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In vitro metabolism of carotenoids, ß carotene and lutein into retinoids in amphibians

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Abstract
Carotenoids are a family of over 600 natural lipid-soluble pigments that are produced within microalgae, phytoplankton, and higher plants. Of these only 50 have provitamin A activity, with the capacity to be transformed into retinol and dehydroretinol. Animals are unable to synthesise carotenoids *de novo*. As animals lack the ability to synthesize vitamin A, they are dependent on dietary intake to provide adequate levels of vitamin A. Vitamin A (retinol) and its naturally occurring and synthetic derivatives are collectively referred to as retinoids. Retinoids are important metabolites of carotenoids that have at least one non-hydroxylated ring system of the β-type, e.g. carotenes (β-carotene, α-carotene, and γ-carotene) and xanthophyls (β-cryptoxanthin and echinenone). The pigmentation and colouration in amphibian occur owing to the deposition of carotenoids from their metabolism by consuming the carotenoids through foods from their habitat or through conversion of these carotenoids into different metabolites during development. The status of the retinoids formed through conversion of carotenoids was examined from the lipid extracts of the carotenoid administered tadpoles of *Duttaphrynus melanostictus* and *Haplobactrachus tigerinus.* The extracts were subjected to UV –VIS spectrophotometer for tentative analysis of the retinoids formed and then by the HPLC procedures for final results. The findings show that larval forms of amphibians of both the species *Duttaphrynus melanostictus* and *Haplobactrachus tigerinus* can convert β–carotene to retinol and lutein to dehydroretinol. The conversion of carotenoids into different retinoids is explained through the mode of cleavage of the carotenoids molecules.

Keywords: carotenoids, retinoids, β–carotene, lutein.

INTRODUCTION

Provitamin A is widely distributed in plants and microorganisms that are capable of carotenoid biosynthesis [1]. Retinoids are important metabolites of carotenoids that have at least one non-hydroxylated ring system of the β-type, e.g. carotenes (β-carotene, α-carotene, and γ-carotene) and xanthophyll (β-cryptoxanthin and echinenone). In most animals, metabolic products of carotene are Vitamin A [2] and its congeners are retinol and dehydroretinol. Retinol is one of the forms of Vitamin A found in marine fishes whereas dehydroretinol is found in freshwater fishes, larval amphibians and fish eating birds and mammals [3]. It was later proved that not only β-carotene is the most important pro-vitamin A but also α and γ carotene, cryptoxanthin and different apo-β-carotenals also have pro-vitamin activities [4] [5]. Besides these, xanthophyll, the oxygenated derivatives of carotenoids such as lutein which occur in green vegetables, plants and animal fats, astaxanthin occurring in crustaceans, molluscs, echinoderms, and fishes, and zeaxanthin and β-cryptoxanthin also displayed the efficiency to be converted into Vitamin A_1 or Vitamin A_2 [6][7][8].

Provitamin A character of carotenoids has been studied in fish, mammals and birds. These findings however do not reveal the conversion of carotenoids, such as β-carotene and lutein in amphibian system. Amphibians interestingly forms the transition group between fishes and other higher animal groups containing dehydroretinol in the larval stages and transforming to retinol during metamorphosis in the adult stages. Amphibians are unable to synthesize carotenoids *de novo.* The pigmentation and colouration in amphibian occur owing to the deposition of carotenoids from their metabolism by consuming the carotenoids through foods from their habitat or through conversion of these carotenoids into different metabolites during development [9][10][11].

The present work describes the *in vitro* metabolism of precursor carotenoids, β –carotene and lutein in two amphibian species *Duttaphrynus melanostictus* and *Haplobatrachus tigerinus.*

MATERIALS AND METHODS

Collection of tadpoles

Climax stage tadpoles of *Duttaphrynus melanostictus* and *Haplobatrachus tigerinus* were collected from wetlands near Hajo region of Kamrup district, Assam, India. The tadpoles were identified following Sahu, 1994 [12]. The animals were reared in aquarium and starved for a week before the experiments were conducted.

Solvents and chemicals

Light petroleum ether (b.p. 40-60^{O}C), diethyl ether, chloroform, absolute ethanol, anhydrous sodium sulphate, aluminium oxide active neutral LR for chromatographic absorption analysis, were

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procured from BDH, Laboratory Chemicals Division, Glaxo Laboratories (India) Pvt. Ltd. Alumina was deactivated, prior to use, by adding the requisite amount of water (usually 5-8 % v/w) in a glass mortar. It was then mixed thoroughly.

Different authentic retinoids samples, such as retinol, dehydroretinol, retinyl propionate β-carotene, lutein, cryptoxanthin, astaxanthin, β-apocarotenals and CAEE samples were supplied by F.Hoffman La Roche, Basel, Switzerland.

Column Chromatography

Chromatographic columns made of Borosilicate glass of different diameters and lengths provided with stopcocks at lower ends were used. Column chromatography was carried on columns (usually 1.5cm × 9.5cm, unless otherwise mentioned) from light petroleum (b.p. 40-60 ^{0}C) solution. Elution of the compounds was carried out with light petroleum alone or mixtures of light petroleum containing increasing proportion of diethyl ether (1-30%, v/v). The elutes were collected in fractions (5-6 ml) and the fractions having similar absorption spectra were pooled together.

Column chromatography of retinoids was done after Goodwin 1951 [13].

UV-vis spectrum:

The UV-visible spectra were recorded in a Beckman DK-2 spectrophotometer. β-carotene, lutein, anhydroluten and 3-dehydroretinol were assayed on the basis of the following $E^{1\%}_{1cm}$ values: 2500, 2200, 2031 and 1455 respectively (all at the point of maximum absorption). The concentration of 3-hydroxyanhdroretinol, rehydrovitamin A_2 and 3-dehydroxyretinol diester was calculated from the absorbance at the point of maximum absorption on the basis of approximate $E^{1\%}_{1cm}$ values of 2500, 2200, 2031 and 1455 respectively (all at the point of maximum absorption). The concentrations of 3-hydroxyandroretinol, rehydrovitamin A_2 and 3-hydroxyretinol diester were calculated from the absorbance at the point of maximum absorption on the basis of approximate $E^{1\%}_{1cm}$ values of 3000, 1500 and 1800 respectively.

HPLC procedure :

The HPLC system includes (water) with column 300 mm × 3.9 mm Nova - Pak C18 (4mm) and a Guard - Pak precolumn module (water 5) were used. Standard retinoids samples (5.0 mg) were dissolved in 100 ml tolune : methanol (1:1) containing 500 mg BHT (butylated hydroxytolune) / litre for producing 50 m/ml standards. These standard stock solutions could be preserved at -20°C for 4 months. HPLC grade solvents, acetonitrile : dichloromethane : methanol : water : propionic acid (71:22:4:2:1, v/v) were used as mobile phase with the flow rate of 1.0 ml/minute in the first 10 minute run, detection of carotenoid pigments was performed at 450 nm and retinol in 352 and 326 nm.

RESULTS:

The fate of β-carotene and lutein were studied in *Duttaphrynus melanostictus* and *Haplobatrachus tigerinus.*

***Incubation of carotenoids with the mucosal protein* :**

The intestinal mucosal regions were scraped out and homogenated and then the protein concentration was determined. The mucosal layers were scraped out, homogenized in ice cold HEPES buffer (1:5 w/v) and centrifuged in 3000 rpm (800 g) at 4^0 C for 30 minutes. The supernatant fractions were collected and the protein content was estimated after Lowery *et al.,* [14]. β -carotene and lutein was purified through 5% deactivated alumina column using light petroleum ether 40-60^0 C as eluent [15].

Mucosal protein (100 µl, 0.15 mg) was incubated in the dark, in glass vials at pH 5-8 and temperature 25-37ºC, using shaking water bath for 1 h with a cocktail containing HEPES buffer 20mM, pH 5-8, 150 mM KCL, β -carotene 0.2 µM dissolved in 10 µl of propylene glycol, NAD (2mM) and dithiothretol (2mM) in a final volume of 1 ml.

In each experiment, two control incubations were taken with no addition of either carotenoids (β -carotene or lutein) or intestinal homogenate.

Metabolic transformation of β-carotene and lutein through in vitro incubation of the intestinal mucosa with the concerned carotenoids.

The *in vitro* reaction for 1h incubation in 37^{0}C, in pH 7 and with different amounts of protein, with the addition of β-carotene and lutein, has been shown below. The HPLC analysis was conducted immediately after the completion of incubation as described in detail by Wang, *et al.*, [16]. The metabolites of β-carotene and lutein were identified and measured from the HPLC and column chromatography of the authentic samples. It has been found that in the absence of β-carotene or lutein and protein, no such products were formed. However, the control experiments were not so much elaborated. Further no detectable amount of cleavage products of either β-carotene or lutein could be identified through HPLC after incubation in the *in vitro* reaction.

METABOLISM OF β-CAROTENE :

Metabolites of β-carotene, after incubation of intestinal mucosal homogenate (1 mg / 60 min / 37^{0}C, pH-7) with β carotene (2 µM) and formation of different metabolites (pmol/h/mg protein) i.e. retinol, retinal, retinoic acid etc. are shown in table 1.

Table 1. Metabolites formed during β-carotene metabolism.

Amphibian Sp. (sex/No./wt.)	Retinoids			Unconverted carotenoids	Recovery % of internal standard
	Retinol	Retinal	Retinoic Acid	β-carotene	94 ± 0.5
D.melanostictus					
Male, n=8, 900-1200g	6 ±0.5	4 ± 0.5	5 ± 0.5	45 ± 1.0	96 ± 0.5
Female, n=7, 900-1350g	5 ± 0.5	7 ±1.0	4 ± 1.2	49 ± 0.5	94 ± 1.0
H.tigerinus					
Male, n=6 780-815 g	11 +1.5	5.5 ± 0.5	5 ± 0.5	53 ± 1.0	92 ± 0.5

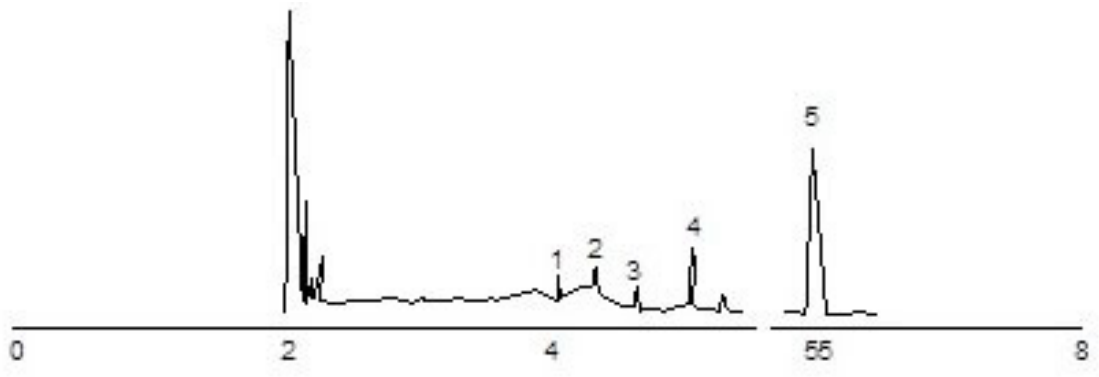

Fig 1. Chromatogram of metabolites of β-carotene: (1) Retinol, (2) Retinal, (3) Retinoic acid, (4) Retinyl propionate, (5) β-carotene.

Metabolism of lutein

Metabolites of lutein, after incubation of intestinal mucosal homogenate (1 mg / 60 min / 37^0 C, pH-7) with lutein (2 µM) and formation of different metabolites (pmol/h/mg protein) i.e. dehydroretinol, 3-hydroxyretinol and 3-hydroxyanhydroretinol etc., are shown in table 2.

Table 2. Metabolites formed during lutein metabolism

Amphibian Sp (sex/No./wt.)	Retinoids			Unconverted carotenoids	Recovery % of internal standard
	Dehydro-Retinol	3-Hydroxy retinol	3-Hyroxyan hydroretinol	Lutein	94 ± 0.5
D.melonistictus					
Male, n=8, 900-1200g	6 ± 0.5	4 ± 0.5	5 ± 0.5	45 ± 0.5	96 ± 0.5
H.tigerinus					
Male, n=6, 80-815 g	11 ± 1.5	5.5 ± 0.5	5 ± 0.5	53 ± 0.5	92 ± 0.5

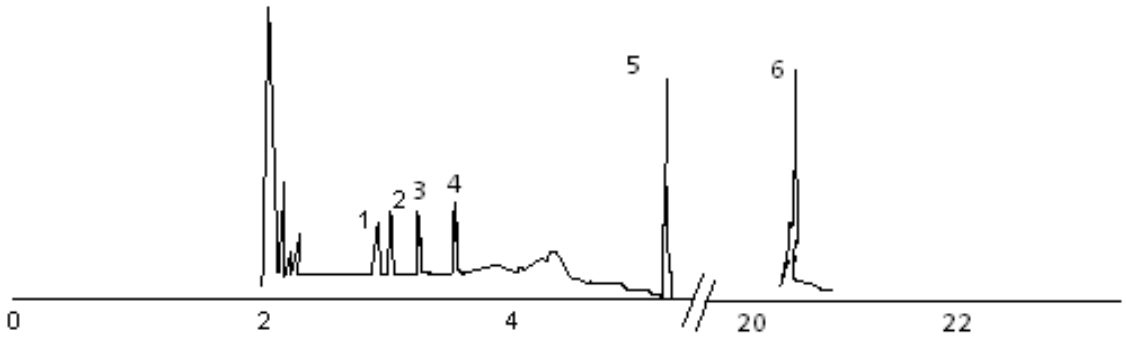

Fig 2. Chromatogram of metabolites of Lutein : (1) Dehydroretinol, (2) 3-hydroxyretinol, (3) 3-hydroxyanhydroretinol, (4) Rehydrovitamin A_2, (5) Retinyl propionate, (6) Lutein.

There are no statistical differences in the yield of metabolites after incubation of β-carotene or lutein with the intestinal homogenate protein from the different species of amphibian.

DISCUSSION

Carotenoids which act as the precursors of both forms of vitamin A provide the bright coloration of fishes, which has attracted the attention of biologists and aqua culturists. This lipid class molecule furnishes the red, orange, yellow, pink etc. colouration of the fish [17][18][19].

Recent work on molecular biology and the detection of the functioning of the enzymes has also clarified many questions regarding the mechanism of the conversion and existence of monoxygenase and dioxygenase enzymes in the conversion of β-carotene into vitamin A [20][21]

In this study on the metabolism of β-carotene, it was found that β-carotene is metabolized directly into retinal, retinol or retinoic acid through central cleavage. Evidence of central cleavage of β-carotene between the 15′ and 15′ carbon atoms yielding retinal as the primary product was reported by Olson and Hayaishi in rat liver [22] and by Goodman and Huang in rat intestine[23].

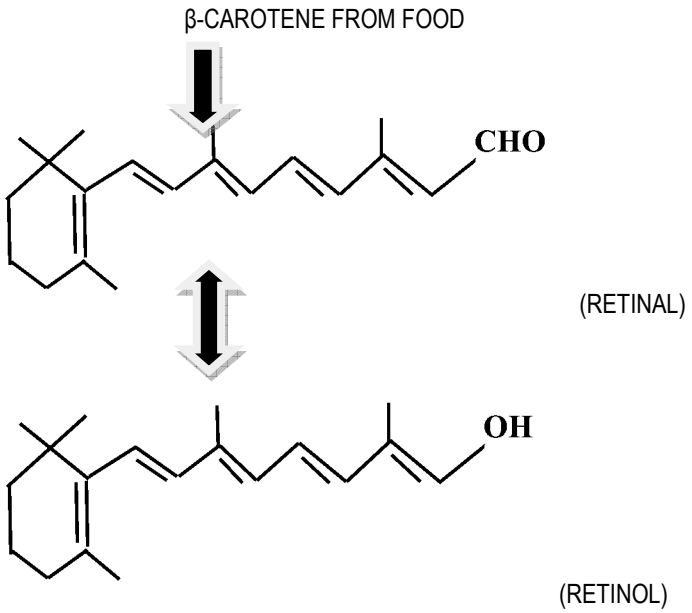

Fig 3. Conversion of β-carotene in amphibian.

In the present study, lutein was found to be the precursor of Vitamin A_2 (dehydroretinol) in *Duttaphrynus melanostictus* and *Haplobatrachus tigerinus*. It has been found that lutein is metabolized into anhydrolutein and later into 3-hydroxyretinol and dehydroretinol. Thus it is probable that anhydrolutein yields dehydroretinol directly (Way 1) or anhydrolutein could be metabolized into 3-hydroxyretinol, 3-hydroxyanhydroretinol and then to dehydroretinol (Way 2).

Lutein has been tested in a number of fishes belonging to various taxonomic statuses like *H.fossilis*, *Mystus* species, *Channa* species and *Labeo* species. In these studies it has been found that lutein is an efficient precursor of dehydroretinol. Goswami, 1984 [24], showed that lutein is metabolized into anhydrolutein and later into 3-hydroxyretinol, 3-hydroxyanhydroretinol and dehydroretinol. In the same experiment with *H.fossilis*, Goswami, 1984, evaluated the metabolism of 3-hydroxyretinol and 3-hydroxyanhydroretinol separately which yielded the formation of dehydroretinol. Budowski, *et al.,* 1963[25], administered anhydrolutein to vitamin A deficient chicks and were able to isolate dehydroretinol from liver. Barua, Singh and Das, 1973[26], described the conversion of lutein into dehydrolutein in freshwater fish, *Saccobranchus fossilis*. They have also shown that both retinol and dehydroretinol can be formed from lutein in *S.fossilis.* Lutein is metabolized into anhydrolutein and later to 3- hydroxyretinol, 3- hydroxyanhydroretinol and dehydroretinol.

The probable pathway of metabolism of lutein in *Duttaphrynus melanostictus* are shown in the fig. 4.

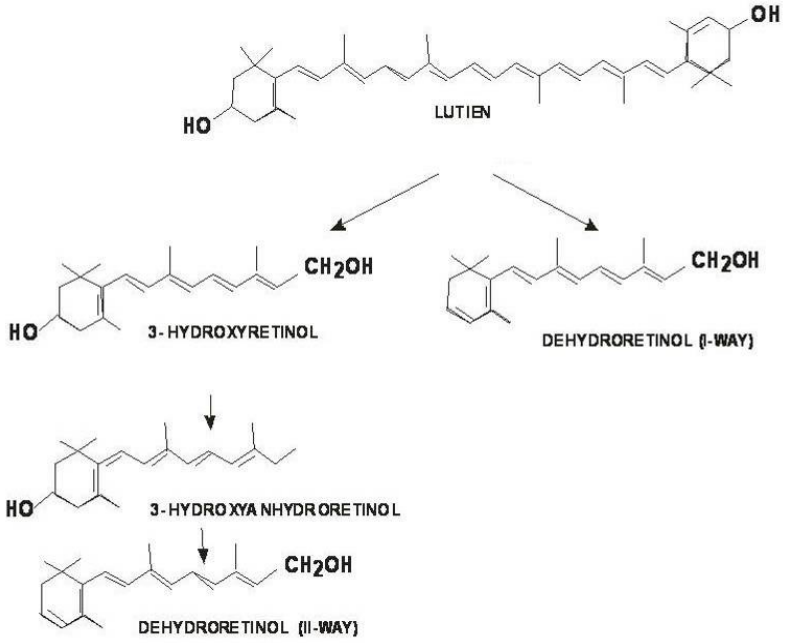

Fig 4. Conversion of lutein in amphibian

CONCLUSION

The experiments dealing with the *in vitro* metabolism of carotenoids showed that

a) β-carotene is metabolized through central cleavage to retinal which later forms retinol and retinoic acid as shown in *Duttaphrynus melanostictus* and *Haplobatrachus tigerinus*.

b) Lutein is metabolized to dehydroretinol either directly through anhydrolutein or through 3-hydroxyretinol and 3-hydroxyanhydroretinol of *Duttaphrynus melanostictus* and *Haplobatrachus tigerinus*.

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