Preparation, Properties and Metabolism of 5,6-Monoepoxy-3-Dehydroretinal

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(Received 4 September 1968)

1. 5,6-Monoepoxy-3-dehydroretinal was synthesized from 3-dehydroretinyl acetate and characterized. 2. When fed to vitamin A-deficient rats, 5,6-monoepoxy-3-dehydroretinal was converted into 5,6-monoepoxy-3-dehydrovitamin A and stored in the liver. 3. It was demonstrated that the rat possesses the necessary enzymes for the reduction and oxidation of 5,6-monoepoxy-3-dehydroretinal to the corresponding alcohol and acid respectively. 4. The biological potency of the epoxy-3-dehydroretinal by the rat-growth assay (determined by USP XIV procedure) was 1.07% of that of vitamin A.

The 5,6-monoepoxy derivatives of vitamin A were found to exert profound growth-promoting activity in vitamin A-deficient rats (Lakshmanan, Jungalwala & Cama, 1965; John, Lakshmanan & Cama, 1967). They were metabolized uniquely without giving rise to vitamin A anywhere in the body, and were utilized as efficiently as the vitamin. The epoxy derivatives of 3-dehydrovitamin A are not known. The present paper describes the preparation, properties and metabolism of 5,6-monoepoxy-3-dehydroretinal, and the assay of its biological potency by the precise USP XIV procedure on male albino rats.

MATERIALS AND METHODS

3-Dehydroretinal was prepared from the liver oil of the Indian freshwater fish *Wallago attu* (Sundaresan & Cama, 1961) and was reduced with LiAlH_4 to 3-dehydroretinol, which was then acetylated with acetyl chloride to give 3-dehydroretinyl acetate. This was purified by chromatography over alumina deactivated with 6% (v/w) of water and had $E_{1\text{cm}}^{1\%}$ 1250 at $350\text{ m}\mu$ in light petroleum (b.p. 40–60°). 3-Dehydroretinyl acetate on reaction with an equimolar quantity of monoperphthalic acid for 12 hr. yielded 5,6-monoepoxy-3-dehydroretinyl acetate. This was purified by chromatography over alumina deactivated with 5% (v/w) of water. The compound crystallized at -30° from light petroleum (b.p. 40–60°). The m.p. of the pale-yellow crystals remained constant at 85–86° through four crystallizations from light petroleum (b.p. 40–60°) (Found: C, 77.1; H, 8.8; $\text{C}_{22}\text{H}_{30}\text{O}_3$ requires C, 77.2; H, 8.8%).

5,6-Monoepoxy-3-dehydroretinyl acetate was saponified with ethanolic 10% (w/v) KOH to give the epoxy alcohol (λ_{max} , 313 $\text{m}\mu$), which, on oxidation with MnO_2 , yielded 5,6-monoepoxy-3-dehydroretinal (λ_{max} , 353 $\text{m}\mu$). This was purified by chromatography over alumina deactivated with 5% (v/w) of water. The compound crystallized at -30° from light petroleum (b.p. 40–60°). The m.p. of the yellow

crystals remained constant at 90–91° through four crystallizations (Found: C, 80.8; H, 8.8; $\text{C}_{20}\text{H}_{26}\text{O}_2$ requires: C, 80.5; H, 8.7%).

5,6-Monoepoxy-3-dehydroretinyl palmitate was prepared from 3-dehydroretinyl palmitate by the same method as that used for the preparation of the corresponding acetate.

The ultraviolet- and visible-absorption spectra of compounds were measured with a Beckman model DU spectrophotometer. The infrared spectrum was measured with a Perkin-Elmer model 137 Infracord spectrometer on a Nujol mull. The nuclear-magnetic-resonance spectrum was recorded on a Varian A-60 spectrometer, with tetramethylsilane as the reference standard, at the National Chemical Laboratory, Poona, India. All other reagents and chemicals were the same as described by Jungalwala & Cama (1965) and Lakshmanan *et al.* (1965).

Oral administration. The compound under study was prepared in refined deodorized groundnut oil containing 0.5% of α -tocopherol, and was stored at -20° . It was administered to the animals with an Agla micrometer syringe (Burroughs Wellcome). For bioassay experiments, 5,6-monoepoxy-3-dehydroretinal (concn. 10 and 15 $\mu\text{g./0.1 ml.}$ of diluent oil) and all-*trans*-retinyl acetate (concn. 0.5 and 1.0 $\mu\text{g./0.1 ml.}$ of diluent oil) solutions were freshly prepared every week.

Preparation of tissue extracts. The procedure followed was essentially that described by Glover, Goodwin & Morton (1948).

Preparation of enzymes. Rat liver alcohol dehydrogenase was prepared by the modified method of Bonnichsen & Brink (1955). Rat liver aldehyde oxidase was prepared as described by Lakshmanan, Vaidyanathan & Cama (1964).

Assay of reductase and oxidase reactions. The reaction mixtures were the same as those described by Lakshmanan *et al.* (1965), except that the substrate used was 5,6-monoepoxy-3-dehydroretinal. The enzyme protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Specific activities are defined as μmoles of 5,6-monoepoxy-3-dehydroretinal utilized/mg. of protein for the time indicated.

Separation and identification of various epoxides of

3-dehydrovitamin A. (a) Adsorption chromatography. A mixture (100 μ g. of each component) of 5,6-monoepoxy-3-dehydroretinyl acetate, 5,6-monoepoxy-3-dehydroretinal and 5,6-monoepoxy-3-dehydroretinoic acid was quantitatively separated by chromatography on a column of alumina deactivated by 7% (v/w) of water. The ester, the aldehyde and the alcohol of 5,6-monoepoxy-3-dehydrovitamin A were eluted successively with 3, 10–12 and 20–25% (v/v) diethyl ether in light petroleum (b.p. 40–60°) respectively. When a mixture of 5,6-monoepoxy-3-dehydroretinal and 5,6-monoepoxy-3-dehydroretinoic acid (100 μ g. of each) was chromatographed on alumina deactivated with 10% (v/v) of water, the aldehyde fraction was completely eluted with light petroleum (b.p. 40–60°); the acid fraction, which was strongly adsorbed on the column, was eluted from the column extruded adsorbent with aq. NH_3 (sp.gr. 0.88)–ethanol (3:7, v/v). The alkaline extract was acidified with 1.0N-acetic acid, and 5,6-monoepoxy-3-dehydroretinoic acid was re-extracted quantitatively with diethyl ether.

(b) Thin-layer chromatography on Kieselgel (E. Merck A.-G.). Chromatograms were developed with 6% (v/v) acetone in light petroleum (b.p. 40–60°) (200 ml.) for about 2 hr. (John, Lakshmanan, Jungalwala & Cama, 1965). The R_F values were: 5,6-monoepoxy-3-dehydroretinyl palmitate, 0.74; 5,6-monoepoxy-3-dehydroretinyl acetate, 0.71; 5,6-monoepoxy-3-dehydroretinal, 0.49; 5,6-monoepoxy-3-dehydroretinol, 0.13; 5,6-monoepoxy-3-dehydroretinoic acid, 0.00. The compounds were identified by comparing their R_F values with those of authentic samples, as well as by co-chromatography with the authentic samples, in the above solvent system. They had characteristic absorption spectra after elution from the chromatograms and gave a specific SbCl_3 colour test.

Determinations. 5,6-Monoepoxy-3-dehydroretinal was measured by the thiobarbituric acid procedure (Futtermann & Saslaw, 1961), 510 $m\mu$ being taken as the absorption maximum. 5,6-Monoepoxy-3-dehydrovitamin A was measured by the SbCl_3 colour-test method, by using $E_{1\text{cm}}^{1\%}$ 1340 at 450 $m\mu$. When 5,6-monoepoxy-3-dehydroretinal and the alcohol could not be separated by chromatography, the former was measured by the thiobarbituric acid procedure (Futtermann & Saslaw, 1961) and the latter by the SbCl_3 colour test by applying the correction formula of Cama, Collins & Morton (1951). 5,6-Monoepoxy-3-dehydroretinoic acid was measured spectrophotometrically by using $E_{1\text{cm}}^{1\%}$

1390 at 340 $m\mu$. For bioassay, male albino rats of this Institute's strain, were made deficient in vitamin A and were then used (Lakshmanan *et al.* 1965).

RESULTS

The spectroscopic properties of the epoxides of 3-dehydrovitamin A are given in Table 1. Epoxidation of 3-dehydrovitamin A results in the hypsochromic shift of λ_{max} , by about 40 $m\mu$ owing to the loss in conjugation of two double bonds. Unlike 5,6-epoxyvitamin A, these compounds do not undergo isomerization to the 5,8-epoxides (furanoid compounds) on treatment with ethanolic hydrochloric acid.

In the infrared-absorption spectrum of 5,6-monoepoxy-3-dehydroretinal, a shoulder at 1252 cm^{-1} was present; this is due to symmetrical stretching of the epoxide ring (Patterson, 1954). The bands at 903 and 843 cm^{-1} also indicate an epoxide structure (Patterson, 1954; Bomstein, 1958). A strong band at 1654 cm^{-1} is attributed to the $\alpha\beta$ -unsaturated $\text{C}=\text{O}$ group in the molecule. The nuclear-magnetic-resonance spectrum shows that the carbon atoms carrying the epoxide bridge do not carry any protons [absence of any signal between 3.0 and 5.5 p.p.m. (4.5–7.0 τ)], as expected from the 5,6-epoxy structure (cf. Jungalwala & Cama, 1965; Morgan & Thompson, 1966). In the lower field (7.4–5.7 p.p.m.; 4.3–2.6 τ) all the eight protons on the double bond are available.

Oral administration. Four vitamin A-deficient rats were given 5,6-monoepoxy-3-dehydroretinal (1.0 mg./rat/day) orally for 7 days. The average gain in weight of the rats during this period was 15 g. They were anaesthetized and killed 24 hr. after the last dose. The livers of the rats were ground with anhydrous sodium sulphate and then extracted with diethyl ether. The solvent was removed *in vacuo* and the oil was dissolved in light petroleum (b.p. 40–60°). Chromatography on

Table 1. Spectroscopic properties of 5,6-monoepoxy-3-dehydroretinyl acetate, 5,6-monoepoxy-3-dehydroretinol and 5,6-monoepoxy-3-dehydroretinal in different solvents

Solvent	5,6-Monoepoxy-3-dehydroretinyl acetate		5,6-Monoepoxy-3-dehydroretinol		5,6-Monoepoxy-3-dehydroretinal	
	$\lambda_{\text{max.}}$ ($m\mu$)	$E_{1\text{cm.}}^{1\%}$	$\lambda_{\text{max.}}$ ($m\mu$)	$E_{1\text{cm.}}^{1\%}$	$\lambda_{\text{max.}}$ ($m\mu$)	$E_{1\text{cm.}}^{1\%}$
Light petroleum (b.p. 40–60°)	313	1854	313	2230	353	1879
Cyclohexane	315	1695	313	2105	355	1650
Chloroform	317	1690	318	2085	366	1550
Ethanol	313	1757	313	2206	367	1526
SbCl_3 colour test	450* (Greenish blue)	1178	450* (Greenish blue)	1340	540† (Violet)	2730

* Initial colour pink, changing to greenish blue (450 $m\mu$) instantaneously.

† Initial colour brick-red, changing to violet (540 $m\mu$) instantaneously.

Table 2. *Stoicheiometry of rat liver alcohol dehydrogenase reaction*

5,6-Monoepoxy-3-dehydroretinal (250 mμmoles) was incubated with increasing concentrations of the enzyme under standard conditions. The results are expressed as mμmoles/reaction mixture after incubation for 1 hr. For details see the text.

Wt. of enzyme (mg.)	5,6-Monoepoxy-3-dehydroretinal utilized.	5,6-Monoepoxy-3-dehydroretinol formed	Specific activity*
0	0	0	
1	15	15	
2	30	30	
3	45	45	
4	60	60	
5	75	75	15.0

* The specific activities towards retinal and 5,6-monoepoxyretinal under identical conditions were found to be 16.1 and 18.3 respectively.

Table 3. *Stoicheiometry of rat liver aldehyde oxidase reaction*

5,6-Monoepoxy-3-dehydroretinal (1.0 μmole) was incubated with increasing concentrations of the enzyme under standard conditions. The results are expressed as mμmoles/reaction mixture after incubation for 30 min. For details see the text.

Wt. of enzyme (mg.)	5,6-Monoepoxy-3-dehydroretinal utilized	5,6-Monoepoxy-3-dehydroretinoic acid formed	Specific activity*
0	0	0	
1.0	39	39	
1.5	59	59	
2.0	78	78	
2.5	98	98	39.0

* The activities towards retinal, 5,6-monoepoxyretinal and 3-dehydroretinal under identical conditions were found to be 273.2, 283.3 and 133.4 respectively.

Fig. 1. Ultraviolet-absorption spectra in light petroleum of 5,6-monoepoxy-3-dehydroretinal (○) and 5,6-monoepoxy-3-dehydroretinoic acid (●) formed enzymically from 5,6-monoepoxy-3-dehydroretinal (Δ).

alumina deactivated with 7% (v/w) of water showed that the liver lipid extract contained both esterified and free 5,6-monoepoxy-3-dehydroretinol. This was confirmed by the behaviour of these substances on thin-layer chromatography (John *et al.* 1965). With antimony trichloride reagent, the lipid extract gave a pink colour, which changed instantaneously to greenish blue with λ_{max}. 450 mμ. The conversion was about 6%.

The metabolism of 5,6-monoepoxy-3-dehydroretinal when administered intraperitoneally was similar to that when it was given orally.

Enzymic reduction of 5,6-monoepoxy-3-dehydroretinal to the corresponding alcohol by rat liver alcohol

dehydrogenase. The formation of 5,6-monoepoxy-3-dehydroretinol was stoicheiometric with the disappearance of 5,6-monoepoxy-3-dehydroretinal (Table 2). The reduction product was characterized by its typical absorption spectrum (Fig. 1), antimony trichloride colour test and chromatographic behaviour. The specific activity of the enzyme towards 5,6-monoepoxy-3-dehydroretinal was 15.0 mμmoles/mg. of protein/hr.

Enzymic oxidation of 5,6-monoepoxy-3-dehydroretinal to the corresponding acid by rat liver aldehyde oxidase. The amount of 5,6-monoepoxy-3-dehydroretinal that disappeared was proportional to the concentration of the enzyme protein (Table 3), the specific activity being 39.2 mμmoles/mg. of protein/30 min. The product of the enzymic reaction was characterized by its typical absorption spectrum (Fig. 1) with λ_{max}. 340 mμ, and was identical with chemically synthesized 5,6-monoepoxy-3-dehydroretinoic acid.

Thus, the studies showed that rats possess the necessary enzymes for the metabolic reduction and oxidation of 5,6-monoepoxy-3-dehydroretinal.

Biological potency of crystalline 5,6-monoepoxy-3-dehydroretinal. The bioassay was carried out in accordance with the USP XIV procedure (Bliss & György, 1951). The growth rates (g./week) over 4 weeks of individual litter mates receiving daily oral doses of 10 and 15 μg. of 5,6-monoepoxy-3-dehydroretinal or of 0.5 and 1.0 μg. of all-*trans*-retinyl acetate were measured. All the negative controls lost considerable weight and most of them died before the end of the assay. The mean weekly gain in weight plotted against the logarithm of dose gave lines for the standard and test doses that were parallel to each other. The biological potency

of 5,6-monoepoxy-3-dehydroretinal, compared with all-*trans*-retinyl acetate, was only 1.07%. Groups of vitamin A-deficient rats were also kept on lower doses of the compound (daily doses of 1.0, 2.0, 2.5 and 5.0 $\mu\text{g.}$). Some of the rats kept on daily doses of 1.0 and 2.0 $\mu\text{g.}$ died by the fourth week of the assay, whereas the rats given 2.5 and 5.0 $\mu\text{g./day}$ survived till the end of the assay; however, they lost weight each week.

DISCUSSION

Oxidation of 3-dehydroretinyl acetate with monoperphthalic acid gave 5,6-monoepoxy-3-dehydroretinyl acetate. On hydrolysis, 5,6-monoepoxy-3-dehydroretinyl acetate yielded 5,6-monoepoxy-3-dehydroretinol, which was readily oxidized with manganese dioxide to 5,6-monoepoxy-3-dehydroretinal. From the ultraviolet, infrared and nuclear-magnetic-resonance spectra of 5,6-monoepoxy-3-dehydroretinal, we conclude that it is a 5,6-epoxide.

5,6-Monoepoxy-3-dehydroretinal was shown to alleviate all the symptoms of vitamin A deficiency in the rat. Since no 3-dehydrovitamin A was detected anywhere in the body, 5,6-monoepoxy-3-dehydroretinal apparently exerted its biological activity *per se*, and not through 3-dehydrovitamin A. When 5,6-monoepoxy-3-dehydroretinal was administered orally or intraperitoneally, 5,6-monoepoxy-3-dehydrovitamin A accumulated in the liver.

It was demonstrated that the rat possesses the necessary enzymes for the conversion of 5,6-monoepoxy-3-dehydroretinal into the corresponding acid and alcohol. The specific activity of rat liver alcohol dehydrogenase towards 5,6-monoepoxy-3-dehydroretinal (Table 2) is slightly less than that towards retinal and 5,6-monoepoxy-retinal. The rate of oxidation of 5,6-monoepoxy-3-dehydroretinal to the corresponding acid by liver aldehyde oxidase is very low compared with that for retinal, 3-dehydroretinal and 5,6-monoepoxyretinal (Table 3).

The biological potency of 5,6-monoepoxy-3-dehydroretinal was only 1.07% of that of all-*trans*-

retinyl acetate. 3-Dehydrovitamin A was shown to be nearly one-third as active as vitamin A (Shantz & Brinkman, 1950; Sundaresan & Cama, 1961). Unlike 5,6-monoepoxyvitamin A, which is almost comparable to vitamin A in its growth-promoting activity, the epoxide of 3-dehydrovitamin A is much less effective than 3-dehydrovitamin A in promoting growth. It may well be that the low activity of the epoxide is due to its poor absorption and utilization.

We thank Dr P. Madhavan Nair, National Chemical Laboratory, Poona, India, for kindly arranging to measure the nuclear-magnetic-resonance spectrum. The technical assistance of Mr C. Venkateshan is gratefully acknowledged.

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