Studies on the Visual Pigment and the Indicator Yellow Analogues formed from 5,6-Monoepoxyretinal

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1. A new visual pigment has been isolated from retinæ of rats maintained on 5,6-monoepoxyretinal for a period of 6 months. 2. Indicator yellow analogues from 5,6-monoepoxyretinal, namely 5,6-monoepoxyretinylidenemethylamine and 5,6-monoepoxyretinal oxime, have been prepared in a homogeneous state, the latter being obtained crystalline. 5,6-Monoepoxyretinylidenedimethylammonium iodide has also been prepared. 3. The spectroscopic properties, analytical data and antimony trichloride colour reactions of these indicator yellow analogues confirm their Schiff base structure. 4. The nature of chromogens formed from 5,6-monoepoxyretinylidenearmino compounds with antimony trichloride and concentrated sulphuric acid is obscure although the chromogens resemble one another very closely. 5. 5,6-Monoepoxyretinylidenemethylamine and 5,6-monoepoxyretinylidenedimethylammonium iodide are very labile, whereas 5,6-monoepoxyretinal oxime is quite stable. 6. Hydrolysis of 5,6-monoepoxyretinylidenemethylamine as a function of pH reveals that, though 5,6-monoepoxyretinylidenemethylammonium ions are stable, the un-ionized compound readily hydrolyses to 5,6-monoepoxyretinal. This hydrolysis can be prevented by excess of un-ionized methylamine and hastened by formaldehyde. 7. The bathochromic shift in the λ_{max} of the 'acid form' of 5,6-monoepoxyretinylidenemethylamine is due to the formation of its ammonium salt. The spectrum of the 'acid form' is identical with that of 5,6-monoepoxyretinylidenedimethylammonium iodide in ethanol. This is also evidence for the quaternary state of the nitrogen atom during the 'acid shift' of 5,6-monoepoxyretinylidenemethylamine. 8. The significance of 5,6-monoepoxyretinal and the corresponding indicator yellow analogues in the formation and properties of a new visual pigment is discussed.

When bleached with white light rhodopsin (visual purple) gives rise to 'indicator yellow', the first stable photo-decomposition product (Dartnall, 1957), which is pale yellow in alkaline solutions and deep yellow in acid media (Lythgoe, 1937). As a result of the classical work of Morton's school retinal (vitamin A aldehyde), the final decomposition product of rhodopsin, is believed to be linked through the amino groups of a protein, opsin, to give rise to visual pigment. In fact, retinal can be linked to many amino compounds such as amines and amino acids as well as to proteins to give products identical with natural indicator yellow derived from rhodopsin (Ball, Collins, Dalvi & Morton, 1949; Pitt, Collins, Morton & Stok, 1955). Bamji, Cama & Sundaresan (1959, 1961) have carried out similar studies on the preparation and properties of the indicator yellow₂ analogues formed from 3-dehydroretinal.

Studies on the visual pigment from retinæ of vitamin A-deficient rats maintained on 5,6-monoepoxyretinal (Lakshmanan, Jungalwala & Cama, 1965) indicated the formation of a new visual pigment. In the present paper we describe preliminary studies on the new visual pigment isolated from vitamin A-deficient rats maintained solely on 5,6-monoepoxyretinal for a period of 6 months, as well as detailed investigations on the preparation and properties of indicator yellow analogues formed from 5,6-monoepoxyretinal, namely 5,6-monoepoxyretinylidenemethylamine and 5,6-monoepoxyretinal oxime.

MATERIALS AND METHODS

Spectrophotometric measurements were carried out in a Beckman model DU spectrophotometer. Light petroleum, spectroscopic ethanol, chloroform and diethyl ether were as described by Jungalwala & Cama (1965). Solutions of SbCl_3 (25%, w/v) in chloroform were freshly prepared before use. Methylamine and hydroxylamine hydrochloride

Studies on the visual pigment from retinæ of

were British Drug Houses Ltd. (Poole, Dorset) AnalaR quality. Formaldehyde was E. Merck (Darmstadt, Germany) reagent quality. Potash alum [$K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O$] (4%) was prepared freshly from the British Drug Houses Ltd. AnalaR reagent. Digitonin was obtained from British Drug Houses Ltd.

Animals. Eight normal albino rats of either sex were made vitamin A-deficient as described by Lakshmanan *et al.* (1965) and then maintained on 5,6-monoepoxyretinal at a dose of 50 μ g./day for 6 months, after which they were dark-adapted for 8–10 hr. before being processed.

Isolation of the visual pigment. Retinae from the dark-adapted animals were dissected out in red light and the visual pigments were isolated from them essentially according to the sucrose flotation method of Collins, Love & Morton (1952) except that treatment with formaldehyde was omitted. For bleaching of the visual pigment, a 100 w bulb was used with a water jacket (approx. 10 cm. thick) for the dissipation of heat.

Buffers. Various pH values were obtained by using HCl for pH 1.0 and 2.0, acetic acid for pH 3.0, 0.2 M-sodium acetate buffer for pH 3.7 and 4.8, 0.2 M-sodium phosphate buffer for pH 5.7, 6.3 and 7.7, 0.2 M-sodium borate buffer for pH 8.6 and KOH for pH 13.0.

5,6-Monoepoxyretinylidenemethylamine. A 100 mg. sample of 5,6-monoepoxyretinal (Jungalwala & Cama, 1965) in light petroleum was bubbled with methylamine gas until the spectrum of the solution showed a distinct change in λ_{max} from 352 to 337 $m\mu$ with a subsidiary peak at 355 $m\mu$. The reaction was regarded as complete when the E_{337}/E_{355} ratio reached a constant value of 1.143. The solution was filtered through Na_2SO_4 to remove traces of moisture and was concentrated *in vacuo*. Attempts to purify this compound by adsorption chromatography led to the formation of dark-red oxidation products. The compound is readily hydrolysed in aqueous media at various pH values and this could be prevented only by the presence of excess of methylamine.

Solutions were prepared in Tween 80 (0.1%, w/v) in various buffers according to the technique of Bliss (1951) as described by Pitt *et al.* (1955). The pH of each solution was checked.

5,6-Monoepoxyretinal oxime. A 37 mg. sample of 5,6-monoepoxyretinal in 5 ml. of ethanol was mixed with a solution of 200 mg. of hydroxylamine hydrochloride in 5 ml. of water containing KOH to bring the pH to 10.5. The reaction was almost complete within 10 min., as shown by a distinct change in λ_{max} of the solution in light petroleum from 352 to 337 $m\mu$ with a subsidiary peak at 355 $m\mu$. The E_{337}/E_{355} ratio remained constant at 1.09 when the reaction time was increased. The reaction mixture, after dilution with water, was thoroughly extracted with diethyl ether, dried over anhydrous Na_2SO_4 , filtered and concentrated *in vacuo*. The final product was dissolved in a small quantity of light petroleum from which 5,6-monoepoxyretinal oxime crystallized out on cooling to -30° . After repeated crystallizations, the pale-yellow crystals melted sharply at 143° (Found: C, 76.25; H, 8.85; N, 4.36. $C_{20}H_{29}NO_2$ requires: C, 76.19; H, 9.21; N, 4.44%).

5,6-Monoepoxyretinylidenemethylammonium iodide. A 20 mg. sample of 5,6-monoepoxyretinylidenemethylamine in light petroleum was evaporated to dryness *in vacuo* and 1.0 ml. of methyl iodide was added. After about 40 min. at room temperature, excess of methyl iodide was removed

by evaporation under reduced pressure and the red residue was washed with light petroleum. The compound failed to crystallize in spite of repeated attempts. It was insoluble in light petroleum but dissolved readily in chloroform and ethanol. The compound had λ_{max} at 450 $m\mu$ in chloroform and at 420 $m\mu$ in ethanol. The compound is unstable in the presence of water: thus, on the addition of water or alkali (mN-KOH) to the ethanolic solution, the compound is transformed immediately into 5,6-monoepoxyretinal.

RESULTS AND DISCUSSION

Vitamin A-deficient rats maintained on 5,6-monoepoxyretinal were cured of xerophthalmia and, unlike animals fed with retinoic acid (Dowling & Wald, 1960), they did not show any defective visual behaviour. However, as facilities for taking electroretinograms of 5,6-monoepoxyretinal-fed rats were not available, the demonstration of their normal visual function could not be unequivocally proved. However, all the rats subsisting on this compound had reddish-brown eyeballs, different from the characteristic glistening red eyeballs of retinyl acetate-fed rats.

Properties of the new visual pigment. The spectrophotometric characteristics of the pigment are depicted in Fig. 1. Although the unbleached pigment shows a broad λ_{max} at 480–495 $m\mu$, the difference spectrum contains two λ_{max} at 480 and 495 $m\mu$. The λ_{max} at 480 $m\mu$ may well be due to a new visual pigment having 5,6-monoepoxyretinal

Fig. 1. Spectra of unbleached (O) and bleached (Δ) visual pigment [in 2% (w/v) digitonin at pH 9.3] from retinae of rats maintained on 5,6-monoepoxyretinal for 6 months. The difference spectrum ($\bullet$) is also shown.

as its chromophore and that at 495m μ may be due to tenaciously held natural rhodopsin. In a comparative experiment under identical conditions, rhodopsin isolated from normal stock colony rats showed an extinction value at its λ_{max} at 495m μ which was almost the same as that observed for the new pigment. 5,6-Monoepoxyretinal failed to support normal reproduction in female rats (M. R. Lakshmanan & H. R. Cama, unpublished work) and consequently the formation and complete replacement of the natural rhodopsin by this pigment could not be unequivocally demonstrated.

An alternative possibility is that the new pigment has two λ_{max} at 480 and 495m μ . Normally, if it were a mixture of two pigments it would exhibit a single λ_{max} at an intermediate wavelength. The fact that two maxima are observed suggests that these may be characteristic of the pigment. Further, the presence of the two peaks could not have been

due to excessive light intensity during bleaching since we used only a 100 w bulb with a water jacket. In fact, the indicator yellow analogues formed from 5,6-monoepoxyretinal also have two-banded spectra (Fig. 2), in contrast with those formed from retinal and 3-dehydroretinal. Further, it is pertinent that even the parent compound, 5,6-monoepoxyvitamin A, has a two-banded spectrum, with λ_{max} at 310 and 325m μ (Jungalwala & Cama, 1965).

We were unable to demonstrate the synthesis *in vitro* of the new visual pigment by combining frog or goat opsin (prepared as described by Herting, Drury & Harris, 1962) with 5,6-monoepoxyretinal according to the procedure of Hubbard & Wald (1952). This may be due either to our failure to effect isomerization of all-*trans*-5,6-monoepoxyretinal to its specific *cis*-isomer in the presence of iodine and light, or to failure to obtain the specific opsin. Hence, unless the synthesis *in vitro* of this pigment is achieved, the true spectrophotometric characteristics of this new pigment cannot be established.

Characteristics of indicator yellow analogues. On condensation with methylamine, 5,6-monoepoxyretinal yielded 5,6-monoepoxyretinylidenemethylamine with the expected shift of λ_{max} in various solvents (Table 1). These shifts are similar to the hypsochromic shifts that occur during conversions of retinal into retinylidenemethylamine (Pitt *et al.* 1955) and 3-dehydroretinal into 3-dehydroretinylidenemethylamine (Cama, Dalvi, Morton & Salah, 1952; Bamji *et al.* 1959). Further, 5,6-monoepoxyretinal oxime and 5,6-monoepoxyretinylidenemethylamine show very similar absorption spectra (Table 1 and Fig. 2). These results suggest that the 5,6-monoepoxyretinylidenemethylamine was reasonably pure and that it has a Schiff base structure, as was proposed by Pitt *et al.* (1955) for retinylidenemethylamine and the oxime. 5,6-Monoepoxyretinylidenemethylamine shows λ_{max} at 415m μ at acid pH and at 360–365m μ at alkaline pH (Fig. 3).

It could be expected that the new pigment, when bleached with white light, would give rise to its

Fig. 2. Absorption spectra of 5,6-monoepoxyretinylidenemethylamine (O), 5,6-monoepoxyretinal oxime (Δ) and 5,6-monoepoxyretinal ($\bullet$) in light petroleum (curves plotted on the basis $E_{\text{max}}=1.0$).

Table 1. Spectroscopic properties of 5,6-monoepoxyretinylidenemethylamine and 5,6-monoepoxyretinal oxime in different solvents

Solvent	5,6-Monoepoxyretinylidenemethylamine		5,6-Monoepoxyretinal oxime	
	λ_{max} (m μ)	$E_{1\%}^{1\text{cm}}$	λ_{max} (m μ)	$E_{1\%}^{1\text{cm}}$
Light petroleum	337	1610	337	1850
	354	1410	355	1697
Ethanol	345	1475	340	1795
			357	1640
Chloroform	345	1428	345	1749

Fig. 3. Absorption spectra of 5,6-monoepoxyretinylidenemethylamine and 5,6-monoepoxyretinal in aqueous media as well as that of 5,6-monoepoxyretinylidenedimethylammonium iodide in ethanol (dispersing agent in aqueous media: Tween 80, 0.1%, w/v). O, 5,6-Monoepoxyretinylidenemethylamine in acetic acid (pH 3.6); Δ , 5,6-monoepoxyretinylidenemethylamine in alkali (pH 10.0); $\bullet$, 5,6-monoepoxyretinylidenemethylamine in alkali (pH 10.0); $\blacktriangle$, 5,6-monoepoxyretinal in alkali (pH 10.0); $\square$, 5,6-monoepoxyretinylidenedimethylammonium iodide.

natural indicator yellow possessing the characteristics of 5,6-monoepoxyretinylidenemethylamine. But, owing to paucity of the photoproduct, it could not be characterized.

Reaction with antimony trichloride reagent. The antimony trichloride reagent gives a fairly stable yellow colour (changing to blackish green on very long standing) with both 5,6-monoepoxyretinylidenemethylamine and the oxime, having $\lambda_{\max}$ at 400–405 and 395 m μ respectively ($E_{1\%}^{1\text{cm}}$ 1690 and 1708 respectively). These two compounds share features that are characteristic of retinylidenemethylamine and the oxime, i.e. they have colour-test $\lambda_{\max}$ at shorter wavelengths, and also have lower $E_{1\%}^{1\text{cm}}$ values, than do those of vitamin A, and they also give rise to stable antimony trichloride chromogens. However, they differ from the retinylidene compounds in that the $\lambda_{\max}$ obtained in the antimony trichloride test are at lower wavelengths. Thus the $\lambda_{\max}$ for retinylidenemethylamine and retinal oxime are at 505 and 480 m μ respectively (Pitt *et al.* 1955). This is presumably because the epoxy derivatives have only four conjugated double bonds. 5,6-Monoepoxyretinylidenedimethylammonium iodide gave a greenish-yellow colour with antimony trichloride, having $\lambda_{\max}$

Table 2. Hydrolysis of 5,6-monoepoxyretinylidenemethylamine in aqueous solutions at different pH values

Tween 80 (final concn. 0.1%) was used as dispersing agent.

pH	$\lambda_{\max}$, immediately after mixing (m μ)	$\lambda_{\max}$, moved to 360–365 m μ after
1.0	375–380	—
2.0	375–380	—
3.6	415	45 min.
4.3	415	35 min.
5.5	375	8 min.
6.6	360–365	—
7.7	360–365	—
8.6	360–365	—
13.0	360–365	—
In the presence of 0.2 M-methylamine		
3.6	345	Hypsochromic shift
6.6	345	Hypsochromic shift
10.0	345	No change
In the presence of 4% formaldehyde		
8.6	365	—

at 425 m μ . Hence, it seems probable that the three antimony trichloride chromogens, namely those of 5,6-monoepoxyretinylidenemethylamine, 5,6-monoepoxyretinal oxime and 5,6-monoepoxyretinylidenedimethylammonium iodide, are similar to one another.

Action of concentrated sulphuric acid on 5,6-monoepoxyretinylidenemethylamine. When treated with concentrated sulphuric acid 5,6-monoepoxyretinylidenemethylamine initially gave a violet chromogen ($\lambda_{\max}$ at 570 m μ) that eventually faded in 5 min. to yellowish orange, having $\lambda_{\max}$ at 435 m μ and an inflexion at about 375–385 m μ . This colour was stable and superficially resembled the corresponding Carr–Price chromogens. Strikingly, the $\lambda_{\max}$ of the initial sulphuric acid chromogen is some 50 m μ higher than that observed for retinylidenemethylamine (Pitt *et al.* 1955).

Hydrolysis of 5,6-monoepoxyretinylidenemethylamine. Jungalwala & Cama (1965) have demonstrated that 5,6-monoepoxyvitamin A compounds are readily isomerized to the corresponding furanoid compounds in the presence of a strong proton donor such as hydrochloric acid. In the presence of hydrochloric acid at pH 1.0 and 2.0, it can be presumed that 5,6-monoepoxyretinylidenemethylammonium chloride is formed first and that it is immediately isomerized to the furanoid compound having $\lambda_{\max}$ at 375–380 m μ (Table 2). Thus it distinctly differs from retinylidenemethylamine, which remains stable as its chloride at pH 1–2 (Morton & Pitt, 1955). However, in the presence of acetic acid (at pH 3.6 and 4.8) 5,6-monoepoxyretinylidenemethylamine existed predominantly

as the acetate ($\lambda_{\max}$ at 415 m μ ; Fig. 3) corresponding to its acid indicator yellow analogue. The acetate slowly hydrolysed to 5,6-monoepoxyretinal with $\lambda_{\max}$ at 360–365 m μ (Table 2). Fig. 4 shows the hydrolysis of the compound at pH 3.6 as a function of time. An isosbestic point at 380 m μ indicates that the effective absorption is due to only two components, as with retinylidenemethylamine (Morton & Pitt, 1955) and 3-dehydroretinylidenemethylamine (Bamji *et al.* 1961). After prolonged storage the solution showed $\lambda_{\max}$ at 340–345 m μ due to isomerization to 5,8-monoepoxyretinal. At that stage it had a spectrum identical with that of 5,8-monoepoxyretinal (Jungalwala & Cama, 1965) as well as with that of 5,6-monoepoxyretinal that

had been treated with hydrochloric acid or acetic acid.

Unlike the retinylidenemethylamine, 5,6-monoepoxyretinylidenemethylamine is extremely unstable not only at neutral pH values but also at alkaline and highly acidic pH values (Table 2). At pH 5.5, the compound shows $\lambda_{\max}$ at 375 m μ even at zero time, which indicates a very rapid formation of 5,6-monoepoxyretinal. Above pH 5.5, the hydrolysis is almost instantaneous and can be prevented only by an excess of methylamine. Thus, in the presence of 0.2 M-methylamine at pH 3.6 and 6.6, the compound gave the characteristic $\lambda_{\max}$ at 345 m μ corresponding to its alkaline indicator yellow analogue (Table 2). On long standing, there was a hypsochromic shift, but the nature of the changes occurring is not known. On the other hand, at pH 10.0, in the presence of 0.2 M-methylamine, $\lambda_{\max}$ at 345 m μ remained stable for at least 24 hr. (Table 2). Excess of methylamine, especially at alkaline pH, effectively stabilizes the highly labile 5,6-monoepoxyretinylidenemethylamine, since the ionization of methylamine is suppressed at high pH. It may be that only un-ionized methylamine is involved in the equilibrium, as has been discussed by Morton & Pitt (1955). The natural alkaline indicator yellow that is formed from the new pigment may be similarly stabilized by amino groups in the retinal opsin and other proteins. On the other hand, the fact that 5,6-monoepoxyretinylidenemethylamine is very rapidly hydrolysed in the presence of 4% formaldehyde at pH 8.6 (Table 2) clearly shows that any conditions which remove free methylamine from the system will accelerate hydrolysis (Pitt *et al.* 1955).

The $\lambda_{\max}$ at 415 and 345 m μ for acid and alkaline 5,6-monoepoxyretinylidenemethylamine show the same relationship as the $\lambda_{\max}$ of the corresponding

Fig. 4. Absorption spectra of 5,6-monoepoxyretinylidenemethylamine at pH 3.6 as a function of time (dispersing agent: Tween 80, 0.1%, w/v): ○, immediately after mixing (zero time); △, after 5 min.; ●, after 10 min.; ▲, after 20 min.; □, after 45 min.

Table 3. Spectroscopic properties of indicator yellow analogues and visual pigments

Compound	No. of conjugated double bonds	λ_{max} of derivatives (m μ)					λ_{max} of visual pigment (m μ)
		Alcohol form in light petroleum	Methylamine compound			Oxime	
			In light petroleum	Acidic	Alkaline		
3-Dehydroretinal	6	350, 287	371	455	385	370	517† (porphyropsin)
Retinal	5	325	355	435	365	356	496‡ (rat rhodopsin, uncorrected)
5,6-Monoepoxyretinal	4	310, 325*	337, 354	415	345	337, 355	480, 495§ (new pigment)

* Jungalwala & Cama (1965).

† Wald, Brown & Smith (1953).

‡ Collins & Morton (1950); Bridges (1959).

§ Present investigation.

analogues formed from retinylidenemethylamine (435 and 365m μ respectively) and 3-dehydro-retinylidenemethylamine (455 and 385m μ respectively). That the nitrogen atom is in the quaternary state during the 'acid shift' of 5,6-monoepoxy-retinylidenemethylamine is further supported by the strong resemblance in spectral characteristics of 5,6-monoepoxyretinylidenedimethylammonium iodide in ethanol and the acid form of 5,6-monoepoxyretinylidenemethylamine (Fig. 3), as well as in their antimony trichloride colour reactions.

With the visual pigments and indicator yellow analogues cited in Table 3, it is evident that for each loss of a conjugated double bond there is a hypsochromic shift of 20m μ in their $\lambda_{\max}$ and that, conversely, with each addition of a conjugated double bond there is a corresponding bathochromic shift.

In conclusion, a two-banded spectrum seems to be a characteristic spectroscopic property of the alcohol form of 5,6-monoepoxyretinal and the corresponding indicator yellow analogues. The subsidiary peak for each of these compounds almost exactly coincides with the main peak of the corresponding vitamin A compound (Table 3). It has not yet proved possible to synthesize the new pigment *in vitro* to see whether this analogy is still maintained.

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