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Intestinal absorption and metabolism of retinoyl β-glucuronide in humans, and of 15-[^{14}C]-retinoyl β-glucuronide in rats of different vitamin A status

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Abstract

In order to prove the hypothesis that humans and animals with adequate vitamin A status do not absorb and metabolize orally administered all-trans retinoyl β-glucuronide, unlabeled retinoyl glucuronide (0.1 mmol) was orally dosed to fasting well-nourished young men. Neither retinoyl glucuronide nor retinoic acid, a possible metabolite, appeared in the blood within 12 h after ingestion. Next, radiolabeled all-trans 15-[^{14}C]-retinoyl β-glucuronide was chemically synthesized by a new procedure, and fed orally to rats of different vitamin A status. Analysis of blood and other tissues 5 or 24 h after the dose, showed the presence of radioactivity (~ 0.5%) in the blood of vitamin A deficient rats, but not in sufficient rats. Livers of all rats contained small, but detectable amounts (0.3 to 1.1% of the dose) of radioactivity. The accumulation of radioactivity in the liver was highest in deficient rats. Analysis of the retinoids showed that the radioactivity in serum and liver was due to retinoic acid formed from retinoyl glucuronide. Within 24 h after the dose, 31 to 40% of the administered radioactivity was excreted in the feces, and 2 to 4.7% of the dose was excreted in the urine. Results of the present studies show that oral administration of retinoyl β-glucuronide did not give rise to detectable changes in blood retinoyl glucuronide and/or retinoic acid concentrations in humans or rats with adequate vitamin A status.

Keywords: Retinoic acid; Retinoyl β-glucuronide; Absorption; Rats; Humans

1. Introduction

Vitamin A is involved in several biological processes including vision, growth, reproduction, embryological development and cell differentiation [1]. The acid forms of vitamin A, all-trans retinoic acid (RA) and its 13-cis isomer are topical and systemic prescription drugs, respectively, for acne [2]. Although very effective, both forms show side effects. RA is very efficient for the treatment of acute promyelocytic leukemia (APL) [3, 4]. But there is limitation in the use of RA in APL therapy because RA, at pharmacological dose level, is toxic, and induces its own metabolism during therapy by increased oxidation via the cytochrome P450 enzyme system [5]. This results in reduction of plasma concentration of RA within a few days of therapy. There is evidence that relapse of APL after RA therapy might be due to lack of effective drug concentration to the leukemic cells. Use of inhibitors of cytochrome P450 has been tried with limited success [6].

All-*trans* retinoyl β-glucuronide (RAG), an endogenous metabolite of vitamin A is biologically as active, but much less toxic than RA (for a review [7]). RAG is effective in the topical treatment of mild to moderate acne with no side effects [8, 9]. Surprisingly, very large oral doses of RAG (0.38 to 1.41 mmol/kg body wt.) were not teratogenic in pregnant Sprague-Dawley (SD) rats [10]. On the other hand, parenterally administered RAG was reported to be highly

Abbreviations used: RAG, retinoyl β-glucuronide; LRAG, 15-[^{14}C]-retinoyl β-glucuronide; RA, retinoic acid; −A, vitamin A-deficient; +A, vitamin A-sufficient.

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teratogenic in National Medical Research Institute (U.K.) (NMRI) strain of mice due to its extensive metabolism to RA [11]. In the Harland strain of mice, subcutaneously administered RAG was only slowly metabolized to RA and there were no adverse signs of toxicity after chronic daily dosing with RAG for about three months [12]. Another study found that intravenously administered RAG was not extensively hydrolyzed to RA in SD rats [13]. Two recent studies, one carried out by administering RAG by IP injection in pregnant Sprague-Dawley rats, and the other carried out by topical application on swine skin showed that RAG was much less toxic than RA [14, 15]. RAG alone, or in combination with 4-chloro-phenyl acetate were shown to exhibit potent antitumor activity in nude mice injected with LA-N-5 cells obtained from the human neuroblastoma cell line [16].

The above studies show that RAG might be a suitable non-toxic alternative to RA. The pharmacology and metabolism of parenterally administered RAG is somewhat known [11–13, 17]. However, if RAG is to be administered orally, there is very little information about its absorption and metabolism in vitamin A sufficient humans and animals. It was reported earlier that orally dosed RAG was converted to RA by vitamin A deficient rats and the resulting RA could be detected in the serum [18–20]. However, the serum of vitamin A sufficient rats dosed similarly with RAG, showed neither RAG nor RA in the serum [19]. Humans may handle RAG differently. Therefore, one of the aims of the present investigation was to determine the fate of an oral dose of RAG in well-nourished adults. Another aim of the study was to synthesize radiolabeled RAG (LRAG) which could be used in rats, and examine, besides blood, liver and other tissues to get a precise information about the fate of an oral dose of RAG.

2. Materials and methods

All work with retinoids and serum were performed in laboratories illuminated with yellow fluorescent light (F40 Gold).

2.1. Chemicals and solvents

Methanol, dichloromethane, 2-propanol, ethyl acetate, glacial acetic acid, diethyl ether, sodium hydride, and ammonium acetate were purchased from Fisher Scientific Co., Fair Lawn, NJ. 1,1′-Carbonyldiimidazole, pyridine and tetrabutylammonium hydroxide were purchased from Fluka (Milwaukee, WI, U.S.A.). All-trans retinyl acetate was purchased from Sigma, St. Louis, MO. RA was obtained from BASF Corporation (Parispanny, NJ, U.S.A.).

2.2. Chemical syntheis of retinoyl β-glucuronide

Unlabeled RAG was synthesized according to a procedure published from this laboratory [18]. For the synthesis of labeled 15-[^{14}C]-RAG (LRAG), the procedure described for unlabeled RAG was followed. First, tetrabutyl ammonium salt of glucuronic acid was prepared by reaction of glucuronic acid with tetrabutyl ammonium hydroxide in methanol. An ampoule containing 250 μl of 15- [^{14}C]-retinoic acid (13.7 mCi/mmol)(Amersham, Chicago, IL, U.S.A.) in benzene was transferred to a test tube, and the solvent was removed under a gentle stream of argon. Unlabeled RA (6.6 μmol = 2 mg) was added to the residue, and the mixture was dissolved in dry pyridine (0.5 mL). Next, 1,1′carbonyldiimidazole (7.4 μmol = 1.2 mg) was added, and the mixture was heated to 60°C and then stirred at room temp. for ~ 4 h until the absorption maximum of the solution shifted to 390 nm from 350 nm. A test drop was examined by TLC on small strips of precoated silica gel (0.25 mm)(Macherey-Nagel, distributed by Brinkmann Instruments, Westbury, NY, U.S.A.) for completion of the reaction. For TLC, a solvent system of hexane:acetone (4:1, v/v) was used. When the reaction was complete, dry tetrabutyl ammonium glucuronate (22.9 μmol = 10 mg) was added, followed immediately by a pinch (40 to 80 μmol = 1 to 2 mg) of sodium hydride. The mixture was stirred at room temp. for ~ 6 h until a test drop showed that there was no other yellow spot other than a yellow spot at the origin. The reaction mixture was cooled in ice, and a mixture of water (0.5 mL) and dilute acetic acid (10%)(0.2 mL) was added. The retinoids were extracted with ethyl acetate 4 times. The pooled extract was washed with water, and then dried over anhydrous sodium sulfate. The solvent was evaporated to dryness, and the residue was dissolved in a few drops of dichloromethane. The solution was subjected to TLC on precoated silica gel plates (20 × 20 cm) with a solvent mixture of hexane/acetone/methanol/dichloromethane (2:1.3:1.2:2, v/v). The main broad yellow band (R_f = 0.32) of LRAG which separated well from traces of RA (R_f = 0.65) was scraped off with a spatula, and LRAG was extruded from the powder with a mixture of methanol: dichloromethane (1:1, v/v). The solvent was evaporated under argon, and the residue was dissolved in a small volume of dichloromethane. The purity of synthesized LRAG was determined by HPLC prior to preparation of the oral dose as described below.

2.3. Human subjects and the oral dose of RAG

Six well nourished young men, all students at Iowa State University, were selected for the study. The use of human subjects in this study was approved by the Human Subjects Review Committee, Iowa State University. The volunteers fasted overnight for 12 h.

Pure RAG (1.05 mmol = 500 mg) was ground to a powder in a mortar with a pestle. Corn oil (5 mL) was added, and the mixture was ground until all the powder dissolved. The oil was transferred to a glass container by means of a pipette, and the mortar was rinsed with corn oil (1 mL x 3) and the rinsings were added to the RAG solution.

Table 1
Recovery (%) of radioactivity from serum, liver, urine and feces of rats of different vitamin A status 5 or 24 after a single oral dose of radiolabeled retinoyl β-glucuronide (LRAG)[1]

	Serum ROL μ(mol/L)[2]	% Dose			
		Serum	Liver	Urine	Feces
Experiment 1: wk 5					
5 h:					
−A[3]	0.63 ± 0.16	0.38 ± 0.08	1.10 ± 0.05	—	—
+A	0.83 ± 0.21	Trace	0.99 ± 0.41	—	—
++A	1.84 ± 0.18	ND	0.50 ± 0.04	—	—
+++A	2.18 ± 0.29	ND	0.30 ± 0.02	—	—
24 h:					
−A	0.63 ± 0.16	ND	0.32	4.72 ± 0.91	39.86 ± 12
+++A	2.18	ND	0.65 ± 0.21	2.76 ± 0.6	31.82 ± 9.1
Experiment 2:					
5 h:					
−A (wk 4)		0.04 ± 0.02	0.62 ± 0.22	—	—
(wk 6)		0.75 ± 0.21	0.49 ± 0.03	—	—
+ cold RAG[4]		Trace	0.31 ± 0.04	—	—
+A		ND	0.55 ± 0.20	—	—
24 h:					
+A		ND	0.10 ± 0.03	1.42 ± 0.41	40.16 ± 4.1

[1] A single dose of LRAG contained 0.09 μmol (680,550 cpm) in Expt. 1 and 0.41 μmol (1,137,963 cpm) in Expt. 2
[2] Values are means ± SD, n = 2/gp (Expt. 1) and n = 3/gp (Expt. 2)
[3] The vitamin A status of rats is indicated by +++A (marginal), +A, ++A, +A (adequate)
[4] The radioactive dose of LRAG was mixed with unlabeled RAG (1 mg/rat)

An aliquot of the solution was analyzed spectrophotometrically [21] and proper dilutions were made to obtain a concentration of 0.1 mmol (47.6 mg) RAG/mL corn oil.

For oral dosing, 1 mL of the corn oil solution of RAG was spread on a slice of bread that was consumed by the human volunteers immediately. This was followed by breakfast that contained no vitamin A or carotenoids. Blood was collected by a phlebotomist of the University Student's Health Center, just before ingestion of RAG (0 h), and 3, 6, 9 and 12 h after ingestion.

2.4. Rats and the oral dose of LRAG

Weanling male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN) through Laboratory Animal Resources, College of Veterinary Medicine, Iowa State University. The rats were housed individually and maintained in accordance with Iowa State University and NIH guidelines under the supervision of Laboratory Animal Resources, College of Veterinary Medicine, Iowa State University. The rats were divided into 4 groups. All rats were fed for 4 to 6 weeks a vitamin A deficient diet (Diet no. 904646, ICN, Cleveland, OH, U.S.A.), and a supplement of corn oil, or corn oil containing retinyl acetate as follows: One group (-A) received 100 μl of corn oil twice weekly; the second (+A), third (++A) and fourth (+++A) group received 100 μl of corn oil containing retinyl acetate (0.75, 2.24, or 6.72 mmol/L, respectively) dissolved in corn oil twice weekly so as to receive 0.02, 0.064 and 0.19 μmol (7, 21 and 63 μg) retinyl acetate/rat/day.

The stock solution of LRAG was prepared as follows: a measured volume of LRAG in dichloromethane was transferred to a mortar, and most of the dichloromethane was allowed to evaporate. Corn oil (2 mL) was added and the mixture was mixed well with a pestle. Trace of dichloromethane that could be present in the oily solution was removed by subjecting to evaporation in a rotary evaporator at 40° under reduced pressure. Proper dilution of the corn oil solution of LRAG was made so as to contain 16126 cpm/μg (Expt. 1) and 3986 cpm/μg (Expt. 2).

In the first experiment (Table 1), each rat after 5 weeks on the diet, was dosed orally with 100 μl of the corn oil solution containing 0.09 μmol (42.2 μg) LRAG (680,550 cpm/rat). In the second experiment, each rat after 4 to 6 weeks on the diet, was dosed orally with 100 μl of the corn oil solution containing 0.41 μmol (194 μg) LRAG (773,300 cpm/rat) (Table 1). A group of another 3 rats were dosed with a mixture of LRAG (0.41 μmol) and unlabeled RAG (2.1 μmol) after 6 weeks on the diet.

Rats used for the 24 h-period of study were transferred to metabolic cages for collection of urine and feces.

The rats were anesthetized by exposure to halothane 5 or 24 h after the dose, and blood was collected from the heart by means of a syringe. Serum was prepared and stored at −78°C until use. Liver and other tissues were collected and stored at –78°C.

2.5. Measurement of radioactivity

An aliquot of the diluted stock solution of LRAG as described above was mixed with Scintiverse BD (Fisher,

Fair Lawn, NJ, U.S.A.), and the radioactivity was counted in a scintillation counter (Packard model 1600 TR).

For determination of the nature of retinoids in serum, tissue extracts, urine and feces, extracts were subjected to HPLC as described below. HPLC fractions were collected every minute in scintillation vials (20 mL) placed in a fraction collector (ISCO model Retriever IV, Lincoln, NE, U.S.A). The fractions were mixed with Scintiverse BD (20 mL) and the radioactivity was counted.

2.6. HPLC

Reversed phase gradient HPLC was carried out on a Waters Associate (Milford, MA) system routinely used in this laboratory [21–23]. A Rainin 3 μ C_{18} Microsorb MV (10 cm long) was used with solvent systems of methanol: water (7:3, v/v, containing 10 mM ammonium acetate) and methanol:dichloromethane (4:1, v/v). The flow rate was 0.6 mL/min.

2.7. Retinoids analysis in serum

Analysis of retinoids in human and rat serum was performed according to published procedure [21–23]. Serum (500 to 1000 μl) was used. The dried extract was dissolved in 2-propanol/dichloromethane (2:1, v/v; 100 μl). An aliquot of 70 μl was injected on to the HPLC system.

2.8. Retinoids analysis in liver and other tissues, feces and urine

The procedure for the extraction of retinoids in liver, other tissues and feces was essentially the same as published before [24]. The dried extract was dissolved in 2-propanol/dichloromethane (2:1, v/v; 100 to 500 μl). An aliquot of 70 to 100 μl was injected on to the HPLC system. Urine was diluted with an equal volume of ethanol, centrifuged for 1 min, and the supernatant was used for analysis.

3. Results

3.1. RAG was not detected in the serum of well nourished humans

Analysis of serum of human volunteers who ingested 0.1 mmol (47.6 mg) RAG showed that the retinoid profiles obtained before the oral dose (0 h) and 3, 6, 9 and 12 h after the dose of RAG remained unchanged. Neither RAG nor any RA formed from it, could be detected in any serum sample. The retinol level in the serum of all the six volunteers was normal ($>$ 1.1 μmol/L).

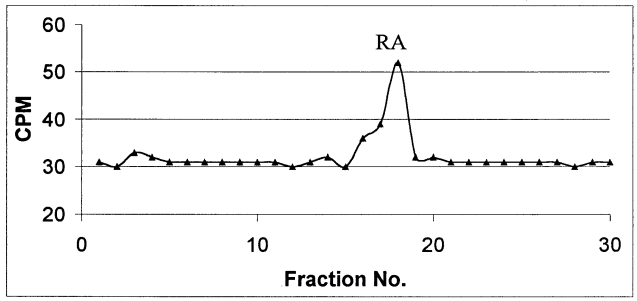

Fig. 1. Radioactive profile of serum extract. HPLC chromatogram of serum extract of a vitamin A-deficient rat showing the radioactive peak of retinoic acid (RA) formed from orally administered retinoyl β-glucuronide (LRAG).

3.2. Retinoids in serum and tissues of rats after oral dose of labeled RAG (LRAG)

3.2.A. Serum retinol

The retinol levels in the serum of rats raised on the vitamin A deficient diet and receiving supplements of corn oil only, or corn oil containing various amounts of retinyl acetate are shown in Table 1. It was found that the rats receiving supplements of corn oil only had a retinol level of 0.63 $\pm$ 0.16 μmol/L. Therefore, these rats were marginally vitamin A deficient (-A) as their retinol level was below 0.7 μmol/L (20 μg/dL). The rats receiving supplements of 0.02, 0.06, and 0.19 μmol retinyl acetate/rat/day showed retinol levels of 0.83 $\pm$ 0.21 (+A rats), 1.84 $\pm$ 0.18 (++A rats) and 2.18 $\pm$ 0.29 (+++A rats) μmol/L, respectively.

3.2.B. Serum RAG and RA

Experiment 1.

The recovery of radioactivity following a single dose of LRAG (0.09 μmol = 680,550 cpm/rat) from serum, liver, urine and feces are presented in Table 1. Five h after a single oral dose of LRAG, the sera of -A rats, showed the presence of 0.38% of the administered dose. The sera of +A rats contained trace of radioactivity, but no radioactivity was detected in the sera of ++A and +++A rats 5 h or 24 h after the dose. HPLC analysis of serum of –A rats revealed that the radioactivity was due to labeled RA formed from LRAG (Fig. 1). The retention times of the labeled RA and the standard RA during HPLC were the same, and both peaks showed identical absorption spectrum with λmax 340 nm as revealed by the photodiode array detector. Thus, the identity of RA in vitamin A deficient rat serum was confirmed.

Experiment 2.

In Experiment 2, the dose size of LRAG was much larger (0.41 μmol = 1,137,963 cpm/rat) than in Expt. 1, and the rats were dosed after 4 or 6 weeks on the vitamin A deficient diet. The recovery of radioactivity as shown in Table 1 was lower in the serum of rats raised for 4 weeks on the vitamin A deficient diet than in serum of rats raised on the same diet for 6 weeks. When the radioactive dose of LRAG was

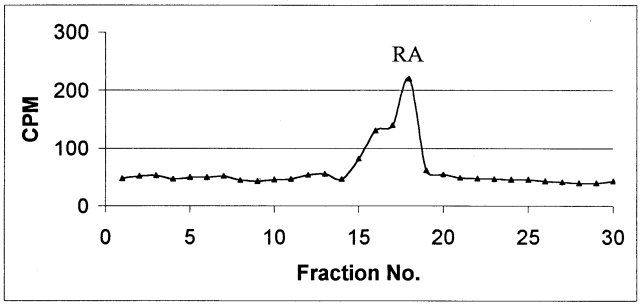

Fig. 2. Radioactive profile of liver extract. HPLC chromatogram of liver extract of a vitamin A-deficient rat showing the radioactive peak of retinoic acid (RA) formed from orally administered retinoyl β-glucuronide (LRAG).

mixed with 1 mg of unlabeled RAG, and administered to –A rats, the absorption of the dose was much higher as judged from the area of RA peak obtained during HPLC of the serum extract. However, due to dilution of LRAG, the recovery of radioactivity was less. The data showed a dose dependent absorption of RAG.

3.2.C. Liver retinoids

Experiment 1.

Unlike serum, the livers of all rats irrespective of the vitamin A status (-A, +A, ++A and +++A) showed the presence of radioactivity 5 h or 24 h after the dose (Table 1). The counts were highest (1.1%) in the livers of –A rat livers. The recovery of radioactivity in the liver decreased as the rats improved their vitamin A status. Analysis by HPLC showed that the radioactivity in liver was due to RA (Fig. 2).

Experiment 2.

As in Expt. 1, increased radioactivity was found in the livers of vitamin A deficient rats than in vitamin A sufficient rats (Table 1). Like serum, the recovery of radioactivity in the liver of rats dosed with a mixture of labeled and unlabeled RAG was less.

3.2.D. Radioactivity in urine and feces

The recovery of radioactivity in urine and feces collected for 24 h after a small (Expt. 1) or large dose (Expt. 2) is shown in Table 1. Less than 5% (1.4 to 4.7%) of the administered dose was excreted in the urine. In the feces, 31.8 to 40.1% of the radioactive dose was excreted within 24 h.

3.2.E. Radioactivity in other tissues

Entire kidney, pancreas, and lung from each rat dosed with LRAG were also extracted and analyzed for the presence of radioactivity. However, the amount of radioactivity was negligible as compared to the background.

4. Discussion

Following ingestion of RAG by well nourished young men with adequate vitamin A status, no significant change in the levels of RAG or RA was noticeable in serum collected 3 to 12 h after the dose. Blood was not collected at earlier points because previous studies on rats showed that the concentration of RA following an oral dose of RAG peaked at 4 h after the dose. If RAG was extensively hydrolyzed to RA during its passage through the GI tract as was observed in NMRI mice [11], it should have been possible to detect significant rise of RA concentration in the blood within the 12 h-study period. But RA was not detected in any of the six volunteers. It was reported earlier from this laboratory that whereas a large oral dose of RAG was not absorbed by rats that had adequate vitamin A status, any small or large oral dose of RAG was hydrolyzed to RA by vitamin A deficient rats, and appeared in the blood [18–20]. Therefore, the result obtained in the present study on humans was not surprising. It would be interesting to test if children with vitamin A deficiency would show the presence of elevated levels of RA in their blood after an oral dose of RAG.

Urine samples of the volunteers who ingested RAG were also collected for 24 h, and analyzed by HPLC, but no detectable amounts of RAG or RA were present (results not presented here).

Because liver and other tissues were not analyzed in the human study, it could not be determined if there were other pathways for the transport and metabolism of orally dosed RAG. If minor metabolites were formed that escaped detection in previous studies involving rats was not known. Therefore, it was decided to use radiolabeled RAG in the next experiment on rats of different vitamin A status. Rats were used because it was possible to locate any radioactivity in other organs. For this, 15-[^{14}C]-RAG was chemically synthesized by a procedure described for the synthesis of unlabeled RAG [18], but different from the first published procedure [25]. Very satisfactory yield of LRAG was obtained by this modified procedure. By use of the radiolabeled LRAG, it was possible to confirm previous finding that orally dosed RAG or RA formed from it, was not detectable in the blood when the vitamin A status was adequate (> 0.7 μmol/L). On the other hand, orally dosed LRAG was absorbed, possibly after it was hydrolyzed to RA in the gut, and could be detected in blood of vitamin A deficient rats. In a previous study it was found that the rate of absorption was dose dependent [20]. In the present study, the amount of radioactivity detected in serum of vitamin A deficient rats was very low due possibly to the low dose size and marginal vitamin A deficiency of the rats in both experiments as compared to previous experiments. In the second experiment in this study shown in Table 1, one test was carried out on 3 rats that were dosed the radioactive LRAG mixed with unlabeled RAG (1 mg/rat). It was found that the amount of total labeled plus unlabeled RA in circulation in blood was much higher, but as a result of dilution of the dose with unlabeled RAG, the amount of radioactivity was much lower than that was observed in similar rats a week before (Table 1). This experiment also supported the

previous observation that the absorption and conversion of RAG to RA was dose dependent.

It was surprising that small but measurable quantities of radioactivity could be found in the livers of all the rats irrespective of their vitamin A status. The amount of radioactivity in the liver was dependent on the vitamin A status. As mentioned before, if RAG was extensively hydrolyzed to RA in the gut, significant amount of RA should have been absorbed and detected in the blood. On the other hand, the urine of both vitamin A deficient and sufficient rats contained some radioactivity (1.4 to 4.7% of the dose). Urinary excretion of radioactivity is indicative of absorption and circulation in blood. Therefore, the possibility of absorption of a small amount of LRAG and its circulation in the blood that escaped detection cannot be ruled out. The enterohepatic circulation of RAG is also known [26].

In one of the studies carried out on vitamin A sufficient pregnant rats, it was found that large doses of RAG (1.4 mmol/ kg body wt) did not cause teratogenicity [10]. Indeed, the fetuses of RAG dosed rats were alive and normal without any sign of birth defects. Surprisingly, the fetuses born to RAG-dosed dams weighed significantly higher than the fetuses of control rats (2.56 g vs. 2.16 g). Lack of teratogenicity in these rats was probably due to lack of extensive intestinal absorption of RAG and subsequent hydrolysis to retinoic acid. On the other hand, the present study showed that small amounts of the oral dose of LRAG could be detected in the livers of both deficient and sufficient rats. Therefore, it is possible that the observed increased weight of the embryos in the tearotogenicity study was due to small amounts of RAG, or RA formed from it, that was available to the developing embryos [10].

The results of the present and previous studies indicate that absorption of orally administered RAG by humans or other animals with adequate levels of vitamin A is not as efficient as other retinoids (retinyl esters or retinoic acid). During vitamin A deficiency, there is a demand for vitamin A from any source. As a result any administered RAG is hydrolyzed to RA, absorbed and goes into circulation. Therefore, the rate of absorption seems to depend on the vitamin A status as shown in rats [19]. Data obtained during past and present studies also indicate that the absorption is very likely dose dependent [20]. Further study on the absorption of RAG in vitamin A deficient humans is warranted because RAG hydrolysis may prove to be a reliable test for the determination of vitamin A status.

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