Metabolism of All-trans-Retinoic Acid and All-trans-Retinyl Acetate

DEMONSTRATION OF COMMON PHYSIOLOGICAL METABOLITES IN RAT SMALL INTESTINAL MUCOSA AND CIRCULATION*

(Received for publication, February 12, 1985)

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The kinetics and metabolism of physiological doses of all-trans-retinoic acid were examined in blood and small intestinal mucosa of vitamin A-depleted rats. A major portion of intrajugularly injected retinoic acid is rapidly (within 2 min) sequestered by tissues; subsequently 13-cis-retinoic acid and polar metabolites are released into circulation. All-trans-retinoic acid appears in small intestinal epithelium within 2 min after dosing and is the major radioactive compound there for at least 2 h. Retinoyl glucuronide and 13-cis-retinoic acid are early metabolites of all-trans-retinoic acid in the small intestine of bile duct-cannulated rats. Retinoyl glucuronide, the major metabolite of retinoic acid in intestinal epithelium, in contrast to other polar metabolites, was not detected in circulation.

An examination of [\textsuperscript{3}H]retinyl acetate metabolites under steady state conditions in vitamin A-repleted rats demonstrates the occurrence of all-trans-retinoic acid and 13-cis-retinoic acid in circulation and in intestinal epithelium, in a pattern similar to that found after injection of retinoic acid into vitamin A-depleted rats. Our data establish that all-trans-retinoic acid, 13-cis-retinoic acid, and retinoyl glucuronide are physiological metabolites of vitamin A in target tissues, and therefore are important candidates as mediators of the biological effect of the vitamin.

Retinoic acid supports the growth and differentiation functions of vitamin A (for reviews see Refs. 1–3) and thus may be the form of vitamin A required for these important somatic functions. Evidence for this view includes the existence of a cellular receptor protein for retinoic acid in vitamin A target tissues (4) and the in vitro demonstration that in differentiation retinoic acid is 100–1000 times more potent than any other known natural form of vitamin A (2).

Although retinoic acid metabolism has been examined in many in vivo animal models (3, 5–11), it is not clear what relationship this might have to actual in vivo conditions at steady state. The demonstration of retinoic acid and its metabolites arising from dietary vitamin A in a normally nourished vitamin A-sufficient animal is essential in order to evaluate biologically active (growth-promoting) metabolites of retinoic acid. Retinoic acid and 5,6-epoxyretinoic acid, a biologically inactive metabolite (12), have been shown to be endogenous metabolites of vitamin A in normal rat kidney (13). The demonstration of endogenous retinoic acid in circulation and in other vitamin A target tissues is lacking; similarly, no data are available on the occurrence of the biologically active retinoic acid metabolites, 13-cis-retinoic acid and retinyl glucuronide (1-D-retinyl-β-D-glucopyranuronic acid), in target tissues of vitamin A-sufficient animals at steady state conditions.

Our studies (14, 15) and subsequent work by others (11, 16, 17) have demonstrated that a various tissues, particularly the small intestinal epithelium, contain significant amounts of 13-cis-retinoic acid. Since 13-cis-retinoic acid is biologically as active as all-trans-retinoic acid (18), 13-cis isomerization of retinoic acid may be associated with biological function. At this time no biochemical information is available on isomerization of retinoic acid in vivo.

A newly characterized metabolite of retinoic acid is retinyl glucuronide (3, 9, 14), originally isolated from bile by Lippel and Olson (19) and recently shown to be the predominant metabolite of retinoic acid in small intestinal epithelium of bile duct cannulated rats (14). Retinyl glucuronide has been demonstrated to be more active than retinoic acid or retinol in modulating differentiation in keratinizing vaginal epithelium (20) and in supporting growth in rats. These findings and subsequent confirmations of occurrence of retinyl glucuronide in other tissues (11) support the concept that retinyl glucuronide may have a pivotal role in metabolism of retinoic acid.

In studies reported here we have focused on identification of the bioactive forms of retinoic acid, specifically, 13-cis-retinoic acid and retinyl glucuronides. We present here, for the first time, evidence that all-trans-retinoic acid, 13-cis-retinoic acid, and retinyl glucuronides are normal vitamin A metabolites in the small intestine under vitamin A steady state conditions. We also demonstrate that all-trans- and 13-cis-retinoic acids are present in circulation and thus are clearly endogenous vitamin A metabolites. Our comparative studies with administered physiological doses of retinoic acid to vitamin A-depleted rats support the view that 13-cis isomerization and glucuronidation of retinoic acid are physiological transformations that are on a metabolic pathway common to retinoic acid and to the reduced forms of vitamin A existing in the body.

EXPERIMENTAL PROCEDURES

Retinoids; Radioisotope Purification; Preparation of Doses—All-trans-[\textsuperscript{11}H]retinoic acid, 1.51 Ci/mmol, was supplied by SRI, Menlo

* This work was supported by United States Department of Agriculture Grants 81-CRCR-1-0700 and 84-CRCR-1-1377 and in part by a grant from Michigan Agricultural Experimental Station. This is Michigan Agricultural Experimental Station Article 11531. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Park, CA. Typically, the preparation was 85-97% all-trans-retinoic acid (RA) upon arrival. Purification was carried out under gold fluorescent lights and in an N₂ atmosphere. Toluene carrier was evaporated and the radioactive retinoic acid was dissolved in a small volume of methanol and purified by HPLC on an analytical reverse-phase column (Whatman ODS-2, 10/25) using mixtures of water and methanol as eluents. The purity was checked by thin layer chromatography (TLC). This system resolves all-trans-retinoic acid from its isomers and other vitamin A compounds. Fractions corresponding to all-trans-retinoic acid were pooled, reduced to dryness by vacuum rotary evaporation, and dissolved in dimethyl sulfoxide or ethanol to give a final concentration of 10 μg of RA/50 μl. A small aliquot was rechromatographed to check purity after removal of dimethyl sulfoxide by lyophilization.

Unlabelled vitamin A compounds and retinoid standards were checked for purity and if necessary, purified as described under "Chromatography."  

All-trans-[11-^H]retinyl acetate, 3.49 Ci/mmol, was obtained from the National Cancer Institute (NCI) and purified as described under "Chromatography." It was found to be 95% pure; impurities were associated with unidentified polar metabolites, eluting at solvent front.

Retinyl acetate for oral dosing was prepared by combining purified all-trans-[11-^H]retinyl acetate with unlabeled crystalline all-trans-retinyl acetate (gift from Hoffmann-La Roche, Inc.) and dissolving them in corn oil.

Labeled retinyl glucuronide was obtained from tissues and bile of stock rats injected with 3 mg of all-trans-retinoic acid containing all-trans-[11-^H]retinoic acid, as described previously (21).

The following unlabeled retinoid standards were used: all-trans-retinol, all-trans-retinyl acetate, all-trans-retinyl palmitate, all-trans-4-oxoretinoic acid, all-trans-5,6-epoxyretinoic acid and 13-cis-retinoic acid (all gifts from Hoffmann-La Roche, Inc.); 4-hydroxyphenylretinamide and 13-cis-ethylretinamide (13-cis-ER) were gifts from NCI. All-trans-retinoic acid was purchased from Eastman. All retinoids were stored under nitrogen at −70°C. All-trans-retinyl linoleate, all-trans-retinyl oleate, and all-trans-retinyl stearate were synthesized from all-trans-retinyl acetate and 3-acyl anhydrides.

Chemicals and Solvents—All chemicals and solvents were of reagent or HPLC grade. Butylated hydroxytoluene, n-propyl gallate, dimethyl sulfoxide, and Na₂EDTA were purchased from Sigma.

Animals—Weanling 20-day-old male Sprague-Davley rats (Holzman Co., Madison, WI) were fed an AIN-76 semipurified diet (22), deficient in vitamin A. Weanling rats consuming this diet are able to utilize their liver stores of vitamin A and develop at a nearly normal rate for 4–6 weeks. At this time their liver vitamin A is depleted, the serum vitamin A concentration has decreased to about one-third of normal, and the animals cease to gain weight (weight plateaus). They are very sensitive to retinoid deficiency (14, 17, 18). Rats were used for experiments when they reached weight plateau stage.

For studies of retinoic acid metabolism, rats were fasted overnight, and then their bile ducts were cannulated under light ether anesthesia. A small dose of all-trans-[1-^H]retinoic acid (8.3–18.5 μCi/μmol), was injected intrajejunally in 50 ml of dimethyl sulfoxide. Rats were retained in restraining cages, their bile was collected under N₂, and their bile ducts were retained in restraining cages, their bile was collected under N₂, and they were killed by cervical dislocation 2, 15, 30, and 45 min after dosing. For studies of vitamin A metabolism under steady state conditions rats were given daily for 14 days by gavage 20 μg of ^H-labeled all-trans-retinyl acetate, specific activity, 476,200 dpm/μg. 70.4 4C\textsuperscript{1} μCi/μmol, in 0.1 ml of corn oil. Rats were killed 48 h after the last oral dose of retinyl acetate. Prior to killing rats were fasted overnight, the bile ducts were cannulated, and bile was collected for 2 h.

Preparation of Extracts of Tissues, Sera, and Bile for Analysis by HPLC—After the rats were killed, blood was collected in tubes containing 0.1 mg of n-propyl gallate, allowed to clot at room temperature for 30 min, and then centrifuged to separate serum and red blood cell fraction. Small intestine was removed, rinsed with ice cold saline, and cut open, and the mucosa was scraped away from the muscle layer with a blunted glass slide. Mucosa, muscle layer, and red blood cell fraction were each homogenized with 2 parts of an aqueous solution containing EDTA and n-propyl gallate, 0.02 mg/ml, and lyophilized. Serum and bile were also lyophilized. The dry samples were extracted twice with methanol and twice with n-hexane in presence of butylated hydroxytoluene, 0.02 mg/ml, and the extracts were evaporated under reduced pressure. In some cases it was necessary to purify the sample by passing it through a Sep-Pak C₁₈ precolumn (Waters Associates). Extracts from samples of retinoic acid and retinoid studies were redissolved in methanol on HPLC. Extracts obtained from samples of retinyl acetate metabolism studies were redissolved in methanolacetone (1:1) and injected on HPLC.

Chromatography—HPLC was accomplished on a C₁₈ reverse-phase column (Whatman ODS-2, 10/25, 25 x 0.46 cm) with a precolumn (7.7 x 0.46 cm containing Whatman C₁₈ Cell ODs pellicular pack). Step gradient elution of retinoid standards (100 ng/2 μl) and biological samples (100–200 μl) was accomplished with mixtures of water and methanol (v/v) containing 0.01 M ammonium acetate and mixtures of CHCl₃ and methanol. The solvent sequence was as follows: H₂O:MeOH (30:70), 11 min; H₂O:MeOH (20:80), 11 min; H₂O:MeOH (12:88), 8 min; and MeOH:CHCl₃ (85:15), 10 min. Fig. 1 illustrates separation of various retinoid standards. UV absorbance was monitored with a Waters model 440 detector (Millipore Waters, Milford, MA).

The eluate was collected in 0.8-ml fractions, dried with a stream of air, redissolved in 0.1 ml of MeOH and ACS scintillation cocktail (Amersham Corp.), and counted in a liquid scintillation spectrophotometer (Packard 3310, Packard Instrument Co.). Radioactivity was plotted as a histogram; identity of radioactive metabolites was established by coelution with standards described under "Experimental Procedures." Quantitation of Metabolites; Recovery of Retinoids; Control Experiments—Since nonspecific isomerization and oxidative degradation of vitamin A molecules are common events associated with processing of biological samples, we were very careful to protect the samples from light, heat, oxidation, and extremes of pH. We also monitored for artifact formation by use of control experiments. Control studies include addition of pure, H-labeled retinoic acids and retinyl glucuronide to unlabeled tissues, sera, and bile and the assessment of recovered radioactivity.

Recoveries for all retinoids were 85–95%. Characteristic degradation products, formed artifically, eluted with epoxyretinoic acids and very polar metabolites. About 0.6–2.8% of 13-cis-retinoic acid was artifically generated from all-trans retinoic acid even after all possible precautions were exercised.

External standardization was carried out with various retinoids; quantitation was achieved during HPLC using the integrated peak area at 340 nm. Standard curves for the retinoids were established as follows: retinoic acid, range 5–100 ng, detection limit, 2 ng; retinol, range 5–200 ng, detection limit, 2 ng; retinyl esters, range 20–20,000 ng, detection limit, 5 ng.

For internal standardization, 4-hydroxybrentinretinamide and 13-cis-retinyl glucuronide were added to samples prior to extraction; their recovery was monitored by HPLC-UV absorbance.

![Fig. 1. Separation of standard retinoids by reverse-phase HPLC. Standards and their respective retention times are as follows:](image-url)

- (1) 4-oxoretinoic acid, 50 ng, 41 min; (2) 5,6-epoxeyretinoic acid, 50 ng, 6.7 min; (3) 13-cis-retinyl glucuronide, 13.6 min; (4) all-trans-retinyl glucuronide, 13.6 min; (5) 13-cis-retinoic acid, 50 ng, 17.4 min; (6) all-trans-retinoic acid, 50 ng, 19.7 min; (7) 4-hydroxybrentinretinamide, 40 ng, 22.8 min; (8) 13-cis-N-ethylretinamide, 35 ng, 27.2 min; (9) all-trans-retinol, 30 ng, 28.8 min; (10) all-trans-retinyl linoleate, 40 ng, 37.3 min; (11) all-trans-retinyl palmitate, 29 ng, 38.5 min; (12) all-trans-retinyl oleate, 25 ng, 38.5 min; (13) all-trans-retinyl stearate, 40 ng, 40.1 min; SF, solvent front. Chromatographic conditions are described under "Experimental Procedures." Dashed lines indicate that standards were estimated by radioactivity measurement.

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2 The abbreviations used are: RA, retinoic acid; RG, retinoyl glucuronide; HPLC, high pressure liquid chromatography.
Retinoids (>2-5 ng) in tissues or plasma could be measured with a UV detector and an electronic peak integrator for calculation of peak areas; collected fractions were also examined for radioactivity in peak areas that co-migrated with standards or endogenous retinoids. For our studies of endogenous vitamin A metabolites, carrier retinoic acids were added to tissue and plasma samples prior to extraction. Low concentrations of retinoids were assessed by radioactivity that co-migrated on HPLC with the appropriate carrier standards.

For studies on metabolism of retinoic acid, quantitation was based on the specific radioactivity of the injected all-trans-RA. Quantitation of vitamin A steady state metabolites from orally administered all-trans-retinyl acetate was based on specific radioactivity of plasma retinol at time of sacrifice, 70.9 μCi/μmol, which was the same as that of administered retinyl acetate.

Incubation with β-D-Glucuronidase—Radioactive metabolites eluting in the retinyl glucuronide region were collected and incubated with β-D-glucuronidase as described earlier (14, 21). The resultant products were rechromatographed.

RESULTS

Kinetics of All-trans-RA and RA Metabolites in Blood—Two minutes after the injection of a physiological dose (8.2-10.0 μg) of 3H-labeled all-trans-RA into vitamin A-depleted bile duct-cannulated rats, only 6% of injected radioactivity remains in circulation (Fig. 2). This rapid decline of circulatory radioactivity continues for 15 min, when only 3% of injected dose is found in circulation. A similar trend of rapid disappearance was also observed when the injected dose was raised to 18 μg (Table I); this observation suggests that injected retinoic acid is immediately (within 0-2 min) sequestered by vitamin A-depleted tissues. Circulatory radioactivity remains low until about 1 h after dosing; at this time it rises and reaches a constant level by about 90 min.

The kinetics of disappearance of plasma radioactivity are very complex (Fig. 2 and Table I). This finding supports the idea that RA is sequestered by more than one target tissue of the vitamin A-depleted animal. Since we used vitamin A-deficient rats and since the animals were bile duct-cannulated, endogenous retinoic acid or any other vitamin A active compounds are present in tissues and in circulation in negligible amounts at the time of dosing and thus will not complicate the kinetics nor will dilute pools of metabolites below the level of detection. Our data on the kinetics of all-trans-RA in plasma are in good agreement with those reported for intact, vitamin A-sufficient rats for the same time frame and dosage range, i.e. 15 min and 30 μg (23).

Analysis of plasma radioactivity reveals that all-trans-RA, 13-cis-RA, and unidentified polar derivatives of retinoic acid are the circulatory forms of injected RA (Fig. 3). At 2 min all-trans-RA is the major (90%) circulatory form; RA content in plasma decreases to 60% of circulating radioactivity by 4 h. During this time there is a rise in polar metabolites, and at 4 h polar metabolites represent 30% of circulating radioactivity.

The crude fraction containing red blood cells was separated from plasma and examined for RA metabolites. This fraction contained from 16 to 20% of radioactivity found in blood (Fig. 2). The pattern of radioactivity in the red blood cell fraction was very similar to that observed in plasma (Fig. 3). The appearance of 13-cis-RA and polar metabolites in the red blood cell fraction followed a pattern similar to that seen in plasma, i.e. the ratio of all-trans-RA and 13-cis-RA was about the same as that found in plasma; the percentage of polar metabolites increased with time and after about 45 min reached a constant value. From these data we conclude that the retinoic acid metabolites found in red blood cell fraction reflect those found in plasma.

We conclude that most of the injected RA is taken up by tissues within 2 min and that metabolites, generated in tissues, are subsequently released into circulation.

Kinetics of 13-cis-RA in Plasma—13-cis-RA was detected in plasma 2 min after injection of all-trans-RA; its concentration reached maximum after 15 min, and thereafter circulatory 13-cis-RA gradually declined (Fig. 3). Our data are consistent with the concept that after a rapid uptake of all-trans-RA into tissues, 13-cis-RA is formed there as an early metabolite; a portion of it is immediately released into circulation. Within 15 min the ratio of circulating all-trans-RA to 13-cis-RA dropped from infinity to 5.5, reflecting an initial rapid generation of 13-cis-RA in tissues; an equilibrium ratio of 20:1 in the circulatory all-trans to 13-cis ratio is reached in about 2 h and prevailed for the remainder of experiment.

![Diagram](image)

**FIG. 2.** Per cent of administered radioactivity in circulation. Plasma values (—) are the combined values from two experiments representing dosage amounts of 8.3 and 10 μg of all-trans-[11-3H]retinoic acid. Red blood cell fraction values (O—O) were obtained after the administration of 10 μg of all-trans-[11-3H]retinonic acid. Values for plasma and red blood cell fractions were calculated as dpm per total blood volume assuming 58 ml of blood/kg of body weight. Numbers in parentheses indicate the number of animals. Data are plotted as the mean ± S.E. or as the average ± S.D. when only two animals were used. Duplicate determinations were made for each animal.

**TABLE I**

<table>
<thead>
<tr>
<th>Dose remaining at time after dosing</th>
<th>2 min</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>17.0 ± 5.1 (4)*</td>
<td>10.3 ± 2.6 (6)</td>
<td>6.9 ± 2.6 (2)</td>
<td>7.4 ± 2.3 (4)</td>
<td>20.5 ± 3.6 (3)</td>
<td>10.5 ± 2.6 (2)</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>6.5 ± 2.2 (5)</td>
<td>2.9 ± 1.1 (4)</td>
<td>2.9 ± 1.8 (2)</td>
<td>2.3 ± 0.6 (4)</td>
<td>3.2 ± 0.6 (4)</td>
<td>1.5 ± 0.7 (2)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate number of animals used. Duplicate determinations were made for each animal. All values are ±S.E., except when 2 animals are used, the values are ±S.D.
from vitamin A-deficient rats was incubated with all-trans-RA using the same experimental conditions, there was no conversion to other metabolic forms of RA. These observations indicate that the red blood cell fraction is not the source of 13-cis-RA and polar metabolites of RA in plasma.

The above control experiments clearly show that the 13-cis-RA found in circulation of dosed rats (Fig. 3) is not formed by factors found in blood. The values reported for all experiments have been corrected for nonspecific isomerization.

Metabolism of All-trans-RA in Small Intestinal Mucosa of Vitamin A-depleted Rats—All-trans-RA appears in the small intestine of bile-duct-cannulated rats very shortly after the intrajugular injection; it was already present in this tissue at the earliest time examined (2 min) and it is the major radioactive compound there for about 2 h (Fig. 4). The multiphasic curve probably reflects the availability of injected RA from circulation.

All-trans-retinoyl glucuronide (RG) is the major metabolite of RA in small intestinal mucosa at all time points examined (Fig. 4). It is first detectable 2 min after the RA dose. There is a rapid rise in RG during the first 30 min; thereafter its increase is slow. At no time was RG detected in the circulation.

Fig. 4 further illustrates that during the first 2–30 min after the intrajugular injection of all-trans-RA there is a rapid conversion of intestinal all-trans-RA to RG and isomerization to 13-cis-RA; these reactions continue, but at a slower rate, throughout the 2 h period studied.

The formation of 13-cis-RG, evident already at 2 min, parallels that of all-trans-RG: there is an initial rapid rise in 13-cis-RG, followed by a slower increase that is sustained. Similarities in the rates of formation of both glucuronides suggest that one enzyme catalyzes both reactions. Fig. 4 also demonstrates that there is a quantitative relationship between 13-cis-RA and 13-cis-RG. It is evident that the metabolism of all-trans-RA in small intestinal epithelium involves 3 initial steps: isomerization to 13-cis-RA, glucuronidation of all-trans-RA, and glucuronidation of 13-cis-RA.

Fig. 4 also shows that there is a gradual rise in polar retinoid acid metabolites other than glucuronides. Small, but variable, amounts of 5,6-epoxy-RA and 4-oxo-RA were detected in the intestinal mucosa at all times. However, most of the radioactive activity eluted with unidentified very polar compounds.

About 1.5% of dose radioactivity was found in the intestinal mucosa 30 min after dosing; a maximum of 2.3% of dose radioactivity was found after 1 h.

Endogenous Metabolites of Vitamin A in Circulation, Small Intestine, and Bile of Retinyl Acetate-maintained Rats—Labeled vitamin A metabolites were examined in rats that had been given daily for 14 days by gavage, 20 μg of [3H]retinyl acetate, specific activity, 70.4 μCi/μmol. This level of vitamin A ingestion satisfies the needs for normal growth and maintenance as well as results in some storage of vitamin A in liver (24). The purpose of the daily replenishment with labeled retinyl acetate was to obtain an equilibration of radioactivity with endogenous body pools of vitamin A, so that we could examine metabolism of vitamin A under steady state conditions. It is well established that equilibration of administered vitamin A with endogenous vitamin A is complete in 4–8 days (3, 10, 25, 26). We found that at time of killing the specific radioactivity of plasma retinol was the same as that of administered retinyl acetate.

Table II summarizes the composition of endogenous vitamin A metabolites in circulation. The major circulatory form is retinol; the amount present in plasma, 40.3 μg/dl, is in the

3 M. H. Zile and M. E. Cullum, unpublished observations.
Common Metabolites from Retinyl Acetate and Retinoic Acid

FIG. 4. Metabolites in small intestinal mucosa of bile duct-cannulated rats after injection of all-trans-[11-3H]retinoic acid. The data represent two experiments using dosage amounts of 10.0 and 18.5 mg of all-trans-[11-3H]retinoic acid. Other experimental details are in the legend to Fig. 3. △—△, all-trans-retinoic acid; O—O, 13-cis-retinoic acid; ■—■, polar metabolites. S.E. for experimental time points 2 min, 15 min, 30 min, 45 min, 1 h, and 2 h for all-trans-retinoic acid are, respectively, 1.9, 0.3, 1.7, 2.1, 2.4, and 2.7; for all-trans-retinol glucuronide are, respectively, 1.1, 7.0, 1.5, and 3.0; for 13-cis-retinol glucuronide are, respectively, 0.3, 0.3, 1.7, 1.3, 0.8, and 0.5; for polar metabolites are, respectively, 1.4, 0.5, 1.7, 1.4, 1.8, and 1.8. Values for the 1.5-h time point represent duplicate determinations from two different animals.

The range of established values for retinol in rat (3, 24, 27).

Plasma also contains a small amount of retinyl palmitate. Furthermore, all-trans-RA and 13-cis-RA are also present in plasma under steady state conditions. Since the animals had been maintained on a vitamin A-deficient diet supplemented with \(^*[11]^\text{H}\)retinyl acetate and since oral administration of retinyl acetate supplements was discontinued 48 h before animals were killed, the vitamin A-associated radioactivity in circulation does not represent newly absorbed vitamin A.

Table II shows the endogenous metabolites present in small intestinal mucosa during steady state vitamin A metabolism. Under these conditions, combined retinyl esters (47%) are the major metabolites; retinol represents the second largest en-

dogenous vitamin A component in this tissue. Intestinal epithelium also has 2.1% all-trans-retinoic acid and 0.9% 13-cis-retinoic acid (ratio of all-trans to 13-cis: 2.3:1.0). Furthermore, retinyl glucuronide (1.2%) and polar metabolites (6.7%), including 4-oxoretinoic acid and 5,6-epoxyretinoic acid, were consistently observed in this epithelial target tissue. Small intestinal muscle layer also contained vitamin A compounds; the major components were retinyl esters and polar metabolites.

The radioactive metabolite peak eluting in the region of standard retinyl glucuronides was incubated with \(\beta\)-d-glucuronidase and the resulting products were rechromatographed. Enzymatic hydrolysis converted 75% of the endogenous retinyl glucuronide to compounds that co-migrated with authentic 13\(^\text{cis}\)s- and all-trans-retinoic acids. These results confirm the identity of endogenous retinyl glucuronides in small intestinal mucosa.

Analysis of biliary vitamin A metabolites (Table II) revealed that under steady state conditions some very polar metabolites were excreted; among those identified by co-chromatography with standards were 4-oxoretinoic acid and 5,6-epoxyretinoic acid. Unaltered retinoic acids, retinyl glucuronides, and retinol, but not retinyl esters, were also detected in bile under our experimental conditions. Some unidentified radioactivity eluting in the region of polar compounds may include retinyl glucuronides.

DISCUSSION

Our work described here focuses on two related questions concerning vitamin A metabolism in intestinal epithelium, a target tissue for vitamin A function (3, 8): What are the metabolites of all-trans-RA immediately after injecting a microgram dose and what are the metabolites of vitamin A (retinyl acetate) at steady state conditions?

For our studies on the metabolism of physiological doses of retinoic acid we used vitamin A-deficient rats, reasoning that, if retinoic acid is the functional form of vitamin A, then in a vitamin A-depleted animal, metabolism of retinoic acid would reflect the need for the vitamin, and thus would correlate to metabolic transformations associated with function.

Retinoic acid was detected in small intestinal mucosa at the earliest time point examined, i.e. 2 min after intravenous

TABLE II

<table>
<thead>
<tr>
<th>Endogenous vitamin A compounds</th>
<th>Concentration*</th>
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<tbody>
<tr>
<td></td>
<td>Concentration*</td>
</tr>
<tr>
<td>Intestinal Intestinal Intestinal Red</td>
<td>Concentration*</td>
</tr>
<tr>
<td>mucosa muscle plasma blood bile</td>
<td>ne/g</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Retinyl stearate</td>
<td>3.5 7.2 ND</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>29.8 22.5 0.8 1.1 ND</td>
</tr>
<tr>
<td>Retinyl linoleate</td>
<td>19.0 7.7 ND ND</td>
</tr>
<tr>
<td>Retinol</td>
<td>47.0 7.4 402.6 28.3 30.9</td>
</tr>
<tr>
<td>All-trans-RA</td>
<td>2.3 1.1 2.7 3.1 7.7</td>
</tr>
<tr>
<td>13-cis-RA</td>
<td>1.0 0.2 0.9 0.6 6.1</td>
</tr>
<tr>
<td>Retinyl glucuronides</td>
<td>1.3 ND ND ND 18.6</td>
</tr>
<tr>
<td>All-trans-5,6-epoxy-RA</td>
<td>7.5 13.0 5.5 2.8 387.2</td>
</tr>
<tr>
<td>All-trans-4-oxo-RA</td>
<td>7.5 13.0 5.5 2.8 387.2</td>
</tr>
<tr>
<td>Polar metabolites</td>
<td>111.4 59.1 412.5 33.9 452.5</td>
</tr>
<tr>
<td>Total retinoids</td>
<td>111.4 59.1 412.5 33.9 452.5</td>
</tr>
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</table>

*Values represent average of two determinations from biological samples obtained from 4 rats 48 h after feeding retinyl acetate and 12 h after fasting. Bile was collected for 2 h prior to sacrifice. Quantitation of the endogenous vitamin A compounds is described under "Experimental Procedures."

Not detected.
dosing and was the major radioactive compound there during the 2-h study. The appearance of all-trans-RA in small intestinal epithelium was accompanied by formation of 13-cis-RA; the amount of 13-cis-RA remained relatively constant during the 2 h after dosing. It appears that all-trans- and 13-cis-retinoic acids in small intestinal epithelium exist in a physiological equilibrium, all-trans being the predominant isomer. This finding confirms data obtained in our initial observations on 13-cis isomerization in small intestinal mucosa (14, 15) and is indirectly supported by subsequent work in other laboratories (11, 16).

Examination of the kinetic pattern of metabolites suggests that glucuronidation of retinoic acid is an early and likely an important event in the vitamin A-depleted intestinal epithelium. The initially rapid conversion of RA to retinolyl glucuronides most likely represents an intense repletion period of the vitamin A-depleted cells; the early metabolites thus may reflect RA metabolism that is intimately associated with RA function in intestinal epithelium. Furthermore, retinolyl glucuronides were found to be the major metabolites of retinoic acid in this tissue. This is in agreement with our earlier studies (14) in which we examined the metabolism of RA at later time points (2-6 h after dosing); those initial observations have been supported by subsequent studies (11). Similar amounts of 13-cis-RA and its glucuronide were present in intestinal epithelium during the 2 h period examined; this suggests stoichiometry for glucuronidation of 13-cis-RA and a possible separate physiological significance of this reaction (equilibrium ratio was 1:1 for the two metabolites).

The second issue addressed in this paper is steady state metabolism of vitamin A. Information on vitamin A metabolism has been obtained by use of retinoic acid in vitamin A-depleted (5-7, 14, 15, 17) and vitamin A-sufficient rats (9, 11, 13, 16, 21, 23, 25, 26). Although earlier studies have demonstrated that acute injected doses of retinol and retinyl acetate can be metabolized to RA in vitamin A-deficient rats (3, 5-10), only recently the occurrence of endogenous retinoic acid was reported in the kidneys of vitamin A-sufficient rats (13). However, no one has demonstrated the generation of RA from dietary vitamin A in small intestine under vitamin A steady state conditions in vitamin A-repleted animals. In our studies reported here we provide evidence of the existence of all-trans-RA, its 13-cis isomer, and their glucuronyl derivatives in vitamin A target tissue, under vitamin A steady state conditions. The evidence is crucial for validation of the postulate that these biologically active (growth-promoting) compounds are on the physiological pathway of vitamin A metabolism and, therefore, clearly have physiological significance.

In our experiments with retinyl acetate-fed rats, the bile ducts of fasted rats were cannulated 2 h prior to sacrifice. Thus, during the last 2 h prior to obtaining intestinal tissue, bile was not secreted into intestinal lumen. Some of the vitamin A metabolites observed in the intestine might have arisen from bile secreted prior to bile duct cannulation. But, this possibility is very unlikely, since the proportions of polar retinoids and retinol in intestine do not reflect those in bile. This observation supports the concept that the endogenous intestinal metabolites observed represent vitamin A compounds involved in maintenance and function of this tissue.

In our present study we demonstrate all-trans-RA (2.7 ng/ml) and 13-cis-RA (0.9 ng/ml) in plasma of rats during vitamin A steady state conditions. Endogenous RA, although detected in human plasma (3 ng/ml (28)) has previously not been detected in the circulation of rats. Our findings thus point to similarity in vitamin A metabolism among higher animals.

An important aspect of our work has been the demonstration of 13-cis-RA as a physiological metabolite during the metabolic transformations of all-trans-RA. 13-cis-RA was detected in small intestinal mucosa and in circulation. Evidence was obtained that the 13-cis-RA present in circulation must have been generated in tissues and then released into circulation. This suggestion is supported by our observation that in vitamin A-depleted rats, intravenous injected all-trans-RA is rapidly removed from circulation. Retinoic acid and its biologically active metabolites were found in small intestinal mucosa as early as 2 min after dosing, suggesting rapid sequestration of retinoic acid by vitamin A-depleted tissues.

It is essential to emphasize that our experimental procedures were designed and executed in such a manner to minimize or eliminate artifact formation, particularly isomerization; therefore, we are confident of the validity of in vivo occurrence of 13-cis-RA. Controversial data concerning physiological occurrence of 13-cis-RA may simply reflect the non-conformity of isolation techniques among various investigators.

At vitamin A steady state conditions, the small intestine of the vitamin A-sufficient rat has various biologically active forms of vitamin A, including retinyl esters, retinol, retinoic acids, and retinoyl glucuronides. Retinol was the major vitamin A compound in small intestine under the described steady state conditions. Retinyl palmitate was the predominant vitamin A ester, but a significant portion of retinyl esters was in the form of retinyl linoleate. The intestinal muscle layer also contained significant amounts of retinyl esters and retinol.

Our work substantiates recent data (13) that retinoic acid is indeed an endogenous metabolite of vitamin A in the rat. Evidence is also presented that 13-cis isomerization and glucuronidation of retinoic acid are metabolic transformations associated with vitamin A steady state conditions in small intestine. About 30% of retinoic acid in small intestinal mucosa is in the 13-cis configuration; the same equilibrium mixture was found in the mucosa of vitamin A-depleted rats 2-6 h after an intravenous dose of all-trans-RA (14). The metabolic pathway of retinoic acid isomerization and glucuronidation that we proposed earlier for vitamin A-depleted bile duct-cannulated rats (14) appears to exist in vitamin A-sufficient rats at steady state conditions. Our studies thus illustrate that the vitamin A-deficient, bile duct-cannulated rat is a valid model for examination of in vivo intestinal metabolism of vitamin A beyond the formation of retinoic acid.

We propose that under steady state conditions all-trans-RA is converted to its 13-cis isomer; both are subsequently glucuronidated. The initially rapid rate of retinolyl glucuronide formation in vitamin A-depleted intestinal mucosa and the subsequent controlled biosynthesis of retinolyl glucuronides suggest an important function of this vitamin A metabolite in intestinal epithelium. Further studies must be conducted to test if glucuronidation of RA is related to the molecular function of retinoic acid.

Acknowledgments-We thank Drs. D. R. Romsoos, J. J. Pestka, and S. Ferguson-Miller for a critical review of the manuscript and J. Hunt for clerical assistance.

REFERENCES
2. Robers, A. B., and Sporn, M. B. (1984) in The Retinoids (Sporn,
Common Metabolites from Retinyl Acetate and Retinoic Acid


19. Lippel, K., and Olson, J. A. (1968) J. Lipid Res. 9, 168–175


