

# Ion-pair high-pressure liquid chromatography of *cis-trans* isomers of retinoic acid in tissues of vitamin A-sufficient rats

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**Abstract** Naturally occurring retinoids were separated by reversed-phase high-pressure liquid chromatography on an octadecylsilane column eluted with acetonitrile-potassium phosphate buffer (pH 7.2) mixtures. The order of elution from a mixture of 500 ng each of the following standards was 4-oxo retinoic acid (RA), retinyl phosphate (RP), 13-*cis* RA, all-*trans* RA, retinol, retinal, retinyl acetate, anhydroretinol, and retinyl palmitate. This method was employed to investigate the *cis-trans* isomerization of RA and its metabolism in vitamin A-sufficient male rats. Rats (200 g) were injected intraperitoneally with 50  $\mu$ Ci of either [10-<sup>3</sup>H]-all-*trans* RA (5.4  $\mu$ g) or [11-<sup>3</sup>H]-13-*cis* RA (8.8  $\mu$ g) and killed after 0.5 and 3 hr. Blood, liver, kidney, small intestines, and testes were removed and lyophilized. All-*trans* RA was converted at 0.5 hr after injection to 13-*cis* RA in all the tissues examined, with the exception of the small intestine; the conversion ranged from 2.4 to 6.9% of the total radioactivity. In addition, all-*trans* RA was converted to metabolites (17.5–47.7%) of greater polarity than 4-oxo RA. After 3 hr, most of the radioactivity (75–90%) was found in the highly polar metabolites. 13-*cis* RA was also partially isomerized to the all-*trans* RA and to the highly polar metabolites by 0.5 and 3 hr after injection. Appreciable radioactivity (10–41%) still resided in the 13-*cis* RA fraction after 3 hr. These results indicate that 13-*cis* RA is partially isomerized to all-*trans* RA and that all-*trans* RA is rapidly metabolized to highly polar compounds in tissues of vitamin A-sufficient rats.—Sundaresan, P. R., and P. V. Bhat. Ion-pair high-pressure liquid chromatography of *cis-trans* isomers of retinoic acid in tissues of vitamin A-sufficient rats. *J. Lipid Res.* 1982. 23: 448–455.

**Supplementary key words** all-*trans* retinoic acid • 13-*cis* retinoic acid • *cis-trans* isomerization • polar metabolites I and II

Retinoic acid (RA) is a potent growth promoter in vitamin A-deficient rats (1) and maintains epithelial differentiation (2). However, it does not support vision or reproduction (3, 4). It has been identified as a natural metabolite of retinol (5, 6) and retinal in the rat (7). Unlike retinol or retinal, it cannot be stored and is rapidly metabolized (8). Recently, its ability to suppress neoplastic development in vivo (9) and in vitro (10) has evinced new interest in a study of its metabolic pathways.

A reversed-phase high-pressure liquid chromatographic method was developed for the separation of various retinoids including retinyl phosphate (RP) and mannosyl retinyl phosphate (MRP) (11).

In the present study, we describe a modification of this method involving ion-pair high-pressure liquid chromatography (HPLC) which permits an investigation of *cis-trans* isomerization of RA in the various tissues of the vitamin A-sufficient rat. Preliminary reports of this work have appeared (12–14).

## MATERIALS AND METHODS

### Chemicals

13-*Cis* and all-*trans* RA, 4-oxo RA, retinol, retinal, and retinyl acetate were gifts (Hoffmann-La Roche Inc., Nutley, NJ). Retinyl phosphate was obtained by chemical synthesis (11). Retinyl palmitate was Type IV, crystalline, synthetic, all-*trans* (Sigma, St. Louis, MO). [10-<sup>3</sup>H]-All-*trans* RA (sp act 2.8 Ci/mmol) was a gift (New England Nuclear, Boston, MA). [11-<sup>3</sup>H]-13-*Cis* RA (SRI International, Menlo Park, CA) had a specific activity of 1.75 Ci/mmol.

The purity of [10-<sup>3</sup>H]-all-*trans* RA and [11-<sup>3</sup>H]-13-*cis* RA was checked by HPLC on an octadecylsilane (ODS) column with acetonitrile-potassium phosphate buffer, pH 7.2, as the mobile phase as described below; the compounds were found to be essentially free of any impurities and were used as such for injection into the rats (Figs. 1 and 2). In one study, [11-<sup>3</sup>H]retinoic acid

Abbreviations: HPLC, high-pressure liquid chromatography; RA, retinoic acid; RP, retinyl phosphate; MRP, mannosyl retinyl phosphate; MXP, mannosyl retinoid phosphate.

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(Hoffmann-La Roche Inc.) was purified on an ODS-2 column, using a methanol-water 80:20 mixture (flow rate 1.0 ml/min). [ $^{11}\text{-}^3\text{H}$ ]-13-*Cis* RA (retention time, 16.5 min) and [ $^{11}\text{-}^3\text{H}$ ]-all-*trans* RA (retention time, 23.0 min) were clearly separated from each other as the standard 13-*cis* and all-*trans* RA. The fractions containing [ $^{11}\text{-}^3\text{H}$ ]-13-*cis* RA and [ $^{11}\text{-}^3\text{H}$ ]-all-*trans* RA from three runs were pooled separately and reduced in volume in vacuo. Approximately 40  $\mu\text{Ci}$  of each isomer was injected into one rat each in this study (the results of this study are presented in Tables 1 and 2).

Solvents were HPLC grade (Waters Associates, Waltham, MA). Anhydroretinol was prepared as described by Dunagin and Olson (15); purity was checked by HPLC.

The rats used in our investigations were of the Sprague-Dawley strain and were weaned on laboratory chow at 3 weeks of age. At the time of killing, they were 8 weeks old, weighed 200 g, and had sufficient vitamin A stores in the liver to be designated as vitamin A-sufficient.

#### High-pressure liquid chromatographic analysis

HPLC was carried out on a programmable liquid chromatography system (Altex Model 322 MP, Altex Scientific Inc., Berkeley, CA) with two Model 100A pumps. The sensitivity of this system is dependent on the recorder with a maximum absorbance unit full scale (AUFs) of 0.01. The ultraviolet spectrophotometer (Model 25, Beckman Instruments, Fullerton, CA) was equipped with a scanning system. For the HPLC study

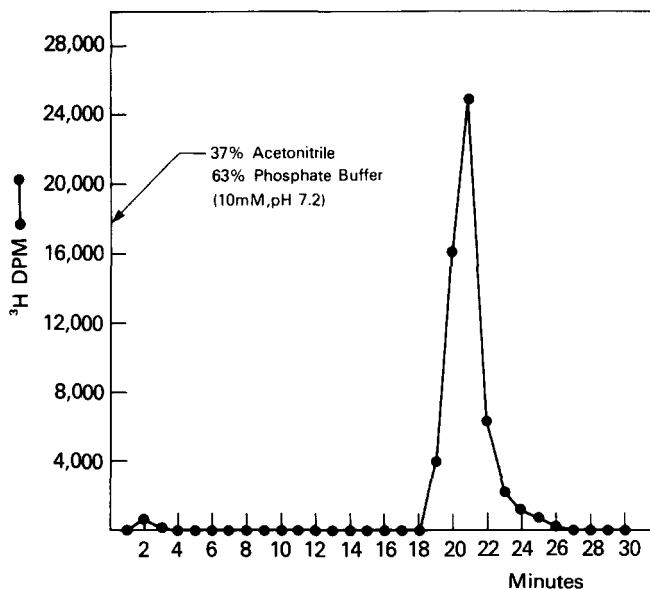


Fig. 1. Reversed-phase HPLC of standard [ $^{10}\text{-}^3\text{H}$ ]-all-*trans* RA. The chromatographic analysis was carried out under the same conditions used for the tissues of radioactive RA-injected animals.

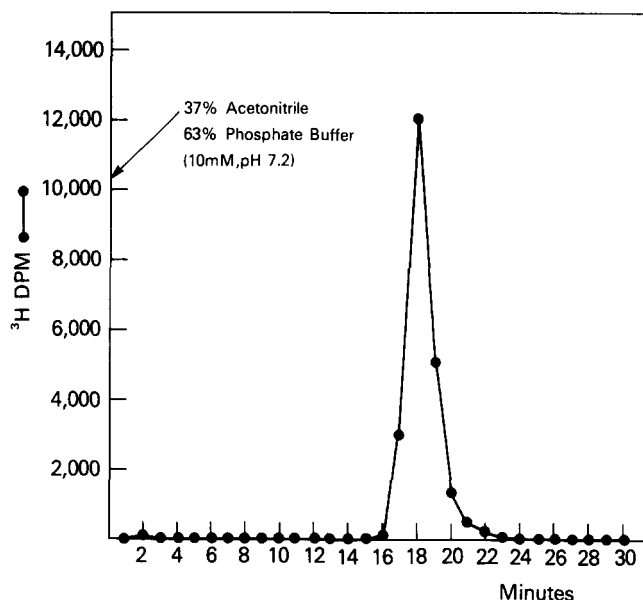


Fig. 2. Reversed-phase HPLC of standard [ $^{11}\text{-}^3\text{H}$ ]-13-*cis* RA. The chromatographic analysis was carried out under the same conditions used for the tissues of radioactive RA-injected animals.

of RA metabolism, a Partisil-10-ODS column (4.6 mm internal diameter  $\times$  25 cm) was used (Whatman Inc., Clifton, NJ). The column was eluted with acetonitrile-10 mM potassium phosphate buffer (pH 7.2) 37:63 at an initial flow rate of 2.2 ml/min for the first 30 min to elute 4-oxo RA, 13-*cis* RA, and all-*trans* RA, and with acetonitrile-potassium phosphate buffer 54:46 at a flow rate of 3.5 ml/min for the following 20 min to elute retinol, retinal, retinyl acetate, and anhydroretinol. The solvent composition was then changed to acetonitrile-water 98:2 and the flow rate was adjusted to 1.5 ml/min for an additional 15 min to elute retinyl palmitate. Ultraviolet absorption was measured in each fraction at 340 nm to allow detection of 4-oxo RA, 13-*cis* RA, all-*trans* RA, anhydroretinol, and retinal in addition to retinol and retinyl palmitate.

#### Metabolic studies in vitamin A-sufficient rats

Studies of the metabolism of radioactive RA in vitamin A-sufficient male rats were carried out by injecting each of two rats intraperitoneally with 50  $\mu\text{Ci}$  of [ $^{10}\text{-}^3\text{H}$ ]-all-*trans* RA (5.4  $\mu\text{g}$ ) and each of two other rats with 50  $\mu\text{Ci}$  of [ $^{11}\text{-}^3\text{H}$ ]-13-*cis* RA (8.8  $\mu\text{g}$ ) in 50  $\mu\text{l}$  of ethanol. Then two rats from the all-*trans* RA group were killed, the first at 0.5 hr and the second at 3 hr after injection, and the same procedure was followed with two rats from the 13-*cis* RA group. Blood, liver, kidney, intestines, and testes were removed from each rat, weighed, and lyophilized. The tissues were extracted with 20 ml of 99% methanol per g of tissue for 30 min at room temperature, with continuous stirring under yellow light. A clear so-

lution was obtained by filtering on a Millipore filter apparatus (Millipore Corp., Bedford, MA) equipped with a Millipore filter. It was dried by flash evaporation and the vacuum was released with nitrogen. The residue was immediately dissolved in about 4 ml of absolute methanol and counted, and an aliquot containing approximately 20,000 cpm and carrier 13-*cis* and all-*trans* RA (500–800 ng each) was injected into the liquid chromatograph.

#### Calculation of the amount of all-*trans* and 13-*cis* retinoic acids and the polar metabolites

The amount of retinoic acid (all-*trans* and 13-*cis*) and the polar metabolites found in the tissues was calculated from the radioactivity appearing in the different fractions after HPLC. The total radioactivity of each appropriate peak was divided by the specific activity of the injected retinoic acid and the weight of the tissue or the volume of blood, and the results were expressed as nanograms of compound per gram of tissue or per ml of blood.

#### Determination of radioactivity

Column samples or aliquots of tissue extracts (less than 1 ml) were counted after adding 15 ml of Aquasol (New England Nuclear). Samples were counted in a liquid scintillation counter (Beckman Model LS-250).

#### Recovery of retinoids after 99% methanol extraction and reversed-phase HPLC

The recovery of retinoids after extraction of the tissues with 99% methanol was 90% of the radioactivity present in the tissues, as determined by solubilization with 0.5 N KOH and measuring the radioactivity in the liquid scintillation counter. The recovery of all vitamin A derivatives, based either on radioactivity or on UV measurements, was of the order of 95%. Recovery was measured after mixing the retinoid with the tissue and following the same extraction procedure as for the tissue from RA-injected animals.

#### Co-crystallization of the radioactive compounds eluted as 13-*cis* RA and all-*trans* RA with carrier 13-*cis* and all-*trans* RA

The eluates comprising the radioactive peaks with retention times characteristic of 13-*cis* and all-*trans* RA were collected separately after HPLC, as described, and pooled to yield approximately 50,000 cpm of each isomer. They were then evaporated to dryness under nitrogen and mixed with 50  $\mu$ g each of the carrier 13-*cis* and all-*trans* isomers. The mixtures were dissolved in 250  $\mu$ l of 1% isopropyl alcohol in hexane. The solvent was reduced in volume to 100  $\mu$ l by evaporation under nitrogen. Both 13-*cis* and all-*trans* RA solutions were held at 4°C for 2–3 hr, after which time crystals of 13-*cis* RA and all-*trans* RA appeared in the tubes. The supernatants were

carefully decanted, and the crystals were washed with a few microliters of 1% isopropyl alcohol in hexane at 4°C and finally dissolved in 250  $\mu$ l of the same solvent. One aliquot was counted for radioactivity and another aliquot (20  $\mu$ l) was used for measuring UV absorbance at 350 nm. The recoveries of radioactivity, 13-*cis* RA, and all-*trans* RA and the specific activities of the crystals after each crystallization (dpm/absorbance unit at 350 nm) were calculated.

## RESULTS

#### Separation of standard retinoids by HPLC

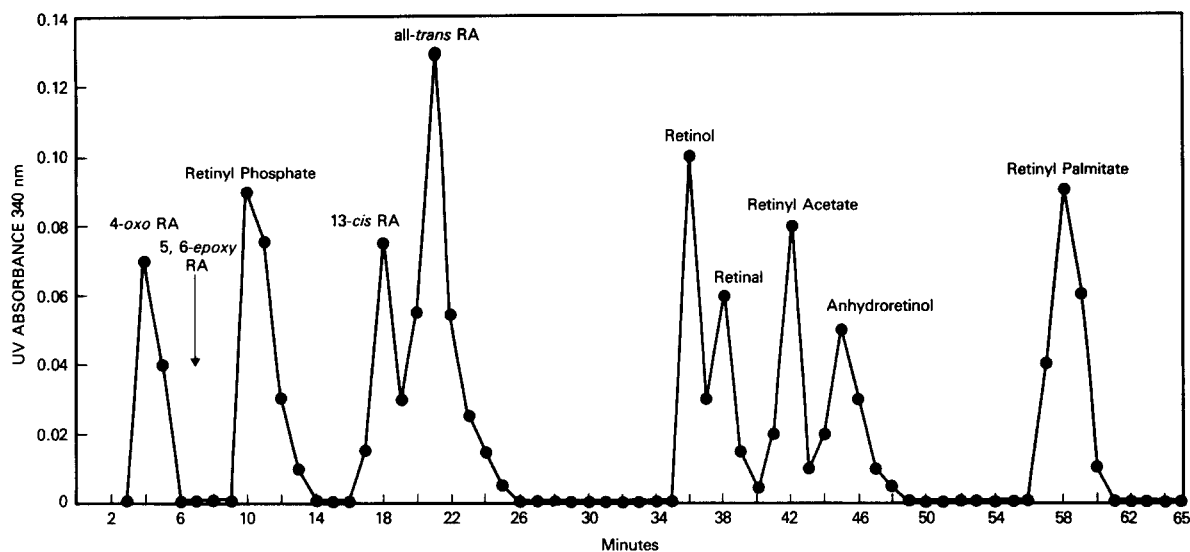
The retinoids were separated by reversed-phase HPLC on an ODS column eluted with acetonitrile–potassium phosphate buffer, pH 7.2, as well as acetonitrile–water mixtures. The order of elution of a mixture of 500–600 ng of each of the following standard retinoids was 4-oxo RA (16, 17), RP, 13-*cis* RA, all-*trans* RA, retinol, retinal, retinyl acetate, anhydroretinol, and retinyl palmitate. A typical chromatogram is illustrated in **Fig. 3**. The position of 5,6-epoxy RA on the chromatogram, which is indicated by the arrow, is based on its retention time (7 min) from another study carried out by one of us (P.V.B.) under identical chromatographic conditions (18). All the standards were in the all-*trans* configuration except 13-*cis* RA. By employing acetonitrile–potassium phosphate buffer, pH 7.2 mixture, 13-*cis* RA and all-*trans* RA were clearly separated.

#### Evidence for absence of new peaks as those occurring in vivo after mixing radioactive retinoic acids with 1 g of liver tissue

**Figs. 4 and 5** represent chromatograms obtained after mixing [ $10\text{-}^3\text{H}$ ]-all-*trans* or [ $11\text{-}^3\text{H}$ ]-13-*cis* RA with 1 g of liver and extracting with 99% methanol, followed by HPLC under the same experimental conditions used for the tissues of RA-injected animals. The results demonstrate that the new peaks are produced in vivo and do not arise during extraction and separation of the metabolites by HPLC.

#### Metabolism of RA in vivo

In the liver of a vitamin A-sufficient rat, a small amount of [ $10\text{-}^3\text{H}$ ]-all-*trans* RA was converted to a compound with retention time characteristic of 13-*cis* RA at 0.5 hr after injection; the conversion was 3.1% of the total radioactivity (**Fig. 6**). In addition, all-*trans* RA was transformed mainly to highly polar metabolites (Peak I, **Fig. 6**). At 0.5 hr after injection, these constituted 17.5% of the total recovered radioactivity. Furthermore, several minor metabolites were observed, as indicated in **Fig. 6**. At 3 hr after injection, most of the radioactivity (87%) was found in the highly polar metabolites (Peak

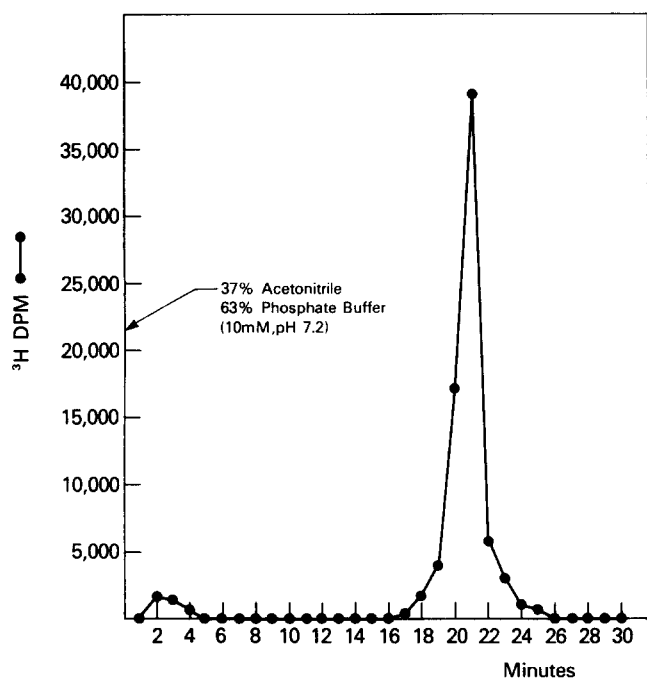


**Fig. 3.** Reversed-phase HPLC separation of standard retinoids. UV absorption was monitored at 340 nm for detection of 4-oxo RA, retinyl phosphate, 13-*cis* RA, all-*trans* RA, retinol, retinal, retinyl acetate, anhydroretinol, and retinyl palmitate. The quantity of each standard was 500–600 ng. The position of 5,6-epoxy RA is indicated by an arrow and is based on its retention time (7 min) from another study carried out by one of us (P.V.B.) under identical chromatographic conditions.

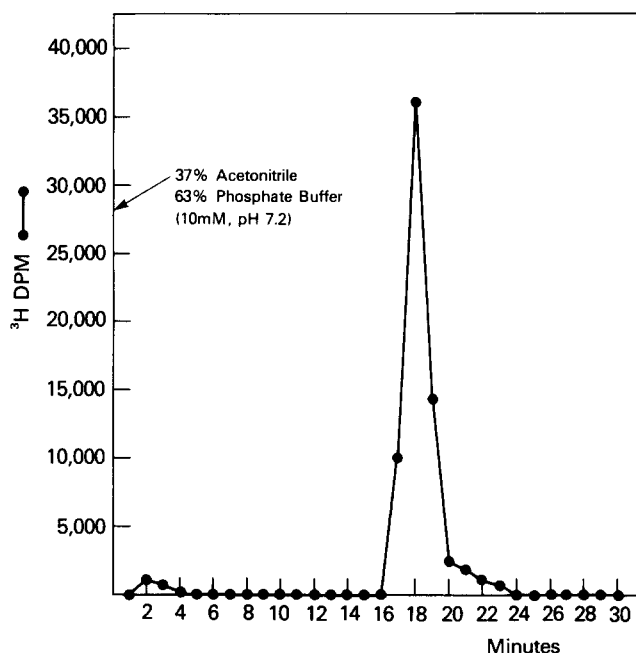
I) with only very small amounts of the total recovered radioactivity (6.2%) remaining as all-*trans* RA and minimal amounts (1.6%) in the fraction with retention time characteristic of 13-*cis* RA.

When [11-<sup>3</sup>H]-13-*cis* RA was injected into a vitamin A-sufficient rat, it was also found to be partially converted to a compound in the liver with retention time characteristic of all-*trans* RA at 0.5 hr after injection

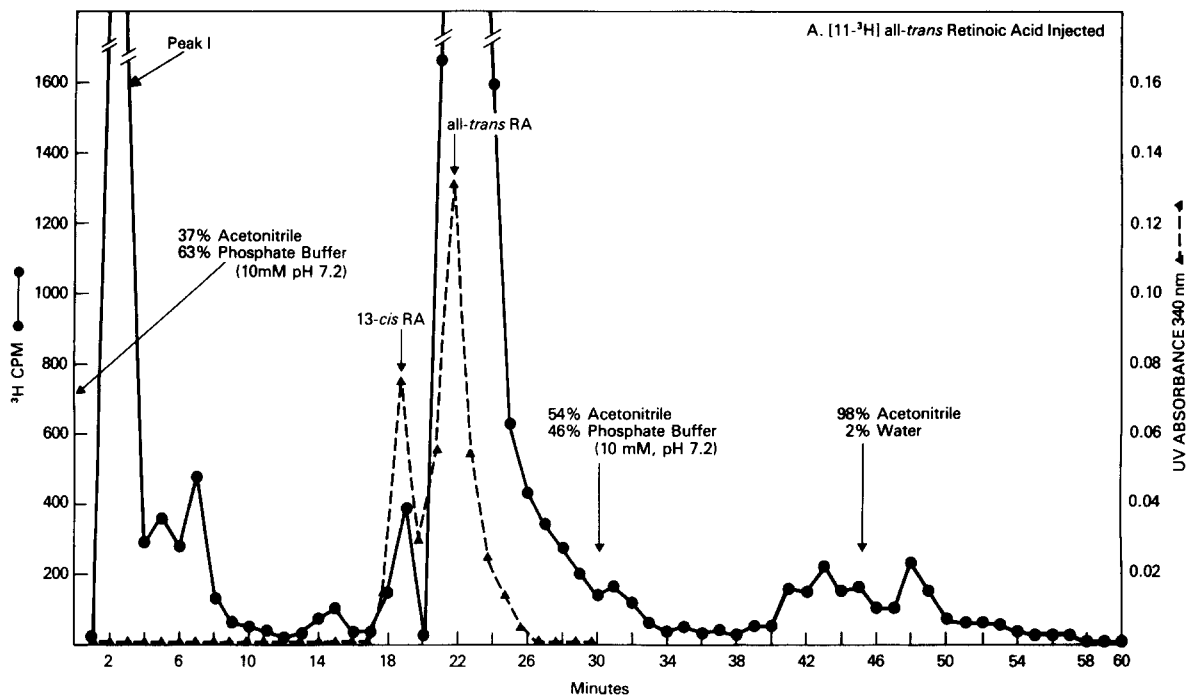
(12%) and to the highly polar metabolites represented by Peak I (14%). In addition, a second peak (Peak II, 22%) representing a compound of greater polarity than 5,6-epoxy all-*trans* RA (18) but less polar than 4-oxo RA was also observed (Fig. 7). At 3 hr after injection, appreciable radioactivity still remained in the 13-*cis* RA fraction (12.5%); a small amount (6.8%) was in a fraction with retention time characteristic of all-*trans* RA, indicating that the conversion of 13-*cis* RA to highly polar



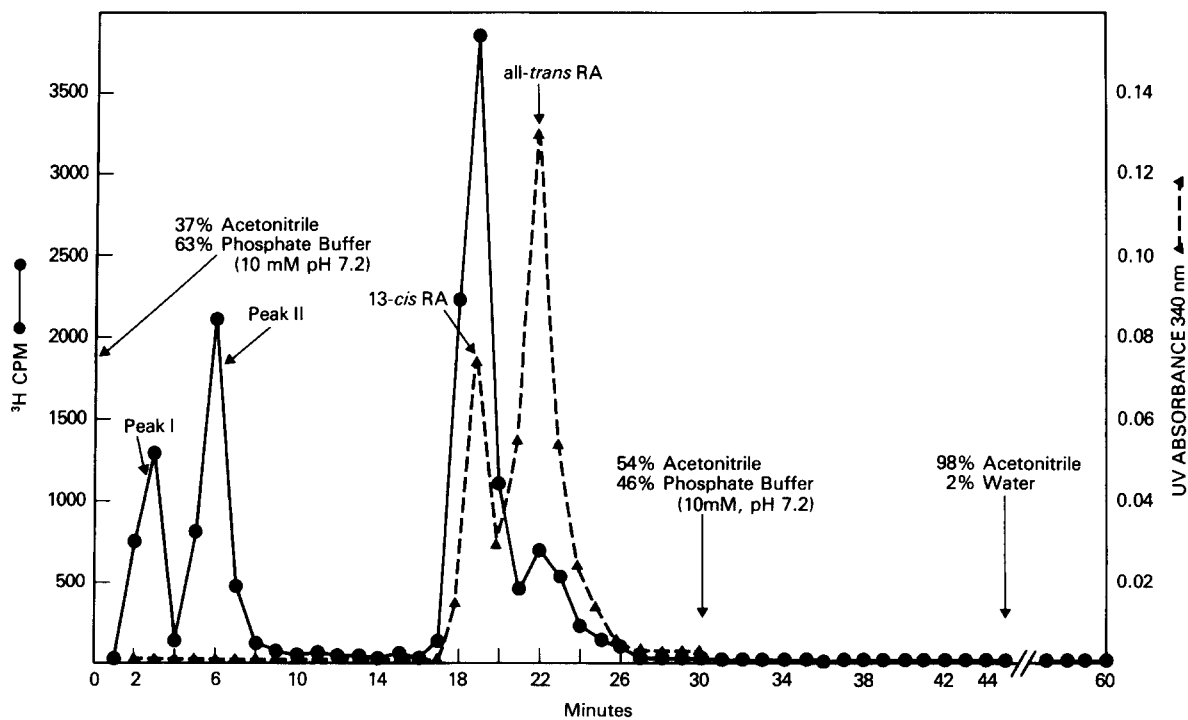
**Fig. 4.** Reversed-phase HPLC of rat liver extract after mixing 5  $\mu$ Ci of [10-<sup>3</sup>H]-all-*trans* RA with 1 g of rat liver and following the same extraction procedure used for the tissues of RA-injected animals.



**Fig. 5.** Reversed-phase HPLC of rat liver extract after mixing 5  $\mu$ Ci of [11-<sup>3</sup>H]-13-*cis* RA with 1 g of liver and following the same extraction procedure used for the tissues of RA-injected animals.



**Fig. 6.** Retinoid metabolism in vitamin A-sufficient rat liver  $\frac{1}{2}$  hr after injection of  $[11\text{-}^3\text{H}]\text{-all-trans}$  RA. Fifty  $\mu\text{Ci}$  of  $\text{all-trans}$  retinoic acid ( $5.4 \mu\text{g}$ ) in  $50 \mu\text{l}$  of ethanol was injected intraperitoneally into a vitamin A-sufficient rat. The rat was killed  $\frac{1}{2}$  hr after injection. Carrier  $\text{all-trans}$  and  $13\text{-cis}$  RA ( $500\text{--}800 \text{ ng}$  each) were added to an aliquot of the tissue extract containing  $20,000 \text{ cpm}$  and the aliquot was analyzed by HPLC as described.



**Fig. 7.** Retinoid metabolism in vitamin A-sufficient rat liver  $\frac{1}{2}$  hr after injection of  $[11\text{-}^3\text{H}]\text{-}13\text{-cis}$  RA. Fifty  $\mu\text{Ci}$  of  $13\text{-cis}$  retinoic acid ( $8.8 \mu\text{g}$ ) in  $50 \mu\text{l}$  of ethanol was injected intraperitoneally into a vitamin A-sufficient rat. The rat was killed  $\frac{1}{2}$  hr after injection. Carrier  $\text{all-trans}$  and  $13\text{-cis}$  RA ( $500\text{--}800 \text{ ng}$  each) were added to an aliquot containing  $20,000 \text{ cpm}$  and the aliquot was analyzed by HPLC as described.

TABLE 1. Co-crystallization of radioactive peak from rat liver with 13-*cis* RA after injection of [10-<sup>3</sup>H]-all-*trans* RA

Solution	Radioactivity in Peak Characteristic of 13- <i>cis</i> RA (dpm)	13- <i>cis</i> RA (absorbance at 350 nm)	% Recovery		Specific Activity (dpm/absorbance unit)
			[ <sup>3</sup> H]	13- <i>cis</i> RA	
Before crystallization	133,019	7.55	100	100	17,618
First crystallization	95,774	6.0	72	79	15,962
Second crystallization	93,114	5.9	70	78	15,782
Third crystallization	92,116	5.8	69	77	15,882

compounds is not as rapid as that of all-*trans* RA. Furthermore, the conversion of 13-*cis* RA into Peaks I and II metabolites increased substantially (Peak I, 40%; Peak II, 33%).

#### Identification of the radioactive peak with retention time characteristic of 13-*cis* RA as predominantly 13-*cis* RA

The eluate comprising the radioactive peak with the characteristic retention time of 13-*cis* RA in HPLC of the liver extract was collected, mixed with carrier 13-*cis* RA, and crystallized from 1% isopropanol in hexane solution. After the first crystallization, 72% of the total radioactivity of the peak and 79% of the added 13-*cis* RA were recovered, indicating that the radioactive peak formed in vivo after all-*trans* RA injection was predominantly 13-*cis* RA. The specific activity of the crystals (15,962 dpm/absorbance unit) remained constant after two more crystallizations, confirming that the main isomer formed in vivo was indeed 13-*cis* RA (Table 1). The remainder of total radioactivity of the peak (28%) probably represents other unidentified *cis* isomers.

#### Identification of the major component of the radioactive peak with retention time similar to all-*trans* RA as all-*trans* RA

The eluate comprising the radioactive peak with the characteristic retention time of all-*trans* RA was obtained after HPLC of the liver extract from the rat injected with [11-<sup>3</sup>H]-13-*cis* RA, mixed with carrier all-*trans* RA, and crystallized from 1% isopropanol in hexane. After the first crystallization, 95% of the radioactivity and 93% of the added all-*trans* RA were recovered (Table 2), indicating that the radioactive peak formed in vivo was mostly all-*trans* RA. In addition, the specific activity of the crystals (23,556 dpm/absorbance unit) remained constant upon recrystallization, confirming

that the main isomer formed in vivo after 13-*cis* RA injection was indeed all-*trans* RA (Table 2). The total radioactivity of the peak (5%) that did not co-crystallize with carrier all-*trans* RA was negligible.

#### Metabolism of RA in kidneys, small intestine, blood, and testes

Metabolites of 13-*cis* and all-*trans* RA were determined in blood, kidneys, small intestine, and testes (Table 3). The results obtained with the liver are also included in Table 3 for comparison. It is evident that at 0.5 hr after injection in all the tissues examined, with the exception of the small intestine, a small amount of all-*trans* RA was converted to 13-*cis* RA in amounts ranging from 0.5 ng/g in the testes to 5.8 ng/g in the liver. In addition, all-*trans* RA was rapidly converted to polar metabolite(s) (I) in all the tissues, the calculated range being 8.2 ng/g to 39.4 ng/g. At 3 hr after injection, polar metabolite(s) I accumulated in the tissues, with the small intestine retaining the highest amounts (283.9 ng/g). Polar metabolite(s) II also accumulated in this tissue, although to a lesser extent than polar metabolite I (Table 3).

#### Conversion of 13-*cis* RA to all-*trans* RA and to polar metabolites I and II

Injection of 13-*cis* RA yielded substantial amounts of all-*trans* RA. The liver was the most active tissue in this isomerization. In addition to polar metabolite(s) I as in the case of all-*trans* RA, a second polar metabolite(s) (metabolite II) was formed in all the tissues examined in amounts varying from 3.3 ng/g in the testes to 180.2 ng/g in the small intestine (Fig. 7).

At 3 hr after injection, substantial amounts of 13-*cis* RA could still be detected in the blood (9.3 ng/ml) and smaller amounts in other tissues. Polar metabolite(s) I increased in the small intestine and the testes, while it

TABLE 2. Co-crystallization of radioactive peak from rat liver extract with all-*trans* RA after injection of [11-<sup>3</sup>H]-13-*cis* RA

Solution	Radioactivity in Peak Characteristic of all- <i>trans</i> RA (dpm)	all- <i>trans</i> RA (absorbance at 350 nm)	% Recovery		Specific Activity (dpm/absorbance unit)
			[ <sup>3</sup> H]	all- <i>trans</i> RA	
Before crystallization	172,329	7.50	100	100	22,977
First crystallization	163,713	6.95	95	93	23,556
Second crystallization	161,989	6.9	94	92	23,477

TABLE 3. Distribution of radioactivity of fractions with retention times characteristic of all-*trans* RA and 13-*cis* RA and of polar metabolites I and II

Fraction	Retinoic Acid Equivalents in				
	Blood ng/ml	Liver ng/g	Kidneys ng/g	Intestine ng/g	Testes ng/g
A. All- <i>trans</i> RA injected (1/2 hr)					
All- <i>trans</i> RA	9.7 (44) <sup>a</sup>	115.3 (62.3)	89.1 (61.0)	48.9 (48.0)	7.7 (36.0)
13- <i>cis</i> RA <sup>b</sup>	1.5 (6.9)	5.8 (3.1)	5.3 (3.6)	— <sup>c</sup>	0.5 (2.4)
Polar metabolite I	8.2 (37.0)	33.5 (17.5)	39.4 (27.0)	29.6 (29.0)	10.2 (47.7)
Polar metabolite II			4.5 (3.1)	5.6 (5.1)	
B. All- <i>trans</i> RA injected (3 hr)					
All- <i>trans</i> RA	0.5 (4.3)	5.6 (6.2)	4.5 (4.8)	8.5 (2.5)	0.5 (4.1)
13- <i>cis</i> RA <sup>b</sup>	0.4 (3.6)	1.5 (1.6)			0.3 (2.2)
Polar metabolite I	9.1 (81.0)	78.8 (87.0)	83.8 (90.0)	283.9 (84.0)	9.5 (75.0)
Polar metabolite II				22.9 (6.8)	
C. 13- <i>cis</i> RA injected (1/2 hr)					
All- <i>trans</i> RA	18.1 (21.0)	23.2 (12.0)	12.5 (12.0)	10.6 (3.2)	0.8 (3.0)
13- <i>cis</i> RA	50.8 (59.0)	92.6 (48.0)	53.0 (51.0)	43.8 (13.2)	8.6 (32.0)
Polar metabolite I	7.2 (8.3)	27.0 (14.0)	14.6 (14.0)	86.1 (26.0)	8.0 (30.0)
Polar metabolite II	8.5 (9.9)	42.4 (22.0)	21.8 (21.0)	180.2 (54.4)	3.3 (12.4)
D. 13- <i>cis</i> RA injected (3 hr)					
All- <i>trans</i> RA	2.2 (9.8)	3.0 (6.8)	4.2 (6.7)	2.6 (0.7)	
13- <i>cis</i> RA	9.3 (41.0)	5.5 (12.5)	7.5 (12.1)	4.1 (1.1)	2.9 (10.0)
Polar metabolite I	3.4 (15.0)	17.6 (40.0)	14.3 (23.0)	177.1 (47.0)	13.4 (47.0)
Polar metabolite II	6.8 (30.0)	14.6 (33.0)	34.9 (56.0)	131.9 (35.0)	6.6 (23.0)

<sup>a</sup> Values in parentheses represent percent total radioactivity in each peak.

<sup>b</sup> The calculated amount of 13-*cis* RA represents 72% 13-*cis* RA and 28% of other unidentified *cis* isomers.

<sup>c</sup> Not detected.

decreased in the liver and blood or remained at the same level in the kidneys as at 0.5 hr. The amounts of polar metabolite(s) II, however, decreased in the blood, liver, and intestines but increased in the kidneys and the testes (Table 3).

## DISCUSSION

The results reported in the present study indicate for the first time that *cis-trans* isomerization of RA takes place reversibly in the tissues of the vitamin A-sufficient rat. The equilibrium of this reversible reaction probably lies toward all-*trans* RA, since the conversion of 13-*cis* RA to all-*trans* RA is greater than that of all-*trans* RA to 13-*cis* RA (Table 3). Previous workers have demonstrated *cis-trans* isomerization of retinol and retinal in the liver and retina of the rat, respectively (19–21). Whether the enzymes catalyzing these isomerizations are identical to those that catalyze *cis-trans* isomerization of RA is not known.

It is reasonable to assume that all-*trans* RA can be transformed in vivo to isomers besides 13-*cis* RA such as 9-*cis*, 11-*cis*, 9,13-di-*cis*, and 11,13-di-*cis* in a manner

analogous to the transformation of all-*trans* retinol. However, the results of our study indicate that the predominant isomer formed from all-*trans* RA in vivo is 13-*cis* RA (Table 1). Similarly, the isomer formed from 13-*cis* RA appears to be mainly all-*trans* RA (Table 2). The possibility of isomerization at the 7,8-*cis* double bond of all-*trans* RA in vivo is remote, since no such *cis* compound has been found to occur naturally either among the known carotenoids or retinoids, and this form of sterically hindered isomer is very labile (22).

In addition to undergoing *cis-trans* isomerization, all-*trans* RA is rapidly metabolized to polar metabolites represented by Peak I (Fig. 6 and Table 3). This peak probably includes phosphorylated derivatives of metabolites of RA, possibly similar to those of retinol, which have been found in intestine (23) and liver (24). In the case of the small intestines and kidney, only small quantities of Peak II are formed.

13-*cis* RA is also metabolized to polar metabolite(s) I. However, the formation of polar metabolite(s) II from 13-*cis* RA is appreciable and seems to be occurring in all the tissues examined. Polar metabolite II is eluted before 5,6-epoxy RA but only after 4-oxo RA (Fig. 7).

Thus, it is possible that this metabolite may include compounds such as 13-*cis* 5,6-epoxy RA. 5,6-Epoxy RA has been shown to be a normal metabolite of RA in vivo (25).

The possibility that non-specific transfer of  $^3\text{H}$  to other molecules may occur in vivo is unlikely since specifically labeled [ $^3\text{H}$ ]-all-*trans* and [ $^3\text{H}$ ]-13-*cis* compounds were employed in this study and very negligible radioactivity was present in non-lipid constituents of the tissues.

The demonstration of a mannosyl phosphoryl derivative of retinol (MRP) in vivo indicated a specialized function for MRP in the biosynthesis of specific glycoproteins (26). Whether retinoic acid can replace retinol in fulfilling such a function was investigated by studying the formation of mannosyl retinoid phosphate (MXP) in 3T12 cells. It was found that MXP was synthesized in 3T12 cells to the extent of 40% of the total metabolites at 40 hr after incubation (18). It may be necessary to initially separate the polar compounds of retinoic acid from nonpolar compounds by HPLC as outlined in the present study and obtain further resolution of the polar compounds before MXP occurring in vivo can be isolated and characterized. ■

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