Preparation, Properties and Metabolism of 5,6-Monoepoxyretinoic Acid

By K. V. JOHN, M. R. LAKSHMANAN AND H. R. CAMA
Department of Biochemistry, Indian Institute of Science, Bangalore 12, India

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1. Methyl retinoate has been converted into methyl 5,6-monoepoxyretinoate by reaction with monoperphthalic acid. The epoxy acid ester on alkaline hydrolysis gave 5,6-monoepoxyretinoic acid. 2. Treatment of the 5,6-monoepoxy compounds with ethanolic hydrochloric acid gave the corresponding 5,8-epoxy (furanoid) compounds. 3. With lithium aluminium hydride, the acid and the ester groups were selectively reduced to primary alcohols. 4. Administration of methyl 5,6-monoepoxyretinoate intraperitoneally and subcutaneously had good growth response in vitamin A-deficient rats. 5. 5,6-Monoepoxyretinoic acid, when given intraperitoneally as the sodium salt, was 157% as active as all-*trans*-retinyl acetate. 6. Methyl 5,6-monoepoxyretinoate was hydrolysed to the epoxy acid by rat-liver homogenate. It had 35% of the biological activity of all-*trans*-retinyl acetate in the rat when given orally.

Several attempts have been made to modify the vitamin A molecule to discover the essential structural requirements of the molecule to fulfil its multifarious functions. The high biological activities of the side-chain-oxidized derivative of vitamin A, i.e. retinoic acid (Van Dorp & Arens, 1946; Malathi, Subba Rao, Seshadri Sastry & Ganguly, 1963), as well as the β -ionine-ring-oxidized derivative, i.e. 5,6-monoepoxyvitamin A (Jungalwala & Cama, 1965; Lakshmanan, Jungalwala & Cama, 1965), suggest that 5,6-monoepoxyretinoic acid, which is oxidized both at the side-chain and at the β -ionine ring, may well be biologically more potent than retinoic acid or 5,6-monoepoxyvitamin A.

There have been several claims of isolation of metabolites of retinoic acid that are biologically more potent than retinoic acid after giving the latter to vitamin A-deficient rats (Wolf, Bergan & Sundaresan, 1963; Yagishita, Sundaresan & Wolf, 1964; Dunagin, Zachman & Olson, 1964; Zile & DeLuca, 1965). None of these compounds has yet been fully characterized or obtained in sufficient amounts to test conclusively for their high biological activity.

The present paper describes the preparation, properties, metabolism and biological potency of 5,6-monoepoxyretinoic acid.

EXPERIMENTAL

Synthetic retinoic acid (Hoffmann-La Roche, Basle, Switzerland) was used for the preparation of methyl reti-

noate. All other reagents and chemicals were as described by Jungalwala & Cama (1965). All melting points are corrected.

Spectroscopic measurements. The ultraviolet- and visible-absorption spectra of compounds were measured with a Beckmann spectrophotometer (model DU). Infrared spectra of substances were taken on a Perkin-Elmer (model 137) Infracord spectrometer as Nujol mull.

Preparation of methyl retinoate. Methyl retinoate was prepared from retinoic acid (1.0g.) by using dimethyl sulphate and NaOH (Heilbron, Jones & Wright, 1936). The reaction mixture was chromatographed on a column (3cm. $\times$ 25cm.) of 100g. of 6% (v/w) water-deactivated alumina. Methyl retinoate was eluted with light petroleum (b.p. 40–60°), and was rechromatographed over 5%-(v/w)-water-deactivated alumina column. The pure ester, having $E_{1\text{cm}}^{1\%}$ 1510 at 352m μ in light petroleum, was obtained (yield 760mg.). It crystallized as yellow needles (m.p. 71°) from light petroleum at –30° (Found: C, 80.0; H, 9.5. C₂₁H₃₀O₂ requires: C, 80.3; H, 9.5%).

Preparation of methyl 5,6-monoepoxyretinoate. A 700mg. sample of methyl retinoate was treated with 580mg. (1:1 molar ratio) of freshly prepared monoperphthalic acid (Böhme, 1955) in 300ml. of dry and peroxide-free diethyl ether. The reaction was followed by checking the spectrum of portions of the reaction mixture. The E_{337}/E_{352} ratio increased until it reached a maximum value of 1.2 after 24hr. If the reaction was continued, this ratio decreased, presumably owing to the formation of other oxidation products. Hence the reaction was stopped after 24hr. by the addition of dilute NaHCO₃ solution. The ethereal layer was washed free of alkali, dried over anhydrous Na₂SO₄ and reduced in volume *in vacuo*. The residue was chromatographed on a column (3cm. $\times$ 25cm.) of 100g. of 5% (v/w) water-deactivated alumina. The yellow band of

methyl retinoate could be eluted with light petroleum, whereas the colourless 337 $m\mu$ -absorbing fraction was eluted with 5% (v/v) diethyl ether in light petroleum. This was rechromatographed on a column (2 cm. $\times$ 25 cm.) of 50 g. of 7%-(v/w)-water-deactivated alumina and had $E_{1\text{cm}}^{1\%}$ 1730 at 337 $m\mu$ in light petroleum. The compound crystallized at -30° from light petroleum; the yellow crystals had a sharp m.p. at 90° , which remained constant even after four crystallizations (yield 300 mg.) (Found: C, 76.3; H, 9.4. $\text{C}_{21}\text{H}_{30}\text{O}_3$ requires: C, 76.4; H, 9.1%).

Reduction of methyl 5,6-monoepoxyretinoate by lithium aluminium hydride. Methyl 5,6-monoepoxyretinoate (20 mg.) was treated with 60 mg. of LiAlH_4 in 40 ml. of dry peroxide-free diethyl ether. The reaction was over in 35 min. and the reaction product was 5,6-monoepoxyretinol (Jungalwala & Cama, 1965).

Preparation of 5,6-monoepoxyretinoic acid. Methyl 5,6-monoepoxyretinoate (30 mg.) was saponified with 1.0 ml. of ethanolic 10% (w/v) KOH for 1 hr. under an atmosphere of nitrogen. The unsaponified ester was extracted with light petroleum. Then 1.0N-acetic acid was added dropwise to the aqueous layer with stirring until the solution was just acidic and the liberated acid was extracted with diethyl ether. The ethereal layer was washed free of acid, dried over Na_2SO_4 , reduced in volume *in vacuo* and chromatographed on a column (2 cm. $\times$ 25 cm.) of 10 g. of silicic acid. Pure 5,6-monoepoxyretinoic acid was eluted with 3% (v/v) diethyl ether in light petroleum and had $E_{1\text{cm}}^{1\%}$ 1408 at 338 $m\mu$ in light petroleum. The yellow compound crystallized from light petroleum at -30° and had a sharp m.p. at 153° , which remained unaltered even after four crystallizations (yield 20 mg.) (Found: C, 75.7; H, 8.7. $\text{C}_{20}\text{H}_{28}\text{O}_3$ requires: C, 76.0; H, 8.9%).

The compound was reduced by LiAlH_4 to 5,6-monoepoxyretinol in 30 min.

Preparation of methyl 5,8-monoepoxyretinoate. A 50 mg. sample of methyl 5,6-monoepoxyretinoate in 5 ml. of ethanol was treated with 1 ml. of ethanolic 0.05N-HCl for 2 min. The hypsochromic shift of λ_{max} by about 30 $m\mu$, from 337 $m\mu$ to 306 $m\mu$, indicated the conversion of methyl 5,6-monoepoxyretinoate into the corresponding furanoid compound. The reaction was stopped by the addition of dilute NaHCO_3 solution and the reaction mixture was extracted with diethyl ether. The ethereal layer was washed free of alkali, dried, reduced in volume *in vacuo* and chromatographed on a column (2 cm. $\times$ 30 cm.) of 30 g. of 7%-(v/w)-water-deactivated alumina. Methyl 5,8-monoepoxyretinoate was eluted with light petroleum and rechromatographed on a column (2 cm. $\times$ 25 cm.) of 25 g. of 6%-(v/w)-water-deactivated alumina, when a homogeneous fraction having λ_{max} at 306 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 1250) was obtained. The concentrate was crystallized from light petroleum; the m.p. of the yellow crystals remained constant at 83° , even after four crystallizations (yield 35 mg.) (Found: C, 76.3; H, 9.5. $\text{C}_{21}\text{H}_{30}\text{O}_3$ requires: C, 76.4; H, 9.1%).

The compound was reduced by LiAlH_4 to 5,8-monoepoxyretinol in 20 min. (Jungalwala & Cama, 1965).

Preparation of 5,8-monoepoxyretinoic acid. 5,8-Monoepoxyretinoic acid was prepared from methyl 5,8-monoepoxyretinoate by alkaline hydrolysis as in the preparation of 5,6-monoepoxyretinoic acid from the corresponding acid methyl ester. The compound was chromatographed on a column of silicic acid and the pure fraction having λ_{max} at 306 $m\mu$ ($E_{1\text{cm}}^{1\%}$ 1320) was crystallized from light petroleum

at -30° . The yellow crystals, after four crystallizations, had a sharp m.p. at 164 – 165° (Found: C, 75.5; H, 8.9. $\text{C}_{20}\text{H}_{28}\text{O}_3$ requires: C, 76.0; H, 8.9%).

Chromatographic behaviour. The R_f values of these compounds on thin-layer chromatography (John, Lakshmanan, Jungalwala & Cama, 1965) and reverse-phase chromatography (Jungalwala & Cama, 1962) respectively were as follows: methyl retinoate, 0.85 and 0.26; methyl 5,6-monoepoxyretinoate, 0.70 and 0.33; methyl 5,8-monoepoxyretinoate, 0.64 and 0.54; 5,6-monoepoxyretinoic acid, 0.0 and 0.94; 5,8-monoepoxyretinoic acid, 0.0 and 1.0.

Metabolic experiments. For oral administration, methyl 5,6-monoepoxyretinoate and retinyl acetate were prepared in refined deodorized groundnut oil containing 0.5% of α -tocopherol (concentrations 0.5 and 1.0 $\mu\text{g.}/0.05\text{ ml.}$ of diluent oil). They were administered to animals with an Agla micrometer syringe (Burroughs Wellcome, Beckenham, Kent). 5,6-Monoepoxyretinoic acid was dissolved in ethanol and neutralized with dilute NaOH. α -Tocopherol was added and, after removing light petroleum, the solution was suitably diluted with 0.1M- Na_2HPO_4 solution, adjusted to pH 10.5 to give the required concentration.

For intraperitoneal administration, the standard and test substances were dissolved in ethanol. 5,6-Monoepoxyretinoic acid was prepared as in oral feeding, but the dilution was made with 0.1M- Na_2HPO_4 solution containing the required amount of Tween 20. α -Tocopherol and Tween 20 were added to the retinyl acetate solution in ethanol, and, after removal of the light petroleum, the solution was suitably diluted with 0.1M- Na_2HPO_4 solution. The final dispersions contained 0.05 mg. of α -tocopherol, 0.3 mg. of Tween 20 and 0.5, 1.0 and 2.0 $\mu\text{g.}$ of the standard and test substances in 0.1 ml. of the solution. The solutions were freshly prepared every 3 days.

Male albino rats of this Institute strain maintained on normal stock colony diet were used, when they reached the plateau stage of vitamin A deficiency (Lakshmanan *et al.* 1965). The biological assays were carried out essentially as described by Bliss & György (1951) according to USP XIV (1950) specifications.

RESULTS

The structures of the parent 5,6- and 5,8-monoepoxyvitamin A compounds have been described previously (Jungalwala & Cama, 1965).

The $E_{1\text{cm}}^{1\%}$ values of these epoxyretinoic acid compounds are higher than those of the corresponding parent compounds as well as the 5,8-furanoid compounds, as is evident from Table 1.

The ultraviolet-absorption spectra of 5,6-monoepoxyretinoic acid in different solvents are shown in Fig. 1.

In the infrared spectra, the shoulder at 3145 cm.^{-1} present in those of the 5,6-monoepoxy compounds was absent from those of the 5,8-monoepoxy compounds and also the parent compounds. A peak at 1718 cm.^{-1} was present in the ester compounds, and is due to the ester carbonyl group. The free acid compounds showed a broad peak at about 2632 cm.^{-1} due to 'OH' of the carboxyl group. The other

Table 1. Spectroscopic properties of various derivatives of retinoic acid

Compound	Light petroleum		Cyclohexane		Ethanol		Chloroform		SbCl ₃ colour test ($E_{1\text{cm}}^{1\%}$ at 530m μ)
	λ_{max} (m μ)	$E_{1\text{cm}}^{1\%}$	λ_{max} (m μ)	$E_{1\text{cm}}^{1\%}$	λ_{max} (m μ)	$E_{1\text{cm}}^{1\%}$	λ_{max} (m μ)	$E_{1\text{cm}}^{1\%}$	
Methyl retinoate	352	1658	353	1148	351	1292	362	1084	—
Methyl 5,6-monoepoxy-retinoate	337	1914	340	1745	342	1532	345	1562	702
Methyl 5,8-monoepoxy-retinoate	306	1406	308	1173	308	1152	312	1157	670
Retinoic acid	351	1510	358	1230	347	1430	363	1260	—
5,6-Monoepoxyretinoic acid	338	1556	345	1340	336	1442	346	1360	532
5,8-Monoepoxyretinoic acid	306	1452	309	1239	298	1257	312	1235	407

Fig. 1. Ultraviolet-absorption spectra of 5,6-monoepoxyretinoic acid in: ○, light petroleum; ●, cyclohexane; △, ethanol; ▲, chloroform.

characteristics were similar to those already reported (Jungalwala & Cama, 1965).

Reaction with antimony trichloride reagent. With antimony trichloride reagent, these epoxy and furanoid derivatives of retinoic acid gave stable pink colour having λ_{max} at 530m μ . The colour development was slower than with other vitamin A compounds. The extinction values reached maximum intensity 1½–2 hr. after adding the reagent and then the colour intensity decreased. The $E_{1\text{cm}}^{1\%}$ values of the antimony trichloride colour test for the various compounds were recorded when the extinction values reached the maximum.

Oral administration. Methyl 5,6-monoepoxyretinoate (1mg./rat) when administered to vitamin A-deficient rats was presumably metabolized so rapidly that attempts to detect it or any of its metabolic derivative in various tissues even at different time-intervals were unsuccessful. However, when 5mg. of the compound was given orally

to a vitamin A-deficient rat, it could be detected in liver 4 hr. after administration.

Intraperitoneal administration. Intraperitoneal administration of methyl 5,6-monoepoxyretinoate at a dosage of 1mg./rat/day for 7 days to four vitamin A-deficient rats failed to show the presence of the compound anywhere in the body. At the same time, all the rats were relieved of vitamin A-deficiency symptoms and gained on an average 20g. in weight during this period.

Subcutaneous administration. A single subcutaneous administration of methyl 5,6-monoepoxyretinoate at as low a dosage as 4 μ g./rat to two vitamin A-deficient rats alleviated their vitamin A-deficiency symptoms within 2 days and led to a gain of 18g. in body weight on an average in a fortnight, after which their weights levelled off. Evidently, it was too small a dose to maintain normal growth of the animal for a prolonged period. However, when a single dose of 1mg. of the compound/rat was administered to two vitamin A-deficient rats, the animals gained on an average 35g. in weight in 4 weeks.

Hydrolysis of methyl 5,6-monoepoxyretinoate by rat-liver homogenate. Sebrell & Harris (1954) have reported that methyl retinoate is readily hydrolysed to free retinoic acid when incubated with rat-liver homogenate (cf. Olson, 1964). Incubation of methyl 5,6-monoepoxyretinoate with rat-liver homogenate also gave 5,6-monoepoxyretinoic acid, as evidenced by its chromatographic behaviour on thin-layer and reverse-phase chromatographic systems.

Biological potency of methyl 5,6-monoepoxyretinoate. The growth rate (g./week) over 4 weeks of individual litter-mate rats receiving methyl 5,6-monoepoxyretinoate and retinyl acetate orally was measured. All the negative controls lost considerable weight and died before the end of the assay. The mean weekly gain in weight (4.0 and 6.1g. for

methyl 5,6-monoepoxyretinoate and 9.6 and 13.0 g. for all-*trans*-retinyl acetate for the daily dosages of 0.5 and 1.0 μ g. of the compounds respectively) plotted against the logarithm of dose gave lines parallel to each other for the standard and test doses. The biological potency of methyl 5,6-monoepoxyretinoate administered orally, as compared with that of retinyl acetate, was found to be 35% (in molar terms).

Biological potency of 5,6-monoepoxyretinoic acid. 5,6-Monoepoxyretinoic acid was administered orally as the sodium salt at dosages of 0.5 and 1.0 μ g., the same standard doses being kept for all-*trans*-retinyl acetate. Each dosage was given to eight vitamin A-deficient rats. All the rats receiving 5,6-monoepoxyretinoic acid decreased in weight and some of them died by the third week of the experiment, while the rats receiving the standard compound gained weight as usual.

Surprisingly, when 5,6-monoepoxyretinoic acid was given intraperitoneally at dosages of 0.5, 1.0 and 2.0 μ g. to vitamin A-deficient rats, the same doses being kept for all-*trans*-retinyl acetate under the same conditions, each dose being given to eight rats, the animals receiving 5,6-monoepoxyretinoic acid showed considerable increase in weight compared with the standards. All the negative controls lost considerable weight and most of them died before the end of the bioassay. The mean weekly gain in weight plotted against the logarithm of the dose gave lines for the standard and test doses that were parallel to each other (Fig. 2). The biopotency of 5,6-monoepoxyretinoic acid as compared with that of all-*trans*-retinyl acetate was 157%.

Fig. 2. Response of male albino rats to all-*trans*-retinyl acetate (○) and 5,6-monoepoxyretinoic acid (●) given intraperitoneally. A, B and C represent the projections of the growth of the rats receiving 0.5, 1.0 and 2.0 μ g. of all-*trans*-retinyl acetate respectively on the growth curve of 5,6-monoepoxyretinoic acid.

Growth of rats maintained on methyl 5,8-monoepoxyretinoate. Methyl 5,8-monoepoxyretinoate (1 μ g./rat/day) was administered orally to eight vitamin A-deficient rats for 4 weeks. The average weight gain (g./week) was 2 g., which was one-third that of rats receiving a similar dose of methyl 5,6-monoepoxyretinoate. Methyl 5,8-monoepoxyretinoate has much less biological activity than the corresponding 5,6-monoepoxy compound.

On administering 5,6-monoepoxyretinoic acid or methyl 5,6-monoepoxyretinoate intraperitoneally or subcutaneously at a single dose of 4.0 μ g./rat/day to vitamin A-deficient rats, their eye lesions were cured. But rats subsisting on 5,6-monoepoxyretinoic acid as the sole vitamin A substitute eventually showed signs of blindness in 2 months, as judged by their physical behaviour. This may be because 5,6-monoepoxyretinoic acid is not reduced *in vivo* to the alcohol or the aldehyde form to take part in vision (Lakshmanan & Cama, 1966). However, this aspect could not be unequivocally proved since facilities for taking an electroretinogram of 5,6-monoepoxyretinoic acid-fed rats were not available.

DISCUSSION

The epoxidation of methyl retinoate results in the hypsochromic shift of the absorption maximum by 15 m μ (Table 1), which is similar to that found for retinyl acetate (Jungalwala & Cama, 1965). Retinoic acid itself, on reaction with monoperphthalic acid, gives rise to the corresponding 5,6-monoepoxide, but, owing to the strong adsorption characteristics of these free acid compounds, they could not be separated on an alumina column. By contrast, methyl retinoate could be easily separated from methyl 5,6-monoepoxyretinoate by chromatography on a column of deactivated alumina and the free epoxy acid could be obtained from the epoxy acid ester on hydrolysis.

There are different reports about the biological activity of retinoic acid, depending on the mode of assay (Arens & Van Dorp, 1946; Van Dorp & Arens, 1946; Boguth, Horn, Soliman & Weiser, 1960; Murray, 1962; Malathi *et al.* 1963; De Man, Van Leeuwen & Roborgh, 1964; Baxter, 1963). The biopotency of retinoic acid in various assays has been reviewed by Ames (1965). Baxter (1963) failed to demonstrate a high degree of biological activity for retinoic acid when administered orally. He attributed the lack of activity to the probable destruction of the compound on storage. In our assays, 5,6-monoepoxyretinoic acid also failed to promote growth of vitamin A-deficient rats by oral administration, even though the compound was freshly prepared every 3 days. The freshly prepared compound, however, when administered by the intraperitoneal route, showed profound biological

activity. This clearly shows that the inability of the compound to exert its biological activity, when administered orally, is not due to its destruction on storage, but may well be due to its destruction during passage through the stomach or due to its rapid excretion from the body.

It is noteworthy that 5,6-monoepoxyretinoic acid is 157% as active as all-*trans*-retinyl acetate when administered intraperitoneally. This is the first time such a high biological activity has been reported for any of the metabolic congeners of vitamin A (cf. Ames, 1965). However, as explained by Thompson (1965) for retinoic acid, the enhanced activity of 5,6-monoepoxyretinoic acid was deduced when the growth response of rats for 5,6-monoepoxyretinoic acid given intraperitoneally was compared with the growth response for retinyl acetate by a similar route. It is apparent that the growth response of retinyl acetate by the intraperitoneal route is comparatively poorer than by the oral route. Nevertheless, when the growth response of 5,6-monoepoxyretinoic acid by the intraperitoneal route is compared with similar response of retinyl acetate by the oral route, a biological activity approximately the same (108%) as that of 5,6-monoepoxyretinoic acid is obtained. Consequently, 5,6-monoepoxyretinoic acid, which is in a more oxidized state than retinoic acid as well as 5,6-monoepoxyretinal, has profound biological activity when administered intraperitoneally and it may well be that it is far ahead of any of these vitamin A derivatives in the proposed scheme of active metabolism of vitamin A (Dowling & Wald, 1960). Wolf *et al.* (1963), after giving retinoic acid to vitamin A-deficient rats, obtained an acidic fraction containing an 'active vitamin A compound' from the intestinal lipid extract. This fraction had an absorption spectrum with inflexions at 310–315 and 335 μ . This may very well be compatible with the fractions containing 5,6-monoepoxyretinoic acid.

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