Origin of some derivatives of retinoic acid found in rat bile

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ABSTRACT After the intraportal injection of retinoic acid-15-^{14}C into rats, all-*trans* methyl retinoate, a *cis* isomer of methyl retinoate, retinoyl β-glucurono-γ-lactone, retinoic acid, and retinoyl β-glucuronide were isolated from methanol extracts of rat bile by chromatography on anion-exchange resin and silicic acid columns and characterized on thin-layer plates of Silica Gel G. On the other hand, when bile was extracted with *n*-butanol or analyzed directly by thin-layer chromatography, only retinoyl β-glucuronide and a very small amount of retinoic acid could be detected. Butanol extracts of the liver and the intestine, however, still contained a small radioactive nonpolar fraction. When retinoyl β-glucuronide was incubated with an anion-exchange resin in the presence of methanol, several nonpolar products appeared. Apparently the methyl retinoate, retinoyl β-glucurono-γ-lactone, and most of the retinoic acid previously found in bile after retinoic acid administration are produced from retinoyl β-glucuronide during the isolation procedure.

KEY WORDS retinoic acid · retinoyl β-glucuronide · methyl esters of retinoic acid · retinoyl β-glucurono-γ-lactone · rat bile · chromatographic artifacts

OF THE VARIOUS METABOLITES of all-*trans* retinoic acid that have been partially purified from various mammalian tissues (1–6), only all-*trans* retinoyl β-glucuronide (7, 8) and 13-*cis* retinoate (9) have been specifically identified. Besides retinoyl β-glucuronide, the major metabolite of intraportally injected retinoic acid-^{14}C in rat bile, three other radioactive fractions have been detected in this laboratory (8): (*a*) a nonpolar fraction (I) containing three components (Ia, Ib, and Ic), which was eluted from an anion-exchange resin with methanol; (*b*) an acidic fraction (IIa), which contained retinoic acid; and (*c*) an acidic polar fraction (IIb), which was eluted before the glucuronides.

In investigating the chemical nature of these fractions, we observed that the presence of several components depended on the isolation procedure employed. Major components of fraction I have now been tentatively identified as retinoyl β-glucurono-γ-lactone (Ia) and as *cis* (Ib) and *trans* (Ic) isomers of methyl retinoate. These compounds apparently are formed from retinoyl β-glucuronide during isolation in the presence of methanol and anion-exchange resins, but not in the presence of *n*-butanol and ammonium sulfate. The possible relationship between these artifacts and other reported metabolites of retinoic acid is discussed.

MATERIALS AND METHODS

Radioactive Compounds and Other Chemicals

All-*trans* retinoic acid-15-^{14}C (11.16 μc/μmole), which was generously donated by Hoffman-La Roche, Inc., Basel, Switzerland, was purified by ion-exchange or silicic acid chromatography. Nonradioactive retinoic acid was obtained from Distillation Products Industries, Rochester, N. Y. and used without further purification. All-*trans* methyl retinoate was synthesized by treatment of retinoic acid with diazomethane (10) and recrystallization of the product from methanol–water 5:1 until the melting point and UV spectrum agreed closely with reported values (11).

Hexamethyldisilazane and trimethylchlorosilane were obtained from Peninsular Chemical Research Co., Gainesville, Fla. Reagent grade solvents were used without purification for column and thin-layer chromatography, but were distilled before being used for spec-

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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trophotometric measurements. The silicic acid used for column chromatography was supplied by Bio-Rad Laboratories, Richmond, Calif. and the Silica Gel G used for thin-layer analyses was obtained from either Research Specialities Co., Richmond, Calif. or E. Merck A.G., Darmstadt, Germany. The anion-exchange resin, AG 2X8, in the acetate form was supplied by Bio-Rad Labs. Bovine and bacterial β-glucuronidase was purchased from Sigma Chemical Co., St. Louis, Mo. Tween 40 (polyoxyethylene sorbitan monopalmitate) was generously provided by Atlas Chemical Industries, Wilmington, Del. Other chemicals were of analytical grade.

Collection and Fractionation of Bile and Tissues

Bile was collected for 24 hr from bile duct-cannulated male rats (Rolfsmeyer Farm, Madison, Wis.) that had been injected intraportally with retinoic acid-15-^{14}C (1.7×10^6 dpm in 5 mg) suspended in 2–5% Tween 40 in 0.1 M phosphate buffer pH 7.4. The collected bile was diluted with 1–4 volumes of methanol and the precipitate was removed by centrifugation. The supernatant solution was passed through an anion-exchange column of Bio-Rad AG 2X8 in the acetate form (1.9 cm I.D. × 5–7 cm high), and the column was developed successively with 100–200-ml portions of methanol, 5% acetic acid in methanol, and 25% acetic acid in methanol. An aliquot of each fraction (I, II, or III) was evaporated under nitrogen, and its UV spectrum was determined from 300 to 400 nm.

Fraction I from the anion-exchange column was evaporated under vacuum at 40–60°C. The residue was suspended in 5–15 ml of *n*-hexane and chromatographed on a column containing 18 g of silicic acid (2 cm I.D. × 15 cm high). The column was developed successively with 200-ml portions of 1% diethyl ether in hexane, 25% ether in hexane, and 100% ether. Fractions of 10 ml were collected, and major components were eluted in the order Ic, Ib, and Ia. The OD at 355 nm and the radioactivity in each fraction were measured. UV spectra of representative fractions were recorded from 300 to 400 nm.

In some cases, acetone was used in place of methanol to dilute the bile and to elute the anion-exchange column. Alternatively, bile was acidified to pH 4.0 with acetic acid, saturated with ammonium sulfate (1.5–2 g/ml of bile), and then extracted with two volumes of *n*-butanol. The liver and intestine, when analyzed, were excised 4 hr after injection, washed with 0.9% saline, and homogenized in 50 ml of water. The homogenates were saturated with ammonium sulfate, acidified, and extracted with butanol in the same manner as described for bile.

Enzymatic Hydrolysis of Fraction Ia

Subfraction Ia, which had been purified by TLC, was solubilized with 0.3% Tween 40 in 3.0 ml of 0.1 M phosphate buffer pH 7.9 which also contained 1.3×10^{-2} M magnesium sulfate, 1.7×10^{-4} M reduced glutathione, and 1.3×10^{-6} M *d*-saccharolactone. This solution was mixed with 1 ml of rat liver microsomes suspended in 0.1 M phosphate buffer pH 7.9. Half of this mixture was inactivated by the addition of 1 volume of methanol, and both tubes were incubated in the dark at 37°C for 30 min in a Dubnoff shaker. The reaction tube was then inactivated with methanol, and denatured protein was removed by centrifugation. The supernatant solutions were chromatographed on thin-layer plates of Silica Gel G in benzene–chloroform–methanol 4:1:1 (3).

Cocrystallization of Fraction Ic with All-trans Methyl Retinoate

Fraction Ic (22 ml) from silicic acid chromatography was mixed with 20.5 mg of all-*trans* methyl retinoate, and the solution was evaporated to dryness under nitrogen. The residue was dissolved in 2.0 ml of methanol, and the radioactivity and OD at 355 nm were determined. Methyl retinoate was recrystallized four times at −10°C from methanol–water 5:1 and the supernatant solutions were discarded. The specific activities of the crystals, in cpm per OD unit at 355 nm, were calculated after each crystallization.

β-Glucuronidase Assay

Microgram quantities of labeled bile metabolites were incubated at 37°C for 30–60 min in the dark with 0.5–1.0 ml of a 1% suspension of either bovine β-glucuronidase in 0.02 M acetate buffer pH 4.6 or bacterial β-glucuronidase in 0.02 M phosphate buffer pH 6.8. 1–4 volumes of either acetone or methanol was added to the control tube before incubation and to the reaction tube afterward. Control tubes without enzyme or with boiled enzyme were also included in some experiments. The resultant supernatant solutions were then analyzed by TLC.

Incubation of Retinoyl β-Glucuronide or of a Butanol Extract of Bile with Anion-Exchange Resin

Retinoyl β-glucuronide which had been purified by TLC, or an evaporated butanol extract of bile, was dissolved in 5 ml of methanol and incubated with 200 mg of anion-exchange resin AG 2X8 in the acetate form. The radioactivity in aliquots of the methanolic solution was measured initially and at various intervals during the subsequent 20 hr of incubation at room temperature in the dark. The final supernatant solution and methanol washes of the resin were pooled and evaporated under

nitrogen. The washed resin was then extracted with 15 ml of 25% acetic acid in methanol, which was also evaporated under nitrogen. The dried residues were dissolved in methanol and analyzed by TLC. A control tube which did not contain the anion-exchange resin was treated in a similar manner.

Light-Induced Isomerization of All-trans Methyl Retinoate

5 mg of all-*trans* methyl retinoate and 2.0–3.0 μg of I_2 in 5 ml of ethanol were irradiated in a 15 ml quartz tube with a 100 watt white light source and continuously stirred with a magnetic stirrer. After 48–72 hr the solution was evaporated to dryness under nitrogen and analyzed by TLC.

Solvent Partition of Retinoyl β-Glucuronide

In order to compare the behavior of retinoyl β-glucuronide with several polar metabolites of retinoic acid isolated by others (2, 4), we studied solvent partition ratios. Ether was pre-equilibrated with an equal volume of either distilled water, 0.01 N hydrochloric acid, 0.1 M phosphate buffer pH 7.0, or 0.01 N sodium hydroxide. 1 ml aliquots from each pair of phases were added to a tube that contained approximately 5000 cpm of dry retinoyl β-glucuronide. After equilibration, 0.1 ml aliquots of both phases were withdrawn and counted. Purified retinoyl β-glucuronide was also partitioned between hexane and 80% ethanol, and the resultant ethanol was partitioned between 64% methanol and ethyl acetate (4). The final ethyl acetate solution was analyzed by TLC.

Tests for Functional Groups in Retinoic Acid Derivatives

Esters present in fractions Ia, Ib, and Ic were saponified in 5% NaOH in 90% ethanol for 1–2 hr at 70°C under nitrogen. The hydrolysate was neutralized with 9 N sulfuric acid and extracted with either hexane or ether. Free carboxyl groups were esterified with diazomethane (10), and free hydroxyl groups were converted to the trimethylsilyl ethers by treatment with hexamethyldisilazane and trimethylchlorosilane in dry pyridine (12). Vicinal free hydroxyl groups were cleaved by exposure to 0.5 M sodium metaperiodate in 0.1 M phosphate buffer pH 6 at room temperature. In all cases the effect of various treatments was assessed by TLC.

Other Procedures

Thin-layer plates of Silica Gel G were prepared by dispersing 45 g of the gel in 90 ml of water and coating 20 × 20 cm glass plates with a layer 0.8 mm thick. Plates were activated at 100°C for 1 hr and stored in a desiccator until used. Gas–liquid chromatography of methyl retinoate and bile fractions took place on a 135 cm column of 100–120 mesh Gas-Chrom P (Applied Science Laboratories Inc., State College, Pa.) coated with 3% SE-30 (methylpolysiloxy gum, General Electric, Schenectady, N.Y.). The flow rate of argon was 50 ml/min with a column outlet pressure of 15 psi. A Research Specialites model 600 chromatograph equipped with an ionization detector was employed. In some experiments the radioactive eluate was collected in anthracene cartridges (Packard Instrument Co., Inc., Downers Grove, Ill.) at 3-min intervals. Spectra were measured in either a Beckman DU or a Zeiss PMQ II spectrophotometer, and column fractions were minitored with a Coleman universal spectrophotometer. Radioactive samples were dissolved in 10 ml of naphthalene–dioxane counting fluid (13) and measured in a Packard Tri-Carb model 500 B or model 574 liquid scintillation counter. A toluene-^{14}C standard solution was added to correct for quenching when required.

RESULTS

Identification of Fraction Ia as Retinoyl β-Glucurono-γ-Lactone

Fraction Ia showed the UV spectrum of retinoic acid (Fig. 1). After saponification the methylated derivative of the acidic moiety had a retention time during GLC identical to that of all-*trans* methyl retinoate. The low R_f values of fraction Ia in some TLC solvent systems, however, (D, F, and G, Table 1) suggested the presence of a polar moiety as well. The presence of hydroxyl groups was established by treatment with hexamethyldisiliazane followed by TLC: this raised the R_f in solvent system D from 0.10 to 0.60. Since no reaction occurred with diazomethane or sodium periodate, free carboxyl and vicinal hydroxyl groups presumably were absent. Nonetheless fraction Ia was partially hydrolyzed by β-glucuronidase. Furthermore, after incubation of

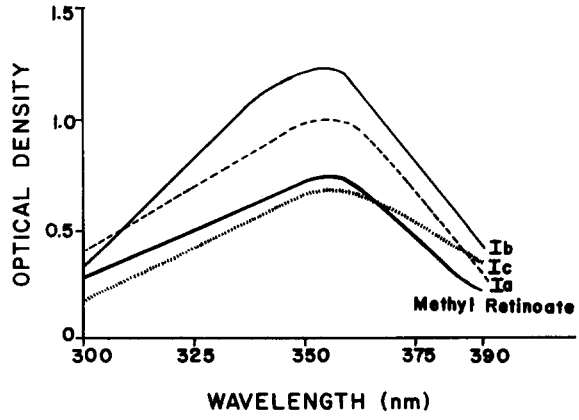

FIG. 1. Spectra of fractions Ia, Ib, Ic, and of all-*trans* methyl retinoate in ethanol.

fraction Ia with rat liver microsomes, a new metabolite appeared which had the same R_f as retinoyl β-glucuronide (0.07 in system A) and now reacted with diazomethane. All of these observations suggest that fraction Ia is retinoyl β-glucurono-γ-lactone.

Tentative Identification of Fraction Ib as a cis Isomer of Methyl Retinoate

Fraction Ib behaved as a neutral compound on an anion-exchange resin column, showed the spectrum of a retinoyl ester, and could be saponified to free retinoic acid. Since its absorption maximum was rather flat in these experiments (Fig. 1), its isomeric structure could not be assigned on the basis of spectra alone. On TLC, however, its R_f was lower than that of all-*trans* methyl retinoate in all the solvent systems examined (Table 1), and was identical with that of one of the photoisomers of all-*trans* methyl retinoate chromatographed in systems A and C. After saponification, the retionic acid moiety of fraction Ib had an R_f of 0.67 in system B, in contrast to the R_f of 0.90 for all-*trans* retinoic acid. Finally, the retention time of the methylated acidic product (i.e. resynthesized Ib) was 15–18 min on GLC compared to 34 min for the all-*trans* isomer. *Cis* isomers of methyl retinoate formed by photoisomerization of all-*trans* methyl retinoate are also known to have lower retention times on GLC than the all-*trans* isomer (8). Clearly, the above characterization is not compelling, but the data are fully in keeping with the possibility that fraction Ib consists of one or more related *cis* isomers of methyl retinoate. Whether this fraction contains 13-*cis* retinoic acid, characterized by Zile, Emerick, and DeLuca (9) in rat liver, has not been ascertained.

Identification of Fraction Ic as All-trans Methyl Retinoate

Fraction Ic and all-*trans* methyl retinoate behaved identically on a silicic acid column developed with 1% ether in hexane, had the same R_f values on thin-layer plates in several solvent systems (Table 1), showed identical retention times of 34.3 ± 0.5 min at 180°C and 5.7 ± 0.1 min at 203° C on the GLC column and possessed essentially the same UV spectra (Fig. 1). Furthermore the specific activity of the crystals remained constant upon repeated crystallization from methanol–water 5:1 of fraction IC with all-*trans* methyl retinoate (Table 2).

Analysis of Butanol Extracts of Bile and Tissues

When bile was acidified, saturated with ammonium sulfate, and extracted with butanol, no radioactive peaks corresponding to fractions Ia, Ib, and Ic could be detected by TLC of the extract. Similarly, radioactive peaks corresponding to fractions Ia, Ib, and Ic could not be detected by TLC (in solvent system A) when acetone instead of methanol was used as a bile diluent, or when untreated bile was analyzed directly by TLC. On the other hand, when butanol extracts of the liver and the intestine were analyzed by TLC, a small amount of nonpolar derivative was present, even though retinoic acid and retinoyl β-glucuronide accounted for 93% of the recovered radioactivity.

TABLE 1 TLC OF RADIOACTIVE COMPONENTS OF FRACTION I FROM RAT BILE AFTER INJECTION OF RETINOIC ACID-15-^{14}C

Solvent System	R_f Values				
	Ia	Ib	Ic	All-*trans* Retinoic Acid	All-*trans* Methyl Retinoate
A. Benzene–chloroform–methanol 4:1:1	0.48	0.67	0.80	0.42	0.83
B. Benzene–chloroform–methanol–acetic acid 5:5:5:1	0.83	0.90	1.00	0.90	1.00
C. Benzene–hexane 4:1	0.03	0.03	0.40	0.00	0.40
D. Chloroform–acetic acid 98:2	0.10	0.50	0.70	0.30	0.70
E. Diethyl ether	0.37	0.84	0.97	0.17	0.97
F. Hexane–ether–acetic acid 80:16:4	0.00	0.23	0.57	0.28	0.60
G. Ethylene dichloride	0.00	0.37	0.58	0.03	0.58

TABLE 2 RECRYSTALLIZATION OF FRACTION Ic WITH ALL-*trans* METHYL RETINOATE

Solution	Observed cpm	% Counting Efficiency	Corrected cpm	OD at 355 nm	Specific Activity (corr. cpm/OD)
1	47,440	33.5	143,000	3370	42
2	37,600	39.8	94,000	2400	39
3	21,940	48.7	45,000	1300	35
4	17,280	51.4	33,000	880	38
5	12,000	58.0	21,000	620	34

Formation of Components of Fraction I from either Retinoyl β-Glucuronide or a Butanol Extract of Bile

When either the butanol extract of bile or purified retinoyl β-glucuronide was incubated for 20 hr with anion-exchange resin AG 2X8 in methanol, radioactive peaks analogous to fractions Ia, Ib, and Ic were detected by TLC in the methanolic supernatant solution and in the eluate (acetic acid–methanol 1:3) of the resin. Incubation with methanol alone yielded no nonpolar compounds.

The yield of fraction I upon treating retinoyl β-glucuronide with the anion-exchange resin was about 12–15% compared with a yield of about 10–16% from chromatographed methanol-treated bile. The distribution of radioactivity among various fractions was also similar. Of the radioactivity recovered in major com-

ponents of fraction I, Ia contained 37%, Ib, 17%, and Ic, 46% from the resin-treated glucuronide, whereas Ia had an average of 33% with a range of 22–49%, Ib, 24% (13–38%), and Ic, 41% (32–50%) in seven experiments on chromatographed methanol-treated bile. Fraction Ib was somewhat more diffuse in the former experiments than in the latter. Similarly, radioactive peaks with R_f values corresponding to fractions Ia, Ib, and Ic were also detected by TLC after a retinoyl β-glucuronide solution had been partitioned by the procedure of Zile and DeLuca (4).

The Probable Absence of Retinoic Acid from Bile

After injection of retinoate into rats, retinoic acid was previously identified as a major component of fraction II, but only from bile diluted with methanol and fractionated by anion-exchange chromatography (8). When untreated bile or a butanol extract of bile was investigated by TLC, on the other hand, no radioactive peak corresponding to retinoic acid was detected. In addition, under conditions in which 57% of free retinoic acid was extracted from an acidified aqueous solution by hexane, less than 2% of the total radioactivity in the bile was extracted. Thus retinoic acid, if present at all in bile, is a very minor component indeed.

Solvent Partition of Retinoyl β-Glucuronide

As expected for a carboxylic acid, ether extracted retinoyl β-glucuronide fairly well from an acidified aqueous phase, less well from distilled water at pH 5.5, and very poorly from neutral or basic solutions (Table 3). Furthermore, less than 1% of radioactive retinoyl β-glucuronide was found in the hexane phase after partitioning it between hexane and 80% ethanol. On the other hand, 93% of the recovered radioactivity was found in the ethyl acetate phase when retinoyl β-glucuronide was partitioned between ethyl acetate and 64% methanol.

TABLE 3 DISTRIBUTION OF RADIOACTIVE RETINOYL β-GLUCURONIDE BETWEEN EQUAL VOLUMES OF ETHER AND VARIOUS AQUEOUS SOLUTIONS

Aqueous Phase	Glucuronide in Ether Phase (*A*)	Glucuronide in Aqueous Phase (*B*)	*K* (= *A*/*B*)
	cpm		
0.01 N HCl	2460	2740	0.90
Distilled water (pH 5.5)	1180	3430	0.34
0.1 M Phosphate buffer (pH 7.0)	260	4780	0.05
0.01 N NaOH	220	5360	0.04

DISCUSSION

The present investigation simplifies our views concerning the metabolism and excretion of retinoic acid in the rat. Apparently retinoyl β-glucuronide is by far the major metabolite of retinoate in bile, if not the sole one; esters of retinoate, the γ-lactone of retinoyl-β-glucuronide, and even retinoic acid itself are produced largely, if not entirely, by hydrolysis and transesterification of the glucuronide during treatment of the bile with methanol in the presence of an anion-exchange resin. These transformations are summarized in Fig. 2. The ability of ion-exchange resins to catalyze transesterification, hydrolysis, and lactonization reactions is well known (14), and the present study stresses the need for caution in the isolation of reactive polar lipids by these conventional procedures. That methanol might release "bound" esters of retinoate from the bile whereas acetone and butanol do not is a possible but unlikely alternative, particularly in view of the similar effects of these solvents on lipoprotein systems.

Small amounts of nonpolar esters of retinoate might, of course, still be formed enzymatically in tissues. Upon butanol extraction of liver and intestine in the present study, for example, a small amount (7%) of the recovered radioactivity appeared in an uncharacterized nonpolar fraction. Furthermore, methyl esters of fatty acids are presumably formed enzymatically in several mammalian tissues (15–17), and retinoic acid might undergo an analogous reaction. By similar reasoning the γ-lactone of retinoyl β-glucuronide might also be a natural product. γ-Lactones do form spontaneoulsy (18) and enzymati-

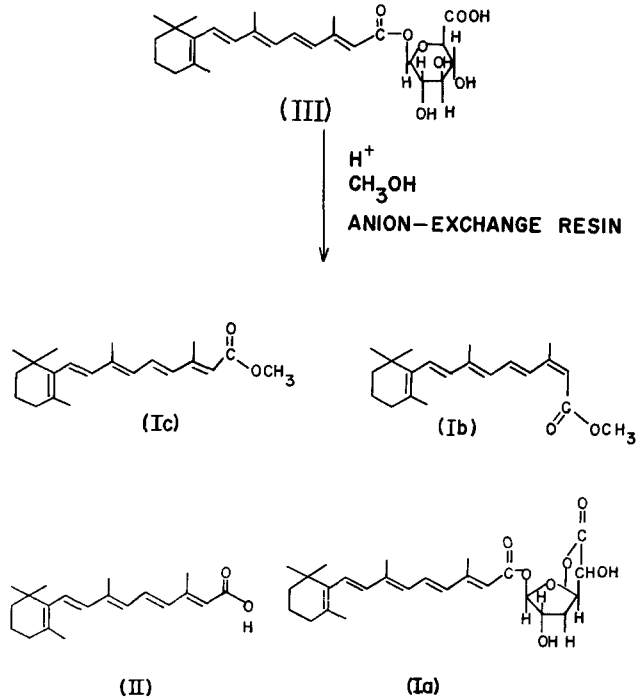

FIG. 2. Summary of resin-catalyzed reactions. Fraction Ib, which is represented here by 13-*cis* methyl retinoate, might be any of several stable *cis* isomers of methyl retinoate.

cally (19–22), although the equilibrium of the reaction favors hydrolysis (19–22). Nonetheless these esters, if formed physiologically in tissues, are clearly not excreted in the bile.

In view of the ready transesterification and hydrolysis of retinoyl β-glucuronide during its isolation, some recently reported but largely uncharacterized metabolites of retinoic acid might fruitfully be discussed. These metabolites are listed in Table 4. The conversion of intraduodenally injected fractions I and II of Zachman and coworkers into biliary retinoyl β-glucuronide (6) can be readily explained in terms of our present findings. Methyl retinoate (fractions Ia and Ib) might readily be hydrolyzed to retinoic acid by a liver esterase (23) and the γ-lactone (Ic) to the glucuronide (III) by a microsomal lactonase. Retinoic acid (II) might then be converted to the glucuronide by the glucuronyl transferase of liver microsomes (24).

Of four radioactive metabolites, namely E-1, E-2, M-1, and M-2, isolated by Zile and Deluca (4) from the liver after the administration of retinoic acid-6,7-^{14}C, only the least polar, E-1, was biologically active. Although at first it appeared to be an ester of a radioactive acidic product (4), fraction E-1 has recently been characterized as 13-*cis* retinoic acid (9). When retinoyl β-glucuronide is treated by the identical isolation procedure, however, several products are formed which behave chromatographically on thin-layer plates of Silica Gel G like fractions Ia, Ib, and Ic. Indeed, the elution pattern for these fractions plus retinoyl β-glucuronide from a silicic acid column corresponds closely with that for fractions E-1, E-2, M-1, and M-2, respectively.

The polar metabolites C and D, isolated from chicken liver and intestine (2) may well be retinoyl β-glucuronide. Fraction C, like retinoyl β-glucuronide, was extracted by ether from an aqueous tissue extract only after acidification, and had biological activity at higher doses but not at lower ones (2, 25). Since retinoyl β-glucuronide is only half removed from an acidified aqueous solution by a single extraction with ether (Table 3), fraction D might also contain the glucuronide.

Compounds 9, 5, and 5-ester, which were isolated from the intestine after administration of retinoate (3), have R_f values on TLC very similar to those of methyl retinoate, retinoic acid, and retinoyl β-glucuronide in the same solvent system (Table 1). Although all of these compounds seemingly possess some growth-stimulating activity (Fig. 1, references 3 and 25), compound 5 was distinguished from retinoic acid in several chromatographic systems and apparently does not contain the terminal C-15 carbon atom of retinoate (3). The decarboxylation of retinoic acid in vivo and in vitro has been demonstrated recently (26, 27).

Although speculation about the possible identity of traces of labeled derivatives isolated from several species by different procedures is always hazardous, several of the cited products may well be identical compounds or isomers, and some may be formed during isolation by transesterification, hydrolysis, or dehydration. Thus particular care must be taken both in the isolation and in the structural analysis of presumed metabolites of vitamin A.

TABLE 4 REPORTED RADIOACTIVE METABOLITES OF RETINOIC ACID

Source	Compound Designation	Bio-activity	Reference
Rat, guinea pig, and hen bile	I, II, III	+	8, 25
Rat liver	E-1	+	4
	E-2, M-1, M-2	−	4
Chick liver	C	+	2
Chick intestine	D	−	2
Rat intestine	5; 5-ester	+	3
	9	−	3
Rat stomach	334	+	1
Rat liver	Acidic lipid factor	+	28
Rat lymph, bile, and liver	Polar acidic and polar nonacidic lymph monoesters	—	29

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