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The effects of pH on the enzymatic formation of β-glucuronides of various retinoids by induced and noninduced microsomal UDPGA-glucuronosyltransferases of several rat tissues in vitro

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All-trans *retinoyl-β-glucuronide, a prominent water-soluble metabolite of all*-trans *retinoic acid (RA) in animals, is formed by the enzymic transfer of the glucuronyl moiety of uridine diphosphoglucuronic acid to RA. Uridine diphosphoglucuronic acid glucuronosyl transferases (UGTs) of microsomal preparations catalyze this reaction. In noninduced rat liver microsomes, maximal activity was observed in the physiologic range (pH 6.9–7.5) for all*-trans*-RA, 9*-cis*-RA, all*-trans*-4-oxo-RA, and the synthetic retinoid tetramethyl, tetrahydro-anthracenyl-benzoic acid. The activities toward 13*-cis*-RA and tetramethyl, tetrahydronaphthenyl-propenyl-benzoic acid were maximal between pH 5.4 and 6.9 and toward acitretin [9-(2′,3′,6′ trimethyl, 4′methoxybenzyl-1′) 3,7 dimethyl, nona-2,4,6,8 tetraenoic acid] at pH 8.4. Several organs catalyze this reaction, but the activities of noninduced microsomes from liver, kidney, and testes were higher than those from intestine and lung. Brain microsomes were inactive. During storage at −80°C, the stability of UGTs varied both with the tissue and the retinoid substrate. 3-Methylcholanthrene both induces UGTs and increases the permeability of microsomal preparations to its substrates. The rates of glucuronidation of the less biologically active retinoids all*-trans*-4-oxo RA and 13*-cis*-RA were increased relatively more (11-fold and 6-fold, respectively) than those of the natural ligands for nuclear transcription factors all*-trans*-RA and 9*-cis*-RA (threefold and twofold, respectively). We conclude that several microsomal UGTs, which are differentially regulated, act on retinoids.* (J. Nutr. Biochem. 9:676–681, 1998)

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Abbreviations used: acitretin, 9-(2′,3′,6′ trimethyl, 4′methoxybenzyl-1′) 3,7 dimethyl, nona-2,4,6,8 tetraenoic acid; acitretin-G, acitretin-glucuronide; BHT, butylated hydroxytoluene; CD-367, tetramethyl, tetrahydro-anthracenyl-benzoic acid; CD-367-G, CD-367 glucuronide; HPLC, high-performance liquid chromatography; 3MC, 3-methylcholanthrene; MES, 2-[N-morpholino]ethanesulfonic acid; NEM, N-ethylmaleimide; 4-oxo-RA, 4-oxoretinoic acid; 4-oxo-RAG, 4-oxoretinoyl β-glucuronide; RA, retinoic acid; RAG, retinoyl β-glucuronide; RAR, retinoic acid receptor; ROL, retinol; RXR, retinoid X receptor; Tris, tris [hydroxymethyl] aminomethane; TTNPB, tetramethyl, tetrahydronaphthenyl-propenyl-benzoic acid; TTNPB-G, TTNPB glucuronide; UDPGA, UDP-glucuronic acid; UGT, UDPGA-glucuronosyl transferase.
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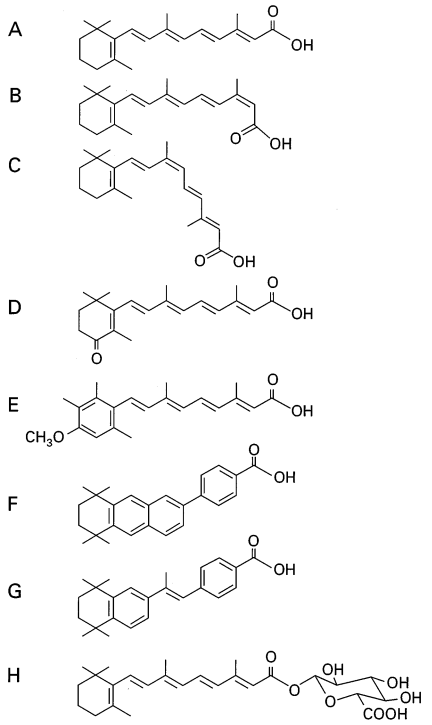

Figure 1 Formulas of retinoids: (*A*) all-*trans*-RA; (*B*) 13-*cis*-RA; (*C*) 9-*cis*-RA; (*D*) all-*trans*-4-oxoRA; (*E*) acitretin; (*F*) CD-367; (*G*) TTNPB; (*H*) all-*trans*-RAG.

Introduction

All-*trans* retinoyl β-glucuronide (RAG) is a prominent water-soluble metabolite of all-*trans* retinoic acid (RA) in humans and other animal species.[1–16] RAG, which also circulates in human plasma, is highly active in inducing growth and cell differentiation, is therapeutic against human acne, and is much less toxic than RA.[10,15,16] Other retinoids, both naturally occurring and synthetic, as well as cis isomers of RA, also form β-glucuronides.[14] Of these other retinoids (*Figure 1*), 13-*cis*-RA is both a commonly found, but generally less active, isomer of RA in tissues and is used as a drug (Accutane®) for treating acne; 9-*cis*-RA is the endogenous ligand for the retinoid X receptor (RXR) group of nuclear retinoid receptors; all-*trans*-4-oxo-RA is a major, but generally less biologically active, metabolite of RA; acitretin [9-(2′,3′,6′ trimethyl, 4′methoxybenzyl-1′) 3,7 dimethyl, nona-2,4,6,8 tetraenoic acid] is a synthetic drug for the treatment of psoriasis; and tetramethyl, tetrahydroanthracenyl-benzoic acid (CD-367) and tetramethyl, tetrahydronaphthenyl-propenyl-benzoic acid (TTNPB) are highly active, but also very toxic, synthetic analogs of RA.[17] The glucuronidation of these compounds is catalyzed by a family of microsomal UDP-glucuronic acid (UDPGA)-glucuronyl transferases (UGT), which can be further induced by pretreatment of the animal with 3-methylcholanthrene (3MC) and with other organic compounds.[14]

To explore further the nature of these reactions, we examined the pH profiles of UGT activities toward a set of seven retinoids in noninduced and 3-MC-induced microsomes of six different rat tissues. As shown in this article, marked differences exist in the relative rates of formation of the glucuronides of various retinoids, in the activities of different tissues, in the pH profiles, in the stability of UGTs toward isomeric RAs, and in the degree of inducibility by 3-MC.

Methods and materials

Chemicals and solvents

Compounds used and their gracious donors or purveyors were: acitretin glucuronide (Dr. Bruno Becker, Iowa State University, Ames, IA USA); CD-367 (Dr. Braham Shroot, Cird-Galderma, Sophia Antipolis, France); 13-*cis*-, 9-*cis*-, all-*trans*-RA, and all-*trans*-4-oxoretinoic acid (Dr. Chris Huselton, Hoffmann-LaRoche, Nutley, NJ USA); acitretin (Prof. Werner Bollag, Hoffmann-LaRoche, Basle, Switzerland); TTNPB (Dr. Hemmige Bhagavan, Hoffmann-LaRoche); all-*trans*-RA, UDPGA, N-ethyl maleimide, 3-MC, 2-[N-morpholino]ethanesulfonic acid (MES) buffer, tris [hydroxymethyl] aminomethane (Tris) buffer, bovine serum albumin, and butylated hydroxytoluene (BHT) (Sigma Chemical Co., St. Louis, MO USA); and solvents for extraction and for high performance liquid chromatography (HPLC; Fisher Scientific Co., Fair Lawn, NJ USA). All other chemicals used were of analytical reagent grade.

The β-glucuronides of the all-*trans*, 13-*cis*, and 9-*cis* isomers of RA and of all-*trans*-4-oxo-RA were synthesized in our laboratory.[18,19]

Animals and treatments

Sprague-Dawley male rats (150–250 g), purchased through Laboratory Animal Resources (Iowa State University), were kept in individual stainless-steel hanging cages and provided with a nutritionally-complete rodent diet containing 6% fat (No. 7002, Harlan Teklad, Indianapolis, IN USA) and with water ad libitum. Rats were housed in a well-ventilated, exclusively rodent animal room at 25°C and 40% relative humidity with a 12-hour light/dark cycle. Animal care was supervised by the staff of Laboratory Animal Resources.

For induction studies, rats were treated with one intraperitoneal injection of 3-MC in corn oil (40 mg/kg body weight) 4 days before sacrifice.[14] Control animals were treated with the vehicle only.

Preparation of microsomes

Rats were euthanized by an intraperitoneal injection of sodium pentobarbital (45 mg/kg body weight). Livers, after being perfused in situ with 10 mL ice-cold 0.25 M sucrose in 3 mM Tris buffer, pH 7.4, were excised, rinsed in fresh perfusion fluid, blotted, and weighed. Kidneys, intestines, lungs, testes, and brain were also excised, freed of connective tissue, rinsed, blotted, and weighed. Each tissue was first minced and then homogenized in 10 vol sucrose-Tris buffer, pH 7.4, by use of a Potter-Elvehjem Teflon homogenizer.[20,21] Microsomes were prepared, frozen in liquid nitrogen, and stored at −80°C as previously described.[14] Protein was measured by the Lowry procedure[22] with bovine serum albumin as the reference standard.

Enzyme assay

The final standard incubation mixture contained 170 μM of the selected retinoid, usually added in 5 μL 100% ethanol, 3 mM of UDPGA, 5 mM N-ethylmaleimide (NEM), 0.002% BHT, 1 mg/mL of microsomal protein, 5 mM $MgCl_2$, and either 50 mM MES buffer (pH 5.4–6.2) or 50 mM Tris buffer (pH 6.9–8.3) in

a final volume of 0.5 mL. UGT activities with MES or Tris buffers at pH 5.4 or 6.2 were comparable. Acitretin was added in 5 μL dimethylsulfoxide, which, like ethanol, was shown to have no effect by itself on the enzyme activity at the concentration used. The reaction was initiated at 37°C by adding UDPGA. The mixture was then incubated for 60 minutes at 37°C under yellow light with gentle shaking. The reaction rate was essentially linear during this period.[14]

The reaction was stopped and retinoids were extracted by adding 3.0 mL ethyl acetate:glacial acetic acid (10:1 v/v). After thorough mixing and centrifugation, 2.5 mL of the 3.0 mL of upper phase were removed and evaporated under argon at 50°C to dryness. Immediately thereafter, the extracted retinoids were dissolved in 100 μL dichloromethane:methanol (1:2 v/v). Retinoids in the dried extract, if not redissolved immediately, are very rapidly oxidized to a mixture of undefined polar products.

Enzyme stability

After storage for 1 year at −80°C, the UGTs in microsomes of liver, kidney, and testes toward 13-*cis*-RA were close to 100% in all cases; toward all-*trans*-RA were 95%, 85%, and 100%, respectively; and toward 9-*cis*-RA were 86%, 64%, and 14%, respectively. Thus, the stability of the UGTs under these conditions was affected both by their tissue of origin and by the specific isomer of RA used as the substrate.

Chromatographic analysis

Aliquots (10 μL) of each redissolved extract were injected onto a reversed-phase 5 μm C_{18} "resolve" column (3.9 mm × 15 cm; Waters Associates, Milford, MA USA) preceded by a C-130 guard column (Upchurch Scientific Co., Oak Harbor, WA USA). For the separation of most acidic retinoids, methanol:water (7.5:2.5 v/v) containing 10 mM ammonium acetate was used isocratically as the eluant at a flow rate of 1 mL/min at 25°C. Eluting solvents, all containing 10 mM ammonium acetate, for other substrates and their products were: all-*trans*-4-oxo-RA, methanol:water (5.5:4.5 v/v), and TTNPB, methanol:water (7:3 v/v). The HPLC system consisted of a 7125 injector (Rheodyne Co., Cotati, CA USA), a model 510 pump (Waters), a V[4] detector (ISCO Co., Lincoln, NE USA), and a CR-4A integrator (Shimadzu Co., Kyoto, Japan). Wavelengths used to detect various compounds were 350 nm for RA-containing compounds, 310 nm for CD-367-containing compounds, and 300 nm for TTNPB-containing compounds. The areas under peaks were standardized against known amounts of reference compounds and corrected for the yield of the internal standard, all-*trans*-retinyl acetate.

Under the HPLC conditions given, retention times were: all-*trans*-RA 8.8 minutes, all-*trans*-RAG 4.9 minutes; 13-*cis*-RA 7.3 minutes, 13-*cis*-RAG 4.1 minutes; 9-*cis*-RA 8.2 minutes, 9-*cis*-RAG 4.7 minutes; all-*trans*-4-oxo-RA 8.1 minutes, all-*trans*-4-oxo-RAG 5.4 minutes; acitretin 5.9 min, acitretin-glucuronide (acitretin-G) 3.5 minutes; CD-367 6.6 minutes, CD-367 glucuronide (CD-367-G) 5.0 minutes; TTNPB 11.3 minutes; and TTNPB glucuronide (TTNPB-G) 7.9 minutes. The important consideration in this assay procedure is the separation of the retinoid substrate from its glucuronide, not the separation of several of the retinoid substrates from each other.

Chromatograms of the formation of 13-*cis*-RAG from 13-*cis*-RA by noninduced and 3MC-induced rat liver microsomes are shown in *Figure 2*. Values for product formation are given as the mean of duplicate samples, which agreed within 10%. No glucuronide peak appeared in the absence of UDPGA, microsomes, or retinoid, or when boiled microsomes were used.[14]

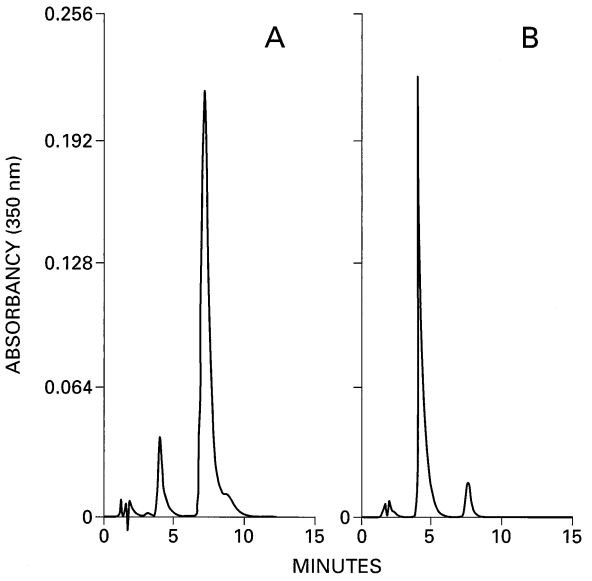

Figure 2 HPLC separation of RAG and RA. The HPLC separation of 13-*cis*-RAG formed from 13-*cis*-RA by noninduced (*A*) and by 3MC-induced (*B*) rat liver microsomes, 60 minutes, pH 6.9. Retention times are: 13-*cis*-RAG, 4.1 minutes and 13-*cis*-RA, 7.3 minutes.

Results

Formation of β-glucuronides of various retinoids as a function of pH by noninduced and 3MC-induced rat liver microsomes

The rates of formation of the β-glucuronides of various retinoids by noninduced liver microsomes at pH values from pH 5.4 to pH 8.3 are shown in *Figure 3*. Two major points might be made: (1) The maximal rates varied 13-fold from 34 nmol/mg protein/h for 9-*cis*-RA to 2.6 nmol/mg protein/h for CD-367. (2) The pH maxima for most retinoids fell in the physiologic range (pH 6.9–7.5). Exceptions were 13-*cis*-RA and TTNPB with more acidic pH maxima and acitretin with a more basic pH maximum.

The pH profiles of rates of β-glucuronidation of various retinoids catalyzed by 3MC-induced microsomes are shown in *Figure 4*. The ratios of maximal UGT activities of induced and noninduced microsomes for various retinoids are: CD-367, 13 times; all-*trans*-4-oxo-RA, 11 times; acitretin, 6 times; 13-*cis*-RA, 6 times; TTNPB, 4 times; all-*trans*-RA, 3 times; and 9-*cis*-RA 2 times. In terms of specific rates, the formation of 13-*cis*-RAG by induced microsomes was at least twofold greater than that of any other retinoid tested. Interestingly, the rate of glucuronidation of the natural ligands for the nuclear retinoid receptors, all-*trans*-RA and 9-*cis*-RA, increased only threefold and twofold, respectively, by treatment with 3MC.

The pH maxima of the glucuronidation rates in 3MC-induced microsomes were shifted to higher pH values. This shift may reflect changes in the permeability and structure of the microsomes as a result of the detergent effects induced by 3MC.[11] However, the relative pH optima of various retinoids in noninduced and induced microsomes were similar but not identical.

All three isomers of retinol (all-*trans*, 9-*cis*, and 13-*cis*) were poor substrates for glucuronidation in both noninduced and 3MC-induced rat liver microsomes [e.g., their rates were <12% those of all-*trans*-RA at all pH values (data not shown)].

Formation of β-glucuronides of RA isomers by noninduced microsomes of other tissues

As shown in *Table 1*, microsomes of the liver, kidney, and testes showed high UGT activity, those of the intestine and lung much less activity, and those of brain no detectable activity. Each tissue also showed a distinct pattern relative to specific isomers: All-*trans*-RA was most active in kidney microsomes, 9-*cis*-RA showed the highest activity in liver and intestinal microsomes, and 13-*cis*-RA was most active in testicular and lung microsomes.

The relative pH profiles of UGT activities toward all-*trans*-RA, 9-*cis*-RA, and 13-*cis*-RA for kidney and testes microsomes are shown in *Figure 5*. For all three isomers, the maximal activity was noted at pH 6.9 in kidney microsomes and at pH 7.6 in testes microsomes. The shape of the pH profiles, however, differed in both tissues for each of the isomers, particularly on the acidic side.

Discussion

Both endogenous and exogenous retinoids are converted in the rat to β-glucuronides by microsomes of the liver, kidney, testes, intestine, and lung, but not detectably of brain. Thus, the formation of retinoid β-glucuronides is a fairly general capability of many tissues. However, each tissue shows specificity in the rates at which different retinoid β-glucuronides are formed. Thus, among naturally occurring isomers of RA, β-glucuronides of the 9-*cis* isomer are formed in vitro most rapidly in the liver and intestine, of the all-*trans* isomer in the kidney, and of the 13-*cis* isomer in the testes and lung. After the oral administration of RAG to vitamin A-sufficient and vitamin A-deficient rats, both RA and RAG were found in most tissues, except for brain, in which some RA was present but RAG was undetectable.[23] Thus, these in vivo observations agree with the in vitro findings reported here.

The β-glucuronidation of retinoids has been most extensively studied with rat liver microsomes.[14] Interestingly, 9-*cis*-RA, the specific ligand for RXR, is the best substrate

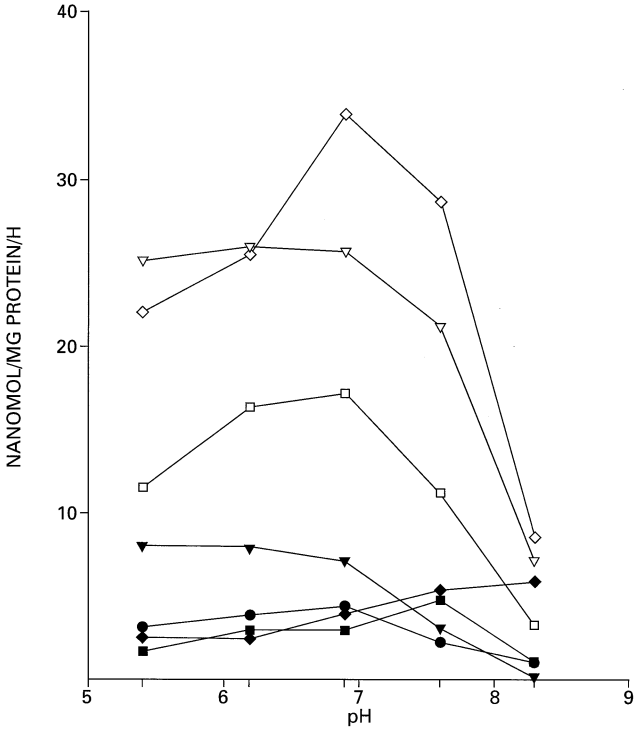

Figure 3 Rates of formation of β-glucuronides of various retinoids as a function of pH in noninduced rat liver microsomes. Each point is the mean value of duplicate samples, which differed by less than 10%. All-*trans*-RAG (□); 9-*cis*-RAG (◇); 13-*cis*-RAG (∇), all-*trans*-4-oxo-RAG (■); acitretin-G (◆); CD-367-G (●); TTNPB-G (▼).

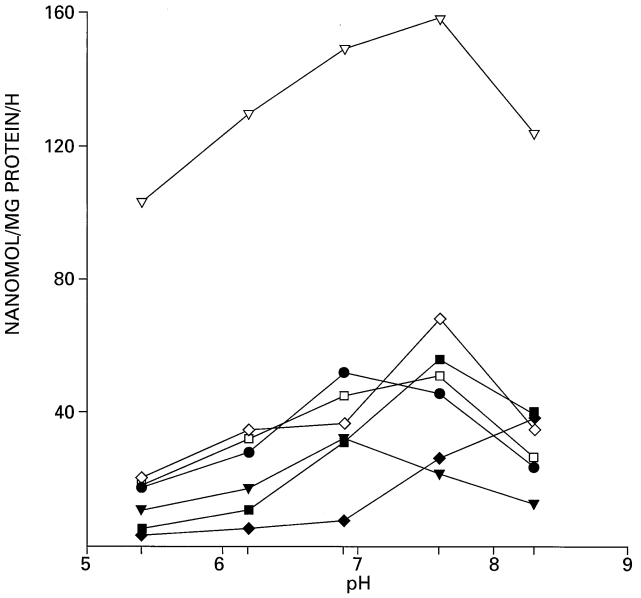

Figure 4 Rates of formation of β-glucuronides of various retinoids as a function of pH in 3MC-induced rat liver microsomes. Each point is the mean value of duplicate samples, which differed by less than 10%. All-*trans*-RAG (□); 9-*cis*-RAG (◇); 13-*cis*-RAG (∇), all-*trans*-4-oxo-RAG (■); acitretin-G (◆); CD-367-G (●); TTNPB-G (▼).

Table 1 Rates of formation of β-glucuronides of retinoic acid (RA) isomers by noninduced tissue microsomes at pH 6.9[1]

Tissue	Formation of β-glucuronides (nmol/mg protein/h)		
	all-*trans*-RA	9-*cis*-RA	13-*cis*-RA
Liver	13.7	25.0	20.9
Kidney	26.0	21.6	15.8
Testis	5.3	13.9	34.9
Intestine	ND	3.9	0.4
Lung	0.2	1.1	4.0
Brain	ND	ND	ND

[1]Standard incubation conditions were used. Values are the mean of duplicate experiments, which differed by <10%.
ND–below the limit of detection (0.1 nmol/mg protein/h).

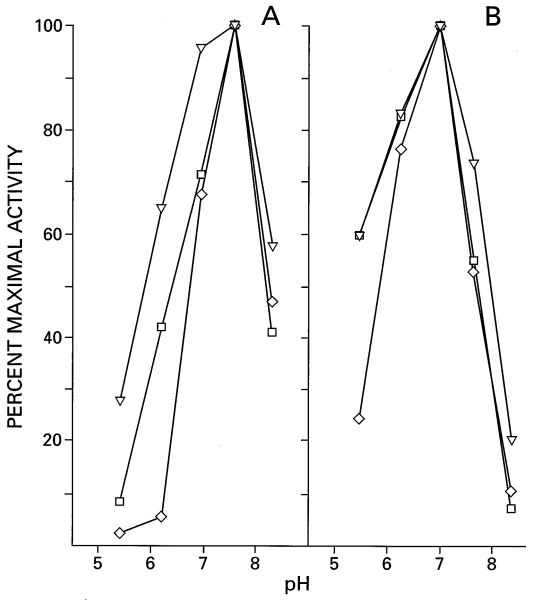

Figure 5 Rates of formation of β-glucuronides of RA isomers as a function of pH in noninduced microsomes of the testis (*A*) and kidney (*B*). Values are plotted as a percentage of the maximal activity, for each pH profile, which occurred at pH 7.6 for testis microsomes and at pH 6.9 for kidney microsomes with respect to all three isomers of RA. Each point is based on the mean value of duplicate samples, which differed by less than 10%. All-*trans*-RAG (□); 9-*cis*-RAG (◇); 13-*cis*-RAG (∇).

for glucuronidation, and all-*trans*-ROL (retinol) is one of the poorest. Although 3MC treatment enhanced UGT activity toward 9-*cis*-RA only twofold, it still was the second most active retinoid, after 13-*cis*-RA. Thus, microsomes might serve to differentiate between RA, which is involved in the regulation of gene expression, and ROL, which is primarily directed toward storage and plasma transport. Each isomer of RA shows its own specificity and extent of binding to various retinoid-binding proteins, including the nuclear receptors for RA, RAR (retinoic acid receptor), and RXR. Glucuronide formation might either enhance the transfer of RA from cytosol to nucleus[15] or divert RA away from the nucleus and target the complex for excretion. Indeed, RAG might directly serve as a donor of retinoyl groups in retinoylation reactions.[15,16,24,25] Thus, different isoforms of microsomal UGTs, each with its own specificity, might control facets of retinoid metabolism and function.

The existence of several UGTs that act differentially on retinoids is increasingly evident. For example, different pH profiles for this set of retinoids were found in both noninduced and 3MC-induced rat liver microsomes. That these pH profiles are primarily affected by factors other than the UGT involved is highly unlikely, inasmuch as the pK values and polarity of all of the acidic retinoids tested are very similar, RAG is stable at all pH values tested, and the addition of 5 mM 1,4-saccharolactone, an inhibitor of β-glucuronidase, had no effect on the amount of RAG formed by washed rat liver microsomes.[14] Furthermore, the response to induction of UGT by 3MC differed greatly with different retinoids; for example, a 13-fold increase of activity toward CD-367, a 6-fold increase toward 13-*cis*-RA, and only a 2-fold increase toward 9-*cis*-RA. The stability of UGT in the frozen state varied with the tissue and the isomer studied; the activity remained high toward 13-*cis*-RA and all-*trans*-RA in frozen microsomes of liver, kidney, and testes, but fell off significantly toward 9-*cis*-RA in a tissue-dependent manner. Furthermore, different isomeric forms of RA were maximally active in different tissues (*Table 1*).

Specific UGTs that act on retinoids are only starting to be isolated and characterized. Thus, Gunn rats, which primarily lack the isoform UGT1.1, form β-glucuronides of all-*trans*-RA and 13-*cis*-RA, but only at 20 to 30% of the rates found in Wistar rats.[26,27] In this regard, a cloned UGT1.1, when expressed in HK293 cells, was shown to be active on all-*trans*-RA and all-*trans*-5,6-epoxy-RA.[27] Furthermore, two UGTs active toward all-*trans*-RA have been separated from the liver microsomes of Wister rats.[26] Clearly, the identification of the specific UGT isoforms involved in the glucuronidation both of endogenous and synthetic retinoids merits further investigation. Although such studies with isolated cloned enzymes can provide crucial information about individual components of the large UGT family,[28,29] more physiologically relevant information may well be provided about retinoid glucuronidation by studies with intact microsomes, as employed here.

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