A Biologically Active Metabolite of Retinoic Acid from Rat Liver

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1. Four major radioactive fractions have been isolated from the livers of vitamin A-deficient rats given $[6,7-^{14}\text{C}_2]$ retinoic acid. 2. At least one of these was more potent than retinoic acid and approximately equal to retinol in the growth assay for vitamin A activity. 3. The biologically active material was chromatographically distinct from retinoic acid, retinol and retinal. 4. Alkaline hydrolysis of this material yielded an acidic compound containing all the radioactivity. 5. The methyl ester of the acidic product was unlike the methyl ester of retinoic acid in its chromatographic behaviour. 6. It is suggested that this metabolite may represent the active form of retinol in its growth-supporting role.

Since the discovery of vitamin A some 50 years ago much has been learned about its chemistry and biochemistry. The structures of the vitamin and its analogues are clearly established, and its role in vision is well understood even at the molecular level (Wald & Hubbard, 1960). Further, the gross physiological effects of the vitamin have been thoroughly described (Moore, 1957). Nevertheless, there remain gaps in our biochemical understanding of this fundamental substance. The biochemical mechanism whereby this vitamin supports growth, development and reproduction in higher animals is still unknown. In a search of it the active form of the vitamin for each of these functions must eventually be identified. Recently attention was focused on this question with the discovery that the acid analogue of vitamin A₁ (retinoic acid) was biologically active (Arens & Van Dorp, 1946a) and could correct all lesions found in vitamin A deficiency except those in the visual cycle (Dowling & Wald, 1960) and in reproduction (Howell, Thompson & Pitt, 1964; Thompson, 1964). The inability to detect retinoic acid in the tissues of animals even after large doses of it (Arens & Van Dorp, 1946b; Sharman, 1949; Refearn, 1960) at first cast doubt on the natural occurrence of this compound. However, there is recent evidence of its presence in blood (Jurkowitz, 1962) and other tissues (Krishnamurthy, Bieri & Andrews, 1963). In addition, enzyme systems capable of generating retinoic acid from retinal have been described (Futerman, 1962; Lakshmanan, Vaidyanathan & Cama, 1964). Consequently, the possibility that retinoic acid itself might be the active form cannot be excluded.

A direct approach to the study of vitamin A

metabolism has become possible with the availability of ^{14}C -labelled retinoic acid. Such an investigation was begun in the Laboratory of Dr G. Wald. We have now been able to demonstrate the conversion *in vivo* of retinoic acid into another substance capable of supporting growth in vitamin A-deficient animals. This material appears to be distinct from known vitamin A compounds and is required at lower dosages than retinoic acid for a growth response when similarly administered. It is the purpose of the present paper to describe these experiments.

MATERIALS AND METHODS

Chemicals. all-*trans*-Retinol, all-*trans*-retinoic acid and all-*trans*-retinal were obtained from Distillation Product Industries, Rochester, N.Y., U.S.A. The methyl ester of retinoic acid was prepared by the methylation of all-*trans*-retinoic acid with diazomethane. A spectrophotometrically pure (u.v. and i.r.) product was obtained by chromatography on alumina columns. Thin-layer chromatography indicated only a single component for retinoic acid and retinal, but retinol gave several components, only one of which gave the characteristic spectrum of pure retinol. The methyl ester of retinoic acid separated into a major and a minor component (probably isomers), both with identical spectra.

All solvents used were redistilled A.R. grade. Skelly B is a petroleum fraction that distils at 65–66°.

$[6,7-^{14}\text{C}_2]$ Retinoic acid was a gift from Hoffmann-La Roche and Co., Basle, Switzerland, and Rochester, N.Y., U.S.A.

Preparation of animals. Male albino Holtzman rats weighing 40–50 g. were obtained at weaning and placed on a vitamin A-deficient purified diet (DeLuca, Manatt, Madsen & Olson, 1963). In about 4 weeks they usually stopped

growing and their weight remained constant for a few days. At this point they were either used for bioassay or were placed on retinoic acid supplements. Supplements and bioassay samples were administered to the animals orally by stomach tube in an equal mixture of oil, ethanol and water.

Crude extract for bioassay. Vitamin A-deficient rats that had been given 50 μ g. of retinoic acid daily for 30 days were not given this supplement for 2 days, then were given 1.5 mg. of [6,7- 14 C]₂retinoic acid (specific activity 0.5 μ C/mg.) by stomach tube and killed by decapitation 3 hr. later. Preliminary findings revealed that at this time the liver contained its greatest radioactivity or 2-5% of the administered dose. The livers were minced in a meat grinder immediately although it was found possible to store them at -10° for at least 30 days without change in results. The minced tissue was dehydrated by grinding thoroughly with 3 parts (by wt.) of anhydrous Na₂SO₄. This was followed by extraction several times with cold (0°) ethanol (2.5 l./100 g. of fresh liver) in a mechanical shaker. After each extraction, the mixture was centrifuged at 1100 g for 10 min. to remove all suspended particles. The supernatant fluids were combined, reduced to one-tenth their volume in a flash evaporator, cooled to -90° in an acetone-solid CO₂ bath and filtered through an ethanol-solid CO₂ filter pad at -72° . At this temperature the sterols are precipitated and are easily removed by filtration. The precipitate was washed several times with cold (-72°) ethanol. The combined filtrate and washings were concentrated to one-tenth their volume in a flash evaporator and used either for bioassay or further purification. All operations were carried out at 0-4° under N₂ and shielded from light whenever possible.

Partition between solvents. The sterol-free filtrate ('crude extract') from the previous step was evaporated in a flash evaporator until an oily residue was left; this was redissolved in a small volume of Skelly B. The material was partitioned between equal volumes of Skelly B and aq. 80% (v/v) ethanol. For an extract representing 100 g. of fresh liver, 1 l. of each solvent was used. The partition was achieved by shaking the solvents mechanically for 15 min. and then allowing the layers to separate. The lower layer was removed and the upper (Skelly B) layer re-extracted with more aq. 80% ethanol. The lower layers were combined, reduced to a small volume and partitioned between ethyl acetate and aq. 64% (v/v) methanol. The aqueous phase was re-extracted with ethyl acetate, and the ethyl acetate phases were combined and evaporated to dryness. The resulting oily residue was dissolved in a small amount of Skelly B and further fractionated on silicic acid columns.

Chromatography on silicic acid columns. Bio-Rad silicic acid (-325 mesh; California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) was activated at 140° for 16 hr. Columns (20 cm. $\times$ 2.5 cm.; hold-up volume 40 ml.) were prepared by slurring 20 g. of silicic acid in Skelly B and allowing the adsorbent to settle by gravity. Crude extract from about ten livers (approx. 100 g. fresh wt.) was adsorbed on the column in Skelly B and the less polar components were eluted with 200 ml. of diethyl ether; the polar components were eluted with 200 ml. of methanol. The two fractions were rechromatographed separately on 40 g. silicic acid columns (60 cm. $\times$ 1.5 cm.) by using a convex gradient elution (Bock & Ling, 1954). For the chromatography of the non-polar components the first solvent system was Skelly B to 20% (v/v) diethyl ether

in Skelly B, and the second solvent system was 20% (v/v) diethyl ether in Skelly B to 100% diethyl ether. For the chromatography of the polar components the solvent systems were 100% diethyl ether to 20% (v/v) methanol in diethyl ether and 20% (v/v) methanol in diethyl ether to 100% methanol. Fractions (10 ml.) were collected for determination of either radioactivity or spectra.

Chromatography on modified DEAE-cellulose. DEAE-cellulose (Carl Schleicher and Schuell Co., Keene, N.H., U.S.A.) was converted into the OH⁻ form as described by Futterman (1962) and poured as an ethanol slurry into a column (30 cm. $\times$ 1.5 cm.). Non-acidic components were eluted with 200 ml. of ethanol; 200 ml. of acidified ethanol (300 ml. of ethanol, 1 ml. of conc. HCl) eluted the acidic components. Fractions (10 ml.) were collected and assayed for radioactivity or extinction at 355 μ .

Adsorption on alumina. Bio-Rad neutral alumina (California Corp. for Biochemical Research) was slurried with hexane and poured into a column (30 cm. $\times$ 1.5 cm.). After the column had been washed with 50 ml. of hexane the substance to be chromatographed was placed on the column in hexane and elution carried out with increasing concentration on diethyl ether in hexane.

Thin-layer chromatography. For thin-layer chromatography the Camag apparatus (Microchemical Specialties Co., Richmond, Calif., U.S.A.) was used. Plain silica gel (Research Specialties Co., Richmond, Calif., U.S.A.) (30 g.) was mixed with 80 ml. of water and spread 25 μ thick over six glass plates (20 cm. $\times$ 20 cm.). After activation at 140° for 8 hr. the plates were stored at 90° until just before use. Materials to be spotted were dissolved in hexane and applied to the plates in 1-2 μ l. of the solvent. The plates were developed at room temperature in the dark, either with benzene (for the chromatography of the methyl ester derivatives) or with methanol-benzene (1:9, v/v) (for retinol, retinal and retinoic acid) as the solvent system. To determine the location of various compounds, small areas of the plate were scraped off and extracted with ethanol, and the adsorbent was sedimented by centrifugation. The clear supernatant was assayed for either spectra or radioactivity. In cases where only radioactivity measurements were desired the scrapings were transferred directly to a vial containing the counting fluid.

Saponification of the biologically active fraction and preparation of a methyl ester derivative. Saponification was carried out in methanolic 6% (w/v) KOH at 60° for 20 min. under N₂. Non-saponifiable matter was extracted with hexane, and the aqueous layer, containing all the radioactivity, was acidified to pH 2 and the radioactivity extracted with fresh hexane. Methylation was carried out with diazomethane in ether as described by Vogel (1956).

Determination of spectra. Ultraviolet and visible spectra were determined with a Cary 11 recording spectrophotometer with redistilled ethanol or spectral-grade hexane (Fisher Scientific Co., Chicago, Ill., U.S.A.) as solvent.

Determination of radioactivity. Measurements of 14 C were carried out in a Packard model 314EX liquid-scintillation counter. For samples in organic phase a toluene counting solution (Herberg, 1960) was used (efficiency, 54%). Aqueous samples were assayed in Bray's (1960) solution (efficiency, 47%). To measure radioactivity in thin-layer silicic acid suspensions a gel was prepared, containing 1500 ml. of dioxan, 300 ml. of water, 150 g. of naphthalene, 7 g. of 2-phenyloxazole and 300 mg. of 1,4-bis-(5-

phenyloxazol-2-yl)benzene to which 4% (by wt.) of Cabosil (Gordon & Wolfe, 1960) (Packard Instrument Co., LaGrange, Ill., U.S.A.) was added (efficiency, 32%). An internal standard of [^{14}C]toluene (Packard Instrument Co.) was used to calculate the efficiency in all cases.

RESULTS

Biological activity of retinoic acid and retinol. Table 1 shows the effect of various doses of retinoic acid on the growth of vitamin A-deficient rats. At least 16 $\mu\text{g.}$ of retinoic acid/day administered in the manner used for the unknown fractions was required for these rats to resume normal growth. Animals receiving either 47 $\mu\text{g.}$ of retinoic acid or 8 $\mu\text{g.}$ of retinol/day had the highest growth rate, but comparable growth rates were usually obtained with doses from 15 $\mu\text{g.}$ to 50 $\mu\text{g.}$ of retinoic acid/day (Table 2).

Biological activity of crude extract. The crude extract was first examined for vitamin A activity by growth assay. The amount of preparation given each animal was calculated on the assumption that the specific radioactivity of the labelled metabolites in the crude extract would be equivalent to that of the parent radioactive retinoic acid. Table 2 shows the growth-promoting effect of the crude extract. After the extract was administered, there was a lag in response for 2-3 days, similar to that experienced with retinoic acid. When 15 $\mu\text{g.}$

equivalents of crude extract were given to the deficient animals, their growth rate was below normal, but 40 $\mu\text{g.}$ doses supported a normal growth rate. Deficient animals receiving an extract from livers of vitamin A-deficient rats that had received no retinoic acid exhibited no growth response.

Isolation of radioactive fractions from the crude extract. When the crude extract was partitioned as described above, most of the radioactivity was

Table 2. Growth response of vitamin A-deficient rats to a crude extract obtained from the livers of rats given retinoic acid

Vitamin A-deficient rats were given daily supplements of the indicated substances in 0.4 ml. of an equal mixture of oil, ethanol and water. The assay period was 10 days. Each value reported is the mean $\pm$ s.e.m.

Supplement	No. of rats in group	Weight gain/day during supplementation (g.)
Control (solvent)	3	-3.0 $\pm$ 0.60
Retinoic acid (15 $\mu\text{g.}$)	3	+3.9 $\pm$ 0.69
Retinoic acid (40 $\mu\text{g.}$)	3	+3.8 $\pm$ 0.31
Retinol (12 $\mu\text{g.}$)	2	+4.1 $\pm$ 0.45
Crude liver extract (40 $\mu\text{g.}$)*	4	+3.4 $\pm$ 0.45
Control liver extract†	4	-3.0 $\pm$ 0.56
Growth rate during the depletion period	19	+4.0 $\pm$ 0.04

* See the Materials and Methods section for the preparation of this extract.

† This extract was prepared in the same way as the crude extract, except that the animals had received no retinoic acid supplements.

Table 1. Growth response of vitamin A-deficient rats to various doses of retinoic acid and retinol

Vitamin A-deficient rats were given supplements of the substances indicated in 0.2 ml. of corn oil every 2 days for a period of at least 24 days. Each value reported is the mean $\pm$ s.e.m. of at least four determinations; the growth rate during the depletion period is the mean $\pm$ s.e.m. of 45 determinations.

Supplement	Weight gain/day during supplementation (g.)
Control (corn oil)*	-6.0 $\pm$ 0.44
Retinoic acid (0.5 $\mu\text{g.}/\text{day}$)*	-5.9 $\pm$ 1.43
Retinoic acid (0.9 $\mu\text{g.}/\text{day}$)*	-0.6 $\pm$ 0.41
Retinoic acid (4.0 $\mu\text{g.}/\text{day}$)*	+0.2 $\pm$ 0.32
Retinoic acid (8.0 $\mu\text{g.}/\text{day}$)†	+0.3 $\pm$ 0.10
Retinoic acid (16.0 $\mu\text{g.}/\text{day}$)	+3.6 $\pm$ 0.13
Retinoic acid (28 $\mu\text{g.}/\text{day}$)	+3.6 $\pm$ 0.32
Retinoic acid (32 $\mu\text{g.}/\text{day}$)	+4.1 $\pm$ 0.33
Retinoic acid (47 $\mu\text{g.}/\text{day}$)	+5.0 $\pm$ 0.37
Retinol (0.8 $\mu\text{g.}/\text{day}$)	+0.2 $\pm$ 0.93
Retinol (8.0 $\mu\text{g.}/\text{day}$)	+5.2 $\pm$ 0.94
Growth rate during depletion period ...	+4.5 $\pm$ 0.04

* These rats died before the assay period had ended.

† These rats died shortly after the assay period ended.

Fig. 1. Separation of radioactive metabolites of retinoic acid on silicic acid columns by using gradient elution. The gradients used (broken lines) were: fraction 0-50, 0-20% ether in Skelly B; fractions 50-100, 20-100% ether in Skelly B; fractions 100-150, 0-20% methanol in ether; fractions 150-200, 20-100% methanol in ether. The elution position of retinoic acid is indicated by the broken peak. Peak E-1 was eluted by 10% ether in Skelly B, peak E-2 by 44% ether in Skelly B, peak M-1 by 10% methanol in ether, and peak M-2 by 55% methanol in ether.

Scheme 1. Outline for the isolation procedure of some metabolites of retinoic acid. For details see the Materials and Methods section and Table 3.

recovered in the ethyl acetate fraction. Chromatography on silicic acid yielded a non-polar fraction E and a polar fraction M, each containing about 50% of the radioactivity. On rechromatography four major ¹⁴C-containing peaks were obtained (Fig. 1). Peak E-1 was eluted with 10% (v/v) diethyl ether in Skelly B, peak E-2 with 44% (v/v) diethyl ether in Skelly B, peak M-1 with 10% (v/v) methanol in diethyl ether, and peak M-2 with 55% (v/v) methanol in diethyl ether. The concentration of components was estimated on the basis of radio-

activity and the specific radioactivity of the original [¹⁴C]retinoic acid. There were also several small radioactive peaks, but they did not yield sufficient quantities for bioassay. The entire isolation procedure is outlined in Scheme 1 and the recovery of radioactivity at each step is given in Table 3.

Bioassay of the isolated radioactive fractions. Table 4 shows the growth of deficient rats receiving the various radioactive fractions isolated by the gradient technique. Only the component E-1 was biologically active at the doses tested. The animals

receiving 10 μ g. of this fraction daily resumed a normal rate of growth. The other three fractions bioassayed were unable to substitute for retinoic acid

Table 3. *Summary of the recovery of 14 C in the various steps during the isolation procedure of metabolites of retinoic acid*

The calculations represent the data obtained in the extraction of 117 livers. The total dose given was 221 mg. of retinoic acid (sp. activity, 1000 disintegrations/min./ μ g. of retinoic acid).

Step	Percentage of administered 14 C
Ethanol extraction	
Ethanol extracts	3.4
Dry residue	0.1
Removal of sterols	
Sterol-free filtrate	3.0
Sterol residue	0.3
First solvent-solvent partition	
Aq. 80% ethanol fraction	3.0
Skelly B fraction	0.0
Second solvent-solvent partition	
Ethyl acetate fraction	2.65
Aqueous fraction	0.35
Batch chromatography on silicic acid	
Ether-eluted non-polar fraction (E)	1.08
Methanol-eluted polar fraction (M)	1.45
Gradient chromatography on silicic acid	
10% Ether in Skelly B eluate (E-1)	0.27
44% Ether in Skelly B eluate (E-2)	0.06
Minor fractions eluted with increasing concentration of ether in Skelly B	0.60
10% Methanol in ether eluate (M-1)	0.45
55% Methanol in ether eluate (M-2)	0.10
Minor fractions eluted with increasing concentration of methanol in ether	0.27
Fractions not chromatographed due to decomposition during handling	0.75

acid in the deficient rat, even when the dose was raised to 20 μ g./day.

When labelled crystalline retinoic acid was added to crude liver extracts from vitamin A-deficient animals and carried through the entire isolation procedure as outlined in Scheme 1, only about 35% of the added retinoic acid was recovered at the end of silicic acid chromatography. In no case did any of the peaks correspond to the four major metabolite peaks described in Fig. 1. No radioactive peaks appeared in the 100% ether to 100% methanol gradient.

Chromatographic comparison of fractions with known compounds. Crystalline retinoic acid, retinal and retinol were chromatographed separately on silicic acid columns by gradient elution. The behaviour of these substances on silicic acid was different from that of the fractions shown in Fig. 1. Retinoic acid was eluted by 14–15% (v/v) diethyl ether in Skelly B and had a spectrum identical with that of crystalline retinoic acid. About 60% of the added retinoic acid was recovered. With retinal, one major peak and one minor peak were obtained, each with a spectrum identical with that of crystalline retinal. The minor peak had 14% of the added retinal, and 57% of the added retinal was recovered in the major peak. When crystalline retinol was chromatographed, a single peak was eluted by 65% (v/v) diethyl ether and this contained 48% of the added retinol. Co-chromatography of the biologically active fraction E-1 and retinoic acid on DEAE-cellulose also demonstrated the difference between these two substances. The unknown substance was not retained on this column at all, whereas retinoic acid was eluted only with the acidified solvent.

Saponification of the biologically active fraction, E-1, resulted in all of the radioactivity becoming water-soluble in the alkaline solution. On acidifica-

Table 4. *Growth response of vitamin A-deficient rats to fractions obtained from the chromatography of a biologically active crude extract containing radioactive metabolites of retinoic acid*

Vitamin A-deficient rats were given daily supplements of the substances indicated in 0.4 ml. of an equal mixture of oil, ethanol and water. The assay period was 10 days. The fractions tested are those obtained from silicic acid columns shown in Table 3 and Fig. 1. Each value reported is the mean $\pm$ S.E.M.

Supplement	No. of animals in group	Weight gain/day during supplementation (g.)
Control (solvent)	6	-5.4 $\pm$ 0.64
Retinoic acid (15 μ g.)	3	+2.7 $\pm$ 0.71
Retinoic acid (48 μ g.)	3	+3.9 $\pm$ 0.29
E-1 fraction (10% ether) (10 μ g.)	4	+3.2 $\pm$ 0.63
E-2 fraction (44% ether) (10 μ g.)	3	-5.4 $\pm$ 0.56
M-1 fraction (10% methanol) (10–20 μ g.)*	3	-4.7 $\pm$ 0.41
M-2 fraction (55% methanol) (10–20 μ g.)*	2†	-5.2 $\pm$ 0.32
Growth rate during depletion	24	+3.9 $\pm$ 0.08

* The dose was increased after 4 days.

† Two other rats died before the assay was completed.

tion, the radioactivity could be extracted into hexane, indicating a free acid. The methyl ester of this free acid could be adsorbed on the neutral alumina columns in hexane and eluted with 10% (v/v) diethyl ether in hexane. On the other hand, the methyl ester of retinoic acid could be eluted with 15% (v/v) diethyl ether in hexane from identical columns. Chromatography of the methylated derivatives on thin-layer silicic acid further demonstrated that the fraction E-1 was not an ester of retinoic acid. The methyl ester of retinoic acid separated into a major component (R_F 0.49) and a minor component (R_F 0.66). Since they had identical u.v. spectra, it seemed probable that they were isomers. A similar observation was made by Dunagin, Zachman & Olson (1964). A comparable amount (5 μ g.) of the E-1 methyl ester had R_F 0.73. It had a white fluorescence and imparted a bleached appearance to the silicic acid. All the radioactivity was entirely associated with the bleached area. The methyl ester of retinoic acid (both components) appeared dark in u.v. light, did not fluoresce and did not bleach the silicic acid.

DISCUSSION

The present experiments demonstrate the conversion *in vivo* of retinoic acid into at least one biologically active metabolite (E-1). This fraction supports growth in vitamin A-deficient rats at least as well as, and probably at lower dosages than, does retinoic acid. Therefore the biological activity of the E-1 fraction cannot be attributed to contamination by the parent retinoic acid. That the E-1 fraction is distinct from retinol, retinal and retinoic acid is illustrated by its chromatographic behaviour on silicic acid. Further evidence that it differs from retinoic acid is given by its behaviour on DEAE-cellulose, which indicates that it lacks a free carboxyl group. An acidic group was generated by alkaline hydrolysis, however, suggesting the possibility that the E-1 fraction might represent merely an ester of retinoic acid. This was apparently ruled out by the different behaviour of the methyl esters of retinoic acid and E-1 in neutral alumina columns and thin-layer silicic acid.

It was shown that none of the metabolites detected could be produced from the original retinoic acid by the isolation procedure, and the high degree of biological activity of the E-1 fraction also provides evidence against the possibility that it is an artifact of the methods used.

The instability of retinoic acid to the isolation procedure raises the question whether the procedure might have destroyed still another biologically active material. Although the precision of growth assays is not great ($\pm 20\%$), the data suggest that at least one-third to one-half of the bio-

logical activity of the crude extract was accounted for by the E-1 fraction. Because we have little information on the stability of the E-1 fraction, the discrepancy might be due to its deterioration. Still another possibility is that some of the minor fractions not tested may also be biologically active. Clearly, these experiments do not exclude the possibility of other vitamin A-active materials in the livers of rats given retinoic acid.

Although not yet examined experimentally, the E-1 metabolite is probably a common product of retinoic acid and retinol metabolism. This substance is conceivably the 'active' form of the vitamin in its basic growth function. Retinal has already been demonstrated to be the 'active' form in the visual scheme. Should these speculations be substantiated, chemical identification and sub-cellular location of the biologically active metabolite might provide an important insight into the basic mechanism of vitamin A action.

The relationship of the presently described metabolites to those reported by others is not clear. There have been reports of water-soluble metabolites of retinoic acid (Krishnamurthy *et al.* 1963; Zachman & Olson, 1964), some possessing biological activity (Rogers, Chang & Johnson, 1963). It is possible that the biologically active metabolite described in the present paper is similar to that reported by Yagishita, Sundaresan & Wolf (1964).

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