Biosynthesis of β-glucuronides of retinol and of retinoic acid in vivo and in vitro

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ABSTRACT After the intraportal injection of retinol-6,7-^{14}C to rats, the *O*-ether derivative of retinol, retinyl β-glucosiduronate, appears in the bile. Both retinoyl β-glucuronide and retinyl β-glucosiduronate are also synthesized in vitro when washed rat liver microsomes are incubated with uridine diphosphoglucuronic acid (UDPGA) and either retinoic acid or retinol, respectively. The synthesis of retinoyl β-glucuronide was also demonstrated in microsomes of the kidney and in particulate fractions of the intestinal mucosa. The glucuronides were characterized by their UV absorption spectra, by their quenching of UV light or fluorescence under it, by their thin-layer chromatographic behavior in two solvent systems, and by the identification of products released during their hydrolysis by β-glucuronidase.

With retinoic acid as the substrate, the UDP glucuronyl transferase of rat liver microsomes had a pH optimum of 7.0, a temperature optimum of 38°C, and a marked dependence on the concentrations of both retinoic acid and UDPGA, but was unaffected by a number of possible inhibitors, protective agents, and competitive substrates. The conversion of retinal to retinoic acid and the synthesis of retinoyl β-glucuronide from retinoic acid could not be detected in whole homogenates, cell fractions, or outer segments of the bovine retina.

KEY WORDS retinol · retinyl β-glucosiduronate · retinoic acid · retinoyl β-glucuronide · rat liver microsomes · glucuronyl transferase · uridine diphosphoglucuronic acid · bile · retina

After the intraportal injection of retinoic acid into rats (1, 2) and into several other species (3), retinoyl β-glucuronide in appreciable quantities is excreted in the bile, partially reabsorbed in the intestinal tract, and re-excreted in the bile (2, 4). Even when small physiological doses (4–6) of retinoic acid, retinal, or retinol are administered, the biliary excretion of retinol derivatives not only occurs, but also appears to be a continuous, controlled process (6).

Although the *O*-ester glucuronide of retinoic acid, retinoyl β-glucuronide, was first identified as a major metabolite of retinoic acid in the rat, glucuronides of the *O*-ether and *N*-glycosyl types are also readily formed in the liver and other internal organs. The possibility that retinol might also form a glucuronide attracted us, and slight differences in the chromatographic behavior of polar derivatives of retinol and retinoic acid found in the bile (see Fig. 2, below; Ref. 4) encouraged us to characterize these biliary metabolites of retinol with care. As a result we report in this paper the isolation of the O-ether derivative of retinol, retinyl β-glucosiduronate, from rat bile, together with its partial characterization.

The formation of β-glucuronides and β-glucosiduronates of a wide variety of substances is catalyzed by one or more of the UDP glucuronate: 1-glucuronyl transferases (EC 2.4.1.17), which are localized largely in the microsomal fraction of the liver, kidney, and intestinal mucosa (7). Uridine diphosphoglucuronic acid (UDPGA) is a necessary cosubstrate in all these reactions. The probability that glucuronide conjugates of retinoic acid and retinol were formed by a similar process seemed compelling; we report here some characteristics of the isolated microsomal conjugating system. A preliminary report of the properties of this enzyme has been published (8).

MATERIALS AND METHODS

Radioactive Compounds and Other Chemicals

All-*trans* retinoic acid-6,7-^{14}C (0.045 μc/μmole), all-*trans*

This paper was drawn in part from a thesis submitted by K. Lippel to the University of Florida in partial fulfillment of the requirements for the Ph.D. degree.

Abbreviations: TLC, thin-layer chromatography; UDPGA, uridine diphosphoglucuronic acid.

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retinoic acid-15-^{14}C (11.16 μc/μmole), and all-*trans* retinol-6,7-^{14}C (2.16 μc/μmole) were generously donated by Hoffman-La Roche, Inc., Basel, Switzerland. The retinoic acid was purified by chromatography on either ion-exchange or silicic acid columns, and the retinol was purified by chromatography on an alumina column. Unlabeled all-*trans* retinol (Distillation Products Industries, Rochester, N.Y.) was used without further purification.

All solvents and chemicals were of analytical reagent grade. Solvents used for spectral measurements were redistilled. NAD$^+$, NADP$^+$, UDPGA, and bovine and bacterial β-glucuronidases were obtained from Sigma Chemical Co., St. Louis, Mo. Reduced glutathione was obtained from the Mann Research Labs Inc., New York, and sodium pyruvate from Calbiochem, Los Angeles, Calif. Tween 40 (polyoxysorbitan monopalmitate) was obtained from Atlas Chemical Industries Inc., Wilmington, Del., and Triton X-100 (octylphenoxypolyethoxy ethanol) from Rohm and Haas Co., Philadelphia, Pa.

Treatment of Bile and Tissues after the Injection of Labeled Retinol in Vivo

4 bile duct-cannulated rats were each injected in their portal vein with 3.0 mg of retinol-6,7-^{14}C (0.015 μc/μmole) in 1 ml of 16% Tween 40 in isotonic saline, and the bile was collected for 24 hr. Thereafter the rats were sacrificed, and the livers and intestines were separately pooled and homogenized in 125 and 80 ml of distilled water, respectively. The pooled bile samples and the homogenates were acidified to pH 4.0 with acetic acid, saturated with ammonium sulfate (1.5 g/ml of solution), and extracted with about an equal volume of *n*-butanol. More than 90% of the radioactivity was extracted into the butanol. Butanol extracts were clarified by centrifugation and were then reduced in volume under nitrogen and applied to thin-layer plates.

Thin-Layer Chromatography

Glass plates 20 × 20 cm were coated with an 800 μ layer of Silica Gel G (Research Specialties Co., Richmond, Calif. or E. Merck A. G., Darmstadt, Germany). Two solvent systems were used: (*a*) benzene–chloroform–methanol–acetic acid 5:5:5:1, termed the 5:5:5:1 system, and (*b*) benzene–chloroform–methanol 4:1:1, called the 4:1:1 system (9).

Retinyl β-glucosiduronate extracted from bile, retinoyl β-glucuronide and retinyl β-glucosiduronate synthesized in vitro, and retinoic acid and retinol released from their corresponding in vitro synthesized glucuronides after incubation with β-glucuronidase were separated on plates developed with the 5:5:5:1 or 4:1:1 systems.

To determine the amount of in vitro glucuronide synthesis we scraped the 1–6 cm segment of the TLC plate into a vial containing 10 ml of naphthalene–dioxane scintillation fluid (10) and counted the radioactivity on a Tri-Carb scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The radioactivity in the 1–6 cm segment of the experimental samples was corrected for the small amount of radioactivity found in the same segment of the control plates.

In other experiments 1 cm segments of the developed plates were counted in separate vials or, alternatively, the separated glucuronides or their hydrolysis products were eluted from the plates with methanol and the methanol extract was analyzed by other methods. The R_f values of retinol, retinoic acid, and their glucuronides are summarized in Table 1.

Homogenization and Fractionation of Tissues

Normal, fed male rats (Rolfsmeyer Farm, Madison, Wis.) weighing 150–300 g were anesthetized with ether. The liver was first perfused with 10–20 ml of cold 0.25 M sucrose, and then was excised, minced, and homogenized in 2–3 volumes of 0.25 M sucrose with a Teflon pestle in a glass homogenizer. The homogenate was fractionated by the procedure of Schneider and Hogeboom (11), and the isolated fractions were washed and resuspended in 25 ml of 0.25 M sucrose. The cellular fractions of kidney were prepared in the same manner. The small intestine was flushed out with 200 ml of cold 0.25 M sucrose and cut into 8-inch segments. Each segment was cut lengthwise, and the mucosa was scraped off and homogenized in 25 ml of 0.25 M sucrose. Most of the mucus was removed by filtration of the homogenate through a 100 mesh nylon screen (12). When only the microsomes of an organ were isolated, 0.01 M phosphate buffer pH 7.4 was used in place of 0.25 M sucrose.

Cow eyes obtained within 15 min after slaughter were iced and used within 1–2 hr. The fat, muscle, and front half of each of 10–20 eyeballs were cut away and the cornea, lens, and vitreous humor were discarded. The back half of the eyeball was everted, and the pink inner layer and the

TABLE 1 R_f VALUES

Compound	Solvent System* 4:1:1	Solvent System* 5:5:5:1
Retinoic acid	0.27 (0.27–0.42)	0.90 (0.85–0.95)
Retinol	0.55 (0.50–0.60)	0.90 (0.85–0.95)
Retinoyl β-glucuronide	0.03 (0.00–0.10)	0.27 (0.20–0.33)
Retinyl β-glucosiduronate	0.05 (0.00–0.13)	0.30 (0.13–0.47)

Figures in parentheses represent range of R_f values observed.
* Solvents were benzene, chloroform, methanol, acetic acid, in that order.

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pigmented outer layer of the retina were removed and separately homogenized in 5–10 ml of either 0.25 M sucrose, when all of the cell fractions were separated as described above (11), or in 0.01 M phosphate buffer pH 7.4, when only the microsomes were isolated. The outer segments of the retina were separated by centrifugation at 1600 *g* for 10 min (13).

Procedures for Incubation in Vitro and for Analysis of Glucuronides

Approximately 0.5–1.0 μc of retinoic acid-15-^{14}C, retinoic acid-6,7-^{14}C, or retinol-6,7-^{14}C was solubilized with 0.1–0.2% Tween 40 in 0.01 M phosphate buffer pH 7.4 or in 0.25 M sucrose. UDPGA (final concentration, approximately 2 mM) and microsomes suspended in 0.1 ml of 0.01 M phosphate buffer pH 7.4 were added at zero time to give a total volume of 1.2–1.3 ml. The mixture was incubated 30–60 min at 37°C in a Dubnoff shaker in the dark. Other cellular fractions were used in place of microsomes in some experiments. The reaction was stopped by the addition of 4 volumes of 0.4% acetic acid in acetone. After the mixture had been centrifuged at 10,000 rpm for 15 min, the clear supernatant solution was evaporated to dryness under nitrogen. The residue was dissolved in methanol and chromatographed on thin-layer plates. Control flasks which did not contain UDPGA were run with each experiment and their contents were similarly analyzed on thin-layer plates.

Assay for Aldehyde Dehydrogenase in Bovine Retina

All-*trans* retinal (10.5 mg) was dissolved in 1.5 ml of *n*-butanol containing 5% Triton X-100, and was carefully diluted to 25 ml with 0.1 M phosphate buffer, pH 7.4. This substrate solution (1.7 ml), 0.1 ml of 50 mM sodium pyruvate, 0.1 ml of either 20 mM NAD$^+$ or 20 mM NADP$^+$, and 0.1 ml of 20 mM reduced glutathione were incubated with 2 ml of either a homogenate, fractions, or outer segments of cow retinas at 37°C for 3 hr in the dark in a shaking bath. The reaction mixture was then extracted four times with 5 volumes of ether–*n*–butanol 9:1. After evaporation of the ether, the butanol layer was passed through an AG-2 X 8 acetate anion-exchange column (1 cm I.D. × 5 cm high). The column was developed with two 10 ml fractions of methanol followed by 10 ml of 5% acetic acid in methanol. The optical density of each tube at 350 mμ was recorded. Retinoic acid is eluted from such a column with 5% acetic acid in methanol.

β-Glucuronidase Assay

Microgram quantities of labeled bile metabolites or of retinoyl β-glycuronide or retinyl β-glucosiduronate synthesized in vitro were incubated at 37°C for 30–60 min in the dark in 0.5–1.0 ml of a 1% suspension of either bacterial β-glucuronidase in 0.02 M acetate buffer pH 4.6 or bovine 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 afterwards. Control tubes with boiled enzyme or without enzyme were also used in some experiments. The extracts, which contained both conjugated and hydrolyzed fractions, were evaporated under nitrogen, dissolved in methanol, and analyzed by TLC for the presence of retinol or retinoic acid.

Other Measurements

Spectra were determined with either a Beckman DU spectrophotometer or a Zeiss PMQ II spectrophotometer. Radioactivity in liquid samples was measured by adding 0.1 ml of the sample solution to 10 ml of naphthalene–dioxane scintillation fluid (10) and counting in either a Model 500B or a Model 574 Tri-Carb scintillation counter. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (14).

RESULTS

Synthesis of Retinyl β-glucosiduronate in Vivo

The butanol extract of the bile collected for 24 hr after the intraportal injection of retinol-6,7-^{14}C showed a spectrum with a detectable maximum around 330 mμ. When analyzed by TLC in the 4:1:1 system, 59% of the recovered radioactivity migrated in the "glucosiduronate" zone, with an R_f between 0 and 0.13 (Fig. 1). In the 5:5:5:1 system, 48% of the recovered radioactivity appeared in a broad peak (R_f 0.13–0.47) in which vitamin A glucuronides are also customarily found. Upon treatment of the butanol extract with β-glucuronidase, most of the released radioactivity migrated with retinol in the 4:1:1 system, with a much smaller portion in the retinoic acid zone (Fig. 2).

As expected, the butanol extract of the pooled livers, upon TLC analysis in the 4:1:1 solvent system, contained mainly retinyl ester (R_f 0.9) with a lesser quantity of retinol (R_f 0.5–0.6). At the most, only traces of polar metabolites were present. The butanol extract of the intestine, on the other hand, contained almost entirely a polar metabolite, as yet not fully characterized, which migrated in the glucuronide zone during TLC in the 4:1:1 solvent system. It is important to note, however, that retinol-6,7-^{14}C was given by intraportal injection in this experiment and not via the intestinal tract.

Synthesis of Retinyl β-Glucosiduronate in Vitro and Its Characterization

When retinol-6,7-^{14}C was incubated with UDPGA and rat liver microsomes, 13.5% of the radioactivity re-

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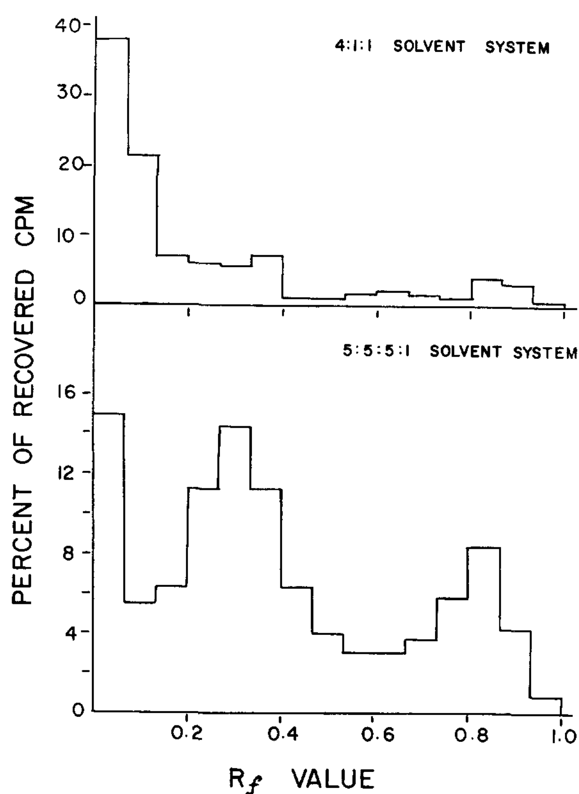

FIG. 1. Separation of biliary products of retinol-6,7-^{14}C on thin-layer plates of Silica Gel G in the 4:1:1 solvent system (above) and 5:5:5:1 solvent system (below). The R_f values of vitamin A glucuronides are 0–0.13 (4:1:1) and 0.13–0.47 (5:5:5:1). The R_f value of retinol and retinoic acid is 0.90 (5:5:5:1).

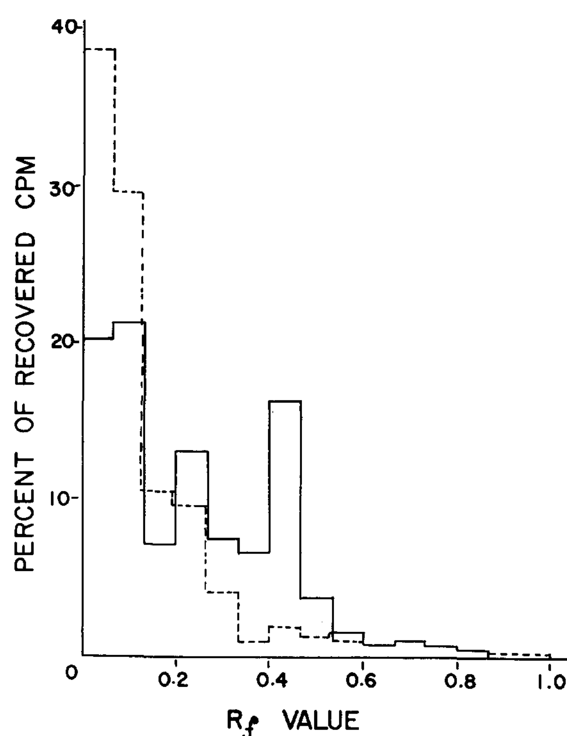

FIG. 2. Analysis by TLC (4:1:1) of a butanol extract of bile treated with β-glucuronidase. R_f values for retinyl β-glucosiduronate, retinoic acid, and retinol in this system are 0.05, 0.3, and 0.5, respectively. Solid line, treated sample; broken line, untreated sample.

covered from a thin-layer chromatogram of the reaction mixture was found in the R_f zone of 0.13, whereas less than 3% of the radioactivity in a sample incubated without UDPGA was detected in this zone (Fig. 3). This UDPGA-dependent polar metabolite was identified as retinyl β-glucosiduronate on the following grounds: (*a*) it was hydrolyzed by β-glucuronidase; (*b*) its TLC spot fluoresced bright yellow when viewed under UV light at 360 mμ, which is characteristic of retinol derivatives and contrasts sharply with the quenching produced by retinal and retinoic acid conjugates; (*c*) the radioactive compound released by β-glucuronidase migrated on TLC with an R_f value identical with that of all-*trans* retinol (0.50) and not with that of retinoic acid (0.27).

Synthesis of Retinoyl β-Glucuronide in Vitro and Its Characterization

The product obtained by incubating UDPGA and labeled retinoic acid with rat liver microsomes was separated from unreacted retinoic acid by TLC on S lica Gel G in the 5:5:5:1 system. In the TLC lane containing the extract from the experimental flask, a dark UV-absorbing spot with an R_f of 0.2–0.33 was present, which contained 4.8% of the recovered radioactivity (Fig. 4). The corresponding area of the control flask did not quench UV light and contained only 0.7% of the recovered counts. In both cases over 90% of the radioactivity migrated with unreacted retinoic acid near the solvent front. The UV absorbing spot was identified as retinoyl β-glucuronide by several means: (a) it had the UV spectrum characteristic of retinoic acid compounds with an absorption maximum at 350 mμ, (*b*) its R_f values were identical with those of retinoyl β-glucuronide formed in vivo in both the 5:5:5:1 (R_f 0.30) and the 4:1:1 (R_f 0.03) solvent systems, and (*c*) it was hydrolyzed by β-glucuronidase, which releases a compound with the chromatographic behavior and spectral properties of retinoic acid.

Distribution of UDP Glucuronyl Transferase in Cell Fractions of Various Tissues

Of various fractions of the liver and kidney, the microsomes clearly contain most of the retinoyl β-glucuronide synthesizing ability (Table 2). Interestingly, no activity was demonstrable in whole homogenates of kidney, although the isolated microsomes were highly active. In the intestine, the activity was broadly distributed among the particulate fractions. Fractionation of this tissue was rendered difficult by the presence of large amounts of mucus, however, and the observed enzyme activity in

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TABLE 2 FORMATION OF RETINOYL β-GLUCURONIDE BY WHOLE HOMOGENATES AND CELL FRACTIONS OF RAT LIVER, KIDNEY, AND INTESTINAL MUCOSA

	Liver	Kidney	Intestine
	mμmoles/hr/mg protein		
Whole homogenate	0.73	0	0.61
Nuclei + debris	0.90	0.06	0.88
Mitochondria	0.76	0.42	0.78
Microsomes	2.59	1.53	0.51
Supernatant fraction	<0.01	0	0.06

Each flask contained 0.75–1.0 μc of retinoic acid-15-^{14}C (0.115 μc/μmole), 2.8 μmoles of UDPGA (5.6 μmoles for intestine), and cell fractions with the following amounts of protein (liver, 0.81–1.02 mg; kidney, 0.09–0.35 mg; intestine, 0.01–0.21 mg) in 1.3 ml of sucrose–0.01 M phosphate buffer pH 7.4. The incubation time at 37°C was 30 min (45 min for intestine).

organelles other than the microsomes probably results from faulty separation.

No retinoyl β-glucuronide could be detected when either the inner or the outer layer of bovine retina was incubated with labeled retinoic acid and UDPGA. In addition, the oxidation of retinal to retinoric acid could not be detected in either whole homogenates or cell fractions of the inner or outer layers of bovine retina in the presence of either NAD$^+$ or NADP$^+$.

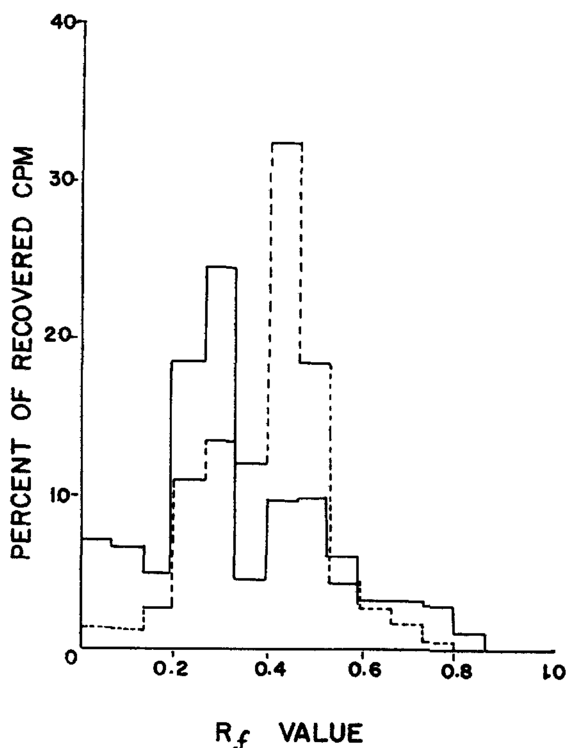

FIG. 3. Distribution of recovered radioactivity on a thin-layer plate (4:1:1) after the incubation of retinol-6,7-^{14}C with rat liver microsomes in the presence (solid line) and absence (broken line) of UDPGA. Under these conditions the R_f values of vitamin A glucuronides, retinoic acid, and retinol are about 0.05, 0.3, and 0.5, respectively.

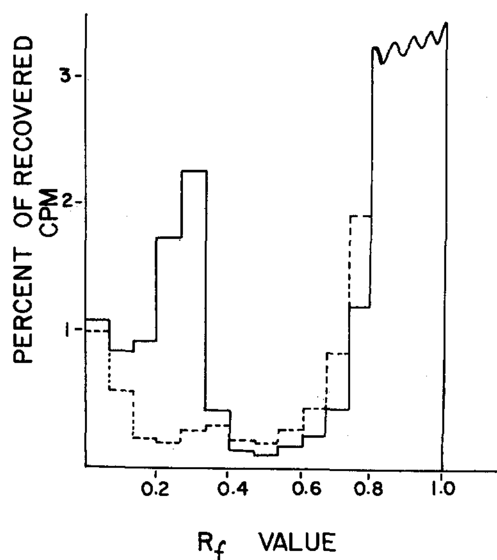

FIG. 4. Distribution of recovered radioactivity on a thin-layer plate (5:5:5:1) after the incubation of labeled retinoic acid with rat liver microsomes in the presence (solid line) and absence (broken line) of UDPGA. The R_f values of retinoyl β-glucuronide and retinoic acid in this system are 0.3 and 0.9, respectively.

Factors Affecting the Synthesis of Retinoyl β-Glucuronide by Rat Liver Microsomes

The effects of enzyme, UDPGA, and retinoic acid concentrations, pH, incubation time, temperature, and several possible inhibitors and competitive substrates on the UDP glucuronyl transferase activity of rat liver microsomes were evaluated.

The rate of formation of retinoyl β-glucuronide was proportional to the amount of enzyme present up to 1 mg of protein, and the enzyme was maximally active at pH 7.0. It was studied over the pH range 5.85–7.40. Although the amount of glucuronide synthesized was found to be proportional to the UDPGA concentration (Fig. 5), the enzyme could not be fully saturated with UDPGA concentrations as high as 10 mM. The formation of glucuronide was similarly dependent on the concentration of retinoic acid (Fig. 6). Higher concentrations of retinoic acid could not be tested effectively because the enzymatic activity was inhibited by the large amount of Tween 40 required to solubilize retinoic acid at these higher concentrations. Glucuronide synthesis increased linearly with time for 30 min (Fig. 7), and was nearly linear up to 60 min. Normally, 30-min incubation periods were used. The optimal temperature for enzyme activity was 38°C; relative activities at other temperatures were 83% at 45°C, 40% at 30°C, and 12% at 22°C.

The UDP glucuronyl transferase that acts on retinoic acid was resistant to the action of many compounds which might well have inhibited or stimulated the reaction (Table 3). Glucuronic acid and γ-glucuronolactone, possible competitive inhibitors for UDPGA, had no effect at

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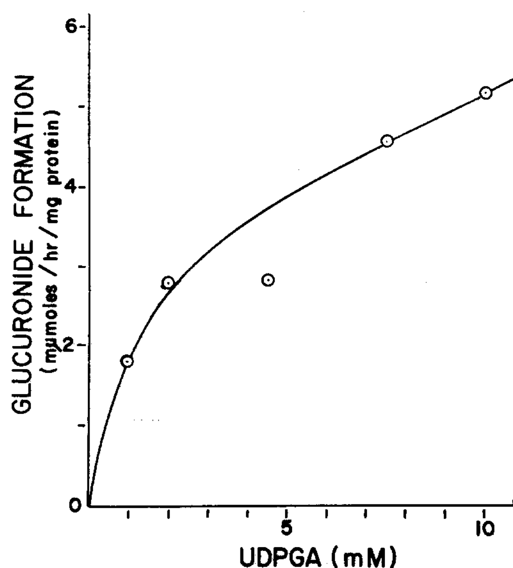

FIG. 5. Synthesis of retinoyl β-glucuronide as a function of UDPGA concentration. Each flask contained 1.0 mM retinoic acid-15-^{14}C (2.04 μc/μmole), 0.45 mg of microsomal protein, and the given concentration of UDPGA in a final volume of 0.6 ml 0.01 M phosphate pH 7.4. Flasks were incubated for 30 min at 37°C. All experimental values are corrected for radioactive contaminants present in the control flask which are equivalent to 1.8 mμmoles of the glucuronide.

5×10^{-5} M. The possibility that newly synthesized retinoyl β-glucuronide might be rapidly hydrolyzed in the microsomal system was tested by the inclusion of *d*-saccharolactone, a known inhibitor of β-glucuronidase. Rather than increasing the yield of the glucuronide, *d*-saccharolactone, if anything, slightly inhibited the reaction. Sodium barbital, an inhibitor of UDPGA pyrophosphatase, and *N*-ethyl maleimide, a powerful sulfhydryl binding reagent, were essentially inert. Rather surprisingly, several water-soluble substrates which also form glucuronides from UDPGA in the presence of microsomes did not depress the synthesis of retinoyl β-glucuronide either (Table 3).

TABLE 3 EFFECT OF VARIOUS COMPOUNDS ON THE SYNTHESIS OF RETINOYL β-GLUCURONIDE BY RAT LIVER MICROSOMES

Added Compound	Percentage of Normal Rate
None	100
Glucuronic acid	95
γ-Glucuronolactone	108
d-Saccharolactone	80
Sodium barbital	101
N-Ethyl maleimide	89
p-Nitrophenol	98
p-Aminobenzoic acid	84
Phenylacetic acid	112

Each flask contained 1.0 mM retinoic acid-15-^{14}C (1.11 μc/μmole), 1.6 mM UDPGA, 0.78 mg of microsomal protein, and 0.05 mM of the cited compound in a final volume of 1.3 ml of 0.01 M phosphate, pH 7.4. The incubation period was 30 or 60 min at 37°C. The rate of retinoyl β-glucuronide formation in the absence of added compounds was 0.93–1.38 mμmoles/hr per mg of protein.

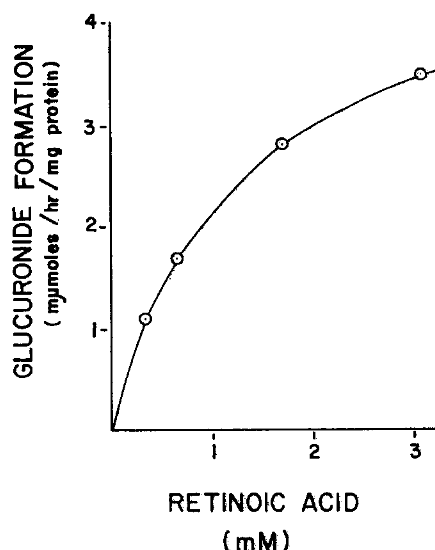

FIG. 6. Synthesis of retinoyl β-glucuronide as a function of retinoic acid concentration. Each flask contained 2.2 mM UDPGA, 0.57 mg of microsomal protein, and the given concentration of retinoic acid-15-^{14}C (3.20 μc/μmole) in a total volume of 1.3 ml of 0.01 M phosphate, pH 7.4. Flasks were incubated for 30 min at 37°C.

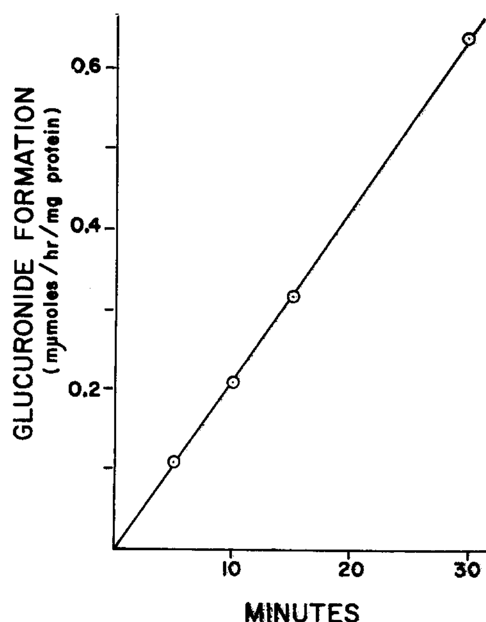

FIG. 7. Synthesis of retinoyl β-glucuronide as a function of incubation time at 37°C. Each flask contained 1.27 mM retinoic acid-15-^{14}C (0.159 μc/μmole), 2.6 mM UDPGA, and 7.96 mg of microsomal protein in a final volume of 2.6 ml of 0.01 M phosphate, pH 7.4. Experimental values are corrected for radioactive contaminants present in the control flask which were equivalent to 0.25 mμmole of the glucuronide.

DISCUSSION

A wide variety of aromatic, alicyclic, and aliphatic compounds are conjugated with glucuronic acid and often excreted as such in vertebrate species. Since administered retinoic acid was shown earlier to be largely excreted in the bile as retinoyl β-glucuronide (1, 2), the fact that ad-

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ministered retinol is excreted in the bile as the analogous *O*-ether derivative, retinyl β-glucosiduronate, is not at all surprising. Although this new metabolite of retinol has not as yet been crystallized and analyzed by rigorous chemical procedures, its identification rests on the following, relatively firm grounds: (*a*) it shows an absorption spectrum with a λ_{max} of 330 mμ; (*b*) it migrates on TLC in the glucuronide fraction in two solvent systems; (*c*) it is hydrolyzed by β-glucuronidase, which releases a product which migrates on TLC with retinol; (*d*) it emits a bright yellow fluorescence when excited with 360 mμ light, a property characteristic of retinol and its conjugates, but not of other compounds in the vitamin A group; and (*e*) it is synthesized from retinol by washed rat liver microsomes in vitro in the presence of UDPGA.

The identification of glucuronic acid conjugates of both retinol and retinoic acid in the bile raises questions concerning their physiological interrelationship. Since retinoic acid is not reduced to retinol biologically, administered retinoate will naturally give rise in the bile only to retinoyl β-glucuronide. Retinal, which may be either oxidized or reduced biologically, has been shown to yield some biliary retinoyl β-glucuronide (2, 15), but also an appreciable quantity of other polar metabolites. In the present study relatively large doses (3.0 mg) of retinol gave predominantly retinyl β-glucosiduronate in the bile, whereas smaller doses (48–58 μg) of retinol, gave, after treatment of the bile with β-glucuronidase, slightly more retinoic acid than retinol; in the latter case half of the radioactivity remained in the bile and was not extracted with chloroform (5). The radioactive derivatives present in the bile after the intravenous injection of chylomicron-bound retinyl ester are cleaved to a significant degree by β-glucuronidase, but the products released were not identified (16). In all likelihood both retinoyl and retinyl conjugates are excreted normally, and the elucidation of factors which control the excretory form of vitamin A under various conditions should excite future interest.

When retinoic acid is administered to rats, water-soluble polar metabolites, which migrate with retinoyl β-glucuronide on TLC and are cleaved by β-glucuronidase, are excreted in the urine, and are found, together with free retinoic acid, in the feces as well (5, 6). If retinyl β-glucosiduronate also proves to be a final excretory product of retinol, the role of retinoic acid in vitamin A metabolism and function is rendered quite uncertain. In any event, retinoic acid can not be considered as an obligatory intermediate in retinol metabolism. Metabolic relationships in the liver may be summarized as follows:

retinyl β-glucosiduronate ⇆ retinol ⇄ retinal →
retinol ⇅ retinyl ester
retinoate ⇄ retinoyl β-glucuronide.

Together with the liver, the kidney and intestine are generally considered to be the major organ sites of glucuronide synthesis (17). In the present case, particulate fractions of all three organs readily catalyzed the formation of retinoyl β-glucuronide from retinoic acid. In view of the known conversion of a moderate but significant portion of dietary β-carotene or retinal to retinoic acid in the gut (18), the subsequent formation of retinoyl β-glucuronide in the small intestine is a probable physiological event. The water-soluble glucuronide might well be transported via the portal blood, which is known to contain appreciable amounts of retinoic acid under certain experimental conditions (18). Whether the intestine also forms retinyl β-glucosiduronate has not yet been tested.

The apparent absence of retinal oxidase and UDP glucuronate:retinoate 1-glucuronyl transferase in various fractions of the retina is of considerable interest. Although formaldehyde is oxidized to formate in the retina (19), the conversion of retinal to retinoate has never been shown (20). Similarly, the formation of glucuronides of anthranilic acid (21) and of *o*-aminophenol (17) could not be demonstrated in tissues of the eye. The apparent absence in the retina of these major routes for the metabolism and excretion of vitamin A may well be one adaptation of this tissue to its need to conserve appreciable concentrations of retinal for action in the visual cycle. The way in which vitamin A is released from the eye is not known.

The demonstrated synthesis of glucuronides of retinol and retinoic acid by washed microsomal preparations of liver, kidney, and intestine opens the way for a more detailed examination of the characteristics of the conjugating system and of its control. It is first instructive, however, to compare the rate of retinoyl β-glycuronide synthesis in vitro and in vivo. In bile-cannulated rats, 10–15% of a dose of 2–3.5 mg of retinoic acid was excreted in the bile during the first 2 hr period (4). By using average values for liver weights and protein content, and by assuming that 80% of the biliary retinoate was glucuronide, we obtain a rough estimate of the rate of retinoyl β-glucuronide synthesis in the liver in vivo, namely 3 mμmoles/hr per mg of protein. Although the microsomal conjugating system was never fully saturated with respect to retinoate and UDPGA in vitro, values above 5 mμmoles of retinoyl β-glucuronide formed per hr per mg of protein were commonly observed in vitro. Clearly many factors may affect the rate both in vivo and in vitro, but the similarities in the observed rates suggest that the system studied in vitro does have a significant relationship to the physiological event. The rate of formation of retinoyl β-glucuronide in vitro also compares favorably with the synthetic rates for glucuronides of testosterone, *o*-aminophenol, and estriol in several tissues (22–24), but is somewhat lower than those for glucuro-

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nides of aniline, *o*-aminophenol, and bilirubin in others (25, 26).

The properties of the conjugating system have been examined most closely in rat liver microsomes with retinoate as the substrate. The enzyme activity shows the expected dependence on the concentrations of retinoate, UDPGA, and enzyme protein, and on the incubation time and temperature. The lack of saturation of the enzyme by high concentrations of UDPGA is reminiscent of the properties of the guinea pig microsomal system in conjugating *p*-nitrophenol (27). A number of potential inhibitors, protective agents, and competitive substrates, which have demonstrated effects in other glucuronide forming systems, were essentially inert at 5×10^{-5} M with respect to the retinoic acid conjugating system. Although the presence in microsomes of a family of transferases with a considerable measure of substrate specificity accords well with recent studies (28), it would be premature to postulate the existence of a specific conjugating enzyme for retinol and retinoic acid.

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