Formation and enterohepatic circulation of metabolites of retinol and retinoic acid in bile duct-cannulated rats

R. D. ZACHMAN, P. E. DUNAGIN, JR., and J. A. OLSON

Department of Biochemistry, College of Medicine, University of Florida, Gainesville, Florida

ABSTRACT Four hours after intraportal injection of retinoic acid-^{14}C into bile duct-cannulated rats, less than 10% of the radioactivity was recovered in the liver, intestine, and kidneys. Within 6 hr, 40% of the radioactivity had appeared in bile. When suspensions of retinol-^{14}C or retinal were similarly injected, 25–35% of the dose was excreted in bile within 24 hr and equivalent amounts were deposited in the liver as retinol ester. The isolated perfused liver also produced these bile metabolites and is probably the major site of their formation in vivo. The intestine may metabolize retinoic acid, however, since some metabolites were found in the intestinal wall and lumen, even in bile duct-cannulated rats.

The bile metabolites of retinol-^{14}C and retinoic acid-^{14}C undergo extensive enterohepatic circulation. The bile radioactivity was not volatilized on boiling at acid pH, was not present in digitonin-precipitated sterols, and did not migrate with bile salts on reversed-phase paper chromatography. Anion-exchange chromatography resolved the metabolites of bile into three fractions containing nonionic compounds, acidic substances like retinoic acid, and more polar acidic derivatives.

KEY WORDS retinal-^{14}C · preparation · retinoic acid · retinol · retinal · metabolism · rat · metabolites · liver · intestine · bile · enterohepatic circulation · anion-exchange chromatography · excretion

MANY ASPECTS of the metabolism of retinol, retinal, and retinoic acid in mammals remain obscure. Dietary retinol and retinal are largely stored in the liver and other organs as retinol ester, whereas administered retinoic acid, which also promotes the growth of retinol-depleted animals, is found only in minute amounts in tissues (1–8), even when large doses are given. Retinoic acid may be formed from retinal by liver enzymes (9–12), however, and recently has been unambiguously identified as a natural product of retinal metabolism in vivo (13). Some metabolites of retinol and retinoic acid found in tissues and excreta have been partially purified (14), but most have not been well characterized (4, 6, 7, 15).

Recently, polar metabolites of retinol and retinoic acid have been found in large amounts in the bile from rat livers perfused with emulsions of these compounds (16). In the present investigation the uptake, metabolism, and disappearance of retinoic acid in various tissues was explored in detail, and the formation and excretion of bile metabolites of retinol and retinoic acid in vivo was described. The bile metabolites are reabsorbed by the intestine and reexcreted in the bile of bile duct-cannulated rats, thereby establishing an enterohepatic circulation of retinol derivatives. A preliminary report of some aspects of this work has appeared (17).

EXPERIMENTAL PROCEDURES

Retinol Substrates

Retinol-6,7-^{14}C and retinoic acid-6,7-^{14}C were supplied by Hoffmann-LaRoche, Inc., Basel, Switzerland. Labeled retinol was further purified by chromatography on deactivated alumina, and retinoic acid was purified either by ion-exchange chromatography or by silicic acid chromatography. Purified retinol-^{14}C was oxidized to retinal in good yield by treatment with activated MnO_2 (18) and the retinal was isolated by alumina chromatography. Nonradioactive crystalline retinol, retinal, and retinoic acid were obtained from Distillation

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Products Industries, Rochester, N. Y., and were used without further purification.

Biological Techniques

Details of the techniques used in this study have been reported (16, 17). In brief, fasted male rats (Rolfsmeyer Farm, Madison, Wis.) weighing from 150 to 300 g were anesthetized and their bile ducts were cannulated. One milliliter of a suspension of the appropriate substrate in 5% Tween 80, Krebs-Ringer bicarbonate buffer, pH 7.4, was injected into the portal vein. Experiments were also carried out in which a suspension of retinol or retinoic acid in 5% Tween 80 (polyoxyethylene sorbitan monooleate) or 0.4% sodium glycocholate solution was injected into the upper duodenum. After the incision had been closed, the animal was placed in a restraining cage and the bile was collected at desired intervals. At predetermined times the animals were killed, various organs were homogenized in 20 volumes of $CHCl_3$–methanol 2:1, and the homogenates were filtered. In contrast to the method of Folch, Lees, and Sloane Stanley (19), the filtrates were not washed with aqueous $CaCl_2$, but were directly evaporated to dryness under reduced pressure, and the residue was taken up in a small volume of methanol.

Ion-Exchange Chromatography of Bile and Tissue Extracts

Methanolic solutions of bile and urine or methanolic extracts of organs were usually chromatographed on columns (1.9 cm i.d. × 6.5 cm) of Bio-Rad AG2-X8 (Bio-Rad Laboratories, Richmond, Calif.) anion-exchange resin (200–400 mesh) in the acetate form. Larger amounts of the extracts were chromatographed on preparative columns (5.0 cm i.d. × 7 cm) of the above resin or of Bio-Rad AG1-X4. The resin bed was equilibrated with methanol before use. Nonionic material was eluted with methanol, and further elution was carried out with increasing concentrations of acetic acid in methanol. The effluent was collected in 10 ml fractions by a Technicon drop-counting fraction collector (Technicon Chromatography Corp., Chauncey, N. Y.). All parts of the system in contact with acetic acid were constructed of Teflon, glass, or stainless steel. Each sample was monitored for absorbance at 350 mμ with a Coleman Universal Spectrophotometer and was further analyzed for radioactivity.

Measurement of Radioactivity

Samples dissolved in organic solvents were plated infinitely thin on aluminum planchets, and assayed in a windowless gas flow Geiger-Müller counter (Nuclear-Chicago Corporation, Des Plaines, Ill.). Alternatively, 0.1–10 ml aliquots of samples were placed in scintillation vials, diluted with either the dioxane–naphthalene scintillation fluid of Bray (20) or the toluene fluid recommended by the Packard Instrument Company [5 g of 2,5-diphenyloxazole and 0.3 g of 2,2-*p*-phenylenebis(5-phenyloxazole) per liter of toluene], and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., La Grange, Ill.). Bray's scintillation fluid was superior in most cases for counting methanol-soluble derivatives of retinol and retinoic acid. In order to correct for the quenching of acetic acid, an internal standard was added to each assay vial. Tubes containing yellowish solutions, which quenched considerably, were always checked with internal standards.

RESULTS

Rate of Appearance of Metabolites of Retinol, Retinal, and Retinoic Acid in Bile

Metabolites of retinol, retinal, and retinoic acid were rapidly excreted into the bile after intravenous injection of these compounds into the portal vein. With similar dosages (2–3.5 mg) of retinoic acid-^{14}C, retinal-^{14}C, and retinol-^{14}C, 10–15% of the radioactivity appeared in the bile within 2 hr (Fig. 1). Within 24 hr, 20–55% of the total injected radioactivity was recovered in bile, depending on the substrate administered. Five days after retinoic acid-^{14}C administration, 95% of the label was recovered in the bile. When lower, more physiological dosages (10–26 μg) of the retinol compounds were administered, the initial rate of appearance and total extent of bile metabolite formation and excretion

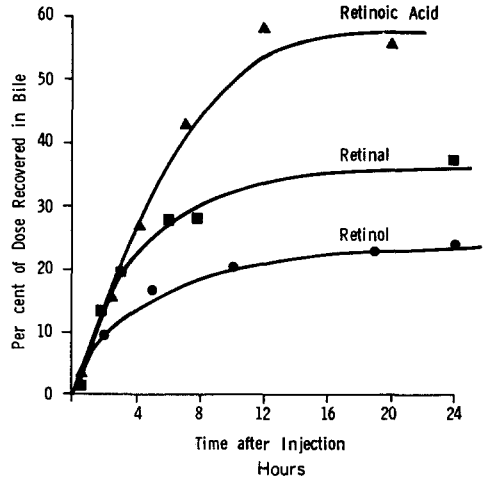

FIG. 1. The cumulative excretion rate of radioactivity into the bile after the intraportal administration of 2–3.5 mg of retinol-^{14}C retinal-^{14}C, or retinoic acid-^{14}C. Plotted values represent averages of 2, 4, and 12 rats, respectively.

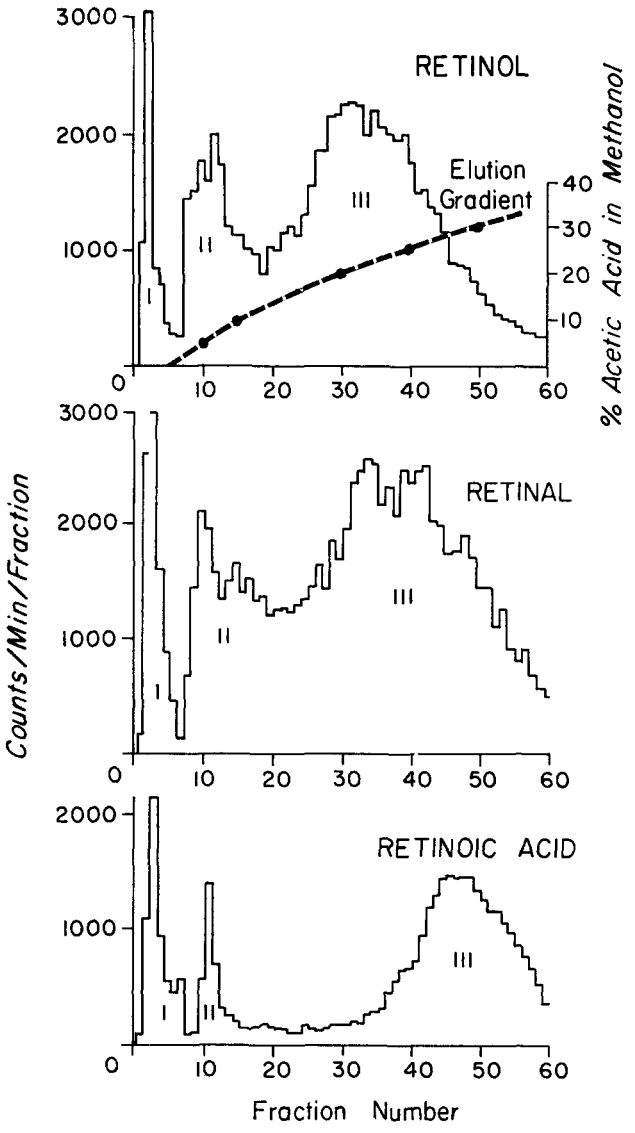

FIG. 2. Separation of bile by ion-exchange chromatography after administration of retinol-^{14}C, retinal-^{14}C, or retinoic acid-^{14}C. Bile was collected for a 6 hr period immediately following intraportal injection of each substrate, diluted with methanol, and chromatographed on an anion-exchange resin with an increasing gradient of acetic acid in methanol.

were similar to those found with larger doses: 40–50% of the radioactivity of the retinoic acid-^{14}C and 18–25% of retinol-^{14}C were excreted within 24 hr. Retinol-deficient rats excreted similar amounts of radioactivity in their bile after receiving labeled retinol or retinoic acid intravenously.

Radioactive metabolites also readily appeared in the bile when retinol-^{14}C or retinoic acid-^{14}C was placed in duodenal sacs or was administered by a stomach tube. In the former series, 26 μg of retinol-^{14}C or 10 μg of retinoic acid-^{14}C suspended with 50 μl of Tween 80 in 1 ml of Krebs-Ringer phosphate, pH 7.4, were injected into a duodenal loop made by loose ligatures at the pylorus and ligament of Treitz. On other occasions 1 ml of a Krebs-Ringer phosphate, 5% Tween 80 suspension of either 60 μg of retinol-^{14}C or 25 μg of retinoic acid-^{14}C were given via stomach tube. Under these conditions 10–15% of the administered radioactivity of either substrate appeared in the bile within 4 hr. Twenty-four hours after injection 20–25% of the label had appeared in the bile.

Properties of the Bile Metabolites

The radioactivity in the bile could be separated into three major radioactive fractions on anion-exchange columns (Fig. 2). Fraction I, eluted with methanol, contained 10–15% of the total radioactivity eluted from the column. Although free retinol, retinal, and retinol ester are quantitatively eluted in this fraction from control columns, their presence in Fraction I of bile could not be clearly demonstrated. Retinoic acid appears in Fraction II. After intraportal injection of labeled retinal or retinoic acid, approximately 30% of the radioactivity in Fraction II of bile was shown to be free retinoic acid by gas–liquid chromatography (13, 21). Fraction III, which was eluted with 30% acetic acid in methanol, contained 60–70% of the total radioactivity of bile. This fraction contains more polar and acidic products of retinol metabolism, including retinoyl β-glucuronide (22, 23).

The radioactivity in bile of rats injected with retinol-^{14}C was not volatilized by boiling under acidic conditions, was not precipitated by digitonin, and was not localized in taurocholate upon chromatography in a reversed-phase paper system (24). About 40% of the radioactivity could be extracted with diethyl ether after the bile was acidified. However, only 2–5% of the radioactivity was extracted by *n*-hexane from either acidic or basic bile solutions. The radioactivity in bile of rats administered either retinol-^{14}C or retinoic acid-^{14}C was dialyzable. Five hours after dialysis against 0.9% NaCl, 70–90% of the radioactivity was lost, and by 24 hr less than 10% remained within the dialysis tubing.

Rate of Disappearance of Metabolites of Retinol, Retinal, and Retinoic Acid from Tissues

After intraportal injection into intact animals, retinoic acid was rapidly eliminated from the liver, intestine, and kidney (Fig. 3). Four hours after the injection of retinoic acid-^{14}C, 6, 2.5, and 0.5% of the total dose was found in the liver, intestine, and kidneys, respectively. Thereafter, the radioactivity in these tissues steadily decreased, and by 40 hr only trace amounts were present.

Extracts of liver and intestine were chromatographed on anion-exchange resins (Table 1). After injection of retinol-^{14}C or retinal-^{14}C, 80–98% of the radioactivity

TABLE 1 DISTRIBUTION OF RADIOACTIVITY IN TISSUES AFTER THE ADMINISTRATION OF LABELED RETINOL, RETINAL, AND RETINOIC ACID TO BILE DUCT-CANNULATED RATS

The distribution of radioactivity in each chromatographic fraction is expressed as a percentage of the total ^{14}C eluted from the anion-exchange column. Recovery of radioactivity in all cases was ⩾80%. For bile the percentage of the injected dose is the cumulative amount excreted in 24 hr. For liver and intestine, the percentage of the injected dose is the amount found in the tissue 24, 6, and 4.5 hr after the administration of retinol, retinal, and retinoic acid, respectively.

Compound Administered (2.5–4.0 mg)	Bile				Liver				Intestine			
	% of Injected Dose	% of Tissue Extract			% of Injected Dose	% of Tissue Extract			% of Injected, Dose	% of Tissue Extract		
		I*	II	III		I	II	III		I	II	III
Retinol-^{14}C	20	10	20	70	24	98	1	1	1.4	78	7	15
Retinal-^{14}C	40	10	19	71	22	97	2	1	0.7	81	10	9
Retinoic acid-^{14}C	55	16	14	70	6	4	79	17	2.3	4	50	46

* Fractions I, II, III as in Fig. 2. Fraction 1 identified as nonpolar metabolites; II, acidic metabolites resembling retinoic acid; III, more polar acidic metabolites.

of liver and intestinal extracts was in Fraction I, further identified on alumina chromatography as retinol ester, and only small amounts of radioactivity were present in acidic metabolites (Fractions II and III). After retinoic acid-^{14}C injection, Fraction II contained 80% of the radioactivity in liver extracts, and 50% of that in intestinal extracts. A small but significant amount of radioactivity (0.25–1.0%) was repeatedly found in the intestinal contents of bile duct-cannulated animals after the administration of any of the three radioactive retinol compounds. Urinary metabolites of bile duct-cannulated animals given retinol and retinal were largely ionic (Fractions II and III), but were not further characterized because of the small amounts present (0.1–0.2% of dose).

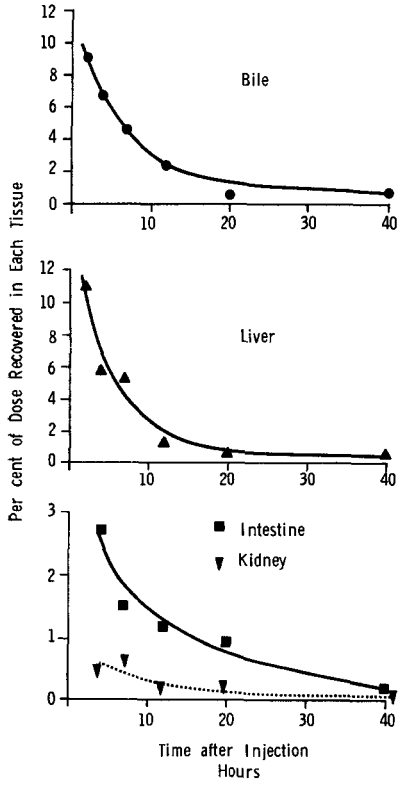

FIG. 3. Effect of time on the recovery of injected radioactive retinoic acid in bile and in various tissues. Two to 3.5 mg were administered to each of 12 rats and the tissues were analyzed for total radioactivity at the specified times.

Enterohepatic Circulation of Bile Metabolites of Retinol and Retinoic Acid

Several rats were given retinoic acid-^{14}C by stomach tube. Thereafter, one rat was cannulated every 12 hr for the following 3 days. Semilogarithmic plots of the amount of the bile radioactivity excreted by these rats in 24 hr against the time of cannulation gave an excretion rate of one-half that obtained from similarly plotting the amount of radioactivity excreted per hour during the 24 hr of cannulation when no recirculation of the bile occurred. This 2-fold rate difference was found with any 24 hr period, suggesting either that the rate of metabolite formation was increased when recirculation of the bile was prevented, or that some reabsorption of the metabolites normally occurred.

When whole bile containing the labeled metabolites of retinol-^{14}C was placed in an intestinal loop of another rat, 30% of this radioactivity was again excreted in the bile within 24 hr. Concomitantly, the radioactivity in the intestinal loop decreased (Fig. 4). Radioactive Fractions I, II, and III were isolated from the bile of animals injected with either radioactive retinol or retinoic acid. After evaporation of the chromatographic solvents, the radioactive residues were individually solubilized in 1 ml of Krebs-Ringer buffer with 0.4% sodium glycocholate, and were placed in duodenal loops of bile duct-cannulated rats. By 24 hr, each of the six recipient rats had excreted in their bile 25–40% of the intraduodenally administered radioactivity. This

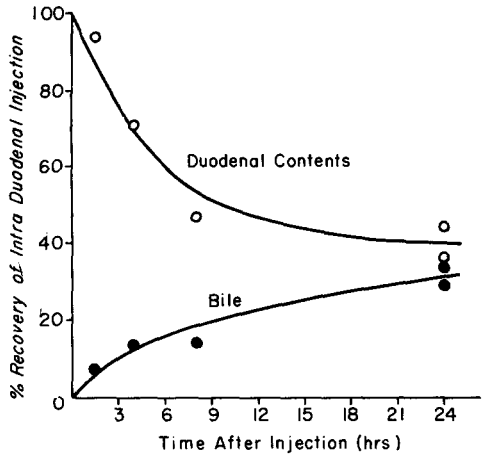

FIG. 4. Enterohepatic circulation of bile metabolites of retinol. Bile labeled with ^{14}C from a rat previously given an intraportal injection of retinol-^{14}C was placed in intestinal loops of bile duct-cannulated rats. Bile was collected from recipient rats for various periods of time and assayed for radioactivity. Contents of the intestinal sac were similarly assayed.

bile was rechromatographed on anion-exchange columns. As shown in Fig. 5, the radioactivity originally in Fraction III was absorbed and reexcreted in the bile as Fraction III, with only trace amounts appearing in Fractions I and II. In contrast, during enterohepatic circulation the radioactivity of Fractions I and II was converted largely into compounds chromatographing in Fraction III. No differences were noted in the metabolism of fractions derived initially from retinol or retinoic acid.

Excretion and Chromatography of Metabolites in the Bile and Perfusate of Isolated Perfused Livers

Isolated livers perfused with retinol-^{14}C or retinoic acid-^{14}C excrete about 10% of the total radioactivity into the bile within 1 hr (16). After perfusion with retinol, the distribution of bile radioactivity determined by ion-exchange chromatography was similar to that observed with whole animals (Fig. 2), except for a somewhat elevated Fraction I. With retinoic acid, chromatograms for the bile of perfused livers again were similar to those obtained from bile in vivo. The pattern of radioactivity in the perfusate, however, was strikingly different than for bile. After 1 hr of perfusion with either retinol-^{14}C or retinoic acid-^{14}C, the perfusate contained virtually no Fraction III. When retinol was the substrate, Fraction I contained most of the radioactivity, and when retinoate was used, Fraction II contained most of the radio-activity as expected.

DISCUSSION

The rapid disappearance of ingested retinoic acid from tissues, observed repeatedly in the past (1–6), was quantitatively analyzed in the present work. Four hours after an intraportal injection of retinoic acid, less than 10% of the injected radioactivity was in the liver, intestine, and kidneys. By 24 hr practically no retinoate remained in these organs. This loss of retinoic acid from tissues was accompanied by a rapid rise in the amount of metabolites of retinoic acid in bile. Forty per cent of the radioactivity of retinoic acid appeared in the bile of bile duct cannulated rats within 6 hr, and 95% of the injected dose was excreted via the bile within 5 days. On the other hand, when retinol-^{14}C or retinal was injected, 25–35% of the dose was deposited in the liver as retinol ester and a roughly equivalent amount was excreted in the bile in 24 hr. Thus, there is some correlation between the oxidation state of the injected compound and the amount of bile metabolite excreted in 24 hr. Goodman, Huang, and Shiratori (25) have recently reported recovering only 8–9% of the radioactivity in bile of rats intravenously injected with suspensions of chylomicrons containing radioactive retinol esters. This smaller amount of radioactivity excreted in the bile may be due to the utilization of the chylomicron form per se, but more probably is related to the fact that the retinol was injected in esterified form. Although the extent that retinol is metabolized by conversion to retinoic acid is unknown, the oxidation of retinal to retinoic acid may be important in determining the amount of bile metabolite produced from each substrate. It is well known that the oxidation of retinal is irreversible (11, 12), whereas esterification of retinol and its oxidation to retinal are not. Indeed, the equilibrium of the alcohol dehydrogenase system which oxidizes retinol to retinal strongly favors reduction (26). In addition, retinol-deficient animals will grow on daily supplements of retinoic acid, but quickly stop growing upon withdrawal of the supplement (27). In any event, biliary excretion is a major route by which metabolites of retinoic acid, retinal, and retinol leave the liver. Similar relative amounts of radioactivity appeared in bile after either physiological (10–26 μg) or high (2–3.5 mg) doses of retinol derivatives. The bile metabolites were readily produced after either intravenous, intraduodenal, or stomach tube administration of retinol and retinoic acid, and appeared in both deficient and normal rats. These facts support the thesis that the excretion of retinol metabolites in the bile is a normal and physiologically significant event, and not the expression of a detoxication phenomenon which is only activated by high intravenous doses of these potentially toxic compounds.

Since the isolated perfused rat liver produced bile metabolites (16) at an initial rate similar to that observed in vivo, the liver is probably the major site of their formation. The intestine, however, may also

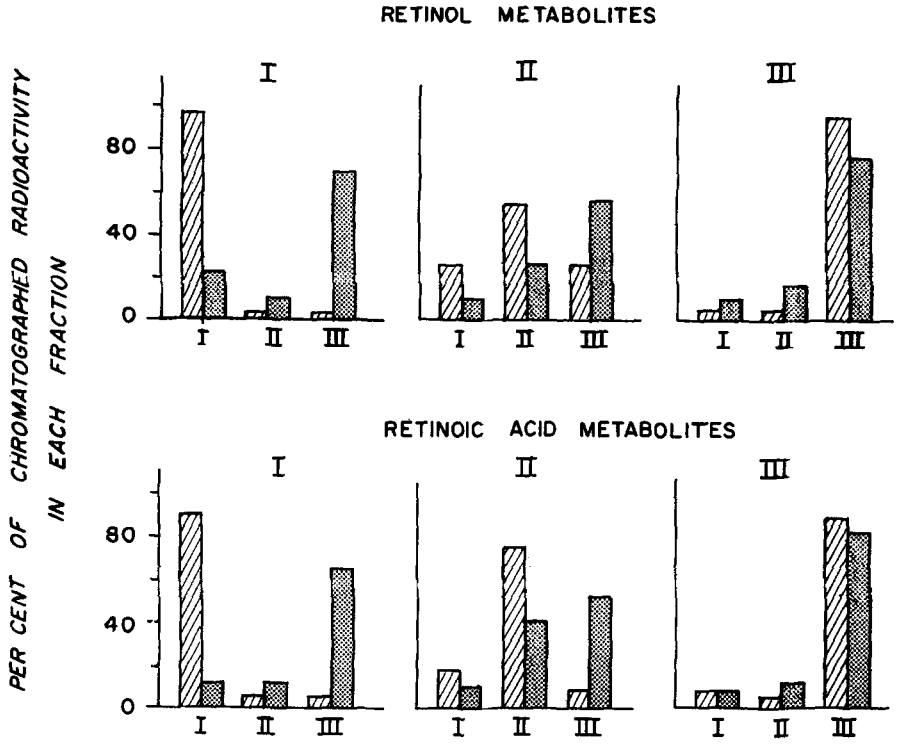

FIG. 5. Enterohepatic circulation of fractionated bile metabolites. Fractions I, II, and III were isolated from the bile of rats which had received intraportal injections of retinol-^{14}C or retinoic acid-^{14}C. Aqueous solutions of each fraction were placed in duodenal loops of bile duct-cannulated rats, and the resultant bile of each recipient rat was chromatographed on anion-exchange columns in the usual manner. The chromatograms of each of the individual fractions before (hatched columns) and after (dotted columns) reabsorption and reexcretion are shown. The initial Fraction II from retinol chromatographed poorly and contained appreciable amounts of Fractions I and III. The metabolism of that fraction is in accord, however, with other observed transformations.

metabolize retinoic acid. Metabolites similar to those in bile were noted in the intestinal wall and lumen, even in bile duct-cannulated rats. These probably were not transported to the intestine via the blood stream, since no significant amounts of metabolite were released into the perfusate in perfused liver experiments. In other laboratories biologically active products of retinoic acid have been isolated from the stomach (7), intestinal tract (8, 14), and liver (4), and water-soluble metabolites of retinol have been found in the plasma, urine, and feces of chickens and rats (6, 15, 28). With the exception of the compounds in the stomach, these metabolites might either arise in the liver and be excreted via the bile into the intestine, or be formed in part directly by the intestine. The metabolites of colon (6, 8) and feces (6, 15), however, may well arise from bile metabolites as a result of oxidation by intestinal bacteria.

The biliary metabolites of retinol compounds have been partially characterized. The bile radioactivity was not volatilized on boiling at acid pH, was not present in digitonin-precipitated sterols, and did not migrate with bile salts on reversed-phase paper chromatography. Apparently, the retinol-6,7-^{14}C compounds were not extensively catabolized to $^{14}CO_2$ or to small radioactive molecules, which then formed fatty acids, steroids, and bile acids (29). The radioactive bile metabolites, being readily dialyzable, are apparently not firmly bound to any large molecule or aggregate in bile. By anion-exchange chromatography the metabolites of bile were separated into three fractions, which contain nonionic compounds (I), acidic substances like retinoic acid (II), and more polar acidic derivatives (III). Whereas the rate of excretion of labeled metabolites in the bile increased with the oxidation state of the compound injected, the distribution of the radioactivity among the fractions was similar for all three retinol-^{14}C compounds (Fig. 2). Fraction I may consist of nonacidic esters of retinol and retinoic acid, and would include degraded nonacidic derivatives of the latter. Although the presence of retinol in bile has been reported (30), the 1% of the radioactivity of our bile metabolites which chromatographed with carrier retinol on alumina has not yet been identified as retinol. Fraction II, in addition to retinoic acid (13),

contains other labeled compounds which have not been characterized. Quantitatively, Fraction III is the most important fraction. Since Fractions I and II are converted into III, the latter contains the final polar and ionic metabolite common to all retinol compounds. The larger portion of this fraction, after retinoic acid-^{14}C administration, has been identified as retinoyl β-glucuronide (22, 23). Nearly half of the bile radioactivity obtained after injection of retinol ester-^{14}C was also hydrolyzed by β-glucuronidase (25). Most of the polar metabolites of tissues described elsewhere would also appear in Fraction III, including Wolf's compound 5 ester, one of the few purified metabolites of retinoic acid (14). The extensive characterization of the bile metabolites is the subject of another communication.

Enterohepatic circulation of biliary components, well-known for cholesterol, bile acids (31), and bile pigments (32), has now been shown to include metabolites of retinol. When whole bile containing labeled retinol metabolites or separate radioactive Fractions I, II, or III were placed in intestinal loops, about 30% of the radioactivity was reexcreted in the bile of the recipient rats within 24 hr. In the process, Fractions I and II were converted to Fraction III. Whether these conserved metabolites of retinol function as feedback inhibitors of their formation as in the cases of cholesterol and bile acids (31), have some function in intestinal physiology, are transport forms of retinoic acid, possess biological activity, or are merely excretion products are possibilities which merit further investigation.

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