All-*trans*-retinoyl β-glucuronide: new procedure for chemical synthesis and its metabolism in vitamin A-deficient rats

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All-*trans*-retinoyl β-glucuronide (RAG) was chemically synthesized in high yields (up to 79 %) by a new procedure involving the reaction of the tetrabutylammonium salt of glucuronic acid with all-*trans*-retinoic acid (RA) *via* the imidazole or triazole derivative. When RAG was fed orally to vitamin A-deficient rats, RA was identified as the major metabolite in the serum within hours of administration of RAG. Very little or no RAG was detected in the serum. Thus RAG, which was not appreciably hydrolysed to RA in vitamin A-sufficient rats [Barua and Olson (1987) Biochem J. **263**, 403–409], was rapidly converted into RA in vitamin A-deficient rats.

INTRODUCTION

All-*trans*-retinoyl β-glucoronide (RAG), first identified as a biliary metabolite of vitamin A [1,2], occurs endogenously in human blood [3]. After administration of all-*trans*-, 13-*cis*- or 9-*cis*-retinoic acid (RA), the respective retinoyl glucuronide or a mixture of retinoyl glucuronide isomers has been characterized as the major plasma metabolite in monkey [4–6], rat [7,8], mouse [5,7], rabbit [9] and man [10]. The small intestine has been shown to be the major site of RAG biosynthesis from RA [8,11]. Like RA, RAG is biologically active in promoting the growth of vitamin A-deficient rats [12,13] and in the induction of differentiation of HL-60 [14–17] and BA-HAN-1C [18] cells, but unlike RA, RAG is much less cytotoxic [14–18] and teratogenic [19]. Like RA, RAG is also effective in the topical treatment of human acne [20], but unlike RA, it does not produce any side effects associated with RA therapy [20].

We reported earlier the first chemical synthesis of RAG in a 30 % yield by a two-step procedure starting from RA via the derivative, retinoyl fluoride [21–23]. The procedure, although highly reproducible, has the following drawbacks: (1) the fluorinating reagent, diethylaminosulphur trifluoride, is corrosive and flammable; (2) retinoyl fluoride must be purified from the crude reaction mixture before it can serve as an effective acylating reagent; (3) yields of RAG never exceeded 30 %. Therefore the method may not be attractive for general laboratory and industrial purposes.

During the conventional synthesis of glucuronides of alcoholic compounds, e.g. retinyl β-glucuronide [24] or morphine glucuronide [25], the functional groups in glucuronic acid are protected, and the 1-OH group is activated by the introduction of bromine. After formation of the glycosyl linkage (R-O-R′), which is stable towards hydrolysis, the protective groups are removed. This procedure is not applicable for the synthesis of glucuronides of carboxylic acids such as RA, however, because the glycosyl linkage (RCOOR′) in the glucuronides of acidic compounds is very labile towards strong acids and bases. As a result, removal of the protective groups also results in the cleavage of the glycosyl linkage to give back the carboxylic acid [18].

Because of the potential of RAG as a drug of therapeutic importance, we have searched for a simpler and more economic method for its synthesis. Our first attempt involved substituting free glucuronic acid for glucose in the reported synthesis of retinoyl β-glucose [26] from retinoyl imidazole or triazole. Because of the acidic nature of glucuronic acid and its insolubility in the reaction solvents, however, RAG could not be synthesized by this route.

In this paper, we report that use of the phase-transfer catalyst, tetrabutylammonium hydroxide, results in the synthesis of glucuronide conjugates of acidic compounds, such as retinoic acid and acitretin, in good yield. Also we demonstrate that RAG is rapidly converted into RA by vitamin A-deficient rats.

EXPERIMENTAL

All experiments were carried out under yellow light illuminated with Gold Fluorescent tubes (Philips F40GO). Serum and retinoids were stored at −20 °C.

Chemicals and solvents

RA was a gift from the BASF Corporation, 1,1′-Carbonyldiimidazole, 1,1′-carbonyldi-(1,2,4-triazole), pyridine and tetrabutylammonium hydroxide 30-hydrate were purchased from Fluka Chemie AG, Buchs, Switzerland. Acetone, methanol, dichloromethane, diethyl ether, ethyl acetate and molecular sieves (type 4A, 8–12 mesh) were purchased from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. Silica gel (40–140 mesh), which was supplied by J. T. Baker Inc., Phillipsburg, NJ, U.S.A., was deactivated with water (4 %, w/v). NaH and glucuronic acid were supplied by Aldrich Chemical Co., Milwaukee, WI, U.S.A. Pyridine and acetone were dried over molecular sieves.

Animal studies

Weanling Sprague–Dawley rats were fed on a vitamin A-deficient diet (ICN, Cleveland, OH, U.S.A.). When the rats ($n = 12$) reached the weight-plateau stage, ten were fed, by means of a Microman positive-displacement pipette (Gilson Medical Electronics, Villiers-le-Bel, France; supplied by Rainin Instruments Co., Woburn, MA, U.S.A.), on a solution of RAG dissolved in corn oil (1 mg in 100 μl per rat) for 1 day, followed by 200 μl of the solution on days 2 and 3. The other two rats, which served

Abbreviations used: RAG, all-*trans*-retinoyl β-glucuronide; RA, all-*trans*-retinoic acid.

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as controls, received corn oil only. After the third dose, the rats were killed 1.5, 2.5, 3.5, 4.5 and 5.5 h later under ether anaesthesia. Blood was collected from the heart by means of a syringe, and centrifuged for 20 min at 500 ***g*** (1500 rev./min). Serum was separated, and whenever possible, was analysed by HPLC on the same day; otherwise serum samples were kept frozen at −72 °C.

Extraction of retinoids

Ethanol (1 ml) containing 0.05 % butylated hydroxytoluene and ethyl acetate (1 ml) was added to serum (0.5 ml) followed by acetic acid (10 %, v/v; 100 μl). After being vortexed for 30 s, the mixture was centrifuged for 1 min at 500 ***g***. The supernatant was pipetted out and saved. The pellet was extracted once with ethyl acetate (1 ml) and once with hexane (1 ml) by vortexing and centrifuging as before. The pooled supernatant solution was vortexed and centrifuged with water (1 ml). The upper organic phase was evaporated to dryness in a stream of argon. The residue was reconstituted in a mixture of dichloromethane/methanol (1:3, v/v; 100 μl), and 40 μl aliquots were analysed by HPLC as described previously [22–24].

Synthesis of tetrabutylammonium glucuronate

Glucuronic acid (2.9 g) was suspended in methanol (25 ml) to which tetrabutylammonium hydroxide 30-hydrate (12 g) was added. The mixture was stirred at room temperature for 1 h until a clear solution resulted. The solvent was removed in a rotary evaporator at 40 °C, thereby yielding a syrup. Addition of acetone (100 ml) resulted in precipitation of the tetrabutylammonium glucuronate. The salt was separated by filtration and washed with acetone. The filtrate and washings, on further concentration in a rotary evaporator, resulted in precipitation of more salt. Tetrabutylammonium glucuronate (5.6 g; 85 %) was obtained as a white powder.

Synthesis of RAG

RA (2 g) was dissolved in pyridine (15 ml) to which 1,1′-carbonyldi-imidazole (1.2 g) was added. The mixture was stirred at room temperature for 1.5 h until the yellow solution turned orange–red and the absorption maximum shifted from 350 to 390 mm. TLC of a test drop of the reaction mixture showed that retinoyl imidazole was the major compound present. To this reaction mixture, tetrabutylammonium glucuronate (3.5 g), pyridine (10 ml) and a pinch (about 20 mg) of NaH were added. The mixture was stirred at room temperature for 4–5 h until a test drop showed that RAG was the major retinoid present.

The reaction was stopped by the careful addition of water. After the solution was made just acidic with acetic acid, retinoids were extracted with ethyl acetate several times until all of the yellow colour disappeared from the aqueous phase. The ethyl acetate extract was washed with water, dried over anhydrous Na_2SO_4, and then evaporated to dryness in a rotary evaporator. The oily residue was dissolved in dichloromethane and chromatographed as described previously [21–24] on a column of silica gel. Development with dichloromethane/diethyl ether (3:1, v/v) resulted in elution of traces of retinoyl imidazole and RA. RAG was then eluted with a mixture of dichloromethane/methanol (1:1, v/v). This eluate was evaporated to dryness in a rotary evaporator to give RAG as a yellow solid (2.5 g; 79 %). In several synthetic runs, the yields ranged from 68 to 79 %.

Reverse-phase HPLC of RAG was performed either on a Waters Resolve 5 μm column (3.9 mm × 15 cm) as described previously [22,23] or on a Rainin Microsorb-MV C_{18} 3 μm column (4.6 mm × 10 cm) with methanol/water (85:15, v/v) containing 10 mM ammonium acetate at a flow rate of 0.8 ml/min. HPLC traces of chromatograms of RAG before and after purification by column chromatography are shown in Figure 1. The final preparation contained traces of a *cis*-isomer and of the α-anomer of RAG, but no RA or other impurities. The physical and chemical properties of RAG prepared by this new procedure was identical with those reported earlier [21–24].

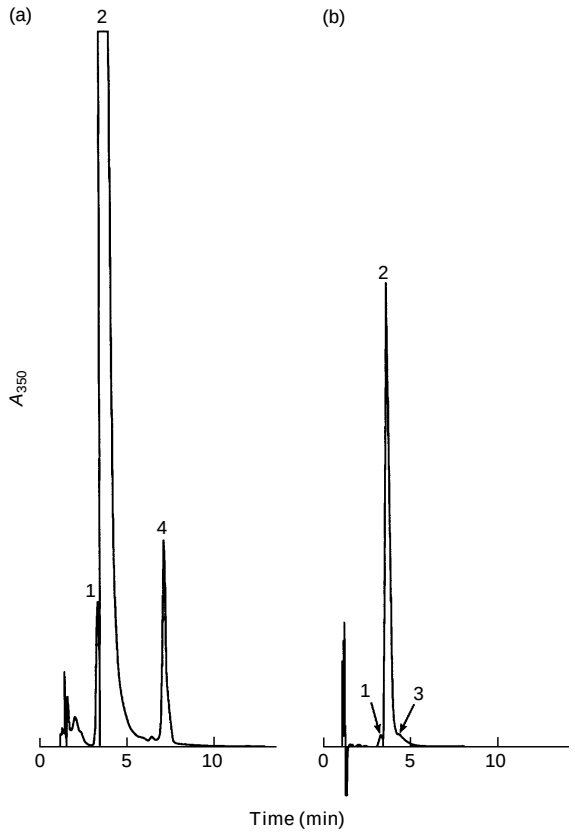
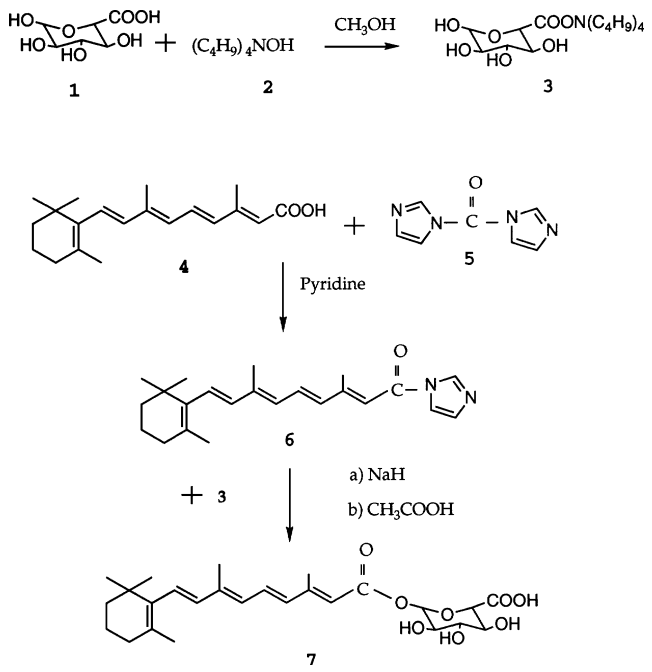


Figure 1 HPLC profile of RAG

Reverse-phase HPLC was carried out on a Rainin Microsorb-MV 3 μm C_{18} column (4.6 mm × 10 cm) by using methanol/water (85:15, v/v) containing 10 mM ammonium acetate at a flow rate of 0.8 ml/min. (**a**) Before purification and (**b**) after purification by column chromatography. Peak identification: 1, *cis*-retinoyl β-glucuronide; 2, RAG; 3, retinoyl α-glucuronide; 4, RA.

RESULTS AND DISCUSSION

RAG (**7**) was synthesized from RA (**4**) *via* its imidazole (**6**) or triazole derivative in high yield (up to 79 %) by a simplified procedure, as shown in Scheme 1. Glucuronic acid (**1**) was first converted into its tetrabutylammonium salt (**3**) which not only protected the free carboxyl group of glucuronic acid but also rendered it soluble in organic solvents. The synthesis of RAG can be carried out in one or two steps as follows. In the two-step procedure, retinoyl imidazole or triazole is first prepared by stirring RA with 1,1′-carbonyldi-imidazole (**5**) or with 1,1′-carbonyldi-(1,2,4-triazole) in dry acetone. It is then purified, and in the second step allowed to react with tetrabutylammonium glucuronate in pyridine or dimethylformamide in the presence of NaH. RAG is thereby produced in about 60 % yield. However, we found that retinoyl imidazole or triazole need not be isolated.



Scheme 1 Chemical synthesis of RAG

1, Glucuronic acid; **2**, tetrabutylammonium hydroxide; **3**, tetrabutylammonium glucuronate; **4**, RA; **5**, 1,1′-carbonyldi-imidazole; **6**, all-*trans*-retinoyl imidazole; **7**, RAG.

Table 1 Metabolites of orally dosed RAG in vitamin A-deficient rats

Vitamin A-deficient rats were given three oral doses of RAG in corn oil (1 mg of RAG day per rat on day 1, and 2 mg/day per rat on days 2 and 3). On day 3, the rats were killed at indicated time points after the last dose, and their serum was analysed by HPLC for metabolites. Abbreviation: ND, not detected.

Rat no.	Time after dose (h)	Concentration (μmol/l) of serum)	
		RAG	RA
1 and 2	0	ND*	ND
3 and 4	1.5	Trace, ND	0.12, 0.21
5 and 6	2.5	0.04, 0.05	0.39, 0.11
7 and 8	3.5	0.01, 0.03	0.03, 0.25
9 and 10	4.5	ND, Trace	0.02, 0.01
11 and 12	5.5	ND	0.01, trace

Therefore, in the one-step procedure, after essentially all of the RA had formed the imidazole (or triazole) derivative, tetrabutylammonium glucuronate and NaH were added to the crude reaction mixture to produce RAG. This resulted in an increase in the yield of RAG up to 79% and a decrease in the synthesis time.

By use of the tetrabutylammonium salt of glucuronic acid, we have also successfully synthesized acitretin glucuronide (results not shown here). Thus the procedure described in this paper should be very useful for the synthesis of the glycosyl linkage between the 1-OH group of glucuronic acid and the COOH group of other compounds.

When RAG was administered orally to vitamin A-deficient rats, it was converted into RA, which was detected in the circulating blood at 1.5 h after the dose (Table 1). The RA concentration peaked in the serum at 2.5–3.5 h and then rapidly decreased.

In another experiment carried out on six vitamin A-sufficient rats, we failed to detect appreciable amounts of radioactivity in the serum of rats within 1–6 h of very small oral doses of ^{3}H-labelled RAG (A. B. Barua and J. A. Olson, unpublished work). It was not clear whether orally administered RAG was not absorbed by vitamin A-sufficient rats or was handled differently from vitamin A-deficient rats. Support of growth in vitamin-A deficient rats by small oral doses of RAG pointed towards its efficient absorption [13]. Experiments are now underway to determine if there are differences in the absorption and metabolism of orally administered RAG in rats with different vitamin A status.

The present finding that orally administered RAG is absorbed and metabolized to RA contrasts with our earlier observations that RAG is not readily hydrolysed to RA in vitamin A-sufficient rats when administered intraperitoneally or orally in large or small doses [19,22]. Although these earlier experiments were not designed in the same way as the current study, serum RA clearly rose after RAG administration in vitamin A-deficient rats but not detectably so in vitamin A-sufficient ones.

A possible explanation is that this hydrolytic reaction, together with others, is regulated by the vitamin A status of the animal. In vitamin A-deficient animals, all possible sources of vitamin A, such as tissue reserves of retinyl ester [27], provitamin A carotenoids [28], and now conjugates of vitamin A, e.g. RAG and possible retinyl β-glucuronide, seem to be expeditiously converted into vitamin A and/or RA. In contrast, in vitamin A-sufficient animals, vitamin A and RA will rather be sequestered by proteins and/or converted into physiologically less active forms. Thus retinol will be preferentially stored as retinyl ester [29], the rate of conversion of provitamin A carotenoids into vitamin A will decrease [28], and the formation of RAG from RA will be enhanced. Because RA is cytotoxic at 10^{-5} M and teratogenic at high doses, whereas RAG is not [14,15,19,20], the formation of RAG from RA may play a protective role under these circumstances.

Thus RAG may serve as a water-soluble transport agent for RA [17], as a physiological controller of RA concentrations in cells and as a non-toxic reservoir of RA that might be mobilized as needed [18,30]. Further studies are needed to clarify the physiological and toxicological roles of RAG.

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