Pergamon

Biochemical Pharmacology, Vol. 47, No. 3, pp. 485–492, 1994.

0006-2952/94 $6.00 + 0.00

GLUCURONIDATION AND ISOMERIZATION OF ALL-*TRANS*- AND 13-*CIS*-RETINOIC ACID BY LIVER MICROSOMES OF PHENOBARBITAL- OR 3-METHYLCHOLANTHRENE-TREATED RATS

JÖRN OLIVER SASS,* ADELHEID FORSTER,† KARL WALTER BOCK† and HEINZ NAU

Institut für Toxikologie und Embryopharmakologie, Freie Universität Berlin, Garystrasse 5, D-14195 Berlin; and †Institut für Toxikologie, Eberhard-Karls-Universität Tübingen, Wilhelmstrasse 56, D-72074 Tübingen, Germany

(*Received* 10 *February* 1993; *accepted* 11 *October* 1993)

Abstract—Glucuronidation and isomerization of all-*trans*-retinoic acid (tr-RA) and 13-*cis*-retinoic acid (13-cis-RA) were investigated in an *in vitro* system using liver microsomes of differently pretreated rats. In agreement with their thermodynamic stability, more retinoic acid was isomerized from the 13-*cis* form to the all-*trans* form than *vice versa*. Also some 9-*cis*-retinoic acid (9-cis-RA) could be found. Isomerization was reduced, but in contrast to glucuronidation was still important if boiled microsomes were used. This supports the view that isomerization can proceed as a non-enzymatic process. 3-Methylcholanthrene (MC) pretreatment of the rats increased the microsomal glucuronidation of 13-cis-RA and tr-RA and the formation of 13-*cis*-retinoyl-β-glucuronide was enhanced up to 7-fold by MC-induced rat microsomes. The rates of glucuronidation by uninduced and phenobarbital-induced rat microsomes differed only slightly. In addition to glucuronides of the applied retinoic acid isomers (13-cis-RA and tr-RA), 9-cis-RA and its glucuronide were found. Induction of retinoid glucuronidation by pretreatment with MC indicates that this metabolic reaction is catalysed by a MC-inducible UGT isozyme. After two recently described pathways (conversions of retinol to retinal and of retinyl methyl ether to retinol) this is a third step of retinoid metabolism, induced by pretreatment with MC. With human microsomes no more than traces of glucuronides were detected; also, incubations with human microsomes resulted in a lower degree of isomerization than with rat microsomal fractions.

Key words: retinoic acid; 9-*cis*-retinoic acid; retinoyl-β-glucuronide; 9-*cis*-retinoyl-β-glucuronide; glucuronidation; isomerization

Retinoic acids (tr-RA‡ and 13-cis-RA, respectively) have become of interest in recent years not only as therapeutic agents in dermatology [1, 2] and oncology (for review see Ref. 3), but also because tr-RA has been suggested as being a morphogen during embryonic development [4]. However, teratogenicity is a serious side effect of retinoids, which may limit the application of these drugs for therapy [5, 6].

Glucuronidation has been observed to be an important metabolic pathway of retinol and retinoids *in vivo* which is significant both in endogenous metabolism and following pharmacological doses [7–13]. In the mouse, glucuronidation of 13-cis-RA to the corresponding retinoyl-β-glucuronide greatly exceeds the glucuronidation of tr-RA to its glucuronide, while the reverse situation exists in the monkey [10, 13].§ Findings from the Berlin laboratory have also shown that the β-glucuronides of tr-RA and 13-cis-RA are prominent metabolites in the plasma of pregnant rats and rabbits following administration of pharmacological doses of the corresponding acids [14, 15]. This is surprising, because drug glucuronides are generally excreted rapidly in urine or bile, and plasma concentrations are low. Recently, additional examples of relatively high plasma concentrations of some drug glucuronides such as morphine-6-glucuronide have been demonstrated [16]. Retinoyl-β-glucuronides have not only attracted attention as metabolites of retinoic acid during a possible detoxification process, but are also being investigated as possible agents for cancer chemoprevention and dermatology [17, 18]. This development is spurred by the hope that the low placental transfer of the glucuronides, as demonstrated in the mouse [10], rat [14] and rabbit [15], will be reflected by low teratogenicity of these glycoconjugates.

Glucuronidation is catalysed by UGTs (EC 2.4.1.17), membrane-bound enzymes which are mainly localized in the endoplasmic reticulum of the liver and various other tissues [16, 19]. Various UGT isozymes have been described which can be induced by either MC or PB [16, 19–21].

In the present study, we have investigated the influence of pretreatment of rats with MC and PB,

* Corresponding author. Tel. (49) 30-8385055; FAX (49) 30-8316139.

‡ Abbreviations: RA, retinoic acid; RAG, retinoyl-β-glucuronide; tr-RA, all-*trans*-retinoic acid; tr-RAG, all-*trans*-retinoyl-β-glucuronide; 13-cis-RA, 13-*cis*-retinoic acid; 13-cis-RAG, 13-*cis*-retinoyl-β-glucuronide; 9-cis-RA, 9-*cis*-retinoic acid; 9-cis-RAG, 9-*cis*-retinoyl-β-glucuronide; PB, phenobarbital, sodium salt; MC, 3-methylcholanthrene; UGT, UDP glucuronosyltransferase; UDPGA, UDP glucuronic acid, disodium salt.

§ Unpublished observations by Tzimas G, Collins MD and Nau H.

respectively, on the *in vitro* glucuronidation of 13-cis-RA and tr-RA by liver microsomes. We have also examined the effects of these microsomes on the *cis–trans* isomerization of retinoic acids. Furthermore, rat microsomal glucuronidation and isomerization have been compared to results obtained with human liver microsomes.

MATERIALS AND METHODS

Chemicals. 13-cis-RA, tr-RA and the corresponding 4-oxo-retinoic acids were supplied by F. Hoffmann-La Roche (Basel, Switzerland). tr-RAG as a reference compound was a gift from Drs A. B. Barua and J. A. Olson, Iowa State University (Ames, Iowa, U.S.A.).

MC, catalase (from bovine liver; EC 1.11.1.6), and glucose oxidase (from *Aspergillus niger*; EC 1.1.3.4) were obtained from Sigma (Deisenhofen, Germany). β-Glucuronidase (from *Escherichia coli*; EC 3.2.1.31) and UDPGA were from Boehringer Mannheim (Mannheim, Germany). Tris was bought from Paerel & Lorei (Frankfurt/M., Germany), Brij 58 from Serva (Heidelberg, Germany). Water (if not specified otherwise) was purified by a Milli-Q water purification system. Methanol (LiChrosolv® gradient grade), DMSO (Uvasol®) and all further chemicals were purchased from Merck (Darmstadt, Germany).

Laboratory precautions. All work with retinoids was performed under dim amber light. Retinoids and their solutions were stored at −20°.

Treatment of animals. Male Wistar rats (body wt: 200 g) were housed individually and received Altromin 1324 (Altromin, Lage, Germany) and tap water *ad lib.* A group of rats received an intraperitoneal injection (40 mg/kg) of MC dissolved in olive oil 4 days before they were killed. Another group of rats received an initial intraperitoneal injection of 100 mg/kg PB (dissolved in water) followed by 0.1% (w/v) PB in the drinking water until they were killed after 4 days. The third group of rats was not treated.

Preparation of microsomes. Rats were killed by decapitation. The livers were removed after perfusion through the portal vein with 40 mL ice-cold 0.9% (w/v) aqueous NaCl solution and microsomes were prepared in a Tris–sucrose buffer (pH 7.4) as described [21]. Human microsomes, which had already shown UGT activity towards paracetamol (approximately 5% of rat microsomal activity if the detergent Brij 58 was applied), were prepared from a human liver (HL18) which was obtained from a 25-year-old female kidney transplant donor shortly after death [22].

The protein concentrations of the microsomal fractions were determined according to Lowry *et al.* [23], using BSA as protein standard.

Assay of retinoic acid glucuronidation. Pilot experiments with and without oxygen removal by a solution containing 10 mM glucose, glucose oxidase (0.01 mg/mL) and catalase (1:5000 dilution of the product supplied by Sigma) [24] showed that removal of oxygen was not necessary.

Incubation mixtures contained 0.1 M Tris–HCl, pH 7.4, 5 mM $MgCl_2$, microsomes (1 mg/mL rat protein or 3 mg/mL human protein) and retinoic acid isomers (3, 30 or 300 μM, added in DMSO, final concentration of the solvent was 10%). The reaction was started by the addition of 3 mM UDPGA. The total sample volume was 100 μL. Samples were incubated at 37° for 30 min. Incubations were stopped by adding three volumes of isopropanol to each sample. The tubes were vortexed immediately, put on ice and centrifuged for 10 min (3000 *g*; 4°). The supernatants were protected from light and stored at −80° until analyses were carried out. In control experiments water was added instead of microsomal preparations or UDPGA. For other controls microsomes that had been boiled for 10 min in a water bath were used or active microsomes were added immediately after the isopropanol treatment of the samples. Incubations with microsomes from untreated controls and PB-treated rats contained Brij 58 (0.2 mg/mg protein), except for the samples used for the investigation of retinoic acid isomerization. Addition of the detergent increased glucuronide formation by approximately 50%. In the incubations with microsomes from MC-treated rats detergent was omitted since the addition of Brij 58 decreased glucuronidation by approximately one third. With a lower Brij 58 concentration (0.05 mg/mg protein) there was a smaller decrease. These differential effects of detergent on activities of untreated or PB-treated rat liver microsomes on the one hand, and on MC microsomes on the other hand may arise because during the 30 min incubation period lysophosphatidylcholines are generated from microsomal phospholipids by the action of lipases [25]. Generation of these endogenous detergents has previously been found to be enhanced in microsomes from MC-treated rats.* Therefore, the addition of Brij 58 may further enhance UGT activity in microsomes of untreated or PB-treated rats. However, in MC microsomes UGT appears to be already fully activated and therefore may be inhibited by the addition of Brij 58.

Retinoid analysis. Solid phase extraction was performed based on a previously described method using a Varian AASP module [26]. In general, 250 μL of the isopropanol-containing supernatants were diluted with three times the volume of an aqueous 2% ammonium acetate solution. This was passed with a nitrogen stream through an AASP-C2 cartridge which had been preconditioned with 3.4 mL methanol and 0.6 mL 2% ammonium acetate solution. The cartridge was washed with 1.5 mL of a mixture of 85% aqueous ammonium acetate solution (0.5%) and 15% acetonitrile to remove polar substances that might interfere, and then loaded onto an AASP autoinjector. Reverse-phase HPLC was performed with the "HPLC system I" which has been described by Eckhoff and Nau [27]. For some of the analyses the gradient was altered to improve resolution of the glucuronide isomers (0 min: 40% B; 8 min: 40% B; 14 min: 83% B; 14.2 min: 99% B; 15 min: 99% B; 15.5 min: 40% B; run time and valve reset time 15.5 min; cycle time 17.5 min). UV detection was carried out both at 340 and 356 nm [28]. A 4-point calibration by peak areas

* Bock KW, unpublished results.

Table 1. Glucuronidation and isomerization of 13-cis-RA by microsomal fractions of rat liver

Treatment	13-cis-RAG* [pmol/(mg protein × min)]	9-cis-RAG [pmol/(mg protein × min)]	tr-RAG [pmol/(mg protein × min)]	Sum of RAG* [pmol/(mg protein × min)]
3 μM				
Untreated	4.4 (1)	13	4.3	21.7 (1)
PB	4.5 ± 0.7 (1)	10 ± 3	4.0 ± 0.2	18.5 (1)
3-MC	32 ± 12 (7)	13 ± 2†		45.0 (2)
30 μM				
3-MC	159 ± 4	50 ± 6†		209
300 μM				
Untreated	349 (1)	48	92	489 (1)
PB	585 ± 27 (2)	111 ± 16	104 ± 15	800 (2)
3-MC	1522 ± 167 (4)	200 ± 18†		1722 (4)

* The induction factors are listed in brackets.

† When 9-cis-RAG and tr-RAG were not completely separated by HPLC, the sum of both isomers is listed.

was done for 13-*cis*- and all-*trans*-4-oxo-retinoic acid, tr-RAG, 13-cis-RA and tr-RA. These retinoids were identified by the retention times as well as absorption at both detection wavelengths. The 13-cis-RAG was already known to elute about 0.4 min prior to the tr-RAG peak [9]. We also identified 13-cis-RAG and 9-cis-RAG by hydrolysis with β-glucuronidase which led to a sharp decrease in the glucuronide peak while the respective aglycon peak increased. To perform this validation the HPLC fraction was collected and purified by rechromatography; then 70 μL of the eluate were incubated for 1 hr at 37° with 140 μL phosphate buffer (pH 6.8) and 70 μL β-glucuronidase suspension. For controls β-glucuronidase was replaced by the same volume of buffer. Such hydrolysis experiments also showed that the extinction coefficient of 13-cis-RAG is comparable to that of tr-RAG, in agreement with previous findings of Creech Kraft *et al.* [10].

RESULTS

Glucuronidation

Glucuronidation studies of RA isomers are complicated by the fact that rapid isomerization of retinoic acids occurs concomitantly. Therefore, the glucuronidation rate of a particular isomer, e.g. 13-cis-RAG, is listed together with RAG formation of the two other isomers (9-cis-RAG and tr-RAG; when no complete separation of the two isomers was achieved, the sum of both is given), and the sum of the three glucuronides (Tables 1 and 2). Three substrate concentrations were used: 3 μM (related to the level of tr-RA in the liver after treatment with pharmacological doses), as well as 30 and 300 μM. Because of concurrent isomerization K_m values could not be determined exactly, but are estimated to be in the range of 10^{-4} M.

Tables 1 and 2 show the formation rates of the glucuronides following incubation with either 13-cis-RA or tr-RA. Incubation with 13-cis-RA led to the formation of far more 13-cis-RAG than tr-RAG from tr-RA. In both cases a notable amount of 9-cis-RAG was formed.

While the rates of glucuronidation obtained with PB-induced rat microsomes differed only slightly from those observed with uninduced rat microsomes, glucuronidation was increased by MC-induced microsomes. With MC-induced rat microsomes glucuronidation in samples containing 13-cis-RA increased by up to seven times for the formation of 13-cis-RAG and up to four times for the total formation of glucuronides compared to incubations with microsomes from untreated rats. In samples with tr-RA, MC-induced microsomes resulted in up to 3-fold increased formation of total glucuronides while tr-RAG production was not more than doubled (Tables 1 and 2). However, tr-RAG could not always be completely separated from 9-cis-RAG. In the case of tr-RA at 300 μM only the formation of 13-cis-RAG appears to be increased by MC pretreatment (Table 2) and there may also be a slight increase in glucuronidation with PB microsomes at the highest concentration of 13-cis-RA (Table 1).

With human microsomes only very low rates of glucuronidation were observed even with a nearly 3-fold higher microsomal protein concentration in the incubation mixture (data not shown).

Isomerization

Isomerization was studied in the absence of Brij 58 with 3 μM of 13-cis-RA and tr-RA. The rate of isomerization decreased if the RA concentration was increased (data not shown).

Isomerization from 13-cis-RA to tr-RA was observed to a far greater extent than from tr-RA to 13-cis-RA: after the incubation, up to 79% of the retinoic acid in 13-cis-RA incubations of rat microsomes was in the all-*trans* form, whereas not more than 21% 13-cis-RA were not found in tr-RA samples (Tables 3 and 4). Isomerization was not affected by UDPGA, but decreased when boiled microsomes were used. If the microsomes were added immediately after isopropanol treatment at the end of the incubation, only low—if any—

Table 2. Glucuronidation and isomerization of tr-RA by microsomal fractions of rat liver

Treatment	13-cis-RAG [pmol/(mg protein × min)]	9-cis-RAG [pmol/(mg protein × min)]	tr-RAG* [pmol/(mg protein × min)]	Sum of RAG* [pmol/(mg protein × min)]
3 μM				
Untreated	3.9 ± 0.7	7.6 ± 1†	(1)	11.5 (1)
PB	2.1 ± 0.5	3.5 ± 1.4	2.5 ± 0.3 (1)	8.1 (1)
3-MC	12	2.8	7.0 (2)	22 (3)
30 μM				
3-MC	76 ± 6	61 ± 7†		137
300 μM				
Untreated	101 ± 7	41.3 ± 3	279 ± 20 (1)	421 (1)
PB	133 ± 9	51.5 ± 1.3	330 ± 8.6 (1)	515 (1)
3-MC	242 ± 29	78.8 ± 18.2	246 ± 56.7 (1)	567 (1)

* The induction factors are listed in brackets.
† When 9-cis-RAG and tr-RAG were not completely separated by HPLC, the sum of both isomers is listed.

Table 3. Isomerization of 13-cis-RA to tr-RA during incubation (30 min; 37°) with rat liver microsomal preparations (without Brij 58)

Incubation conditions	13-cis-RA (nM)	tr-RA (nM)	$\frac{[\text{tr-RA}] \times 100}{[\text{13-cis-RA}] + [\text{tr-RA}]}$ (%)
Complete*			
Untreated rats	775 ± 38	1373 ± 54	63.9
PB-treated rats	839 ± 193	1529 ± 88	64.6
MC-treated rats	326 ± 19	1227 ± 46	79.0
Without UDPGA†			
Untreated rats	802 ± 64	1429 ± 35	64.1
PB-treated rats	866 ± 53	1533 ± 96	63.9
MC-treated rats	577 ± 36	1625 ± 46	73.8
Microsomes added after incubation‡			
Untreated rats	2761 ± 115	184 ± 10	6.25
PB-treated rats	2645 ± 124	316 ± 13	10.7
MC-treated rats	2675 ± 89	305 ± 28	10.2
Boiled microsomes§			
Untreated rats	2268 ± 88	582 ± 70	20.4
PB-treated rats	1685 ± 90	810 ± 26	32.5
MC-treated rats	1537 ± 135	961 ± 62	38.5
Without microsomes‖	2788 ± 36	203 ± 14	6.79

Values are means ± SD; N = 3–5.
* Complete incubation mix.
† Control: water instead of UDPGA solution.
‡ Control: addition of microsomes after isopropanol treatment of samples.
§ Control: microsomes boiled before addition.
‖ Control: water added instead of microsomes.

isomerization was observed. Possibly MC-induced microsomes showed slightly enhanced 13-*cis* to all-*trans* isomerization compared to the other rat microsomes. There was no significant difference in retinoic acid isomerization between microsomes of untreated and PB-treated rats. Due to enhanced glucuronidation, retinoic acid concentration of the samples with active MC-induced microsomes were reduced; this, however, did not alter markedly the percentage of isomerization (Tables 3 and 4).

In all microsome-containing samples, with the exception of the control incubations to which retinoic acid was added after isopropanol treatment, a small HPLC peak could be detected between those of 13-cis-RA and all-trans-RA. This peak coelutes with 9-cis-RA. When the peak was collected and

Table 4. Isomerization of tr-RA to 13-cis-RA during incubation (30 min; 37°) with rat liver microsomal preparations (without Brij 58)

Incubation conditions	tr-RA (nM)	13-cis-RA (nM)	$\frac{[13\text{-cis-RA}] \times 100}{[tr\text{-RA}] + [13\text{-cis-RA}]}$ (%)
Complete*			
Untreated rats	2266 ± 80	441 ± 32	16.3
PB-treated rats	2199 ± 104	551 ± 16	20.0
MC-treated rats	1676 ± 53	357 ± 26	17.6
Without UDPGA†			
Untreated rats	2106 ± 177	430 ± 38	17.0
PB-treated rats	2179 ± 67	582 ± 22	21.1
MC-treated rats	1924 ± 64	497 ± 14	20.5
Microsomes added after incubation‡			
Untreated rats	2494 ± 98	67.4 ± 13.2	2.63
PB-treated rats	2680 ± 180	170 ± 22	5.96
MC-treated rats	2880 ± 156	92.1 ± 9.7	3.10
Boiled microsomes§			
Untreated rats	2300 ± 255	307 ± 73	11.8
PB-treated rats	2559 ± 146	362 ± 31	12.4
MC-treated rats	2175 ± 23	472 ± 22	17.8
Without microsomes‖	2925 ± 184	92.4 ± 10.7	3.06

Values are means ± SD; N = 4–5.
*†‡§‖ See legend to Table 3.

purified by rechromatography, it showed the same ratio of absorbance at 340 and 356 nm as the pure 9-*cis* reference compound.

With human microsomal preparations less isomerization was found than with rat microsomal fractions (Table 5). As expected, due to the absence of NADPH and oxygen, neither in samples with human microsomes nor with rat microsomes could significant amounts of 4-hydroxy- or 4-oxo-retinoic acids be detected.

DISCUSSION

We have investigated the glucuronidation of the retinoic acids *in vitro* to gain more information on this conjugation reaction, and have used rat microsomal incubations as a model system. We show that both 13-cis- and tr-RA acid are conjugated with glucuronic acid, but glucuronidation of 13-cis-RA yields about twice as much glucuronide as tr-RA. Furthermore, our study shows that 13-cis-RAG is a quantitatively important metabolite of tr-RA. These findings correlate well with previous results obtained *in vivo* in the mouse: after pharmacological doses the glucuronidation of 13-cis-RA was more extensive than the glucuronidation of the *trans* isomer [10].* Also, 13-cis-RAG has been found to be a major metabolite of 13-cis-RA in rat bile, whereas tr-RAG was reported to be only a minor biliary metabolite after dosage of tr-RA [29, 30]. Neither the formation of 9-cis-RAG nor production of 9-cis-RA by isolated microsomes has been described before.

The retinoyl-β-glucuronides may have vitamin A/retinoid activity as demonstrated *in vivo* and with *in vitro* assays [17, 18]. It has been found previously that the glucuronides of 13-cis- and tr-RA are present in the embryo of the mouse, rat and rabbit in very low (or even undetectable) concentrations, compared with corresponding plasma levels [10–12, 14, 15].* The retinoid-like activity of these conjugates, together with their apparent limited placental transfer, highlights the fact that active retinoids with low teratogenicity may become a possibility.

Our results indicate that tr- and 13-cis-RA are glucuronidated by a MC-inducible UGT isozyme, while PB has no major influence on the glucuronidation of these retinoic acids. Investigations by Bank *et al.* [31], concerning the *in vitro* glucuronidation of all-trans-RA by microsomes of TCDD-treated rats, which were performed with a rather high concentration (100 μM) of tr-RA, also support these results [31]. This is an unexpected finding since MC has been proposed generally to induce glucuronidation of planar phenols, whereas PB-inducible UDP glucuronysyltransferase catalyses the glucuronidation of more bulky molecules [16, 19]. Although El Mouelhi and Bock [32] have shown that naproxen [2-(6-methoxy-2-naphthyl) propionic acid] can be glucuronidated by both MC- and PB-inducible isozymes. tr- and 13-cis-RA, although not planar molecules, apparently also fit the structural requirements of an MC-inducible UGT isozyme which catalyses their glucuronidation. However, there are indications that PB pretreatment leads to an increase in glucuronidation at high concentrations of 13-cis-RA (Table 1). Recently it has been shown that MC can also induce enzyme activities of rat

* Unpublished observations by Tzimas G, Collins MD and Nau H.

Table 5. Isomerization of 13-cis-RA to tr-RA and *vice versa* during incubation (30 min; 37°) with a microsomal preparation from single human liver (without Brij 58)

Incubation conditions	Incubations with 13-cis-RA			Incubation with tr-RA		
	13-cis-RA (nM)	tr-RA (nM)	[tr-RA] × 100 / [13-cis-RA] + [tr-RA] (%)	tr-RA (nM)	13-cis-RA (nM)	[13-cis-RA] × 100 / [tr-RA] + [13-cis-RA] (%)
Complete*	1602 ± 114	1132 ± 78	41.4	2682 ± 41	361 ± 16	11.9
Without UDPGA†	1297 ± 95	1489 ± 92	53.4	2641 ± 19	378 ± 6	12.5
Microsomes added after incubation‡	2493 ± 156	401 ± 20	13.9	2762 ± 58	80.9 ± 3.4	2.85
Boiled microsomes§	2074 ± 88	628 ± 48	23.2	2436 ± 108	355 ± 34	12.7
Without microsomes‖	2788 ± 36	203 ± 14	6.79	2925 ± 184	92.4 ± 10.7	3.06

Values are means ± SD; N = 4–5.
*†‡§‖ See legend to Table 3.

microsomes at other steps of retinoid metabolism: the conversions of retinol to retinal and of retinyl methyl ether to retinol are also induced by MC [33, 34].

In contrast to rat microsomes, incubations of human microsomes exhibited only minor or undetectable glucuronidation activities. This is in agreement with the situation *in vivo*: in plasma of a patient treated with 13-cis-RA only small amounts of retinoyl-glucuronides were found, while very high concentrations of glucuronides were present in the plasma of 13-cis-RA-treated rats [13, 14]. This species difference in glucuronidation may also be one reason for the high clearance of 13-cis-RA in rodents as compared to humans [35, 36].

Cis–trans isomerization of retinoic acid has been suggested to play an important role, especially for the teratogenesis of 13-cis-RA [10, 37]. In the present study a high percentage of retinoic acid isomerization was observed. In samples with 13-cis-RA, the concentrations of tr-RA determined after incubation with microsomes greatly exceeded even the levels of the applied 13-cis-RA. This is compatible with the thermodynamic stability of retinoid isomers: the all-*trans* molecules are more stable than the corresponding 13-*cis* isomers [38, 39]. In glucuronidation samples (especially with 13-cis-RA) less total retinoic acid was measured than in controls, because glucuronidation resulted in a decrease in the acids.

Isomerization was reduced but still important—especially for the 13-cis-RA samples—if boiled microsomes were used for the incubations. This supports the suggestion by Shih *et al.* [40] that isomerization can proceed as a non-enzymatic process. These authors advocate the hypothesis that isomerization is catalysed by sulfhydryl groups which are mostly oxidized during the heating process [40, 41]. Sulfhydryl groups are present in the proteins of the microsomal preparations. The decreased rate of isomerization at higher retinoic acid concentrations suggests the saturability of this process.

It may be that there are different isozymes/mechanisms responsible for the glucuronidation of 13-cis-RA and tr-RA or that these two retinoic acid isomers differ in regard to their affinities for the UDP glucuronosyltransferase responsible. It was not expected that much 13-cis-RAG and also 9-cis-RAG would be found even after incubation with tr-RA, although by analogy to retinol and retinoic acids tr-RAG should be the most stable glucuronide (Tables 1 and 2) [38, 39]. After incubation with 13-cis-RA more 13-RAG and 9-cis-RAG than tr-RAG were formed, although tr-RA was the main retinoic acid isomer after incubation (Tables 1 and 3). It is therefore unlikely that a high degree of isomerization occurred after the glucuronidation, because in that case tr-RAG should have been the predominant isomer. We will investigate further whether retinoyl-glucuronides are less sensitive towards isomerization than retinoic acids.

Our findings indicate that 13-cis-RAG, the major metabolite of 13-cis-RA in the rat, is formed to a greater extent and possibly in a different way than the all-*trans* isomer (after incubation with the corresponding acids). Furthermore, the formation

of rather high amounts of 9-cis-RAG emphasizes the importance of investigation into the metabolism of 9-cis-RA *in vivo* and *in vitro*. To obtain more information about retinoic acid isomerization experimental conditions should be established which allow glucuronidation to proceed at a decreased rate of isomerization. It would also be interesting to mirror the *in vivo* situation with an *in vitro* assay able to form both 4-oxo-retinoic acids and retinoyl-β-glucuronides.

Acknowledgements—We thank Mr H. Gschaidmeier and Ms C. Plum for their help with the experiments. This work was supported by the Deutsche Forschungsgemeinschaft, Sfb 174, C6.

REFERENCES

1. Peck GL, Ohlsen TG, Yoder FW, Strauss JS, Downing DT, Pandya M, Butkus D and Arnaud-Battandier J, Prolonged remissions of cystic acne and conglobate acne with 13-cis-retinoic acid. *N Engl J Med* **300**: 329–333, 1979.
2. Thorne EG, Topical tretinoin research: an historical perspective. *J Int Med Res* **18** (Suppl 3): 18C–25C, 1990.
3. Smith MA, Parkinson DR, Cheson BD and Friedman MA, Retinoids in cancer therapy. *J Clin Oncol* **10**: 839–964, 1992.
4. Franceschi RT, Retinoic acid: morphogen or more mysteries? *Nutr Rev* **50**: 19–20, 1992.
5. Rosa FW, Wilk AL and Kelsey FO, Teratogen update: vitamin A congeners. *Teratology* **33**: 355–364, 1986.
6. Kochhar DM, Kraft J and Nau H, Teratogenicity and disposition of various retinoids *in vivo* and *in vitro*. In: *Pharmacokinetics in Teratogenesis* (Eds. Nau H and Scott WJ Jr), Vol. 2, pp. 173–187. CRC Press, Boca Raton, FL, 1987.
7. Barua AB and Olson JA, Retinoyl-β-glucuronide: an endogenous compound of human blood. *Am J Clin Nutr* **43**: 481–485, 1986.
8. Zile MH, Inhorn RC and DeLuca HF, Metabolites of all-trans-retinoic acid in bile: identification of all-trans- and 13-cis-retinoyl glucuronides. *J Biol Chem* **257**: 3537–3543, 1982.
9. Eckhoff C, Wittfoht W, Nau H and Slikker Jr W, Characterization of oxidized and glucuronidated metabolites of retinol in monkey plasma by thermospray liquid chromatography/mass spectrometry. *Biomed Mass Spectrom* **19**: 428–433, 1990.
10. Creech Kraft J, Eckhoff C, Kochhar DM, Bochert G, Chahoud I and Nau H, Isotretinoin (13-cis-retinoic acid) metabolism, cis–trans isomerization, glucuronidation, and transfer to the mouse embryo: consequences for teratogenicity. *Teratogen Carcinogen Mutagen* **11**: 21–30, 1991.
11. Tzimas G, Collins MD, Hummler H and Nau H, Transplacental pharmacokinetics of all-trans-retinoic acid in the rat: comparison between single and multiple oral application. *Teratology* **46**: 36, 1992.
12. Tzimas G, Bürgin H, Collins MD, Hummler H and Nau H, Pharmacokinetics of all-trans-retinoic acid in pregnant rabbits after single and multiple oral administration. *Teratology* **46**: 37, 1992.
13. Creech Kraft J, Slikker Jr W, Bailey JR, Roberts LG, Fischer B, Wittfoht W and Nau H, Plasma pharmacokinetics and metabolism of 13-cis- and all-trans-retinoic acid in the cynomolgus monkey and the identification of 13-cis- and all-trans-retinoyl-β-glucuronides. A comparison to one human case study with isotretinoin. *Drug Metab Dispos* **19**: 317–324, 1991.
14. Collins MD, Tzimas G, Hummler H and Nau H, Comparative transplacental pharmacokinetics of all-trans-retinoic acid, 13-cis-retinoic acid and retinyl palmitate in the rat following daily administrations of equipotent teratogenic doses. *Teratology* **47**: 11, 1993.
15. Tzimas G, Bürgin H, Collins MD, Hummler H and Nau H, The high sensitivity of the rabbit to the teratogenic effects of 13-cis-retinoic acid is a consequence of prolonged exposure of the embryo to 13-cis-retinoic acid and 13-cis-4-oxo-retinoic acid, and not of isomerization to all-trans-retinoic acid. *Arch Toxicol*, in press.
16. Mulder GJ, Glucuronidation and its role in regulation of biological activity of drugs. *Annu Rev Pharmacol Toxicol* **32**: 25–49, 1992.
17. Mehta RG, Barua AB, Olson JA and Moon RC, Effects of retinoid glucuronides on mammary gland development in organ culture. *Oncology* **48**: 505–509, 1991.
18. Olson JA, Moon RC, Anders MW, Fenselau C and Shane B, Enhancement of biological activity by conjugation reactions. *J Nutr* **122** (3S): 615–624, 1992.
19. Jansen PLM, Mulder GJ, Burchell B and Bock KW, New developments in glucuronidation research: report of a workshop on "Glucuronidation, its role in health and disease". *Hepatology* **15**: 532–544, 1992.
20. Bock KW, Josting D, Lilienblum W and Pfeil H, Purification of rat-liver microsomal UDP-glucuronyltransferase: separation of two enzyme forms inducible by 3-methylcholanthrene or phenobarbital. *Eur J Biochem* **98**: 19–26, 1979.
21. Bock KW, Fröhling W, Remmer H and Rexer B, Effects of phenobarbital and 3-methylcholanthrene on substrate specificity of rat liver microsomal UDP-glucuronyltransferase. *Biochim Biophys Acta* **327**: 46–56, 1973.
22. Bock KW, Forster A, Gschaidmeier H, Brück M, Münzel P, Schareck W, Fournel-Gigleux S and Burchell B, Paracetamol glucuronidation and human phenol UDP-glucuronosyltransferase. *Biochem Pharmacol* **45**: 1809–1814, 1993.
23. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
24. Werringloer J and Kawano S, Cytochrome b_5 and the integrated microsomal electron transport system. In: *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (Eds. Coon MJ, Conney AH, Estabrook RW, Gelboin HV, Gillette JR and O'Brien PJ), Vol. 1, pp. 469–476. Academic Press, New York, 1980.
25. Dutton GJ, *Glucuronidation of Drugs and Other Compounds*. CRC Press, Boca Raton, FL, 1980.
26. Collins MD, Eckhoff C, Chahoud I, Bochert G and Nau H, 4-Methylpyrazole partially ameliorated the teratogenicity of retinol and reduced the metabolic formation of all-trans retinoic acid in the mouse. *Arch Toxicol* **66**: 652–659, 1992.
27. Eckhoff C and Nau H, Identification and quantitation of all-trans- and 13-cis-retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. *J Lipid Res* **31**: 1445–1454, 1990.
28. Eckhoff C and Nau H, Vitamin A supplementation increases levels of retinoic acid compounds in human plasma: possible implications for teratogenesis. *Arch Toxicol* **64**: 502–503, 1990.
29. Swanson BN, Frolik CA, Zaharevitz DW, Roller PP and Sporn MB, Dose-dependent kinetics of all-trans-retinoic acid in rats: plasma levels and excretion into bile, urine, and faeces. *Biochem Pharmacol* **30**: 107–113, 1981.
30. Meloche S and Besner J-G, Metabolism of isotretinoin: biliary excretion of isotretinoin glucuronide in the rat. *Drug Metab Dispos* **14**: 246–249, 1986.

31. Bank PA, Salyers KL and Zile MH, Effect of tetrachlorodibenzo-*p*-dioxin (TCDD) on the glucuronidation of retinoic acid in the rat. *Biochim Biophys Acta* **993**: 1–6, 1989.
32. El Mouelhi M and Bock KW, Stereoselective (*S*)- and (*R*)-naproxen glucuronosyltransferases of rat liver. *Drug Metab Dispos* **19**: 304–308, 1991.
33. Shih TW and Hill DL, Conversion of retinol to retinal by rat liver microsomes. *Drug Metab Dispos* **19**: 332–335, 1991.
34. Shih TW, Shealy YF and Hill DL, Conversion of retinoid ethers to alcohols by enzymatic activity present in rat liver microsomes. *Drug Metab Dispos* **19**: 336–339, 1991.
35. Nau H, Creech Kraft J, Eckhoff C and Löfberg B, Interpretation of retinoid teratogenesis by transplacental pharmacokinetics. In: *Pharmacology of Retinoids in the Skin* (Eds. Reichert U and Shroot B), Vol. 3, pp. 165–173. Karger, Basel, 1989.
36. Nau H, Correlation of transplacental and maternal pharmacokinetics of retinoids during organogenesis with teratogenesis. In: *Methods in Enzymology* (Ed. Packer L), Vol. 190, part B, pp. 437–448. Academic Press, San Diego, CA, 1990.
37. Creech Kraft J, Löfberg B, Chahoud I, Bochert G and Nau H, Teratogenicity and placental transfer of all-trans-, 13-cis-, 4-oxo-all-trans-, and 4-oxo-13-cis-retinoic acid after administration of a low oral dose during organogenesis in mice. *Toxicol Appl Pharmacol* **100**: 162–176, 1989.
38. Robeson CD and Baxter JG, Neovitamin A. *J Am Chem Soc* **69**: 136–141, 1947.
39. Rando RR, The chemistry of vitamin A and vision. *Angew Chem Int Ed Engl* **29**: 461–480, 1990.
40. Shih TW, Shealy YF, Strother DL and Hill DL, Nonenzymatic isomerization of all-trans- and 13-cis-retinoids catalyzed by sulfhydryl groups. *Drug Metab Dispos* **14**: 698–702, 1986.
41. Hamm R and Hofmann K, Changes in the sulphydryl and disulphide groups in beef muscle proteins during heating. *Nature* **207**: 1269–1271, 1965.