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Glucuronidation of Retinoic Acid by
Small Intestinal and Liver Microsomes

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GLUCURONIDATION OF RETINOIC ACID BY SMALL INTESTINAL AND LIVER MICROSOMES

By

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ABSTRACT

GLUCURONIDATION OF RETINOIC ACID BY SMALL INTESTINAL AND LIVER MICROSOMES

BY

Kevin Lyndon Salyers

We studied and compared the kinetics of retinoic acid glucuronidation by UDP-glucuronosyltransferase in the small intestine and liver. Due to phospholipid dependency of this enzyme, the reaction was conducted in a liposomal system. The apparent Km for retinoic acid with small intestinal microsomes was 123 uM and the apparent Vmax, 46 pmol/min/mg of microsomal protein. The apparent Km for retinoic acid of liver microsomes was 182 uM and the apparent Vmax, 55 pmole/min/mg protein. The differences in Vmax and Km between the tissues were significant at $p < 0.05$. Retinoyl-β-glucuronide was identified by cochromatography with authentic retinoyl-β-glucuronide, hydrolysis with β-glucuronidase, and negative ion mass spectrophotometry. The similarity in the kinetics of retinoic acid glucuronidation in small intestine and liver suggests that the reaction is catalyzed by the same enzyme and that retinoyl-β-glucuronide is an important metabolite of vitamin A in both tissues.

To my wife Renee'
for her patience
and her love.

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LIST OF ABBREVIATIONS

BHT	butylated hydroxytoluene
CRABP	cellular retinoic acid-binding protein
CRBP	cellular retinol-binding protein
^{3}H-RA	all-trans-(11-^{3}H) retinoic acid
^{3}H-RG	all-trans-(11-^{3}H) retinoyl-β-D-glucuronide
MFO	mixed function oxidase
NaAc	sodium acetate
NADH	nicotinamide adenine dinucleotide
RA	all-trans retinoic acid
RAL	all-trans retinal
RBP	retinol binding protein
REH	retinyl ester hydrolase
RG	all-trans retinoyl-β-D-glucuronide
ROL	all-trans retinol
SL	saccharo-1,4-lactone
TTR	transthyretin
UDP	uridine 5'-diphosphate
UDPGA	uridine 5'-diphospho-glucuronic acid
UDPGT	uridine 5'-diphosphate-glucuronosyltransferase

INTRODUCTION

Vitamin A is an essential micronutrient which is required for normal development, growth, reproductive capacity, and vision. The term "vitamin A" is used generically to include all natural compounds which exhibit the biological activity of retinol. Retinoic acid (RA) is a physiological metabolite of retinol (1); it sustains body growth and differentiation of epithelial tissues, but unlike retinol, does not support visual and reproductive functions (2-4). One of the first metabolites of retinoic acid to be identified was retinoyl-β-glucuronide (RG) (5). The conjugate of retinoic acid and glucuronic acid has been detected in the bile (5), urine (6), intestinal mucosa (7), liver (8), and in human blood (9). In the liver, RG occurs at the same level whether physiological or pharmacological levels of RA are administered (8,10). In the intestinal mucosa of bile duct-cannulated rats, RG is the major metabolite of all-trans-RA. Although RG was originally considered primarily an excretion product, it has subsequently been shown to be as active as RA in promoting growth of vitamin A deficient rats (6) and in causing epithelial differentiation in a rat vaginal-smear assay (11).

The conjugation of retinoic acid with uridine diphosphate-glucuronic acid (UDPGA) is catalyzed by uridine

diphosphate-glucuronosyltransferase (UDPGT) (12). This enzyme belongs to a family of integral membrane proteins with the majority of their activity located in the endoplasmic reticulum (13). The regulation of UDPGT activity in vivo is unknown. However, its catalytic activity in vitro can be altered by membrane perturbing processes such as mechanical disruption treatment with detergents, proteases or phospholipases (14). The lipid environment of UDPGT appears to be an integral part of its regulatory mechanism (15). The dependence of UDPGT activity on the presence of phospholipids, especially phosphatidylcholine (PC), has been clearly demonstrated by partial or complete loss of enzyme activity during purification (14,16,17). The residual activity of pure, delipidated enzyme can be enhanced as much as 700-fold by reconstitution with appropriate phospholipids (17,18). In addition, affinities for substrates, catalytic specificity, and cooperative binding of UDPGA depend on the physical properties of the lipids used to reconstitute the pure enzyme (17-20).

A valuble approach to characterizing enzymes is kinetic analysis. The kinetic parameters Km and Vmax are useful in differentiating isoenzymes and the mechanism(s) at their active site(s), estimating the intracellular substrate concentration, and elucidating an in vivo role of the enzyme in metabolism.

The purpose of this study was to test the hypothesis that the apparent kinetic parameters for small intestinal and liver microsomal UDP-glucuronosyltransferase are significantly different toward the substrate all-trans-retinoic acid. A significant difference may indicate different physiological roles for the enzyme in the metabolism of retinoic acid in different tissues.

The specific objectives of the study were:

1) To develop in vitro reaction conditions which mimic the native membrane environment of UDPGT.

2) To determine and compare the apparent kinetic parameters of the small intestinal and liver microsomal UDPGT- catalyzed glucuronidation of retinoic acid.

3) To compare the apparent kinetic parameters for retinoyl UDPGT in the liver between a liposomal and a soluble system.

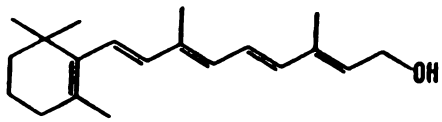
REVIEW OF VITAMIN A

Vitamin A is essential for growth, vision, reproduction, maintenance of differentiated epithelia, and mucus secretion in higher animals. Except for the role of retinal (RAL) in the visual cycle, which has been well characterized at the molecular level (21), very little is known about the biochemical function of vitamin A. This review will summarize the literature on the metabolism of the natural forms of vitamin A. Several natural forms of vitamin A and their derivatives are shown in Fig. 1.

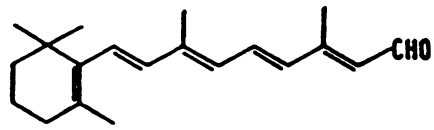
A. Dietary Sources and Absorption of Vitamin A

The major source of vitamin A in the human diet is plant carotenoids, or pro-vitamin A compounds, such as β-carotene. The absorption of β-carotene by the intestine requires the presence of fats, bile salts, and vitamin E (22). After absorption into the cells of the small intestine, the enzyme 15,15'-dioxygenase cleaves β-carotene at the central double bond to form two molecules of RAL (23). The RAL produced in this reaction is reduced to form retinol (ROL) by nicotinamide adenine dinucleotide (NADH) and aldehyde reductase (24). Other dietary sources of vitamin A, obtained only from animal-derived foods, are retinyl esters and ROL. The absorption of retinyl esters requires bile salts, fats, dietary protein and vitamin E

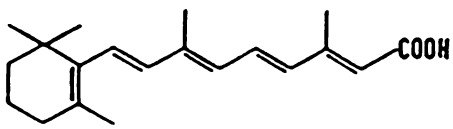
Figure 1. Structural Formulas and Nomenclature of Selected Retinoids.



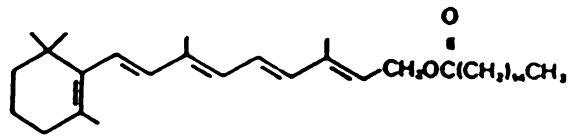
Retinol



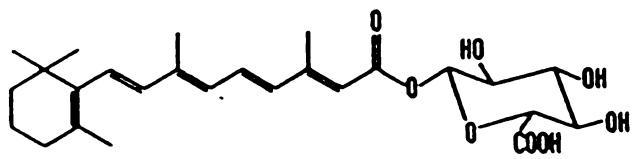
Retinal



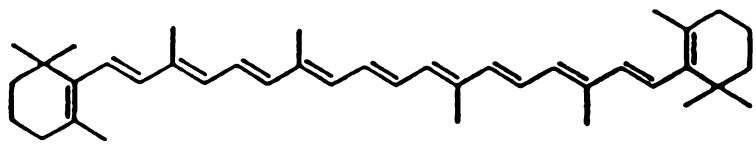
Retinoic Acid



Retinyl Palmitate



Retinoyl-B-Glucuronide



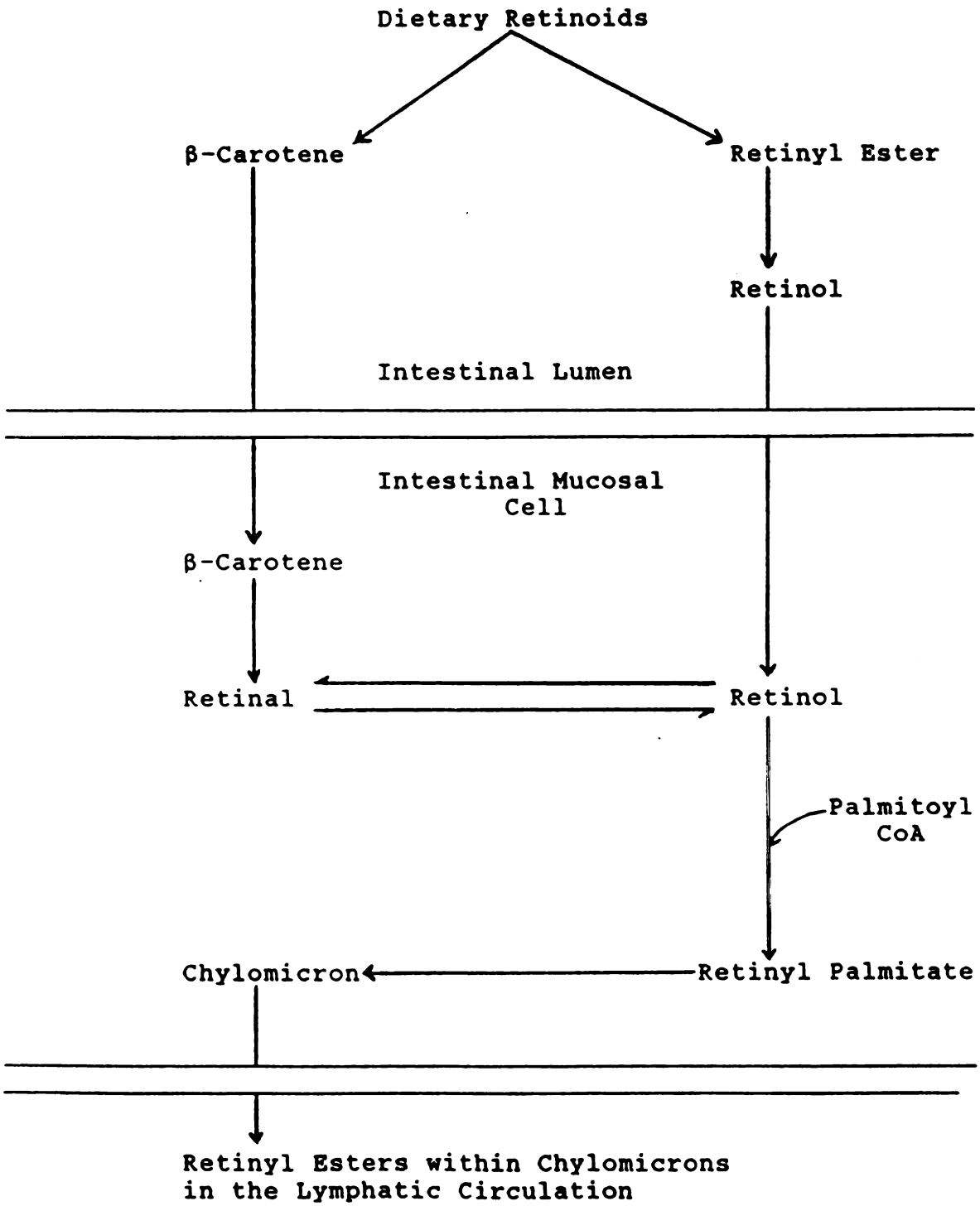
B-Carotene

(22). Dietary retinyl esters are hydrolyzed in the lumen of the intestine by digestive esterases (25); so the actual form entering the mucosal cell is ROL.

Retinol from either plant or animal dietary sources is re-esterified in the intestinal mucosal cell and the resulting retinyl esters associate with chylomicrons and enter the lymphatic system for transport through the general circulation. The absorption of dietary retinoids is shown in Fig. 2. In the plasma, chylomicrons acquire C and E apolipoproteins which activate lipoprotein lipases (26). Lipolysis of triglycerides result in the formation of smaller lipoprotein particles called chylomicron remnants (26). The chylomicron remnants reaching the liver are rapidly taken up by the parenchymal cells (27). The retinyl esters are hydrolysed and re-esterified, primarily with palmitic acid, for storage in the liver. Retinyl esters are stored both in parenchymal and fat-storing liver cells (28). The role of these different liver cell types has not been defined entirely. Approximately 90% of the body's vitamin A is stored in the liver as retinyl esters.

For release from storage, retinyl esters are hydrolyzed by retinyl ester hydrolase (REH), and mobilized as ROL bound to retinol binding protein [RBP] (29). Retinol-RBP is transported in plasma as a 1:1 molar complex with transthyretin (TTR) (30). These proteins serve to transport vitamin A from the liver to peripheral sites of action. Retinol mobilization and delivery to target tissues are

Figure 2. Absorption of Dietary Retinoids



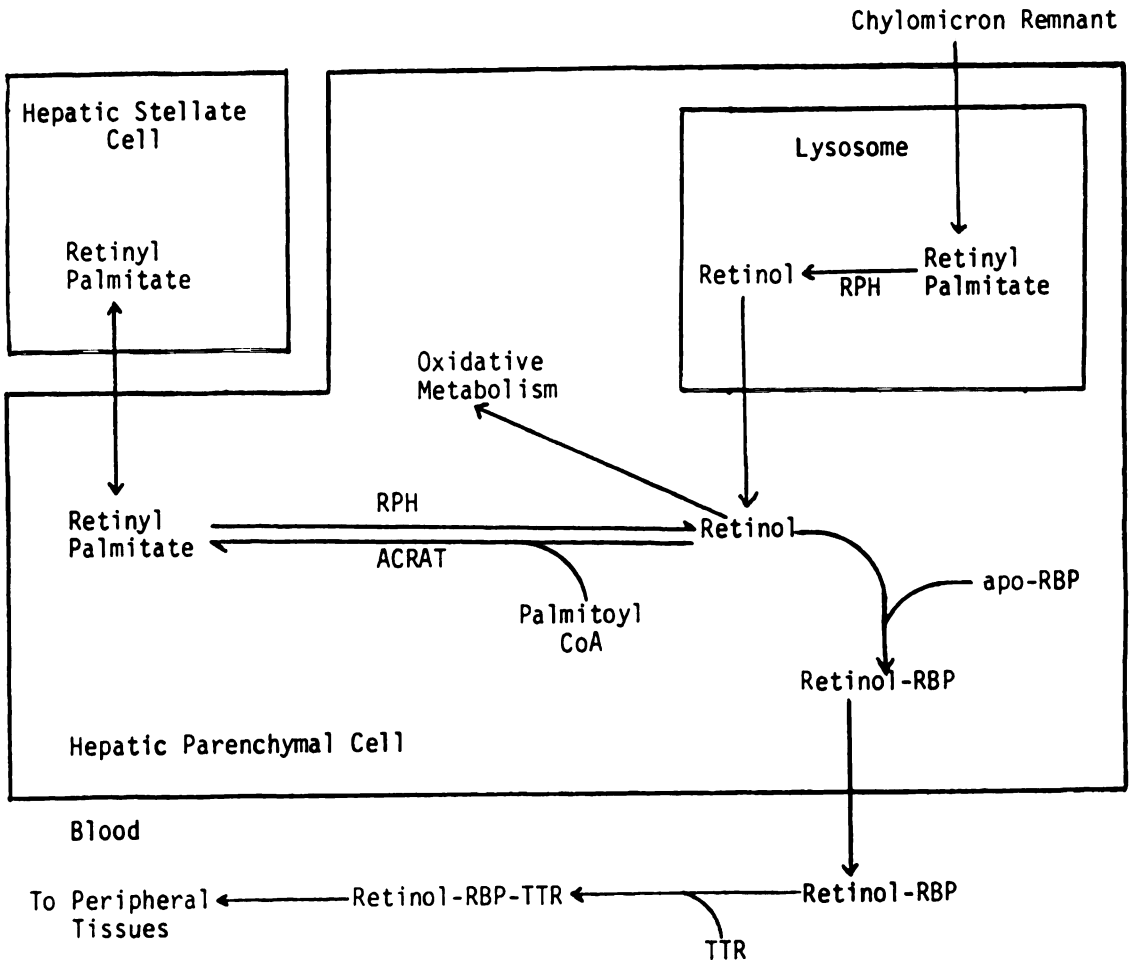
highly regulated processes which may be regulated by the rate of RBP synthesis, or the activity of REH. Hepatic uptake, storage, and mobilization of vitamin A are shown in Fig. 3.

Cellular uptake of ROL from plasma has been demonstrated in cells from bovine epithelium (31), monkey small intestine (32), cornea (33), liver parenchyma (34), and human placenta syncytiotrophoblast (35). However, the roles of RBP and TTR in cellular uptake of ROL are not clearly defined. Once in the cell, ROL is bound to another protein, cellular retinol-binding protein (CRBP) (36). Retinoic acid (RA), a biologically active metabolite of vitamin A, is also bound in the cell to a specific protein, cellular retinoic acid-binding protein (CRABP) (37). CRABP has a more limited tissue distribution than CRBP; abscence of CRABP in rat liver is currently under investigation (38).

It has been suggested that CRBP may be involved in the interaction of ROL with binding sites on the nucleus (39). The proposed mechanism involves the translocation of the ligand, mediated by a cytoplasmic-specific binding protein (receptor), into the nucleus. Pure CRBP complexed with radioactive ROL has been shown to transfer the vitamin into the nucleus to specific binding sites on the chromatin without itself remaining bound to the chromosomal material (39).

Recently, two separate laboratories have cloned the nuclear receptor for RA (40,41). The protein is highly

Figure 3. Hepatic Uptake, Mobilization and Storage of Vitamin A



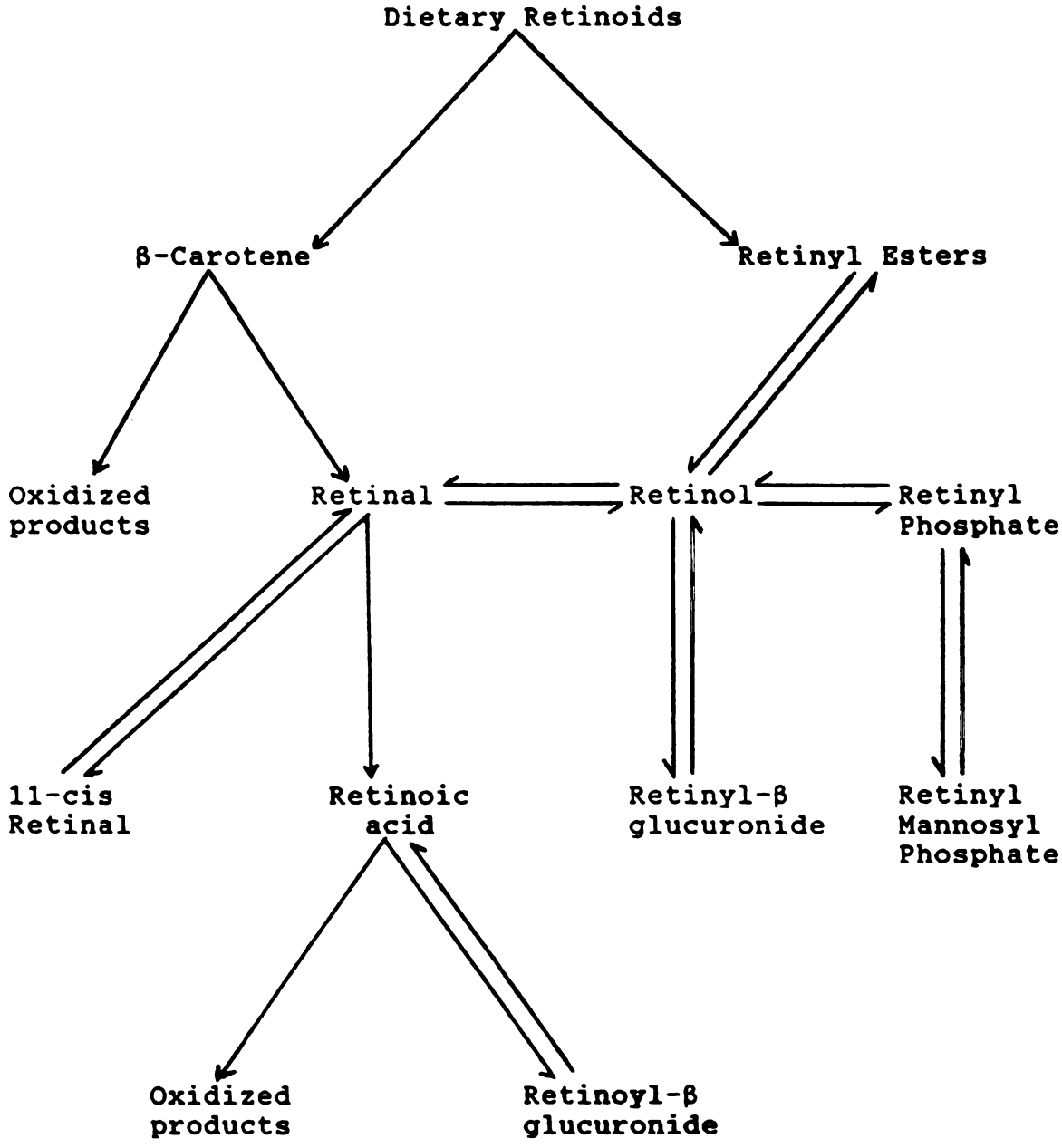
ACRAT: Acyl-CoA Retinol Acyltransferase
RBP: Retinol Binding Protein
RPH: Retinyl Palmitate Hydrolase
TTR: Transthyretin

homologous to the receptors for steroid and thyroid hormones, and vitamin D_3 (40,41). The authors suggest that the molecular mechanisms of vitamin A involvement in embryonic development, differentiation and tumor cell growth, are similar to those of other members belonging to this nuclear receptor family.

B. Intracellular Metabolism of Retinol

The first step in vitamin A metabolism is the reversible oxidation of ROL to RAL. In the eye, all-trans RAL is isomerized to 11-CIS RAL and utilized as a chromophore in the visual cycle (21). Figure 4 is a schematic representation of dietary retinoid metabolism. In most other tissues, irreversible oxidation of RAL to RA can occur (3). Retinoic acid supports growth in animals, maintenance of epithelial tissue and bone, but is inactive in vision and mammalian reproduction (2-4). Unlike ROL, RA is not stored in the liver (42), making it preferable for pharmacologic use. Retinoic acid is at least 10 times more active than ROL in suppressing keratinization of tracheas in organ culture (43). Retinoic acid is formed in the intestine, kidney, and liver, and transported in the plasma bound to serum albumin (44). Recently, RA was found in human blood (3 ng/ml) (45,46); the source was not determined.

Figure 4. Vitamin A Metabolism



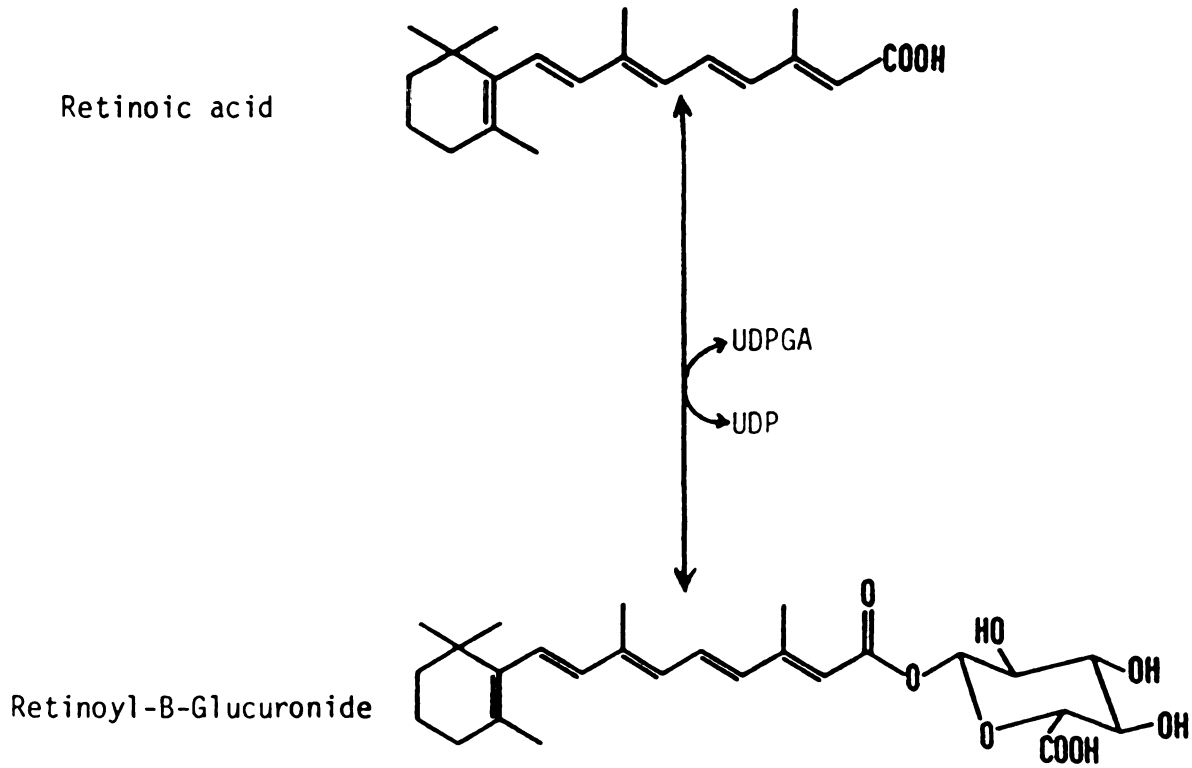
One of the next possible metabolic steps in RA metabolism is the isomerization of all-trans RA to form 13-cis-RA (47); this reaction is reversible (48). Except for a role in the visual cycle, the biological significance of isomerization reactions in mammalian tissues is unknown. In tracheal organ culture, 13-cis-RA has similar biological activity to the all-trans isomer (49). Both isomers of RA can be oxidized, or hydroxylated, at the C-4 position of the cyclohexenyl ring to yield 4-oxoretinoic acid and 4-hydroxyretinoic acid (7,49). The enzyme required for the in vitro synthesis of 4-hydroxyretinoic acid from RA is located in microsomes of the hamster liver and intestinal mucosa. This microsomal enzyme requires NADPH, O_2, and is inhibited by carbon monoxide (50). This suggests that the enzyme belongs to a class of mixed function oxidases containing cytochrome P-450 (50,51). A dehydrogenase further oxidizes 4-hydroxy to 4-oxoretinoic acid; it requires NAD, is not inhibited by CO, and does not require O_2 (51). Both 4-hydroxy and 4-oxoretinoic acid are less than one-tenth as active as RA in causing epithelial differentiation in the trachael organ-culture assay (52). Therefore, both 4-hydroxy and 4-oxoretinoic acid may be early metabolites which lead to the elimination of retinoic acid from the body.

Another oxidation pathway of RA is epoxidation of the cyclohexenyl ring at the 5,6 position. In vitro studies reveal the presence of 5,6-epoxyretinoic acid in homogenates of rat kidney, small intestine, mucosa, and liver (53). In

other studies it has been shown to have biological activity and promotes adhesive properties in transformed cultured mouse fibroblast (54), acts as an antitumor promoter (55), and maintains epithelial differentiation in tracheal organ culture (56). However, in vivo, 5,6 epoxyretinoic acid has only 0.5 % of the activity in growth maintenance compared to ROL (57), and may be an elimination metabolite, or it may have an unknown role in metabolism.

One of the first metabolites of RA to be identified was retinoyl-β-glucuronide (RG) (5). Figure 5 is a schematic representation of the conjugation of RA. Conjugates of all-trans and 13-cis-RA with glucuronic acid were first detected in the bile (5), and more recently in the urine (6), intestinal mucosa (7), liver (8), and in human blood (9). In the liver, RG occurs at the same level whether physiological or pharmacological levels of RA are administered (8,10). Retinoyl-β-glucuronide is the major metabolite of all-trans-RA in intestinal mucosa of bile duct-cannulated rats (7). The intestinal epithelium, a target organ for vitamin A, can receive ROL from the circulation or diet, oxidize it to RA, isomerize and/or glucuronidate it for metabolic functions or excretion. Although RG was originally considered primarily an excretion product, it has been shown to be as active as RA in promoting growth of vitamin A-deficient rats (6) and in causing epithelial differentiation in a rat vaginal-smear assay (11). Also, RG is equally as effective as RA in

Figure 5. Glucuronidation of Retinoic Acid.

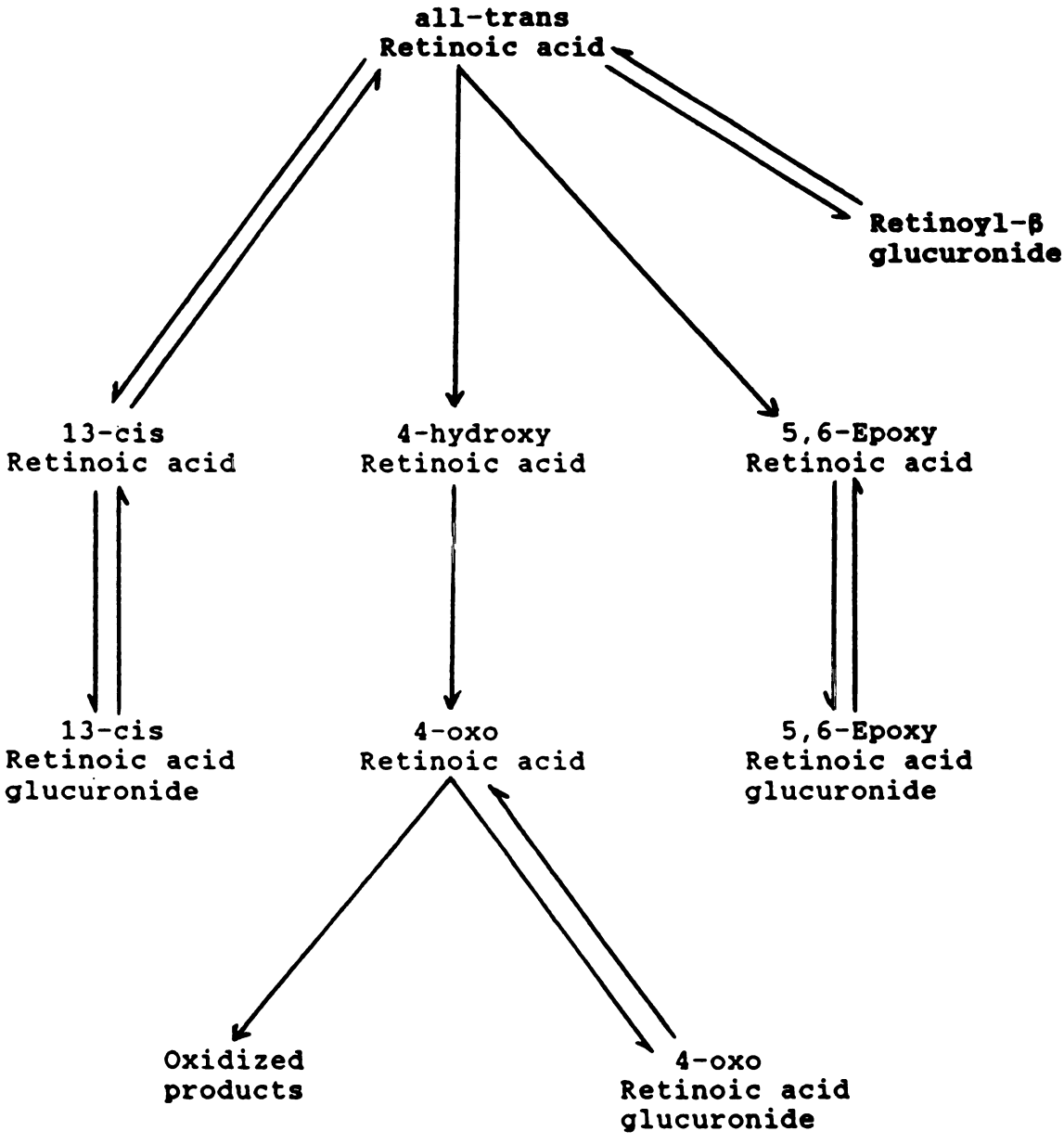


prohibiting HL-60 cell proliferation and inducing their differentiation into mature granulocytes (58).

Retinol can also be conjugated with UDP-glucuronic acid by UDP-glucuronosyltransferase in liver microsomes (8,59), and probably in other tissues. Retinyl-β-D-glucuronide was first identified in bile (8) and recently in human blood (9). As with retinoyl-β-glucuronide, retinyl-β-glucuronide was originally thought to be an excretion product, but has been shown to be as effective as retinyl acetate in a rat growth assay, when injected intraperitoneally, but not when administered orally (60). Other vitamin A derivatives can be glucuronidated: 5,6-epoxyretinoyl-glucuronide was found in the small intestine (61), and 4-oxoretinoyl-glucuronide, in the bile (62). Both of these glucuronides are considered to be elimination products. Figure 6 is a diagram of retinoic acid metabolism.

Glucuronides are generally considered to be water soluble elimination derivatives, however, retinoyl and retinyl glucuronide both have been demonstrated to be biologically active (6,11,58,60). Although the role of the glucuronides in vitamin A action is unknown, they may serve in the following ways. Glucuronidation may be a solubilization mechanism which would enable the hydrophobic retinoyl moiety to partition into hydrophilic cellular compartments. Secondly, glucuronidation is reversible; β-glucuronidase and UDP-glucuronosyltransferase both catalyze the hydrolysis of the glucuronides. Thus, the glucuronides

Figure 6. Metabolism of Retinoic acid



could be temporary intracellular storage forms of the active retinoyl moiety. Thirdly, glucuronidation of RA and ROL may be necessary for intercellular transfer; both glucuronides are found in human plasma (9). In this way, the glucuronides could be feedback signals to control ROL-RBP release from the liver. Lastly, the glucuronides could act on cellular differentiation via undefined mechanisms.

In conclusion, the metabolism of vitamin A in vivo, as well as in vitro, is a complex process that produces many metabolites which vary in biological activity. Further characterization of metabolic pathways would help define the biological role of vitamin A. There exist many derivatives of vitamin A, natural and synthetic, which have not been discussed. For a complete review of this subject see Ref 63.

REVIEW OF GLUCURONIDATION

Many endogenous compounds, such as bilirubin and steroid hormones, as well as exogenous compounds, such as drugs, are nonpolar. To aid in their excretion, these compounds are converted into more polar compounds. The changing of polarity may involve oxidation, reduction, or hydrolysis by enzyme systems. These altered compounds may then be conjugated with glucuronic acid, sulfate, glutathione, or glycine. The more polar conjugates are usually readily excreted into the urine and bile. However, conjugation may also yield biologically active compounds, or activated compounds with greatly increased toxicity (64).

Glucuronidation is the most common type of conjugation in mammalian metabolism; it is catalyzed by a family of enzymes, uridine diphosphate-glucuronosyltransferase(s) (UDPGT), which transfer the glucuronic acid moiety from uridine diphosphate-α-D-glucuronic acid (UDPGA) to an aglycone. The reaction mechanism is thought to be an SN_2 type of nucleophilic displacement of the UDP moiety with resulting inversion (65).

Many of the aglycones for UDPGT are the products of the mixed-function oxidase (MFO) systems, also located in the

endoplasmic reticulum. The MFOs are responsible for the oxidation of xenobiotics, steroids, fatty acids, vitamin A, and numerous other endogenous compounds. Many of these oxidized derivatives are then suitable for glucuronide bond formation. Among these are phenols and alcohols (66), carboxylic acids (67), amines (68), thiol derivatives (69), and acetylenic derivatives (70). Numerous endogenous compounds have been shown to be glucuronidated, including bile acids (71), steroid hormones (72), vitamin A (12), catecholamines (73), and thyroxine (74).

The majority of mammalian UDPGT activity is located in the endoplasmic reticulum (13). However, activity has also been described in the Golgi apparatus (13), plasma membrane (13,75), nuclear envelope (13,76), and possibly in the mitochondria (77). UDPGT is an integral membrane protein; the orientation of its catalytic site (s) (cytosolic and/or luminal exposure) is currently debated. Depending on the species and substrate used, UDPGT activity has been found in the skin (78), kidney (79), lung (80), liver (67), gastrointestinal mucosa (81), spleen, brain, heart, thymus (82), and the adrenal gland (83).

While the liver plays an important role in glucuronidation, extrahepatic glucuronidation may be the more logical method of dealing with ingested toxic molecules and endogenous metabolites, rather than allowing them to be absorbed and circulated unchanged to the liver. Intestinal

UDPGT and other metabolizing enzymes serve as the first line of defense against xenobiotics at their site of entry.

Glucuronides of endogenous and exogenous compounds are excreted by the liver into the blood or bile. Metabolites in the bile enter the intestine where they may be hydrolyzed by β-glucuronidase (bacterial or lysosomal), or UDPGT. Once hydrolyzed in the intestine, they may become re-glucuronidated. This process of glucuronidation and hydrolysis may have a significant effect on the pharmacokinetics of many endogenous and exogenous compounds.

A. Modulation of UDPGT Activity

The regulation of UDPGT activity in vivo is unknown. However, its activity in vitro can be increased by membrane-perturbing processes such as mechanical disruption, aging, freezing, thawing, treatment with detergents, organic solvents, certain ions, proteases, or phospholipases (64). The same phenomenon has been observed with many other microsomal enzymes. Experiments comparing in vivo and in vitro hepatic or intestinal microsomal UDPGT activity indicate that the enzyme is not fully expressed (84). It is not known if latency is an intrinsic property of UDPGT. However, it has been suggested that latency may occur during the isolation of microsomes (85).

The lipid environment of UDPGT appears to be an integral part of its regulatory mechanism (15). Thus,

treatment of microsomes with detergents or phospholipases alter the catalytic activity of its substrates (14). The dependence of UDPGT activity on the presence of phospholipids, especially phosphatidylcholine (PC), has been clearly demonstrated by partial or complete loss of enzyme activity during purification (14,16,17). When 98% of the phospholipid was separated from rat liver microsomal protein only 0-6% of p-nitrophenol-glucuronosyltransferase activity remained (86). However, by incubating the lipid-free enzyme with liposomes 30-45% of the original activity was restored (86). Another study showed a 40-100% increase in activity of p-nitrophenol-glucuronosyltransferase when incubating a phospholipid-free enzyme with phosphatidyl and lysophosphatidyl-choline (87). In another study, the residual activity of pure, delipidated enzyme was enhanced as much as 700-fold by reconstitution with appropriate phospholipids (17,18). In addition, affinities for substrates, catalytic specificity, and cooperative binding of UDPGA depend on the physical properties of the lipids used to reconstitute the pure enzyme (17-20).

B. Heterogeneity of UDPGT

It has been demonstrated that UDPGT activities are mediated by a family of enzymes. There are four approaches which have been used to determine UDPGT heterogeneity. One approach is based on selective induction of UDPGT by phenobarbital (PB) or by 3-methylcholanthrene (3-MC)

(88,89). The substrates for the 3-MC induced enzyme form are termed GT-1 substrates; examples are p-nitrophenol, 1-naphthol and 2-aminophenol (90,91). The substrates for the PB induced enzyme form are termed GT-2 substrates; examples include morphine, phenolphthalein, and chloramphenicol (90,91). In response to polycyclic hydrocarbons, the GT-1 enzyme form is more easily induced than the GT-2 enzyme form (88,89). GT-1 and GT-2 substrates can also be separated in two groups on the basis of their molecular size and shape (92,93). A study involving 31 different aglycones of various structures, categorized substrates by their molecular thickness. Flat substrates less than 4.5 A thick, are glucuronidated by GT-1 form, while those greater than 4.5 A are glucuronidated by GT-2 (94). However, GT-1 and GT-2 substrates do not include many endogenous compounds such as steroids, hormones, and bilirubin (95).

Another approach has been to follow developmental patterns of hepatic glucuronidation (90). The GT-1 enzyme activity induced by 3-MC reaches adult levels during the late fetal stage of development, while the GT-2 enzyme activity induced by PB reaches adult activity two days after birth (90). The late fetal or GT-1 substrates include planar and aromatic compounds such as phenols, naphthols, and hydroxylated coumarins (90), while the neonatal or GT-2 substrates include estradiol, phenolphthalein, and morphine (90).

A more direct approach in determining the heterogeneity of UDPGT has been the purification of the enzyme. In the early attempts on the purification of UDPGT it was found that solubilization of the membranes in which UDPGT resides can considerably alter the structure of the enzyme (66). Recently, several investigators have been able to purify to homogeneity several hepatic UDPGT's, using nonionic detergents, diethylaminoethyl (DEAE) sephadex and affinity chromatography. The specific rat liver UDPGT isoenzymes are UDPGT-bilirubin [57,000] (96), UDPGT-testosterone [58,000] (97), UDPGT-esterone/P-nitrophenol [59,000] (98), and UDPGT-morphine [56,000] (91). The nonspecific UDPGT isoenzymes are much more numerous.

Purification of UDPGT's has assisted in the resolution of heterogeneity; however, it has also brought considerable attention to the influence of phospholipids on catalytic activity of these enzymes (14-16,20,86,96,99). Microsomal UDPGT's kinetic and regulatory properties are modified by treatments that alter the structure or composition of the phospholipid region of the membrane (14,96,100). During the purification process, the removal of phospholipids by DEAE-cellulose chromatography partially inactivates enzyme activity (91,96). However, by adding lecithin liposomes to each eluted fraction from the DEAE-cellulose column, 95% of UDPGT activity toward bilirubin and greater than 50% of the activity towards 4-nitrophenol can be recovered (96). In another study, phosphatidyl-choline was added to the

chromatography buffers, and both the recovery and the activity of UDPGT towards morphine were greatly improved (91). The addition of detergent to solubilize the microsomes also increased the Km for morphine by 14-fold over the Km of native microsomes (91). Presently there is considereable debate as to the effect of phospholipids on UDPGT activity. They may affect the enzyme directly or by controlling the substrate and/or cosubstrate accessibility (64).

Kinetic analysis of purified enzymes is a classical approach to determining properties of enzymes. Enzyme kinetics can help to differentiate isoenzymes and the mechanism at their active site(s) and allosteric sites, estimating the intracellular substrate concentration, and a possible in vivo role of the enzyme in metabolism. The major difficulty with determining the kinetic properties of UDPGT has been the purification process. Purification techniques have improved, but this has not aided the characterization of the isoenzymes, primarily because during purification, there is a loss of the essential native phospholipid environment. The addition of phospholipids to the delipidated enzyme may not restore catalytic properties of endogenous UDPGT, or it may affect substrate specificity.

MATERIALS AND METHODS

A. Materials

B-Glucuronidase (bovine type B-10), uridine 5'-Diphospho-glucuronyltransferase (bovine, liver, type 111), uridine 5'-Diphospho-Glucuronic acid [sodium salt] (UDPGA), uridine 5'-Diphosphate [sodium salt] (UDP), L- phosphatidyl choline [from egg yolk, type 1x-E] (PC), Brilliant Blue R, sodium dodecyl sulfate, butylated hydroxy toluene (BHT), all-trans-retinoic acid [type XX] (RA), sodium acetate (NaAc), and Trizma base were obtained from Sigma Chemical Company, St. Louis, Missouri. Ethylenedinitrilo-Tetraacetic acid (disodium salt), and glycerol were purchased from Mallinckrodt Inc., Paris, Kentucky. Saccharo-1,4-lactone-monohydrate (SL) was purchased from Calbiochem-Behring corporation, LaJolla, California. Sepharose 4-B (CL) was obtained from Pharmacia Inc., Piscataway, New Jersey. All-trans-[11-^{3}H]-retinoic acid (^{3}H-RA) was purchased from Amersham Corp., Arlington Hts., IL. (35.9 Ci/mmol). Both all-trans-retinoyl-B-D-glucuronide (RG) and tritiated all-trans retinoyl-B-D-glucuronide (^{3}H-RG) were generously supplied by Dr. A. Barua, (Iowa State University). All other chemicals and solvents were of HPLC or analytical reagent grade.

B. Animals

Twenty-four 200 g female Sprague-Dawley rats were obtained from the Holtzman Company, Madison, Wisconsin. The rats were housed separately at a temperature between 20-25 °C, with approximately 40% humidity, and 12 hr light and 12 hr dark cycle. All rats were fed ad libitum an AIN-76 semipurified vitamin A deficient diet supplemented with 30 ug/day of retinyl acetate for 2 weeks. The rats were randomly assigned to three groups of eight rats and sacrificed. To remove the blood from the livers, each liver was perfused with 50 ml of ice cold 0.9% NaCl. To remove intestinal contents, small intestines were perfused with ice cold 0.9% NaCl.

Livers from each group of eight rats were pooled as were the small intestines from each group. Microsomes were prepared immediately from these two tissues and stored at -70 °C under N_2. All microsomes were used within 30 days. The enzyme activities were measured and compared for liver and small intestine from a single group of rats; thus, each group of rats represents a single observation.

C. Isolation of Small Intestinal Mucosa

The rats were sacrificed by cervical dislocation. The entire small intestine, from the pyloric valve to the ileocolic valve, was removed and immediately flushed free of

intestinal contents with ice cold 0.9% saline solution. All further work was done on ice. The intestinal mucosa was scraped free from the intestinal wall with a glass slide. The mucosae from each group of eight rats were pooled. The three groups of eight rats each represent single observations.

D. Preparation of Microsomes

Following the isolation and pooling of intestinal mucosa and of livers, all further procedures were done on ice. The pooled livers were cut into small pieces. All of the following procedures were done to both tissues, but separately. The pooled tissues were added to 3 volumes of 0.25 M sucrose, which contained 10 mM Tris-chloride, pH 7.4. and homogenized with a glass Potter-Elvehjem (Thomas, type C 144-25) homogenizer. Microsomes were prepared by aggregation with calcium, by the method of Schenkman and Cinti (101). Homogenates were centrifuged for 5 min. at 600 x g. The precipitates were discarded and the supernatant was centrifuged for 10 min. at 12,000 x g. The precipitate was again discarded and $CaCl_2$ was added to the post-mitochondrial supernatant to contain 8mM $CaCl_2$ which results in an aggregation of microsomes. The aggregate was sedimented by centrifuging at 25,000 x g for 15 min. After this centrifugation the supernatant was discarded and the pellet washed once with 350 ml of 150 mM KCl-10 mM Tris-HCl, pH 7.4, centrifuged at 25,000 x g for 15 min., and the

microsomal pellet homogenized with an equal volume of glycerol and stored at -70 °C. Before microsomes were used, glycerol was removed by homogenizing with 0.025 M Tris buffer, pH 7.4 and centrifuging at 105,000 x g for 90 minutes. The microsomal pellets were pooled and homogenized with 0.025 M Tris buffer, pH 7.4, with a 1.0 ml Wheaton homogenizer. Protein concentration of the microsomes was determined by a modified Lowry method (102).

E. Preparation of Substrates

For the conjugation reaction, all-trans-RA and tritium labelled all-trans-RA were purified to greater than 99% purity and re-examined by HPLC prior to use. Chromatographic conditions are reported in the chromatography section of Methods. The tritiated and unlabelled RA were added together, and the concentration and specific activities determined. The concentration of ^{3}H-RA varied from 75-100 uM; specific activity ranged from 15-20 uCi/umole.

For the deconjugation reaction, both the tritiated and unlabelled RG were purified to greater than 99% purity and re-examined by HPLC prior to use. The chromatographic conditions and the addition of unlabelled RG to tritiated RG were similar to RA. However, the concentration of ^{3}H-RG varied from 300-400 uM and the specific activity ranged from 5-10 uCi/umole.

F. Preparation of Liposomes

Small unilamellar vesicles (liposomes) incorporating either ^{3}H-RA or ^{3}H-RG into the bilayer were prepared as described by Finkelstein and Weissman (104). All liposomal components are listed as final reaction concentrations. For the conjugation reactions, phosphotidyl choline (PC) (50 mM) and ^{3}H-RA (5-1000 uM) were dissolved in chloroform containing butylated hydroxytoluene (BHT). The organic solvent was removed by evaporation in a rotor vaporator under reduced pressure. Tris-HCl buffer (25 mM, pH 7.4 at 37 °C), containing 10 mM UDPGA was added, and the flask was hand-swirled until the dried PC-^{3}H-RA film was removed from the flask and the mixture formed a milky emulsion. For the deconjugation reactions, crude PC (50 mM) and ^{3}H-RG were dissolved in chloroform containing BHT. The organic solvent was removed by evaporation in a rotor vaporator under reduced pressure. Sodium acetate buffer (40 mM, pH 5.1 at 37 °C) containing 10 mM UDP and 70 mM SL was added, and the flask hand-swirled until the dried PC-^{3}H-RG film was removed from the flask and the mixture formed a milky emulsion. To obtain liposomes that are homogeneous in size, the milky emulsions were sonicated (separately) for 15 minutes on ice with a Branson W-350 sonifier, continuous at a setting of 50 watts using a microtip probe. The vesicle dispersion was

then centrifuged at 105,000 x g for 90 minutes to remove titanium probe particles. The radiolabeled liposomes were stored at 4 °C in the dark, or under yellow lighting and used within 6 hours.

G. Characterization of Liposomes

Characterization of liposomes was by a modification of the procedure described by Whitmer et.al. (103). The homogeneity of ^{3}H-RA liposomes was demonstrated chromatographically on a Sephrose 4B column. The column was equilibrated at room temperature with 25 mM Tris-HCl buffer (pH 7.4 at 22 °C). Fraction volumes of 2.0 ml were collected. Liposomes (1.0 ml) containing 50 mM PC, 100 uM ^{3}H-RA (15.78 uCi/umole), 10mM UDPGA, and 3.0 mg Coomasie Blue, were applied to a column of Sepharose 4B. The absorption of each collected fraction was read in a Beckman model 35 spectrophotometer at 565 nM, and radioactivity was measured with a Packard 4000 liquid scintillation counter.

H. Enzyme Reaction

Each reaction mixture contained the liposomal preparation described in the methods section. Tris-HCl buffer (25 mM, pH 7.4 at 37 °C), was used for the conjugation reactions, and NaAc buffer (40 mM, pH 5.1 at 37 °C) for the deconjugation reactions. For controls, liver and small intestinal microsomes were denatured by boiling

for 30 minutes. Liposomes, buffer, and two glass beads were preincubated for 15 minutes in a shaking 37 °C water bath. The enzymatic reation was initiated by the addition of either 0.6 mg/ml of small intestinal or liver microsomes, and reaction vials were incubated for 0-60 minutes in a 37 °C shaking water bath. The reaction was terminated by the addition of 3.0 ml of ice cold methanol; nitrogen was added and the reaction vial capped. The enzyme reactions were stored at 20 °C in the dark and analyzed within 4 days.

I. Chromatography

Chromatography was performed on a C-18 reverse-phase analytical column (Whatman ODS-3, 0.46 x 25cm) and a precolumn containing Whatman CO:PELL ODS support. The chromatography system utilizes a fixed wavelength detector, (Waters, Model 440), and a chromatography pump, (Waters, M6000). Elution of retinoid standards and experimental samples was accomplished with mixtures of water and methanol containing 0.01 M ammonium acetate. One ml fractions were collected from experimental samples for measurement of radiolabelled retinoids by scintillation counting.

J. Extraction Procedure

All samples were prepared for HPLC analysis in the following manner: centrifuge sample at a setting of 7 in a Model CL centrifuge (International Equipment Co., Needham Hts., Mass.) for 5 minutes, remove the supernatant. Add 1ml of methanol to the precipitate, vortex for 5 minutes and re-centrifuge for 5 minutes. Transfer this supernatant to the supernatant of the first centrifugation, and evaporate to near dryness with a stream of nitrogen. Next, quantitatively transfer the sample to a 1.0 ml volumetric flask; and add methanol to 1.0 ml final volume. From this known volume aliquots were analyzed by HPLC or scintillation counting.

K. B-Glucuronidase Assay

To identify RG, RG was isolated from enzyme reactions, purified by HPLC, and then incubated with β-glucuronidase (105). Each 1.0 ml reaction mixture contained 20 ug BSA, 25,000 units β-glucuronidase (type B-10), 3.0 ug ^{3}H-RG, and 50 mM NaAc buffer, pH 4.5 (final reaction concentration). Each reaction was carried out in duplicate. For control reactions, β-glucuronidase was denatured by boiling for 30 minutes. Reactions were initiated by adding live or denatured β-glucuronidase to all other components in the

reaction vials. All reactions were incubated for 1 hour in a 37 °C water shaking bath. The reactions were terminated by adding 3 volumes of ice cold methanol; nitrogen was added, vials capped, and stored at -20 °C until analysed. The extraction, HPLC analysis, and scintillation counting are described in Methods.

L. Scintillation Counting

Each 1.0 ml HPLC fraction was dried with a stream of air and 4.0 ml of 3a20 scintillation fluid (Research Products International Corp. Mount Prospect, IL) was added. Tritium content of each fraction was determined with a Packard 4000, (Packard Instrument Co.) liquid scintillation counter.

M. Statistical Analysis

Analysis of statistical significance between the small intestinal and liver microsomal enzymes was performed using the Student t-test as previously described (106). Double-reciprocal plot analysis of the kinetic data was done as described elsewhere (107).

N. Mass Spectra

Mass spectral analysis was performed on a JEOL HX110 mass spectrometer (Michigan State University-National Institute of Health mass spectral facilities). The matrix used for the negative ion fast atom bombardment (FAB) spectra was triethanolamine.

O. Coulter Counter

Microsomes and liposomes were sized with a model Z_{B1} Coulter Counter, (Coulter electronics Inc.,Hialeah, FL), a 256 Coulter Channelyzer, and plotted with a x-y recorder II.

P. Wilman 4 Program

WILMAN 4 is an IBM computer program was used to calculate the estimates, Km, V and Km/V from initial velocity measurements according to one of four statistical methods. The program is available from the Marketing Division, Instructional Media Center, Michigan State University, East Lansing, MI.

RESULTS

A. Identification of Retinoyl-B-Glucuronide.

A typical HPLC profile of authentic retinoid standards is shown in Fig. 7. Radioactivity that co-eluted with the RG standard were isolated from the reaction mixtures of both small intestine and liver. The isolated metabolite was incubated with bovine liver β-glucuronidase, an enzyme which specifically hydrolyzes β-glycosidic linkages. The radiolabelled product from the enzymatic hydrolysis co-eluted with RA standard (Fig. 8); this provided indirect evidence that the isolated metabolite is a retinoic acid conjugate with a β-glycosidic linkage. Direct evidence for the identification of RG was obtained by mass spectra analysis. The negative ion fast atom bombardment (FAB) spectrum (Fig. 9) showed a prominent ion at M/Z 475; this represents the deprotonated molecular ion of RG.

B. Characterization of ^{3}H-RA Liposomes.

The homogeneity of PC liposomes in the 105,000 x g supernatant was demonstrated chromatographically on Sepharose 4B (Fig. 10). Coomassie Blue labeled-PC and radiolabeled RA co-eluted in a single narrow peak at approximately 40% of the bed volume. This provides evidence

Fig. 7 Reverse-phase HPLC profile of RA and RG standards.

Chromatography was performed on a C-18 reverse-phase analytical column (see Methods). Elution of retinoid standard solutions and experimental samples was accomplished with mixtures of water and methanol containing 0.01 M ammonium acetate. SF indicates solvent front.

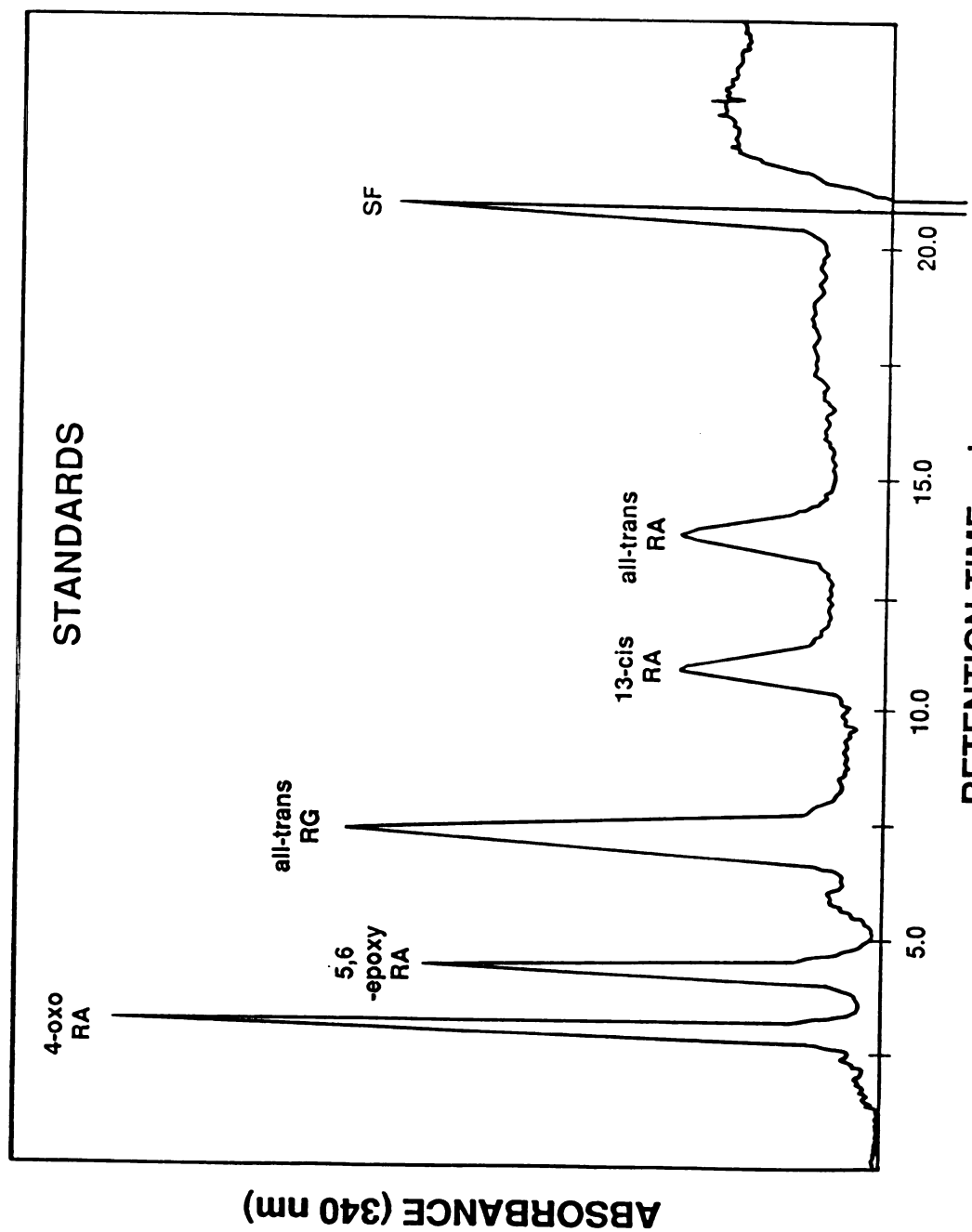


Figure 7. Reverse-phase HPLC profile of RA and RG standards.

Fig. 8 Identification of the reaction product as RG by β-glucuronidase assay.

Radioactivity that co-eluted with retinoyl-β-glucuronide standard was isolated from the reaction mixtures of both small intestine and liver. The isolated metabolite was incubated with 25,000 units β-glucuronidase (——). For control reactions, the enzyme was heat denatured (----) prior to incubation.

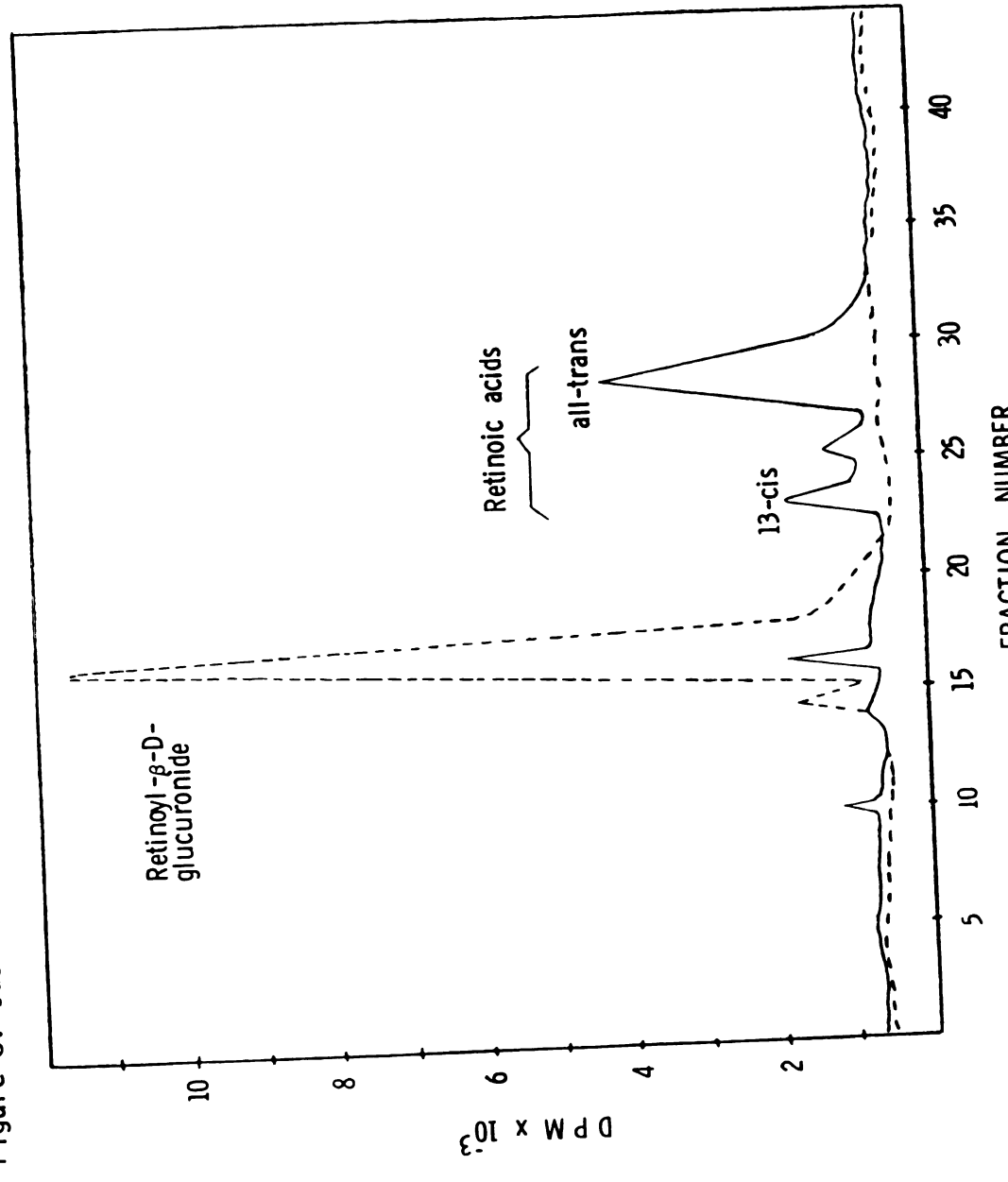


Figure 8. Identification of the reaction product as RG by B-glucuronidase assay.

Fig. 9 Identification of the reaction product RG by negative-ion mass spectroscopy.

Fractions tentatively identified as RG by cochromatography with authentic RG and β-glucuronidase assay were isolated from reaction mixtures. The isolated metabolite was further characterized by negative ion mass spectra analysis. The negative ion FAB spectrum displays a prominent ion at M/Z 475; this represents the deprotonated molecular ion of RG. The matrix used was triethanolamine.

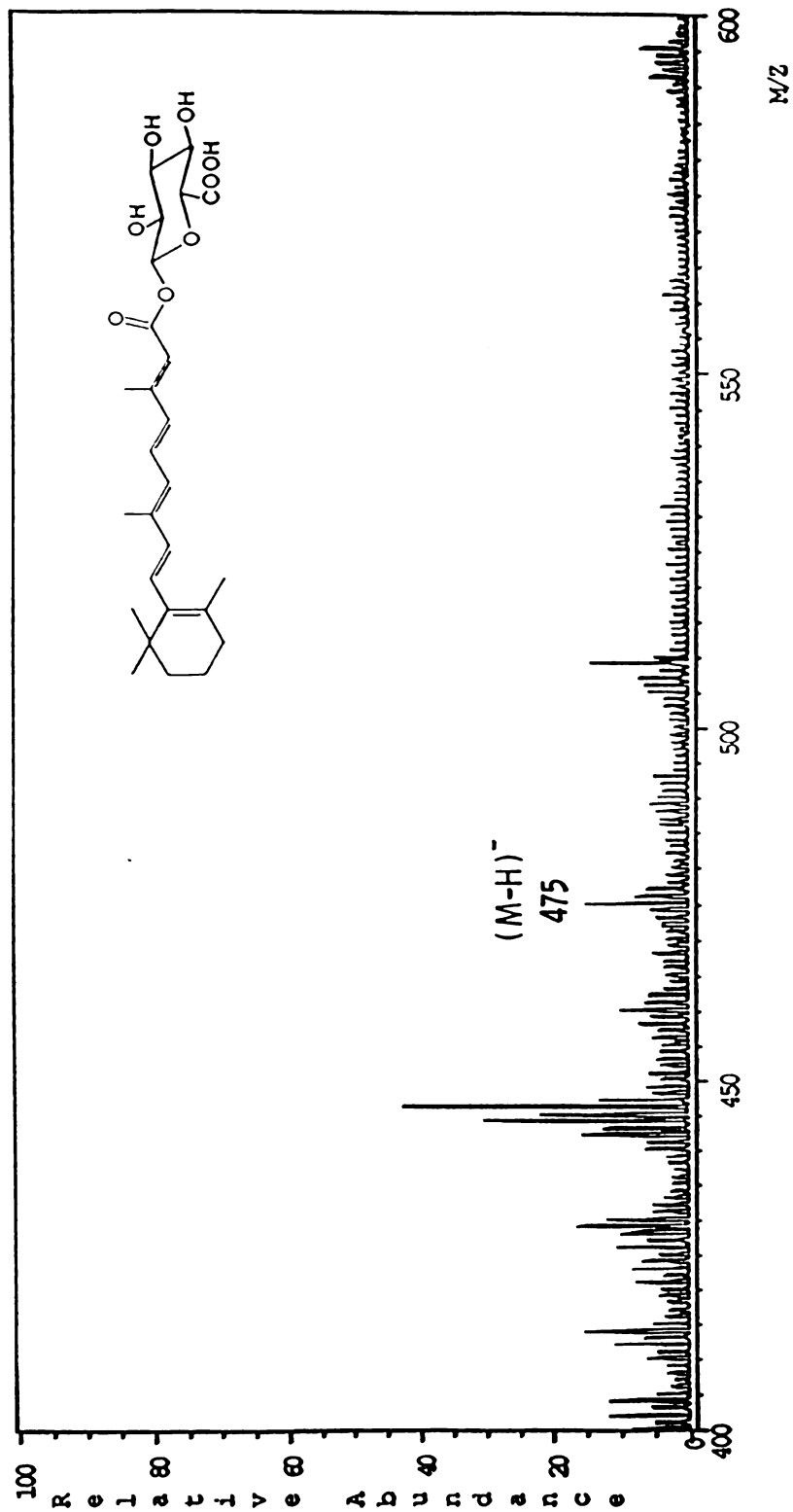


Figure 9. Identification of the reaction product RG by negative-ion mass spectroscopy.

Fig. 10 Chromatography of ^{3}H-RA liposomes on Sepharose 4B.

Liposomes (1.0 ml) containing 58 mg PC, 3.0 mg Coomassie Blue, 10 mM UDPGA, and 23.5 ug of ^{3}H-RA (17,021 dpms/ug) were applied to a column (56 x 1.5 cm) of Sepharose 4B, equilibrated and eluted with 25 mM Tris-HCl buffer (pH 7.4 at 22 C). Collected fraction volumes were 2.0 ml, and radioactivity recovery in the liposome peak was 91%.

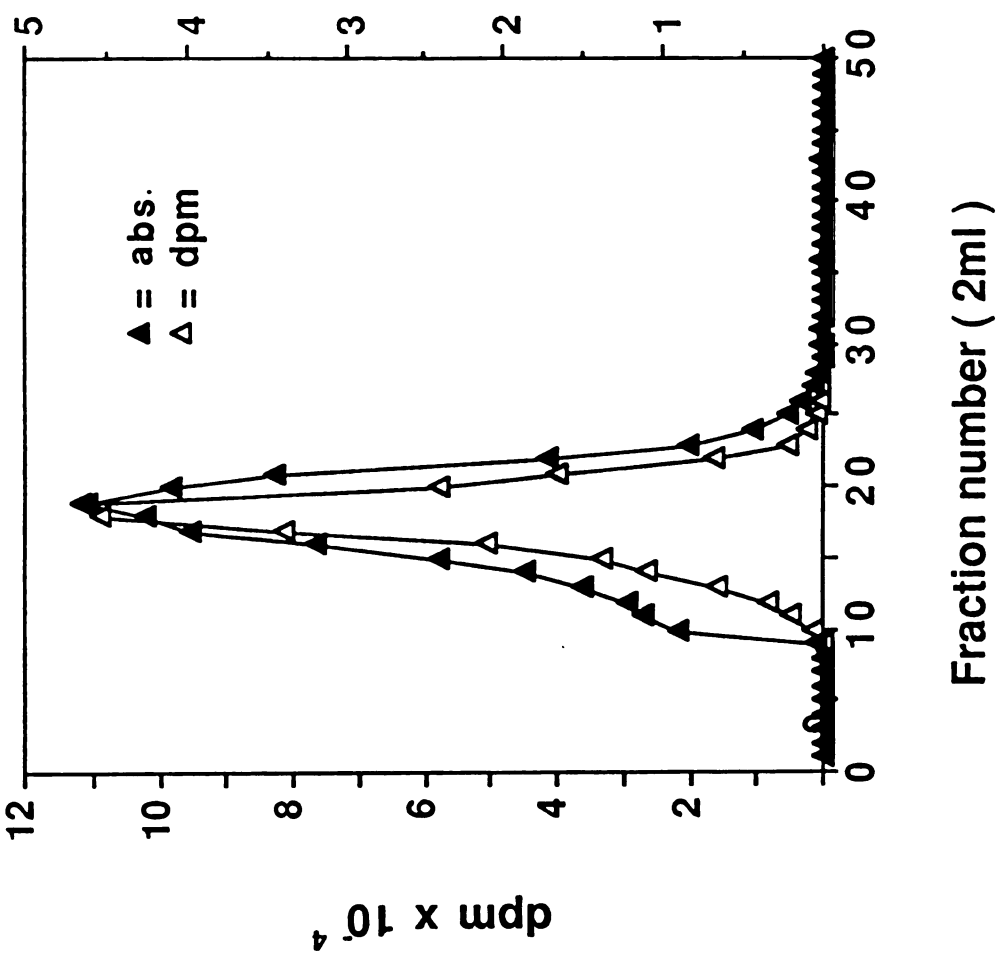


Figure 10. Chromatography of ^{3}H-RA liposomes on Sepharose 4B.

that the radiolabeled RA was associated with the liposomes because free ^{3}H-RA in Tris buffer was insoluble and aggregated on the Sephrose 4B gel surface. The recovery of radiolabeled RA in the liposomal fraction was 91%. The size of the liposomes were determined with a Coulter counter and found to be approximately 1.0 uM in diameter.

C. Determination of Optimal Incubation Period of Small Intestinal mucosa.

The percent conversion of ^{3}H-RA to ^{3}H-RG was measured at five different incubation periods for three separate experiments (Fig. 11-B). In all liposomal reaction mixtures, substrate concentration was 100 uM, microsomal protein concentration was 0.6 mg/ml, all other reaction conditions were constant as described in Methods. The percent conversion of ^{3}H-RA to ^{3}H-RG was linear between 0 and 30 min., from 30-60 min. percent conversion reaches plateau. Thirty minutes was determined as the optimal incubation period and was used for the kinetic analysis of RG production.

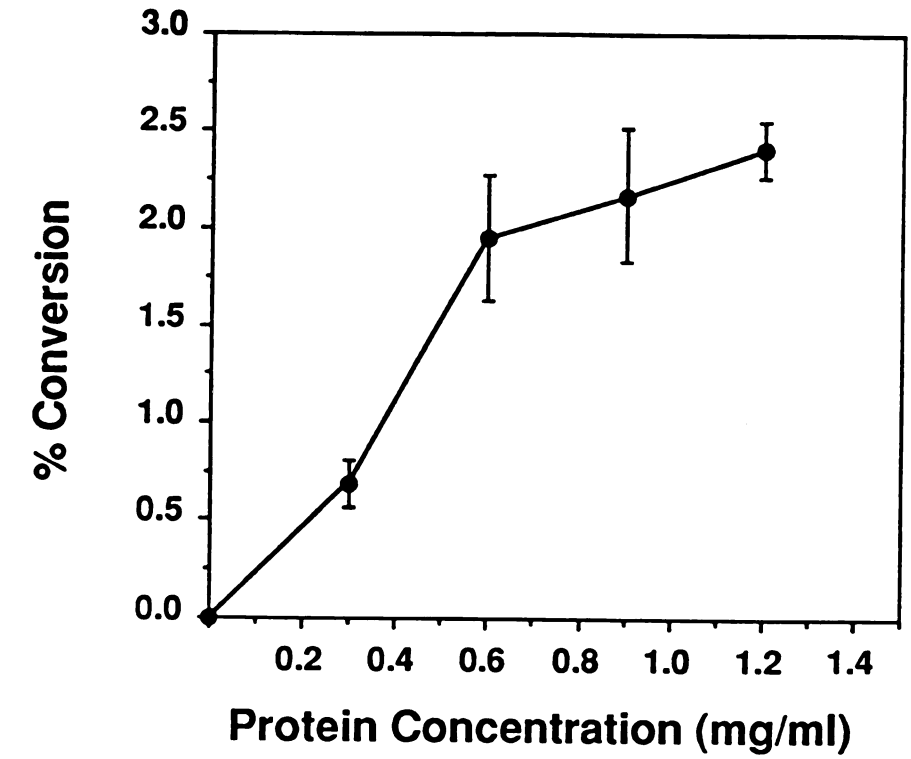
D. Determination of Optimal Microsomal Protein Concentration of Small Intestinal Mucosa.

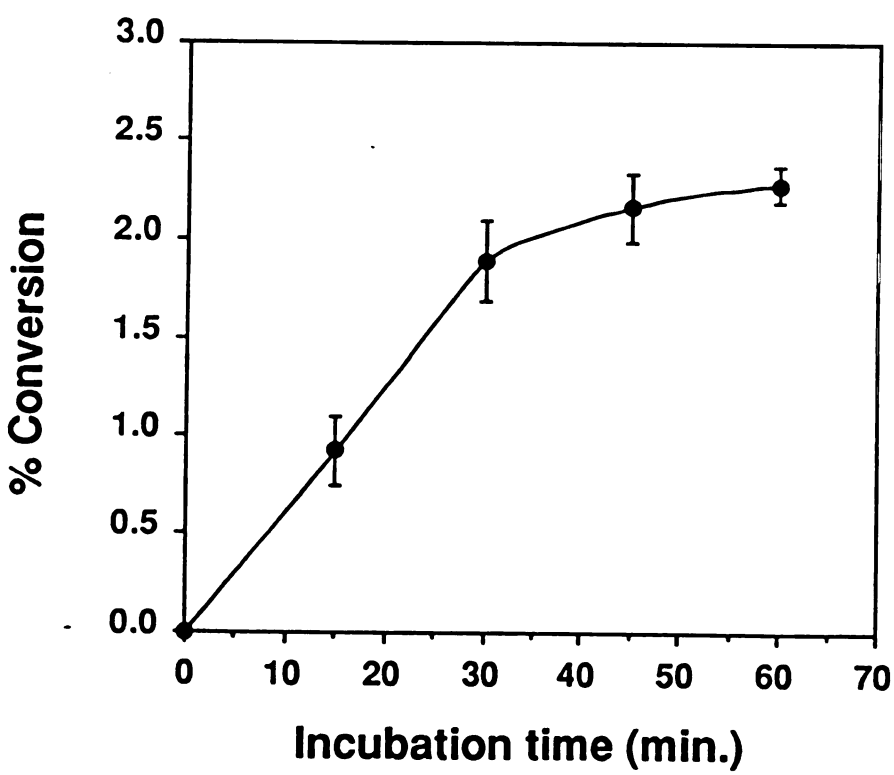
The percent conversion of ^{3}H-RA to ^{3}H-RG was measured at five different microsomal protein concentrations for

Fig. 11 Determination of glucuronidation of RA by small intestinal mucosa: optimal microsomal protein concentration and incubation period.

The percent conversion of ^{3}H-RA to ^{3}H-RG was measured for three separate experiments (A) five different microsomal protein concentrations for 30 min. and (B) five different incubation periods at 0.6 mg/ml protein concentration. In all reaction mixtures substrate concentration was 100 uM; all other reaction conditions were constant as described in Methods.

Figure 11. Determination of glucuronidation of RA by small intestinal mucosa.





three separate experiments (Fig. 11-A). In all liposomal reaction mixtures 100 uM substrate concentration and 30 minute incubation periods were used; all other reaction conditions were constant as described in Methods. The percent conversion of ^{3}H-RA to ^{3}H-RG was linear between 0-0.6 mg/ml microsomal protein concentration; from 0.9-1.2 mg/ml the percent conversion reaches plateau . The optimal microsomal protein concentration was determined to be 0.6 mg/ml and was used for the kinetic analysis of RG production in both liver and small intestine.

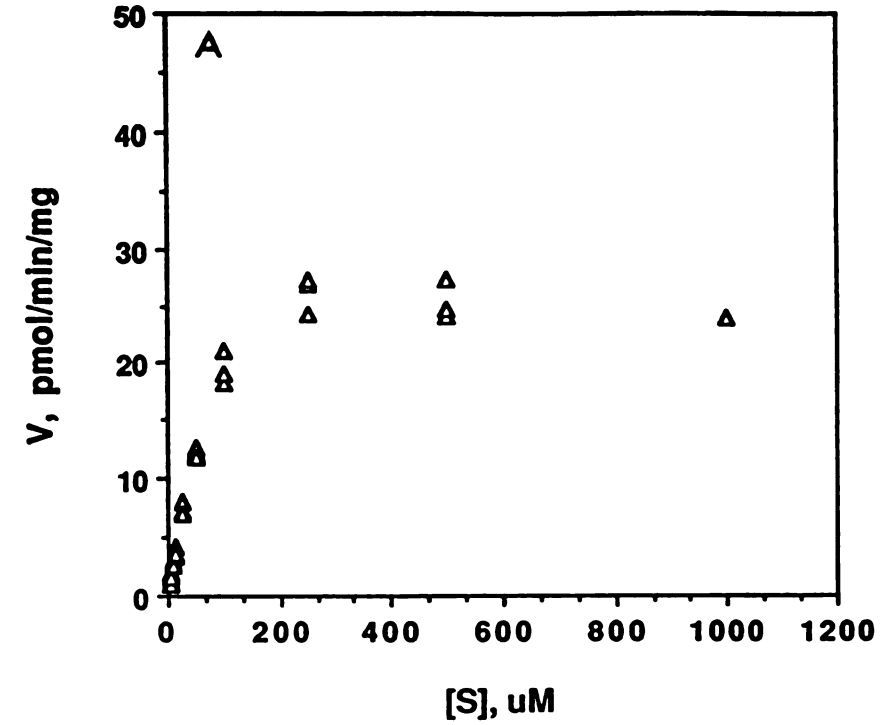
E. Determination of Initial Reaction Rates.

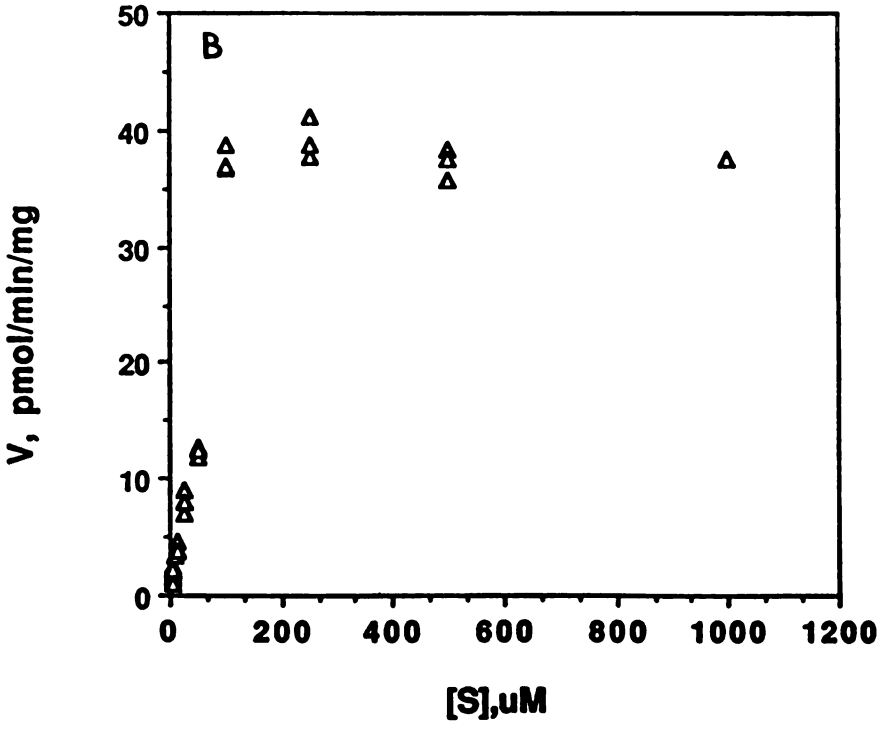
Initial reaction rates for UDPGT in liver and intestinal mucosal microsomes were determined for ten different substrate concentrations in each of the three separate experiments; pH, length of incubation, co-substrate and protein concentrations were held constant (Fig. 12-A,B). The initial reaction rate was found to be linear between 5-250 uM substrate concentration for liver microsomes and 5-100 uM for small intestinal microsomes. At higher substrate concentrations, the enzyme appeared to be saturated with substrate; at these conditions the reaction will not follow Michaelis-Menten kinetics.

Fig. 12 Determination of initial reaction rates.

Initial reaction rates for production of ^{3}H-RG were determined at 10 different ^{3}H-RA concentrations for 3 separate experiments, while protein and co-substrate concentrations, pH and incubation period were all held constant. (A) liver microsomal protein (0.6 mg/ml), (B) small intestinal microsomal protein (0.6 mg/ml).

Figure 12. Determination of initial reaction rates.





F. Kinetic Analysis of UDPGT Toward the Substrate RA.

The apparent kinetic constants were determined for small intestinal and liver microsomal UDPGT in vitamin A-sufficient female rats. Only data which had first order characteristics were used to obtain double-reciprocal plots (Fig. 13-A,B); linearity of this reaction implies characteristics of Michaelis-Menten kinetics. Table 1 gives the kinetic parameters calculated from the results obtained in three separate experiments with each type of the microsomal preparation. The two calculation methods used (see Methods) yielded values which were in good agreement. The apparent Km for RA with small intestinal microsomes was determined to be 182.8 uM and apparent Vmax, was 55.8 pmole/min/mg. The apparent Km for RA of liver microsomes was determined to be 123.6 uM; apparent Vmax, was 46.4 pmole/min/mg. The difference in the kinetic parameters between the two tissues was significant at $p < 0.05$.

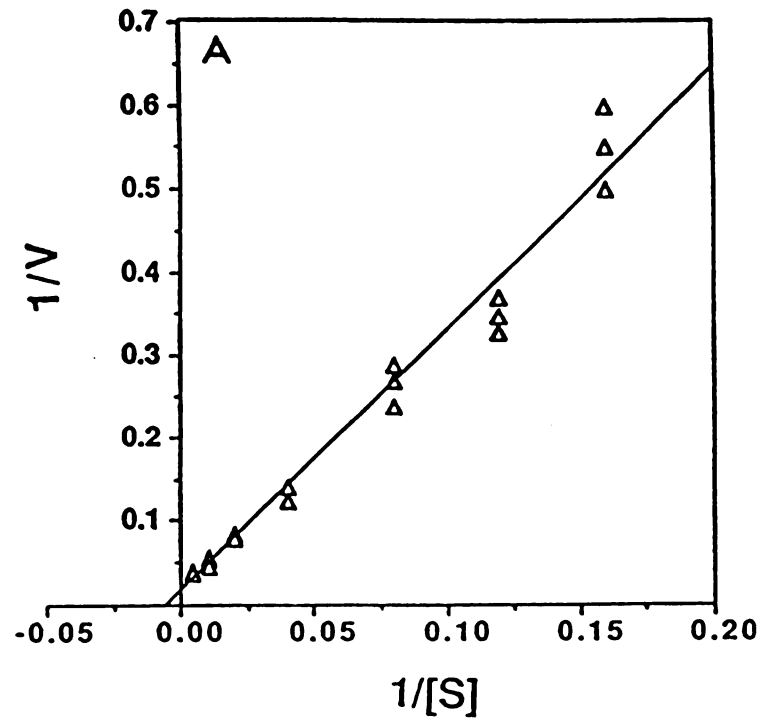
G. Deconjugation of RG Catalyzed by Microsomal UDPGT.

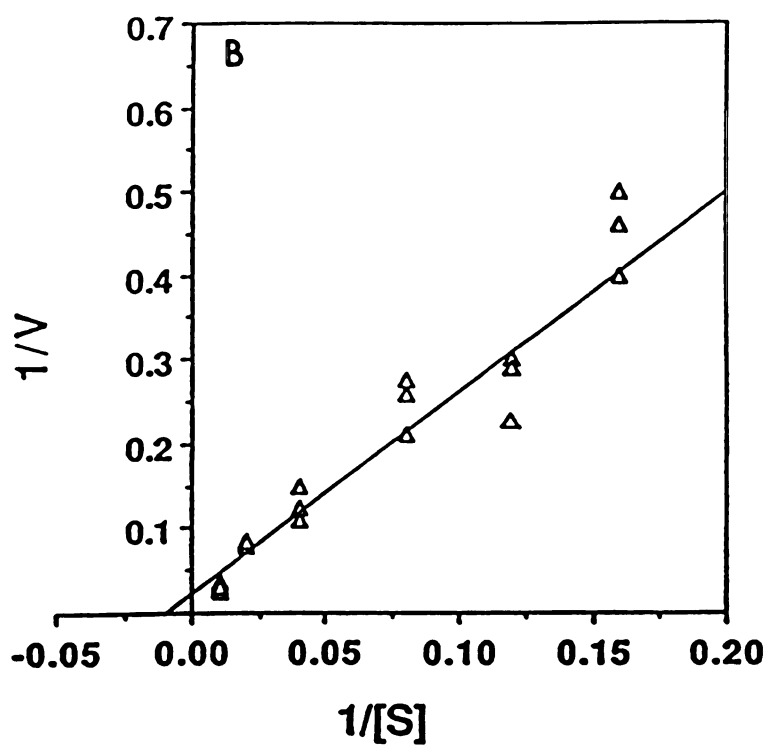
The data in Table 2 represent preliminary results for the deconjugation of radiolabeled RG using small intestinal and liver microsomes. This reaction is optimal at low pH (pH 5.1), which is also favorable for β-glucuronidase

Fig. 13 Double-reciprocal plot of initial reaction rates for production of RG catalyzed by small intestinal and liver microsomal UDPGT.

Each data point represents the initial velocity of RG synthesis for three separate experiments. Substrate concentrations range from 5-1000 uM; only those substrate concentrations which were determined to give first order reaction rates by the Haldane equation are plotted. Apparent kinetic parameters were determined by linear regression analysis. (A) liver microsomal protein (0.6 mg/ml) was used as the source of UDPGT; and (B) small intestine microsomal protein (0.6 mg/ml) was used as the source of UDPGT, all other assay conditions are described in Methods.

Figure 13. Double-reciprocal plot of initial reaction rates for production of RG.





activity, an ubiquitous enzyme generally found within the lysosomes. There was 0.9-1.3% deconjugation of RG by the rat small intestinal microsomes; incubation of bovine liver UDPGT with RG resulted in 12.9% deconjugation of RG. Using rat intestinal microsomes and conditions of constant substrate, co-substrate and protein concentrations, the deconjugation rate was decreased 67% in the presence of saccharo lactone, a β-glucuronidase inhibitor (Table 2). These results suggest that both UDPGT and β-glucuronidase may compete with UDPGT for the substrate RG.

Table 1. Kinetic parameters of small intestinal and liver microsomal UDPGT.

Small Intestinal Microsomal UDPGT

Experiment	Km (uM)[a]	Vmax (pmole/min/mg)[a]	Km (uM)[b]	Vmax (pmole/min/mg)[b]
#1	128.2	46.7	123.5	40.5
#2	111.1	47.5	92.3	32.5
#3	131.6	45.0	99.4	33.7
Derived $\bar{X}$	123.6 ± 10.9	46.4 ± 1.3	105.1 ± 16.4	35.6 ± 4.3

Liver Microsomal UDPGT

Experiment	Km (uM)[a]	Vmax (pmole/min/mg)[a]	Km (uM)[b]	Vmax (pmole/min/mg)[b]
#1	200.0	59.0	202.7	67.7
#2	181.8	54.8	177.1	65.0
#3	166.7	53.5	152.1	61.3
Derived $\bar{X}$	182.8 ± 16.7	55.8 ± 2.9	177.3 ± 25.3	64.7 ± 3.2

a = Data derived from Lineweaver-Burk plot.
b = Data derived from WILMAN4 computer program.

Table 2. Deconjugation of ^{3}H-RG by small intestinal and liver microsomal UDPGT.

UDPGT source	protein mg/ml	^{3}H-[RG]	[SL]	V pmole/min/mg	% conversion
Rat intestinal microsomes	2	40 uM	----	1.2	1.3
Rat intestinal microsomes	2	40 uM	70 uM	0.8	0.9
Bovine liver microsomes (Sigma type III)	24	2 uM	-----	0.1	12.9

All reaction conditions are described in Methods.

DISCUSSION

Glucuronidation is generally considered to be a detoxification mechanism for both endogenous and exogenous substances. It was, therefore, of interest to find that retinoyl-β-glucuronide represented a major metabolite of retinoic acid, the active form of vitamin A, in bile duct cannulated rats (8,10). In order to determine the physiological significance of this biologically fully active metabolite of retinoic acid (2-4), we have examined the kinetic parameters of glucuronidation of retinoic acid in small intestinal mucosa, a target tissue for vitamin A action, and compared them to those in the liver, the major site of storage of vitamin A esters, and also the major organ for detoxification reactions.

Enzyme purification is the classical approach to understanding the function of an enzyme and its regulation; this applies to both membrane-bound and soluble enzymes. However, due to the lipid-dependence of membrane-bound enzymes, they must be reconstitiuted with phospholipids. Reconstituting a purified membrane-bound enzyme is extremely difficult because it depends on duplication in vitro of the chemical and physical environment of the enzyme in its natural membrane. The reconstitution system should include duplicating the surface charge of the microsome, asymmetry

of lipids across the bilayer, and the complex lipid-protein composition.

Glucuronidation of RA is catalyzed by UDPGT. This enzyme is an integral component of the microsomal membrane and its lipid environment appears to be an important part of the regulatory mechanism of UDPGT (15). Treatment of microsomes with detergents or phopholipases alters the catalytic activity of the enzyme (14). The kinetic parameters of membrane-bound UDPGT toward a specific substrate differ dramatically with the choice of detergent and concentration of the detergent (14,64).Moreover, delipidation of a partially purified preparation of enzyme leads to a marked decrease in activity, which can be restored by adding phospholipids, particularly phosphotidyl choline (86,99). A novel experimental system to study UDPGT without the use of solubilizing agents has been the incorporation of the substrate into liposomes, artificial cellular membranes prepared from phospholipids, which more closely mimic the native environment of microsomal UDPGT. An example of the use of a liposomal system in kinetic analysis is the determination of the apparent Km of bilirubin-UDPGT (103). The Km determined in this system was found to be very similar to that of a previous study in which the physiological concentration of bilirubin was determined (108). Due to the phospholipid dependency of UDPGT, my experimental approach in determining apparent

kinetic parameters has been with liposomes. Phosphatidyl choline was selected as a component for the artificial membrane described here because it is the major lipid component of mammalian endoplasmic reticulum.

Kinetic analysis for production of retinoyl-β-glucuronide by liver microsomal UDPGT gave an apparent Km of 182.8 uM and Vmax of 55.8 pmole/min./mg. The Km and Vmax determined in a liposomal system are approximately three times higher than the previously determined apparent kinetic parameters (12), even though in both studies the same enzyme source was used. However, in the previous study (12) detergent was used to solubilize the microsomal membranes; this may account for the difference in kinetic parameters as noted in other studies (14-17). The apparent kinetic parameters for production of RG by small intestinal microsomal UDPGT were determined to be : Km = 123.6 uM and Vmax = 46.4 pmole/min./mg. Kinetic parameters of RA glucuronidation in the small intestinal mucosa have not been reported previously. The studies described here represent the only kinetic parameters determined for the in vitro biosynthesis of RG by microsomal UDPGT in small intestinal mucosa. Data from three separate experiments were used (Table 1) to statistically compare the kinetic parameters of the different tissues, the kinetic parameters between tissues were calculated to be significantly different at $p < 0.05$.

The physiological significance of retinoic acid glucuronidation is not clear. While glucuronidation is an accepted mechanism for solubilization and subsequent elimination of many endogenous degradation products, the formation of retinoyl-β-glucuronide in a target tissue such as the small intestine may represent a temporary cellular storage, or transport mechanism, of the relatively lipid-soluble retinoic acid, prior to its utilization for cellular functions.

Although the studies demonstrate that the Km for intestinal glucuronidation of retinoic acid was lower (33%) than that in the liver, the difference is not of a magnitude that warrants an interpretation that these enzymes are different isozymes. Further studies are needed to determine if retinoic acid UDPGT in the tissues is a constitutive enzyme and whether there is another isozyme associated with the detoxification of retinoic acid.

During the in vitro biosynthesis of RG other metabolites were formed. Since a microsomal preparation was the enzyme source, the presence of other enzymes could not be excluded. Two metabolites present in all reaction mixtures were 13-cis RG and 13-cis-RA. This could be due either to the action of an isomerase or a non-enzymatic reaction due to mechanical agitation. Another metabolite, more polar than RG, and co-eluting with authentic 5,6-epoxy retinoic acid, was also present in most reaction mixtures.

Another metabolite more polar than 4-oxo and 5,6-epoxy retinoic acids was not identified. These unidentified metabolites may be 4-oxoretinoyl-β-glucuronide or other unknown retinoic acid derivatives. In addition to the above reactions, I also considered the reverse reaction, deconjugation of RG. I examined the deconjugation of RG in the liposomal system. Deconjugation is catalyzed by UDPGT and β-glucuronidase, and the reaction is optimal at low pH (pH 5.1). The data in Table 2 represents preliminary results for the deconjugation of radiolabeled RG using small intestinal and liver microsomes.

The physiological role of RA glucuronidation in the small intestine is not clear. Retinoyl-β-glucuronide has been shown to be as active as RA in promoting growth of vitamin A-deficient rats (6), inducing epithelial differentiation in a rat vaginal-smear assay (11), and in inhibiting HL-60 cell proliferation and inducing their differentiation into mature granulocytes (58). The rationale that RG is deconjugated to free RA prior to its biological action is logical and cannot be ruled out by previous studies.

The role of RG in vitamin A action is unknown. It is possible to suggest the following functions: 1) RG, similarly to other endogenous glucuronides could be water soluble elimination derivatives; however, retinoyl-β-glucuronide has been demonstrated to be biologically active

(43,47,48); 2) glucuronidation of retinoic acid may be a solubilization mechanism which would enable the hydrophobic retinoyl moiety to partition into hydrophilic cellular compartments; 3) glucuronidation is reversible, the deconjugation is catalzyed by both UDPGT and β-glucuronidase. In this way, RG may serve as a temporary intracellular storage form of the active retinoyl moiety; 4) Since RG is found in human plasma it may be necessary for intercellular transfer; 5) the glucuronides in circulation could be a feedback signal to control retinol-retinol binding protein release from the liver, and RG may act on its own on cellular differentiation in a yet undefined manner.

CONCLUSION

This study has demonstrated the in vitro glucuronidation of retinoc acid by liver and small intestinal microsomal UDP-glucuronosyltransferase (s) in liposomes. The kinetic parameters were determined for each tissue, statistically compared, and found to be significantly different. The magnitude of the Km of retinoic acid specific UDPGT in both tissues was in the range that suggests similar metabolic roles for the enzyme in the two tissues. The Km and Vmax determined for liver in this liposomal system are approximately three times higher than previously determined apparent kinetic parameters for liver, using detergent (12), even though both studies used the same enzyme source. This suggests that the use of detergents in membrane-bound enzyme assays should be questioned. It appears that liposomes represent a more appropriate system to assay this enzyme. Further studies with a purified enzyme will aid in a complete understanding of the regulation and function of UDPGT in vitamin A metabolism. However, the in vitro assay system will need to duplicate the chemical and physical environment of the enzyme in its natural membrane.

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