Occurrence of 3-Hydroxyretinol in the Freshwater Fish *Bagarius bagarius* and *Wallago attu*

ISOLATION AND SYNTHESIS

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A new chromogen that absorbs maximally at 329 nm was frequently found in the liver oils of the freshwater fish *Bagarius bagarius* and *Wallago attu*. It was shown to be a diester of 3-hydroxyretinol. 3-Hydroxyretinol was found to be a metabolite of lutein during the biosynthesis of 3-dehydroretinol in a freshwater fish. A new method for the chemical synthesis of 3-hydroxyretinol from methyl 3-dehydroretinoate is described. The possible pathway for the conversion of lutein into 3-dehydroretinol *in vivo* is discussed.

Any structural change in retinol, except at C-15, considerably diminishes its vitamin A-type activity. The only change of substituents at C-1 to C-14 that does not considerably lower the growth-promoting activity of retinol is further dehydrogenation; the only naturally occurring compounds of this type are those of the vitamin A₂ (3-dehydroretinol) series with an extra double bond at the 3,4-position of the β -ionone ring (Pitt, 1971). Retinol is found in large amounts in the liver of saltwater fish, whereas 3-dehydroretinol is found in the liver of freshwater fish. It is now well established that β -carotene and a number of other related carotenoids can act as pro-(vitamin A), and that the transformation of β -carotene into retinol occurs in the intestine via retinal. However, the biosynthesis of 3-dehydroretinol has not been elucidated. Barua *et al.* (1973) and Barua & Das (1975) have shown that lutein (β -carotene-3,3'-diol) can act as an efficient pro-(vitamin A₂) in the freshwater fish *Saccobranchus fossilis*. During this conversion of lutein into 3-dehydroretinol, anhydro-lutein (3,4-didehydro- β -caroten-3'-ol) has been isolated as an intermediate that is also an efficient precursor of 3-dehydroretinol (Barua & Das, 1975). A compound resembling retinol has been isolated during the conversion of lutein and also of anhydro-lutein into 3-dehydroretinol in the fish. This compound exhibits an absorption maximum at 327–330 nm and the u.v. spectrum is similar to that of retinol, but differs from retinol in exhibiting an absorption maximum at 675 nm in the SbCl₃ colour test (Das, 1974). The presence of a similar compound in the liver oil of *Wallago attu* was reported by Balasundaram *et al.* (1956, 1958). This compound, which closely resembles retinol, has been frequently observed in the liver oils of several other freshwater fish, namely *Bagarius bagarius*, *Wallago attu*, *Silonia silonia* and *Mystus seenghala* (Nayar, 1965;

Goswami, 1970; Ghosh, 1972). In the present paper we report evidence that the compound is 3-hydroxyretinol, and that it is a metabolite of lutein in the fish. A new method of chemical synthesis of the compound is described.

Experimental

Materials

Light petroleum (b.p. 40–60°C and b.p. 60–66°C), acetone, methanol (AnalaR) and benzene were from BDH [supplied by Glaxo Laboratories (India), Bombay, India]. Cyclohexane was from BDH Chemicals, Poole, Dorset, U.K. Diethyl ether was freshly distilled over iron prepared by reduction (supplied by Riedel De Haen, Germany) to remove peroxides. Chloroform and carbon tetrachloride were stored over anhydrous CaCl₂, and were distilled before use. Synthetic retinoic acid was a gift from Hoffman-La Roche (Roche Products, Bombay, India).

SbCl₃ reagent (Carr–Price reagent) was prepared by dissolving 100 g of SbCl₃ (AnalaR, BDH) in 400 ml of pure dry chloroform. Alumina for chromatographic analysis (Brockmann grade 1) was from BDH. It was deactivated by adding 5% (v/w) of water. MnO₂ (precipitated) was from BDH. Silica gel G for t.l.c. was from E. Merck A.-G., Darmstadt, Germany. MgO for chromatographic adsorption analysis (BDH) was activated by heating at 135°C for 3 h before use. A mixture of MgO/Celite 535 (Koch–Light, Colnbrook, Bucks., U.K.) (1:1, w/w) was used for column chromatography. Other chemicals were obtained as follows: H₂O₂ (30%, w/w; i.e. '100 volume'; Sarabhai Merck Chemicals Pvt., Wadi Wadi, Baroda, India); NaBH₄ and LiAlH₄ (Koch–Light, Colnbrook, Bucks., U.K.); OsO₄ (Johnson Matthey and Co., Wembley, Middx., U.K.); bromine (Merck). *NN*-Dimethylaniline (BDH) was purified by adding

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one-half its volume of acetic anhydride and refluxing the solution for 3 h. It was then distilled and pure dimethylaniline was collected at 193–195°C. Other reagents were of AnalaR quality.

Methods

Column chromatography was carried out on columns (usually 1.5 cm × 9.5 cm, unless otherwise mentioned) of alumina from light petroleum (b.p. 60–66°C) solution. (Light petroleum used throughout the present studies had b.p. 60–66°C unless otherwise stated.) Elution of the compounds was carried out with light petroleum alone or mixtures of light petroleum containing increasing proportions of diethyl ether (1–30%, v/v). The eluates were collected in fractions (5–6 ml) and the fractions having similar absorption spectra were pooled together.

The u.v. and visible spectra were recorded in a Beckmann DK-2 spectrophotometer. β -Carotene, lutein, anhydrolutein and 3-dehydroretinol were assayed on the basis of the following $A_{1\text{cm}}^{1\%}$ values: 2500, 2200, 2031 and 1455 respectively (all at the point of maximum absorption). The concentration of 3-hydroxyanhydroretinol, rehydrovitamin A₂ and 3-hydroxyretinol diester was calculated from the absorbance at the point of maximum absorption on the basis of approx. $A_{1\text{cm}}^{1\%}$ values of 3000, 1500 and 1800 respectively. The i.r. spectra were recorded in a Carl-Zeiss Jena UR-10 i.r. spectrophotometer. The n.m.r. (nuclear-magnetic-resonance) spectra were recorded in a Varian 60 MHz n.m.r. spectrometer at the Indian Institute of Science, Bangalore-560012, India.

Results

Chromatography of *B. bagarius* and *W. attu* fish liver oils: isolation of 3-hydroxyretinol diester

The livers (90 g wet wt.) of three *B. bagarius* fish were pooled, minced and ground with anhydrous

Na₂SO₄ in a glass mortar, extracted with light petroleum (b.p. 40–60°C) and filtered. The extraction was repeated until the extract was insensitive to SbCl₃ reagent. The combined extracts were concentrated under reduced pressure and the concentrate was evaporated to dryness *in vacuo*. The crude oil (2.0 g) was chromatographed on an alumina column (3 cm × 18 cm). The compounds eluted by light petroleum and mixtures of light petroleum and diethyl ether are shown in Table 1. Similarly the liver oil was extracted from 62 g (wet wt.) of only one *W. attu* and subjected to column chromatography in the same way as described above. The compounds that were recovered from the liver oil are shown in Table 1.

3-Hydroxyretinol diester

The fraction eluted with light petroleum containing 1–2% (v/v) of diethyl ether has been characterized as the diester of 3-hydroxyretinol (λ_{max} , 330 nm). This was rechromatographed on a column (1.5 cm × 9.5 cm) of 15 g of alumina. The yellow zone was eluted with light petroleum containing 1–2% (v/v) of diethyl ether. The eluate was collected in fractions (5 ml). The purified product had the following properties: λ_{max} , 328 nm in light petroleum, 329 nm in ethanol; the SbCl₃ reaction resulted in λ_{max} , 675 nm. The i.r. spectrum showed bands at 965, 1130, 1180, 1280 and 1720 cm⁻¹. The bands at 1280 and 1720 cm⁻¹ were assigned to the ester carbonyl group. That the compound was an ester was further supported by its behaviour on an alumina column and the appearance of a strong band in the i.r. spectrum at 3300 cm⁻¹ after saponification (see below).

Saponification of the diester of 3-hydroxyretinol

The ester (100 mg) was saponified with 100 ml of 10% (w/v) methanolic KOH at 50°C for 30 min.

Table 1. Chromatographic separation of vitamin A derivatives present in freshwater fish liver oils
Oils extracted from 90 g (wet wt.) of three pooled livers of *B. bagarius* and 62 g (wet wt.) of one liver of *W. attu* were chromatographed on columns (3 cm × 18 cm) of alumina and eluted with light petroleum and light petroleum containing increasing proportions of diethyl ether. The compounds that were recovered are listed in order of decreasing adsorption affinity.

Compound	λ_{max} values in light petroleum (nm)	Content of vitamin A derivatives			
		Wt. of chromatographic fraction from <i>B. bagarius</i>		Wt. of chromatographic fraction from <i>W. attu</i>	
		(mg)	(μ g)*	(mg)	(μ g)*
'433 Compound'	433, 410, 389, 369, 349, 333	98	150	Not calculated	
3-Dehydroretinyl ester	350, 287, 276	315	176 000	196	85 900
3-Hydroxyretinol diester	330	177	90 700	75	9080
3-Hydroxyanhydroretinol	387, 365, 348, ~333	197	886	16	205
Rehydrovitamin A ₂	387, 365, 348, ~333	106	113 000	70	80 000
3-Dehydroretinol	350, 287, 276	79	6130	120	9500

* Calculated on the basis of $A_{1\text{cm}}^{1\%}$ values shown in the Experimental section.

It was then extracted with peroxide-free diethyl ether, washed several times with water to remove the alkali, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was dissolved in 2ml of light petroleum and was chromatographed on a column (1.5cm $\times$ 5.5cm) of 10g of alumina. The strongly adsorbed yellow band of 3-hydroxyretinol was eluted with light petroleum containing 30% (v/v) of diethyl ether. The diethyl ether was evaporated *in vacuo* and the residue (80mg) was dissolved in light petroleum (b.p. 40–60°C) and gave λ_{max} 328nm. The i.r. spectrum of the compound showed bands at 970, 1040, 1180, 1310 and 3300 cm^{-1} . The band at 3300 cm^{-1} was assigned to the hydroxy-group. The n.m.r. spectrum showed signals at δ (p.p.m.) 1.45 (3H, CH_3 at C-1), 2.2 (3H, CH_3 at C-5), 2.4 (3H, CH_3 at C-9), 3.1 (3H, CH_3 at C-13), 3.7 (1H, OH at C-15) and 5.0 (1H, OH at C-3).

Oxidation of 3-hydroxyretinol: preparation of 3-hydroxyretinal

3-Hydroxyretinol (30mg) obtained in the above experiment was dissolved in 5ml of light petroleum at 50°C and passed through a column (2cm $\times$ 4cm) of 2g of activated MnO_2 . The u.v.-absorption maximum of the solution changed from 330nm to 355nm. The light petroleum solution was concentrated under reduced pressure to about 2ml and the solution was passed for a second time through the same column of MnO_2 . The absorption maximum of the solution changed to 368nm. The first yellow zone (2.2mg) formed during column chromatography was eluted with light petroleum and showed λ_{max} 389, 367, 351, ~333nm in light petroleum. The second zone (3.02mg) eluted with light petroleum containing 6–8% (v/v) of diethyl ether showed absorption maxima at 403, 383, 363 and ~343nm. The third dark-yellow main zone (16.0mg) was eluted with light petroleum containing 20–25% (v/v) of diethyl ether. The eluate was collected in fractions (10ml). The first fractions showed λ_{max} 369nm in light petroleum, whereas the latter fractions showed absorption peaks at 335, 371 and ~355nm. The upper zone (4.6mg), eluted with light petroleum containing 30% (v/v) of diethyl ether, showed a λ_{max} 326nm.

The fractions exhibiting a λ_{max} 369nm in light petroleum contained 3-hydroxyretinal. It was purified further by chromatography on an alumina column (1.5cm $\times$ 5.5cm). The yellow band of 3-hydroxyretinal was eluted with light petroleum containing 20–25% (v/v) of diethyl ether. The purified product (13mg) showed λ_{max} 369nm in light petroleum and at 380nm in methanol. The bands at 3300 and 1630 cm^{-1} observed in the i.r. spectrum of the purified product were assigned to hydroxy and carbonyl groups respectively. 3-Hydroxyretinal obtained thus, and 3-hydroxyretinal obtained synthetically by the oxida-

tion of zeaxanthin (β,β -carotene-3,3'-diol) by the method of Barua & Barua (1966), were co-chromatographed as follows: two spots, one of the 3-hydroxyretinal obtained by the oxidation of 3-hydroxyretinol and another of 3-hydroxyretinal obtained by the oxidation of zeaxanthin, and a third spot containing a mixture of the two, were applied to a silica-gel plate (0.25mm thick) and subjected to t.l.c. The solvent system used was acetone/light petroleum (3:7, v/v). The mixed spot did not separate and all the three spots moved the same distance (R_F 0.39).

Isolation of 3-hydroxyretinol diester from vitamin A-depleted fish after oral administration of lutein and anhydrolutein

Vitamin A-depleted *S. fossilis* fish were given lutein or anhydrolutein as described by Barua *et al.* (1973) and Barua & Das (1975).

Isolation from fish after oral administration of lutein. About 160–200 μg of lutein/day per fish was administered to vitamin A-depleted fish for 10 days. The amount of lutein administered (Table 2) is only approximate as a small amount of lutein always escaped from the fish through the opercular openings. The livers were minced and ground with anhydrous Na_2SO_4 . The lipids were extracted with light petroleum (b.p. 40–60°C) until the extract did not show any blue colour with SbCl_3 reagent. The bulked extract was evaporated to dryness under decreased pressure; the lipid residue was weighed and dissolved in 2ml of light petroleum. It was then chromatographed on a column (2cm $\times$ 14cm) of 50g of alumina. The compounds that were eluted by the method of Barua & Das (1975) have been characterized and are shown in Table 2. The fraction showing λ_{max} 330nm contained 3-hydroxyretinol diester, which was characterized as described below.

Isolation from fish after oral administration of anhydrolutein. About 250–277 μg of anhydrolutein/day per fish was administered to vitamin A-depleted fish for 20–30 days. The amount of anhydrolutein administered (Table 3) is only approximate as a small amount of anhydrolutein always escaped from the fish through the opercular openings. The livers were minced and ground with anhydrous Na_2SO_4 . The lipids were extracted with light petroleum (b.p. 40–60°C) until the extract was insensitive to SbCl_3 reagent. The extracts were pooled and evaporated to dryness. The residue was weighed and dissolved in 2ml of light petroleum. This solution was chromatographed on a column (2cm $\times$ 14cm) of alumina. The compounds that were eluted are shown in Table 3.

The fraction containing 3-hydroxyretinol diester (λ_{max} 330nm; obtained from fish given lutein and anhydrolutein) was purified further by chromatography on a column (1.5cm $\times$ 5.5cm) of alumina.

Table 2. *Metabolism of lutein in S. fossilis*

Lutein was administered to vitamin A-depleted *S. fossilis* and the metabolites of lutein were isolated after 10 days of lutein treatment and separated by column chromatography as described in the text.

No. of fish	Approx. amount of lutein administered (μg)	Amount isolated (μg)*			
		3-Dehydroretinyl ester	Anhydrolutein ester	3-Hydroxyretinol diester	Lutein
10	2000	95.5	4.0	6.12	15.5
14	1600	61.08	7.9	10.09	4.2
15	2000	61.1	10.34	4.1	20.5

* Calculated on the basis of $A_{1\text{cm}}^{1\%}$ values shown in the Experimental section.

Table 3. *Chromatographic separation of liver oil of vitamin A-depleted fish after oral administration of anhydrolutein.* Oils extracted from the livers of vitamin A-depleted fish (nine fish in Expt. 1 and ten fish in Expt. 2), after oral administration of approx. 2500 μg of anhydrolutein for 30 days (Expt. 1) or 20 days (Expt. 2), were chromatographed on columns (2 cm $\times$ 14 cm) of 50 g of alumina and different compounds were eluted with light petroleum and light petroleum containing an increasing proportion of diethyl ether.

Fraction no.	Eluent	λ_{max} in light petroleum (nm)	SbCl_3 blue colour (nm)	Identified compound	Amount isolated (μg)*
1	Light petroleum	474, 450, 420	590	β -Carotene (residual)	7.5† 8.0‡
2	Light petroleum	350, 287, 276	693	3-Dehydroretinyl ester	66.5† 73.2‡
3	2% Diethyl ether in light petroleum	460	—	Anhydrolutein ester	49.2† 48.1‡
4	5% Diethyl ether in light petroleum	330	675	3-Hydroxyretinol diester	13.9† 16.5‡
5	20% Diethyl ether in light petroleum	474, 448, 418	—	Unidentified	11.1† 10.1‡

* Calculated on the basis of $A_{1\text{cm}}^{1\%}$ values shown in the Experimental section.

† Expt. 1.

‡ Expt. 2.

The yellow zone, which was eluted with light petroleum containing 1–2% (v/v) of diethyl ether, showed a λ_{max} at 330 nm. 3-Hydroxyretinol diester isolated from *B. bagarius* fish liver oil and those isolated from vitamin A-depleted fish after oral administration of lutein and anhydrolutein were co-chromatographed on a silica-gel plate (0.25 mm thick) with a solvent system of acetone/light petroleum (1:9, v/v). No separation of the mixed spot was obtained and the R_F value was 0.54 for all the three spots, thus showing them to be identical.

3-Hydroxyretinol diester obtained as above was saponified as described above, purified by column chromatography and then subjected to t.l.c. as follows. Three spots of 3-hydroxyretinol, one isolated from *B. bagarius* fish liver oil, one obtained from liver oil from fish that had been administered lutein, another obtained from liver oil from fish that had been administered anhydrolutein and a mixed spot containing all these three were applied on a silica-gel plate (0.25 mm thick). The solvent system used was

acetone/light petroleum (3:7, v/v). The mixed spot did not separate and all the three spots moved the same distance (R_F 0.21).

Synthesis of 3-hydroxyretinol

Synthesis from retinoic acid. (i) Preparation of methyl 3-dehydroretinoate from methyl retinoate. Methyl retinoate (prepared from retinoic acid by reaction with methyl iodide) was converted into methyl 3-dehydroretinoate as described by Rowland & Bluhm (1967) for the preparation of 3,4-didehydroionone from β -ionone with the following modification: methyl retinoate (50 mg) was dissolved in 10 ml of carbon tetrachloride. Br_2 (0.0544 g) in 6 ml of carbon tetrachloride was added at -5 to -10°C over a period of 3 h. The bromine was added dropwise with shaking and care was taken to carry out the reaction in the absence of water. Moreover the Br_2 was added at regular intervals so that the colour of Br_2 did not persist. The mixture was warmed to 35 – 40°C and the carbontetrachloride removed *in vacuo*. *NN*-Dimethyl-

aniline (10 ml) was added to the residue and the mixture was heated at 90–95°C for 1 h. After cooling to 25°C, 50 ml of light petroleum (b.p. 40–60°C) and about 30 ml of water was added. The light petroleum solution was separated and washed several times with 200 ml of 6M-HCl, then four times with water. The solution was dried over anhydrous Na₂SO₄ and the solvent removed *in vacuo*. The residue was dissolved in 2 ml of light petroleum and chromatographed on a column of alumina. The first light-yellow band of unreacted methyl retinoate was eluted with light petroleum. The major deep-yellow band of methyl 3-dehydroretinoate absorbing at 367 nm, with an absorbance inflexion at 305 nm in light petroleum, was eluted with 1% (v/v) diethyl ether in light petroleum. The yield was 28 mg; the m.p. of the acid was 178–180°C.

(ii) Preparation of methyl 3-methoxyretinoate. By using the procedure outlined by Surmatis & Thommen (1967) for the preparation of 3-methoxy- β -ionone, 58 mg of methyl 3-dehydroretinoate was dissolved in 7 ml of methanol at 50°C. A cold (5°C) solution of 0.6 ml of 36M-H₂SO₄ in 65 ml of methanol (5°C) was added to this methanolic solution at 0––5°C. The mixture was kept at 0°C for 24 h. The absorption maxima of the crude methanolic solution changed from 367 nm to 350 nm. It was then poured into a separating funnel that contained ice and 5 ml of 0.1M-NaOH, and the mixture was extracted with diethyl ether. The diethyl ether layer was washed several times with water to remove methanol. The diethyl ether layer was then dried over anhydrous Na₂SO₄ and the solvent evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of light petroleum and chromatographed on an alumina column. The first yellow zone that was eluted with light petroleum was the unchanged methyl 3-dehydroretinoate, which gave $\lambda_{\max}$ 365 nm in light petroleum. The second pink zone of methyl 3-methoxyretinoate, which was eluted with 2% (v/v) of diethyl ether in light petroleum, showed a $\lambda_{\max}$ at 345 nm in light petroleum. The yield was 36.85 mg. The i.r. spectrum showed bands at 965, 1030, 1100, 1150, 1240, 1310, 1590, 1710 and 1740 cm⁻¹. The band at 1150 cm⁻¹ was assigned to the methoxy group in the compound. The n.m.r. spectrum exhibited signals at δ (p.p.m.) 1.24 (3H, CH₃ at C-1), 1.79 (3H, CH₃ at C-5), 1.97 (3H, CH₃ at C-9), 2.30 (3H, CH₃ at C-13), 3.24 (3H, CH₃ of ester group) and 3.3 (3H, O-CH₃).

(iii) Preparation of methyl 3-acetoxyretinoate. Methyl 3-methoxyretinoate was converted into methyl 3-acetoxyretinoate by the procedure outlined by Ganem & Small (1974) for the preparation of cholesteryl acetate with the following modification: methyl 3-methoxyretinoate (41 mg) was dissolved in 4 ml of ethyl acetate with shaking at 40°C. After cooling to 25°C, 0.2 ml of a solution of anhydrous FeCl₃ (5 mg) dissolved in 4 ml of acetic anhydride was

added with a pipette, with shaking. The solution was kept at 25°C for 4 h. At the end of this period the mixture was poured into 20 ml of water and methyl 3-acetoxyretinoate was extracted three times with diethyl ether. The combined extract was washed several times with water, dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of light petroleum and chromatographed on an alumina column. The fast-moving yellow zone of methyl 3-acetoxyretinoate was eluted with light petroleum (30 mg) and showed $\lambda_{\max}$ 350 nm in light petroleum. The n.m.r. spectrum exhibited signals at δ (p.p.m.) 1.27 (3H, CH₃ at C-1), 1.66 (3H, CH₃ at C-5), 2.00 (3H, CH₃ at C-9), 2.33 (3H, CH₃ at C-13) and 2.97 (6H, singlet, acetoxy and carboxymethyl groups).

(iv) Preparation of 3-hydroxyretinol. Methyl 3-acetoxyretinoate (72 mg) was treated with 100 mg of LiAlH₄ in 10 ml of dry peroxide-free diethyl ether at 0°C. The reaction was complete in 5 min. Excess of LiAlH₄ was decomposed with water and the reaction product extracted with diethyl ether, washed with water, dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo*. The residue was dissolved in light petroleum and chromatographed on an alumina column (1.5 cm $\times$ 5.5 cm). The strongly adsorbed yellow band of 3-hydroxyretinol (yellow fluorescence in u.v. light) was extruded, and was eluted with diethyl ether. It showed an absorption maximum at 329 nm in light petroleum. The yield was 54 mg.

Synthesis from zeaxanthin. (i) Preparation of 3-hydroxyretinal. The procedure described by Barua & Barua (1966) was used. Zeaxanthin (100 mg) was dissolved in diethyl ether (250 ml), and 15 g of anhydrous Na₂SO₄ and 2 ml of 2% (w/v) OsO₄ solution were added with stirring. After about 15 min, 30 ml of 30% (v/v) H₂O₂ solution was added until the solution became yellow. 3-Hydroxyretinal was isolated and purified by chromatography as described by Barua & Barua (1966).

3-Hydroxyretinal was further purified by chromatography on a column (1.5 cm $\times$ 12.5 cm) of 10 g of MgO/Celite (1:1, w/w). The purified product (25.49 mg) showed $\lambda_{\max}$ 368 nm in light petroleum and $\lambda_{\max}$ 378 in methanol.

(ii) Preparation of 3-hydroxyretinol. 3-Hydroxyretinal (10 mg) was reduced with NaBH₄ as described by Barua & Barua (1966) and purified by chromatography on an alumina column. The purified product (6.8 mg) showed $\lambda_{\max}$ 328 nm in light petroleum. The SbCl₃-reagent product showed $\lambda_{\max}$ at 675 nm.

Co-chromatography of naturally occurring 3-hydroxyretinol and synthetic 3-hydroxyretinol

Three spots, one of naturally occurring 3-hydroxyretinol (isolated from *B. bagarius* fish liver oil), one of 3-hydroxyretinol prepared from zeaxanthin and

one of 3-hydroxyretinol synthesized from retinoic acid, and a fourth spot containing a mixture of all these three compounds were applied to a silica-gel plate (0.25 mm thick) and subjected to t.l.c. with acetone/light petroleum (3:7, v/v) as the solvent system. The mixed spot did not separate and all the four spots moved the same distance (R_F 0.20).

Preparation of 3-methoxyanhydroretinol and 3-hydroxyanhydroretinol

3-Hydroxyretinol (10 mg) was treated with 10 ml of dry methanolic 0.03 M-HCl. The mixture was kept in the dark for 40 min. The solution was treated with 0.6 M- Na_2CO_3 and the product was extracted with light petroleum. It was then washed with water and adjusted to pH 7, dried over anhydrous Na_2SO_4 and the solvent evaporated *in vacuo*. Chromatography of the crude solution on an alumina column resulted in the separation of two zones.

3-Methoxyanhydroretinol. The main yellow zone of 3-methoxyanhydroretinol eluted with light petroleum showed λ_{max} . 390, 369, 351 and ~ 334 nm in light petroleum (b.p. 40–60°C); a green colour with λ_{max} . 693 nm was produced with SbCl_3 reagent.

Mixed chromatography of this 3-methoxyanhydroretinol with the 3-methoxyanhydroretinol prepared from dehydroretinol with dry methanolic HCl, run on a silica-gel plate (0.25 mm thick) with acetone/light

petroleum (3:17, v/v) as the solvent system, did not result in any separation of the mixed spot (R_F 0.69).

3-Hydroxyanhydroretinol. The second yellow zone of 3-hydroxyanhydroretinol was eluted with light petroleum containing 10% (v/v) of diethyl ether. It showed λ_{max} . 390, 369 and 351 nm with an inflexion at 334 nm. Co-chromatography of 3-hydroxyanhydroretinol with naturally occurring 3-hydroxyanhydroretinol (isolated from *B. bagarius* fish liver oil) on a silica-gel plate (0.25 mm thick) with acetone/light petroleum (3:17, v/v) showed them to be identical (R_F 0.24).

Discussion

A new chromogen exhibiting a λ_{max} . at 330 nm was observed by Balasundaram *et al.* (1956, 1958) in the liver oil of *W. attu*. The compound closely resembles retinol. Subsequently it has been shown to occur in the liver oils of several other freshwater fish (Nayar, 1965; Goswami, 1970; Ghosh, 1972). During the chromatographic separation of liver oil from *B. bagarius* on a column of deactivated alumina, this '330 compound' was eluted only after 3-dehydroretinyl ester. Moreover, in the SbCl_3 colour test it absorbed at 675 nm instead of 620 nm, characteristic of retinol, revealing it to be different from retinyl ester. Saponification of the '330 compound' resulted, without any change in the u.v. spectrum, in the for-

Scheme 1. Possible main pathway of biosynthesis of 3-dehydroretinol

mation of the free alcohol that was, however, unstable. Study of the i.r. spectrum of the naturally occurring compound and of its hydrolysis product revealed that the naturally occurring compound was an ester with both hydroxy groups esterified.

The oral administration of lutein or of anhydrolutein to the vitamin A-depleted *S. fossilis* also led to the formation of the '330 compound' along with the 3-dehydroretinyl ester.

It has been shown that *S. fossilis* can synthesize 3-dehydroretinyl ester from lutein, and that anhydro-

lutein is an intermediate in this biosynthesis (Barua *et al.*, 1973; Barua & Das, 1975). If the structures of lutein (I) and of anhydrolutein (II) are compared (Scheme 1), then any cleavage of the anhydrolutein molecule, whether by central cleavage at the 15,15'-double bond or by cleavage by β -oxidation from both ends, should result in the formation of both 3-hydroxyretinol (III) and 3-dehydroretinol (IV). Since the chromophoric systems in retinol and 3-hydroxyretinol are the same, both compounds should show similar u.v.-absorption spectra. It was, therefore, suggested that the '330 compound' might be 3-hydroxyretinol. Adsorption affinity during column chromatography on alumina or t.l.c. on silica-gel supported this view. To arrive at such a structure, the properties of '330 compound' were compared with synthetic 3-hydroxyretinol. 3-Hydroxyretinol was synthesized from zeaxanthin by a procedure described by Barua & Barua (1966). It was also synthesized from methyl 3-dehydroretinoate (VI) by a new route (Scheme 2). The spectroscopic properties of methyl 3-dehydroretinoate and the various intermediates in the synthesis of 3-hydroxyretinol are shown in Table 4. The present procedure for the preparation of 3-hydroxyretinol from methyl retinoate (overall yield from methyl retinoate is 19.34%) has the advantage over the previous method (Barua & Barua, 1966) (overall yield from zeaxanthin is 17.34%). Since yield is greater and the procedure avoids the use of OsO_4 . Since zeaxanthin is not easily available, the present method for the preparation of 3-hydroxyretinol from methyl retinoate (V), which can be prepared in one step from retinol (Barua & Ghosh, 1973), is more convenient. The present procedure for the introduction of a hydroxy group at C-3 in retinol should provide a new route for the synthesis of 3-oxygenated carotenoids that are in general synthesized from isophorone via 2,4,4-trimethyl-7,10-dioxaspiro[4,5]decan-3-one (Mayer & Isler, 1971). Some difficulties were encountered in the synthesis of 3-hydroxyretinol. First, in the preparation of methyl 3-dehydroretinoate (VI) from methyl retinoate by bromination followed by dehydrobromination, the bromination of methyl retinoate in carbon tetrachloride has to be carried out at -10°C and the amount of Br_2 added was such that the colour of Br_2 did not persist. Secondly, during the preparation of methyl 3-methoxyretinoate (VII), H_2SO_4 had to be added at 0°C with shaking, otherwise the reaction mixture turned greenish-yellow resulting in the formation of other products. Greatest difficulty was encountered in the preparation of methyl 3-acetoxyretinoate (VIII). At the early stage of the preparation of this compound, solid anhydrous FeCl_3 was directly added and the solution was stirred for 1 h. This often resulted in the decomposition of the acetoxy compound, possibly due to a slight excess of FeCl_3 .

Scheme 2. Chemical synthesis of 3-hydroxyretinol

Table 4. Spectroscopic properties of methyl 3-dehydroretinoate, methyl 3-methoxyretinoate, methyl 3-acetoxyretinoate and 3-hydroxyretinol

	$\lambda_{\max.}$ (nm)			
	Light petroleum as solvent	Benzene as solvent	Methanol as solvent	SbCl ₃ product
Methyl 3-dehydroretinoate	367	373	371	645
Methyl 3-methoxyretinoate	351	358	352	630
Methyl 3-acetoxyretinoate	350	354	352	630
3-Hydroxyretinol	329	334	330	675;

added and the yields were low. This difficulty has now been overcome by dropwise addition of a dilute solution of anhydrous FeCl₃ in acetic anhydride.

The synthetic 3-hydroxyretinol prepared from retinoic acid and also from zeaxanthin and the naturally occurring 3-hydroxyretinol were identical: their adsorption affinities on deactivated alumina column or on silica-gel plates were the same and mixed chromatography did not result in any resolution. The 3-hydroxyretinol structure was further supported by oxidation of the compound with MnO₂ to give 3-hydroxyretinal. Although the u.v.-absorption spectrum of 3-hydroxyretinal was similar to retinal, the compound differed from retinal in exhibiting a strong absorption at 3300 cm⁻¹ in the i.r. spectrum.

Furthermore, treatment of either naturally occurring or synthetic 3-hydroxyretinol with dry methanolic HCl gave 3-methoxyanhydroretinol, which had a $\lambda_{\max.}$ of 693 nm in the SbCl₃ reaction. Two anhydrovitamin A₂ compounds were formed having different adsorption properties on alumina. One of these compounds was similar to synthetic 3-methoxyanhydroretinol prepared from 3-dehydroretinol by treatment with dry methanolic HCl. The other compound, which is somewhat more strongly adsorbed than 3-methoxyanhydroretinol on an alumina column, has similar chromatographic adsorption to the naturally-occurring 3-hydroxyanhydroretinol isolated from the liver oil of *B. bagarius*.

The appearance of this second form of anhydrovitamin A in the liver oils of certain freshwater fish (Balasundaram *et al.*, 1956, 1958; Barua & Nayar, 1963), for which a 3-hydroxyanhydroretinol structure has been proposed (Barua & Barua, 1966; Barua & Nayar, 1963), can be easily explained by the presence of 3-hydroxyretinol that can arise from lutein. The isolation of 3-hydroxyretinol from the livers of vitamin A-depleted fish after oral administration of lutein or anhydrolutein has therefore led us to suggest the probable main pathway for the formation of 3-dehydroretinol in the freshwater fish *S. fossilis*

shown in Scheme 1. Furthermore, the natural occurrence of 3-hydroxyretinol and of 3-hydroxyanhydroretinol (naturally occurring 3-hydroxyanhydroretinol) can be easily explained if lutein is considered as a precursor of 3-dehydroretinol.

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