Metabolic Studies on Retinoic Acid in the Rat

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The nature of metabolites in the urine arising from differentially labelled retinoic acid was investigated after injection of physiological doses into retinol-deficient rats. Distribution of radioactivity after partition of urine into ether-soluble, acidic and water-soluble fractions revealed that there were at least six metabolites in urine. Of these, the major metabolite(s) was one lacking both C-14 and C-15 of retinoic acid. Enzymic or alkaline hydrolysis of acidic and water-soluble fractions did not release any retinoic acid, thus indicating that retinoyl β -glucuronide was not present in urine in significant amounts.

Retinoic acid has been known to have biological activity in retinol-deficient rats ever since its isolation as an intermediate in retinol synthesis (Arens & Van Dorp, 1946; Van Dorp & Arens, 1946; Malathi, Subba Rao, Seshadri Sastry & Ganguly, 1963). However, it was found to be inactive in vision (Dowling & Wald, 1960) and reproduction (Thompson, Howell & Pitt, 1964). The conversion of retinal into retinol by retinal reductase (Futterman & Saslaw, 1961; Wald & Hubbard, 1949; Bliss, 1951) and the transformation of retinal into retinoic acid by retinal oxidase (Dmitrovskii, 1961; Futterman, 1962; Elder & Topper, 1962; Bamji, Mahadevan, Lakshmanan & Murthy, 1962) have both been demonstrated in liver and other tissues. It has been suggested that the functions of retinol other than those concerned with vision and reproduction would require conversion of retinal into the acid form (Dowling & Wald, 1960). Thus the current hypothesis is that retinoic acid is itself, or is a precursor of, the systemically active form of retinol.

In more recent studies, retinoic acid has been found to undergo oxidative decarboxylation (Sundaresan & Therriault, 1968; Roberts & DeLuca, 1967) and isomerization (Zile, Emerick & DeLuca, 1967). In addition, it has been shown to conjugate with glucuronic acid and occur in the bile as retinoyl β -glucuronide (Dunagin, Meadows & Olson, 1965). Further, the transformation of retinoic acid into a biologically active acidic metabolite has been reported (Yagishita, Sundaresan & Wolf, 1964). However, our knowledge of the excretory products of retinoic acid metabolism is still fragmentary. The present paper deals with the nature of urinary excretory products of retinoic acid after injection of physiological doses of [15-¹⁴C]-, [14-¹⁴C]- and [6,7-¹⁴C₂]-retinoic acid into retinol-

deficient rats. A preliminary report of this investigation has appeared (Sundaresan & Bhagavan, 1970).

MATERIALS AND METHODS

[15-¹⁴C]Retinoic acid and [6,7-¹⁴C₂]retinoic acid were obtained through the courtesy of Professor O. Wiss of Hoffmann-La Roche and Co., Basle, Switzerland. [14-¹⁴C]Retinoic acid was purchased from Tracerlab, Waltham, Mass., U.S.A. All radioactive compounds were purified by column chromatography on washed DEAE-cellulose by elution with 1% (v/v) HCl in ethanol (Futterman, 1962). The solvent containing the pure compound was evaporated under N₂, the residue was dissolved in 0.1 M-NaOH and the pH was adjusted to 8.5. The non-radioactive all-*trans*-retinoic acid was purified in the same manner as the radioactive compound. The radioactive compounds were diluted with appropriate amounts of purified all-*trans*-retinoic acid so that a total of 10 μ g of retinoic acid was injected into each rat.

The concentration of retinoic acid in all solutions was determined spectrophotometrically with a Zeiss PMQII spectrophotometer, by using an $E_{1\text{cm}}^{1\%}$ value of 1500 at 350 nm in ethanol (Kofler & Rubin, 1960). The radioactivity was determined with a Nuclear-Chicago model 723 liquid-scintillation counter.

Preparation of animals and administration of retinoic acid. Retinol deficiency was produced by feeding weanling male rats of the Sprague-Dawley strain with a purified retinol-deficient diet as described by Sundaresan, Winters & Therriault (1967), with a slight modification. The pups in this study were derived from mothers that were kept on the retinol-deficient diet during the period of lactation. When animals reached a plateau in growth rate in about 6 weeks, three groups of four animals each, comparable in body weight, were used in the experiment. Each group was injected with 10 μ g of retinoic acid per rat in aqueous solution (pH 8.5), either [15-¹⁴C]retinoic acid (0.1467 μ Ci), [14-¹⁴C]retinoic acid (0.1926 μ Ci) or [6,7-¹⁴C₂]retinoic acid (0.1520 μ Ci), and placed in open metabolism cages. Urines

were collected for 24h under toluene and stored frozen until analysis.

Extraction of metabolites from urine. Total volumes of urine were measured and the radioactivity in a sample of the urine (0.5ml) was determined by using 10ml of counting solution and 5ml of ethanol. The counting solution consisted of 5g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/1 of toluene. The counting efficiency for ^{14}C in urine was 58% by the channels-ratio method.

The remainder of the urine sample was extracted four times with equal volumes of diethyl ether. This was designated the ether-soluble fraction. The aqueous residue was then acidified with dil. HCl to pH2, re-extracted four times with equal volumes of diethyl ether and the ether extracts were combined to give the acid fraction. The remaining aqueous residue was designated the water-soluble fraction. Samples of each fraction were counted for radioactivity in 1ml of ethanol and 10ml of counting solution.

Silicic acid chromatography of water-soluble fractions. The water-soluble fractions obtained after fractionation of urine were evaporated under reduced pressure at room temperature in a rotary evaporator. The residue was extracted with 40ml of methanol. The methanol-insoluble residue was removed by centrifugation. The precipitate was washed twice with 5ml of methanol and the washings were added to the original supernatant. The radioactivity of the precipitate was negligible. The methanol extract was concentrated to a small volume of about 4ml. Silicic acid (BioSil A; 100–200 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was washed with methanol, acetone and diethyl ether and heat-activated at 120°C for 24h. Silicic acid (3g) was added to the methanol extract and the solvent was carefully removed under reduced pressure. Columns were prepared from 15g of the silicic acid in iso-octane (2,2,4-trimethyl-pentane). The water-soluble fraction adsorbed on the silicic acid was then added to the top of the silicic acid column, in iso-octane. Stepwise gradient elution was performed, beginning with diethyl ether (Fig. 1), and 25ml fractions were collected. The radioactivity of a sample of each fraction was determined.

β -Glucuronidase treatment of acidic and water-soluble fractions of urine. Samples of $^{15}\text{-}^{14}\text{C}$ -labelled acidic fractions (containing about 6000d.p.m.) were dried under N_2 and dissolved in 1ml of an aqueous solution of Tween 80 (0.34% final concentration). This was treated with 0.5ml of bacterial glucuronidase (1850 units) and 2ml of 20 mM- KH_2PO_4 - Na_2HPO_4 buffer, pH 6.8, and incubated at 37°C for 30 min. Control tubes had the same incubation mixture

in addition to 1ml of ethanol, which was added before incubation. The reaction was stopped by addition of 1ml of ethanol and the mixture diluted to 15ml by addition of water. The mixture was extracted three times with equal volumes of diethyl ether. The ether was evaporated under N_2 and the residue taken up in 0.2ml of acetone. From each tube 75 μl was chromatographed on Whatman no. 1 filter paper, with butan-1-ol saturated with 3M-formic acid as the solvent (descending). $^{15}\text{-}^{14}\text{C}$ -labelled water-soluble fractions were also treated with bovine β -glucuronidase and analysed as described above.

Alkaline hydrolysis of acidic fractions from urine. $^{15}\text{-}^{14}\text{C}$ -labelled acidic fractions (containing about 6000d.p.m.) in ethanol were saponified with 2ml of ethanolic 10% (w/v) KOH for 1h under N_2 at 40°C. After saponification, an equal volume of water was added and the mixture extracted with 40ml of diethyl ether, which was discarded. The aqueous fraction was then acidified with dil. HCl to pH3 and re-extracted three times with 40ml of diethyl ether. The combined ether extracts were concentrated under reduced pressure and taken up in a small volume of acetone. Both this fraction and an untreated acidic fraction were analysed by t.l.c. on silica gel-coated glass fibre in a system of benzene-chloroform-methanol (4:1:1, by vol.) (Yagishita *et al.* 1964).

RESULTS

The distribution of radioactivity in ether-soluble, acidic and water-soluble fractions is presented in Table 1. Recovery of the total radioactivity of the urine in the different fractions was 94–98%. Examination of the distribution of radioactivity shows that there is no significant difference between the ether-soluble fractions from [$^{15}\text{-}^{14}\text{C}$]-, [$^{14}\text{-}^{14}\text{C}$]- and [$^{6,7}\text{-}^{14}\text{C}_2$]-retinoic acid-treated animals. In the acidic fractions there is a significant difference between [$^{15}\text{-}^{14}\text{C}$]- and [$^{14}\text{-}^{14}\text{C}$]-retinoic acid ($P < 0.01$). Among the water-soluble fractions there is a significant difference between [$^{15}\text{-}^{14}\text{C}$]- and [$^{14}\text{-}^{14}\text{C}$]-retinoic acid ($P < 0.01$), and a much greater difference between $^{14}\text{-}^{14}\text{C}$ - and $^{6,7}\text{-}^{14}\text{C}_2$ -labels ($P < 0.01$).

The patterns obtained on chromatography of water-soluble fractions on silicic acid columns are shown in Fig. 1. The total radioactivity obtained for each fraction has been computed as a percentage of the injected dose. Recovery of the radioactivity applied on the column was in the range 86–106%.

Table 1. Distribution of metabolites of [^{14}C]retinoic acid in the urine

Each rat received 10 μg of labelled retinoic acid intraperitoneally. 'Total' values were obtained by direct radioactivity determination. The results are given as means $\pm$ S.E.M. for four animals in each group.

Label	Dose (μCi)	Percentage of radioactive dose excreted			
		Ether-soluble	Acidic	Water-soluble	Total
$^{15}\text{-}^{14}\text{C}$	0.1467	3.23 ± 0.5	9.39 ± 0.8	7.41 ± 0.4	21.14 ± 1.3
$^{14}\text{-}^{14}\text{C}$	0.1926	5.70 ± 0.9	15.34 ± 0.8	12.13 ± 0.6	33.84 ± 1.2
$^{6,7}\text{-}^{14}\text{C}_2$	0.1520	5.63 ± 0.4	12.24 ± 0.3	30.00 ± 2.3	49.89 ± 2.1

Fig. 1. Separation of water-soluble metabolites in rat urine by silicic acid column chromatography. (a) $[15-^{14}\text{C}]$ -Retinoic acid; (b) $[14-^{14}\text{C}]$ retinoic acid; (c) $[6,7-^{14}\text{C}_2]$ -retinoic acid. The fraction nos., composition and volume of the eluting solvent in stepwise elution were as follows: 1–4, diethyl ether (100 ml); 5–8, 20% (v/v) ethanol in diethyl ether (100 ml); 9–13, 50% (v/v) ethanol in diethyl ether (125 ml); 14–17, 75% (v/v) ethanol in diethyl ether (100 ml); 18–21, ethanol (100 ml); 22–25, 50% (v/v) ethanol in methanol (100 ml); 26–29, methanol (100 ml). The eluate was collected in 25 ml fractions.

The pattern of elution of $6,7-^{14}\text{C}_2$ -labelled water-soluble fraction is different from that of the $15-^{14}\text{C}$ - or $14-^{14}\text{C}$ -label. Four prominent peaks appeared with the $6,7-^{14}\text{C}_2$ -label, in contrast to three major peaks with both the $15-^{14}\text{C}$ - and $14-^{14}\text{C}$ -labels.

The difference in the pattern of elution was observed more clearly when the two major peaks from the $6,7-^{14}\text{C}_2$ -labelled fraction, eluted with 50% (v/v) ethanol and 75% (v/v) ethanol in diethyl ether consecutively, were separated by paper chromatography with butan-1-ol saturated with 3M-formic acid. The first radioactive spot from the $6,7-^{14}\text{C}_2$ -label corresponded to the major radioactive zone from the $15-^{14}\text{C}$ -label (R_F 0.75), but the second radioactive area (R_F 0.64) did not correspond to any from the $15-^{14}\text{C}$ -label. The R_F of retinoic acid standard in this system was 0.93. These chromatographic results thus indicate marked differences between the metabolites of $6,7-^{14}\text{C}_2$ -labelled water-soluble fraction and those of either the $15-^{14}\text{C}$ - or $14-^{14}\text{C}$ -labelled compound in the urine. The results on β -glucuronidase treatment of acidic or water-soluble fractions of $15-^{14}\text{C}$ -label showed

that there was no release of radioactivity from the fractions after hydrolysis. Chromatography of the acidic fractions before and after hydrolysis in a solvent containing butan-1-ol saturated with formic acid did not show any significant difference in radioactivity distribution in different areas. In addition to β -glucuronidase treatment, acidic fractions from $[15-^{14}\text{C}]$ retinoic acid were subjected to alkaline hydrolysis, which again did not reveal any significant difference in radioactivities of fractions obtained before and after hydrolysis. The ether-soluble fractions from the three labels were not tested for retinoyl β -glucuronidase because of the report that the glucuronide is extracted very poorly by ether from neutral or basic solutions (Lippel & Olson, 1968).

DISCUSSION

Although it has been shown that metabolites of retinol are excreted mainly in the faeces (Nath & Olson, 1967) after a lag of approx. 24 h (Sundaresan & Therriault, 1968), another important route of excretion of metabolites is the urine. By using differentially labelled retinoic acid in physiological doses, a considerably greater percentage of the injected dose has been found to be excreted in the urine with $[6,7-^{14}\text{C}_2]$ retinoic acid than with the $14-^{14}\text{C}$ - or $15-^{14}\text{C}$ -labelled compound. These results on the overall excretion in the urine are in general agreement with previous results (Roberts & DeLuca, 1967), except for the finding that a higher percentage of injected dose was recovered from $[14-^{14}\text{C}]$ - than from $[15-^{14}\text{C}]$ -retinoic acid during 24 h. The apparent difference could be due to the nutritional status of the retinol-deficient animals, and also to the mode of administration of retinoic acid.

Examination of radioactivity distribution in urine (Table 1) reveals distinct features of metabolism and excretion of retinoic acid. In accordance with previous results on rapid decarboxylation of retinoic acid (Sundaresan & Therriault, 1968), the least amount of radioactivity is excreted in the urine on administration of $[15-^{14}\text{C}]$ retinoic acid, and the highest percentage of excretion is found with the $6,7-^{14}\text{C}_2$ -label. Further, the results on the distribution of radioactivity in ether-soluble, acidic and water-soluble fractions indicate the possibility of at least six metabolites of retinoic acid in the urine. This number is based on the assumption that the radioactivity appearing in urine from $[15-^{14}\text{C}]$ -retinoic acid represents a molecule with an intact side chain and a substituted cyclohexene ring. On this basis, the nature of metabolites could be represented as follows: 1, ether-soluble, possibly common to all three labels, 3–6% of the injected dose; 2, acidic, has C-15, C-14, C-6 and C-7, 9.4%

of the injected dose; 3, acidic, has only C-14, C-6 and C-7, 3–6% of the dose; 4, water-soluble, has an intact side chain, 7.4% of the dose; 5, water-soluble, has only C-14, C-6 and C-7, 4.5% of the dose; 6, water-soluble, has only C-6 and C-7, 18% of the dose. It is thus apparent that this may constitute the major metabolite(s) of retinoic acid.

Silicic acid chromatography of water-soluble fractions indicated that the metabolites of [6,7-¹⁴C]₂retinoic acid are different from those of [15-¹⁴C]- and [14-¹⁴C]-retinoic acid. However, these differences were not observed by Geison & Johnson (1970), which could be due to the poor recoveries from silicic acid columns.

Nath & Olson (1967) found that an appreciable percentage of radioactivity in urine appeared in the retinoyl β -glucuronide zone, with a small amount in the retinoic acid area. However, Emerick, Zile & DeLuca (1967) did not demonstrate the presence of retinoic acid, although they found radioactivity in the chloroform extract after treatment of urine with β -glucuronidase. These reports prompted us to examine the presence of retinoyl β -glucuronide in urine. Enzymic or alkaline hydrolysis of 15-¹⁴C-labelled water-soluble fraction or acidic fraction did not release any retinoic acid. Under similar experimental conditions retinoyl β -glucuronide was hydrolysed to free retinoic acid by β -glucuronidase (Dunagin, Zachman & Olson, 1966).

Our results indicate that a product(s) of decarboxylation at C-15 is excreted in the urine to an extent of 11% of an injected dose of retinoic acid. About 18% of the injected dose has been shown to represent a moiety (or moieties) lacking both C-15 and C-14 of retinoic acid. Retinoyl β -glucuronide is not present in urine in significant amounts. Whether or not retinoyl β -glucuronide will be found in appreciable quantities, after massive doses of retinoic acid, as a product of detoxication is open for further investigation.

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