Tissue Distribution of Retinol and its Metabolites after Administration of Double-Labelled Retinol

By P. R. SUNDARESAN and G. M. SUNDARESAN

Lipids Laboratory, Research Institute, St. Joseph Hospital, Lancaster, Pa. 17604, U.S.A.

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The tissue concentrations and distribution of radioactivity present in retinol and its metabolites were investigated in vitamin A-deficient rats 24 h after injection of physiological doses (10 µg) of [6,7-¹⁴C₂, 11,12-³H₂]retinol. The highest concentration of radioactivity was observed in the adrenals, followed by kidney, spleen, liver, intestine and blood. The total radioactivity was greatest in urine, followed in descending order by liver, kidney, blood and intestine. The ¹⁴C/³H ratios of crude light-petroleum extracts in the liver, intestines, lungs, heart and faeces were similar to the ratio of the injected retinol dispersion. However, the ¹⁴C/³H ratios in the adrenals, kidney, spleen, blood, brain and urine were quite different from that of injected retinol. Alumina chromatography of the kidney and intestinal extracts demonstrated that retinol and retinyl palmitate are the principal forms of vitamin A present. However, alumina chromatography of the liver extract did not reveal the presence of retinol but yielded a major compound with a low ¹⁴C/³H ratio. That this compound was not retinol was shown by its inability to react with ethanolic HCl to yield anhydoretinol. The distribution of radioactivity in ether-soluble, acidic and water-soluble fractions of urine indicated that most of the radioactivity was present in the acidic and water-soluble fractions. The ¹⁴C/³H ratios in ether-soluble and acidic fractions were higher than that of injected retinol, whereas in the water-soluble fraction the ratio was similar to the injected material.

Wolf *et al.* (1957) reported the synthesis and distribution of [14-¹⁴C]retinol in the rat after administration of the compound as a Tween emulsion. It was found that high percentages of the injected dose of retinol resided in the liver and carcass non-saponifiable fractions (9.3 and 9.9% respectively) 24 h after the injection. Further, approx. 5% of the injected dose was recovered in the expired CO₂ and 4–12% in water-soluble compounds of urine. A detailed study by Goodman *et al.* (1965) described the tissue distribution and metabolism of retinol when injected in the form of chylomicrons containing newly absorbed retinol. In a brief report, Coward & Thompson (1967) indicated that the kidney is the tissue with the highest radioactivity, after administration of small doses of [15-¹⁴C]retinyl acetate to vitamin A-deficient rats maintained on retinoic acid-supplemented diets. Moore (1957) had previously reported on the distribution and storage of retinol in tissues such as kidney, lung, adrenals, retina and plasma when administered in small doses. Smith *et al.* (1973) investigated the distribution of retinoic acid in different tissues of the rat. However, our knowledge of the metabolism of retinol in tissues when injected in small doses is fragmentary. The present paper describes our work on the tissue concentrations and distribution of retinol and its metabolites, when physiological doses of double-labelled

retinol [6,7-¹⁴C₂, 11,12-³H₂]retinol are injected into vitamin A-deficient rats. Preliminary reports of this investigation have been published (Sundaresan & Sundaresan, 1972a,b).

Materials and Methods

[6,7-¹⁴C₂]Retinol and [11,12-³H₂]retinol were obtained through the courtesy of Dr. O. Wiss of Hoffmann-La Roche and Co., Basle, Switzerland. Neutral alumina (Brockmann activity 1; 80–200 mesh), suitable for chromatography, was purchased from Fisher Scientific Co., Philadelphia, Pa., U.S.A. The solvents used were of reagent grade and distilled before use. All radioactive compounds were purified by column chromatography on 10% (v/w) water-deactivated alumina columns. The concentration of retinol was determined spectrophotometrically with a Zeiss PMQ II spectrophotometer by using the $E_{1\%}^{1\text{cm}}$ value of 1800 at 325 nm in ethanol (Cama *et al.*, 1951). The radioactivity of the double-labelled retinol solution was determined with a Nuclear-Chicago model 723 liquid-scintillation counter.

Vitamin A deficiency in animals and administration of retinol

Vitamin A deficiency was produced in specially prepared weanling male rats of the Sprague-Dawley

strain as described by Sundaresan & Bhagavan (1971) and Sundaresan & Sundaresan (1973). When the rats reached a plateau in growth rate, they were supplemented with a solution of retinoic acid ($100\text{ }\mu\text{g}/0.1\text{ ml}$) in peanut oil twice weekly for 3 weeks. The animals began to lose weight when this supplementation was withdrawn for 1 week. At this time, they were injected with $10\text{ }\mu\text{g}$ of double-labelled retinol as a 15% Tween emulsion in physiological saline (0.9% NaCl) containing $117\text{ }\mu\text{g}$ of α -tocopherol/ml; the specific radioactivities of [^{14}C]retinol and [^3H]retinol were $20.03\text{ }\mu\text{Ci}/\text{mg}$ and $165.11\text{ }\mu\text{Ci}/\text{mg}$ respectively and the $^{14}\text{C}/^3\text{H}$ ratio of the retinol dispersion was 0.121. The rats were then placed in open metabolism cages and urine and faeces were collected for 24 h and stored at -20°C until analysis. Urine was collected in bottles containing 5 ml each of toluene.

Extraction of retinol metabolites from urine, blood and other tissues

At 24 h after injection of retinol in Tween dispersion the rats were anaesthetized with chloroform and blood was withdrawn into oxalated syringes. Tissues were quickly removed, weighed and extracted with light petroleum ($30\text{--}60^\circ\text{C}$) after being ground with anhydrous Na_2SO_4 . The procedure for extraction of metabolites from urine was as reported by Sundaresan & Bhagavan (1971). For blood, equal volumes of ethanol were added to the blood samples and thoroughly mixed in a VirTis homogenizer before extraction with 3 vol. of diethyl ether. The radioactivity in 1.0 ml portions of urine and tissue extracts was determined in 10 ml of counting solution consisting of toluene and Triton X-100 in a ratio of 2:1. The toluene solution contained 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene per litre of toluene. The counting efficiency was 50% for ^{14}C and 10% for ^3H in an optimized double-labelled counting system. Internal standards for ^3H and ^{14}C were added in sequence to each sample and the samples recounted after each addition to determine the respective isotope efficiencies.

Column chromatography of tissue extracts

The liver, kidney and intestinal extracts were chosen for column chromatography on a 10% (v/w) water-deactivated alumina column as described by Sundaresan & Theriault (1969). Three radioactive peaks were obtained by stepwise elution of the column with 2, 10 and 50% (v/v) acetone in light petroleum ($30\text{--}60^\circ\text{C}$) respectively. Under identical conditions, non-radioactive retinyl palmitate and retinol in small amounts ($5\text{--}20\text{ }\mu\text{g}$) on a 10% (v/w) water-deactivated alumina column were eluted with 2 and 10% (v/v) acetone in light petroleum ($30\text{--}60^\circ\text{C}$) respectively.

Treatment of the fraction eluted with 10% acetone with ethanolic HCl

The radioactive fraction obtained by elution with 10% (v/v) acetone in petroleum ether during chromatography of the liver extract, was evaporated to dryness under N_2 and taken up in 1 ml of 95% ethanol. It was subsequently added to 1 mg of non-radioactive retinol in 1 ml of 95% ethanol and treated with ethanolic HCl (0.195 M) for 15 min. After terminating the reaction by addition of Na_2CO_3 , the mixture was extracted with light petroleum ($30\text{--}60^\circ\text{C}$). The extract was washed with distilled water and reduced in volume *in vacuo*. It was subsequently subjected to column chromatography on 10% (v/w) water-deactivated alumina. The fractions obtained by stepwise elution with light petroleum ($30\text{--}60^\circ\text{C}$), 2, 10 and 50% acetone in light petroleum ($30\text{--}60^\circ\text{C}$) were analysed spectrophotometrically as well as for radioactivity. Anhydroretinol, obtained as a yellow fraction by elution with light petroleum, was identified by its typical maxima at 350, 370 and 390 nm in light petroleum.

Chromatography of the light-petroleum extract of injected retinol solution

In a control experiment, a sample of Tween emulsion of retinol was extracted three times with light petroleum ($30\text{--}60^\circ\text{C}$) immediately after solubilization of retinol and the extract, after reduction in volume *in vacuo*, was chromatographed on 10% (v/w) deactivated alumina as described above.

Results

In Table 1, the distribution of radioactivity (d.p.m./g or ml), amount of retinol (ng of retinol equivalents/tissue) as well as the $^{14}\text{C}/^3\text{H}$ ratios in different tissues of the rat are given. The highest concentration of radioactivity, corresponding to 326 ng of retinol equivalents/g wet wt. of tissue, was seen in the adrenals. This was followed in descending order by kidney, spleen, liver, intestine and blood. The total radioactivity, expressed as ng of retinol equivalents/tissue, was greatest in urine, followed by liver, kidney, blood and intestine. Though the concentration of retinol and metabolites was highest in the adrenals, the contents in this tissue amounted to only 10 ng of retinol equivalents. The $^{14}\text{C}/^3\text{H}$ ratios in intestine, lungs, heart and faeces were similar to the ratio of the injected material. However, the ratios in the adrenals, kidney, spleen, blood and urine were very different from that of injected retinol.

Table 2 gives the distribution of radioactivity in ether-soluble, acidic and water-soluble fractions of urine with the corresponding $^{14}\text{C}/^3\text{H}$ ratios. The higher $^{14}\text{C}/^3\text{H}$ ratio (1.4) observed for whole urine

(Table 1) was mainly due to the contributions of ether-soluble and acidic metabolites, which had high $^{14}\text{C}/^3\text{H}$ ratios. The $^{14}\text{C}/^3\text{H}$ ratios of the water-soluble metabolites were similar to the injected material.

The results of column chromatography on the liver extract are presented in Fig. 1. Three peaks of radioactivity were obtained by elution with 2, 10 and 50% (v/v) acetone in light petroleum respectively. Similar peaks were also obtained from the kidney and intestinal extracts after column chromatography.

In the experiments with liver, the major radioactive fraction was shown to have a $^{14}\text{C}/^3\text{H}$ ratio of

0.4, whereas the other two peaks had ratios similar to the injection ratio (Fig. 1). The major radioactive peak did not behave like retinol as indicated by its reaction with ethanolic HCl after addition of non-radioactive retinol as carrier to this fraction. No radioactivity was associated with anhydroretinol which was eluted with light petroleum in subsequent column chromatography on deactivated alumina. Anhydroretinol was readily identified in this experi-

Table 1. *Distribution of radioactivity in tissues of rats injected with [6,7- $^{14}\text{C}_2$]retinol and [11,12- $^3\text{H}_2$]retinol*

Each rat received 10 μg of double-labelled retinol intraperitoneally. The total amount of retinol and its metabolites was calculated by dividing the total d.p.m. of ^{14}C obtained for each tissue by the ^{14}C specific radioactivity of injected retinol (44.48 d.p.m./ng) and expressed as ng of retinol equivalents/tissue. The results are given as means $\pm$ S.E.M. for five animals. The $^{14}\text{C}/^3\text{H}$ ratio of injected retinol is taken as 1. The values for the blood are given as d.p.m./ml and the total volume of blood is computed as 7% of the body weight (twice the value for plasma volume as 3.5% of the body weight; Goodman *et al.*, 1965). The values for urine and faeces represent total d.p.m. in these samples.

Tissue	^{14}C (d.p.m./g or ml)	Amount of retinol and metabolites (ng of retinol equivalents/tissue)	$^{14}\text{C}/^3\text{H}$ ratio
Adrenals	14501 $\pm$ 623	10 $\pm$ 0.5	1.5 $\pm$ 0.1
Kidney	9641 $\pm$ 1550	353 $\pm$ 44	1.4 $\pm$ 0.2
Spleen	5856 $\pm$ 457	49 $\pm$ 4	0.43 $\pm$ 0.02
Liver	3079 $\pm$ 1022	501 $\pm$ 119	0.8 $\pm$ 0.2
Intestine	1829 $\pm$ 140	204 $\pm$ 15	1.0 $\pm$ 0.1
Blood	861 $\pm$ 83	292 $\pm$ 24	0.13 $\pm$ 0.01
Lungs	504 $\pm$ 26	14 $\pm$ 0.8	1.2 $\pm$ 0.1
Heart	281 $\pm$ 23	5 $\pm$ 0.6	1.2 $\pm$ 0.1
Brain	139 $\pm$ 16	6 $\pm$ 0.7	0.45 $\pm$ 0.05
Urine	27850 $\pm$ 1650	626 $\pm$ 36	1.4 $\pm$ 0.1
Faeces	1214 $\pm$ 257	27 $\pm$ 6	0.9 $\pm$ 0.1

Fig. 1. *Alumina chromatography of rat liver extract*

The fraction numbers, composition and volume of the eluting solvent in stepwise elution were as follows: 1, light petroleum (30–60°C) (25 ml); 2–4, 2% (v/v) acetone in light petroleum (30–60°C) (75 ml); 5–8, 10% (v/v) acetone in light petroleum (30–60°C) (100 ml); 9–13, 50% (v/v) acetone in light petroleum (30–60°C) (125 ml). The eluate was collected in 25 ml fractions. The values in parentheses with an asterisk refer to the $^{14}\text{C}/^3\text{H}$ ratios of the major radioactive fractions. The $^{14}\text{C}/^3\text{H}$ ratio of the injected retinol is taken as 1.

Table 2. *Distribution of metabolites from double-labelled retinol in the urine*

Each rat received 10 μg of double-labelled retinol intraperitoneally. The results are given as percentage of total radioactivity excreted and as means $\pm$ S.E.M. for five animals in each group. The $^{14}\text{C}/^3\text{H}$ injection ratio is taken as 1.

	% of total radioactivity excreted			
	Ether-soluble	Acidic	Water-soluble	Total
[6,7- $^{14}\text{C}_2$]Retinol	13.6 $\pm$ 2.7	48.6 $\pm$ 2.4	37.7 $\pm$ 1.9	100
[11,12- $^3\text{H}_2$]Retinol	12.7 $\pm$ 1.8	40.6 $\pm$ 2.1	46.7 $\pm$ 2.4	100
$^{14}\text{C}/^3\text{H}$ ratio	1.37 $\pm$ 0.1	1.58 $\pm$ 0.3	1.08 $\pm$ 0.1	1.42 $\pm$ 0.1

Fig. 2. Alumina chromatography of the extract of injected retinol dispersion

1, Light petroleum (30–60°C) (25 ml), 2–5, 2% (v/v) acetone in light petroleum (30–60°C) (100 ml); 6–9, 10% (v/v) acetone in light petroleum (30–60°C) (100 ml); 10–13, 50% (v/v) acetone in light petroleum (30–60°C) (100 ml). For explanation of the values in parentheses, refer to Fig. 1.

ment by its typical absorption maxima at 350, 370 and 390 nm in light petroleum.

Experiments with the kidney extract also indicated the presence of a peak eluted with 10% (v/v) acetone–light petroleum. However, this peak had a ${}^{14}\text{C}/{}^3\text{H}$ ratio of 0.9 whereas the two minor peaks eluted with 2 and 50% (v/v) acetone–light petroleum had ratios of 1.1 and 0.8 respectively. In this study, the main peak reacted with ethanolic HCl to yield anhydroretinol, which was characterized by its absorption maximum in light petroleum as well as by its radioactivity.

In experiments with the intestinal extract, three radioactive peaks were again observed. However, the major peak was eluted with 2% (v/v) acetone–light petroleum and had a ${}^{14}\text{C}/{}^3\text{H}$ ratio of 1.1. The 10 and 50% acetone–light petroleum fractions also had ${}^{14}\text{C}/{}^3\text{H}$ ratios of 0.9 and 1.7 respectively. In this investigation, the major radioactive fraction, after saponification with KOH, reacted with ethanolic HCl to yield anhydroretinol.

Chromatography of the light-petroleum extract of the injected retinol solution under identical experimental conditions yielded four peaks (Fig. 2). This indicated that immediately after solubilization of retinol with Tween and physiological saline under

N_2 and subdued light, small amounts of artifacts were formed even in the presence of α -tocopherol. However, the main fraction representing 90% of the total radioactivity recovered from the column, had a ${}^{14}\text{C}/{}^3\text{H}$ ratio of 0.9. The two minor peaks eluted with 2 and 50% (v/v) acetone in light petroleum had ${}^{14}\text{C}/{}^3\text{H}$ ratios of 1.3 and 1.0 respectively, whereas the peak eluted with petroleum ether had a ${}^{14}\text{C}/{}^3\text{H}$ ratio of 0.8. In addition, the major radioactive peak reacted with ethanolic HCl to yield anhydroretinol, which was characterized as described above.

Discussion

It has been observed by many investigators in the past that a considerable time elapses between exhaustion of reserves of vitamin A as measured chemically and the appearance of deficiency symptoms in the rat (Dann, 1932; Baumann *et al.*, 1932). Further, there is a considerable gap between the daily dose of vitamin A which is necessary for good growth and the dose which is necessary to cause accumulation of the vitamin in the liver (Moore, 1957). When single doses of retinol are administered daily to depleted rats, at least $30\mu\text{g}$ of retinol seem to disappear before measurable storage begins. The fate of small doses of retinol after oral or intraperitoneal administration has not been studied in detail. With the use of double-labelled retinol, it has been possible to gain additional information on the metabolism of physiological doses of retinol.

The highest concentration of radioactivity observed in the adrenal glands (Table 1) confirms previous observations by Willmer & Laughland (1957) who found similar radioactivity in the adrenals after administration of β - $[{}^{14}\text{C}]$ carotene. However, our data do not agree with the report of Goodman *et al.* (1965), who found only negligible radioactivity in the adrenals after administration of $[{}^{15}\text{-}{}^{14}\text{C}]$ retinol in chylomicrons intravenously. The discrepancy between the results obtained in our study and those of Goodman *et al.* (1965) could be due to the vitamin A status of the animals used. Goodman *et al.* (1965) probably observed only a small amount of radioactivity in the adrenals because they used vitamin A-sufficient animals in their investigations.

The role of retinol in steroid synthesis has been controversial (Sundaresan, 1972). The activity of the enzyme $\Delta^5\text{-}3\beta$ -hydroxy steroid dehydrogenase in pair-fed control rats did not differ significantly from that in vitamin A-deficient animals (Rogers & Bieri, 1968). However, Granguad *et al.* (1969) and Juneja *et al.* (1966) found lowered activity of $\Delta^5\text{-}3\beta$ -hydroxy steroid dehydrogenase in the deficient animals. More recently, Piziak & Gawienowski (1972) have suggested that vitamin A may be required for efficient hormone production in the placenta of the guinea pig. Despite these contradictory results, the

rapid uptake of retinol and its maintenance during the experimental period of 24h, as observed in the present study and that of Willmer & Laughland (1957), in the adrenals of the deficient animals may suggest an important function of retinol in this organ.

Our data on the concentrations of retinol and its metabolites in tissues such as liver, kidney, intestine, blood, heart and brain are in general agreement with those of Coward & Thompson (1967). The total amounts of retinol and its metabolites are highest in the liver, indicating that liver plays a major role in the metabolism of retinol (Table 1). In addition, the significant uptake of retinol in the kidney suggests that this organ also contributes to a significant extent to the metabolism of retinol. It is noteworthy that the major portion of radioactivity in the kidney is due to the presence of retinol. Further, significant amounts of retinol and its metabolites are found in the plasma and intestine.

Although most of the administered vitamin A is normally found in the liver, the intestine and kidney are known to be target organs for vitamin A (DeLuca *et al.*, 1969; Kleiner-Bossaler & DeLuca, 1971). Detailed analysis of retinol and its metabolites in liver, intestine and kidney was carried out by alumina chromatography. In our studies, the liver extracts examined have consistently shown the presence of three radioactive peaks (Fig. 1). Whereas the two minor peaks have $^{14}\text{C}/^3\text{H}$ ratios similar to the injection ratio, the main radioactive fraction has a $^{14}\text{C}/^3\text{H}$ ratio of only 0.4. It is possible that the low $^{14}\text{C}/^3\text{H}$ ratio could arise from a preferential utilization of ^{14}C -labelled or non-tritiated molecules compared with tritiated molecules of retinol by the vitamin A-deficient rat. If this is the case, more of the tritiated molecules will accumulate in the liver 24h after the injection, thus causing a lower $^{14}\text{C}/^3\text{H}$ ratio than 1. In support of this conclusion, Goodman *et al.* (1966) reported isotopic discrimination against $[^3\text{H}]$ -retinol during both the chemical oxidation of retinol to retinal as well as the enzymic oxidation of retinal to retinoic acid. In these studies, the $^3\text{H}/^{14}\text{C}$ ratio of the product, retinal, increased after each oxidation step. In addition, it is possible that the retinol with a low $^{14}\text{C}/^3\text{H}$ ratio might be converted into a metabolite with a low $^{14}\text{C}/^3\text{H}$ ratio.

On the other hand, the low $^{14}\text{C}/^3\text{H}$ ratio could be due to the removal of ^3H from the side chain of retinol and incorporation into other lipids to a greater extent than ^{14}C . However, this conclusion is excluded by preliminary results obtained in the liver with $[15\text{-}^{14}\text{C}, 11,12\text{-}^3\text{H}_2]$ retinol. The major liver fraction in this case has a $^{14}\text{C}/^3\text{H}$ ratio of 0.3, similar to the ratio (0.4) obtained with $[6,7\text{-}^{14}\text{C}_2, 11,12\text{-}^3\text{H}_2]$ retinol. If the alternative explanation were valid, then the intact side chain of retinol would have to be incorporated into other lipids to the same extent to provide a similar $^{14}\text{C}/^3\text{H}$ ratio, whether the ring-

labelled or side-chain-labelled $[^{14}\text{C}]$ retinol is used and such a possibility is remote. Further, the low $^{14}\text{C}/^3\text{H}$ ratio cannot be attributed to poor recovery since the recoveries for ^{14}C and ^3H were 90–95% and 80–85% respectively of total d.p.m. applied to the column. It should be also noted that the main radioactive fraction of the liver extract is not an artifact obtained during chromatography, since a fraction obtained under identical conditions of chromatography of the extract of injected retinol solution has been characterized as retinol. This has been demonstrated by the $^{14}\text{C}/^3\text{H}$ ratio (0.9) as well as by the reaction of this fraction with ethanolic HCl to yield anhydroretinol (Fig. 2).

The major radioactive peaks obtained by chromatography of kidney and intestinal extracts have been identified as retinol. This was evidenced by the ability of the radioactive fractions of the kidney and the intestine (after saponification) to react with ethanolic HCl to yield anhydroretinol. Thus retinol predominates in the kidney whereas retinyl palmitate is the principal form of vitamin A in the intestines.

The distribution of radioactivity in ether-soluble, acidic and water-soluble metabolites of urine (Table 2) indicates that a major portion of the excreted radioactivity resided in the acidic and water-soluble fractions (49 and 38% of the excreted ^{14}C dose), whereas only a small but significant portion appeared in the ether-soluble metabolites. This is somewhat in contrast with the distribution of radioactivity found in urine fractions of $[6,7\text{-}^{14}\text{C}_2]$ retinoic acid-injected rats (Sundaresan & Bhagavan, 1971). The higher rate of metabolism of retinoic acid compared with retinol might account for differences in the excretory patterns of these compounds in urine.

Our results indicate that a metabolite of retinol which is resistant to chemical dehydration by ethanolic HCl, accumulates in the liver 24h after injection of double-labelled retinol in physiological doses. Whether this metabolite is an intermediate in the metabolism and function of vitamin A is not at present clear.

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