Br. J. Nutr. (1975), **33**, 319

Occurrence and conversion of anhydrolutein into dehydroretinol in a freshwater fish

By A. B. BARUA and R. C. DAS*

Department of Chemistry, Gauhati University, Gauhati 781014, *Assam, India*

(*Received* 25 *February* 1974 – *Accepted* 30 *July* 1974)

1. Lutein and anhydrolutein have been isolated from liver oils of freshly caught *Saccobranchus fossilis*.

2. *S. fossilis* initially contained more dehydroretinol than retinol; administration of anhydrolutein to the vitamin A-depleted fish resulted in the accumulation of dehydroretinol.

3. Anhydrolutein has also been isolated from the liver oils of lutein-treated, vitamin A-depleted fish.

4. The pathway of the conversion of lutein into dehydroretinol is discussed.

The mechanism of dehydroretinol (vitamin A_2) biosynthesis in freshwater fish has not been completely elucidated. There are claims that β-carotene (Morton & Creed, 1939) and astaxanthin (Grangaud & Moatti, 1958*a*, *b*) serve as the precursors of dehydroretinol in freshwater fish. Other claims that anhydrolutein (3′-hydroxy-3,4-dehydro-β-carotene) serves as the precursor (Budowski & Gross, 1965; Savithry, Krishna Mallia & Cama, 1972), are based on experiments done, not with fish, but with chicks and rats which do not usually contain dehydroretinol. Recent work done in the authors' laboratory suggests that lutein is a natural carotenoid that can act as a precursor of dehydroretinol (Barua, Singh & Das, 1973). However, the exact mechanism for this conversion is not known. The present work is an attempt to find a possible pathway for the conversion of lutein into dehydroretinol. We have demonstrated that anhydrolutein is present naturally in the liver oil of *Saccobranchus fossilis* and can be formed from lutein. Anhydrolutein is also an effective precursor of dehydroretinol, which suggests that the conversion of lutein into dehydroretinol may involve anhydrolutein as an intermediate.

MATERIALS AND METHODS

Light petroleum (b.p. 40°–60°), cyclohexane, acetone and benzene were supplied by British Drug Houses (Glaxo Laboratories (India) Ltd), Bombay. Other solvents were purified by the methods described previously (Barua *et al.* 1973).

Reagents

p-Toluene sulphonic acid was obtained from Riedel-De Haen AG, Germany. Other reagents were described previously by Barua *et al.* (1973). Magnesium oxide, for chromatographic adsorption analysis (British Drug Houses Ltd, Poole, Dorset), and Celite 545 (Koch–Light Laboratories, Colnbrook, Bucks.) were used for chromato-

* Present address: Department of Chemistry, Shillong College, Shillong 3, Meghalaya, India.

graphy. Silica gel (supplied by Chemo Synthetics, Calcutta, India) (0·25 mm thickness) was used for thin-layer chromatography. Two solvent systems, cyclohexane–diethyl ether (80:20, v/v) (Bolliger & Konig, 1969) and light petroleum–benzene–ethanol (60:30:10, by vol.), were used.

Preparation of lutein and anhydrolutein

Fresh leaves of *Zea mays* (collected from Shillong, India) were used as the source of lutein.

Fresh leaves (800 g) were ground, using an electric blender, with 2 l ethanol added gradually. The ethanolic solution was saponified with KOH (200 g) at 60° for 2 h. Lutein was then isolated and characterized by the procedure described previously (Barua *et al.* 1973).

Anhydrolutein was prepared from lutein by the method of Budowski, Ascarelli, Gross & Nir (1963) with the following modifications. *p*-Toluene sulphonic acid (50 mg) was dissolved in 100 ml benzene by refluxing for 30 min at 70°. The hot solution of *p*-toluene sulphonic acid was added gradually to 100 ml lutein in benzene (1 g/l). The solution was warmed to 80° and examined spectrophotometrically at intervals of 5 min. When the product showed a single peak at 460–465 nm, the reaction mixture was immediately cooled and neutralized with a dilute solution of $NaHCO_3$. The product was extracted into diethyl ether, washed with water, dried over anyhdrous Na_2SO_4 and the solvent was removed by evaporation under reduced pressure. The residue was dissolved in light petroleum (10 ml) and applied to a deactivated alumina (50 ml water/kg) column. Anhydrolutein appeared as a red zone below the small dark-yellow zone of unconverted lutein and was eluted with light petroleum–diethyl ether (100:5, v/v) and collected in fractions of about 10 ml. The solutions which produced a single peak at 460 nm were pooled. For further purification, anhydrolutein was chromatographed on a column of MgO–Celite (10:10, w/w). Anhydrolutein, which appeared as a red zone, was eluted with light petroleum–acetone (100:5, v/v).

The R_F for purified anhydrolutein chromatographed on silica gel, using cyclohexane–diethyl ether as the solvent system, was 0·46, without further resolution; an R_F of 0·74 was obtained with the light petroleum–benzene–ethanol solvent system. Anhydrolutein was crystallized from light petroleum at $-5°$. The extinction maximum for the crystalline sample was 460 nm in light petroleum, 475 nm in chloroform and 465 nm in benzene.

Spectroscopic examination and estimation of vitamin A and carotenoids

The visible and ultraviolet spectra of the solutions were obtained using a spectrophotometer (Model DK-2; Beckman Instrument Co. Inc., Fullerton, California, USA). Retinol and dehydroretinol contents were estimated by the procedure described by Barua *et al.* (1973). Anhydrolutein was estimated using the value 2031 for the extinction coefficient ($E^{1\%}_{10\,mm}$) at 460 nm in light petroleum (Savithry *et al.* 1972). Lutein and other carotenoids were estimated by using values 2200 and 2500 for the $E^{1\%}_{10\,mm}$ at 445 nm and at the wavelength of maximum extinction respectively.

Table 1. *Retinol and dehydroretinol content of the liver of* Saccobranchus fossilis *either freshly caught or vitamin A-depleted or after administration of anhydrolutein*

Batch no.	No. of days of depletion	Anhydrolutein treatment: Period (d)	Anhydrolutein treatment: Method	No. of fish	Total wt of fish (g)	Extinction maxima (nm): Liver oil in light petroleum (b.p. 40°–60°)	Extinction maxima (nm): $SbCl_3$–liver oil complex	Calculated* content (μg/fish) of: Dehydroretinol	Calculated* content (μg/fish) of: Retinol
					Freshly caught				
1	—	—	—	2	20	478, 450, 335, 286, 276	690, 620	23·2	6·4†
2	—	—	—	3	22		690, 620	18·6	4·3†
3	—	—	—	5	48		690, 620	19·2	6·1†
					Vitamin A-depleted				
1	150	—	—	2	12	450, 335	nd	—	—
2	180	—	—	2	9		nd	—	—
3	180	—	—	2	15		nd	—	—
					After administration of anhydrolutein				
1	—	7	Orally with meat	2	11	474, 448, 335, 286, ~276	690	3·6	—
1	—	10	Orally with meat	3	13	460, 335, 286, ~276	690	44·8	—
2	—	20	Orally with meat	4	15	455, 335, 286, ~276	690	5·8	—
2	—	25	Orally with meat	3	10	455, 335, 286, ~276	690	10·2	—
2	—	10	Orally with meat	3	9	460, 335, 286, ~276	690	2·9	—
2	—	10	Orally with meat	2	14	460, 335, 286, ~276	690	11·3	—
2	—	27	Orally with meat	2	9	455, 335, 286, ~276	690	11·8	—
3	—	30	Orally with meat	9	41	460, 335, 286, ~276	690	4·1	—
2	—	2	Subcutaneous injection	2	20	474, 450, 335, 286, ~276	690	4·5	—
3	—	1·5	Subcutaneous injection	1	7	460, 335, 286, ~276	690	5·5	—

nd, not detected.

* Calculated from values for the extinction of the $SbCl_3$ complex.

† Corrected according to the procedure outlined by Cama & Morton (1953).

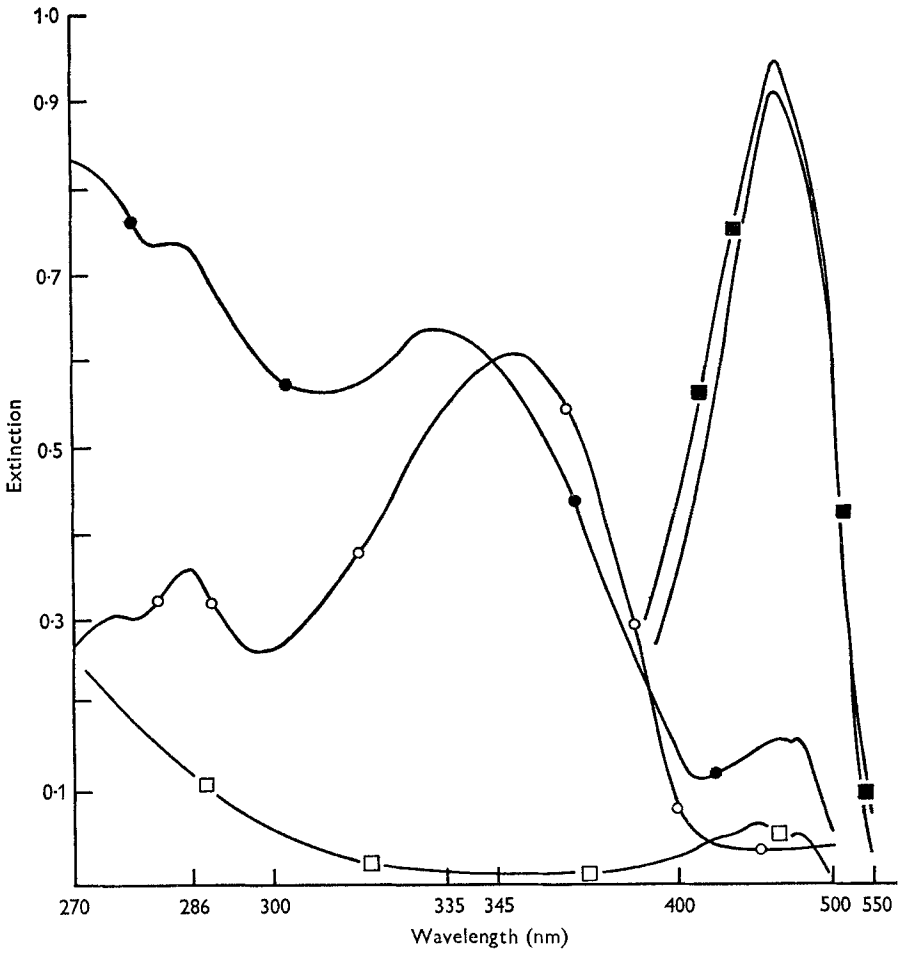

Fig. 1. Visible and ultraviolet spectra, in light petroleum (b.p. 40°–60°), of liver oils from *Saccobranchus fossilis*. Vitamin A-depleted fish (□——□); vitamin A-depleted fish, after oral administration of anhydrolutein (●——●); dehydroretinyl ester, isolated by separation on a deactivated alumina column, from liver oils from anhydrolutein-treated fish (○——○); anhydrolutein, isolated by column chromatography, from liver oil of freshly caught fish (——); anhydrolutein prepared from lutein (■——■).

Administration of anhydrolutein and lutein

Crystalline lutein or anhydrolutein was dissolved in a few drops of groundnut oil, mixed with goat meat and given orally to the fish. For subcutaneous injection, the carotenoid was mixed with Tween-80 (Calcutta Chemical Co. Ltd, Calcutta, India), treated with a few ml water and stirred to get a homogenous dispersion and this was injected into the fish using an Agla micrometer syringe (Wellcome Foundation Ltd, London).

Extraction of liver oil

Extraction of the oil from the liver or the alimentary canal was done using light petroleum as described by Barua *et al.* (1973).

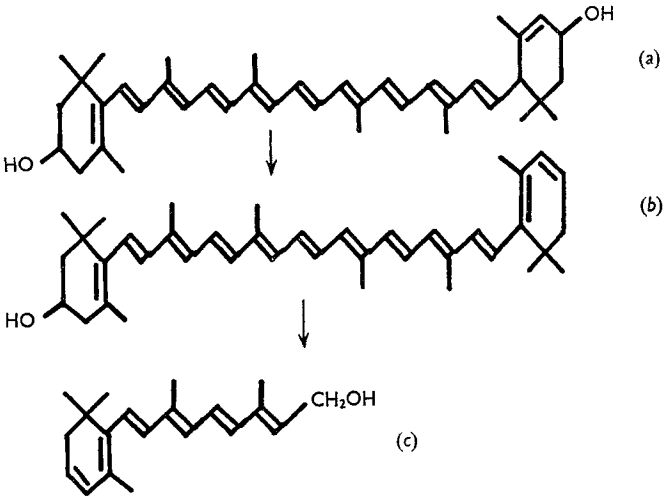

Fig. 2. Reaction scheme 1 showing the structures of (*a*) lutein, (*b*) anhydrolutein and (*c*) dehydroretinol.

Fish

S. (*Heteropneustes*) *fossilis* was used and the fish were depleted of vitamin A by giving a diet of rice and goat meat, using the procedure outlined by Barua *et al.* (1973).

RESULTS AND DISCUSSION

The liver oil of freshly caught *S. fossilis* was found to contain mainly dehydroretinol, with a small amount of retinol. When these fish were maintained on a diet of rice and goat meat, their vitamin A content gradually decreased and was negligible by the end of 5–6 months (Table 1); the extinction at 320–350 nm of liver oils from these fish was low (Fig. 1) and no colour was produced with antimony chloride reagent (Table 1).

Oral administration of anhydrolutein to the vitamin A-depleted fish for a few days resulted in the accumulation of dehydroretinol which was identified by its ultraviolet spectrum (extinction maxima at 335, 286 and approximately 276 nm; Table 1, Fig. 1), which was similar to that of the liver oil of freshly caught *S. fossilis*. In addition, the formation of dehydroretinol was confirmed by the production of a blue complex with $SbCl_3$, which had a maximum extinction at 690 nm (Table 1). There was also conversion of anhydrolutein into dehydroretinol when the former was administered subcutaneously to the vitamin A-depleted fish (Table 1). Column chromatography on deactivated alumina of the liver oil from fish given anhydrolutein resulted in the separation of dehydroretinyl ester (extinction maxima 345–350 (broad), 286 and 276 nm (Fig. 1); 693 nm for the $SbCl_3$ complex), and unconverted anhydrolutein (extinction maximum 460 nm), together with three other unidentified compounds. It has been reported recently from this laboratory that lutein is the natural carotenoid that can serve as a precursor of dehydroretinol (Barua *et al.* 1973). It was also reported that the allylic hydroxyl group of lutein can be eliminated as water, with the consequent introduction of a double bond, followed by rearrangement of the double bonds

Table 2. *Results of the separation on deactivated alumina columns of constituents of liver oil from freshly caught* Saccobranchus fossilis

(Results for a group of thirty-eight fish, total body-weight 1350 g, total liver weight 12·7 g)

Fraction no.	Eluant	Extinction maxima (nm) in light petroleum (b.p. 40°–60°)	Compound	Amount (μg)
1	Light petroleum (b.p. 40°–60°)	474, 450, 424	β-carotene	1822
2	Light petroleum	345, 287, 276	Dehydroretinyl ester	1458*
3	Light petroleum–diethyl ether (100:2, v/v) Fast	472, 448, 424	Unidentified	86
4	Light petroleum–diethyl ether (100:2, v/v) Slow	460	Anhydrolutein ester	45
5	Light petroleum–diethyl ether (100:8, v/v)	472, 446, 420	Unidentified	36·6
6	Light petroleum–diethyl ether (100:10, v/v)	470, 445, 420	Unidentified	198
7	Light petroleum–diethyl ether (100:12, v/v)	472, 445, 422	Lutein ester	1375
8	Light petroleum–diethyl ether (100:15, v v)	476, 448, 424	Unidentified	396
9	Light petroleum–diethyl ether (100:20, v/v)	470, 442, 420	Unidentified	243·2
10	Diethyl ether (after extrusion)	472, 448, 424	Unidentified	40·8

* Calculated from values for the extinction of $SbCl_3$ complex.

to form anhydrolutein. Once anhydrolutein is formed it may give rise to dehydroretinol. The biosynthesis of dehydroretinol from lutein could, therefore, be represented by reaction scheme 1 (Fig. 2).

Savithry *et al.* (1972), during their study of the conversion of anhydrolutein to dehydroretinol, recovered some anhydrolutein from rats after they had been given this compound. During the present study also, some anhydrolutein was recovered after it had been fed to the vitamin A-depleted fish. Therefore, if reaction scheme 1 correctly describes the biosynthesis of dehydroretinol, it should be possible to isolate, even if in small amounts, some anhydrolutein from freshwater fish containing predominantly dehydroretinol. We therefore studied the natural occurrence of anhydrolutein in these fish. We selected *S. fossilis* which we obtained as freshly caught, fairly big fish from local fish dealers. The liver oil was extracted with light petroleum and the concentrated extract was chromatographed on deactivated alumina columns. The fractions obtained were characterized and the results are shown in Table 2. The liver oil from freshly caught *S. fossilis* contained, in addition to dehydroretinol, anhydrolutein and lutein (as esters). The anhydrolutein ester was converted into the free alcohol which had an extinction maximum at 460 nm in light petroleum, 465 nm in cyclohexane and 475 nm in chloroform. The naturally occurring anhydrolutein (as the alcohol) was further characterized by mixed chromatography using anhydrolutein, prepared from lutein by treatment with *p*-toluene sulphonic acid. Column and thin-layer chromatography did not result in any resolution of the two compounds, establishing their identity. Thin-layer chromatography of naturally occurring anhydro-

Table 3. *Results of the separation on deactivated alumina columns of liver oil from lutein-treated* Saccobranchus fossilis

(Results for a group of fifteen fish, total body-weight 60 g, total liver weight 1·51 g)

Fraction no.	Eluant	Extinction maxima (nm) in light petroleum (b.p. 40°–60°)	Compound	Amount (μg)
1	Light petroleum (b.p. 40°–60°)	474, 450, 424	Residual(?) β-carotene	7·7
2	Light petroleum	345, 286, 276	Dehydroretinyl ester	61·06*
3	Light petroleum–diethyl ether (100:2, v/v)	460	Anhydrolutein ester	10·34
4	Light petroleum–diethyl ether (100:5, v/v)	330	Unidentified	Very little present
5	Light petroleum–diethyl ether (100:12–14, v/v)	476, 445, 422	Lutein ester	20·46

* Calculated from values for the extinction of $SbCl_3$ complex.

Table 4. *Recovery of dehydroretinol, anhydrolutein and unconverted lutein from liver oil from lutein-treated, vitamin A-depleted* Saccobranchus fossilis *after separation on deactivated alumina columns*

Expt no.	No. of fish	Period (d) of		Time between last dose and death (h)	Approximate lutein dose (μg)	Amount recovered (μg)		
		Depletion	Lutein treatment			Dehydroretinol*	Anhydrolutein	Lutein
1	14	135	7	16	1500	37·1	10·6	5·7
2	15	150	8	16	1600	61·1	7·9	4·2
3	10	180	10	3	2000	95·5	4·0	15·5
4	15	180	10	3	2000	61·1	10·34	20·5

* Calculated from values for the extinction of $SbCl_3$ complex.

lutein (as the alcohol), anhydrolutein prepared from lutein, and liver oil (unsaponified) of freshly caught *S. fossilis*, using silica gel and the solvent system, cyclohexane–diethyl ether (80:20, v/v), resulted in the detection of anhydrolutein (R_F 0·46) in the fish liver oil. The possibility of the formation of anhydrolutein from lutein as an artifact during the isolation procedure can be ruled out, because lutein from *Z. mays* leaves and anhydrolutein from liver oil of *S. fossilis* were isolated under identical conditions. If anhydrolutein was formed from lutein as an artifact in the liver oil, it would have been possible to detect some anhydrolutein during the isolation of lutein also: we failed to detect any. Lutein was identified in *S. fossilis* liver oil by thin-layer chromatography of the unsaponified liver oil together with authentic lutein using silica gel, when it remained at the origin with cyclohexane–diethyl ether (80:20, v/v) and had an R_F of 0·53 with light petroleum–benzene–ethanol (60:30:10, by vol.). Further, lutein isolated from fish liver oil was converted into the free alcohol by saponification with alcoholic KOH. The naturally occurring sample of lutein and

authentic lutein could not be separated by mixed chromatography on columns of deactivated alumina.

There has been no previous convincing report of the occurrence of anhydrolutein in the plant kingdom. Budowski, Ascarelli, Gross, Nir & Bondi (1964) reported the presence of anhydrolutein in acidulated soya-bean soapstock and showed that anhydrolutein was an artifact formed from lutein during the acidulation of raw soapstock. The present report on the isolation of anhydrolutein from the liver oil of freshly caught *S. fossilis*, therefore, indicates that dietary lutein may be transformed into anhydrolutein by the fish.

To confirm this view experiments were done in which lutein was fed to vitamin A-depleted fish. The results of a preliminary experiment (Table 4, Expt 1) indicated that a compound was present which had an extinction maximum at 460 nm, but no emphasis was placed on the correct identity of this compound. Further experiments were, therefore, done to confirm the identity of this compound and these established that lutein-treated, vitamin A-depleted fish contained some anhydrolutein in addition to dehydroretinol and unconverted lutein. The different compounds which were isolated after giving lutein to the vitamin A-depleted fish are shown in Table 3. Anhydrolutein was identified by the examination of the visible spectra in different organic solvents and by thin-layer chromatography using silica gel. Three mixtures were examined: one contained anhydrolutein (as the alcohol) isolated from the liver oil from lutein-treated fish, after saponification, another contained anhydrolutein prepared chemically from lutein and the third contained a mixture of these two. All the compounds had an R_F of 0·46 in cyclohexane–diethyl ether (80:20, v/v) and an R_F of 0·74 in light petroleum–benzene–ethanol (60:30:10, by vol.), and the mixture was not resolved. The other compounds shown in Table 3 were similarly identified, after saponification where necessary, by their R_F values after thin-layer chromatography.

The recoveries for lutein, anhydrolutein and dehydroretinyl ester from lutein-treated, vitamin A-depleted *S. fossilis* are shown in Table 4. As reported in our earlier paper (Barua *et al.* 1973) the method of oral administration of lutein was such that a considerable quantity of the carotenoid was lost during feeding and therefore only the approximate amount of lutein or anhydrolutein fed to the fish was known.

The isolation of anhydrolutein from liver oils of freshly caught fish as well as from lutein-treated, vitamin A-depleted fish, therefore, supports our view that lutein is the natural carotenoid that can act as a precursor of dehydroretinol, and that the intermediate in this transformation is anhydrolutein (Fig. 2).

The authors thank Professor N. N. Siddhanta, Head of the Department of Chemistry, for providing the necessary facilities.

REFERENCES

Barua, A. B., Singh, H. & Das, R. C. (1973). *Br. J. Nutr.* **30**, 1.
Bolliger, H. R. & Konig, A. (1969). In *Thin-Layer Chromatography* 2nd ed., p. 264 [E. Stahl, editor]. London: George Allen & Unwin.
Budowski, P., Ascarelli, I., Gross, J. & Nir, I. (1963). *Science, N.Y.* **142**, 969.
Budowski, P., Ascarelli, I., Gross, J., Nir, I. & Bondi, A. (1964). *J. Am. Oil Chem. Soc.* **41**, 441.
Budowski, P. & Gross, J. (1965). *Nature, Lond.* **206**, 1254.
Cama, H. R. & Morton, R. A. (1953). *Analyst, Lond.* **78**, 74.
Grangaud, R. & Moatti, J.-P. (1958*a*). *C. r. Séanc. Soc. Biol.* **152**, 1235.
Grangaud, R. & Moatti, J.-P. (1958*b*). *C. r. Séanc. Soc. Biol.* **152**, 1245.
Morton, R. A. & Creed, R. H. (1939). *Biochem. J.* **33**, 318.
Savithry, K. N., Krishna Mallia, A. & Cama, H. R. (1972). *Indian J. Biochem. Biophys.* **9**, 325.

Printed in Great Britain