Oxidation of Zeaxanthin

ISOLATION AND PROPERTIES OF 3-HYDROXYRETINENE

By R. K. BARUA AND A. B. BARUA

Department of Chemistry, University of Gauhati, Gauhati 14, Assam, India

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1. Anthers of *Delonix regia* flowers are a rich source of zeaxanthin. 2. Oxidation of zeaxanthin with hydrogen peroxide in the presence of osmium tetroxide results in the formation of 3-hydroxyretinene as one of the products. 3. 3-Hydroxyretinene gives 3-acetoxyretinene with acetic anhydride, and an oxime with hydroxylamine hydrochloride. 4. Reduction of 3-hydroxyretinene with sodium borohydride yields 3-hydroxyvitamin A. 5. Treatment of 3-hydroxyvitamin A with dry ethanolic hydrogen chloride results in the formation of 3-ethoxy-anhydrovitamin A (anhydrovitamin A₂) and a compound that resembles naturally occurring anhydrovitamin A₂.

Zeaxanthin (3,3'-dihydroxy- β -carotene) and its dipalmitate, physalene, occur widely in Nature and have also been synthesized by Isler *et al.* (1956a, 1957) and Isler, Montavon, Ruegg, Saucy & Zeller (1956b). Jungalwala & Cama (1962) found that 90% of the total carotenoids in the anthers of *Delonix regia* (Gul Mohr) flowers is zeaxanthin. These anthers are a rich source of zeaxanthin. Several xanthophylls including zeaxanthin and lutein have been shown to be absorbed unchanged by the animal body, but it is not yet known whether they play an essential role in animal physiology. Earlier reports by Brockmann & Volker (1934) showed that deficiency of hydroxylated carotenoids rather than hydrocarbon carotenoids leads to the formation of white feathers after moulting and of white yolks in eggs of canaries. Patel, Mehl & Deuel (1951) failed to get any evidence of the appearance of vitamin A after administration of zeaxanthin by stomach tube in vitamin A-deficient rats. These workers gave intraperitoneal injections of an aqueous colloidal solution of zeaxanthin to rats and observed that appreciable quantities of unchanged zeaxanthin were absorbed by the liver, but there was no trace of vitamin A formation. In a study of the transport of carotenoids across the intestines of rats and chickens by Ganguly, Krishnamurthy & Mahadevan (1959), it was shown that lutein is absorbed into the intestinal-mucosa muscle and tissue, but is not converted into vitamin A. A recent report of the conversion of carotenoids like anhydrolutein (3'-hydroxy-3,4-dehydro- β -carotene) and 3,4:3',4'-bisdehydro- β -carotene into vitamin A₂ in rats (Budowski & Gross, 1965) is of considerable interest as the biogenesis of vitamin A₂ is still unknown.

Several chemical studies have been made on the oxidation of zeaxanthin. Oxidation of zeaxanthin with ozone and potassium permanganate resulted in the formation of $\alpha\alpha$ -dimethylsuccinic acid (Karrer, Helfenstein, Wehrli & Wettstein, 1930). Karrer, Ruegger & Solmsen (1938) obtained β -citaurin by partial oxidative degradation of zeaxanthin with potassium permanganate. Oxidation of zeaxanthin with manganese dioxide resulted in the formation of rhodoxanthin (Entschel & Karrer, 1959).

In none of the above studies, however, has the formation of 3-hydroxyretinene been reported. In a study of the oxidation of zeaxanthin similar to the oxidation of β -carotene with hydrogen peroxide-osmium tetroxide (Wendler, Rosenblum & Tishler, 1950), 3-hydroxyretinene has now been isolated in 16–30% yield as one of the products of oxidation. 3-Hydroxyretinene was characterized from a study of its u.v. and i.r. spectra and by preparing derivatives like 3-hydroxyvitamin A and 3-acetoxyretinene, whose i.r. spectra were also studied. 3-Hydroxyvitamin A is of special interest as it gives anhydrovitamin A₂ (3-ethoxyanhydrovitamin A₁) and a compound in spectroscopic and chromatographic behaviour identical with naturally occurring anhydrovitamin A₂ reported by Balasundaram, Bamji, Cama, Sundaresan & Varma (1958) and by Barua & Nayar (1963).

MATERIALS AND METHODS

Light petroleum (b.p. 40–60°), ethanol, chloroform and diethyl ether were purified by the methods described by Barua & Rao (1964). Benzene was washed with conc. H₂SO₄ and then refluxed with alkaline KMnO₄, washed

with water, dried with anhydrous CaCl_2 and finally distilled at 78° . E. Merck A.-G. (Darmstadt, Germany) alumina for chromatography, standardized according to Brockmann, was suitably deactivated by the addition of 5–8% (v/w) of water. H_2O_2 (30%, w/w; i.e. '100 volume'; E. Merck A.-G.), NaBH_4 (L. Light and Co. Ltd., Colnbrook, Bucks.), OsO_4 (Johnson, Matthey and Co. Ltd., Wembley, Middlesex) and SbCl_3 (AnalaR; British Drug Houses Ltd., Poole, Dorset) were obtained from the sources indicated. Other reagents were A.R. quality.

All the u.v. and visible spectra were recorded in a Beckman DK-2 spectrophotometer. The i.r. spectra were recorded in a Carl Zeiss UR-10 infrared spectrophotometer. The i.r. spectrum of 3-hydroxyretinene presented in this paper was recorded in the National Chemical Laboratory, Poona, India, with a Perkin-Elmer Infracord instrument. All the spectra given in this paper were traced from original automatically recorded spectra.

EXPERIMENTAL

Preparation of zeaxanthin. Fresh anthers (20 g.) of *D. regia* flowers were placed in light petroleum. The deep-red extract was decanted and extraction was continued until the extract was colourless. The combined extract was concentrated as far as possible by distillation under reduced pressure. The concentrate was next treated with an equal volume of methanolic 10% (w/v) KOH and refluxed at 60° for 30 min. The mixture was kept at room temperature overnight. Water was then added to bring the concentration of methanol to about 90% (v/v) and two layers separated. The upper light-petroleum layer was washed several times with aq. 90% (v/v) methanol and the washings were added to the methanol layer. The latter solution, containing the hypophasic pigments, was washed several times with light petroleum. It was then diluted with a large volume of water and the pigments were extracted with diethyl ether. The ethereal extract was washed with water to neutrality, dried over anhydrous Na_2SO_4 and finally evaporated to dryness by distillation under reduced pressure. The residue was dissolved in benzene and then light petroleum was added so that the ratio of benzene to light petroleum was 1:5 (v/v).

The solution was subjected to chromatography on 50 g. of alumina weakened by adding water (5%, v/w) and the chromatogram was developed with the same solvent mixture. After extrusion of the column, the main red zone (containing zeaxanthin) formed at the top was eluted with diethyl ether containing 5% (v/v) of methanol. The eluate was evaporated to dryness *in vacuo*. Zeaxanthin was crystallized from methanol. Yellow crystals (clusters) separated, m.p. 205° (uncorr.), λ_{max} 427 (inflection), 451 and 480 m μ (in ethanol). The yield was 35 mg.

The flowers of *D. regia* also proved to be a good source of zeaxanthin and about 10 mg. of the carotenoid was obtained/100 g. of dry flowers.

Oxidation of zeaxanthin. By following substantially the same procedure as laid down by Wendler *et al.* (1950) for the oxidation of β -carotene, zeaxanthin (50 mg.) was dissolved in ether (approx. 200 ml.) in a 1 l. three-necked flask equipped with a mechanical stirrer and a dropping funnel. About 5 g. of anhydrous Na_2SO_4 was added to the solution and 2 ml. of 2% (w/v) OsO_4 solution was added slowly and with stirring. After about 15 min. H_2O_2 solution was

added slowly and with vigorous stirring until the colour of the solution changed from the original red to orange-yellow (approx. 15 ml. of 30% H_2O_2 was required). At this point the reaction mixture was cooled in an ice bath and aq. 5% (w/v) NaHCO_3 solution was added. After the effervescence had subsided, the solution was decanted, the Na_2SO_4 cake was washed with ether and the ethereal solution was washed with aq. KOH until the aqueous layer was colourless. Finally the solution was washed with water to neutrality, dried over anhydrous Na_2SO_4 and the ether removed *in vacuo*. The red oily residue was dissolved in light petroleum.

The oxidation product was next chromatographed on 25 g. of alumina (plus 8% of water). Two main zones separated at the top: a yellow zone and an orange-yellow zone above it. The yellow zone was eluted with light petroleum containing 10% (v/v) of ether. Then the orange-yellow zone was eluted with light petroleum containing 15–20% (v/v) of ether. The eluate was collected in 10 ml. fractions. The first few fractions showed absorption peaks at 330 and 365 m μ whereas the latter fractions showed λ_{max} 368 m μ (solvent light petroleum).

Isolation of 3-hydroxyretinene from the zeaxanthin oxidation products. The fractions exhibiting λ_{max} 368 m μ and containing 3-hydroxyretinene were pooled. The solvent was removed *in vacuo* and the orange-red residue (13 mg.) was dissolved in light petroleum and rechromatographed. The purified product had the following properties: λ_{max} 368 m μ in light petroleum, 375 m μ in ethanol (Fig. 1); the SbCl_3

Fig. 1. Absorption spectra of 3-hydroxyretinene prepared by oxidation of zeaxanthin in ethanol (—) and light petroleum (●—●), and 3-acetoxyretinene prepared from 3-hydroxyretinene in ethanol (■—■) and light petroleum (▲—▲).

Fig. 2. Infrared spectrum of 3-hydroxyretinene (in Nujol mull).

reaction resulted in $\lambda_{\max}$ 635 m μ with a shoulder at 700 m μ . The i.r. spectrum (Fig. 2) in Nujol showed bands at 3440 and 1652 cm $^{-1}$ assigned to hydroxyl and carbonyl groups respectively.

Repetition of the oxidation experiment resulted in the isolation of 3-hydroxyretinene in 16–30% yield.

Preparation of 3-hydroxyretinene oxime. 3-Hydroxyretinene (5 mg.) was dissolved in a few drops of ethanol. Hydroxylamine hydrochloride (0.5 g.) was dissolved in water (2 ml.) and a few drops of aq. NaOH solution were added. This solution was poured on to the ethanolic solution and the mixture warmed at 60° for 5 min. with shaking. It was then cooled in ice to 0° and kept at this temperature for about 1 hr. 3-Hydroxyretinene oxime separated as yellow crystals, which were separated by filtration, washed with water and then dissolved in ethanol ($\lambda_{\max}$ 358 m μ in ethanol).

Preparation of 3-acetoxyretinene. 3-Hydroxyretinene (10 mg.) was dissolved in pyridine (2.5 ml.) and acetic anhydride (1 ml.) was added. The solution was refluxed at 70° for 1 hr., then cooled, diluted with water and the product extracted with light petroleum. The extract was washed with water several times, dried over anhydrous Na₂SO₄ and then evaporated to dryness *in vacuo*. The residue was dissolved in light petroleum (2 ml.) and chromatographed on alumina (20 g., plus 8% of water). A main yellow zone containing 3-acetoxyretinene moved down very slowly and was eluted with light petroleum containing 5% (v/v) of ether. The eluate was evaporated to dryness and the residue was dissolved in light petroleum. 3-Acetoxyretinene exhibited $\lambda_{\max}$ 369 m μ (in light petroleum) and 376 m μ (in ethanol) (Fig. 1). The i.r. spectrum (Fig. 4) in Nujol showed bands at 1750, 1675 and 1250 cm $^{-1}$ assigned to acetate C=O stretching, aldehyde C=O stretching and acetate C–O stretching vibrations respectively.

Preparation of 3-hydroxyvitamin A. 3-Hydroxyretinene (10 mg.) was dissolved in ethanol (10 ml.). The solution was cooled in an ice bath, solid NaBH₄ (approx. 5 mg.) added and the mixture shaken. Within 2 min. the solution turned almost colourless. At this point water was added and the product extracted with ether. The ethereal layer was washed with water to neutrality, dried over anhydrous

Fig. 3. Absorption spectra of 3-hydroxyvitamin A (—) prepared from 3-hydroxyretinene and 3-ethoxyanhydrovitamin A (●—●) and 3-hydroxyanhydrovitamin A (▲—▲) prepared from 3-hydroxyvitamin A in light petroleum.

Na₂SO₄ and ether was removed under reduced pressure. The residue was dissolved in light petroleum.

The product was chromatographed on 10 g. of alumina (plus 8% of water). The main pale-yellow zone (bright yellow fluorescence under u.v. light) was eluted with light petroleum containing 30–35% (v/v) of ether. The eluate,

Fig. 4. Infrared spectra of (a) 3-acetoxyretinene (in Nujol mull) and (b) 3-hydroxyvitamin A (in thin film).

containing 3-hydroxyvitamin A, was evaporated to dryness *in vacuo* and the residue dissolved in light petroleum. The following properties were noted: the u.v. spectrum showed $\lambda_{\max}$ 325m μ (in light petroleum or in ethanol) (Fig. 3); with Carr-Price reagent the compound produced a blue colour, $\lambda_{\max}$ 625 and 585m μ ; in the i.r. spectrum (thin film) absorption bands were observed at 3400 (O-H stretching vibration), 1382, 1130 and 1045cm $^{-1}$ (C-O stretching and O-H in-plane deformations in primary and secondary alcohols) (Fig. 4).

Preparation of 3-ethoxyanhydrovitamin A and 3-hydroxyanhydrovitamin A. 3-Hydroxyvitamin A (10mg.) was treated with dry ethanolic HCl (0.03N, 10ml.). The mixture was kept in the dark for 40 min. There was deepening of the colour. The solution was treated with aq. Na₂CO₃ and the product was extracted with light petroleum. It was then washed with water to neutrality, dried over anhydrous Na₂SO₄ and the solvent removed *in vacuo*. The residue was dissolved in light petroleum (2ml.).

The solution was poured on to a column (1.5cm. $\times$ 15cm.) of alumina (plus 5% of water) and the chromatogram was developed with light petroleum. A main yellow zone and a small yellow zone separated.

(a) 3-Ethoxyanhydrovitamin A₁. The main yellow zone moved down slowly and was eluted with light petroleum. This zone contained 3-ethoxyanhydrovitamin A₁: $\lambda_{\max}$ 335 (inflexion), 350, 370 and 390m μ (in light petroleum) (Fig. 3). With the Carr-Price reagent a green colour with $\lambda_{\max}$ 693m μ was produced. In the i.r. spectrum a sharp band was observed at 1100cm $^{-1}$ assigned to an ether group.

Mixed chromatography of this synthetic anhydrovitamin A₂ with anhydrovitamin A₂ (prepared from vitamin A₂ with dry ethanolic HCl) on alumina (plus 5% of water) did not result in any separation of zones on development with light petroleum.

(b) 3-Hydroxyanhydrovitamin A. After the elution of 3-ethoxyanhydrovitamin A, the small yellow zone was eluted with light petroleum containing 10% (v/v) of ether.

The solvent was removed *in vacuo*, the residue was dissolved in light petroleum and the spectral properties were studied. It showed $\lambda_{\max}$ 335 (inflexion), 350, 370 and 390m μ (in light petroleum) (Fig. 3), and with SbCl₃ a band with $\lambda_{\max}$ 693m μ .

Mixed chromatography of this synthetic anhydrovitamin A₂ (3-hydroxyanhydrovitamin A) with naturally occurring anhydrovitamin A₂ (isolated from *Bagarius bagarius* fish-liver oil) on a column (1.5cm. $\times$ 15cm.) of alumina (plus 5% of water) and development with light petroleum followed by light petroleum containing 2-3% (v/v) of ether did not result in any separation of zones. The single zone formed moved slowly and was finally eluted with light petroleum containing 10% (v/v) of ether.

Preparation and properties of 3-acetoxyvitamin A. 3-Acetoxyretinene (5mg.) was reduced with NaBH₄ in the same way as described for 3-hydroxyretinene above. 3-Acetoxyvitamin A was eluted from a column of alumina (plus 8% of water) with light petroleum containing 20% (v/v) of ether. The substance showed $\lambda_{\max}$ 325m μ (in light petroleum) in the u.v. spectrum.

3-Acetoxyvitamin A was treated with dry ethanolic HCl in the same way as described for 3-hydroxyvitamin A above. The resulting product, however, did not furnish any anhydrovitamin A.

DISCUSSION

In agreement with Jungalwala & Cama (1962) it has been found that zeaxanthin is the principal carotenoid in the anthers of *D. regia* flowers. Contrary to the report of Karrer, Eugster & Faust (1950) the lutein content of the anthers was found to be very small compared with that of zeaxanthin.

Much work has been done on the oxidation of β -carotene, especially to locate the sites of oxidative attack. Wendler *et al.* (1950) stated that the

oxidation of β -carotene with hydrogen peroxide in the presence of catalytic amounts of osmium tetroxide should proceed to a major extent at the unsaturated bonds 11-12, 11'-12' and 15-15', which are least substituted and sterically most accessible. Of these the 15-15' position is the least hindered as there are no methyl groups in its vicinity and should therefore be more susceptible to oxidative attack. Using the Wendler *et al.* (1950) procedure Glover & Redfearn (1954) made a detailed study of the oxidation of β -carotene and obtained evidence indicating that the penultimate bond in the conjugated system was the one preferentially attacked by the reagent followed by the stepwise oxidation of the product to form the whole series of β -apocarotenals including retinene.

As expected, evidence was obtained in the present work for the formation of 3-hydroxyretinene when zeaxanthin was oxidized with hydrogen peroxide in the presence of a catalytic amount of osmium tetroxide. In some of the experiments the lower yellow zone formed on chromatography of the oxidation products of zeaxanthin contained a compound spectroscopically similar to oxoretinene. This compound exhibits $\lambda_{\max}$ 380 m μ with a shoulder at 293 m μ (in ethanol). Probably 3-hydroxyretinene, which is formed first, is subjected to a second attack on the β -ionone ring and the CH(OH) group at position 3 is oxidized to a C=O group, thereby yielding some oxoretinene. During chromatography of the oxidation products of zeaxanthin, the orange-yellow zone formed at the top contains 3-hydroxyretinene. This zone is very often contaminated with another compound exhibiting $\lambda_{\max}$ 330-335 m μ . The two did not separate well and it is therefore preferable to follow Barua & Morton (1949) in using the technique of 'flowing' chromatography, when the first few fractions carry the '335 compound', and the later fractions the 3-hydroxyretinene without contamination by the '335 compound'.

It is concluded that the compound exhibiting $\lambda_{\max}$ 375 m μ is 3-hydroxyretinene for the following reasons: (i) It forms an oxime with hydroxylamine hydrochloride, indicating the presence of a carbonyl group, and an acetoxy derivative with acetic anhydride, revealing the presence of a hydroxyl group. (ii) Its adsorption affinity on deactivated alumina is much greater than that of retinene. Whereas retinene can be eluted from such a column with light petroleum alone, this hydroxy compound can only be eluted with light petroleum containing 15-20% (v/v) of ether. (iii) The reduction product of 3-hydroxyretinene, i.e. 3-hydroxyvitamin A, gives, on treatment with dry ethanolic hydrogen chloride, anhydrovitamin A₂, exhibiting $\lambda_{\max}$ 693 m μ in the Carr-Price reaction. The 3-hydroxyretinene structure is further confirmed

from a study of the i.r. spectrum of the compound. Sharp bands observed at 3440 and 1652 cm.⁻¹ are assigned to hydroxyl and carbonyl groups respectively. Henbest, Jones & Owen (1957) also reported peaks at 3430 and 1660 cm.⁻¹ for the 4-hydroxyretinene that they prepared from vitamin A₁. Conversion of 3-hydroxyretinene into 3-acetoxyretinene and examination of the i.r. spectrum of the latter revealed sharp bands at 1750, 1675 and 1250 cm.⁻¹, which are assigned to acetate carbonyl, aldehyde carbonyl and acetate C-O vibrations respectively.

3-Hydroxyvitamin A prepared from 3-hydroxyretinene has stronger adsorption affinity than vitamin A₁ on a weakened alumina column. Whereas vitamin A₁ can be eluted from such a column with light petroleum containing 20% (v/v) of ether this hydroxyvitamin can only be eluted with light petroleum containing 30-35% (v/v) of ether. Moreover, unlike vitamin A₁, which gives anhydrovitamin A₁, this hydroxyvitamin A gives anhydrovitamin A₂ ($\lambda_{\max}$ 693 m μ in the Carr-Price reaction) on treatment with dry ethanolic hydrogen chloride. Two such anhydrovitamins A₂ are formed having different adsorption properties on alumina columns. One of these anhydrovitamins A₂ is similar to synthetic anhydrovitamin A₂ (3-ethoxyanhydrovitamin A) prepared from vitamin A₂ by treatment with dry ethanolic hydrogen chloride. This was confirmed by carrying out mixed chromatography of the two compounds, when a single zone is obtained. Moreover, examination of the i.r. spectrum of this anhydrovitamin A₂ prepared from 3-hydroxyvitamin A shows a sharp band at 1100 cm.⁻¹ assigned to an ether group (Henbest, Jones, Owen & Thaller, 1955).

The other anhydrovitamin A₂, which is somewhat more strongly adsorbed than 3-ethoxyanhydrovitamin A on an alumina column, has similar chromatographic adsorption to the naturally occurring anhydrovitamin A₂ isolated from fish-liver oil by Barua & Nayar (1963). It is probable that when 3-hydroxyvitamin A (I) is treated with ethanolic hydrogen chloride the end hydroxyl group of the side chain is removed and that there is a rearrangement of the double bonds to produce anhydrovitamin A with the hydroxyl group still intact in the β -ionone ring; or otherwise there is formation of 3-hydroxyanhydrovitamin A (II). Compound (II) then suffers dehydroxylation at the 3-position in the β -ionone ring, producing a positive charge there (compound III), and an ethoxy group enters the ring, forming stable 3-ethoxyanhydrovitamin A, i.e. anhydrovitamin A₂ (IV). If, however, the latter reaction does not go to completion, then both 3-hydroxyanhydrovitamin A and 3-ethoxyanhydrovitamin A will appear. The reaction can probably be represented as shown in Scheme 1.

Scheme 1.

In agreement with this scheme, it is observed that, when the reaction of 3-hydroxyvitamin A with dry hydrogen chloride is prolonged to about 60 min., the second form of anhydrovitamin A₂ (hydroxyanhydrovitamin A) is not formed. From the above reaction scheme, a 3-hydroxyanhydrovitamin A structure (II) is proposed for the second anhydrovitamin A₂, which has stronger adsorption affinity on the alumina column. For the same reason, a 3-hydroxyanhydrovitamin A₁ structure can be proposed for the naturally occurring anhydrovitamin A₂.

A study of the i.r. spectrum of the compound would have revealed the presence of the hydroxyl group, but unfortunately non-availability of zeaxanthin in greater quantity and the difficulty in controlling the reaction with dry hydrogen chloride made it impossible to study the i.r. spectrum.

An attempt to obtain 3-hydroxyanhydrovitamin A by a different reaction scheme was also made. 3-Acetoxyretinene, which is prepared from 3-hydroxyretinene, is first reduced to give 3-acetoxyvitamin A. It was thought that 3-acetoxyvitamin A would produce 3-acetoxyanhydrovitamin A. This latter compound will give 3-hydroxyanhydrovitamin A on hydrolysis. But, contrary to our expectation, 3-acetoxyvitamin A did not furnish any anhydrovitamin A on treatment with dry ethanolic hydrogen chloride.

The identification of naturally occurring anhydrovitamin A₂ as 3-hydroxyanhydrovitamin A₁ should be regarded as tentative until further evidence be forthcoming to confirm it.

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