Isomerization of all-*trans*-retinoic acid to 9-*cis*-retinoic acid

Jonathan URBACH and Robert R. RANDO*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, U.S.A.

The discovery of the biological activity of 9-*cis*-retinoic acid raises questions as to its mode of biosynthesis. A simple mechanism involves the direct isomerization of all-*trans*-retinoic acid to 9-*cis*-retinoic acid. It is shown here that bovine liver membranes, but not supernatant fractions, can isomerize all-*trans*-retinoic acid into 9-*cis*-retinoic acid and 13-*cis*-retinoic acid. The concentration of 9-*cis*-retinoic acid generated approaches its equilibrium concentration, which is determined here to be approximately 15%. However, the isomerization process could not be shown to be saturable, and is first-order in all-*trans*-retinoic acid in the concentration range measured (8.3 nM to 3 μM). Isomerization reactions measured using bovine liver microsomes appear to be mediated by thiol groups, as they can be blocked by group-specific thiol-blocking reagents such as *N*-ethylmaleimide. It is interesting to note that the non-stereospecific behaviour observed here mimics what is observed when all-*trans*-retinoic acid is applied to cells. Finally, significant formation of 9-*cis*-retinoids was not found when the reaction was carried out with liver microsomes and either all-*trans*-retinol or all-*trans*-retinal.

INTRODUCTION

Mono-*cis* isomers of retinoids play important biological roles. Two noteworthy examples are 11-*cis*-retinal and 9-*cis*-retinoic acid. The visual pigment rhodopsin contains 11-*cis*-retinal as the light-absorbing chromophore, adducted to an active-site lysine residue via protonated Schiff base formation [1]. The sensation of light is initiated when a photon is absorbed by rhodopsin, leading to the *cis*-to-*trans* isomerization of the chromophore [2]. The retinoic acids affect many different biological processes and are probably of great importance developmentally [3–10]. Recently, it has been shown that 9-*cis*-retinoic acid produces biological effects distinct, at least in degree, from those produced by all-*trans*-retinoic acid [11–13]. Moreover, 9-*cis*-retinoic acid binds to a subset of previously characterized receptors, known as RXR receptors, in addition to the RAR receptors, which are also known to be activated by all-*trans*-retinoic acid [14,15].

The discovery of 9-*cis*-retinoic acid as a biological mediator immediately provokes interest as to its mode of biosynthesis. A possible simple mechanism involves the direct isomerization of all-*trans*-retinoic acid to 9-*cis*-retinoic acid. Indeed, experiments with COS-1 and S2 cells appeared to demonstrate that all-*trans*-retinoic acid could be, at least partially, converted into 9-*cis*-retinoic acid and 13-*cis*-retinoic acid [11–13]. As the biological reduction of retinoic acids to retinals or retinols is unknown, this result would imply that there is a direct interconversion at the retinoic acid stage of oxidation. In the present paper, the catalysed isomerization of all-*trans*-retinoic acid by liver microsomes and chemical catalysts is described.

EXPERIMENTAL

Materials

Retinoic acid, 13-*cis*-retinoic acid and 9-*cis*-retinal were purchased from Sigma Chemical Co. All-*trans*-retinol was purchased from the Fluka Chemical Co. ^{3}H-labelled retinoids, [11,12-^{3}H]retinoic acid and [11,12-^{3}H]retinol were purchased from New England Nuclear. 9-*cis*-Retinoic acid was prepared from 9-*cis*-retinal or 9-*cis*-retinol by NaCN-catalysed MnO_2 oxidation in methanol followed by hydrolysis of the resulting methyl ester [16,17]. 9,13-Di-*cis*-retinoic acid was prepared by h.p.l.c. purification from a photoisomerate of all-*trans*-retinoic acid. Its identity was established by n.m.r. spectroscopy, and by comparing it with known spectra [18]. Fresh bovine liver was purchased from Research 87 (Revere, MA, U.S.A.).

Preparation and fractionation of bovine liver homogenates

Bovine liver (40 g) was placed in a Waring blender, and 200 ml of buffer containing 50 mM sodium phosphate, pH 7.4, and 0.2 mM dithiothreitol was added. The liver was homogenized at high speed with two 45 s bursts. The homogenate was then transferred to centrifuge tubes and centrifuged for 30 min at 750 *g*. The supernatant was then centrifuged at 50000 *g* for 2 h. The 50000 *g* pellet, which was the microsomal fraction, contained the greatest isomerization activity per mg of protein, as demonstrated below, and was used for all experiments unless otherwise indicated.

Assays for microsomally catalysed isomerization

In a Microfuge tube, 100 μl of 500 mM buffer was combined with 400 μl of sample. To initiate the reaction, 1.0 μCi of all-*trans*-retinoic acid (50 Ci/mmol) in 100 μl of 5% BSA was added, and the reaction mixture was incubated for 1 or 3 h under dim red light at 23 °C with gentle shaking. Reactions were stopped by the addition of 850 μl of chloroform/methanol/formic acid (70:30:1, by vol.), shaking, then centrifuging to separate the layers. The organic layers were then removed, and concentrated *in vacuo*. To the residue was added 5 μl of chloroform followed by 100 μl of acetonitrile/methanol/water/formic acid (23:7:10:0.2, by vol.) containing a photoisomerate of retinoic acid. This suspension was centrifuged to pellet any solids. The supernatant was then used for h.p.l.c. analysis.

H.p.l.c. analysis of isomerization reaction mixtures

Extracts from isomerization assay reaction mixtures were analysed by h.p.l.c. on a Beckman Ultrasphere IP reversed-phase

* To whom correspondence should be addressed.

column. The mixtures were eluted in a solvent containing acetonitrile/methanol/water/acetic acid (23:7:10:0.2, by vol.) at a flow rate of 1.5 ml/min. The u.v. detector was set at 350 nm and a Berthold h.p.l.c. radioactivity monitor (model LB 506 C1) was used to measure ^3H disintegrations. After each run, peaks corresponding to 9-*cis*-retinoic acid and all-*trans*-retinoic acid were integrated using Berthold software so that the relative peak sizes could be determined. Radioactive isomer peaks were identified by comparing the retention times of isomers in a mixture formed by photoisomerization of all-*trans*-retinoic acid. The identities of these isomeric peaks were established by co-injection of the photoisomerate with authentic samples of the various isomeric retinoic acids.

RESULTS

It had previously been shown that S2 cells and COS-1 cells could isomerize all-*trans*-retinoic acid to generate 9-*cis*-retinoic acid and 13-*cis*-retinoic acid [11–13]. Moreover, 9-*cis*-retinoic acid had been found to occur in liver and kidney [11]. Therefore initial experiments aimed at uncovering an *in vitro* isomerization activity were conducted on liver homogenates. All-*trans*-retinoic acid (40 nM) was incubated with various fractions from bovine liver for 1 h. The rates of formation of 9-*cis*-retinoic acid were measured, and are shown in Table 1. Significant amounts of 9-*cis*-retinoic acid were formed, along with 13-*cis*-retinoic acid and 9,13-di-*cis*-retinoic acid, when the incubations were carried out with a liver microsomal preparation. The maximum amount of 9-*cis*-retinoic acid formed appeared to be approximately 10% of total retinoic acid isomers. A reaction time course which establishes this is shown in Figure 1. The 9-*cis*-retinoic acid-forming activity appeared to be only membrane-associated, as only a relatively small amount of activity was found in supernatant fractions (Table 1).

That the peak identified as 9-*cis*-retinoic acid was authentic was independently verified. First, the putative 9-*cis*-retinoic acid was collected, purified and found to be coeluted with authentic 9-*cis*-retinoic acid (Figure 2a). Esterification of the material with diazomethane produced a compound which was coeluted with authentic methyl 9-*cis*-retinoate (Figure 2b), and, finally, reduction of the radioactive ester with lithium aluminium hydride generated a compound that was coeluted with authentic 9-*cis*-retinol (Figure 2c). These chemical conversion studies established that the microsomally generated 9-*cis*-retinoic acid was authentic.

A study of the effects of buffer and pH on the isomerization process was then undertaken. As shown in Table 2, sodium borate buffer at pH 9 appeared to afford the greatest amount of microsome-mediated isomerization when compared with background control values (heat-inactivated microsomes). A typical h.p.l.c. trace from an assay conducted in 100 mM sodium borate, pH 9, is shown in Figure 3.

Although the experiments reported above appear to mimic what has been observed when all-*trans*-retinoic acid is added to certain cells [11–13], the observation that the isomerization process is non-stereospecific suggests that it might not be enzyme mediated. If the isomerization process is enzyme mediated, it ought to be saturable with respect to substrate. As concentrations of all-*trans*-[^3H]retinoic acid were increased, the rate of 9-*cis*-[^3H]retinoic acid generated remained the same, indicating that the reaction remained first order with respect to all-*trans*-retinoic acid, rather than showing saturation (Figure 4). Overall, these experiments suggest that the observed synthesis of 9-*cis*-retinoic acid is probably not enzyme-mediated. This, of course, is consistent with the observed lack of stereospecificity in the isomerization process.

The experiments described above demonstrate the catalysed formation of 9-*cis*-retinoic acid from all-*trans*-retinoic acid. It was of interest to determine whether isomerization could be achieved in the absence of liver membranes. When all-*trans*-retinoic acid was incubated with 1% BSA and 1% SDS in combination, isomerization was effected. It is interesting to note that no appreciable isomerization occurred when all-*trans*-retinoic acid was incubated with 1% BSA or 1% SDS alone. As the isomerization of all-*trans*-retinoic acid was effected by chemical catalysis, the equilibrium constant for the isomerization could readily be determined. An h.p.l.c. trace showing the equilibrium mixture generated from isomerizing radioactive all-*trans*-retinoic acid with BSA and SDS for 12 h is shown in Figure 5. Equilibrium was effected beginning with pure 13-*cis*-retinoic

Table 1 Assays of liver homogenate fractions

The catalytic capacity of various bovine liver fractions to effect isomerization of all-*trans*-retinoic acid to 9-*cis*-retinoic acid was measured using the following procedure: 1 μCi (44 nM) of all-*trans*-retinoic acid (50 Ci/mmol) in 100 μl of 5% BSA was added to 500 μl portions of liver homogenate fractions in 50 mM sodium phosphate, pH 7.4. Reactions were incubated for 1 h at 23 °C with shaking. Exposure to light was avoided. Reaction mixtures were extracted and analysed as described in the Experimental section. The experiments were for single representative determinations which were independently repeated several times with similar results.

Fraction	9-*cis*-Retinoic acid (%)	Protein (mg)	Rate of 9-*cis*-retinoic acid formation (fmol/min per mg)
Heat-inactivated homogenate	1.0	6.4	0.5
Crude homogenate	1.2	6.4	0.6
600 *g* supernatant	1.3	5.6	0.8
600 *g* pellet	3.1	1.6	6.6
500000 *g* supernatant	1.2	4.2	1.0
Microsomal fraction	3.0	0.8	12.8

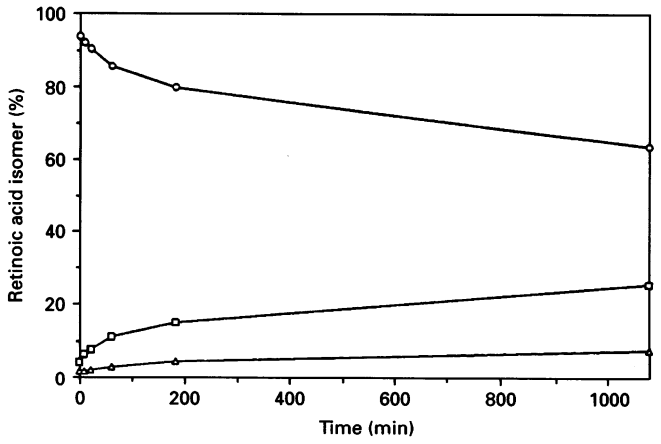


Figure 1 Time course of formation of retinoic acid isomers from 1 μCi (44 nM) of all-*trans*-retinoic acid (50 Ci/mmol) catalysed by bovine liver microsomes showing percentage of 13-*cis*- and 9,13-di-*cis*-retinoic acids (□), 9-*cis*-retinoic acid (△) and all-*trans*-retinoic acid (○)

Portions of microsomal pellet in 500 μl of 50 mM sodium phosphate, pH 7.4, containing approx. 3 mg of protein, were assayed by the standard procedure with incubation times ranging from 0 min (extraction solvent added before microsomes) to 18 h.

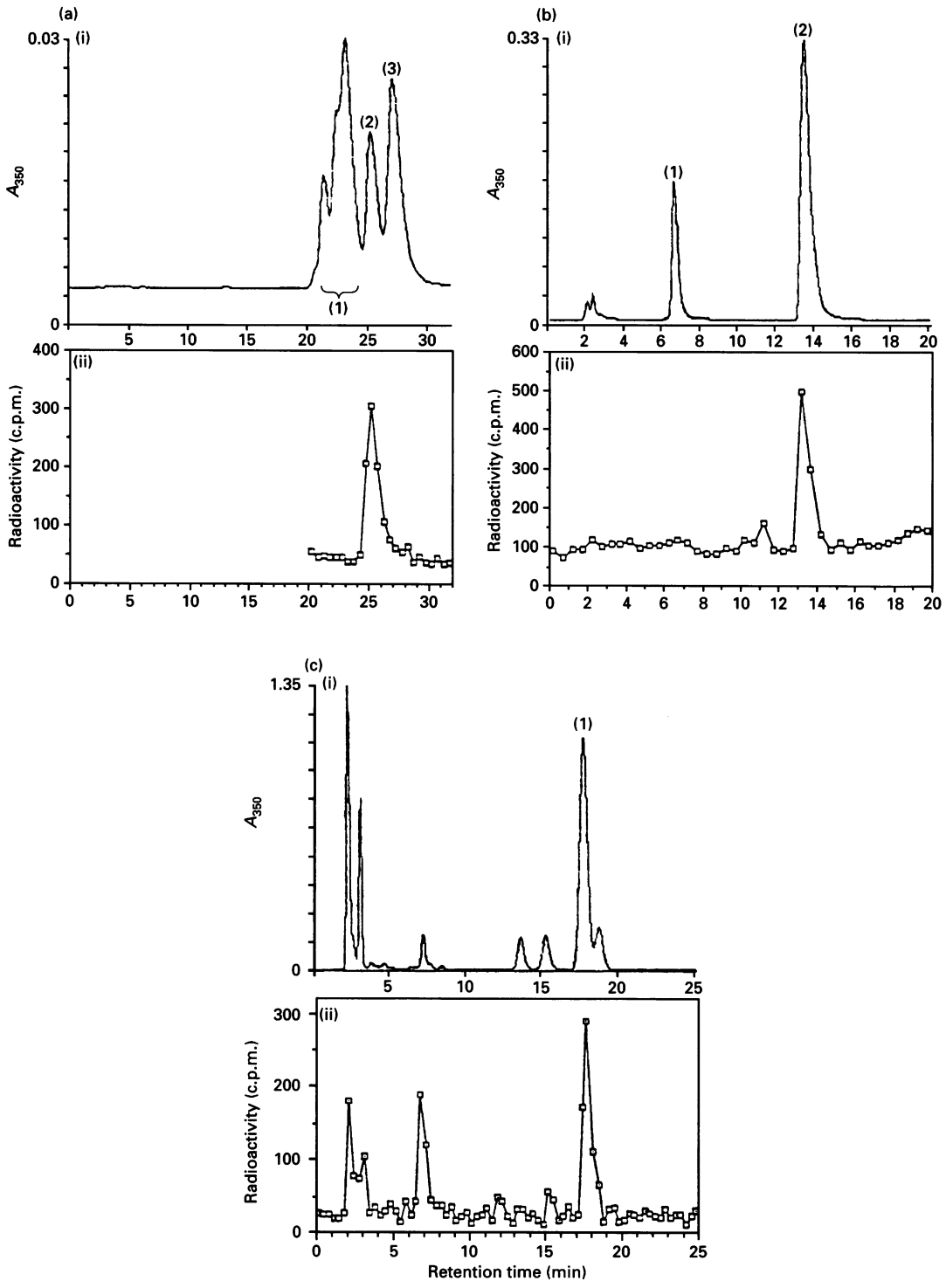


Figure 2 Verification of the structure of 9-*cis*-retinoic acid purified from microsomally catalysed isomerization of all-*trans*-retinoic acid

(**a**) (i) Photoisomerate of all-*trans*-retinoic acid co-injected with (ii) purified ^{3}H-labelled metabolite coeluted with 9-*cis*-retinoic acid in the photoisomerate mixture. Peaks are as follows: (1) 13-*cis* and 9,13-di-*cis*; (2) 9-*cis*; (3) all-*trans*. H.p.l.c. conditions were as follows: solvent, acetonitrile/methanol/water/acetic acid (23:7:10:0.2, by vol.); flow rate, 1.5 ml/min; column, Beckman Ultrasphere IP (5 μM) (C18). (**b**) H.p.l.c. analysis of methylated metabolite showing (i) u.v. trace of co-injected (1) 9-*cis*-retinoic acid and (2) methyl 9-*cis*-retinoate; and (ii) counts from methylated metabolite of 11,12-all-*trans*-[^{3}H]retinoic acid. H.p.l.c. conditions were as follows: solvent, acetonitrile/methanol/water/acetic acid (80:10:10:1, by vol.); flow rate, 1.5 ml/min; column, Beckman Ultrasphere IP (5 μm) (C18). (**c**) (i) H.p.l.c. trace showing u.v.-absorption profile of the product mixture from the lithium aluminium hydride reduction of authentic methyl 9-*cis*-retinoate and (ii) counts from the product mixture of the reduced methylated metabolite of 11,12-all-*trans*-[^{3}H]retinoic acid. Peak (1) is 9-*cis*-retinol. H.p.l.c. conditions were as follows: solvent, hexane/ethyl acetate/propan-2-ol (190:10:1, by vol.); flow rate, 1.5 ml/min; column: LiChrosorb Si60 (10 μm).

Table 2 Assays for comparison of buffer conditions

The effects of buffer and pH on the isomerizing activity of bovine liver microsomes were studied using the following procedure. Microsomal pellets prepared by the standard procedure in 50 mM phosphate buffer, pH 7.4, were resuspended in ice-cold distilled water. To 400 μl portions of this suspension, containing approx. 1.2 mg of protein, were added 100 μl of 500 mM buffer and 1 μCi (44 nM) of all-*trans*-retinoic acid (50 Ci/mmol). Reaction mixtures were incubated with shaking for 3 h, then extracted and analysed as described in the Experimental section. The experiments were for single representative determinations which were independently repeated several times with similar results.

Buffer	9-*cis*-Retinoic acid product from fresh microsomes (%) (a)	9-*cis*-Retinoic acid product from heat-inactivated microsomes (%) (b)	a − b
83 mM Sodium acetate, pH 4	1.6	0.9	0.7
83 mM Sodium acetate, pH 5	2.2	1.0	1.2
83 mM Sodium acetate, pH 6	4.0	0.8	3.2
83 mM Sodium phosphate, pH 6	2.4	1.1	1.3
83 mM Sodium phosphate, pH 7	3.8	1.2	2.6
83 mM Sodium phosphate, pH 8	4.3	0.9	3.4
83 mM Sodium borate, pH 8	3.1	0.7	2.4
83 mM Sodium borate, pH 9	5.5	0.6	4.9
83 mM Sodium borate, pH 10	3.2	0.8	2.4
83 mM Tris/HCl, pH 7	5.9	2.9	3.0
83 mM Tris/HCl, pH 8	6.5	3.0	3.5
83 mM Tris/HCl, pH 9	5.8	2.4	3.4

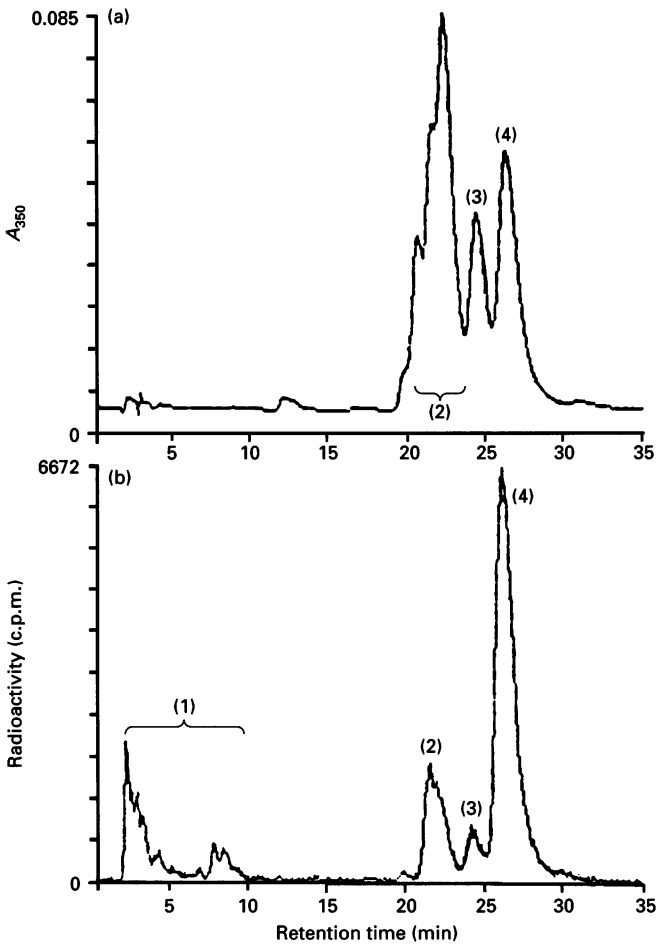


Figure 3 Example of isomerization assay, typical h.p.l.c. trace (a) showing isomers in co-injected retinoic acid photoisomerate by u.v. and (b) ^{3}H counts from product mixture of microsome-catalysed isomerization of 11,12-[^{3}H]retinoic acid

Peaks are as follows: (1) oxidation products; (2) 13-*cis* and 9,13-di-*cis*; (3) 9-*cis*; (4) all-*trans*. H.p.l.c. conditions were as follows: solvent, acetonitrile/methanol/water/acetic acid (23:7:10:0.2, by vol.); flow rate, 1.5 ml/min; column, Beckman Ultrasphere IP (5 μm) (C18).

acid, pure 9-*cis*-retinoic acid and pure all-*trans*-[^{3}H]retinoic acid. The percentages of the isomers at equilibrium are shown in Table 3. It is noteworthy that at equilibrium there is approximately 15% 9-*cis*-retinoic acid. However, the liver membrane preparation was not able to achieve the equilibrium concentration of 9-*cis*-retinoic acid, possibly because of the low rate of isomerization coupled with competing chemical processes.

The mechanism of the catalysis of the isomerization has not been investigated in detail, but it appears likely that thiol groups on the proteins are important. For example, thiol-blocking reagents, such as *N*-ethylmaleimide and *p*-hydroxymercuribenzoate, virtually abolish isomerase activity catalysed by either liver microsomes or BSA or SDS. In addition, mercaptans by themselves catalyse the isomerization of retinoic acid.

Finally, it is also possible that the relevant biological isomerization in the biosynthesis of 9-*cis*-retinoic acid occurs at the alcohol or aldehyde oxidation states. However, studies with liver microsomes and chick embryo extracts showed no catalysed isomerization with either all-*trans*-retinol or all-*trans*-retinal. Therefore in the studies described here only the isomerization of the retinoic acids proved to be biologically catalysed.

DISCUSSION

Previously published experiments have demonstrated that 9-*cis*-retinoic acid is a novel effector molecule which expresses its activity through RXR receptors [11–13]. These observations suggest questions concerning the mode of biosynthesis of this retinoid. The simplest mechanism for the isomerization reaction involves a direct isomerization at the retinoic acid level of oxidation. A mechanism of this type should be relatively simple to demonstrate, as the retinoic acids are not known to be reduced to retinals or retinols. Therefore direct experiments with all-*trans*-retinoic acid as substrate should be possible without the concern of further processing of the retinoic acid to retinals or retinols.

In the search for an enzyme that can catalyse the relevant isomerization, the isomeric equilibrium position of the retinoic acids must be considered. In the case of 11-*cis*-retinoids, an energy source is required to drive the process, as these latter retinoids only account for approximately 0.1% of an equilibrium mixture [19]. Equilibration of the retinoic acids with SDS and BSA showed 9-*cis*-retinoic acid to be a relatively major isomer at equilibrium, accounting for approx. 14.8% of the equilibrium mixture (3.3 kJ/mol relative to all-*trans*-retinoic acid). These results are consistent with previous studies which have indicated that in retinals and retinyl esters, 9-*cis*-isomers account for

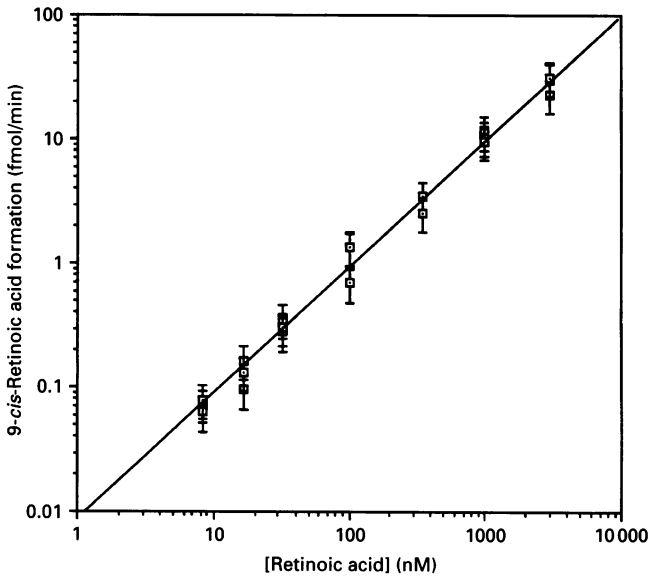


Figure 4 First-order rate of 9-*cis*-retinoic acid formation

Solutions were prepared containing all-*trans*-[^{3}H]retinoic acid (0.25–1.0 μCi per sample) along with various amounts of unlabelled retinoic acid in 100 μl of 100 mM sodium borate, pH 9. To these solutions was added 500 μl of bovine liver microsomes containing 2.7 mg of protein in 100 mM sodium borate, pH 9, giving each reaction mixture a total volume of 600 μl. Final concentrations of retinoic acid ranged from 8.3 nM to 3 μM. Reaction mixtures were incubated for 3 h and then extracted with 850 μl of chloroform/methanol/formic acid (70:30:1, by vol.). 10 μl of a solution containing 33 nCi of [^{3}H]methyl palmitate, and a retinoic acid photoisomerate was added. Reaction mixtures were analysed by h.p.l.c., and the rates of formation were calculated. The error bars represent standard deviations from the mean for separate triplicate experiments.

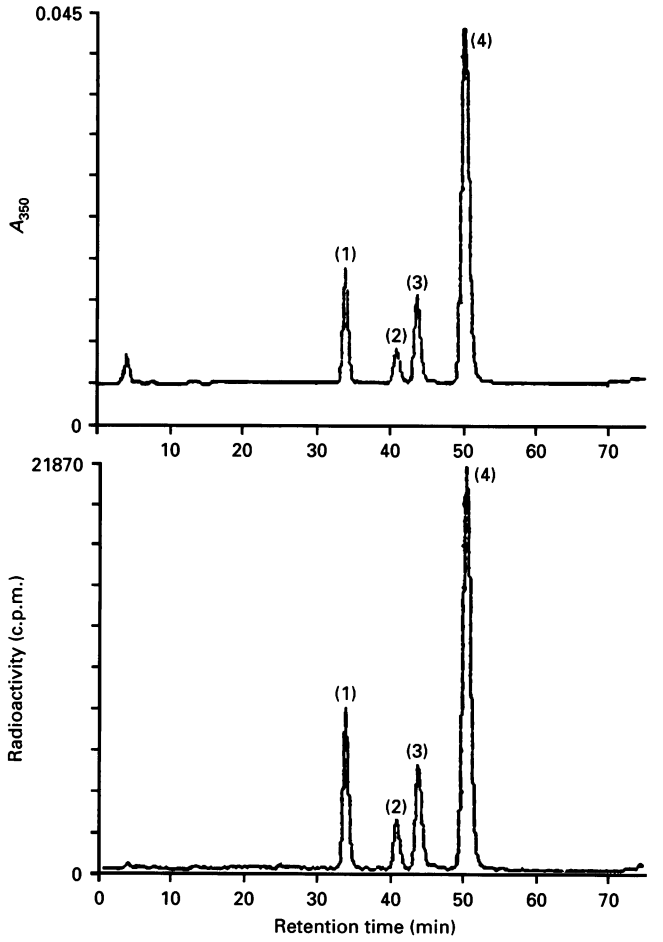


Figure 5 H.p.l.c. trace showing the product mixture from equilibration of 11,12-all-*trans*-[^{3}H]retinoic acid

11,12-All-*trans*-[^{3}H]retinoic acid (10 nmol; 1 μCi; 10 Ci/mol) was dissolved in 2 μl of 1 M NaOH and 98 μl of water; then 400 μl of 1% BSA/1% SDS was added. The reaction mixture was shaken for 12 h at 23 °C, 22 h in the dark, then extracted according to the standard protocol and analysed by h.p.l.c. Peaks are: (1) 13-*cis*; (2) 9,13-di-*cis*; (3) 9-*cis*; (4) all-*trans*. H.p.l.c. conditions were as follows: solvent, hexane/octan-1-ol (199:1, v/v) with 10 mM trifluoroacetic acid; flow rate, 1.5 ml/min; column, two LiChrosorb Si60 (5 μm) columns in series.

10–14% of the equilibrium mixture [19]. Unlike the case with 11-*cis* analogues, an energy source would probably not be obligatory in the formation of 9-*cis*-retinoids, given the stability of these compounds. It is also not at all obvious how energy might be transduced in the retinoic acid series, as it can be in the retinol series [20,21].

The studies reported here show that a membrane preparation from bovine liver can generate the putative biological effector molecule 9-*cis*-retinoic acid from added all-*trans*-retinoic acid (Scheme 1). This catalysis, however, is non-stereospecific, because 13-*cis*-retinoic acid is also generated. As 13-*cis*-retinoic acid has no known biological function, the reason for its biosynthesis is obscure at this point. However, the observed non-stereospecific isomerization process appears to mimic closely what is observed when all-*trans*-retinoic acid is added to COS-1 or S2 cells in culture [11–13]. Here, large amounts of 13-*cis*-retinoic acid were generated in addition to 9-*cis*-retinoic acid.

Although it was not possible to determine directly if the liver microsome-mediated isomerization is catalysed by enzyme, several features of the reaction suggest that it is not. As described above, the process is non-stereospecific, and, in addition, it appears to be slow. The rate of formation of 9-*cis*-retinoic acid observed here is sluggish, having a velocity of 0.78 nmol/h per mg of protein at 44 nM all-*trans*-retinoic acid and at 23 °C and pH 9. Also, less than the equilibrium concentration of 9-*cis*-retinoic acid was achieved after isomerization. The fact that the equilibrium concentration of 9-*cis*-retinoic acid was not achieved may simply be a reflection of the relatively low rate of isomerization, and of the possibility that other chemical processes may occur which lead to a steady-state situation. To test more extensively whether the biosynthesis of 9-*cis*-retinoic acid is enzyme-mediated, we needed to determine whether or not the isomerization reaction was saturable or not. The isomerization rate of increasing concentrations (8.3 nM to 3 μM) of all-*trans*-[^{3}H]retinoic acid was measured in these experiments. As 9-*cis*-retinoic acid typically binds to its receptors in the nanomolar range, there seemed to be no need to exceed micromolar concentrations in this experiment [11–13]. Moreover, the plasma concentration of all-*trans*-retinoic acid has been measured to be only 12 nM [22]. The rate of 9-*cis*-retinoic acid formation remained first order with respect to all-*trans*-retinoic acid, rather than showing saturation. As the critical micellar concentration for all-*trans*-retinoic acid has been estimated to be approximately 1 μM, there also does not appear to be any effect of the physical state of the retinoic acid on the isomerization rate [23].

Although the mechanism of the liver microsome-catalysed isomerization of the retinoic acids has not been explored, several experimental observations bear on this issue. The rate of microsome-mediated catalysis appears to be maximal at pH

Table 3 Equilibrium percentages of retinoic acid isomers

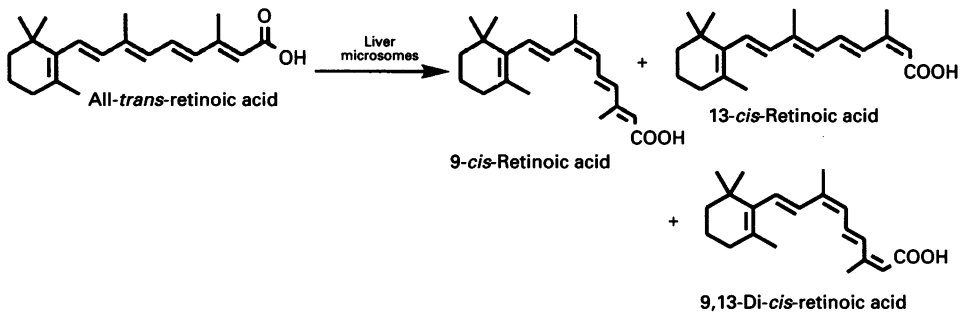
Portions containing 10 nmol of all-*trans*-retinoic acid to which had been added 1 μCi of all-*trans*-[^{3}H]retinoic acid (50 μCi/mmol) were placed in siliconized Microfuge tubes and taken up in 100 μl portions of 20 mM NaOH. To this was added 400 μl of 1% BSA/1% SDS. Reaction mixtures were extracted after various time intervals and analysed by h.p.l.c. After approx. 6 h, equilibrium was reached as determined by integration of radioactivity peaks associated with each individual retinoic acid isomer. The equilibrium percentages presented here were the averages of percentages from four data points at 7 h and two data points at 12 h. The equilibrium percentages were further confirmed by equilibration starting from the 13-*cis* and 9-*cis* isomers. The resulting product mixtures were analysed by h.p.l.c. Integrations of isomer peaks in the elution profiles at 350 nm were the same as those for reactions starting with all-*trans*-retinoic acid, within experimental error. The h.p.l.c. solvent was hexane/octan-1-ol (199:1, v/v) with 10 mM trifluoroacetic acid, run through two LiChrosorb Si60 (5 μm) columns in series at a flow rate of 1.5 ml/min. These conditions had the advantage that they could resolve the 13-*cis* and the 9,13-di-*cis* isomers of retinoic acid. Such a separation was not attainable on C18 columns, which are more generally used to spearate retinoic acid isomers.

Isomer	Equilibrium percentage	ΔG^0 relative to all-*trans* (kJ/mol)
13-*cis*	17.2±1.1	3.3
9,13-di-*cis*	6.7±0.5	5.0
9-*cis*	14.8±0.6	3.3
All-*trans*	61.4±1.3	–

approx. 9. On the surface it would appear that this should have little to do with the pK_A of retinoic acid, which has been measured to be between 3.8 and 4 at low retinoic acid concentrations, such as those employed here [24]. However, the pK_A of all-*trans*-retinoic acid has been measured to be considerably higher (approx. 8) in membranes and at concentrations exceeding 1 μM, concentrations at which micelles may be formed [23]. Furthermore, the isomerization process can be mimicked by simple chemical means, such as the addition of SDS with BSA at pH 9. We have found that thiol-blocking reagents, such as *N*-ethylmaleimide, strongly inhibit microsome-mediated isomerization, suggesting that catalysis of the isomerization is mediated by thiol groups (J. Urbach and R. R. Rando, unpublished work). Moreover, *N*-ethylmaleimide also blocks the above-mentioned chemical catalysis of isomerization, showing that it too is probably thiol-mediated. The pH- and thiol-dependences of the isomerization reaction might then suggest that the isomerization reaction is mediated by a thiol anion, which, along with an anomalously high pK_A for retinoic acid, would be expected to generate a pH versus rate maximum. If a base-catalysed Michael-addition mechanism of catalysis were operative, protonated retinoic acid would be expected to be the Michael acceptor and the thiolate anion would be the Michael donor. Further experiments are needed to sort out specifics of the catalytic mechanism, but the origins of the observed catalysis appear relatively straightforward.

As the retinoic acids cannot be biologically reduced, the isomerization process observed here is not ascribable to isomerization at the retinol or retinal states of oxidation. It is, in principle, possible that a biologically significant isomerization process, which eventually provides 9-*cis*-retinoic acid, occurs with either all-*trans*-retinal or all-*trans*-retinol as substrate, as there are well-described pathways for the oxidation of retinols and retinals to retinoic acids [25]. Along these lines it is noteworthy that the observed isomerization occurring in the biosynthesis of 11-*cis*-retinal occurs at the alcohol level of oxidation [26,27]. Here, all-*trans*-retinol (vitamin A) is first esterified by lecithin retinol acyltransferase to generate an all-*trans*-retinyl ester [20,21]. The all-*trans*-retinyl ester is then processed by an isomerohydrolase enzyme to generate 11-*cis*-retinol, which is readily oxidized to produce 11-*cis*-retinal [20,21]. This unusual energy-requiring mechanism is needed because of the thermodynamic instability of 11-*cis*-retinal, which accounts for only 0.1% of a retinal equilibrium mixture [19].

However, neither all-*trans*-retinol nor all-*trans*-retinal was specifically isomerized to its 9-*cis* congeners by either bovine liver microsomes or chick embryo homogenates. The latter tissue is known to possess RXR receptors, and presumably produces the endogenous 9-*cis*-retinoic acid effector for this receptor [11]. Other than the retinoic acid-isomerizing process described here, no other mechanism for the biosynthesis of 9-*cis*-retinoids has been described. It is certainly possible that the process described here is the biologically significant one. After all, it mimics the reaction produced when all-*trans*-retinoic acid is applied to cells. If there is another more biologically relevant process going on, its demonstration will be confounded by the process described here. There are other possibilities that need to be considered as well. Although unlikely, it is possible that 9-*cis*-retinoic acid is not the naturally occurring RXR effector, in which case there is no need for an enzyme-mediated route to it. Another possible mechanism involves the oxidative processing of dietary 9-*cis*-β-carotene to 9-*cis*-retinoic acid in animals, obviating the requirement for an isomerization reaction in the retinoid series. It is an established fact that 9-*cis*-β-carotene is found in plants [28–31]. In addition, 9-*cis*-retinol and 9-*cis*-β-carotene have been found in human serum and in milk products [28–31]. This raises the possibility that 9-*cis*-retinoic acid and other 9-*cis*-retinoids may be generated from ingested 9-*cis*-β-carotene in the same way that all-*trans*-retinoids can be generated from all-*trans*-β-caro-



Scheme 1 Retinoic acid isomerization catalysed by liver microsomes

tene. Thus 9-*cis*-β-carotene may play the same provitamin role with respect to 9-*cis*-retinoic acid as all-*trans*-β-carotene does with respect to all-*trans*-retinoic acid.

This work was financially supported by the U.S. Public Health Service N.I.H. grant EY-04096. We thank Dr. G. Eichele and Dr. C. Thaller for the chick embryo extracts.

REFERENCES

1 Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S.-L., Rao, J. K. M. and Argos, P. (1983) Biophys. Struct. Mech. **9**, 235–244
2 Hubbard, R. and Kropf, A. (1958) Ann. N.Y. Acad. Sci. **44**, 130–139
3 Brockes, J. P. (1990) Nature (London) **345**, 766–768
4 Eichele, G. (1989) Trends Genet. **5**, 246–251
5 Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A. and Evans, R. M. (1992) Nature (London) **358**, 771–774
6 Lotan, R. (1980) Biochim. Biophys. Acta **605**, 33–91
7 Matsushiuma, Y., Kawachi, E., Tanaka, H., Kagechika, H., Hashimoto, Y. and Shudo, K. (1992) Biochem. Biophys. Res. Commun. **189**, 1136–1142
8 Sherman, M. I. (ed.) (1986) Retinoids and Cell Differentiation, CRC Press, Boca Raton, FL
9 Summerbell, D. and Madden, M. (1990) Trends Neurosci. **13**, 142–147
10 Thaller, C. and Eichele, G. (1990) Nature (London) **345**, 815–819
11 Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M. and Thaller, C. (1992) Cell **68**, 397–406
12 Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenburger, M., Lovey, A. and Grippo, J. F. (1992) Nature (London) **355**, 359–361
13 Durand, B., Saunders, M., Leroy, P., Leid, M. and Chambon, P. (1992) Cell **71**, 73–85
14 Allenby, G., Bocquel, M. T., Saunders, M., Kazmer, S., Speck, J., Rosenburger, M., Lovey, A., Kastner, P., Grippo, J. F., Chambon, P. and Levin, A. A. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 30–34
15 Lehmann, J. M., Jong, L., Fanjul, A., Cameron, J. F., Lu, X. P., Haefner, P., Dawson, M. I. and Pfahl, M. (1992) Science **258**, 1944–1946
16 Barua, A. B. and Ghosh, M. C. (1972) Tetrahedron Lett. **18**, 1823–1825
17 Corey, E. J., Gilman, N. W. and Ganem, B. E. (1972) J. Am. Chem. Soc. **90**, 5616–5617
18 Liu, R. S. and Asato, A. E. (1984) Tetrahedron **40**, 1931–1969
19 Rando, R. R. and Chang, A. (1983) J. Am. Chem. Soc. **105**, 2879–2882
20 Rando, R. R. (1991) Biochemistry **30**, 595–602
21 Rando, R. R. (1991b) J. Bioenerg. Biomembr. **23**, 133–146
22 De Leenheer, A. P., Lambert, W. E. and Claeys, I. (1982) J. Lipid. Res. **23**, 1362–1367
23 Noy, N. (1992) Biochim. Biophys. Acta **1106**, 151–158
24 Steele, C. E., Marlow, R., Turton, J. and Hicks, R. M. (1987) Br. J. Exp. Med. **68**, 215–223
25 Posch, K. C., Burns, R. D. and Napoli, J. L. (1992) J. Biol. Chem. **267**, 19676–19682
26 Bernstein, P. S. and Rando, R. R. (1986) Biochemistry **25**, 6473–6478
27 Bernstein, P. S., Law, W. C. and Rando, R. R. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1849–1853
28 Ben-Amotz, A., Mokady, S. and Avron, M. (1988) Br. J. Nutr. **59**, 443–449
29 Ben-Amotz, A., Mokady, S., Edelstein, S. and Avron, M. (1989) J. Nutr. **119**, 1013–1019
30 Ollilainen, V., Heinonen, M., Linkola, E., Varo, P. and Koivistoinen, P. (1989) J. Dairy Sci. **72**, 2257–2265
31 Stahl, W., Schwartz, W, Sundquist, A. R. and Sies, H. (1992) Arch. Biochem. Biophys. **294**, 173–177

Received 6 August 1993/7 October 1993; accepted 3 November 1993