Structure and Synthesis of Naturally Occurring Anhydrovitamin A₂

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The structure of naturally occurring anhydrovitamin A₂ was elucidated as 3-hydroxy-anhydroretinol from study of its u.v.-visible, i.r., n.m.r. and mass spectra. The structure has been confirmed by a chemical synthesis. *Saccobranchus fossilis*, a freshwater fish, can convert 3-hydroxyretinol into 3-hydroxyanhydroretinol, which in turn is converted into 3-dehydroretinol. Chemical conversion of 3-hydroxyretinol into 3-dehydroretinol was also carried out.

Anhydroretinol (anhydrovitamin A₁) is formed when retinol is treated with ethanolic HCl (Shantz *et al.*, 1943) or with toluene-*p*-sulphonic acid (Shantz, 1950). Anhydroretinol shows an absorption triplet at 392, 371 and 351 nm in its u.v. spectrum. Similar treatment of 3-dehydroretinol (3,4-didehydroretinol) with ethanolic HCl gives anhydrovitamin A₂, exhibiting similar absorption bands in the u.v. region (Shantz, 1948; Farrar *et al.*, 1952). Further studies by Henbest *et al.* (1955) have shown that anhydrovitamin A₂ may have a 3-ethoxyanhydroretinol or 3-methoxyanhydroretinol structure, depending on the nature of the solvent used in its preparation.

During the course of an investigation on 3-dehydroretinol, Balasundaram *et al.* (1958) reported the natural occurrence of a compound exhibiting the same absorption triplet as anhydrovitamin A₂ in the liver oils of the freshwater fish *Wallago attu*. The presence of a similar compound in the liver oil of *Bagarius bagarius* was also reported by Barua & Nayar (1963). The natural and synthetic anhydrovitamins A₂ possessed exactly similar absorption bands in their u.v. spectra, but they differed in chromatographic behaviour, the naturally occurring compound being more strongly adsorbed on a deactivated-alumina column. The distribution ratios between light petroleum (b.p. 60–66°C) and aq. 95% (v/v) methanol of naturally occurring anhydrovitamin A₂ and chemically prepared anhydrovitamin A₂ (3-ethoxyanhydroretinol) were also different (Balasundaram *et al.*, 1958). From these observations Balasundaram *et al.* (1958) reported that the naturally occurring anhydrovitamin A₂ differed from the synthetic anhydrovitamin A₂ (3-ethoxyanhydroretinol) in not having an ethoxy group, but having a different substituent in the β -ionone ring or possibly elsewhere. Although Barua & Barua (1966) and Barua & Nayar (1966) have proposed a 3-hydroxyanhydroretinol structure, the structure has not so far been confirmed.

It has been shown by Barua *et al.* (1977) that the oral administration of lutein ($\beta\epsilon$ -carotene-3,3'-diol) or anhydrolutein (3,4-didehydro- β -caroten-3'-ol) to the vitamin A-depleted *Saccobranchus fossilis* led to the formation of 3-hydroxyretinol along with 3,4-didehydroretinol. It is probable that the naturally occurring anhydrovitamin A₂ for which a 3-hydroxyanhydroretinol structure has been proposed by Barua & Barua (1966) and Barua & Nayar (1966) can arise from 3-hydroxyretinol in a similar manner to the way in which anhydroretinol is formed from retinol. A study was therefore made of the metabolism of 3-hydroxyretinol in vitamin A-depleted *S. fossilis*. In the present paper we report the evidence that the naturally occurring anhydrovitamin A₂ is 3-hydroxyanhydroretinol and that it is formed from 3-hydroxyretinol in the freshwater fish *S. fossilis*. The fish is able to convert 3-hydroxyanhydroretinol into 3-dehydroretinol. Further we report the chemical conversion as well as biological transformation by *S. fossilis* of 3-hydroxyretinol into 3-dehydroretinol.

Experimental

Materials

α -Tocopheryl acetate was from Glaxo Laboratories, Bombay, India. LiAlH₄ was from Alfa Products, Beverly, MA, U.S.A. The sources of other chemicals and solvents used are described by Barua *et al.* (1977). Light petroleum used throughout the present investigation had b.p. 40–60°C unless otherwise stated. The diester of 3-hydroxyretinol and 3-hydroxyanhydroretinol were isolated from the liver oils of *B. bagarius*, *W. attu* and *Mystus seenghala* as described by Barua *et al.* (1977).

Methods

The u.v. and visible spectra were recorded in a Beckman DK-2 spectrophotometer. The concentrations of β -carotene, 3-dehydroretinol, the diester

of 3-hydroxyretinol, 3-hydroxyanhydroretinol and rehydrovitamin A₂ were calculated from the absorption values used by Barua *et al.* (1977). '433 Compound' was assayed on the basis of the $A_{1\text{cm}}^{1\%}$ value of 1468 (Ghosh, 1972) at the point of maximum absorption. The i.r. spectra were recorded in a Carl Zeiss Jena UR-10 i.r. spectrophotometer. The n.m.r. spectra were recorded in a Varian 60MHz n.m.r. spectrometer at the Indian Institute of Science, Bangalore-560012, India, and Regional Research Laboratory, Jorhat, India. The mass spectra were recorded at the Regional Research Laboratory, Jorhat, India.

Fish and fish diet

Freshly caught *S. fossilis* fish were kept alive in tanks as described previously by Barua *et al.* (1973) and made vitamin A-depleted by keeping them on a special fish diet devoid of carotenoids and vitamin A, prepared according to the composition given in Table 1 (D. R. Fraser, personal communication). The diet was prepared as follows: CM-cellulose was dissolved in water at 25°C and then gelatin was added with stirring. The mixture was heated on a water bath at 80°C until all the gelatin had dissolved. The mixture was allowed to cool to 40°C and then the casein/dextrin/vitamin mixture and salt mixture were mixed together and added with stirring to the gelatin solution. The mixture was stirred well to remove any lumps that were formed. A mixture containing

vitamins E and D was dissolved in refined groundnut oil to give a final volume of 20ml and the mixture was added to the diet and stirred well. The whole mixture was poured into a shallow dish and kept at 4°C for 1–2h, during which time the diet solidified to the consistency of cheese. The diet was kept in an airtight container at 4°C. Whenever required, it was cut into small pieces and supplied to the fish.

Administration of diester of 3-hydroxyretinol and 3-hydroxyanhydroretinol

A suspension of diester of 3-hydroxyretinol in Tween-80 was mixed with goat meat and orally administered to the vitamin A-depleted fish as described by Barua *et al.* (1973). 3-Hydroxyanhydroretinol was dissolved in 0.2ml of Tween-80 containing aq. 40% (w/v) α -tocopheryl acetate. It was then mixed with goat meat and orally administered to the vitamin A-depleted fish as described by Barua *et al.* (1973).

Isolation of carotenoids and vitamin A

The fish were killed by a blow to the head and the livers were ground to powder in a glass mortar along with acid-washed sea sand and anhydrous Na₂SO₄. The lipids were extracted four to five times with light petroleum until the extract gave no colour with SbCl₃ reagent. The bulk extract was evaporated to dryness *in vacuo*, and the residue dissolved in 2ml of light petroleum was subjected to column chromatography.

Column chromatography of carotenoids and vitamin A

Column chromatography was carried out on columns (1cm×7cm and 1.5cm×5.5cm, unless otherwise mentioned) of water-deactivated (5%, v/w) alumina. The mixture of compounds or extract was dissolved in a small volume (about 2ml) of light petroleum (b.p. 60–66°C) and poured into the column, which was developed with light petroleum (b.p. 60–66°C). The compounds were eluted with light petroleum (b.p. 60–66°C) or mixtures of light petroleum containing various proportions of diethyl ether (1–35%, v/v).

T.l.c. of carotenoids and vitamin A

The compounds separated by column chromatography were purified by t.l.c. on silica-gel plates (0.25mm thick). The compounds dissolved in light petroleum were applied to the plates as strips and not as spots. The following solvent systems were used for resolution of the different compounds: 5% (v/v) acetone in light petroleum for 3-dehydroretinyl ester (R_F 0.76); 10% (v/v) acetone in light petroleum for diester of 3-hydroxyretinol and methyl 3-dehydroretinoate (R_F 0.68); 20% (v/v) acetone in light

Table 1. *Composition of vitamin A-deficient diet*
The diet is identical with that recommended by Dr. D. R. Fraser (personal communication) except for the omission of linolenic acid (1g).

Ingredient	Amount (g)
CM-cellulose	2
Water	200
Gelatin	12
Acid-precipitated casein	38
White dextrin	28
Vitamin mixture*	7
Salt mixture†	4
Fat-soluble vitamins*	8

* Composition of the vitamin mixture: thiamin hydrochloride (250mg), riboflavin (1g), pyridoxine hydrochloride (250mg), choline chloride (25g), nicotinamide (3.75g), calcium pantothenate (2.5g), inositol (10g), biotin, (25mg), folic acid (75mg), L-ascorbic acid (5g), cellulose powder (300g). Vitamin D (1.4mg) and α -tocopheryl acetate (800mg) were dissolved in 20ml of refined groundnut oil.

† Composition of the salt mixture: CaCO₃ (205g), CaHPO₄ (325g), Na₂HPO₄ (185g), KCl (205g), MgSO₄·H₂O (70g), MnSO₄·H₂O (4.5g), ferric citrate (4.35g), CuSO₄ (375mg), ZnCO₃ (75mg), KIO₃ (25mg).

petroleum for 3-dehydroretinal (R_F 0.64); 20% (v/v) ethyl acetate in cyclohexane for 3-acetoxyanhydroretinol (R_F 0.75), 3-methoxyanhydroretinol (R_F 0.44) and 3-hydroxyanhydroretinol (R_F 0.21); 30% (v/v) acetone in light petroleum for 3-dehydroretinol (R_F 0.53). The separated bands were immediately transferred into flasks each containing about 10ml of peroxide-free diethyl ether. The diethyl ether extracts were filtered and evaporated to dryness *in vacuo* and the residues were dissolved in light petroleum. The spectral properties and R_F values of the naturally occurring compounds and those prepared synthetically were compared with authentic compounds.

3-Dehydroretinyl ester formed was further characterized as follows: first it was saponified with methanolic 10% (w/v) KOH. The 3-dehydroretinol obtained was then converted into 3-methoxyanhydroretinol by the procedure described by Henbest *et al.* (1955). 3-Dehydroretinol was also converted into 3-dehydroretinal by oxidation with activated MnO₂ (Ball *et al.*, 1948).

Results

Chromatography of *B. bagarius*, *W. attu* and *M. seenghala* liver oils: isolation of 3-hydroxyanhydroretinol

The liver oil (5.2g) from three livers (52g wet wt.) of *B. bagarius* was chromatographed on an alumina column (3cm×12cm) and the different compounds that were separated are shown in Table 2. Similarly the liver oil was extracted from 50g (wet wt.) of four *W. attu* fish and 30g (wet wt.) of one *M. seenghala* fish and subjected to column chromatography (Table 2).

3-Hydroxyanhydroretinol

The fractions eluted with light petroleum containing 8–10% (v/v) diethyl ether have been characterized as 3-hydroxyanhydroretinol (λ_{max} 387, 366, 350, ~333 nm in light petroleum). This was rechromatographed on an alumina column and the 3-hydroxyanhydroretinol fraction thus obtained was purified further by t.l.c. The purified compound was dissolved in a small quantity (2–4ml) of methanol and left at –5°C to 0°C overnight. Colourless sterols precipitated. These were removed by cold filtration and the process was repeated until there was no further precipitation. The methanolic solution was concentrated to a volume of about 5ml and further treated with an equal volume of ethanolic 2% (w/v) digitonin and kept at 0°C for 24h. No precipitate was obtained. About 50ml of water was added and the product extracted with diethyl ether. The diethyl ether layer was washed 3 to 4 times with water, dried

Table 2. Chromatographic separation of vitamin A derivatives present in freshwater-fish liver oils

Oils extracted from livers of *W. attu*, *B. bagarius* and *M. seenghala* were chromatographed on columns (3cm×18cm) of alumina and eluted with light petroleum and light petroleum containing various proportions of diethyl ether. The compounds that were recovered in various experiments are shown. Weights of compounds were calculated on the basis of $A_{1cm}^{1\%}$ values shown in the Experimental section.

Fish	No. of livers	Wet weight of the livers (g)	Weight of the oil (g)	Content of vitamin A derivatives (μ g)						
				'433 Compound'	β -Carotene	3-Dehydro-retinyl ester	Diester of 3-hydroxyretinol	3-Hydroxy-anhydroretinol	Rehydrovitamin A ₂	3-Dehydro-retinol
<i>W. attu</i>	2	60	2.37	—	—	53 100	3180	5580	1390	—
	2	90	4.37	919	—	6790	6040	2100	1570	—
	4	50	5.32	5370	266	55 000	25 500	7600	1000	—
	3	30	1.93	—	—	7420	1320	36	60	—
	2	52	—	6690	—	53 600	7250	13 200	2730	—
<i>B. bagarius</i>	1	30	1.54	9970	—	61 600	8770	8380	1130	—
	3	52	5.19	1800	—	9070	—	96 700	50 200	—
	1	70	2.69	31	—	34 000	6280	1000	87	2230
<i>M. seenghala</i>	1	8	1.81	274	131	2550	1630	393	2000	—
	1	—	5.40	—	—	70 800	39 300	1470	6850	—
	1	—	1.82	—	—	10 500	1690	1470	937	—

over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was dissolved in light petroleum. The u.v.-visible spectra of the purified compound and its SbCl_3 -reaction product were recorded as shown in Table 3. The i.r. and n.m.r. spectra of the purified compound were also recorded (Tables 4 and 5). Though the fragmentation pattern in the mass

spectrum of the naturally occurring compound was in good agreement with the expected values for a 3-hydroxyanhydroretinol structure, the M^+ ion value was obtained at 386, which may be due to the presence of a trace amount of sterol, probably cholesterol. To demonstrate the absence of an ester group and the presence of a free hydroxy group in the

Table 3. *Spectroscopic properties of 3-methoxyanhydroretinol, 3-acetoxyanhydroretinol, 3-hydroxyanhydroretinol and 3,5-dinitrobenzoate of 3-hydroxyanhydroretinol*
Details of the preparation of the compounds are given in the Experimental section.

Compound	$\lambda_{\text{max.}}$ (nm)			
	Light petroleum as solvent	Ethanol as solvent	Benzene as solvent	SbCl_3 product
3-Methoxyanhydroretinol	389, 367, 350, ~333	389, 370, 350, ~333	395, 374, 355, ~338	693
3-Acetoxyanhydroretinol	388, 367, 350, ~333	388, 367, 350, ~333	397, 375, 357, ~340	690
3-Hydroxyanhydroretinol	386, 366, 349, ~333	386, 365, 348, ~333	396, 374, 356, ~340	690
3,5-Dinitrobenzoate of naturally occurring 3-hydroxyanhydroretinol	390, 370, 351, 260, 255.5, 249.5, 244.5, ~239	390, 370, 351, 260, 255.5, 249.5, 244.5, ~239	—	—

Table 4. *I.r. spectra of 3-methoxyanhydroretinol, 3-acetoxyanhydroretinol, 3-hydroxyanhydroretinol and 3,5-dinitrobenzoate of 3-hydroxyanhydroretinol as prepared in the Experimental section*

Compound	Stretching frequency (cm^{-1})	
	Naturally occurring or prepared from the naturally occurring compound	Synthetic compound
3-Methoxyanhydroretinol	—	720, 800, 850, 950, 1160
3-Acetoxyanhydroretinol	725, 970, 1170, 1740	720, 970, 1150, 1710
3-Hydroxyanhydroretinol	730, 960, 1170, 3400	745, 960, 1150, 3230
3,5-Dinitrobenzoate of 3-hydroxyanhydroretinol	730, 860, 970, 1150, 1730,	730, 860, 970, 1150, 1720

Table 5. *Chemical shifts in p.p.m. for naturally occurring 3-hydroxyanhydroretinol and synthetic 3-hydroxyanhydroretinol prepared as described in the Experimental section*
The solvent was $[\text{D}^2\text{H}]\text{chloroform}$.

Compound	δ (p.p.m.)					
	Methyl protons				Hydroxy proton at C-3	Others
	C-1	C-5	C-9	C-13		
Naturally occurring 3-hydroxyanhydroretinol	1.24	2.8	1.44	1.9	3.50	5.28 (t, C-15 methylene proton), 7.18 (s, olefinic protons at C-7, C-8, C-10, C-11 and C-12)
Synthetic 3-hydroxyanhydroretinol	1.24	2.82	1.39	1.94	3.54	5.27 (t, C-15 methylene protons), 7.18 (s, olefinic protons at C-7, C-8, C-10, C-11 and C-12)

compound the following experiment was carried out.

The purified anhydro compound (45mg) was saponified with 100ml of ethanolic 15% (w/v) KOH at 60–70°C for 45min. The saponified product was then co-chromatographed on a t.l.c. plate along with naturally occurring 3-hydroxyanhydroretinol. No resolution of the mixed spot was observed. Under u.v. light a single dark-brown fluorescent spot was observed. The presence of a free hydroxy group was further confirmed by acetylation and benzylation of the purified naturally occurring anhydro compound.

Preparation of 3-acetoxyanhydroretinol

A portion (18mg) of 3-hydroxyanhydroretinol (isolated from *B. bagarius* liver oil) dissolved in 2ml of pyridine was acetylated with 0.1ml of acetyl chloride dissolved in 2ml of pyridine and was kept at 0 to –5°C for 15min and then at 25–30°C for 2h. The acetylated product was then subjected to t.l.c. The u.v.-visible and i.r. spectra of the pure 3-acetoxyanhydroretinol were recorded (Tables 3 and 4).

Preparation of 3,5-dinitrobenzoate of naturally occurring 3-hydroxyanhydroretinol

To 176mg of 3-hydroxyanhydroretinol (isolated from *W. attu* liver oil) dissolved in 2ml of pyridine was added 29mg of 3,5-dinitrobenzoyl chloride and the mixture heated to 90°C for 40min, then cooled. A saturated solution of NaHCO₃ was added with stirring. The brown solid was washed with NaHCO₃ solution followed by water. The crude product was recrystallized from hot aq. 90% (v/v) ethanol. The 3,5-dinitrobenzoyl derivative showed m.p. 130°C. The u.v. and i.r. spectra of the benzyolated derivative were recorded (Tables 3 and 4). The yield of the compound after recrystallization was 115mg.

Chemical synthesis of 3-hydroxyanhydroretinol from 3-dehydroretinol

Preparation of 3-dehydroretinol. Methyl 3-dehydroretinoate was prepared from methyl retinoate as described by Barua *et al.* (1977). Methyl 3-dehydroretinoate was reduced to 3-dehydroretinol with LiAlH₄ in the same way as described in the preparation of 3-hydroxyretinol (Barua *et al.*, 1977).

Method 1. Preparation of 3-hydroxyanhydroretinol. This was prepared by the procedure outlined by R. K. Barua & K. Goswami (unpublished work) as follows. A 40mg portion of 3,4-didehydroretinol was dissolved in 29ml of light petroleum and was treated with 20ml of light petroleum saturated with HCl for 35min at 25–30°C. The crude product was extracted and chromatographed on a column of 10g of alumina.

The second zone, containing 3-hydroxyanhydroretinol, was eluted with light petroleum containing 8–10% (v/v) diethyl ether and was further purified by t.l.c. The yield of the compound was 2.6mg.

Method 2. (a) Preparation of 3-methoxyanhydroretinol. This was prepared by the procedure outlined by Henbest *et al.* (1955). For this 36mg of 3-dehydroretinol was dissolved in 20ml of pure dry methanol and was treated with 10ml of dry methanolic 0.03M-HCl for 35min at 25–30°C. The crude product was extracted and chromatographed on a column of 10g of alumina. The second zone, containing 3-methoxyanhydroretinol, was eluted with light petroleum and was further purified by t.l.c. The u.v. and i.r. spectra of the purified product were recorded (Tables 3 and 4). The yield of the compound was 19mg.

(b) Preparation of 3-acetoxyanhydroretinol. 3-Methoxyanhydroretinol was converted into 3-acetoxyanhydroretinol by the procedure described by Barua *et al.* (1977). First 54mg of 3-methoxyanhydroretinol was dissolved in 4ml of ethyl acetate. Then 0.2ml of a solution of anhydrous FeCl₃ (2mg) dissolved in 2ml of freshly distilled acetic anhydride was added dropwise with continuous stirring. The reaction was carried out for 90min at 25–30°C. On chromatography on a column (1.5cm × 5cm) of 7g of alumina, the first zone eluted with light petroleum showed λ_{max} . 388, 367, 350 and ~333nm in light petroleum, and the SbCl₃-reaction product had λ_{max} . 690nm. The product on further purification by t.l.c. moved faster (R_F 0.75) than the corresponding 3-methoxyanhydroretinol (R_F 0.44). The compound on co-chromatography with the acetylated derivative of naturally occurring 3-hydroxyanhydroretinol showed no resolution (R_F 0.75). 3-Acetoxyanhydroretinol was further characterized by its i.r. spectrum (Table 4). The yield of the compound was 54mg.

(c) Preparation of 3-hydroxyanhydroretinol. 3-Acetoxyanhydroretinol (70mg) was dissolved in 20ml of dry peroxide-free diethyl ether and was treated with 20mg of LiAlH₄ at 0°C. Reaction was carried out for 25min. Excess of LiAlH₄ was decomposed with water and the product extracted with diethyl ether. The extract was dried over anhydrous Na₂SO₄ and the solvent evaporated *in vacuo*. The residue was dissolved in 1ml of light petroleum and was chromatographed on a column of 10g of alumina. The strongly adsorbed zone of 3-hydroxyanhydroretinol was eluted with light petroleum containing 8–10% (v/v) diethyl ether and was further purified by t.l.c. The purified product was characterized by u.v. (Table 3), i.r. (Table 4), n.m.r. (Table 5) and mass spectra. The mass spectrum of the synthetic compound revealed a molecular-ion value of 284, which is in good agreement with its molecular formula (C₂₀H₂₈O). The yield of the compound was 49mg.

Metabolism of 3-hydroxyanhydroretinol in *S. fossilis*

The initial reserves of vitamin A were depleted by keeping *S. fossilis* on a vitamin A-deficient diet for 4 months, after which they were given 3-hydroxyanhydroretinol as shown in Table 6.

About 30–189 μg of 3-hydroxyanhydroretinol per fish on alternate days was administered to vitamin A-depleted fish for 45 days. The fish were killed 24h after the last dose and the liver oil was subjected to column chromatography. The first and the second zones, containing β -carotene and 3-dehydroretinyl ester respectively, were eluted with light petroleum. The third zone, containing 3-hydroxyanhydroretinol, was eluted with light petroleum containing 8–10% (v/v) diethyl ether. Table 6 shows the amounts of the various compounds that were separated and estimated spectrophotometrically.

Metabolism of 3-hydroxyretinol in *S. fossilis*

In these experiments most of the initial reserves of vitamin A were depleted by keeping *S. fossilis* on a vitamin A-deficient diet for 4 months, after which they were given diester of 3-hydroxyretinol as indicated in Table 7.

About 58–88 μg of diester of 3-hydroxyretinol per fish was administered on alternate days to vitamin

A-depleted fish for 30 days. The fish were killed 12–24h after the last dose, and the livers were removed, extracted and chromatographed on a column of 10g of alumina. First β -carotene was eluted with light petroleum, followed by 3-dehydroretinyl ester. Diester of 3-hydroxyretinol was eluted with light petroleum containing 2% (v/v) diethyl ether. 3-Hydroxyanhydroretinol was eluted with light petroleum containing 8–10% (v/v) diethyl ether. Several experiments were carried out and the amounts of the different metabolites thus obtained were determined spectrophotometrically and are shown in Table 7.

Chemical conversion of 3-hydroxyretinol into 3-dehydroretinol, 3-dehydroretinal and methyl 3-dehydroretinoate

By using the procedure outlined by Barua *et al.* (1969) for the preparation of methyl retinoate, 3-hydroxyretinol was mixed with NaCN (3g) and MnO_2 in methanol (150ml) and kept at 25°C for 19–44h. The solution was filtered, water added to it and the product extracted with diethyl ether. The diethyl ether layer was washed several times with water to remove methanol, and was then dried over anhydrous Na_2SO_4 and the solvent evaporated to dryness *in vacuo*. The residue was dissolved in 2ml of light petroleum and chromatographed on a column

Table 6. Metabolism of 3-hydroxyanhydroretinol in *S. fossilis*

In each experiment six vitamin A-depleted *S. fossilis* fish were given for 45 days the indicated amounts of 3-hydroxyanhydroretinol dissolved in 0.2ml of Tween 80 containing 40% (w/v) of α -tocopheryl acetate mixed with goat meat. The different metabolites were isolated and separated by chromatography as described in the text. Values are calculated on the basis of $A_{1\text{cm}}^{1\%}$ values shown in the Experimental section.

Expt. no.	Approx. amount of 3-hydroxyanhydroretinol administered (μg)	Total amount isolated (μg)		
		β -Carotene	3-Dehydroretinyl ester	3-Hydroxyanhydroretinol
1	4000	26.4	118.7	6.0
2	25000	27.2	412.5	11.6

Table 7. Metabolism of 3-hydroxyretinol in *S. fossilis*

Vitamin A-depleted *S. fossilis* were given for 30 days (Expts. 1–4) and for 15 days (Expt. 5) the indicated amounts of diester of 3-hydroxyretinol dissolved in 0.2ml of Tween 80 mixed with goat meat. The negative controls received goat meat mixed with Tween 80. The different metabolites were isolated and separated by chromatography. The amounts were calculated on the basis of $A_{1\text{cm}}^{1\%}$ values as shown by Barua *et al.* (1977).

Expt. no.	Number of fish	Approx. amount of diester of 3-hydroxyretinol administered (μg)	Total amount isolated (μg)		
			3-Dehydroretinyl ester	Diester of 3-hydroxyretinol	3-Hydroxyanhydroretinol
1	5	6700	45.3	27.2	—
2	6	5200	64.9	22.0	—
3	3	4181	58.7	30.33	—
4	3	4181	40.6	4.977	290.0
5	2	434.8	8.65	1.55	5.0

of 7g of alumina. Any methyl 3-dehydroretinoate formed was eluted with light petroleum. Light petroleum containing 10% (v/v) diethyl ether was used for elution of 3-dehydroretinal. 3-Dehydroretinol was eluted with light petroleum containing 20–25% (v/v) diethyl ether. 3-Hydroxyretinol was eluted with light petroleum containing 30–35% (v/v) diethyl ether. The respective fractions were further purified by t.l.c. 3-Dehydroretinol obtained was characterized as described in the Experimental section. 3-Dehydroretinal was characterized by conversion into 3-dehydroretinol by reduction with NaBH₄. Methyl 3-dehydroretinoate was reduced with LiAlH₄ when 3-dehydroretinol was obtained. The experiment was repeated and the results are shown in Table 8.

Discussion

The existence in the liver oil of *W. attu* of a naturally occurring anhydrovitamin A₂ different from the chemically prepared anhydrovitamin A₂ (3-ethoxy- or 3-methoxy-anhydroretinol) was first reported by Balasundaram *et al.* (1958). The occurrence of this naturally occurring anhydrovitamin A₂ in the liver oil of *B. bagarius* was subsequently reported by Barua & Nayar (1963). Barua & Nayer (1966) reported that chemical oxidation of the compound with MnO₂ resulted in the formation of 3-dehydroretinal and proposed a 3-hydroxyanhydroretinol structure for the naturally occurring compound. Barua & Barua (1966) found that treatment of 3-hydroxyretinol prepared from zeaxanthin with ethanolic HCl gave 3-ethoxyanhydroretinol as the main product, but little of a compound similar to the naturally occurring anhydrovitamin A₂ was also formed. On the basis of this, they had also proposed a 3-hydroxyanhydroretinol structure for the naturally occurring anhydrovitamin A₂. During the present study we have found that the naturally occurring anhydrovitamin A₂ is present in the liver oils of *M. seenghala*, besides *W. attu* and *B. bagarius*. Among these, both *B. bagarius* and *W. attu* fish liver oils have been found to be the rich source of the naturally occurring anhydrovitamin A₂. However, it was observed that even these fish liver oils occasionally contain very little of the anhydro compound (Table 2). An attempt was made to confirm the 3-hydroxyanhydroretinol structure proposed by Barua & Barua (1966) and Barua & Nayar (1966). It was found that the naturally occurring compound is always accompanied by a large quantity of sterols, which could be separated, except for a trace (as indicated by the mass spectrum), by repeatedly cooling the methanolic solution followed by treatment with digitonin. Although the i.r. and n.m.r. spectra indicated the absence of any sterol, the mass

Table 8. Chemical oxidation of 3-hydroxyretinol
3-Hydroxyretinol was oxidized with MnO₂ and NaCN in methanol. The different compounds formed were isolated by chromatography as described in the text.

Expt. no.	Amount of 3-hydroxyretinol (mg)	Reaction time (h)	MnO ₂ taken (g)	Amount obtained (mg)			
				3-Dehydroretinol	3-Dehydroretinal	Methyl 3-dehydroretinoate	3-Hydroxyretinol
1	138	39	18.2	—	61	45	—
2	158	39	18.9	—	15	105	17
3	78	40	10.8	24	—	13	24
4	24	19	10.7	13	—	—	—
5	102	20	12.3	20	—	—	67
6	198	40	12.03	78	—	42	23
7	112.5	42	11.2	53	—	39	11
8	112.5	44	11.92	82	—	48	14
9	45	6*	6.4	11	—	13	3

* In this experiment the mixture was continuously stirred.

spectrum showed that it contained a trace of sterol, probably cholesterol, as an M^+ value at 386 was obtained. Repeated attempts to remove the last trace of sterol from the naturally occurring anhydro compound have not so far been successful. However, the fragmentation pattern in the mass spectrum of the naturally occurring anhydrovitamin A_2 agreed with the theoretical m/e values. We therefore attempted a synthesis of the compound assuming a 3-hydroxy-anhydroretinol structure for the naturally occurring anhydrovitamin A_2 . We first prepared the compound by the procedure outlined by R. K. Barua & K. Goswami (unpublished work), by treatment of 3-dehydroretinol with light petroleum saturated with HCl. The yields were not very satisfactory, and therefore we tried a different route similar to the one reported for the synthesis of 3-hydroxyretinol by Barua *et al.* (1977). In this procedure, 3-methoxyanhydroretinol was first prepared from 3-dehydroretinol and subsequently converted into 3-hydroxyanhydroretinol through 3-acetoxyanhydroretinol. The overall yield was found to be 36.9%. Comparison of the properties of the synthetic 3-hydroxyanhydroretinol with the naturally occurring anhydrovitamin A_2 revealed that they are identical. In the i.r. spectra of the naturally occurring and the synthetic anhydrovitamins A_2 broad bands at 3400 and 3230 cm^{-1} respectively were observed for the O-H stretching frequency; bands at 1170 and 1150 cm^{-1} respectively were also observed, which were assigned to the C-O stretching of the secondary alcoholic group at the C-3 position of the β -ionone ring. The n.m.r. spectra of the naturally occurring and synthetic 3-hydroxyanhydroretinol showed signals for the proton of the hydroxy group at C-3 and for the protons of the end methylene group at C-15. The n.m.r. spectrum is therefore indicative of a 3-hydroxyanhydroretinol structure. In the mass spectrum of the synthetic 3-hydroxyanhydroretinol the m/e values of the major mass fragments obtained are 137, 163, 203, 239, 267, which agreed with the expected m/e values of 137, 163, 203, 238 and 266. The acetyl derivatives of both the naturally occurring and the synthetic 3-hydroxyanhydroretinols were prepared. The i.r. spectrum showed bands at 1170 and 1740 cm^{-1} respectively for the C-O and C=O stretching frequency of the acetate group. The 3,5-dinitrobenzoyl derivative of the naturally occurring and synthetic 3-hydroxyanhydroretinol had m.p. 130°C. Further, the λ_{max} at 266, 255.5, 249.5, 244.5 and $\sim 239\text{nm}$ in the u.v. spectrum of the 3,5-dinitrobenzoyl derivatives showed the presence of the benzene ring in the compound. The melting point of the mixture of the benzoyl derivatives of both the naturally occurring and the chemically prepared 3-hydroxyanhydroretinols was also found to be 130°C, without any lowering of melting point, thus proving that the two compounds are the same.

The liver oils also contained rehydrovitamin A_2 , whose structure is not clearly known. According to Bamji *et al.* (1962) it is formed in the rat from 3-ethoxyanhydroretinol; the rehydro compound, however, does not contain any ethoxy substituent. Although no detailed study has been made during the present investigation, it appears that rehydrovitamin A_2 is not an artifact of the isolation by isomerization of 3-dehydroretinol, as repeated treatment of the latter compound with light petroleum, concentration of the solution and chromatography on deactivated alumina column did not result in appearance of any rehydrovitamin A_2 . Further investigation will be necessary to establish that rehydrovitamin A_2 is not an artifact from other constituents of the liver oil during the isolation procedure and to establish its structure.

The possible pathway of formation of the naturally occurring 3-hydroxyanhydroretinol from lutein in the fish has been suggested from this laboratory (Barua *et al.*, 1977): anhydrolutein first formed from lutein in the fish is cleaved into 3-dehydroretinol and 3-hydroxyretinol. 3-Hydroxyanhydroretinol may then be formed from 3-hydroxyretinol in the same way as anhydroretinol is formed from retinol. During the present investigation, we therefore decided to study the metabolism of 3-hydroxyretinol in the fish. 3-Hydroxyretinol was orally administered to the vitamin A-depleted *S. fossilis* for a period of 30 days and we expected 3-hydroxyanhydroretinol to be formed. But, surprisingly, instead of 3-hydroxyanhydroretinol, 3-dehydroretinyl ester was formed and isolated. However, in two of the experiments 3-hydroxyanhydroretinol was also isolated along with 3-dehydroretinyl ester, whereas in the rest of the three experiments no 3-hydroxyanhydroretinol was detected and the amounts of unconverted diester of 3-hydroxyretinol recovered were extremely low. Isolation of 3-hydroxyanhydroretinol from the liver oils of vitamin A-depleted *S. fossilis* given 3-hydroxyretinol suggest that 3-hydroxyretinol is first converted into 3-hydroxyanhydroretinol and then into 3-dehydroretinol. An attempt was therefore made to examine whether 3-dehydroretinol was formed from the orally administered 3-hydroxyanhydroretinol. Two experiments were carried out where 3-hydroxyanhydroretinol was administered to the vitamin A-depleted *S. fossilis*, and it was possible to isolate 3-dehydroretinyl ester. It therefore appears that the biosynthesis of 3-dehydroretinol from 3-hydroxyretinol proceeds via 3-hydroxyanhydroretinol.

With a view to preparing methyl 3-hydroxyretinoate by oxidative esterification of 3-hydroxyretinol in the same way as methyl retinoate was prepared from retinol (Barua & Ghosh, 1972), 3-hydroxyretinol was oxidized with MnO_2 in the presence of methanol and NaCN. However, instead of the expected methyl 3-hydroxyretinoate, 3-dehydroretinol was formed. By prolonging the oxidation

time, it was, however, possible to oxidize it further to 3-dehydroretinol and subsequently to methyl 3-dehydroretinoate. This chemical conversion of 3-hydroxyretinol into 3-dehydroretinol further supports the formation of 3-dehydroretinol from 3-hydroxyretinol in *S. fossilis*.

Studies on the metabolism of 3-hydroxyretinol in *S. fossilis* therefore show that 3-dehydroretinol may appear in the freshwater fish *S. fossilis* by two different routes. It may be formed from lutein via anhydrolutein directly (Barua *et al.*, 1977) and from 3-hydroxyretinol via 3-hydroxyanhydroretinol (Scheme 1). Although nothing definite about the mechanism of cleavage of lutein into 3-dehydroretinol and 3-hydroxyretinol, whether by central cleavage or by β -oxidation, can be concluded, from the present investigation it appears that the conversion of lutein into 3-dehydroretinol possibly takes place by a central-cleavage mechanism. First anhydrolutein is formed from lutein, which is then centrally cleaved into 3-dehydroretinol and 3-hydroxyretinol. Isolation of both 3-dehydroretinol and 3-hydroxyretinol by Barua *et al.* (1977) points towards a central cleavage of anhydrolutein. However, if anhydrolutein is oxidized by central cleavage of the C-15-C-15' double bond to give rise to two

molecules, one of 3-dehydroretinol and the other of 3-hydroxyretinol, it was difficult to explain in the previous study (Barua *et al.*, 1977) why the amount of 3-hydroxyretinol was much less than that of 3-dehydroretinol formed from lutein. It was also difficult to explain why the quantity of 3-dehydroretinol is higher than that of 3-hydroxyretinol in the liver oils of freshly caught *B. bagarius*. From the present investigation it can possibly be explained why 3-dehydroretinol and 3-hydroxyretinol do not occur in equal amounts in the liver oils of fresh-water fish. It is probable that initially both 3-dehydroretinol and 3-hydroxyretinol are formed from anhydrolutein in equal amounts, but further conversion of 3-hydroxyretinol into either 3-dehydroretinol or 3-hydroxyanhydroretinol results in less 3-hydroxyretinol.

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References

- Balasundaram, S., Bamji, M. S., Cama, H. R., Sundaresan, P. R. & Varma, T. N. R. (1958) *J. Biol. Chem.* **238**, 827-832
 Ball, S., Goodwin, T. W. & Morton, R. A. (1948) *Biochem. J.* **42**, 516-523
 Bamji, M. S., Cama, H. R. & Sundaresan, P. R. (1962) *J. Biol. Chem.* **237**, 2747-2749
 Barua, A. B. & Ghosh, M. C. (1972) *Tetrahedron Lett.* **18**, 1823-1825
 Barua, A. B., Ghosh, M. C. & Goswami, K. (1969) *Biochem. J.* **113**, 447
 Barua, A. B., Singh, H. T. & Das, R. C. (1973) *Br. J. Nutr.* **30**, 1-12
 Barua, A. B., Das, R. C. & Verma, K. (1977) *Biochem. J.* **168**, 557-564
 Barua, R. K. & Barua, A. B. (1966) *Biochem. J.* **101**, 250-255
 Barua, R. K. & Nayar, P. G. (1963) *Curr. Sci.* **32**, 257
 Barua, R. K. & Nayar, P. G. (1966) *Biochem. J.* **101**, 302-307
 Farrar, K. R., Hamlet, J. C., Henbest, H. B. & Jones, E. R. H. (1952) *J. Chem. Soc.* 2657-2668
 Ghosh, M. C. (1972) Ph.D. Thesis, University of Gauhati
 Henbest, H. B., Jones, E. R. H., Owen, T. C. & Thaller, V. (1955) *J. Chem. Soc.* 2763-2765
 Shantz, E. M. (1948) *Science* **108**, 417
 Shantz, E. M. (1950) *J. Biol. Chem.* **182**, 515-524
 Shantz, E. M., Cawley, J. D. & Embree, N. D. (1943) *J. Am. Chem. Soc.* **65**, 901-906

Scheme 1. Alternative pathway of formation of 3-dehydroretinol in *S. fossilis*