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Formation of Retinoic Acid from Retinol in the Rat

By R. J. EMERICK,* MAIJA ZILE and H. F. DeLUCA
Department of Biochemistry, University of Wisconsin, Madison, Wis. 53706, U.S.A.

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1. The formation *in vivo* of retinoic acid from microgram quantities of intrajugularly administered [15-^{14}C]retinol was demonstrated in the rat. 2. Endogenously formed retinoic acid (about 0·1 μg./rat) was found in liver, and to a much smaller extent in intestine, 12hr. after retinol administration. 3. Excretion of some of the endogenously formed retinoic acid occurred in the bile of bile-duct-cannulated rats. 4. Excretion of unaltered retinoic acid in the urine of intact rats did not occur even after the intrajugular administration of preformed retinoic acid.

Retinoic acid supports growth (Arens & van Dorp, 1946; Dowling & Wald, 1960) but not the visual cycle (Dowling & Wald, 1960) or reproduction (Howell, Thompson & Pitt, 1964; Thompson, 1964) in animals consuming diets otherwise devoid of vitamin A. Little if any retinoic acid is found in tissues a few hours after relatively large (milligram) doses (Sharman, 1949; Yagishita, Sundaresan & Wolf, 1964; Redfearn, 1960; Zile & DeLuca, 1965; Jurkowitz, 1962; Krishnamurthy, Bieri & Andrews, 1963), and the rapid excretion of retinoic acid and its metabolites in the bile of bile-duct-cannulated rats has been demonstrated (Dunagin, Meadows & Olson, 1965; Zachman, Dunagin & Olson, 1966).

Although some attempts to demonstrate retinoic acid formation from retinol or retinal *in vivo* have been unsuccessful (Krishnamurthy *et al.* 1963; Wright, 1960), the parenteral administration of milligram quantities of retinal has been shown to result in the rapid appearance of small amounts of retinoic acid in rat tissues (Deshmukh, Malathi & Ganguly, 1965) and bile (Dunagin, Zachman & Olson, 1964). Whether retinoic acid is formed only in systems in which abnormally large amounts of the aldehyde retinal are introduced, or is a normal product of retinol metabolism, has heretofore been a subject of speculation. The results reported in the present paper provide conclusive evidence for the formation of retinoic acid from microgram quantities of retinol *in vivo*.

MATERIALS AND METHODS

Chemicals. The all-*trans* and *cis* isomers of retinoic acid and retinol were obtained from Distillation Products Industries, Rochester, N.Y., U.S.A. The *cis* isomers of retinoic acid, *cis* isomers of retinol and [15-^{14}C]retinoic acid were gifts from Hoffmann–La Roche and Co., Basle, Switzerland, and Nutley, N.J., U.S.A. [15-^{14}C]Retinyl acetate and [15-^{14}C]retinol were obtained from Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A. Chromatography of [15-^{14}C]retinol on a silicic acid column under the conditions described below yielded a major radioactive fraction that co-chromatographed with non-radioactive all-*trans*-retinol, and a very minor fraction that was eluted slightly earlier and previously shown to include *cis* isomers of retinol. No trace of retinoic acid was found.

Skelly B, a petroleum fraction, was redistilled at 65–66°. All other solvents and chemicals were A.R. grade.

Animals. Male albino Holtzman rats were obtained at weaning and placed on a vitamin A-deficient diet (DeLuca, Manatt, Madsen & Olson, 1963). After about 4 weeks, when they ceased to gain in weight, they were given thrice-weekly oral supplements of 100 μg. of retinoic acid in cottonseed oil until they were used in the experiments about 2–3 weeks later, when they weighed 225–280g.

Administration and collection of radioactive materials. The radioactive vitamin A compounds used in these experiments were administered by injection into the jugular vein, under light ether anaesthesia, in 0·25ml. of an aqueous suspension containing 20% of ethanol, 0·1% of Tween 40 (polyoxyethylene sorbitan monopalmitate) and 0·9% of NaCl. For bile-duct-cannulated rats, the doses were administered approx. 12hr. after the operation.

Bile or urine, when obtained, was collected in vials maintained in an ice bath at 0°. Approx. 25 μg. of all-*trans*-retinoic acid and 0·25mg. of α-tocopherol in 0·1ml. of chloroform were added to each of the collection vials initially. The materials collected were transferred once (urine) or twice (bile) daily to a freezer at −17° and stored until further treatment. Various tissues were obtained 12hr. after administration of the radioactive vitamin A.

Treatment with β-glucuronidase. Diluted bile or urine, to which 230–700 μg. of carrier all-*trans*-retinoic acid and 2–7mg. of α-tocopherol in 1–3ml. of chloroform had been added, was adjusted to pH7 with 0·66M-KH_2PO_4 and

* Present address: Station Biochemistry Department, South Dakota State University, Brookings, S.Dak., U.S.A.

0·66M-Na_2HPO_4 (0·1M-phosphate final concentration). After addition of 8000 units (bile, one rat) or 25000 units (bile, four rats; urine, two rats) of β-glucuronidase (bacterial, type 2; Sigma Chemical Co., St Louis, Mo., U.S.A.) the mixtures were incubated in the dark at 37° for 2hr. under N_2.

Extraction of metabolites. Diluted bile or urine previously treated with β-glucuronidase, or an aqueous 25% homogenate of liver or intestine, was extracted by shaking it in a separatory funnel with methanol–chloroform (water–methanol–chloroform; 0·8:2:1, by vol.). An additional volume each of water and chloroform was added to the monophase mixture to facilitate a separation into two phases (Bligh & Dyer, 1959). The chloroform phase was removed, evaporated to dryness under reduced pressure and redissolved in Skelly B before chromatography.

Saponification of biological materials. Extracts or fractions, when saponified, were made to 5% (w/v) KOH in approx. 50% (v/v) aqueous methanol and refluxed under N_2 for 30min. The alkaline saponification mixture was transferred to a separatory funnel and extracted with 1vol. of chloroform for recovery of retinol, or acidified to pH3 with HCl and re-extracted with 1vol. of chloroform for recovery of retinoic acid. Both chloroform extracts were washed with aqueous 50% (v/v) methanol, and portions were counted to determine radioactivity. Carrier retinol or retinoic acid added to the mixture before saponification was recovered in the chloroform extracts.

Chromatography on silicic acid columns. The column used for all chromatographic separations in these studies was a multibore column having a total length of 60cm. with four sections varying in diameter from 13mm. for the top section to 4·5mm. for the bottom section, with an approximate ratio 1·4:1 for adjacent sections (Fischer & Kabara, 1964). Columns were prepared from a slurry of 15g. of <325 mesh, heat-activated, silicic acid (Bio-Rad silicic acid; Calbiochem Corp., Los Angeles, Calif., U.S.A.) in Skelly B. Extracts of bile, urine or tissue were placed on the column in Skelly B and chromatographed with a 250ml. convex-gradient elution (Bock & Ling, 1954) consisting of Skelly B to 15% (v/v) diethyl ether in Skelly B (gradient 1). A second 250ml. gradient, when used, consisted of 15–35% (v/v) diethyl ether in Skelly B unless otherwise indicated. Columns were developed in the cold (approx. 5°) under 8lb./in.^{2} pressure with a flow rate of about 1ml./min., and fractions of 10ml. volume were collected.

Partitioning of acidic and non-acidic fractions. The acidic and non-acidic components in extracts or chromatographic eluates were separated by partitioning them between equal volumes of diethyl ether and 0·1N-NaOH. The organic phase containing non-acidic materials was removed. After being washed with another volume of diethyl ether, the aqueous phase was acidified to about pH3 with 2N-HCl and extracted with 2vol. of diethyl ether. This ether extract contained all of the carrier retinoic acid originally present.

Reduction of retinoic acid with lithium aluminium hydride. Isolated radioactive and carrier retinoic acids were reduced with $LiAlH_4$ by the method described by Wendler, Slates, Trenner & Tishler (1951). Yields of 40–80% retinol were obtained.

Determination of radioactivity. Measurements of ^{14}C were made in a Packard model 314EX liquid-scintillation spectrometer. A toluene counting solution (Herberg, 1960) giving a counting efficiency of 60–63% was used for samples in the organic phase. A dioxan counting solution (Zile & DeLuca, 1965), modified by omitting the thixotropic gel, was used for aqueous samples (counting efficiency 30–35%). For bile or urine, 0·1ml. fractions were counted; 1–2ml. portions or the entire volume of chromatographic fractions were counted. If the entire fraction was used, it was evaporated to dryness before the addition of counting solution.

Spectrophotometric measurements. Extinction measurements were made of chromatographic fractions with a Beckman model DU spectrophotometer. Ultraviolet spectra were determined in ethanol or hexane with a Cary model 15 recording spectrophotometer. Spectra were obtained for all vitamin A compounds immediately before use and for all peak fractions of metabolites obtained by chromatography.

RESULTS

Identification of retinoic acid in liver and intestine after retinol administration. Chloroform–methanol extraction of livers or intestines obtained from three rats 12hr. after the intrajugular administration of 54μg. of [15-^{14}C]retinol yielded most of the radioactivity in the chloroform phase, as shown in Table 1. In the initial chromatography of the extracts with added carrier retinoic acid on silicic acid columns, a fraction containing principally radioactive retinol esters trailed and tended to

Table 1. *Retinoic acid present in tissues after intrajugular administration of* [15-^{14}C]*retinol*

Tissue*	No. of pooled rats	Radioactivity (% of dose)	Radioactivity extracted with chloroform (%)	Endogenous retinoic acid (% of dose†)	Endogenous retinoic acid (μg./rat)
Liver	3	39	99	0·20	0·11
Intestine	3	1	67	0·03	0·02
Bile‡	4	12	51§	0·36	0·18

* Liver and intestine obtained 12hr. after dosage.
† Approx. 50μg. doses.
‡ Bile collected for average of 80hr. after dosage.
§ After glucuronidase treatment.

overshadow the small amounts of radioactive retinoic acid that may have been present. However, rechromatography of combined fractions containing the retinoic acid from the acidic portion resulted in identical elution patterns for carrier retinoic acid and associated radioactivity as shown in Fig. 1 for liver. Ultraviolet spectra further confirmed that this fraction was retinoic acid. Similar data of a lower magnitude were obtained with the intestinal preparation.

Reduction of the liver retinoic acid fraction with lithium aluminium hydride and subsequent chromatography of the products on silicic acid showed that the radioactivity co-chromatographing with carrier retinoic acid before reduction now co-chromatographed with the resultant retinol (Fig. 2). The latter was eluted in two fractions in the second gradient.

The two peaks representing *cis* (peak 1) and all-*trans* (peak 2) isomers of retinol had u.v.-spectral characteristics of 13-*cis*-retinol (328mμ) and all-*trans*-retinol (325mμ) respectively. The two-peak elution pattern obtained in this chromatogram was reproduced by reduction of a similarly small quantity of light-treated retinoic acid (all-*trans*-retinoic acid exposed to laboratory light for 2hr.). Chromatography of pure isomers of retinol showed that 13-*cis*-retinol is eluted with and is indistinguishable from the peak of *cis*-retinol formed in this manner. However, 9,13-di-*cis*-retinol is also eluted in this area and its separation from 13-*cis*-retinol is incomplete under these conditions. Robeson & Baxter (1947) have shown 13-*cis*-retinol to be more resistant to oxidation than all-*trans*-retinol. This may explain the relative predominance of the *cis* isomers in these preparations.

The specific activity 2274 disintegrations/min./μmole of retinoic acid before reduction remained essentially unchanged (2343 disintegrations/min./μmole) after being reduced and rechromatographed as retinol. From these data, the amount of endogenously formed retinoic acid present in the rat livers amounted to 0·2% of the dose or 0·11μg./rat, and that present in intestine was only about 15% of the amount found in liver.

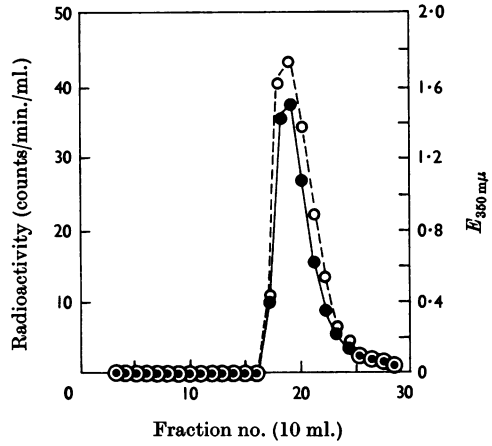

Fig. 1. Chromatography of liver retinoic acid and carrier isolated from three rats given 54μg. of [15-^{14}C]retinol 12hr. previously. ●, E_{350}; ○, radioactivity.

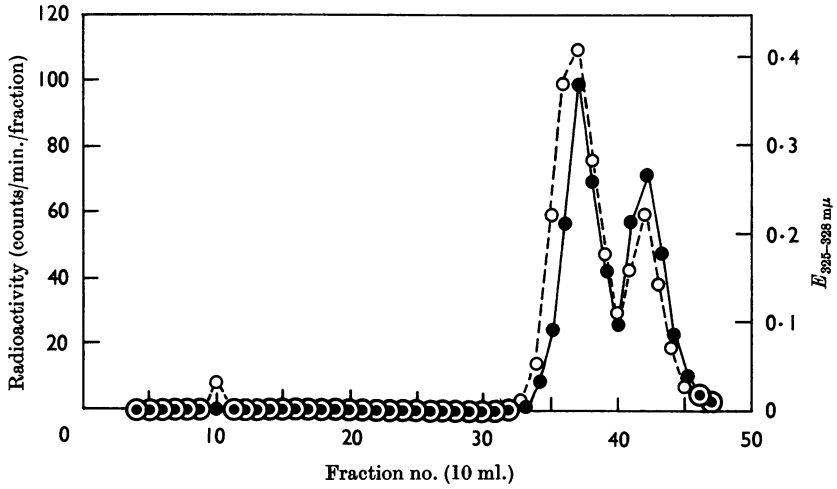

Fig. 2. Chromatography of isolated liver retinoic acid and carrier (peak of Fig. 1) after reduction to retinol. ●, $E_{325-328}$; ○, radioactivity. The two-peak elution pattern represents *cis* (peak 1) and *trans* (peak 2) isomers of retinol.

Excretion of endogenously formed retinoic acid in bile after administration of retinol. Four bile-duct-cannulated rats injected with 48–54μg. of [15-^{14}C]-retinol gave an average bile flow of 0·6ml./rat/hr. Biliary excretion of the radioactivity during the 70–94hr. (average 80hr.) collection period amounted to 11·0–14·3% (average 12·1%) of the administered radioactivity. After 36–40hr., excretion of radioactivity in the bile appeared to be linear and was equivalent to approx. 1μg. of the injected retinol/24hr. Treatment of the entire collection of bile with β-glucuronidase and extraction with chloroform–methanol gave 51% of the radioactivity in the chloroform phase (Table 1). The material contained in the chloroform phase, including the non-radioactive all-*trans*-retinoic acid carrier added during the collection period, was chromatographed on a silicic acid column. In accordance with the procedure used for tissue extracts, the acidic fraction obtained by partitioning the retinoic acid peak against 0·1N-sodium hydroxide was rechromatographed. This chromatogram is shown in Fig. 3. With the exception of a minor shoulder on the trailing edge, radioactivity associated with the peak chromatographed very similarly to the carrier retinoic acid.

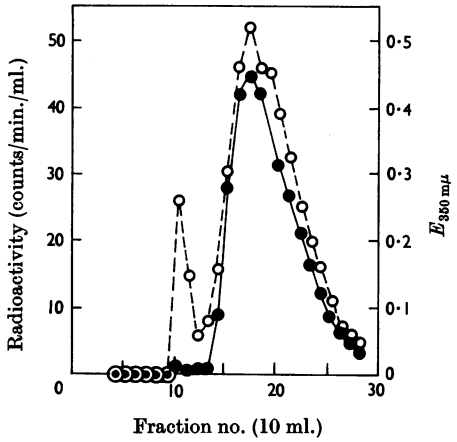

Fig. 3. Chromatography of biliary retinoic acid and carrier isolated from bile obtained from four bile-duct-cannulated rats during an 80hr. period after the intrajugular administration of 48–54μg. of [15-^{14}C]retinol. ●, E_{350}; ○, radioactivity.

After reduction of the retinoic acid fraction and associated radioactivity with lithium aluminium hydride, the radioactivity previously appearing as retinoic acid now co-chromatographed with the resultant retinol. The two-peak (*cis* and *trans* isomers) elution pattern, as shown in Fig. 4, was the same as that obtained with similar preparations from liver, as described earlier.

Before reduction, the retinoic acid fraction had a specific activity 9120 disintegrations/min./μmole of retinoic acid. After reduction, specific activity of the resultant retinol (both peaks; Fig. 4) was 9180 disintegrations/min./μmole. From the final recovery of the carrier retinoic acid as retinol, endogenous retinoic acid excreted in the bile over

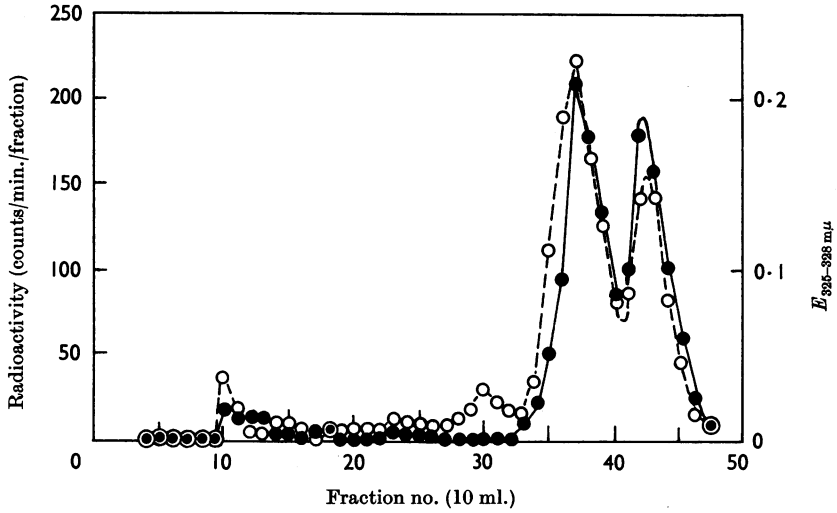

Fig. 4. Chromatography of isolated biliary retinoic acid and carrier (peak of Fig. 3) after reduction to retinol. ●, $E_{325-328}$; ○, radioactivity. The two-peak elution pattern represents *cis* (peak 1) and *trans* (peak 2) isomers of retinol.

Table 2. *Excretion of* ^{14}C *in urine after intrajugular injection of* [15-^{14}C]*retinoic acid*

Rat no.	Percentage of administered ^{14}C recovered in urine		
	0–24hr.	24–48hr.	48–72hr.
1	31·4	2·2	1·0
2	22·9	2·2	1·1
Average	27·1	2·2	1·0

an 80hr. period was equivalent to 0·36% of the administered dose of retinol. This compared very favourably with the amount of retinoic acid that appeared to be formed from a comparable dosage of retinyl acetate in a preliminary study (not reported).

Retinol excreted in bile. The non-acidic fraction of the major bile peak obtained during the initial bile chromatography was saponified in methanolic potassium hydroxide. The radioactivity associated with this fraction remained non-acidic and ether-soluble after saponification. On silicic acid, the radioactivity subsequently chromatographed in close association with all-*trans*-retinol. From the recovery of carrier retinol, which was added before saponification, radioactive retinol present in the bile in a saponifiable form was equivalent to 0·23% of the administered [15-^{14}C]retinol.

Examination of urine for retinoic acid excretion. Three 24hr. urine samples were collected from two rats after intrajugular injection of 58 μg. of [15-^{14}C]-retinoic acid. Excretion data are shown in Table 2. An average of 27·1% of the administered radioactivity was recovered in the urine during the first 24hr. This was about 10 times the amount excreted during the next 48hr. In view of its high radioactivity, only the urine excreted during the first 24hr. was fractionated to determine the extent of retinoic acid excretion via the urine.

After treatment with β-glucuronidase and extraction with chloroform–methanol, 9% of the radioactivity remained in the chloroform phase. Chromatography of this fraction on silicic acid yielded a sharp peak of radioactivity chromatographing with carrier retinoic acid, but it was of such a small magnitude (equivalent to about 0·02% of dose) that it could not be characterized further. Saponification of the aqueous methanol phase yielded no additional chloroform-soluble radioactivity that could be eluted from silicic acid with gradient 1.

DISCUSSION

The presence of retinoic acid as a product of retinol metabolism *in vivo* has been conclusively demonstrated in liver and intestine of intact rats and in the bile of bile-duct-cannulated rats. A quantity of retinoic acid approximating to a total of 0·13 μg./rat was found in liver and intestine combined 12hr. after retinol administration. Of this amount, about 85% was found in the liver.

From reports of Sharman (1949), Yagishita *et al.* (1964), Zile & DeLuca (1965) and others on the rapid disappearance of administered retinoic acid, a rapid turnover rate for retinoic acid in animal tissues is certain. In view of the apparent rapid turnover rate of retinoic acid, the approx. 0·13 μg. of retinoic acid/rat found in liver and intestine is consistent with the well-established critical dose range 0·15–1·2 μg. of retinol/rat/day (Harris, 1960), and is of a magnitude that indicates that metabolism of retinol to retinoic acid may represent a major metabolic pathway that could be involved in the manifestation of its growth-promoting activity.

Retinoic acid, approximating to 0·36% of a 50 μg. dose in 69–80hr., was excreted in the bile after the intrajugular administration of [15-^{14}C]retinol. The data of Zachman *et al.* (1966), indicating biliary excretion of unaltered retinoic acid or its glucuronic acid conjugate in quantities representing only a portion of an injected dose of retinoic acid, suggest that the retinoic acid recovered in the current experiments from administered retinol may represent only a fraction of that formed *in vivo*. The presence of retinoic acid in bile or animal tissues as a normal product of retinol metabolism has not been previously reported.

No identifiable amount of retinoic acid was found in the urine of intact rats after the intrajugular injection of 58 μg. of [15-^{14}C]retinoic acid. However, 27 and 3·2% of the radioactivity was excreted in the urine in the first 24hr. and in the next 48hr. respectively. The urinary excretory products appeared largely as water-soluble non-saponifiable metabolites. Although Zachman *et al.* (1966) recovered 95% of the radioactivity from injected [6,7-$^{14}C_2$]retinoic acid in bile of bile-duct-cannulated rats, they reported that retinoic acid metabolites previously excreted in the bile can be reabsorbed and converted into more polar products, which presumably could be excreted in the urine of intact rats. Owing to the failure to find retinoic acid in the urine of intact rats after administration of preformed retinoic acid, no attempt was made to detect urinary retinoic acid after retinol administration.

In the present study, only 12·1% of the radioactivity of the [15-^{14}C]retinol dose was recovered in the bile in 80hr. In contrast, Zachman *et al.* (1966) used [6,7-$^{14}C_2$]retinol and recovered 18–25% of the label in bile in 24hr. This difference is explained to some degree by the data obtained by

Roberts & DeLuca (1967), which show that, in comparison with the C-6 and C-7 positions, a greater portion of the terminal (C-15) carbon of vitamin A compounds is recovered in expired carbon dioxide.

The presence of retinol in bile, based on a non-specific colorimetric assay, has been reported by Bochdal & Hruba (1962). However, bile pooled from four rats injected with [15-^{14}C]retinol contained only a small quantity (0·23% of the dose in 80hr.) of a compound that chromatographed in gradient 1 as a retinol ester before saponification, and chromatographed with carrier retinol in gradient 2 after saponification. Similarly, Zachman *et al.* (1966) have reported that 1% of the radioactivity of bile metabolites from vitamin A compounds chromatographed with carrier retinol on alumina.

These data demonstrate conclusively that retinoic acid is formed as a normal metabolite of retinol metabolism in the rat, and is present in liver and to a lesser degree in intestine. Although bile and urine may contain similar polar fractions originating from vitamin A, only bile in these studies appeared to contain an identifiable amount of excreted retinoic acid that had not undergone major molecular alterations. That excretion of retinoic acid or retinol in the bile may represent a conservation of these compounds appears unlikely in view of the relatively small amounts present.

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