Chemical synthesis of all-*trans*-[11-³H]retinoyl β -glucuronide and its metabolism in rats *in vivo*

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All-*trans*-[11-³H]retinoyl β -glucuronide (RAG) was synthesized in a single step from all-*trans*-[11-³H]retinoyl fluoride, with a 24 % yield. After its intraperitoneal injection into rats, RAG was detected in the blood, liver, intestine and kidney during the following 24 h period. Although the concentration of radiolabelled metabolites decreased with time, RAG predominated at nearly all times in nearly all tissues. Small amounts of retinoic acid (RA) were also universally present, together with unidentified polar metabolites and small amounts of non-polar esters of RA. The major excretion products of RAG in faeces and urine were RA and polar metabolites. Thus RAG, although converted in part to RA *in vivo*, persists as a major component in blood and tissues for at least 24 h. These observations support the concept that the retinoid β -glucuronides might serve a physiologically significant role in the function of vitamin A.

INTRODUCTION

The glucuronide conjugates of retinol (retinyl β -glucuronide; ROG) and of retinoic acid (retinoyl β -glucuronide; RAG) show interesting biological actions; namely, both support well the growth of vitamin A-deficient rats (Nath & Olson, 1967; Barua & Olson, 1985a, 1987a) and inhibit the induction of neoplastic changes in mammary gland cultures (Mehta *et al.*, 1987). RAG is at least as effective as retinoic acid (RA) in inducing the cellular differentiation of vaginal epithelia (Sietsema & DeLuca, 1982), and both stimulate the granulocytic differentiation of HL-60 promyelocytic leukaemia cells much better than does retinol (Gallup *et al.*, 1987; Zile *et al.*, 1987). Retinoid β -glucuronides are also found in human blood (Barua & Olson, 1986; Barua *et al.*, 1989) and in a variety of tissues (Zile *et al.*, 1982), as well as in bile (Dunagin *et al.*, 1966). While acting as effective inducers of differentiation, the cytotoxicity of the two glucuronides towards HL-60 cells is at least 50 % less than that of the parent compounds (Gallup *et al.*, 1987; Zile *et al.*, 1987). RAG and ROG are also much less toxic than RA in the chondrogenesis suppression assay (D. Kochhar, unpublished work). Although all-*trans*-RA shows 90–100 % teratogenic activity in rats or mice at a dose level of 100 mg/kg body weight, all-*trans*-RAG and -ROG are not demonstrably teratogenic at the same or an even higher dose level (Gunning *et al.*, 1989). If RAG was rapidly converted to RA, the same degree of teratogenicity should have been observed in the RAG-treated rats. The purpose of the present study was to determine whether RAG stimulated rat growth and cellular differentiation *in vivo* as an intact compound or only after its hydrolysis to RA. To investigate the metabolism of RAG directly, we have now synthesized tritiated RAG in one step from retinoyl fluoride and have investigated its fate in rats *in vivo*. A preliminary

communication concerning this study has previously appeared (Barua & Olson, 1987b).

MATERIALS AND METHODS

Chemicals and solvents

All-*trans*-[11-³H]RA was obtained from SRI International, Menlo Park, CA, U.S.A., through the courtesy of the National Cancer Institute, Bethesda, MD, U.S.A. All-*trans*-RA, all-*trans*-retinyl acetate and β -glucuronidase from *Escherichia coli* (activity 570 000 units/g) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Methyl retinoate was prepared by reacting RA with diazomethane; methyl 5,6-epoxyretinoate and methyl 4-oxoretinoate were prepared from methyl retinoate by oxidation with MnO₂; methyl 4-hydroxyretinoate was prepared by reduction of methyl 4-oxoretinoate with NaBH₄; and the corresponding free acids were prepared by hydrolysis of the methyl esters with base (Barua & Ghosh, 1972). The fluorinating reagent (diethylaminosulphurtrifluoride), D-glucuronic acid, and Diazald (for preparation of diazomethane) were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. All other chemicals were either analytical or reagent grade.

H.p.l.c.

Retinoids were separated on a Waters Associates (Milford, MA, U.S.A.) 5 μ m C₁₈ column by a reversed-phase linear gradient h.p.l.c. procedure, as described previously (Barua *et al.*, 1988). The solvent gradient was run from CH₃OH/water (17:8, v/v) containing 10 mM-ammonium acetate to CH₃OH/CH₂Cl₂ (4:1, v/v) at a flow rate of 1 ml/min for a 20 min period, followed by isocratic elution with the latter solvent for an additional 10 min. Absorbance was monitored at 350 nm. The

Abbreviations used: ROG, retinyl β -glucuronide; RAG, retinoyl β -glucuronide; RA, retinoic acid; RAF, retinoyl fluoride; BHT, butylated hydroxytoluene.

Fig. 1. H.p.l.c. profile of standard retinoids

Reversed-phase h.p.l.c. was carried out on a Waters 5 μ m C₁₈ column (3.5 mm $\times$ 15 cm) by using a linear gradient of CH₃OH/water (17: 8, v/v) containing 10 mM-ammonium acetate to CH₃OH/CH₂Cl₂ (4:1, v/v) at a flow rate of 1 ml/min for 20 min. Abbreviations: ORA, 4-oxoretinoic acid; OHRA, 4-hydroxyretinoic acid; RAG, retinoyl β -glucuronide; RA, retinoic acid; OMR, methyl 4-oxoretinoate; ROL, retinol; EMR, methyl 5,6-epoxy-retinoate; MR, methyl retinoate; RP, retinyl palmitate.

separation of reference retinoids in this system is depicted in Fig. 1.

Synthesis of all-*trans*-[11-³H]RAG

[11-³H]RA (183 μ g containing 5.98×10^8 d.p.m.) in toluene was evaporated to dryness in a stream of argon, and the residue was redissolved in diethyl ether (1 ml). This labelled RA was added with mixing to a solution of unlabelled RA (10 μ mol) dissolved in diethyl ether (10 ml), followed by cooling in a bath of dry ice and acetone. Diethylaminosulphurtrifluoride (10.2 μ mol) dissolved in diethyl ether (1 ml), after being similarly cooled, was added to the RA solution. The resultant yellow solution, which contained [11-³H]retinoyl fluoride (RAF), was evaporated to dryness under argon, and the residue was dissolved in diethyl ether (200 μ l). Tritiated RAF was purified by t.l.c. on a silica gel plate (0.25 mm thick, 20 cm $\times$ 20 cm) developed with hexane/acetone (4:1, v/v) (Barua & Olson, 1983). The yellow RAF band moved slightly behind the solvent front, leaving polar side-products at the origin. The RAF band was scraped off and extracted with diethyl ether, which was filtered through a Whatman no.1 filter paper. The silica gel particles were then extracted several more times with small volumes of diethyl ether. The pooled ether extract was evaporated to dryness under argon, and the residue of RAF was dissolved in acetone (4 ml).

D-Glucuronic acid (150 μ mol) was dissolved in water (2 ml) containing NaHCO₃ (350 μ mol) and added to the RAF solution, and the mixture was stirred at room temperature under argon for 16 h. The formation of RAG was monitored by t.l.c. by using hexane/acetone (2:1, v/v) as the developing solvent. RAG appeared at the origin as a yellow spot, and RAF moved almost with the solvent front. If a considerable amount of RAF was still present at 16 h, more water (2 ml) was added and the

mixture was stirred for an additional 3–6 h. The solution was diluted with water (6 ml), neutralized by adding dropwise a solution of acetic acid in water (1:10, v/v), and extracted with ethyl acetate three or four times. The extract was washed with water and was then centrifuged briefly to separate water from the ethyl acetate phase. Ethyl acetate was removed under argon, and the residue was dissolved in methanol (100 μ l). Portions of 20 μ l were purified isocratically by h.p.l.c. on the 5 μ m column with a solvent mixture of CH₃OH/water (7:3, v/v) containing 10 mM-ammonium acetate at a flow rate of 1.5 ml/min (Barua & Olson, 1985b). All-*trans*-RAG separated as the major peak from traces of the α -anomer and *cis*-isomers. The all-*trans*-RAG fractions were pooled, diluted with an equal volume of water, made just acidic with a few drops of dilute acetic acid, and extracted with ethyl acetate five times. The pooled extract, after washing with water (2 ml) and centrifuging, was evaporated under argon. The residue was dissolved in methanol, and the amount of RAG was estimated from its absorption at 360 nm ($A_{1\text{cm}}^{1\%}$ 1065). The specific activity of all-*trans*-[11-³H]RAG (1277 μ g, yield 24.4%) was found to be 1.19×10^5 d.p.m./ μ g. Aliquots of the methanol solution were evaporated to dryness under argon, and the residue was dissolved in saline solution (0.85%, w/v) to give a concentration of 70.6 nmol of RAG/0.4 ml of saline.

Animal studies

Male Sprague–Dawley rats ($n = 6$) weighing approx. 200 g and raised on a normal rat diet were used. Each rat received by intraperitoneal injection [³H]RAG (70.6 nmol containing a total of 4.0×10^6 d.p.m. in 0.4 ml of saline). The rats were killed under ether anaesthesia at 0.5, 1, 3, 6, 12 or 24 h after the injection. Blood was collected by cardiac puncture with a syringe and allowed to clot. Serum was separated by centrifugation. The liver, small intestine and kidney were removed and washed with saline. Serum and tissues were stored at -20°C until analysis. The urine and faeces of one of the rats kept in a metabolic cage were also collected for 24 h. Urine and faeces were stored at -20°C .

Extraction of retinoids

Serum. A 0.5 ml portion was treated with ethanol (1 ml) to which 10 μ l of butylated hydroxytoluene (BHT) (0.1%, w/v) and ethyl acetate (2 ml) were added. The mixture was vortexed and centrifuged briefly. The pellet was extracted once more with ethyl acetate, and the pooled extract was diluted with water (2 ml), vortexed and centrifuged. After removal of the upper organic phase, the lower aqueous layer was treated with 50 μ l of 1 M-acetic acid and extracted twice with ethyl acetate (2 $\times$ 1 ml) and finally with hexane (1 ml). The pooled organic phase was washed with water (1 ml), vortexed, centrifuged and evaporated under argon. The residue was dissolved in 100 μ l of CH₃OH/CH₂Cl₂ (3:1, v/v). Portions (50–75 μ l) were analysed by h.p.l.c.

Other tissues. Portions of liver (1 g) and small intestine (2 g) and individual kidneys were each ground to a powder with anhydrous Na₂SO₄ and extracted first with ethyl acetate (30 ml) and then with CH₂Cl₂ (3 $\times$ 30 ml). Each filtered extract was evaporated nearly to dryness in a rotary evaporator. The residue was dissolved in CH₂Cl₂ (2 ml), the flask was rinsed with CH₂Cl₂ (2 $\times$ 1 ml), and

the pooled extracts in a conical tube were evaporated in a stream of argon to dryness. The residue was dissolved in $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$ (3:1, v/v, 100–200 μl). Portions (50–100 μl) were analysed by h.p.l.c.

Faeces and urine. The faeces (13.3 g), collected for 24 h after the injection of RAG, were ground to a powder. A 2 g portion was extracted with CH_2Cl_2 (6 $\times$ 5 ml). The extract was evaporated, and the residue was prepared for h.p.l.c. analysis as described for other tissues. An aliquot (50 μl) of the final solution (200 μl) was injected. Urine (200 μl) was treated with 200 μl of methanol, and the solution was centrifuged. A 100 μl aliquot was analysed by h.p.l.c.

Other chemical procedures

Alkaline hydrolysis. RAG in serum and tissue extracts was hydrolysed by treatment with methanolic KOH in either the presence or absence of water as follows. (a) Aqueous hydrolysis: minced tissue (1 g) was treated with methanol (2 ml, containing 0.1% BHT), methanolic KOH (10%, w/v; 2 ml) and water (5 ml). The solution was refluxed for 15 min and, after the addition of water (5 ml), for 5 min more. The cooled solution was made just acidic with 1 M-HCl, and the retinoids were extracted with ethyl acetate (3 $\times$ 5 ml). After being washed with water, the extract was prepared for h.p.l.c. analysis as already described. (b) Non-aqueous hydrolysis: RAG (100 μg in 100 μl of CH_3OH) or the extracted residue from 0.5 ml of serum or from 1 g of other tissues was dissolved in CH_3OH (5 ml). Methanolic KOH (10%, v/v; 100 μl) was added to the solution, and the mixture was refluxed for 5 min. The solution was cooled to room temperature, diluted with water (5 ml), made just acidic with 1 M-HCl, and extracted with ethyl acetate (3 $\times$ 2 ml). The extracted residue was prepared for h.p.l.c. analysis as already described.

Methylation with CH_2N_2 . RAG (100 μg in 100 μl of methanol) or the concentrated extract (100 μl) from 0.5 ml of serum or from 1 g of other tissues was evaporated to dryness under argon and dissolved in diethyl ether (500 μl). An ethereal solution of CH_2N_2 (approx. 10 mg in 2 ml) was added and the mixture was left at room temperature for 30 min. The solvent was removed under argon. The residue was dissolved in $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$ (3:1, v/v; 100 μl) and analysed by h.p.l.c.

Action of β -glucuronidase. RAG (10 μg in 100 μl of methanol) or a portion of tissue extracts was evaporated to dryness under argon and dissolved or suspended in sodium acetate buffer (0.2 M, pH 6.8, 2 ml). The mixture was incubated in the presence or absence of β -glucuronidase (10 mg) at 37 °C for 1–2 h. The retinoids were extracted with ethyl acetate (2 $\times$ 1 ml) and finally with hexane (1 ml). The pooled extract was evaporated to dryness under argon. The residue was dissolved in 100 μl of $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$ (3:1, v/v) and analysed by h.p.l.c.

Determination of radioactivity. Portions (50–100 μl) of extracts from serum or other tissues as well as chromatographic fractions (1 ml) were each mixed with 5 ml of Scintiverse (Fischer Scientific Co., Fair Lawn, NJ, U.S.A.). Their ^3H content was then quantitatively measured by use of an LS-7500 scintillation counter (Beckman Instrument Co., Fullerton, CA, U.S.A.).

Background and quench corrections were made by using H number values of known solvent mixtures.

Recoveries of radioactivity from serum and various tissues were calculated on the basis of the total ^3H counts (d.p.m.) present in a particular extract relative to the total ^3H counts (d.p.m.) in the administered dose.

RESULTS

Synthesis of [11- ^3H]RAG

When a solution of all-*trans*-[11- ^3H]RAF in acetone was stirred with a solution of glucuronic acid in water in the presence of sodium bicarbonate, RAG was formed in good yield. All-*trans*-[11- ^3H]RAG was always accompanied by small amounts of the α -anomer and of *cis*-isomers, which were separated by h.p.l.c. (Barua & Olson, 1985b).

Distribution of radioactivity in blood, various tissues and excreta of rats after an intraperitoneal injection of [11- ^3H]RAG

The distribution of radioactivity at various time in serum and in other tissues of rats dosed with [11- ^3H]RAG is shown in Table 1. Administered RAG entered the circulation quickly, as indicated by the peak in radioactivity and RAG concentration 30 min after the injection (Table 1). Thereafter, the concentration of radioactivity in the blood fell slowly with time. In the liver, the concentration of radiolabelled metabolites reached a peak within 1 h but then fell slowly (Table 1). In the small intestine and kidney, the concentrations of radioactivity reached maxima at 3 h and thereafter fell (Table 1). Approx. 35% of the administered dose of [11- ^3H]RAG was excreted in the faeces and urine during the 24 h period (Table 1).

Nature of radioactive compounds in serum, tissues and excreta

Serum. At 0.5 h, the major radioactive retinoid present in the serum was injected RAG, along with small quantities of RA and unidentified polar compounds (Figs. 2a and 3). Although the radioactivity and con-

Table 1. Distribution of radioactivity in serum, other tissues, faeces and urine of rats at various times after an intraperitoneal dose of [11- ^3H]RAG

Rats were given 70.6 nmol of [11- ^3H]RAG in saline by intraperitoneal injection and were killed at various times afterwards. The retinoids were extracted, and the radioactivity associated with each organ was determined. The volume of serum was assumed to be 4% of the body wt.

Time (h)	Distribution of ^3H (% of dose)					
	Serum	Liver	Small intestine	Kidney	Faeces	Urine
0.5	15.26	4.49	0.92	0.25	—	—
1	5.63	7.86	2.4	0.34	—	—
3	3.76	1.93	3.75	0.38	—	—
6	2.15	4.64	0.85	0.1	—	—
12	1.41	1.24	0.37	0.1	—	—
24	1.11	2.18	0.26	0.08	23.0	12.1

Fig. 2. Time course of appearance of radiolabelled metabolites of RAG in various tissues of rats given [11-³H]RAG

Rats were given 70.6 nmol of [11-³H]RAG in saline by intraperitoneal injection and were killed 0.5, 1, 3, 6, 12 or 24 h after the dose. Serum (a), liver (b), small intestine (c) and kidney (d) were analysed by reversed-phase gradient h.p.l.c. for all metabolites. The concentration of each metabolite was determined from the radioactivity associated with each. Abbreviations: POL, polar metabolites; RAG, retinoyl β -glucuronide; RA, retinoic acid; MR, methyl retinoate.

concentration of metabolites in the serum decreased with time, radioactive RAG predominated at all times during the entire 24 h period. The RA concentration was always low; the ratio of RAG/RA in serum during the 24 h period is shown in Table 2. A non-polar ester of retinoic acid, possibly methyl or ethyl retinoate, was also observed in the serum in small constant amounts during the entire period of study.

Liver. The concentration of radioactivity in the liver reached a peak within 1 h and then fell (Table 1). Except at 1 and 3 h, RAG was predominant in the liver at all times during the study (Fig. 2b). RA was present in the liver at all times and was the major metabolite of RAG at 1 h. The ratio of RAG/RA in the liver during the 24 h period of study is shown in Table 2. Considerable amounts of radioactivity were also associated with some polar compound(s) that eluted with the void volume. A non-polar metabolite of retinoic acid, presumably an ester, was also present in small concentrations.

Small intestine. RAG was the major radioactive compound present in small intestine at all times, except at 24 h (Fig. 2c). Polar compounds were present in significant amounts throughout the study, and were most predominant at 24 h. RA was always present and the amount remained significant up to 3 h. The concentration of RAG reached a maximum at 3 h and then dropped rapidly. The RAG/RA ratio fluctuated in the small intestine (Table 2).

Kidney. The concentration of RAG peaked at 1 h, and except at 3 h when polar metabolites predominated, RAG was the predominant radiolabelled compound in kidney at all times (Fig. 2d). Appreciable amounts of an unidentified polar metabolite and a small amount of RA were always present. The ratio of RAG/RA was highest in kidney (Table 2). A small amount of an unidentified radioactive metabolite of intermediate polarity between RA and methyl retinoate, not shown in Fig. 2, was observed in both the kidney and small intestine, but not in the liver or serum.

Faeces. The predominant metabolite of RAG excreted in the faeces during the 24 h period was RA (Fig. 4a). Radioactivity also appeared in the polar fraction (1–6 min) and in a fraction eluting just after RA (14–16 min). RAG was not identified in the faeces.

Urine. The polar fraction (1–6 min) of urine contained most of the radioactivity (Fig. 4b). A significant amount of RA, but only traces of RAG, were also present in the urine (Fig. 4b).

Characterization of RAG and RA in blood and other tissues

The retention times of radiolabelled RAG and RA in the blood and other tissues were identical with those of standards. Furthermore, the absorption spectrum of the RAG peak fraction isolated from serum at 0.5 h was the same as that of standard RAG.

When serum or other tissue extracts were incubated with β -glucuronidase for 1–2 h and then analysed by h.p.l.c., the radioactivity associated with the RAG peak decreased by 50–90%, and the radioactivity associated

Fig. 3. H.p.l.c. profiles of $[11\text{-}^3\text{H}]$ retinoids from the sera of rats

Samples were analysed 0.5, 1, 3, 6, 12 and 24 h after an intraperitoneal dose of $[11\text{-}^3\text{H}]$ RAG. POL, polar metabolite; RAG, retinoyl β -glucuronide; RA, retinoic acid; MR, methyl retinoate.

with the RA peak increased proportionately, thereby indicating the formation of RA from RAG.

Treatment of a mixture of RAG and RA in diethyl ether with diazomethane gave their corresponding methyl esters. Methylated RAG eluted at 16.4 min, and methyl retinoate eluted at 21.9 min. Similar treatment of a liver extract in ether gave the same products (Fig. 5a).

Hydrolysis of standard RAG or of RAG present in serum or other tissues with aqueous methanolic KOH expectedly resulted in the formation of RA (Fig. 5b). When the reaction time was short and the conditions

were essentially anhydrous, however, methyl retinoate rather than RA was the major product (Fig. 5c).

DISCUSSION

A two-step procedure for the synthesis of all-*trans*-RAG from RAF was reported earlier from this laboratory (Barua & Olson, 1985b). We have now simplified the procedure by substituting glucuronic acid for 6,3-glucuronolactone. By reacting modest quantities of RAF (10 mg–1 g) with a large excess of glucuronic acid in the

Table 2. The ratio of RAG/RA present in blood and various tissues at different times after injection of $[11\text{-}^3\text{H}]$ RA

Rats were killed at various times following an intraperitoneal dose of $[^3\text{H}]$ RAG. Retinoids were extracted from serum and other tissues and analysed by h.p.l.c. The concentrations (nmol/ml of serum or nmol/g of tissue) of RAG and RA were determined from the radioactivity associated with each.

Tissue	Time after injection (h)...	RAG/RA					
		0.5	1	3	6	12	24
Serum		7.7	8.5	6.1	6.7	7.6	8.6
Liver		1.4	0.8	1.1	2.8	2.0	2.8
Small intestine		11.0	8.4	4.9	11.5	14.0	8.0
Kidney		25.0	40.0	24.1	26.0	30.0	22.5

Fig. 4. H.p.l.c. profiles of $[11\text{-}^3\text{H}]$ retinoids from faeces (a) and urine (b)

Faeces and urine were collected for 24 h after an intraperitoneal dose of $[11\text{-}^3\text{H}]$ RAG. POL, polar metabolite.

presence of sodium bicarbonate in aqueous acetone, 50–60% yields of RAG were obtained in a single step.

When smaller quantities of RAF (100–1000 μg) are used, as in the synthesis of radiolabelled RAG described in this paper, the yield is somewhat lower (20–40%). Nonetheless, the current procedure, because of its simplicity and rapidity, is clearly an improvement over that described earlier (Barua & Olson, 1985b).

The question that we set out to answer was whether RAG stimulated rat growth and cellular differentiation *in vivo* as an intact compound or only after its hydrolysis to RA. Our observations do not provide a clear response to that question. During a 24 h period, RAG remained as the predominant compound of blood, small intestine and kidney (Table 2). RAG also predominated in the liver, except at 1 h when RA predominated over RAG (Table 2). On the other hand, RAG was clearly hydrolysed in part to RA as well as being converted to both more polar and less polar metabolites. The more polar metabolites, which were found in significant amounts in all tissues studied, have not yet been characterized. The less polar metabolites show retention time like methyl or ethyl retinoate and, upon hydrolysis in base, are converted to RA. Methyl retinoate and ethyl retinoate have earlier been identified as endogenous metabolites of RA (Dunagin *et al.*, 1966; Miller & DeLuca, 1985).

From Table 2, it can be seen that the ratio of RAG/RA remained more or less constant in the blood and in the kidney (except at 1 h), whereas the ratio fluctuated in the

Fig. 5. H.p.l.c. profiles after methylation and hydrolysis of $[11\text{-}^3\text{H}]$ retinoids from the liver of a rat

Extracts of liver from a rat killed 1 h after a dose of $[11\text{-}^3\text{H}]$ RAG were methylated with diazomethane to form the methyl ester of RAG (MRAG) and methyl retinoate (MR) (a); and hydrolysed with KOH under aqueous conditions to form RA (b); and under anhydrous conditions to form a mixture of RA and MR (c).

liver and in the small intestine. Some general facets of the dynamics of RAG metabolism can possibly be derived from these observations. Intraperitoneally administered RAG rapidly appears in the blood, from which it is removed primarily by the liver and by the small intestine. On the basis of the relative amounts of metabolites present, the hydrolysis of RAG to RA seems to occur most readily in the liver, less rapidly in the intestine, and slowest in the kidney. Because both liver and intestine can form RAG from RA, some of the RAG found in these tissues may well be produced by a cycle of hydrolysis and resynthesis. It can be seen from Table 1 that some oscillations in the total radioactivity in the liver was observed. Because only one rat was used at each time

point, the observed difference in the total radioactivity in the liver may be due to individual variation between rats. Because such a variation was not observed in the blood and other tissues of the same rat, the oscillations in total radioactivity may not be real. Further study of this phenomenon is warranted.

Metabolites of vitamin A, but very little retinol itself, are excreted in both the faeces and urine. Interestingly, very little RAG was excreted by either route; rather, RA was the major excretory form in the faeces and, accompanied by a predominant polar fraction, was an important metabolite in urine. Although RAG might well be hydrolysed to RA by intestinal bacteria, the earlier suggestion that glucuronides of vitamin A are formed primarily to facilitate their excretion is weakened by these observations. The chemical nature and possible biological activity of the polar metabolites of RAG present in tissues and urine are now being investigated.

RAG and ROG are metabolized differently (Barua *et al.*, 1988). Although intraperitoneally injected ROG and RAG both appear quickly in blood, ROG is concentrated more rapidly in the intestine and RAG in the liver. ROG is hydrolysed *in vivo* to retinol, which appears predominantly in the liver, but also in the intestine as retinyl ester. Furthermore, roughly half of the vitamin A ester formed in the liver from ROG was 5,6-epoxyretinyl esters (Barua *et al.*, 1988). Thus, the glucuronide form of retinol seemingly enhanced epoxidation. In contrast, only small amounts of non-polar esters of RA, such as methyl retinoate, are formed from released RA. Finally, whereas RAG predominated in blood during the whole study period, ROG was more rapidly cleared.

The observations that retinoid β -glucuronides are endogenous, water-soluble metabolites of retinol and RA, show significant biological activity both *in vivo* and *in vitro*, and, at least in the case of RAG, are not major excretion products, raises questions concerning their possible physiological roles in the function of vitamin A. Because RAG is present naturally in various tissues and blood, administered RAG persists as a major metabolite in the blood and tissues for at least 24 h, and administered RAG is hydrolysed at a relatively slow rate to RA, RAG may well play an important physiological role in the overall functions of vitamin A rather than being merely an excretion product. Being water-soluble, RAG may facilitate transport of RA to target tissues. It is also possible that RAG serves as an active intermediate in the retinoylation of nuclear proteins (Takahashi & Breitman,

1989). Future work will be aimed at establishing the possible role of RAG in the functions of vitamin A.

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