Biochem. J. (1966) 101, 835

The Preparation and Biological Activity of Methyl 5,6-Epoxyretinoate

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(*Received* 27 *June* 1966)

1. Oxidation of methyl retinoate with monoperphthalic acid gave methyl 5,6-epoxyretinoate, obtained as pale-yellow crystals, m.p. 89°. 2. The structure of the epoxide was confirmed by its ultraviolet, infrared, nuclear-magnetic-resonance and mass spectra. 3. The biological properties of the epoxide were investigated in male and female rats, and were found to be qualitatively similar to those of retinoic acid and methyl retinoate. 4. When administered to male rats reared on a vitamin A-free diet, the epoxide permitted growth although it did not maintain good general health. 5. Rats given a vitamin A-free diet and supplements of the epoxide had degenerate testes. 6. Female rats, maintained on a vitamin A-free diet containing retinoic acid and given supplements of the epoxide during pregnancy, resorbed their foetuses and failed to deliver litters. 7. The threshold of the electroretinogram response in male rats reared on a vitamin A-free diet with supplements of the epoxide was elevated above normal and was similar to that of rats maintained with methyl retinoate. 8. The oral administration of the epoxy acid to rats did not result in the accumulation of the corresponding epoxy alcohol in their livers.

Epoxides of retinyl acetate, retinol and retinal have been prepared and found to have biological activity (Lakshmanan, Jungalwala & Cama, 1965; Jungalwala & Cama, 1965). Retinyl acetate was converted into a 5,6-epoxide by reaction with monoperphthalic acid. The 5,6-epoxyretinyl acetate was saponified to give the epoxy alcohol (5,6-epoxyretinol), which, on oxidation with manganese dioxide, yielded the corresponding epoxy aldehyde (5,6-epoxyretinal). The 5,6-epoxides isomerized in the presence of mineral acid to furanoid 5,8-epoxides.

In a bioassay based on rat growth, 5,6-epoxyretinal was shown to have biological activity equal to that of retinyl acetate, but no evidence was obtained to suggest that the epoxides reverted to the true vitamins. Metabolic studies indicated that interconversions took place analogous to those known to occur among the parent compounds: thus (*a*) 5,6-epoxyretinal was reduced to 5,6-epoxyretinol, which was then stored in the liver as an epoxy ester, and (*b*) enzymes were obtained that, *in vitro*, oxidized 5,6-epoxyretinal to the corresponding 5,6-epoxyretinoic acid.

There have been no reports of the preparation of crystalline epoxides of retinoic acid, and Jungalwala & Cama (1965) did not describe biological tests on their crude material obtained by enzymic methods.

Retinoic acid is known to have biological activity in the rat when assayed in growth tests (van Dorp & Arens, 1946; Malathi, Subba Rao, Seshadri Sastry & Ganguly, 1963), although it does not maintain vision (Dowling & Wald, 1960) or reproduction (Thompson, Howell & Pitt, 1964). It has been suggested that the biological properties of retinol and retinal, other than those concerned with vision and reproduction, could require conversion into the acid (Dowling & Wald, 1960). An investigation of the biological properties of 5,6-epoxyretinoic acid therefore seemed necessary as it might support or refute a similar mechanism with respect to the epoxides. This approach was further encouraged by the suggestion of Lakshmanan *et al.* (1965) that the epoxy acid might be a normal metabolite of vitamin A.

Preliminary experiments revealed that an epoxide of methyl retinoate could be prepared by direct reaction with monoperphthalic acid. In the present paper we describe the preparation and properties of the substance (I) together with the results of biological tests of its efficacy for rats.

EXPERIMENTAL

Materials. Crystalline methyl retinoate was prepared from retinoic acid by the method of Robeson (1952). Monoperphthalic acid was prepared from phthalic anhydride and hydrogen peroxide (Royals & Harrell, 1955). Diethyl ether was dried over sodium wire, and was redistilled from reduced iron immediately before use. Benzene and light petroleum (b.p. 40–60°) were dried over sodium

wire and redistilled. Other solvents were spectroscopically pure.

Spectroscopic measurements. Ultraviolet spectra were determined in cyclohexane solution with a Unicam SP.800 recording spectrophotometer unless otherwise stated. Infrared spectra were measured in KBr disks with a Perkin–Elmer 237 recording spectrometer. Nuclear-magnetic-resonance (n.m.r.) spectra were recorded in carbon tetrachloride solution with a Varian A–60 instrument, with tetramethylsilane as a reference standard. Mass spectra were recorded with an Associated Electrical Industries MS 9 mass spectrometer.

Preparation of epoxides of methyl retinoate. The method was similar to that used by Jungalwala & Cama (1965) in the preparation of 5,6-epoxyretinyl acetate. In a typical preparation, methyl retinoate was mixed at room temperature with monoperphthalic acid in ether solution in a 1:1 molar ratio. The conversion into the epoxide was followed spectrophotometrically until the $\lambda_{max.}$ 350 mμ changed to $\lambda_{max.}$ 337 mμ, when an equal volume of aq. 10% (w/v) $NaHCO_3$ was added to stop the reaction. The ether layer was washed several times with distilled water, dehydrated over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was dissolved in light petroleum and chromatographed on a column of acid-washed alumina (Spence) deactivated by the addition of 5% (v/w) of water. By using increasing concentrations of diethyl ether in light petroleum as eluents, unchanged methyl retinoate was eluted in the 2%-ether fraction ($\lambda_{max.}$ 354 mμ), methyl 5,6-epoxyretinoate was eluted in the 4–6%-ether fraction ($\lambda_{max.}$ 340 mμ) and methyl 5,8-epoxyretinoate was eluted in the 100%-ether fraction ($\lambda_{max.}$ 307 mμ). The crude samples of methyl 5,6-epoxyretinoate were rechromatographed on columns of alumina and were then recrystallized several times from methanol to give pale-yellow crystals, m.p. 89°. In cyclohexane, the substance showed two absorption peaks at 342 and 356 mμ ($E^{1\%}_{1cm.}$ 1670 and 1290 respectively) with a shoulder at 325–330 mμ. The yield of crystalline 5,6-epoxide varied in different preparations from 10 to 60%.

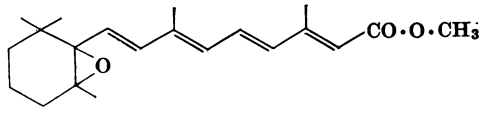
Basal vitamin A-free diet. The basal diet consisted of (percentages): sucrose, 58·3; casein, 20; arachis oil, 4; cellulose, 3; dried brewer's yeast, 8; methionine, 0·5; choline chloride, 0·2; minerals, 4; B vitamins in glucose, 1; fat-soluble vitamins in oil, 1. The minerals were Salts G of Fox & Briggs (1960) with the following additions to each 60 kg.: $Na_2MoO_4,2H_2O$, 5 g.; Na_2SeO_3, 200 mg.; NaF, 500 mg.; $CoCl_2,6H_2O$, 2 g.; H_3BO_3, 10 g.; $K_2Al_2(SO_4)_4,24H_2O$, 50 g.; $Na_2SiO_3,9H_2O$, 50 g. The oil solution of fat-soluble vitamins in arachis oil contained: 1 mg. of ergocalciferol and 15 g. of α-tocopheryl acetate/kg. The B vitamin mix in glucose contained (g./kg.): inositol, 40; calcium pantothenate, 5; nicotinic acid, 2; *p*-aminobenzoic acid, 1; riboflavine, 1; pyridoxine hydrochloride, 0·5; thiamine hydrochloride, 0·5; menadione, 0·5; folic acid, 0·1; vitamin B_{12}, 0·005; biotin, 0·003. Supplements of retinyl acetate, methyl retinoate and methyl 5,6-epoxyretinoate were dissolved in arachis oil and were administered orally with a pipette.

Biological tests with methyl 5,6-epoxyretinoate in male rats. Weanling hooded male rats (32) were given the unsupplemented basal vitamin A-free diet for 3 weeks. They were then divided into four groups (A, B, C and D) each containing eight rats. All the rats remained on the basal vitamin A-free diet, but those in groups B, C and D were given supplements of methyl 5,6-epoxyretinoate, methyl retinoate and retinyl acetate respectively. These supplements were given three times weekly so as to provide the equivalent of 100 μg. of the crystalline substances/rat/day.

Body weights were recorded at regular intervals.

After a total of 104 days on the diet the threshold of the electroretinogram response was determined in surviving animals. After this measurement, the rats were killed and their testes and seminal vesicles were removed and weighed. Smears were made from the epididymal ducts for microscopic examination, and livers were removed and frozen for subsequent chemical analysis.

Measurement of the electroretinogram threshold. Rats were dark-adapted for 12 hr. in a mechanically ventilated light-tight cabinet. All subsequent handling of the animals was either in total darkness or in dim red light. The rats were anaesthetized with Nembutal and their pupils were dilated with Cocaine–homatropine eye drops B.P. Each animal was placed in a special holder, which maintained the head on one side with an eye orbit protruding upwards. The positioned animal was then placed in a metal box into which could be focused a beam of light from a tungsten lamp. This beam was interrupted by a manually operated shutter and by a modified camera shutter with a range of fixed speeds. A photocell, positioned to respond to stray light and coupled to an oscilloscope, was used to time the duration of the test flashes. Flashes of light for the threshold determinations were controlled by manual operation of the shutter and lasted at least 0·1 sec. The intensity of the light-beam was varied by means of neutral-density filters covering a range of density of 5·8 in steps of 0·2. The



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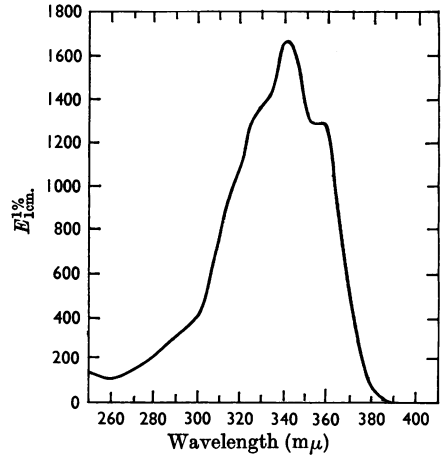

Fig. 1. Ultraviolet spectrum of methyl 5,6-epoxyretinoate in cyclohexane.

electroretinogram signal was obtained from two Ag/AgCl electrodes placed respectively on the edge of the cornea and on the cheek, and was amplified in a capacity-coupled amplifier (frequency response 3 decibels down at 0·2 cyc./sec. and 2 kcyc./sec.) and displayed on a Solartron CD1400 oscilloscope system. The threshold was taken to be the minimum light-intensity producing an electroretinogram response distinguishable from the preparation noise. The latter, in the absence of low-frequency components due to laboured respiration, was usually 10–20 μv.

Tests on pregnant female rats. Female hooded rats were reared from weaning on the basal vitamin A-free diet supplemented with retinoic acid (3 μg./g. of diet). After 14 weeks they were placed in cages with normal males of the same strain, and when mated, as judged by the presence of spermatozoa in the vaginal smear, they were distributed into three groups (E, F and G). Those in group E (five rats) were given supplements of retinyl acetate, and those in group F (nine rats) were given supplements of methyl 5,6-epoxyretinoate. The supplements were given daily during pregnancy and each dose contained 100 μg. of the crystalline substance. All the pregnant animals, including those in group G (six rats), were continued on the basal diet supplemented with retinoic acid.

RESULTS

Chemical and physical properties of methyl 5,6-epoxyretinoate. The epoxide prepared from methyl retinoate had absorption peaks in cyclohexane at 342 and 356 mμ with a shoulder at 325–330 mμ (Fig. 1). From the position of the absorption

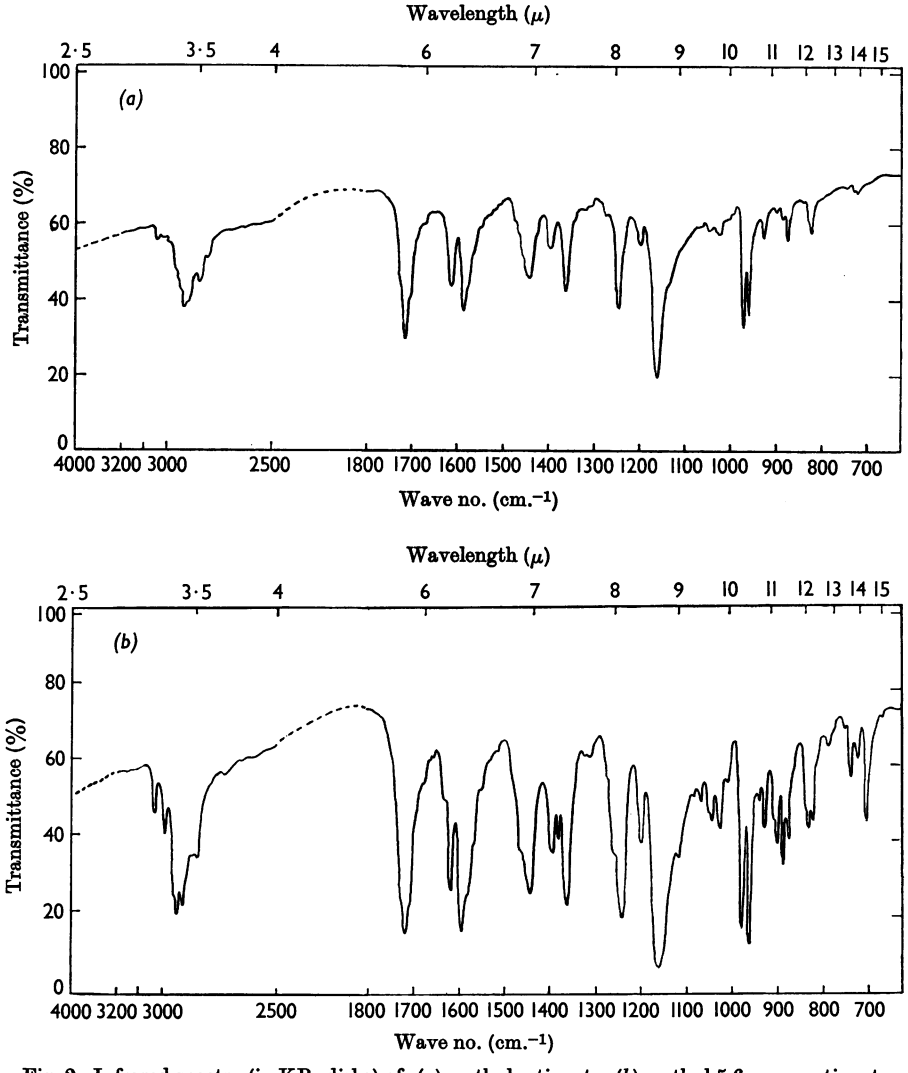

Fig. 2. Infrared spectra (in KBr disks) of: (*a*) methyl retinoate; (*b*) methyl 5,6-epoxyretinoate.

maximum four double bonds must remain conjugated with the carbonyl group. As was to be expected, the location of the main infrared-absorption bands was similar in the epoxide and in methyl retinoate (Fig. 2). This was perhaps the most significant feature of the infrared data. We consider it unwise to attempt an unambiguous interpretation of fine structure in the infrared spectra of vitamin A derivatives as these substances are unstable and samples soon become contaminated with impurities and isomers. We have, however, compared the spectrum of the epoxide with that of methyl retinoate, and the differences agreed with published data on epoxide formation.

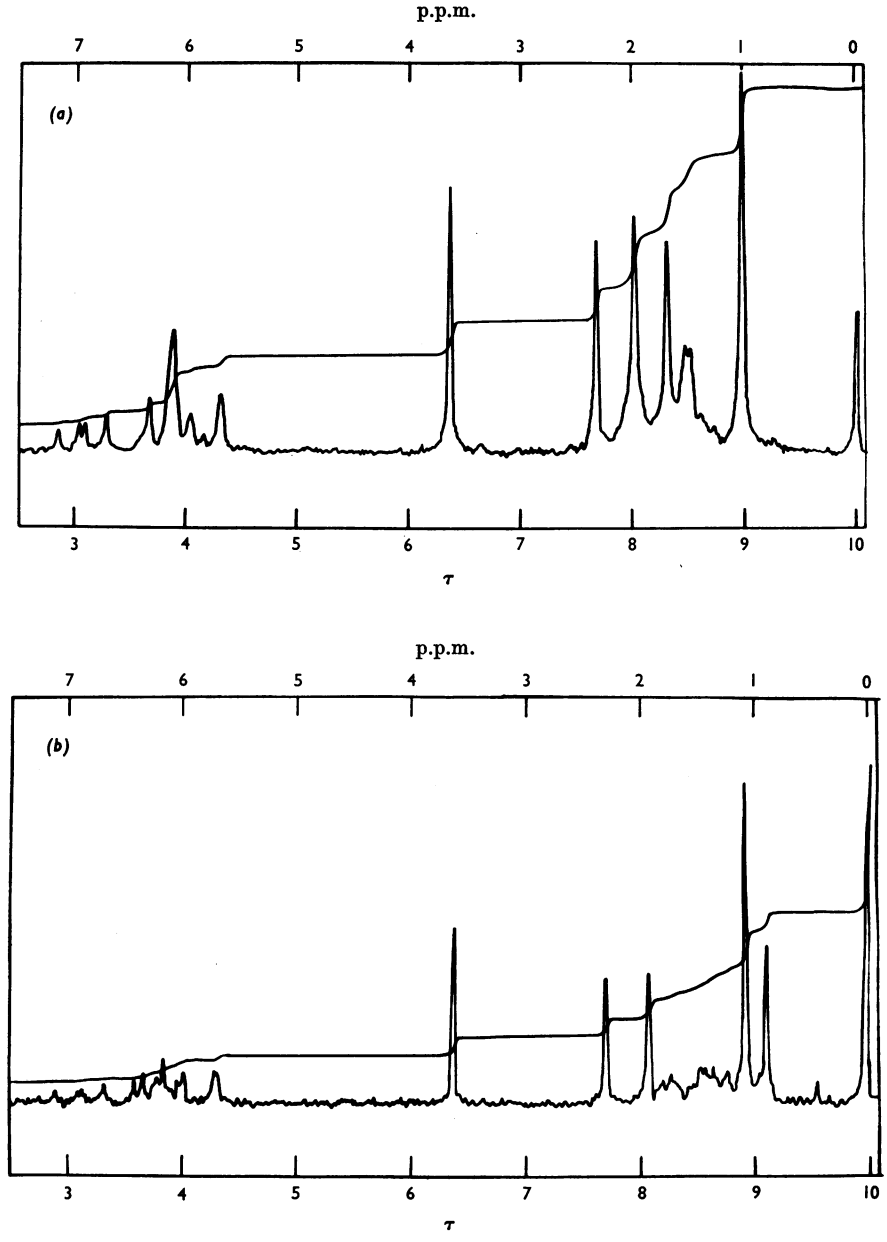

Fig. 3. Nuclear-magnetic-resonance spectra with integration of: (*a*) methyl retinoate; (*b*) methyl 5,6-epoxyretinoate.

In the infrared spectra of the epoxides they prepared, Jungalwala & Cama (1965) report a shoulder at 3145cm.$^{-1}$, which they associate with a 5,6-epoxide structure. We failed to find this band in our spectra, and we conclude that these authors were relating it to a 3040 cm.$^{-1}$ band, due to epoxymethine C–H stretching, which is discussed in the literature cited (Henbest, Meakins, Nicholls & Taylor, 1957). It should be noted, however, that 5,6-epoxides of vitamin A derivatives do not contain a relevant C–H bond. We have noted in the spectrum of methyl retinoate a weak band at 3040cm.$^{-1}$, which is much more prominent in the spectrum of the corresponding epoxide; the reason for this difference remains obscure. The shoulder at 1260cm.$^{-1}$ in the spectrum of our epoxide corresponds to the so-called 8μ band suggested by Patterson (1954) to be due to symmetrical stretching of the epoxide ring. Similarly, the bands at 899 and 830cm.$^{-1}$ correspond respectively to the 11μ and 12μ bands discussed by Patterson (1954) and Bomstein (1958) as being indicative of an epoxide structure. Bomstein (1958) also suggests that there are bands in the 14–15μ region associated with the epoxide ring. These are present at 700cm.$^{-1}$ in the spectrum of methyl 5,6-epoxyretinoate.

The difference observed between the n.m.r. spectra of methyl retinoate and its 5,6-epoxide (Fig. 3) is consistent with the structural change resulting from the introduction of a 5,6-epoxide group into the methyl retinoate molecule. The integration curve shows that all 30 protons present in methyl retinoate remain in the epoxide, thus eliminating the possibility of a 2,3-epoxide or a 3,4-epoxide. The absence of a signal in the range 4·5–7·4τ (2·6–5·5 p.p.m.), apart from the signal at 6·35τ (3·65 p.p.m.) due to the methyl protons on the ester group, shows that the carbon atoms carrying the oxide bridge do not carry any protons (cf. Jungalwala & Cama, 1965). The region 2·8–4·4τ (5·6–7·2 p.p.m.) represents the six protons on the double bonds. The sharp signals at 7·7τ (2·3 p.p.m.) and 8·05τ (1·95 p.p.m.) are assigned to the methyl protons at C-13 and C-9. However, in the spectrum of the parent compound, the 8·05τ band probably contains the signal from the C-4 protons, as well as those from one of the side-chain methyl groups. In the epoxide, as the ring protons are no longer close to a conjugated system, their signal shifts to join those of the other ring protons at 8·0–9·0τ (1·0–2·0 p.p.m.). The protons of the *gem*-dimethyl group at C-1 give a sharp signal at 9·0τ (1·0 p.p.m.) in methyl retinoate, and at 8·9τ (1·1 p.p.m.) in the epoxide. The protons of the C-5 methyl group at 8·3τ (1·7 p.p.m.) in methyl retinoate shift to a higher field (9·1τ, 0·9 p.p.m.) in the spectrum of the epoxide. This is due to the magnetic influence of the 7,8-double bond, and again indicates a 5,6-epoxide structure (cf. Jungalwala & Cama, 1965).

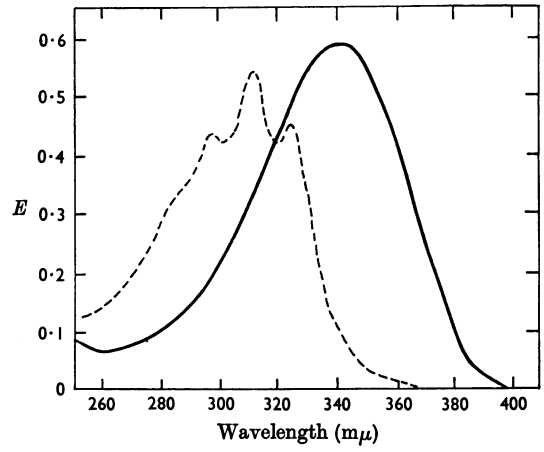

Fig. 4. Ultraviolet spectra in ethanol of: ——, methyl 5,6-epoxyretinoate (4μg./ml.); – – –, product of reduction of methyl 5,6-epoxyretinoate with $LiAlH_4$.

Mass spectrometry of methyl retinoate and methyl 5,6-epoxyretinoate confirmed the expected molecular weights, 314 and 330, consistent with their suggested structures. Addition of mineral acid to 5,6-epoxides of carotenoids (Karrer, 1945), retinol, retinyl acetate and retinal (Jungalwala & Cama, 1965) catalyses their isomerization to furanoid 5,8-epoxides. When traces of acid were added to ethanolic solutions of the methyl 5,6-epoxyretinoate the ultraviolet spectrum changed to $\lambda_{max.}$ 308mμ with shoulders at 295 and 325mμ, confirming that a similar isomerization had occurred. Reduction of methyl 5,6-epoxyretinoate with lithium aluminium hydride produced a substance with absorption maxima at 293, 310 and 324mμ in ethanol (Fig. 4). This is considered to be due to the formation of the corresponding alcohol, 5,6-epoxyretinol. The spectroscopic data agree with those of Jungalwala & Cama (1965), who prepared this substance by a different chemical method, and we have identified a substance with a similar absorption spectrum in the livers of rats given 5,6-epoxyretinal (cf. also Lakshmanan *et al.* 1965). Methyl retinoate and methyl 5,6-epoxyretinoate could be separated on thin plates of Kieselgel G with benzene as a solvent system, and the 5,6-epoxide (R_F 0·23) stained green after spraying with a solution of phosphomolybdic acid in ethanol, whereas methyl retinoate (R_F 0·45) stained blue.

Biological activity in male rats. The rats given the unsupplemented diet (group A) developed

classical signs of vitamin A deficiency. They had arrested growth, laboured breathing, encrusted eyes and xerophthalmia, and all were dead or moribund after a total of 5 weeks on the experiment. Typical vitamin A-deficiency lesions, such as distended intestines, small bluish oedematous testes, infected salivary glands and marked emaciation, were found at post-mortem examination.

The rats given either methyl retinoate or retinyl acetate (groups C and D) grew at a normal rate and appeared superficially to be normal. The only marked difference in the external appearance of these animals was the soft feminine fur of those given methyl retinoate, a characteristic noted by Thompson *et al.* (1964). At the end of the experiment the testes of the rats given methyl retinoate were small and oedematous (Table 1) and smears of the contents of the epididymides revealed only a few spermatozoa. In contrast, the testes from the retinyl acetate-supplemented animals were normal in size and appearance and the epididymal smears contained many spermatozoa.

The rats in group B given supplements of methyl 5,6-epoxyretinoate at first grew well; however, as the experiment continued their body weights fell gradually below those of the animals in groups C and D (Fig. 5) and their condition became noticeably inferior to that of the controls. Their appearance

Table 1. *Results of biological tests on male rats maintained on a vitamin A-deficient diet either unsupplemented or with supplements of methyl 5,6-epoxyretinoate, methyl retinoate or retinyl acetate*

Group	Supplement (100 μg./rat/day)	Total time on diet before examination or death (days)	Body wt. (g.)	Testes wt. (g.)	Seminal vesicle wt. (g.)	Liver retinol (μg./g.)	log(Electro-retinogram threshold) (see the text)
A	None	42	88				
		36*	80				
		42	67				
		22*	125	—	—	—	—
		42	144				
		37*	91				
		29*	135				
		40*	129				
B	Methyl 5,6-epoxy-retinoate	70*	137	—	—	0	—
		104	204	0·97	0·45	0	2·4
		104	226	1·15	0·83	0	2·0
		104†	158	0·68	0·19	0	—
		104†	163	—	—	0	—
		63*	199	—	—	0	—
		104	188	0·92	0·78	0	2·0
		104	168	0·73	0·67	0	4·6
C	Methyl retinoate	104	360	1·51	1·60	0	1·6
		104	282	0·99	1·96	0	1·8
		104	310	1·38	1·79	0	1·6
		104	262	0·95	1·03	0	1·4
		104	257	1·00	1·43	0	1·4
		104†	302	1·08	1·37	0	—
		104	352	0·79	1·31	0	2·0
		104	254	1·43	1·63	0	2·2
D	Retinyl acetate	104	306	2·27	1·72	440	0·2
		104	328	2·20	1·81	203	0·6
		104	284	2·24	1·40	384	−0·4
		104	272	2·26	1·56	431	0·0
		104	335	2·70	1·54	316	−0·2
		104†	196‡	1·95	0·52	512	—
		104	266	2·25	1·45	306	0·6
		104	294	2·82	1·58	141	0·2

* Rat died during the experiment.
† Rat died under anaesthetic during electroretinogram measurement.
‡ Pneumonic rat.

suggested an incipient deficiency of vitamin A although there was no indication of xerophthalmia. Three rats in this group died during the experiment after intercurrent infection and those surviving were afflicted with respiratory disease. At post-mortem examinations all the rats in this group were found to have small oedematous testes and scant epididymal smears were obtained similar to those from the animals in group C. Spectroscopic examination of the unsaponifiable lipid from the livers of animals in group D given retinyl acetate revealed substantial quantities of retinol (Table 1); similar tests on the livers from the rats from the other two groups failed to detect either retinol or any of its epoxides.

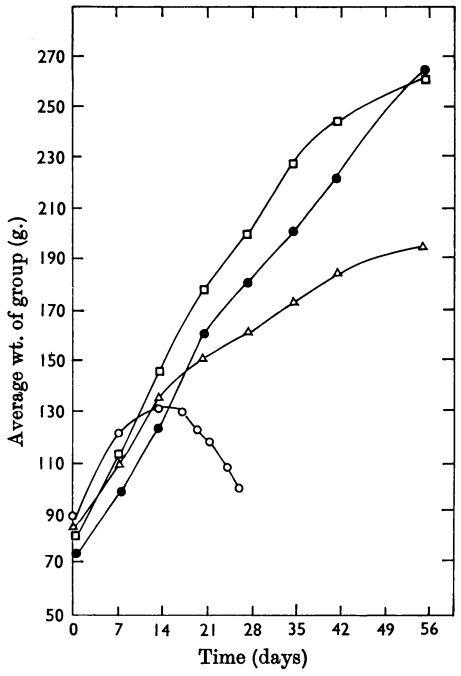

Fig. 5. Growth curves of male rats given a vitamin A-deficient diet supplemented with: ○, no supplement; △, methyl 5,6-epoxyretinoate; □, methyl retinoate; ●, retinyl acetate.

Electroretinogram threshold measurements. The log(threshold intensity) values obtained in these experiments are expressed relative to that found for normal rats of this strain, which is arbitrarily given the value 0 (Table 1). The threshold in the rats in group D given retinyl acetate was thus normal; however, light-intensities 25–200-fold greater were required for an electroretinogram response from those given methyl retinoate. We have regularly found elevations of the threshold of this magnitude in hooded rats of this age given vitamin A-deficient diets containing retinoic acid, and by this time their rhodopsin concentrations are 30–50% of the normal (cf. Dowling & Wald, 1960). The vision of the rats from group B given methyl 5,6-epoxyretinoate was found to have deteriorated more rapidly than that of the animals given methyl retinoate, probably because of their long-standing general debility.

Reproduction in females. The outcome of pregnancy in each of the rats tested is given in Table 2. The animals given retinyl acetate delivered normal healthy litters, whereas those given only retinoic acid showed profuse vaginal bleeding from the fifteenth day of pregnancy and failed to deliver litters, in confirmation of previous findings (Thompson *et al.* 1964). Those given the epoxide also bled from the vagina and neither delivered litters nor aborted any foetuses.

DISCUSSION

Following the preparation and characterization of a series of monoepoxides of several vitamin A derivatives by Jungalwala & Cama (1965) we have investigated the epoxides of retinoic acid. A crystalline substance was obtained from methyl retinoate after reaction with monoperphthalic acid. The molecular weight of the derivative was consistent with a monoepoxide structure, and from the ultraviolet, infrared and n.m.r. spectra we conclude that it is a 5,6-epoxide (methyl 5,6-epoxyretinoate).

The epoxide was found to have biological properties that were qualitatively similar to those of retinoic acid. In rats it permitted growth but neither reproduction nor vision. Thus male rats maintained on the epoxide had degenerate testes

Table 2. *Effects of supplements of retinyl acetate or methyl 5,6-epoxyretinoate on the outcome of pregnancies in rats reared on a vitamin A-deficient diet containing retinoic acid*

Group	Supplement during pregnancy (100 μg./rat/day)	No. of rats mated	Litters produced	No. of rats resorbing foetuses	No. of pups in each litter
E	Retinyl acetate	5	5	0	9; 5; 10; 10; 6
F	Methyl 5,6-epoxyretinoate	9	0	9	
G	None	6	0	6	

and elevated electroretinogram thresholds. Pregnant female rats maintained on retinoic acid given supplements of the epoxide resorbed their foetuses. As epoxyretinol was not detected in the liver non-saponifiable material of rats given the epoxy acid ester it is concluded that the methyl 5,6-epoxyretinoate is not reduced *in vivo* to either the corresponding aldehyde or alcohol. Although a daily supplement of 100 μg. of the epoxide prevented the characteristic overt signs of vitamin A deficiency in rats, the growth and general health of these animals was not normal. Periodic spectrophotometric checks of the solutions used to dose the animals have eliminated the possibility that this difference was due to deterioration of the epoxide on storage. Although we have not made bioassay measurements with small quantities of the epoxide it is clear that its biological activity is inferior to that of retinoic acid and 5,6-epoxyretinal. Lakshmanan *et al.* (1965) suggested that some of the biologically active metabolites of vitamin A isolated recently (Wolf, Bergan & Sundaresan, 1963) might be epoxides of retinoic acid, and stated that: 'These observations imply that 5,6-epoxyretinoic acid is far ahead of vitamin A itself in the proposed scheme of the general metabolism of vitamin A (Dowling & Wald, 1960)'. Our results do not support the suggestion that the 5,6-epoxyretinoic acid is a normal intermediate in the formation of an active form of retinoic acid, as such a substance should be at least as effective biologically as retinoic acid itself. It is, however, possible that the low activity of the epoxide in our experiments was due to poor absorption or instability in the digestive tract. Another possible explanation is that the epoxy acid has weak biological activity by virtue of either its structural similarity to or a limited conversion into some more active substance such as retinoic acid.

Although there is little evidence to suggest that 5,6- or 5,8-epoxides are obligatory intermediates in the metabolism of vitamin A, their biological properties are not completely without interest or significance. Thus it is possible that they can be formed *in vivo* in either normal or pathological tissues. Epoxides may also be produced by atmospheric oxidation of retinol, retinyl esters or retinoic acid derivatives during chemical manipulation of tissue extracts or storage of fortified diets. We have, in fact, found evidence for the existence of 5,6- and 5,8-epoxides in our stored samples of retinoic acid. The production of these and other biologically active artifacts in vitamin A metabolism experiments must therefore be considered, especially when the isolation of a small quantity of an 'active form' of vitamin A is attempted.

We thank Professor R. A. Morton, F.R.S., for his encouragement and advice, Miss P. McMahon and Mrs K. McLaughlin for technical assistance, Mr D. R. Lewin for data on rhodopsin concentrations in our rats, Dr O. Isler of Hoffmann–La Roche and Co., Basle, for gifts of retinoic acid, and Mr J. Barkley of the Department of Organic Chemistry, University of Liverpool, for measuring the nuclear-magnetic-resonance and mass spectra. This work was supported in part by U.S. Public Health Service Grant AM-05282-021. B. M. is in receipt of a Medical Research Council Research Studentship and J. N. T. is a British Egg Marketing Board Fellow.

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