shown).

A plot of the relationship between microsomal protein and ethyl retinoate formation approached linearity between 0 and 2.5 mg of protein per ml, clearly showing microsomal dependence (results not shown).

Table 1. Effect of ethanol concentration on ethyl retinoate formation

Final ethanol concentration, %	nmol per assay
10.0	3.52 ± 0.21
7.5	2.75 ± 0.14
5.0	1.46 ± 0.27
2.5	0.63 ± 0.03
1.0	0.36 ± 0.16
None	ND

Conditions were as described in the text except that the ethanol concentration of the reaction mixture was varied as shown. Each datum point represents triplicate determinations. ND, not detectable. Data are presented as mean ± SEM.

Effect of Temperature, pH, Oxygen, Sulfhydryl-Reducing Reagents, and Reducing Cofactors on Ethyl Retinoate Formation. As expected, the results of the temperature dependence study on ethyl retinoate formation showed a typical temperature-dependent profile, with maximal activity observed at 37°C (data not shown). However, the reaction was not affected by changes in pH between 6.5 and 8.5 (data not shown). Replacement of air with either argon or 95% O_2/5% CO_2 did not affect ethyl retinoate production, as might be expected (data not shown). The reaction was not affected by the addition of a sulhydryl-reducing agent, 5 mM dithiothreitol (results not shown). Addition of a NADPH-generating system (5 mM glucose 6-phosphate/500 μM NADPH/2 units of glucose-6-phosphate dehydrogenase) or a NADH-generating system (1.7 M ethanol/500 μM NAD^+/2 units of alcohol dehydrogenase) had no effect on ethyl retinoate formation, as expected.

Dependence of Ethyl Retinoate Production on Coenzyme A and a Source of ATP. Activation of a carboxylic acid moiety is necessary in the esterification of all-*trans*-retinoic acid; therefore, an ATP requirement might be expected. The activated form of the carboxylic group is usually either a phosphate mixed anhydride or a coenzyme A-activated intermediate. Table 2 shows that with the addition of an ATP source and coenzyme A, ethyl retinoate formation was greatly increased. There was no apparent enhancement of metabolite production with the addition of coenzyme A or an ATP source alone. The marked dependence of this reaction on coenzyme A and ATP was further examined. Maximal ethyl retinoate formation was observed at 100 μM coenzyme A (data not shown). A decrease in ethyl retinoate production at 1–10 mM coenzyme A may be due to the detergent-like activity of coenzyme A.

Table 3 shows the effect of coenzyme A and ATP addition on the total enzyme activity and on the specific activity in various cellular fractions. Coenzyme A increased total enzyme activity in all fractions (P_1, P_2, and S_2) and in all combinations. The greatest total activity in any single fraction was noted in P_1 and P_2. However, the microsomal pellet (P_2) had the highest specific activity of all fractions examined.

DISCUSSION

Sietsema and DeLuca (8) initially noted a nonpolar metabolite of retinoic acid generated *in vitro* by kidney microsomes. This metabolite was not identified. The authors suggested that this material may have been generated by acid-catalyzed esterification of all-*trans*-retinoic acid. Stephens-Jarnagin *et al.* observed the production of several nonpolar metabolites of all-*trans*-retinoic acid in F9 teratocarcinoma cells (unpublished results). A prominent metabolite was determined to be an ester of retinoic acid. This metabolite is likely to be ethyl retinoate because of comigration with ethyl retinoate, and base hydrolysis yielded retinoic acid.

Table 2. Effect of ATP or coenzyme A (or both) on ethyl retinoate formation

Reaction conditions	nmol per assay
Control	1.98 ± 0.06
+ Coenzyme A	1.88 ± 0.11
+ ATP	1.87 ± 0.23
+ Coenzyme A + ATP	10.12 ± 0.14

Conditions were as described in the text except that an ATP-regenerating system or coenzyme A (or both) was added to the reaction mixture as shown. Each datum point represents triplicate determinations. Data are presented as mean ± SEM.

Table 3. Subcellular location of coenzyme A-stimulated ethyl retinoate formation

Fraction	Total enzyme activity, nmol per assay		Specific activity, nmol/mg of protein		
	With CoA	Without CoA	With CoA	Without CoA	Ratio*
Whole homogenate	2.26	1.10	0.51	0.21	2.05
$P_1 + P_2 + S_2$	2.75	0.77	0.55	0.14	3.57
$P_1 + P_2$	2.51	0.80	0.81	0.26	3.14
$P_1 + S_2$	2.00	0.51	0.47	0.12	3.92
$P_2 + S_2$	1.94	0.45	0.62	0.15	4.30
P_1	1.73	0.43	0.67	0.17	4.02
P_2	1.82	0.40	1.23	0.27	4.55
S_2	0.32	0.02	0.20	0.01	16.0

Fresh hepatic tissue was used. Each assay contained 250 μM retinoic acid, 5 mM saccharic acid 1,4-lactone, 0.04% Tween 40, 10% ethanol, 25 mM sucrose, 50 mM Tris buffer, an ATP-generating system, and the liver tissue fraction(s) shown; 100 μM coenzyme A was present or absent, as indicated. Incubation time was 30 min. *With CoA/without CoA (column 2/column 3).

The purpose of this study was to determine the structure and the mode of production of this nonpolar metabolite of liver microsomes. This metabolite is clearly ethyl retinoate because it comigrates with ethyl (but not methyl) retinoate, it yields all-*trans*-retinoic acid on alkaline hydrolysis, and the gas chromatograph/mass spectral analysis shows it to be identical with synthetic ethyl retinoate. This metabolite has not been observed during *in vivo* metabolism of all-*trans*-retinoic acid studies (2, 3). When examining metabolism *in vivo*, the dosing vehicle for retinoic acid was dimethyl sulfoxide or propylene glycol (or both). Since ethanol does not occur naturally in mammals, it is not surprising that ethyl retinoate has not been observed *in vivo*.

An obvious question is what is the *in vivo* significance of the ethyl retinoate reaction. Perhaps another alcoholic function is the natural acceptor of retinoic acid in the reaction. Possible acceptors are the hydroxyl groups on glycerol, mono- and diglycerides, glycerolphosphate, carbohydrates, amino acids (serine, threonine, and tyrosine), and possibly even amino groups. Such reactions could be of importance to retinoic acid function or be related to its inactivation.

A significant finding was that the formation of ethyl retinoate was greatly increased by the addition of coenzyme A and an ATP source. These results suggest very strongly the existence of a retinoyl-coenzyme A intermediate, which may be a key donor of the retinoyl moiety or be a key intermediate in the metabolism of retinoic acid. We have yet to determine if the second step of a coenzyme A-mediated pathway (retinoyl-coenzyme A to ethyl retinoate) involves an enzymatic process or is through a nonspecific nucleophilic attack. Isolation of retinoyl-coenzyme A from our reaction mixture or through chemical synthesis will allow us to directly answer this question as well as determine what is the natural acceptor of retinoyl-coenzyme A. These results may provide new insight into retinoic acid metabolism and function.

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1. Wolf, G. (1980) in *Nutrition and the Adult*, eds. Alfin-Slater, R. B. & Kritchevsky, D. (Plenum, NY), Vol. 3B, pp 97–204.
2. Stephens-Jarnagin, A., Sietsema, W. K., Miller, D. A. & DeLuca, H. F. (1983) *Arch. Biochem. Biophys.* **220,** 502–508.
3. Silva, D. P., Jr., & DeLuca, H. F. (1982) *Biochem. J.* **206,** 33–41.
4. Skare, K. L., Sietsema, W. K. & DeLuca, H. F. (1982) *J. Nutr.* **112,** 1626–1630.
5. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.
6. Lippel, K. & Olson, J. A. (1968) *J. Lipid Res.* **9,** 580–586.
7. Zile, M. H., Inhorn, R. C. & DeLuca, H. F. (1982) *J. Biol. Chem.* **257,** 3537–3543.
8. Sietsema, W. K. & DeLuca, H. F. (1979) *Biochem. Biophys. Res. Commun.* **90,** 1091–1097.
9. Miller, D. A. & DeLuca, H. F. (1985) *Arch. Biochem. Biophys.*, in press.