Rapid Papers

(Pages 133–144)

Formation of Vitamin A in a Freshwater Fish

ISOLATION OF RETINOIC ACID

By A. B. BARUA and U. C. GOSWAMI*

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

(Received 3 May 1977)

The intestines of freshly caught *Saccobranhus fossilis* (a freshwater fish that contains dehydroretinol) became free from carotenoids and from vitamin A when the fish were starved for about 20 days. When β -carotene was administered to such fish, retinoic acid could be isolated from the intestines after approx. 4 h. When lutein was administered to such fish, dehydroretinol and 3-hydroxyretinol could be isolated from the intestines after approx. 5 h.

Vitamin A₂ (dehydroretinol) is the usual form of vitamin A found in most freshwater fish. The origin of vitamin A₂, which differs from retinol in possessing an extra double bond in the 3,4-position of the β -ionone ring, is not clearly known, and the subject has been reviewed by Ganguly & Murthy (1967). Barua *et al.* (1973) and Barua & Das (1975) have reported the transformation of lutein (3,3'-dihydroxy- α -carotene) into dehydroretinol via anhydrolutein in the freshwater fish *Saccobranhus fossilis*. The freshly caught *S. fossilis* may contain retinol (although very often it is absent), and studies (Barua *et al.*, 1973) made on vitamin A-depleted fish failed to show the appearance of any retinol (as judged by liver-storage test) after administration of β -carotene to the fish. This is surprising, since β -carotene is the best pro-(vitamin A) known. Attempts were therefore made to trace the origin of retinol in the fish. We report in the present paper that β -carotene is converted by the fish into retinoic acid and in some cases into retinol. Further, lutein has been shown to be converted into dehydroretinol in the apparently normal and healthy fish.

Materials and Methods

Solvents and chemicals

The sources of β -carotene, lutein and of other chemicals and solvents used have been described before (Barua *et al.*, 1973).

Fish

Freshly caught *Saccobranhus* (*Heteropneustes*) *fossilis* were procured from local fish dealers and were kept in large glass tanks containing tap water. The

fish were starved for 15–20 days and only the intestines were examined for residual carotenoids and vitamin A. When the intestines were found to be free from any carotenoids or vitamin A or other food particles after 15–20 days, the fish were used to study the metabolism of carotenoids.

Administration of carotenoids

An aqueous suspension of carotenoids in Tween 80 was prepared by the procedure described by Barua *et al.* (1973), and 0.1–0.5 ml of the solution was administered directly into the intestine either through the mouth or through the anal opening, by means of an Agla micrometer syringe.

Extraction of lipids

Only the intestines were used to detect residual carotenoids or vitamin A before the experiment or to detect the appearance of vitamin A after the administration of carotenoids. The fish were killed by decapitation after anaesthesia with diethyl ether. The intestines were ground to a powder in a glass mortar with sea-sand and anhydrous Na₂SO₄ and the lipids were extracted four or five times with approx. 5 ml portions (per intestine) of light petroleum (b.p. 40–60°C). The bulk extract (approx. 20–25 ml/intestine) was evaporated to dryness *in vacuo* and the residue was dissolved in a known volume of light petroleum (b.p. 40–60°C) (3–5 ml). A Beckman DK 2 spectrophotometer was used to record the u.v.- and the visible-absorption spectra of the extract (after the required dilution where necessary) and to measure the SbCl₃ colour maxima (Barua *et al.*, 1973).

Column chromatography of lipid extracts

This was carried out on water-deactivated (8%, v/w) Brockmann grade 1 alumina [for chromatographic analysis; BDH, supplied by Glaxo Labora-

* Present address: Department of Zoology, University of Gauhati, Gauhati 781014, Assam, India.

tories (India), Bombay, India] columns (usually 10g, 1.5cm×5.5cm). Carotenoids and vitamin A were eluted with light petroleum (b.p. 40–60°C) or with mixtures of light petroleum containing increasing proportions (1–20%, v/v) of diethyl ether. Fractions of volume 1–2ml of the eluate were collected.

Retinoic acid was adsorbed very strongly on deactivated alumina. It was therefore eluted, after extrusion, with ethanol/aq. NH_3 (sp.gr. 0.88) (8:1, v/v). The eluate was acidified with aqueous 6M-HCl, with cooling, and retinoic acid was extracted with diethyl ether (Barua & Barua, 1964).

Results

The intestines from *S. fossilis* (which had been starved for 15–20 days) were free from carotenoids and from vitamin A as judged from the u.v.- and the visible-absorption spectra of the intestine extract (Fig. 1); the SbCl_3 reaction performed on such extracts failed to show any colour. When this condition was reached, the fish were given β -carotene (about 500 $\mu\text{g}/\text{fish}$) and the intestines were examined for any vitamin A over the period 0.5h to 20 days. In the 168 experiments carried out over this period, retinoic acid could be detected and characterized in

Fig. 1. Absorption spectra of intestine extracts. Absorption spectra in light petroleum (b.p. 40–60°C) of: intestine extracts (in 3ml) from five *S. fossilis* after starvation for 15 days (---), indicating the absence of carotenoids and of vitamin A; intestine extracts (in 10ml) from five *S. fossilis* after the administration of β -carotene after starvation for 15 days, indicating the appearance of retinoic acid (—); retinoic acid (-.-) (in 10ml) isolated from the intestines of three *S. fossilis* after the administration of β -carotene after starvation for 15 days.

only 12 experiments, in all of which the fish were killed between 4 and 6h after the administration of β -carotene. Each experiment was done on pooled samples of intestine from various numbers of fish. The u.v.- and visible-absorption spectra of these 12 crude extracts showed the spectra characteristic of β -carotene and retinoic acid (Fig. 1). Chromatographic separation of the crude extract resulted in the isolation of pure retinoic acid, λ_{max} at 350nm (in ethanol), which changed to 337nm on addition of NaOH and again to 350nm on addition of HCl; the SbCl_3 colour gave a λ_{max} at 570nm. For further identification of retinoic acid, the crude intestine extract showing λ_{max} at 350nm was evaporated to dryness at room temperature *in vacuo* and the residue was extracted twice with 10ml portions of approx. 5% (w/v) NaHCO_3 . The bicarbonate extract was acidified with 6M-HCl, with cooling, and retinoic acid was extracted with diethyl ether. The solvent was removed *in vacuo* and the residue was dissolved in light petroleum (b.p. 40–60°C). This solution showed a λ_{max} at 350nm, and the SbCl_3 colour gave a λ_{max} at 570nm, thus conclusively proving that this substance was acidic and that it was retinoic acid.

The identity of retinoic acid isolated as described above was further confirmed by converting it into methyl retinoate. Samples of the retinoic acid isolated from fish intestines in several of the experiments were pooled and dissolved in ethyl acetate (5ml). To this was added anhydrous K_2CO_3 (50mg) and methyl iodide (0.02ml), and the mixture was warmed at 50°C for 1h. Water was added, and the methyl retinoate was extracted with diethyl ether. The extract was applied to alumina and the methyl retinoate was eluted with light petroleum (b.p. 40–60°C). Methyl retinoate thus obtained showed a λ_{max} at 350nm in light petroleum (b.p. 40–60°C). Treatment of the methyl retinoate in diethyl ether with LiAlH_4 at 0°C resulted in formation of retinol, as judged from the λ_{max} at 325nm of the reduced product.

In only two of the experiments with β -carotene was it possible to detect retinol, as judged from the u.v.-absorption spectrum (λ_{max} at 325nm in light petroleum) and SbCl_3 colour (λ_{max} at 615nm). As the amount of the crude extract was very small in both the experiments, chromatographic separation was not attempted.

Table 1 shows the amounts of β -carotene administered to the fish together with the amounts of retinoic acid isolated and of retinol formed. It should be mentioned that the whole of the β -carotene solution administered was not retained by the fish and that a small portion always came out through the opercular or the anal openings. The amounts of β -carotene actually taken up by the fish were much less than is shown in Table 1.

Some 27 experiments were carried out in which

Table 1. Isolation of retinoic acid from the intestines of *S. fossilis* after administration of β -carotene

After starvation of the fish for 15–20 days, the intestines of *S. fossilis* became free from carotenoids and vitamin A. When this condition was reached, an aqueous suspension of β -carotene in Tween 80 was administered in one single dose to each fish. The fish were killed at the times indicated after the dose, and the intestine extracts were chromatographed on alumina as described in the text. The amounts of retinoic acid isolated and of retinol formed from β -carotene are indicated. β -Carotene, retinoic acid and retinol were measured by their gross absorbance at 450, 350 and 325 nm respectively on the basis of $A_{10\text{nm}}^{1\%}$ values of 2500 for β -carotene, 1500 for retinoic acid and 1832 for retinol. The results are expressed for pooled samples of intestine from the number of fish indicated. ND, Not detectable.

No. of fish used	Total β -carotene administered (μg)	Time between administration and killing (h)	Total β -carotene recovered (μg)	Total retinoic acid isolated (μg)	Total retinol formed (μg)
3	1488	4	424	54.6	ND
5	2900	4	664	26.6	ND
12	6120	4	1760	180	ND
3	1530	4	240	51.3	ND
15	8040	4	2560	114.6	ND
20	9920	4	2440	170	ND
2	992	4	14.6*	ND	53.8*
5	2900	4	14*	ND	25.3*
3	1108	4	—	94.6	ND
4	7584	4.5	720	61.3	ND
3	1740	5	224	28.3	ND
2	992	5.5	176	24	ND
3	1530	5.5	17.6	64.6	ND
5	2900	6	—	41.3	ND

* Calculated from gross absorbance of the crude intestine extract at 450 nm (for β -carotene) and 325 nm (for retinol). Chromatographic separation was not done.

lutein was administered into the intestine of *S. fossilis* whose alimentary canals were free from carotenoids or vitamin A. Intestines of such fish given lutein showed the presence of dehydroretinol in 11 of the experiments, in all of which the fish were killed between 4 and 6 h after the administration of lutein. The u.v.-absorption spectra were characteristic of dehydroretinol (λ_{max} , 345–350, 287 and 276 nm in light petroleum, b.p. 40–60°C) and the SbCl_3 colour maximum was obtained at 690 nm. No retinoic acid or retinol was detected in these experiments.

Chromatography of such intestine extracts on deactivated alumina resulted in separation of pure dehydroretinyl ester, a small amount of dehydroretinol and a compound characterized as 3-hydroxyretinol (A. B. Barua, R. C. Das & K. Kohli, unpublished work).

Discussion

In attempts to study the conversion of β -carotene into vitamin A in the freshwater fish *S. fossilis*, it was of interest to isolate retinoic acid from the intestines of fish given β -carotene. Retinoic acid was detected only in those experiments where the experimental fish were killed between 4 and 6 h after administration of β -carotene. This is the first report on the isolation of retinoic acid from the intestine of fish given β -

carotene. Retinoic acid may be formed from either retinal by further oxidation (Dmitrovsky, 1961; Elder & Topper, 1961; Mahadevan *et al.*, 1962; Futterman, 1962) or apocarotenals via apocarotenoid acids (Sharma *et al.*, 1976). We were unable to detect any retinal or apocarotenoid acid during the present study. It may be that the quantities formed were too small to be detected.

However, our failure to isolate any dehydroretinol from the fish given β -carotene in the present study and also in the previous study by the liver-storage test (Barua *et al.*, 1973) suggests that the biosynthesis of dehydroretinol from β -carotene is not a major pathway.

Previous studies on the biosynthesis of dehydroretinol made on the same fish (Barua *et al.*, 1973; Barua & Das, 1975) revealed that the fish can convert dietary lutein into anhydrolutein, which is then cleaved into dehydroretinol. These studies were made by the liver-storage test in fish made vitamin A-depleted by feeding them on a diet of rice and goat meat. Apparently the diet was lacking in many of the essential nutrients given to the fish for 3–5 months, and might adversely affect the normal metabolism of carotenoids. The present finding confirms that in apparently normal and healthy fish lutein is a precursor of dehydroretinol.

Chromatography of the liver oil of the freshly caught *S. fossilis* was carried out several times and the presence of retinol was observed only occasionally, whereas in most liver oils retinol was not detected (also see Barua & Das, 1975). We failed to detect both retinol and retinoic acid in all the experiments in the previous study (Barua *et al.*, 1973) and in most of the experiments in the present study, which were aimed at studying the metabolism of β -carotene in the fish. By contrast, the conversion of lutein into dehydroretinol was observed in many of the experiments in the present and the previous studies (Barua *et al.*, 1973). This strongly suggests that, like lutein, β -carotene was also utilized by the fish, usually giving rise to retinoic acid rather than retinol; the retinoic acid, however, frequently escaped detection because of its rapid disappearance from the intestine. It is surprising that the freshly caught *S. fossilis* store large quantities of β -carotene, but no retinol (Barua & Das, 1975).

We thank Professor N. N. Siddhanta, Head of the Department of Chemistry, for providing facilities and for

letting U. C. G. work in the University laboratory, and thank Professor J. Ganguly of the Indian Institute of Science, Bangalore, for useful discussions. Financial assistance from the University Grants Commission, India, to U. C. G. is gratefully acknowledged.

References

- Barua, A. B. & Das, R. C. (1975) *Br. J. Nutr.* **33**, 319–327
- Barua, A. B., Singh, H. T. & Das, R. C. (1973) *Br. J. Nutr.* **30**, 1–12
- Barua, R. K. & Barua, A. B. (1964) *Biochem. J.* **92**, 21c–22c
- Dmitrovsky, A. A. (1961) *Biokhimiya* **26**, 225–233
- Elder, T. D. & Topper, J. Y. (1961) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **20**, 196
- Futterman, S. (1962) *J. Biol. Chem.* **237**, 677–680
- Ganguly, J. & Murthy, S. K. (1967) in *The Vitamins* (Sebrell, W. H., Jr. & Harris, R. S., eds.), vol. 1, 2nd edn., pp. 146–148, Academic Press, New York and London
- Mahadevan, S., Murthy, S. K. & Ganguly, J. (1962) *Biochem. J.* **85**, 326–331
- Sharma, R. V., Mathur, S. N. & Ganguly, J. (1976) *Biochem. J.* **158**, 377–383