1986

Studies on the Metabolism of Astaxanthin in the Rainbow Trout (*Salmo gairdneri*)

Abdulrhaman Salih Al-Khalifah
University of Rhode Island

Follow this and additional works at: https://digitalcommons.uri.edu/oa_diss

Recommended Citation

Al-Khalifah, Abdulrhaman Salih, "Studies on the Metabolism of Astaxanthin in the Rainbow Trout (*Salmo gairdneri*)" (1986). *Open Access Dissertations*. Paper 553.
https://digitalcommons.uri.edu/oa_diss/553

This Dissertation is brought to you for free and open access by DigitalCommons@URI. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu.

STUDIES ON THE METABOLISM OF ASTAXANTHIN
IN THE RAINBOW TROUT (Salmo gairdneri)

BY

ABDULRHMAN SALIH AL-KHALIFAH

A DISSERTATION SUBMITTED IN PARTIAL
FULLFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOLOGICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

1986

DOCTOR OF PHILOSOPHY DISSERTATION

OF

ABDULRHMAN SALIH AL-KHALIFAH

Approved:

Dissertation Committee

Major Professor

Herbert J. Simpson

Marjorie Caldwell

H Glenn Gray

A. Amick

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

1986

ABSTRACT

Racemic astaxanthin was fed to rainbow trout (Salmo gairdneri) for 2, 4, and 6 weeks. The fish showed a bright pink coloration of the skin and flesh; the highest amount of astaxanthin (162 ug/100g) was found in the skin of fish fed the test diet for six weeks. Lutein, 3-epilutein, and zeaxanthin were also detected in the flesh and skin; it was concluded that astaxanthin was converted to zeaxanthin in the skin. The mean vitamin A content of the liver was determined; the ratio of vitamin A₁:vitamin A₂ was approximately 1:3. There was no significant difference in the vitamin A content of the control and experimental fish; this proves that astaxanthin was not functioning as a vitamin A precursor in vitamin-A-rich rainbow trout.

Retinol and 3, 4-dehydroretinol were extracted from the intestine of rainbow trout low in vitamin A, after force feeding with astaxanthin using a feeding tube. Antibiotic-treated fish had no marked difference in vitamin A content compared with a control group that received no antibiotic. This proves that astaxanthin was converted to vitamin A in fish depleted of vitamin A, that microorganisms were not involved in the conversion, and that conversion occurred in the intestine. An in vitro study using ³H 3S, 3S'- astaxanthin incubated with

duodenal and ileal segments of the intestine provided HPLC and radioisotope data, which showed that rainbow trout were able to bioconvert astaxanthin to vitamin A. The results confirmed earlier observations.

I sincerely thank my major professor, Dr. Charles J. Simpson, for his guidance and encouragement during my study. Special thanks are also extended to Dr. J. Caldwell and H. G. Gray who also served as committee members.

My most sincere thanks and gratitude are offered to my wife, for her patience and sacrifice and for all my family. Special thanks are extended to William L. Smith and Dr. J. G. Gray, for their help and assistance during the study.

I thank my fellow researchers, Vicky and Gregory for their support.

Lastly, I am grateful to my government for providing me with a scholarship and to the United States Education Mission for their support and encouragement.

ACKNOWLEDGEMENTS

I sincerely thank my major professor, Dr. Kenneth L. Simpson, for his guidance and encouragement during my study. Special thanks are also expressed to Drs. M.J. Caldwell and H.G. Gray who also served on my committee.

My most sincere thanks and gratitude are offered to my wife, for her patience and sacrifice and for all my family. Special thanks are expressed to Hoffman-La Roche and Co. Ltd., Basle, Switzerland, for providing astaxanthin.

I thank my fellow researchers, Paura and Henery for their support.

Lastly, I am grateful to my government for providing me with a scholarship and to the Saudi Arabian Educational Mission for their support and encouragement.

PREFACE

This dissertation is written in the manuscript format. The style followed is that recommended for publication in Comparative Biochemistry and Physiology. The text consists of three manuscripts:

- I. Pigmentation and metabolic transformation of
of astaxanthin in rainbow trout (Salmo gairdneri).
- II. Conversion of astaxanthin to vitamin A₁ and A₂
in rainbow trout.
- III. In vitro metabolism of ³H₂ - 3S, 3S⁻ astaxanthin
in rainbow trout.

There are three appendices: Appendix A contains a literature review of the pigmentation and metabolism of astaxanthin in fish. Appendix B describes laboratory aquaria for trout. Appendix C contains a bibliography of the whole dissertation.

TABLE OF CONTENTS

	Page
ABSTRACT.....	ii
ACKNOWLEDGEMENTS.....	iv
PREFACE.....	v
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
MANUSCRIPT I	
ABSTRACT.....	2
INTRODUCTION.....	3
MATERIALS AND METHODS.....	5
RESULTS.....	10
DISCUSSION.....	11
REFERENCES.....	14
MANUSCRIPT II	
ABSTRACT.....	27
INTRODUCTION.....	28
MATERIALS AND METHODS.....	30
RESULTS.....	34
DISCUSSION.....	34
REFERENCES.....	37

MANUSCRIPT III

ABSTRACT.....	46
INTRODUCTION.....	47
MATERIALS AND METHODS.....	50
RESULTS.....	53
DISCUSSION.....	54
REFERENCES.....	57

APPENDIX A

LITERATURE REVIEW.....	74
------------------------	----

APPENDIX B

LABORATORY AQUARIA FOR TROUT.....	97
-----------------------------------	----

APPENDIX C

BIBLIOGRAPHY.....	101
-------------------	-----

LIST OF TABLES

MANUSCRIPT I

Table 1.	Concentration of carotenoids in skin and flesh of rainbow trout.....	22
2.	Mean vitamin A ₁ and A ₂ content of liver and blood of rainbow trout....	23

MANUSCRIPT II

Table 1.	Amount of vitamin A isolated from the intestines of rainbow trout, raised on avitamin A-deficient diet.....	39
----------	---	----

MANUSCRIPT III

Table 1.	Radioactivity isolated from the intestine after incubation with ³ H-astaxanthin.....	61
----------	---	----

LIST OF FIGURES

MANUSCRIPT I

- Figure 1. HPLC chromatogram of retinol in rainbow trout liver..... 24

MANUSCRIPT II

- Figure 1. Flow chart of experimental design..... 40
2. HPLC chromatogram of retinol in rainbow trout intestine..... 42
3. Suggested mechanism for the formation of retinol and 3, 4-dehydroretinol from astaxanthin in fish..... 44

MANUSCRIPT III

- Figure 1. Gross morphology of the digestive tract of the rainbow trout..... 63
2. Diagram of the intestine of the trout.. 65
3. Eversion of the fish intestine..... 67
4. Filling the intestinal sac..... 69
5. Intestinal sac apparatus (after Smith and Lane, 1979)..... 71
6. Identification of ^3H label following incubation of a sample of fish duodenum for 60 min with $^3\text{H}_2$ - 3S, 3S'-astaxanthin..... 73

APPENDIX A

- Figure 1. Metabolic pathway from astaxanthin to zeaxanthin in matured chum salmon..... 85
2. Metabolic pathway from astaxanthin to tunaxanthin in yellowtail..... 88
3. Metabolic pathway of astaxanthin in rainbow trout..... 91
4. Suggested mechanism for the formation of retinol and 3, 4-dehydroretinol from astaxanthin in fish..... 96

APPENDIX B

- Figure 1. Laboratory aquaria.....100

MANUSCRIPT I

PIGMENTATION AND METABOLIC TRANSFORMATION OF ASTAXANTHIN IN RAINBOW TROUT

(SALMO GAIARDNERI)

ABDULRHMAN S. AL-KHALIFAH

DEPARTMENT OF FOOD SCIENCE AND NUTRITION

UNIVERSITY OF RHODE ISLAND

KINGSTON, RHODE ISLAND 02881 (USA)

ABSTRACT

Rainbow trout (salmo gairdneri) with an average weight of 90 g were divided into four groups - a control and three experimental groups, each fed pigmented diets with racemic astaxanthin (3S, 3S'), (3S, 3R), (3R, 3R) (1:2:1) at a dose of 100 mg/kg of diet for two, four or six weeks.

The experimental fish showed a bright pink coloration in the skin and flesh, while control fish were gray. Carotenoids were deposited mainly in the skin; a small amount was found in the flesh. The highest amount of astaxanthin (162 ug/100g) was found in the skin of fish fed the test diet for six weeks.

Lutein, 3'-epilutein, and zeaxanthin were also detected in the flesh and skin. Zeaxanthin was higher than other carotenoids in the skin of the six week group, while the other carotenoids were about the same for all four groups. This proves that there was a conversion of astaxanthin to zeaxanthin.

The mean vitamin A content of the liver was determined, and the ratio of vitamin A₁:vitamin A₂ was approximately 1:3. There was no significant difference in the vitamin A content of the control and experimental tissue. This is an indication that astaxanthin was not functioning as vitamin A precursors in the liver of vitamin A-rich rainbow trout.

Only vitamin A₁ was detected in the blood and the quantity ranged from 6-7 ug/100 ml of serum.

Introduction

Carotenoids are the main pigment of many aquatic animals, especially farmed fish. In salmonids such as rainbow trout, astaxanthin (3,3'-dihydroxy- β,β -carotene, 4,4'-dione) is responsible for the typically pink color of the flesh. The astaxanthin is of dietary origin (Steven 1947) and is important in the cultivation of salmon and trout. In aquaculture, astaxanthin is commonly used as pigmenters (Bauenfeind 1976; Simpson et al. 1981). It is generally assumed that carotenoids cannot be synthesized de novo by animals, but many marine animals have the metabolic capacity to modify dietary carotenoids structurally (Liaaen-Jensen 1978; Tanaka 1976a, b).

Vitamin A has long been known as an essential nutrient for animals. The major source of vitamin A is the plant carotenoids, mainly β -carotene. About two thirds of the vitamin A necessary in human nutrition is supplied by β -carotene (William 1971). Animals, including humans, can not synthesize carotene but can convert some carotene to vitamin A (Krause and Mahan 1979).

The provitamin A character of carotenoids has been extensively studied in mammals and birds. In fish and in aquatic animals generally, the question of the conversion of carotenoids into vitamin A has remained relatively obscure, being further complicated by the presence of

vitamin A₂, which frequently predominates over vitamin A₁ in freshwater fish.

Earlier reports indicated that both vitamins A₁ and A₂ can be formed from a common precursor such as β -carotene (Morton and Creed 1939) or cryptoxanthin (Goswami 1984). It has been shown that 3-dehydroretinol can be formed from lutein via anhydrolutein in several species of freshwater fish (Barua et al. 1973, 1979; Barua and Das 1975; Barua and Goswami 1977). Thus it was established that lutein acted as one of the best precursors of 3,4-dehydroretinol. Recently, astaxanthin was reported as a precursor of vitamin A in depleted freshwater fish (Schiedt et al. 1985; Gross 1966 et al.).

Some reports have been made of carotenoid metabolism in marine fish. Studies on pigmentation of yellowtail and red sea bream with krill oil indicated that astaxanthin might be converted into tunaxanthin (Fujita et al. 1983a, b). Miki et al. (1984) reported that dietary astaxanthin esters were converted to idoxanthin and doradexanthin. Metabolism of carotenoids has also been reported in freshwater fish: gold fish and red carp (Hata and Hata 1972a,b, 1975, 1976; Tanaka et al. 1976a,b; Hsu et al. 1972; Matsuno et al. 1979, 1981), tilapia (Matsuno 1980; Matsuno and Katsuyama 1982), cat-fish (Matsuno and Nagata 1980), and rainbow trout (Schiedt 1985, 1986).

In our study, we determined the retention and

distribution of carotenoids in the skin, flesh, liver, and blood of rainbow trout (Salmo gairdneri).

Materials and methods

Rainbow trout Salmo gairdneri with an initial weight of 35 gm were purchased from Plymouth Rock Trout Company (Plymouth, MA). The fish were acclimated for three months on the commercial trout feed that they had previously been fed. Then they were divided into four groups - a control and three experimental groups, each fed a pigmented diet for either two, four or six weeks. Each group had 15 fish with an average weight of 90 g and was maintained in a 470-liter fiberglass tank supplied with aerated fresh water. The water temperature was kept at 11-13°C and the oxygen level at 8 ppm. The fish were fed at a rate of 2% of their body weight per day.

Fish diets

The control diet was a commercial trout feed (Ziegler Bros. Feed Mills Inc., Pennsylvania).

The test diet was prepared using the commercial trout feed. The feed was mixed with 100 mg/kg of racemic astaxanthin (F. Hoffman-LaRoche Ltd., Basle, Switzerland); 20 g of gelatin was dissolved in 400 ml of hot water and thoroughly mixed with the astaxanthin-treated feed. The mixture was shaped into "spaghetti" by forcing it through

a grinder fitted with a plate with small openings. The spaghetti was air-dried at -20°C for twenty minutes and broken into small pellets. The test diet was refrigerated until used.

Sampling.

At the end of every feeding study, the fish were anesthetized with a MS-222 (Sigma Chemical Co., St. Louis, MO) solution, and blood samples were taken at the caudal peduncle using heparinized syringes. The fish were sacrificed immediately after the blood sample and the livers were pooled. The fish and livers were stored at -20°C for further treatment and analysis. The blood samples were centrifuged at 1300 xg for 15 minutes in a cold room. The plasma was then withdrawn and placed in 2-ml screw-cap vials; the head space was filled with nitrogen and the vials were tightly screwed. The plasma was stored at -20°C for further analysis.

Carotenoid analysis.

Skin and Flesh.

The frozen fish were thawed, cleaned and washed, and the head and entrails were removed. The skin and fins were carefully separated from the flesh. The flesh was minced with a knife. The pigments were extracted separately from the skin and from the flesh with acetone

in a Waring blender. The acetone extracts were then diluted with water, and the lipids including the carotenoids were extracted with petroleum ether until all pigments were removed. The mixture was evaporated and the residue was then separated on a Microcel-C column using 1-4% acetone in petroleum ether as developing solvent.

The astaxanthin content was determined in the respective diester, monoester, and free fractions using $E_{1\text{ cm}}^{1\%} = 1910$ for calculating the astaxanthin equivalent (Schiedt, 1986). The yellow xanthophyll fractions were saponified with a solution of 15% KOH in methanol. The alkaline mixture was left at room temperature in the dark under nitrogen for 16 hrs. After the saponification was completed, the pigments in each sample were transferred into a mixture of petroleum ether and ethyl ether by the addition of water. The ether solutions were combined in a separatory funnel and washed repeatedly with water until they were free of alkali. Lutein, 3'-epilutein and zeaxanthin were separated by HPLC using acetonitrile and methanol (95:5) at a flow rate of 1.5 ml/min.

Liver.

The frozen livers were thawed, dehydrated with MgSO_4 and extracted with acetone. The extract was filtered through filter paper and the filtrate was evaporated. The residue was re-dissolved in a certain volume of hexane.

The approximate vitamin A content ($A_1 + A_2$) was then determined by spectrophotometry using $\lambda_{\max} = 328$ for vitamin A in hexane, assuming an average $E_{1\%}^{1\text{cm}} = 1500$ (Schiedt 1986). Subsequently the lipid extract was saponified. Finally, the residue was dissolved in a chromatographic solvent system, ACCN, DCM, ME0H (70:20:10), and this solution was applied to the HPLC at a flow rate of 0.5 ml/min.

Blood.

For the analysis of carotenoids in plasma, 1 ml of plasma was pipetted into a test tube. One ml of ethanol was added, and the tube was shaken for 20 sec, 1.2 ml of hexane was added, and the tube was shaken vigorously for 40 sec. The mixture was then centrifuged, and 1 ml of the hexane phase was transferred to a second test tube. This tube was placed in a water bath at 60°C under N_2 to evaporate the hexane. The lipid was dissolved by mixing it for 30 seconds with 0.5 ml of HPLC mobile phase solution, ACCN:DCM:ME0H (70:20:10), and this solution was injected into the HPLC at a flow rate of 1.5 ml/min.

Instrumentation.

The HPLC system was a Waters model 6000-A solvent delivery system (Waters Assoc., Milford, MA), a Waters U6K injection system, and a Perkin-Elmer LC-85

spectrophotometric detector connected to a Perkin-Elmer LC1-100 integrator (Norwalk, CT). The column contained a C-18 10-micron reverse phase packing and was 25 cm in length x 4 mm in diameter (E. Merck, West Germany).

Solvents.

Chromatographic mobile phases consisted of mixtures of HPLC-grade acetonitrile (ACCN), dichloromethane (DCM), and methanol (MeOH) (Fisher Scientific, Medford, MA). All solvents were filtered through a <0.45 μ m membrane filter (Gelman, Ann Arbor, MI). Acetone and petroleum ether (PE) were distilled before use.

Standardization.

Crystalline retinol (vitamin A) and β -carotene were purchased from Sigma Chemical Co. (St. Louis, MO). Crystalline dehydroretinol (vitamin A₂) was provided as a gift by Hoffmann La-Roche (Switzerland). Lutein, 3-epilutein and zeaxanthin were extracted and isolated from spinach (Yamamoto et al. 1962), mackerel (Motsuno et al. 1980), and corn (Quackenbush et al. 1961), respectively.

Retinol was purified several times according to the procedure described by Bieri et al. (1976). β -carotene, lutein, 3-epilutein and zeaxanthin were purified several times on a MgO column (Tanaka et al. 1981) and recrystallized (Davies 1976). Purified retinol and

carotenoids were dissolved in a chromatographic solvent system and injected into the HPLC instrument. Standard curves were generated by a linear relationship between carotenoids and retinol concentration and peak area.

Statistical analysis

Variation among samples were tested for significance with analysis of variance (Little et al. 1978).

Results

Rainbow trout fed the test diet containing racemic astaxanthin for two weeks showed a slight pink coloration in the skin and fins. The fish fed the test diet for four and six weeks had strongly colored red skin, while the fish on the control diet were gray.

The carotenoid composition of each group of fish at the end of the study is given in Tables 1 and 2. Table 1 shows the carotenoid levels in the skin of fish from each group. The highest amount of total astaxanthin (162 ug/100g) was found in the skin of fish fed the test diet for six weeks. No astaxanthin was detected in the control fish. Zeaxanthin and 3'-epilutein were also detected in the skin. The 3'-epilutein levels were about the same for all four groups, while zeaxanthin increased with increasing exposure to astaxanthin.

The carotenoid compositions of the flesh are also

presented in Table 1. Free astaxanthin was found to be the predominant pigment in all three experimental groups. Lutein and zeaxanthin were found in small quantities. No astaxanthin was detected in the control group.

A HPLC chromatogram of vitamin A extracted from the liver of rainbow trout is shown in Figure 1. The mean vitamin A content of the liver is presented in Table 2. The ratio of A₁:A₂ was approximately 1:3. There was no significant difference in the vitamin A content of the livers of the control and experimental fish. Table 2 also shows the vitamin A content of the blood. Only vitamin A₁ was determined and the quantity ranged from 6-7 ug/100 ml of plasma. No lutein or zeaxanthin was detected in the liver or blood. β -carotene was not detected in either liver, blood, skin or flesh of rainbow trout.

Discussion

In all four groups, most of the astaxanthin and other carotenoids accumulated in the skin and flesh. The final astaxanthin concentration in the flesh and skin of rainbow trout was in agreement with findings of Kamata (1976, 1985), Lambertesen and Braekkan (1979), and Torrisen and Simpson et al. (1981); higher values were obtained by Foss et al. (1984) and Johnson et al. (1980). The discrepancies may be ascribed to different experimental and environmental conditions. The dietary carotenoid

concentration and duration of feeding may influence the deposition rate (Choubert and Luquet, 1982). It is also known that sexual maturity plays an important role in pigmentation. In the present experiment no sexual maturation was observed. Fish size is another limiting factor for pigmentation (Deufel 1965; Choubert and Luquet 1975). The presence of free astaxanthin in the flesh and astaxanthin ester in the skin of the fish could indicate that astaxanthin was incorporated in the free form into the flesh and later transferred to skin, to where it was esterified and stored.

The absolute content and relative composition of the yellow xanthophyll was the same for the control group as for the three experimental groups except in the skin where there was a greater content of zeaxanthin. This proves that there is metabolism of the administered racemic astaxanthin into zeaxanthin. This conversion is a reverse reaction of the keto-oxidation of zeaxanthin in many animals (Goodwin 1971; Thommen 1971; Buchecker 1981) and in fish (Hata et al. 1971; Tanaka et al. 1976a, b). The conversion of astaxanthin to zeaxanthin was found also in other marine and freshwater fish (Kitahria 1985; Miki et al. 1985; Scheidt 1985).

The 3'-epilutein was not greater in the astaxanthin-supplemented groups; hence it must be assumed that dietary lutein is first deposited in the flesh and possibly

epimerized in the skin to 3-epilutin. There were no significant differences in vitamin A content of liver and blood, indicating that astaxanthin was not converted to vitamin A in the vitamin-A-saturated fish, in which dietary retinol is dehydrogenated to A₂ in the intestinal wall.

- Watanabe, T. (1971). *Journal of Nutrition* 101: 1-10.
- Watanabe, T. (1972). *Journal of Nutrition* 102: 1-10.
- Watanabe, T. (1973). *Journal of Nutrition* 103: 1-10.
- Watanabe, T. (1974). *Journal of Nutrition* 104: 1-10.
- Watanabe, T. (1975). *Journal of Nutrition* 105: 1-10.
- Watanabe, T. (1976). *Journal of Nutrition* 106: 1-10.
- Watanabe, T. (1977). *Journal of Nutrition* 107: 1-10.
- Watanabe, T. (1978). *Journal of Nutrition* 108: 1-10.
- Watanabe, T. (1979). *Journal of Nutrition* 109: 1-10.
- Watanabe, T. (1980). *Journal of Nutrition* 110: 1-10.
- Watanabe, T. (1981). *Journal of Nutrition* 111: 1-10.
- Watanabe, T. (1982). *Journal of Nutrition* 112: 1-10.
- Watanabe, T. (1983). *Journal of Nutrition* 113: 1-10.
- Watanabe, T. (1984). *Journal of Nutrition* 114: 1-10.
- Watanabe, T. (1985). *Journal of Nutrition* 115: 1-10.
- Watanabe, T. (1986). *Journal of Nutrition* 116: 1-10.
- Watanabe, T. (1987). *Journal of Nutrition* 117: 1-10.
- Watanabe, T. (1988). *Journal of Nutrition* 118: 1-10.
- Watanabe, T. (1989). *Journal of Nutrition* 119: 1-10.
- Watanabe, T. (1990). *Journal of Nutrition* 120: 1-10.
- Watanabe, T. (1991). *Journal of Nutrition* 121: 1-10.
- Watanabe, T. (1992). *Journal of Nutrition* 122: 1-10.
- Watanabe, T. (1993). *Journal of Nutrition* 123: 1-10.
- Watanabe, T. (1994). *Journal of Nutrition* 124: 1-10.
- Watanabe, T. (1995). *Journal of Nutrition* 125: 1-10.
- Watanabe, T. (1996). *Journal of Nutrition* 126: 1-10.
- Watanabe, T. (1997). *Journal of Nutrition* 127: 1-10.
- Watanabe, T. (1998). *Journal of Nutrition* 128: 1-10.
- Watanabe, T. (1999). *Journal of Nutrition* 129: 1-10.
- Watanabe, T. (2000). *Journal of Nutrition* 130: 1-10.
- Watanabe, T. (2001). *Journal of Nutrition* 131: 1-10.
- Watanabe, T. (2002). *Journal of Nutrition* 132: 1-10.
- Watanabe, T. (2003). *Journal of Nutrition* 133: 1-10.
- Watanabe, T. (2004). *Journal of Nutrition* 134: 1-10.
- Watanabe, T. (2005). *Journal of Nutrition* 135: 1-10.
- Watanabe, T. (2006). *Journal of Nutrition* 136: 1-10.
- Watanabe, T. (2007). *Journal of Nutrition* 137: 1-10.
- Watanabe, T. (2008). *Journal of Nutrition* 138: 1-10.
- Watanabe, T. (2009). *Journal of Nutrition* 139: 1-10.
- Watanabe, T. (2010). *Journal of Nutrition* 140: 1-10.
- Watanabe, T. (2011). *Journal of Nutrition* 141: 1-10.
- Watanabe, T. (2012). *Journal of Nutrition* 142: 1-10.
- Watanabe, T. (2013). *Journal of Nutrition* 143: 1-10.
- Watanabe, T. (2014). *Journal of Nutrition* 144: 1-10.
- Watanabe, T. (2015). *Journal of Nutrition* 145: 1-10.
- Watanabe, T. (2016). *Journal of Nutrition* 146: 1-10.
- Watanabe, T. (2017). *Journal of Nutrition* 147: 1-10.
- Watanabe, T. (2018). *Journal of Nutrition* 148: 1-10.
- Watanabe, T. (2019). *Journal of Nutrition* 149: 1-10.
- Watanabe, T. (2020). *Journal of Nutrition* 150: 1-10.

REFERENCES

- Barua, A., and Das, R. C., 1975. Occurrence and Conversion of anhydrolutein into dehydroretinol in a fresh water fish., British Journal of Nutrition 33 p. 319-327.
- Barua, A., Goswami, U., 1977, Formation of vitamin A in a fresh water fish, Biochem. J. v. 164 p. 133-136.
- Barua, A.B., Singh, H.T., and Das, R.C. 1973, Conversion of lutein into dehydre by fresh water fish saccobranthus fossilis Br. J. N. 30-P-12.
- Bauernfeind, J.C. (1976) Canthaxanthin: a pigmenter for salmonids Prog. Fish. Cult. 38, 180-183.
- Bieri, J.G., Tolliver, T.J., and Catignani, G.L., 1979, Simultaneous determination of -tocopherol and retinol in plasma or red blood cell by high pressure liquid chromatography. Am. J. Clin. Nut. 32:2143.
- Buchecker R. (1981) Carotenoid Chemistry and Biochemistry (Edited by Britton G. and Goodwin T.W.), pp. 175-181, Pergamon Press, Oxford.
- Choubert, G. Jr. & Luquet, P. (1975) Feeding test of capelin oil, (Mallotus villosus) on rainbow troupe: Amount and qualities of carotenoid content in the muscle skin. Ann. Hydrobiol. 6, 123-130.
- Choubert, G. and Luquet, P., 1975. Nature des carotenoides fixes au niveau de la peau et du muscle de la truite arc-en-ciel ayant ingere de l'huile rouge de capelan. Ann. Hydro-biol.,

6:123-130.

- Choubert, G. and Luquet, P., 1982. Fixation et retention musculaire de la canthaxanthine par la truite arc-en-ciel. *Ann. Zootech.*, 31:1-10.
- Choubert, G. and Luquet, P. 1983, Utilization of shrimp meal for rainbow trout (Salmo gairdneri Rich.) pigmentation. Influence of fat content of the diet. *Aquaculture*, 32:19-26.
- Davies, B.H. (1976) Carotenoids. In Chemistry and Biochemistry of Plant Pigments (Edited by Goodwin, T.W.) Vol. 2, pp. 38-165. Academic Press, N.Y. and London.
- Duefel, J. (1965) Pigmentierungsversuche mit-Canthaxanthin bei Regenbogenforellen. Arch. Fish. Wiss. 16, 125-132.
- Foss, P. Storebakken, T., Schiedt, K., Liaaen-Hensen, S., Austreng, E. & Streiff, K. (1984) Carotenoids in diets for salmonids. 1. Pigmentation of rainbow trout with individual optical isomers of astaxanthin on comparison with canthaxanthin. Aquaculture 41, 213-226.
- Fujita T., Satake M., Hikichi S., Takeda M., Shimeno S., Kuwabara H., Miki W., Yamaguchi K. and Konosu S. (1983a) Pigmentation of cultured yellowtail with krill oil. *Bull. Jap. Soc. scient. Fish.* 49, 1595-1600.
- Fujita T., Satake M., Watanabe T., Kitajima C., Miki W., Yamaguchi, K. and Konosu S. (1983b) Pigmentation of cultured red sea bream with astaxanthin diester purified from krill oil. *Bull. Jap. Soc. scient. Fish.* 49, 1855-1861.

- Georgiev, G.S., 1971, Carotenoids and vitamin A content in *Salmo irideus* eggs and their significance in the initial periods of the embryogenesis, *Folia Balcanica*, VII (a) p. 1-10.
- Goodwin, T.W. (1971). Carotenoids (Edited by Isler), pp. 641-656.
- Goswami, B. et al. 1984 (Abst.). Studies on the metabolism of astaxanthin in the fresh water fish, Heteropneustes fossilis, 7th International Sym. on Carotenoids.
- Goswami, U. and Barua, A., 1981, Origin of Retinol in freshwater fish, *current science* V. 50 (3)^P 150-151.
- Goswami, U. and Bhattacharjee, S., 1982 Biosynthesis of 3-Dehydroretinol:Metabolism of Lutein (BE-Carotene-3,3 Diol). in *clarias batrachus* and *ompok pabo*, *Biochemistry international*, V. 5 (4) p. 545-552.
- Goswami, V., 1984, Metabolism of cryptoaxauthin in freshwater fish, *British Journal of Nutrition* V. 52, 575-581.
- Gross, J. and Budowski, P. 1966, Conversion of carotenoids in to vitamin A₁ and A₂ in two species of fresh water fish. *Biochem. J.* 101 747-754.
- Hata, M., Hata, M. and Onishi, T., 1973, Conversion of β -carotene and retinol₁ to retinol₂ in freshwater fish, *Tohoku Journal of Agri. Research* V. 24 (4)^P 197-204.
- Hata, M. and Hata M. (1972a) Carotenoid pigments in goldfish-IV. Carotenoid metabolism *Bull. Jap. Soc. scient. Fish*, 38, 331-338.

- Hata M., and Hata M. (1972b) Carotenoid pigments in goldfish-V. Conversion of zeaxanthin to astaxanthin. Bull. Jap. Soc. scient. Fish, 38, 339-343.
- Hata M. and Hata M (1975) Carotenoid metabolism in fancy red carp, *Cyprinus carpio*-I. Administration of carotenoids. Bull. Jap. Soc. scient. Fish 41, 653-655.
- Hata M. and Hata M. (1976) Carotenoid metabolism in fancy red carp, *Cyprinus carpio*-II. Metabolism of C-zeaxanthin. Bull. Jap. Soc. scient. Fish. 42, 203-205.
- Johnson, E.A., Villa, T.G. and Lewis, M.J. 1980. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. Aquaculture, 20:123-134.
- Kamata, T. (1976) The pigmentation of rainbow trout, *Salmo gairdneri*. Master's Thesis, University of Rhode Island, Department of Food and Resource Chemistry.
- Kamata, T., Neamtu, G.G. & Simpson, K.L. 1986 Manuscript II. Utilization of *Adonis aestivalis* as a dietary pigment source for rainbow trout, *Salmo gairdneri* Ph.D. Diss. University of Rhode Island.
- Kithara T. (1983) Behavior of carotenoids in the chum salmon (*O. keta*) during anadromous migration. Comp. Biochem. Physiol. 76B, 97-101.
- Kitahara T. (1984) Behavior of carotenoids in the chum salmon (*O. keta*) during development. Bull. Jap. Soc. scient. Fish. 50, 531-536.

- Krause, M.V. and Mahan, L.K. (1979). Food Nutrition and Diet Therapy. 6th ed. W.R. Saunders Company, Philadelphia, London, Toronto.
- Kuo, H.C., Lee, T-C., Kamata, T. & Simpson, K.L. (1976) Red crab processing waste as carotenoid source for rainbow trout. Alimenta 15, 47-51.
- Lambertsen, G. & Braekkan, O.R. (1971) Method of analysis of astaxanthin and its occurrence in some marine products. J. Sci. Food Agric. 22, 99-101.
- Liaaen-Jense, S., 1978. Marine carotenoids in : P. Scheuer (Editor), Marine natural products, chemical and biological perspectives, Vo. 2. Academic Press, New York. pp. 2-73.
- Little, T., Hills, F., 1978, Agrigultural Experimentation Design and analysis, John Wiley and Sons, N.Y.
- Matsuno, T., Matsutaka, H., Katsuyama, M., and Nagata, S., 1980. Occurance of 3-epilutein from fish. Bull. Jap. Soc. Scient. Fish. 46 337-340.
- Matsuno T., Nagata S., Iwahashi M., Koike T. and Okada M. (1979) Intensification of color of fancy red carp with zeaxanthin and mysoxanthophyll, major carotenoid constituents of Spirulina. Bull. Jap. Soc. scient. Fish, 45, 627-632.
- Matsuno T., Matsutaka H. and Nagata S. (1981) Metabolism of lutein and zeaxanthin to ketocarotenoids in goldfish Carassius auratus. Bull. Jap. Soc. scient. Fish, 47, 605-611.

- Matsuno T. and Nagata S. (1980) Biosynthesis of characteristic, principal carotenoids of Japanese common catfish parasiloxanthin (7', 8'-dihydro-zeaxanthin) and 7,8-dihydro-parasiloxanthin. Bull. Jap. Soc. scient. Fish, 46, 1363-1367.
- Matsuno T., Katsuyama M., Iwashii M., Koike T. and Okada M. (1980a) Intensification of color of red Tilapia with lutein rhodoxanthin and spirulina. Bull. Jap. Soc. scient. Fish. 46, 479-482.
- Miki, W., Yamaguchi, K., Konosu, S., Takane, T., Satake, M., Fujita, T., Kuwabara, H., Shimeno, S., and Takeda, M., 1985. Origin of tunaxanthin in the integument of yellowtail (*Seriola quinqueradiata*), Comp. Biochem. Physiol, Vol. 80B No. 2 pp. 195-201.
- Miki W., Yamaguchi K., Konosu S. and Watanabe T. (1984) Metabolism of dietary carotenoids in eggs of red sea bream. Comp. Biochem. Physiol. 77B, 665-668.
- Morton, R.A. & Greed, R.H. (1939). Biochem. J. 33, 318.
- Quackenbush, F., Firch, J., Rabourn, W., McQuistan, M., Petzold, E., and Kargi, T., 1961, Analysis of Carotenoids in Corn Grain, Agricultural and Food Chemistry, V. 9(2) pp. 132-135.
- Schiedt, K., Vecchi, M., and Blinz, E., 1986. Astaxanthin and its metabolites in wild rainbow trout (*Salmo gairdneri*, Comp. Biochem. physiol. v. 83b No. 1 pp. 9-12.

- Schiedt, K., Leuenberger, F.J., Vecchi, M. & Glinz, E. (1985) Absorption, retention and metabolic transformation of carotenoids in rainbow trout, salmon and chicken. Pure & Appl. Chem. 57, 685-692.
- Simpson, K.L., Katayama, T. & Chichester, C.O. (1981). Carotenoids in fish feeds. In carotenoids as colorants and vitamin A precursor (Edited by Bauernfeind, J.C.) pp. 463-538. Academic Press, N.Y., London.
- Steven, D.M. (1947) Carotenoid pigmentation of trout. Nature 160, 540-541.
- Tanaka, Y., Katayama, T., Simpson, K., 1981. Stability of carotenoids on silica gel and other adsorbents, Bulletin of the Japanese Soc. of Sci. Fisheries 47(6) pp. 799-811.
- Tanaka, Y., Simpson, K., and Chichester, C.O. (1976b) The biosynthesis of astaxanthin-XX. The carotenoids in marine red fish and metabolism of the carotenoids in red sea bream, *Chrysophyrus major* Temminch and Schlegel. Bull. Jap. Soc. scient. Fish, 12, 1177-1182.
- Tanaka T., Katayama T., Simpson K.L. and Chichester C.O. (1976a) The biosynthesis of astaxanthin-XIX. The distribution of -doradexanthin and the metabolism of the carotenoids in goldfish. Bull. Jap. Soc. scient. Fish. 42, 885-891.
- Thommen H. (1971) Carotenoids (Edited by Isler O.), pp. 637-656. Birkhauser Verlag. Basel und Stuttgart.

- Torrissen, O. and Braekkan, O.R., 1979. The utilization of astaxanthin forms by rainbow trout (Salmo gairdneri) Proc. World Symp. Finfish Nutr. Fishfeed Technol., Hamburg, 20-23 June 1978. Vol. II. Heenemann, Berlin, pp. 377-382.
- Torrissen O., Tidemann, E. Hansen, F. and Raa, J., 1981. Ensiling in acid - method to stabilize astaxanthin in shrimp processing byproducts and improve uptake of this pigment by rainbow trout (Salmo gairdneri). Aquaculture, 26:77-83.
- Torrissen, O. & Braekkan, O.R. (1979) The utilization of axtaxanthin forms by rainbow trout (Salmo gairdneri). Proc. World Symp. Finfish Nutr. Fishfeed Technol. Hamburg, vol. II, pp. 377-383. Heememan, Berlin.
- Williams, S.R., 1971. Nutrition and Diet Therapy. 3rd Ed. The C.V. Mosby Company, Saint Louis.
- Yamamoto. H.Y. Chichester, C.O. and Nakayama, T. 1962. Biosynthetic origin of oxygen in the leaf xanthophylls Arch. Biochem. Biophys. 96:645.

Table 1. Concentration of Carotenoids in skin and flesh of rainbow trout (ug/100g)

Type of Sample	Carotenoid	Control	Period fed test diet		
			2 weeks	4 weeks	6 weeks
Skin	3-epilutein	30.0 ⁽¹⁾	30.0	40.0	40.0*
	zeaxanthin	10.0	15.0	18.0	27.0*
	astaxanthin	-	10.0	9.0	12.0
	astaxanthin monoester	-	12.0	16.0	30.0
	astaxanthin diester	-	40.0	80.0	120.0
	TOTAL astaxanthin	-	62.0	105.0	162.0
Flesh	Lutein	1.0	0.9	1.0	1.0
	Zeaxanthin	0.7	0.7	0.7	0.7
	astaxanthin	-	61.0	72.0	90.0

(1) Data presented as mean for six replicate, for value with superscripts are significantly different at 0.05.

Table 2. Mean vitamin A₁ and A₂ content of liver and blood of rainbow trout

Type of Sample	Carotenoid	Control	2 weeks	4 weeks	6 weeks
Liver (1)	A ₂	70	68	70	70
	A ₁	24	25	24	25
	Total Vit. A	94	93	94	95
Blood (2)	Vitamin A ₁	7	6	6	.7

(1) content as ug/g

(2) content as ug/100 ml

Figure 1. HPLC chromatogram of reticulin in rainbow trout liver. Peak: 1. dehydrated (A); 2. A-reticulin (A). Condition: ACN:DM:MEOH = 70:20:10; flow rate, 0.5 ml/min.

Figure 1. HPLC chromatogram of retinol in rainbow trout liver. Peak: 1, dehydroretinol (A_2); 2, A-retinol (A_1). Condition: ACCN:DCM:MEOH = 70:20:10; flow rate, 0.5 ml/min.

MANUSCRIPT II

CONVERSION OF ASTAXANTHIN TO VITAMINS A₁ AND A₂ IN
RAINBOW TROUT (SALMO GAIRDNERI)

ABDULRHMAN S. AL-KHALIFAH

DEPARTMENT OF FOOD SCIENCE AND NUTRITION
UNIVERSITY OF RHODE ISLAND
KINGSTON, RHODE ISLAND 02881 (USA)

ABSTRACT

Rainbow trout (Salmo gairdneri) weighing approximately 500 g were fed for 35 days on a diet free of vitamin A and carotenoids. A control group received no further treatment, was sacrificed, and had a low vitamin A content in the intestines. One group received tetracycline during the last 7 days of feeding. This and another group were force-fed 100 ug of astaxanthin, dissolved in a volume of Tween 20, directly into the stomach through a feeding tube. The fish were then sacrificed after seven hours. Vitamin A₁ and A₂ were determined using HPLC. The results showed that astaxanthin increased the vitamin A content in the intestine. This indicates that astaxanthin converted into vitamin A. There was no marked difference in vitamin A content between the two experimental groups. This shows that microorganisms do not seem to be involved in the conversion. The astaxanthin uptake was about 10 percent.

Introduction

Animals differ in their intestinal metabolism and uptake of carotenoids through the intestinal wall. Mammals, for example, preferentially absorb β -carotene, while fish and birds absorb the 3,4-oxygenated xanthophylls much better.

Several carotenoids capable of acting as provitamin A are known (Moore 1957), and it has been conclusively established that a number of carotenoids with at least one unsubstituted β -ionone ring and a fully conjugated isoprenoid side chain can serve as the precursor of retinol in mammalian and avian species.

Fish and birds utilize the oxygenated carotenoids; xanthophylls are indicated as possible vitamin A₁ and A₂ precursors in freshwater fish. Earlier reports indicated that both vitamin A₁ and A₂ can be formed from a common precursor such as β -carotene (Morton 1939; Hata 1973). Various authors claim that in several species of freshwater fish lutein is a precursor of dehydroretinol via anhydrolutein (Barua et al. 1973, 1977, 1979; Goswami et al. 1981, 1982, 1983, 1984). Gross et al. (1966) found evidence of conversion of astaxanthin, canthxanthin and zeaxanthin via β -carotene to vitamin A₁ and A₂ in guppies and platies. Goswami (1984) found that astaxanthin is converted to β -carotene in the intestinal wall of H. fossilis. Schiedt et al. (1985) proved that

astaxanthin could be a precursor of vitamins A₁ and A₂ in vitamin-A-depleted freshwater fish. The formation of vitamin A₂ from astaxanthin occurs in many steps (Granqued et al. 1957). The purpose of this study was to determine whether the conversion was caused by microorganisms or if it was a property of the fish intestine itself.

Materials and Methods

Animals

Rainbow trout (Salmo gairdneri) weighing approximately 500 g were obtained from the fish culture unit of the University of Rhode Island. Fish were maintained at $14 \pm 2^{\circ}\text{C}$ in fiberglass tanks. All the fish were fed for 35 days on a diet free of vitamin A and carotenoids. One group (Group 1; 4 trout) was then sacrificed. Another group (Group 2; 8) was force-fed astaxanthin and then sacrificed after 7 hours. A third group (Group 3; 8 trout) received tetracycline mixed with the feed during the last 7 days of the 35 days, was then force-fed astaxanthin, and sacrificed after 7 hours. The experimental design is shown schematically in Figure 1.

Antibiotic administration

Tetracycline hydrochloride (purchased from the Sigma Chemical Co., St. Louis, MO) was mixed with the feed (600 mg/kg). One group of trout was fed the diet daily for the last 7 days of the experiment.

Astaxanthin administration

Astaxanthin was dissolved in a certain volume of Tween 20. Fish were anesthetized with MS222 (Sigma Chemical Co., St. Louis, MO). For each fish, 100 ug of astaxanthin, prepared as above, was administered directly

into the stomach through a feeding tube attached to an injection syringe. Seven hours after administering the astaxanthin, fish were sacrificed and the intestines were pooled.

Analytical methods

The intestines of each fish were removed, cut into small pieces, and ground with anhydrous sodium sulfate. Carotenoids and retinol were extracted with acetone. The carotenoids were transferred to diethyl ether by the addition of distilled water. The aqueous phase was extracted with ether several times. The combined ether fractions were washed with distilled water and dried over anhydrous sodium sulfate. Then the solvent was removed on a rotary evaporator to obtain an oily substance. Astaxanthin content was determined by the method of Al-Khalifah and Simpson (1986). The sample was saponified (Davis 1976). Finally, the residue was dissolved in a chromatographic solvent system, Acetonitrile (ACCN), dichloromethane (DCM) and methanol (MEOH) (70:20:10) and was injected into the HPLC at a flow rate of 0.5 ml/min.

Standard and standard curve

Retinol (vitamin A₁) was purchased from Sigma Chemical Company (St. Louis, MO); dehydroretinol (vitamin A₂) was a gift from Hoffmann La-Roche Co. (Switzerland). The retinol was purified by alumina column chromatography (Bieri et al. 1979). Retinol was purified several times until the HPLC chromatogram showed no peaks except for vitamin A.

For quantification, retinol and dehydroretinol were

dissolved in petroleum ether, and the concentration was measured by spectrophotometer at $\lambda_{\text{max}}=328$. The extinction coefficient values in petroleum ether are 1832 and 1455 for retinol and dehydroretinol, respectively (Barua 1973).

Petroleum ether was evaporated in a rotary evaporator and the residue was reconstituted in the chromatographic solvent system. The resulting retinol solution was injected into the HPLC and a linear relationship was obtained between vitamin A concentration and peak area.

Liquid chromatography

The HPLC instrumentation consisted of a Waters model 6000A solvent delivery system (Waters Assoc., Milford, MA), a Waters U6K injector system, and a Perkins-Elmer LC-85 spectrophotometric detector connected to a Perkins-Elmer LC1-100 integrator (Norwalk, CT). A 10- μ m, C-18 stainless steel column (25 X 0.4 cm) was used (Merck, Germany).

The chromatographic mobile phases were mixtures of HPLC-grade acetonitrile, dichloromethane and methanol (Fisher Scientific, Medford, MA). All solvents were filtered through a <0.45 μ m membrane filter (Gelman, Ann Arbor, MI).

Results

A chromatogram of an intestine extract is presented in Figure 2 and shows the two peaks of vitamins A₁ and A₂. The content of carotenoids isolated from the intestine are shown in Table 1. The intestines of Group 1 rainbow trout, which had been fed the vitamin-A-free diet for 35 days, were free from carotenoids and vitamin A in the ileal portion, but not in the pyloric caeca (duodenum). As shown in Table 1, only about 10% of the astaxanthin was taken up by the fish.

Astaxanthin increased the vitamin A content of the intestines of Group 2 and Group 3, compared with that of Group 1. However, there was no marked difference in vitamin A content between the two astaxanthin-treated groups. The ratio of A₁:A₂ was approximately 1:2 in both Group 2 and Group 3.

Discussion

The role of 3, 4-oxy carotenoids as precursors of vitamin A is revealed in this study. The marked difference between the levels of vitamin A in the intestine before and after astaxanthin was administered indicated that astaxanthin was converted into vitamin A. This is evidence that astaxanthin undergoes biological reduction and cleavage to yield vitamin A. This observation is in agreement with earlier findings

(Grangaud 1962; Gross et al. 1966; Schiedt et al. 1985).

The possible formation of 3,4-dehydroretinol (Vitamin A₂) from astaxanthin in rainbow trout was also investigated in the present study. It was found that astaxanthin can serve as a precursor of 3,4-dehydroretinol in the intestine of these fish. It was concluded that retinol is the immediate precursor of 3,4-dehydroretinol. This conclusion is in agreement with the finding of Hata et al. (1973), Lambertsen et al. (1969), and Schiedt (1985). The metabolism of carotenoids to yield 3,4-dehydroretinol in freshwater fish is currently of great interest. Earlier studies suggested that xanthophylls based on β -carotene are precursors (Gross et al. 1966), while a more recent view is that lutein (β, ϵ -carotene, 3,3' diol) is the precursor in H. Fossillis, Channa striatus (Barua et al. 1973, 1977; Goswami et al. 1981), and Mystus vittatus (Goswami 1984). Cryptoxanthin can also be the precursor in H. Fossillis and Channa striatus (Goswami 1984).

From studies of administering astaxanthin to Gambusia holbrooki, Granguad et al. (1962) determined that the probable steps leading from astaxanthin to vitamin A₁ and A₂ are as follows (Figure 2):

- a. reduction of astaxanthin to β -carotene
- b. conversion of β -carotene to vitamin A₁
- c. dehydrogenation of vitamin A₁ to A₂

This metabolism involved many steps.

In this study using antibiotic-treated fish, it was shown that a microorganism does not seem to be involved at any steps of the reduction or cleavage. Also, it was proven that the conversion occurred in the intestine of the fish.

The astaxanthin absorption rate of 8 to 10% (Table 1) agrees very well with that of previous studies (Goswami 1984; Schiedt 1985).

The ratio of $A_1:A_2$ of about 1:2 is different from the ratio of 1:4 found by Schiedt (1985). The difference could be explained by the time between astaxanthin administration and sacrifice (7 hours) in our study. Perhaps equilibrium was not achieved in that time period.

REFERENCES

- Al-Khalifa and Simpson, in preparation, 1986.
- Barua, A. and DAS, R., 1975, British Journal of Nutrition 33, 319-327.
- Barua, A., Singh, H., and DAS, R. (1973), British Journal of Nutrition 30, 1-12.
- Barua, A., DAS, R., and Verma, K., (1977) Biochemical Journal 168, 557-564.
- Bieri, J.G., Tolliver, T.J. and Catiganani, G.L. 1979. Am. J. Clin. Nut. 32, 2143-
- Davis, B.H. 1976, Chemistry and Biochemistry of plant pigments (Goodwin, Ed.) Vol. 2, pp. 38-165. Academic Press, N.Y.
- Goswami, V.C. and Barua A. (1981) Current Science 50, 150-151.
- Goswami, V.B., Bhattacharjee (1982) Biochemistry International 3, 545-552.
- Goswami, V., (1983). Indian Journal of Animal Science 53, 50-52.
- Goswami, V., (1984) British J. of Nutrition 52, 575-581.
- Goswami, B. Baurva, A., (1984). 7th International Symp. of Carotenoid, Munich, Germany.
- Grangaud, R., Vignais, P., Massonet, R. and Moutti, J. (1957) Biol. Soc. Chem, Biol. Paris 39, 1271.
- Grangaud, R., (1962), C.R. Acad. Sci. 254, 1579-581.
- Gross, W., and Budowski, P. (1966) Biochem. J. 101, 747-754.
- Hata, M. Hata, M., and Onishi, T. 1973; Tohoku T. Agric. Res. 24, 197 11

- Lambertsen, G. and Olaf, R. 1969. In vivo conversion of vitamin A₁ to vitamin A₂ Acta Chem. Scand. 23(3) 1063-1064.
- Moore, T., (1957) "Vitamin A". Amsterdam, Elsevier N.Y.
- Morton, R.A. and Greed, R.H. 1939 Biochem. J. 33, 318-324.
- Schiedt, K., Levenberger, F.J., Vecchi M., and Glinz E., 1985. Pure and Appl. Chemistry 57, 685-692.
- Weedon, B. C. L (1971). In "Carotenoids" (Ilsler, ed.) pp. 29-59. Birkhaeuser, Basel.

Table 1. Amount of vitamin A isolated from the intestine of rainbow trout, raised on vitamin A-deficient diet.

Exp. No.	No. of Fish	Amount of astaxanthin administered	Total Vitamin A(ug)			Astaxanthin Isolated (ug)
			vitamin A ₁	vitamin A ₂	total vitamin A	
Group 1	4	0	64	140	204	-
Group 2	8	800	191	420	611	89
Group 3	8	800	174	430	603	96

- 1 control
- 2 astaxanthin
- 3 astaxanthin and tetracycline

Figure 1. Flowchart of experimental design.

Figure 2. HPLC chromatogram of retinol in rainbow trout intestine. Peak, 1. dehydroretinol (A_2); 2. retinol (A_1) condition. ACCN:DCM:MEOH = 70:20:10. Flow rate, 0.5 ml/min.

Figure 3. Suggested mechanism for the formation of retinol and 3,4-dehydroretinol from astaxanthin in fish.

MANUSCRIPT III

IN VITRO METABOLISM OF ^3H -3S, ^3S -ASTAXANTHIN IN RAINBOW
TROUT (SALMO GAIARDNERI)

ABDULRHMAN S. AL-KHALIFAH

DEPARTMENT OF FOOD SCIENCE AND NUTRITION
UNIVERSITY OF RHODE ISLAND
KINGSTON, RHODE ISLAND 02881 (USA)

ABSTRACT

In vitro uptake and metabolism of ^3H -3S, 3S-astaxanthin in the intestine of rainbow trout (Salmo gairdneri) were investigated by incubating amiceller that contained labelled astaxanthin for 60 min. at $14 \pm 1^\circ\text{C}$ through strips of duodenum and everted sacs. Samples of intestine extract were analyzed by HPLC. It was found that 12.3 percent of the astaxanthin was taken up by the duodenum, but only 3 percent by the iliem. Retinol was detected, but it was very low in both intestinal segments. This indicates that very little of the astaxanthin was metabolized to retinol during the incubation period.

The HPLC and radio isotope data, proved that rainbow trout were able to convert astaxanthin to vitamin A.

Introduction

Despite the widespread distribution of freshwater fish and their often significant economic value, advances in our understanding of their nutritional physiology have been limited to a preliminary description of metabolic processes involving amino acids (Marcotte et al. 1984; Habibi 1984; Yamamoto 1966; Hokazono 1979) and lipids (Yamamoto 1966; Sir et al. 1981; Ezeasor and Stokoe 1981; Bauermeister et al. 1979). One of the least studied areas of freshwater fish nutritional physiology is nutrient absorption and metabolism.

We studied the absorption and metabolism of carotenoids in rainbow trout (Salmo gairdneri). Carotenoids are fat-soluble pigments related to vitamin A. Of the carotenoids, the most important naturally occurring vitamin A precursor for animals is β -carotene (Sporn et al. 1984) and dehydroretinal (vitamin A₂) for freshwater fish (Baru et al. 1977; Hata et al. 1973). Since β -carotene is the best-known carotenoid, more information is available on its digestion, absorption and deposition in tissue (Sporn et al. 1984). However, astaxanthin (3, 3'-dihydroxy β, β -carotene-4, 4'-dione) is the most commonly occurring carotenoid in aquatic organisms.

It has been claimed that astaxanthin can act as a vitamin A precursor. The biosynthesis of vitamin A from astaxanthin was first conclusively demonstrated by

Grangaud et al. (1962). They fed astaxanthin to vitamin-A-deficient fish and found a definite increase in the vitamin A content of the fish livers. Since then, the conversion of astaxanthin to vitamin A has been demonstrated in a variety of freshwater fish (Gross et al. 1966; Schiedt et al. 1985; Al-Khalifa 1986).

Several investigators have studied the metabolism of β -carotene in rat intestine (Olson 1964; Hollander 1977; Hollander 1981; El Gorab 1975), in freshwater fish (Hata 1973), and in different tissues of animals. However, little information is available on the metabolism of astaxanthin, and its absorption in the intestine.

In studying astaxanthin conversion in rainbow trout, Al-khalifa and Simpson (1986) observed an almost complete inhibition of the conversion when fish were fed a vitamin-A-rich diet. They also found that when astaxanthin was administered by a feeding tube to fish fed a diet free of vitamin A, the vitamin A content of the intestine of the fish increased. Furthermore, when the fish were given Tetracycline, an antibiotic, the conversion rate was not affected.

The intestine performs complex physiological functions and it is a target tissue for astaxanthin. Thus, the metabolism of astaxanthin in intestinal tissue is important in understanding its mode of action.

The digestive tract of rainbow trout consists of a

foregut (mouth, esophagus, and stomach), midgut (duodenum and ileum), and hindgut (rectum and anus) (Weinreb and Bilstad 1955; and Burnstock 1959). The gross anatomy of the digestive tract is shown in Figure 1. The short muscular esophagus leads to the J-shaped stomach, which consists of a cardiac, intermediate, and pyloric region. The pyloric valve leads to the ascending or anterior portion of the intestine. This region has pouch-like extensions, called the pyloric caeca. The remainder of the intestine consists of the midgut region and the rectum.

The midgut is generally considered important in lipid and carotenoid absorption. The two midgut regions may differ greatly in their absorption and metabolism. Different numbers of cell types in the two regions may produce different functional properties. Yamamoto et al. (1966), Sir et al. (1981) and Ezensor et al. (1981) demonstrated that the duodenal portion is the major site of lipid absorption.

In our study, we exposed the duodenal and ileal segments of rainbow trout to labelled astaxanthin and analyzed them for metabolites to compare the uptake and metabolism of astaxanthin by the two portions of the intestine. Our findings provide a basis for using the intestine to search for biologically active astaxanthin metabolites.

Materials and Methods

Rainbow trout (Salmo gairdneri) weighing approximately 500 g were obtained from the fish culture unit of the University of Rhode Island. They were maintained in a circulating system at $12 \pm 1^{\circ}\text{C}$. The photoperiod was 12 hours of light and 12 hours of darkness.

Trout were stunned by a blow to the head and their spinal cords were then severed. An incision was made from the anal vent anteriorly along the midline of the belly, exposing the viscera. The digestive tract was removed. Intestinal contents were expressed with a pair of forceps. Fat and mesentery adhering to the serosal surface were removed. The gut was washed with buffer solution. The gut was cut into the duodenal and ileal segments and immediately placed in a Petri dish containing the buffer solution in continuous equilibration with 95% O_2 -5% CO_2 .

Ileal Segment.

The ileal portion was everted by the method of Smith and Lane (1971) (Fig. 3). The gut was firmly tied to the applicator with a thread and everted by gently forcing. The everted intestine was cut free with a razor blade and removed from the applicator after one end of the everted gut was firmly tied with a thread. The everted intestine sac preparation was then firmly tied to a Pyrex tube

modified from Crance and Wilsing (1958) filled with buffer solution (on the serosal side) (Fig. 4), and suspended in 7-10 ml of the incubation medium (containing labeled astaxanthin) (the mucosal side) (Fig. 5). The entire apparatus was incubated in a water bath at $14 \pm 1^\circ\text{C}$ for 60 min. A mixture of 95% O_2 -5% CO_2 was bubbled through the mucosal buffer medium. The gas flow rate was adjusted to avoid physical damage to the gut mucosa.

Duodenal segment (pyloric caeca)

Since pyloric caeca (Fig. 2) have many diverticula, the duodenum (none-everted) was prepared for incubation as strips (Schachter et al. 1960). The gut wall was slit longitudinally along the antimesenteric side, and the segment was flattened with the serosal side facing down. The rectangular segment was then sliced through the entire thickness of the gut wall with a sharp blade, once longitudinally and three times horizontally. They were randomly divided and incubated in 25 ml Erlenmeyer flasks containing incubation medium for 60 min.

Media: The buffer solution used was a Ringer solution for trout of composition (g/l), NaCl 7.37 g, KCl 0.31 g, CaCl_2 0.17 g, MgSO_4 0.14 g, KH_2PO_4 0.46 g, and Na_2PO_4 2.02 g, (pH 7.3). The incubation medium was the buffer solution to which was added 0.3% Tween 20, 0.1%

glucose, 4.4 mg astaxanthin (specific activity 189 ucu/mg, gift from Hoffman La-Roche, Switzerland), and a given amount of bile acid (Olson, 1964).

Analysis

After exposure to incubation medium with labelled astaxanthin, the intestinal section was washed three times in warm buffer solution to remove radioactivity adhering to the surface. Then the section was homogenized in 100 ml of a hexane-ethanol (3:1) mixture containing the following carriers: astaxanthin, retinol ester, and retinol. The hexane phase was evaporated to dryness under a stream of nitrogen and dissolved in a known volume of hexane. An aliquot was taken and the radioactivity was measured. A portion of the sample, usually $2-6 \times 10^4$ dpm, was then used for the separation of astaxanthin metabolism by HPLC. All operations were carried out under incandescent light or in the dark.

High Performance Liquid Chromatography (HPLC)

The HPLC instrumentation consisted of a Waters model 6000A solvent delivery system (Waters Assoc., Milford, MA), a Waters U6K injector system, and a Perkins-Elmer LC-85 spectrophotometric detector connected to a Perkins-Elmer LC1-100 integrator (Norwalk, CT). A 10 μ m C-18 stainless steel column (25 X 0.4 cm) was used (Merck,

Germany).

The chromatographic mobile phases were mixtures of HPLC-grade acetonitrile, dichloromethane, and methanol; (Fisher Scientific, Medford, MA). All solvents were filtered through a <0.45 mm membrane filter (Gelman, Ann Arbor, MI).

Determination of Radioactivity

Fractions were collected from the column in 20-ml vials; 10 ml of scintillation fluid, Aquasol II (New England Nuclear, Boston, MA), was added to each vial. Each vial was then counted for ^3H radioactivity for 10 minutes in a Packard Tricarb Scintillation Counter model 3255 on the $^3\text{H}/^3\text{H}_0$ mode. The counts per minute [CPM] value for each ^3H fraction was then corrected for sample counting efficiency using an internal standard procedure and converted to disintegrations per minute [dpm].

Results

In this study the duodenal and ileal portions of intestine were mounted separately to compare uptake and metabolism of $^3\text{H}_2$, -3S, 3S'-astaxanthin. Samples of intestine were analyzed by HPLC to determine the extent to which the label had been modified during transport. Table 1 shows the distribution of ^3H in the two midgut segments of rainbow trout intestine after 60 min. of incubation in

labelled astaxanthin. The primary labelled compound present was identified as astaxanthin, by elution with front solvent. 12.3% of the radioactivity was taken up by the duodenum (pyloric caeca) while the ileal portion took up only 3%. Retinol was very low for both intestinal segments, indicating that very little of the astaxanthin was metabolized to retinol during the incubation period. This was identified as retinol by comparison with elution of labeled and non-labeled standard. The chromatogram of the duodenum is similar to that of the ileum. An HPLC chromatogram of the extract of the duodenum is shown in Figure 6.

Discussion

Most studies favor the concept that micellar phase is the preferred physiological state for most efficient absorption of lipids from the intestinal lumen. The fat-soluble carotenoids are insoluble in aqueous solution but soluble in lipid solvent. They are similar in their physical characteristics to other important biological compounds such as cholesterol and fatty acids.

Since the intestinal lumen is an aqueous environment, absorption of fat-soluble carotenoids is accomplished by interaction of bile acids with the carotenoids. The bile acids possess hydrophobic and hydrophilic ends. Above a critical micellar concentration, bile acids form

aggregates called micelles. The micellar particles present the polar ends of the bile acid toward the aqueous environment, and the non-polar portion toward the interior of the particles. The non-polar interior of the micellar particles form an ideal environment for solubilizing fat-soluble carotenoids. Adding detergent oil to bile acid micellar solutions expands the micelles and thereby increase the solubilization of fat-soluble carotenoids within the micellar particles.

This study confirmed earlier observations (Granguard 1962; Schiedt 1985) that freshwater fish were able to convert astaxanthin to retinol. However, only very small amounts were converted. This could be explained by low activity of astaxanthin as a source of vitamin A. Conversion of carotenoids that have a retinol structure at one or both ends (for example, β -carotene or cryptoxanthin) has been demonstrated in many animal species. Thus, fish possess an enzyme system that catalyzes the cleaving of carotenoids into retinol.

It was reasonable, though, to speculate that since they had the ability to convert astaxanthin to vitamin A, freshwater fish might supply vitamin A by this conversion.

When fresh water fish are faced with a serious shortage of vitamin A in the diet, they could synthesize the vitamin A they required from the carotenoid source available to them and thus compensate for any deficiency

of vitamin A in the diet. Under normal conditions, however, this conversion will not happen. Isolating the enzyme system responsible for reduction and conversion is an area for future research.

It was shown that 12.3% of the astaxanthin was taken up by pyloric caeca while only 3% by the ileum. This difference may have occurred because the absorption surface areas were different. This is an important parameter. Bergut et al. (1975) and Ash (1980) demonstrated that these portions of the intestine had comparable lengths but different uptake surface areas. In our study, the uptake capacity of the duodenum (pyloric caeca) was higher than that of the ileum. This is in agreement with other studies, which demonstrated that the duodenum is the major site of lipid uptake (Yamamoto 1966; Sire et al. 1981; Ezensor and Stokoe 1981).

In summary, the present work provided HPLC and radio isotope data to confirm that rainbow trout are able to bioconvert astaxanthin to vitamin A. The results confirmed earlier observations that the major pigment in aquatic animals is converted to vitamin A.

REFERENCES

- Al-Khalifa A. and Simpson, K. In preparation, 1986.
- Ash, R. (1980). Hydrolytic capacity of the trout (Salmo gairdneri) intestinal mucosa with respect to three specific dipeptides. Comp. Bio. Chem. Physiol. 65B, 173-176.
- Barua, A., Goswami, U., 1977, Formation of vitamin A in freshwater fish, Biochem. J. v. 164, p. 133-136.
- Bauermeister, A. & EM, Pirie, B.J.S. Tsargent, J.R. (1979). An electron microscopic study of lipid absorption in the pyloric caeca of rainbow trout (Salmo gairdneri) fed with ester-rich zooplankton. Cell Tissue Res. 200, 475-486.
- Bergot, P., Solari, A. and Luguët, P. (1975). Comparaison des surfaces absorbantes des caeca pyloriques et de intestin. Chez La Truite arc-en-ciel (Salmo gairdnerii). Ann Hydrobiol. 6, 27-43.
- Burnstock, G. 1959. The morphology of the gut of the brown trout (Salmo trutta). Quart. J. Micor. Sci. 100(2) 183-198.
- Crane, R., and Wilson, H., 1958. In vitro method for the study of the rate of intestinal absorption of sugar. J. App. Physiol. 12, 154-156.
- El-Gorab, M., Underwood, B. and Loerch, J. 1975. The roles of bile salts in the uptake of β -carotene and retinol by rat everted gut sac.

- Ezensor, D.N. and Stokoe W.M. (1981). Light and electron microscopic studies of the absorption cells of the intestine, caeca and rectum of the adult rainbow trout, Salmo gairdneri, J. Fish Biol. 18, 527-544.
- Granguard, R. 1962. Conversion in vitro de l'astaxanthine en vitamin A par l'intestin de Gambusia holbrooki Grd. C.R. Acad. Sci. 254, 579-581.
- Gross, J. and Budowski, P., 1966. Conversion of carotenoids into vitamin A₁ and A₂ in two species of freshwater fish. Biochem. J. 101, p. 747-754.
- Habibi, H. and Bernard, W. (1984). Intestinal transport of leucine in intact and gonadectomized under-yearling rainbow trout, Salmo gairdneri, Comp. Bioch. Phy. Vol. 79A, No. 3, pp. 349-352.
- Hata, M., Hata, M., and Onishi, T., 1973. Conversion of β -carotene and retinol₁ to retinol₂ in freshwater fish, Tohoku J. of Agri. Res. v. 24(4), p. 197-204.
- Hokazano, S. 1979. Biochemical studies on amino acid metabolism in fish, M.S. Thesis, Kagoshima University of Japan.
- Hollander, D., and Muralldhara, K.S. 1977. Vitamin A₁ intestinal absorption in vivo: influence of liminal factors on transport. Am. J. Physiol. 232(5) E471-477.

- Hollander, D., 1981. Intestinal absorption of Vitamins A, E, D, and K. J. of Laboratory and Clinical Medicine, V. 97-4-449.
- Marcotte, G. and Nove LA (1984). In vitro intestinal absorption of glycine and l-alanine by rainbow trout, Salmo gairdneri, Comp. Biochem. Physiol. Vol. 79A No. 2, pp. 209-213.
- Olson, J. 1964a, The biosynthesis and metabolism of carotenoids and retinol (vitamin A), J. of Lipid Res. [5], p. 281-299.
- Olson, J. 1964b, The effect of bile and bile salts on the uptake and cleavage of β -carotene into retinol ester (vitamin A ester) by intestinal slices, J. of Lipid Res. [5], p. 402-408.
- Schachter, D., Dowdie, E., and Schenker, H., 1960, Accumulation of Ca45 by slices of small intestine. Am. J. Physiol 198, 275-279.
- Schiedt, K., Levenberger, F.J. Vecchi, M., and Glinz E, (1985). Absorption, retention and metabolic transformation of carotenoids in rainbow trout, salmon and chicken, Pure and Appl. Chem. 57, 685-692.
- Sire, M.F., Lutton C. and Vernier, J.M. (1981). New views on intestinal absorption of lipids in teleostreams fishes: an ultra-structural and biochemical study in the rainbow trout. J. Lipid Res. 22 (81-94).

Smith, R., Lane, C., (1971). Amino acid transport by the fish intestine. Exp. in Physiol. and Biochem. 4, 93-103.

Sporn, M., Roberts, A., Goodman, D., 1984, The Retinoids, Academic Press, N.Y.

Ward, W.E. 1968. The uptake of amino acids by intestinal segment of the trout, Salmo gairdneri. Ph.D. Thesis of College of Arts and Sciences, Kent State University. Ohio, U.S.A.

Weinreb, E.L., and N.M. Bilstad (1955). Histology of digestive tract and adjacent structures of the rainbow trout (Salmo gairdneri). Copeia (3): 194-204.

Wilson, T.H. and Wiseman G. (1954). The use of sacs of everted small intestine for study of the transference of substance from the mucosal to serosal surface. J. Physiol, Lond. 123, 116-125.

Yamamoto, T. (1966). An electron microscopic study of the columnar epithelial cell in the intestine of freshwater teleosts; gold fish (Carasius auratus) and rainbow trout (Salmo irrideus). Z. Zellforsch, Mikrosk Anat. 72, 66-87.

Table 1. Radioactivity isolated from the intestine after incubation with ³H-astaxanthin

Portion of intestine	Pigment	Radioactivity (dpm)	% uptake
pyloric caeca (duodenal)	astaxanthin	125000	12.3
	retinol	670	
ileal	astaxanthin	31500	3.0
	retinol	125	

Figure 1. Gross morphology of the digestive tract of the rainbow trout, Salmo gairdneri

Figure 2. Diagram of the intestine of the trout: duodenal (A); ileal (B); and rectum (C) portions, pyloric valve (P.V.) and anus (A).

Figure 3. Eversion of the fish intestine. One end of the gut is securely tied to the glass rod applicator (a, b). The gut is gently forced over the tied portion and onto the applicator (c, d). The everted intestine is cut free (e).

Figure 4. Filling the intestinal sac. (a) A loop of thread is placed around a length of intestine which has been carefully drained and tied shut. (b) A polyethylene syringe tip is inserted into the sac and the overhand knot is tightened. (c) The intestine is filled. (d) The syringe tip is removed and the knot is tightened further.

Figure 5. Intestinal sac apparatus (after Smith and Lane,
1971)

Figure 6. Identification of ^3H label following incubation of representative of fish duodenal for 60 min. with ^3H , 3S, 3S, astaxanthin

--- represents radioactivity
— represents chromatogram obtained from injection of non label standard on the column.

Peak: 1, astaxanthin; 2, retinol

APPENDIX A LITERATURE REVIEW

4,4'-diaminodiphenyl ether (4,4'-DDO) is a colorless, crystalline solid which melts at 110°C. It is soluble in many organic solvents, including benzene, chloroform, and carbon tetrachloride. The compound is used in the synthesis of various dyes and pigments. It is also a component of certain polymers. The structure of 4,4'-DDO is shown below.

4,4'-Diaminodiphenyl ether (4,4'-DDO) was first isolated from the urine of a patient with a rare metabolic disorder. It was later identified as the product of the oxidation of 4,4'-diaminodiphenyl ether (4,4'-DDO) by the enzyme 4,4'-diaminodiphenyl ether oxidase. The structure of 4,4'-DDO is shown below.

4,4'-Diaminodiphenyl ether (4,4'-DDO) was first isolated from the urine of a patient with a rare metabolic disorder. It was later identified as the product of the oxidation of 4,4'-diaminodiphenyl ether (4,4'-DDO) by the enzyme 4,4'-diaminodiphenyl ether oxidase. The structure of 4,4'-DDO is shown below.

Astaxanthin ($C_{40}H_{52}O_4$:3,3'-dihydroxy-BB-carotene-4,4'-dione) is the most commonly occurring carotenoid in aquatic organisms. It has two carbonyl groups, eleven conjugated ethylenic double bonds and two hydroxy groups which make esterification possible. The pink to red coloring of many echinoderms and crustaceans, the skin of several fishes, and the meat of many salmonid fish consist partly or wholly of astaxanthin and its esters (Fox, 1957; Simpson et al., 1981; Straub, 1976).

Astaxanthin was first isolated from the shell of lobster as the form of astacene (B,B-carotene-3,3',4,4'-tetraone) by Kuhn and Lederer (1933). Later astaxanthin was isolated from the lobster egg (Kuhn and Sorensen, 1938a,b) and the structure of 3,3'-dihydroxy-B,B-carotene-4,4'-dione was determined based on the structure of astacene which was determined by Karrer et al. (1934, 1935).

Since astaxanthin was isolated from the lobster (Kuhn and Lederer, 1933), it has been found in a number of organisms. It was originally believed that astaxanthin was a typical animal carotenoid. However, the isolation of astaxanthin from the green algae, Haematococcus pluvialis (Tischer, 1938), the flagellate, Euglena heliorubescens (Tischer, 1941), the yeast, Phaffia rhodozyma (Andrews et al., 1976) and the higher plant,

Adonis annua L. (Seybold and Goodwin, 1958; Egger, 1965) and *A. aestivalis* (Neamtu et al., 1966) disproved the view that astaxanthin is purely an animal pigment.

Pigmentation of Salmonids:

Carotenoids are the main pigment of many aquatic animals, in Salmonids such as trout. Astaxanthin is responsible for the typically red color of the flesh. The coloration of salmonids has become of interest in the cultivation and important factor affecting consumer's acceptance.

The astaxanthin is of dietary origin (Steven 1947) and has received great interest in the cultivation of salmon and trout. In aquaculture, astaxanthin and canthxanthin are commonly used as pigmenters (Bauernfeind, 1976, Simpson, 1981).

Pigmentation studies with astaxanthin for rainbow trout use crustacen waste material as pigment source. It has been reported that 90-95% of the pigments in red crab processing waste (Kuo et al., 1976), shrimp meal waste (Lambertsen and Braekkan, 1971; Kamata et al., 1976; Choubert et al., 1983), crawfish (Chen and Meyers, 1982) are in some form of astaxanthin.

Spinelli et al. (1974) fed red crab meat to 7-month-old rainbow trout for 60 days and they observed that the fish had a hue similar to the reddish cast of ocean-caught

salmon.

Saito and Regier (1971) fed diets containing 20 and 30% shrimp waste and 20% crab waste. After 8 weeks on the 30% diet, brook trout were similar in color to those fed for 12 weeks on 20% shrimp meal. Petersen et al. (1966) fed raw crayfish and a crayfish extract to rainbow and brown trout. Although the amount of pigment in the diet was adjusted to be equal, the extract provided better results. Steel (1971) studied several diets and he observed that shrimp waste meal and pigment extract were most effective for pigmentation of trout. He also noted that the most rapid pigmentation was obtained with shrimp meal extract. Kuo et al. (1976) fed rainbow trout diets containing 20% freeze-dried Atlantic red crab, Geryon quinquedens waste and pigment extract. Fish fed the crab waste diet grew as fast as the controls, but there was no significant coloring effect over the controls. On the other hand, fish fed carotenoid extract from the crab meal showed an excellent red coloration. Kamata et al. (1976) fed rainbow trout diets consisting of shrimp protein and pigment extract from the protein absorbed on a commercial trout ration. The results indicated that the protein or pigment extract could be used as a pigment and a protein source for Salmonids, raised in aquaculture.

Choubert and Luquet (1983) fed rainbow trout diets containing 30% shrimp meal, this caused a reddish

pigmentation of the skin and muscle.

The pigmentation of trout with pigment extracts from plant sources has been reported by several authors. Peterson et al. (1966) fed paprika extract to rainbow trout. They observed that fish deposited undesirable yellow pigments in the skin. Lee et al. (1978) used extracts of marigold and squash flower as a pigment source for trout. They reported that the fish fed with squash flower extract showed better coloration, but the fish fed with marigold extract had undesirable yellowish coloration. Kamata et al (1977) studied the utilization of Hyppophae rhamnoides oil (5 mg% pigment in diet) as a pigment source for rainbow trout. After a 4-week feeding period, some red coloration was observed. Kamata et al. (1986) fed the flower, Adonis aestivalis and its pigment extract to rainbow trout. They found that the fish fed with adonis extract showed excellent pigmentation. However, a direct feeding of the flower caused high mortality possibly because of some toxic compound in it.

A number of investigators have used crystalline carotenoids for pigmentation of salmonids. Steven (1947) fed B-carotene to trout, but he could not get a good coloration. The use of crystalline B-carotene has not been successful. Saito and Regier, 1971; Deufel, 1965; Schmidt and Baker, 1969; Steel, 1971; Bauernfeind, 1976. These investigators used canthaxanthin for salmonid

culture and cantaxanthin could rapidly be taken up by trout and salmon in flesh and skin.

Astaxanthin is the best pigment source for salmonids, crustaceans and sea bream. Torrissen and Braekkan (1979) and Kamata and Simpson (1976) reported on the feeding of pure astaxanthin, its mono- and diester, and astacene, to rainbow trout. These reports taken together show that the skin preferentially takes up the esters and deposits all fed forms as the esters. Astaxanthin is found only in the flesh as the free form. Regardless of the form fed to the fish, either esterified or deesterified, the esters are deposited in the skin and the free form deposited in the flesh. Schiedt et al. (1985) studied absorption and pigmenting efficiency of astaxanthin, canthaxanthin and zeaxanthin in rainbow trout. They reported that astaxanthin was the best pigments, followed by canthaxanthin and astaxanthin dipalmitate. Foss et al. (1984), and Storebakken et al. (1985) also reported that optical isomers of astaxanthin were more efficacious than canthaxanthin in pigmenting the flesh of rainbow trout and salmon. Foss et al. (1984) investigated the efficiency of optical isomers of astaxanthin in trout, and they found that the same utilization was present in all three astaxanthin isomers. Schiedt et al. (1985) carried out similar experiments. They found equal absorption and deposition of "racemic" and (3S,2'S)-astaxanthin and a

slightly increased deposition of (3R,3'S)-compared with (3S,2'S)-astaxanthin. Both groups also found that no epimerization occurred in trout after administration of free (3S,3'S)-, (3R,3'R)- and (3R,3'S)-astaxanthin.

Formation of Astaxanthin in Fish

It has been concluded by Goodwin (1950) that carotenoids are manufactured de novo only in the plant world. Since animals are thought not capable of de novo synthesis of carotenoids, these compounds must be supplied in the diet, but many marine animals have metabolic capacity to modify structurally dietary carotenoid (Liaaen, 1978). Carotenoids from food can be converted into astaxanthin in some aquatic animals.

Several authors have reported that goldfish is capable of the conversion of carotenoids. Katayama et al. (1970a, 1970b) reported that lutein was converted to astaxanthin in Carassius auratus, these authors proposed that astaxanthin was formed from lutein via - α -doradexanthin and β -doradexanthin. This conversion was further confirmed by Hsu et al. (1972) using ^{14}C -lutein.

Hata et al. (1971) reported that most of the fresh water red fish belong to cyprinidal species. They can convert zeaxanthin to astaxanthin (zeaxanthin $\rightarrow$ doradexanthin $\rightarrow$ astaxanthin, but cannot convert cantaxanthin and β -carotene to astaxanthin. Rodriguez et

al. (1973) reported that astaxanthin level of the fish (C. auratus) was significantly increased when β -carotene, isocryptoxanthin, echinenone or canthaxanthin was fed.

Lobster, prawn and crab can convert β -carotene into astaxanthin through isocryptoxanthin, echinenone, 4-hydroxy-4-Keto- β -carotene, canthaxanthin and phoenicoxanthin, Katayama et al. (1972, 1973, 1974).

Carotenoids from food can be converted into astaxanthin in some aquatic animals; for example, the prawn (Penaeus japonicus), lobster (Panulius japonicus) and crab (Portunus trituberculatus). The crustacea can convert β -carotene and zeaxanthin to astaxanthin. The proposed metabolic pathway in these animals is as follows:
 β -carotene $\rightarrow$ echinenone $\rightarrow$ canthaxanthin $\rightarrow$ phoenicoxanthin $\rightarrow$ astaxanthin, and zeaxanthin $\rightarrow$ β -doradexanthin $\rightarrow$ astaxanthin (Tanaka 1978).

Louis et al. (1983) demonstrated that lobsters are able to use dietary β -carotene, echinenone and canthaxanthin as precursors in the production of their primary tissue carotenoid, astaxanthin, and this capability is consistent with generalized crustacean biosynthetic scheme of β -carotene $\rightarrow$ echinenone $\rightarrow$ canthaxanthin $\rightarrow$ astaxanthin or β -cryptoxanthin $\rightarrow$ zeaxanthin $\rightarrow$ astaxanthin.

Matsuno et al. (1984) reported that spindle shell Fusinus perplexus can convert β -carotene to 3S,3S-

astaxanthin via echinenone, 4^h hydroxyechinenone, canthaxanthin, and (3S) phoenicoxanthin.

Salmonids, on the other hand, cannot convert any carotenoid to astaxanthin (Tanaka 1978). They can only transfer astaxanthin from food organisms to their own tissues. Peterson et al. (1966) suggested that α -carotene was not converted into canthaxanthin in significant amount of rainbow trout. Hata and Hata (1975) reported that rainbow trout have no ability to oxidize lutein, zeaxanthin and α -carotene into astaxanthin, and that the accumulated astaxanthin in the muscle of salmonid is from those ingested.

Metabolic Pathways of Astaxanthin in Fish

Metabolic pathway of astaxanthin conversion in fish are reverse reaction of keto-oxidation of zeaxanthin or β -carotene to astaxanthin in many animals. This metabolic route for reduction of astaxanthin, is a new area, and has only now been investigated.

Fujita et al. (1983), cultured red sea bream with astaxanthin diester Purified from krill oil. This indicates that astaxanthin diester has been metabolized to tunaxanthin via β -carotenetriol, zeaxanthin and 3^h-epilutein.

Chum salmon appear to be unique in that astaxanthin in the muscle is converted to zeaxanthin in the skin

during the period preceding spawning (Kitahara 1983). These conversions (Figure 1) of astaxanthin $\Rightarrow$ 4 keto zeaxanthin (adonixanthin) $\Rightarrow \beta$ -carotene-triol $\Rightarrow$ zeaxanthin has not been found in other fishes and is a phenomenon peculiar to salmon during anadromous migration.

Kitahara, 1984, studied the behavior of carotenoids in the chum salmon (*Oncorhynchus keta*). The author suggested that during development, astaxanthin which was in the free state in the beginning was gradually reduced, and was converted into other xanthophylls of the ester type. Goswami, B. et al. (1984) studied the metabolism of astaxanthin in the fresh water fish (Heteropneustes fossilis). It was found that astaxanthin was converted into β - β -carotene in the intestine and deposited in liver.

Miki et al. (1984) studied the metabolism of dietary β -carotene, canthaxanthin, astaxanthin diester, astaxanthin monoester, and astaxanthin in eggs of Red Sea bream. He reported that only keto and non-esterified carotenoids were detected in the eggs, canthaxanthin was transferred into the eggs but not β -carotene. Astaxanthin esters were converted largely to non-esterified astaxanthin and partly to doradexanthin. He presumed that idoxanthin present in the eggs was derived from non-esterified astaxanthin in the diet.

Figure 1. Metabolic pathway from astaxanthin to zeaxanthin in matured chum salmon

Fujita et al. (1983) presume that astaxanthin esters in the krill oil was metabolized to tunaxanthin fraction via β -carotene triol, zeaxanthin, and 3'-epilutein, in yellow tail fish.

Miki et al. (1985) examined the bioconversion of astaxanthin and lutein in the yellow tail fish. He presumed that astaxanthin was metabolized to tunaxanthins via β -carotene-triol, zeaxanthin and 3'-epilutein. The overall pathway is given in Figure 2.

Matsuno et al. (1985) Studied the reductive metabolic pathways for (3S,3'S) astaxanthin in fish. He concluded that these pathways can be divided into two groups: (i) those that metabolize (3S,3'S)-astaxanthin into (3R,3'R)-zeaxanthin via (3S,4RS,3'R)- $\beta\beta$ -carotene-3,4,3'-triol (i.e. mackerel and yellowtail); (ii) those that metabolize (3S,3'S)-astaxanthin into tunaxanthin A, B, and C [(3S,6S,3'S,6'S)-, (3R,6S,3'S,6'S)- and (3R,6S,3'R,6'S) - $\epsilon\epsilon$ -carotene-3,3' diol, respectively via (3R,3'R)-zeaxanthin, (3R,6'S)-3-dihydroxy- $\beta\epsilon$ -caroten-3'-one and (6S,6'S)-carotene-3,3'-dione (i.e. dolphin and flying fish).

Schiedt, et al. (1985), investigated the metabolism of astaxanthin, and canthaxanthin, in skin of rainbow trout and atlantic salmon (Salmo salar). They found that astaxanthin was converted to deepoxyneoxanthin via β -adonixanthin, zeaxanthin, and antheraxanthin shown in

Figure 2. Metabolic pathway from astaxanthin to tunaxanthin in yellowtail

Figure 3 in rainbow trout while in Atlantic salmon only to zeaxanthin via idoxanthin or β -adonixanthin. Canthaxanthin was converted to β -carotene via echinenone in both rainbow trout and atlantic salmon.

Carotenoids as Vitamin A Precursors in Fish

Several carotenoids are capable of acting as provitamin A precursors. It has been conclusively established that a number of carotenoids with at least one unsubstituted β -ion ring and fully conjugated isoprenoid side chain can serve as the precursor of retinol in fish. Earlier reports indicated that both vitamin A₁ and A₂ can be formed from a common precursor such as β -carotene.

Hata, et al. (1973) study in vivo and in vitro conversion of β -carotene to retinol₁ and retinol₂ in two fresh water fish species (funa) carassius auratus and (Raigyo) Chana argus cantor, using the radio isotope tracer technique, it was found that retinol₁ and retinol₂ were synthesized from β -carotene, and the ratio was different between the two fish species.

Goswami et al. (1981), study the metabolism of β -carotene in fresh water fish Channa gachua, it was concluded from the studies that fish are capable of the conversion of β -carotene into either retinol or retinoic acid, but are unable to convert β -carotene into dehydroretinol.

Figure 3. Metabolic pathway of astaxanthin in rainbow trout

Barua, et al. (1977) examined the bio conversion of β -carotene and lutein in fresh water fish Saccobranthus fossilis, which had been starvated for 20 days. He presumed that only retinoic acid could be isolated from the intestine for fish fed β -carotene, while dehydroretinol and 3-hydroxyretinol could be isolated from the intestines of fish fed lutein.

Barua, et al. (1973). Study the conversion of lutein into dehydroretinol by Saccobranthus fossilis depleted in vitamin A. It found that β -carotene and zeaxanthin were not converted to dehydroretinol, while lutein was converted.

Barua, et al. (1975), reported that lutein can act as a precursor of dehydroretinol, and anhydrolutein can be intermediate in this transformation, in Saccobranthus fossilis, depleted in vitamin A.

Goswami, et al. 1982, study the metabolism of lutein (β , ϵ -carotene-3,3'-diol) in clarias batrachus and ompok pabo it was found that lutein is centrally cleaved into dehydroretinol via anhydrolutein, 3-dehydroxyretinol and 3-hydroxyanhydrolutein in both species.

Goswami, et al. (1984), reported that lutein can serve as an efficient precursor of dehydroretinol in the fresh water fish Heteropneustes fossilis, with anhydro lutein (3',4'-didehydro- β - β -caroten-3-ol) an intermediate in this transformation.

Goswami (1984), study the metabolism of cryptoxanthin in fresh water fish. Channa gachua, Labeo boga (retinol-rich) and Heteropneustes fossilis (dehydroretinol-rich). it was concluded that retinol-rich fresh water fish can convert cryptoxanthin into retinol and no 3-dehydroretinol or 3-hydroxyretinol and 3-dehydroretinol were isolated from the vitamin-A deficient H. Fossilis, after administration of cryptoxanthin.

Grangaud et al. (1957, 1962), Study the conversion of astaxanthin to vitamin A in vitamin A depleted Gambusia holbrooki, using vivo and vitro study, they concluded that astaxanthin converted to retinol and the probable steps as follows (Figure 4) (a) reduction of astaxanthin to β -carotene, (b) conversion of β -carotene into vitamin A₁ (or retinol), (c) dehydrogenation of vitamin A₁ to A₂ (or of retinal to 3,4-dehydroretinal).

Gross et al. (1966), examine the conversion of some carotenoids into vitamin A₁ and A₂ in guppies (Lebistes reticulatus) and platies (Xiphophours variatus), they were found to form vitamin A from β -carotene and from its oxygen-containing derivatives isozeaxanthin, canthaxanthin and astaxanthin. A slight conversion into vitamin A₂ occurred. Lutein was devoid of provitamin A properties.

Schiedt et al. (1985), studied the conversion of astaxanthin, canthaxanthin and zeaxanthin in vitamin A-depleted rainbow trout, it was found that these

carotenoids, could be a precursors of vitamin A₁ and A₂ in freshwater fish.

Lambertsen et al. (1969) confirm the conversion of vitamin A₁ to vitamin A₂ in young rainbow trout.

Figure 4. Suggested mechanism for the formation of retinol and 3,4 dehydroretinol from astaxanthin in fish

APPENDIX B

LABORATORY AQUARIA FOR TROUT

The 125-gallon fiberglass tanks, which were constructed with a water nozzle, circulation system and water pump, were used for culture of trout. The water was filtered in each tank by means of a fine mesh of 1/16 inch and 1/32 inch to give satisfactory water quality. The water was filtered by passing it through a fine mesh of 1/16 inch and 1/32 inch. The waste materials (feces, food, etc.) were collected in the center of the bottom of the tank and the materials were carried out by water through a hole of 1/16 inch pipe. Furthermore, this hot water could carry in some amount of oxygen. The water level in the tank was kept turbulent water. The temperature of the water was approximately 11°C.

The water level in each tank was kept at a constant level by means of a pump and air line and air could supply an amount of oxygen to the water level.

The water level in each tank was kept at a constant level by means of a pump and air line and air could supply an amount of oxygen to the water level. In the water level, there were some holes and some holes. As mentioned above, the water level was kept at a constant level by means of a pump and air line and air could supply an amount of oxygen to the water level. Thus the water level in each tank was kept at a constant level by means of a pump and air line and air could supply an amount of oxygen to the water level. The water level in each tank was kept at a constant level by means of a pump and air line and air could supply an amount of oxygen to the water level.

Fish Culture system

The 125-gallon fiberglass tanks, which were equipped with a water nozzle, aeration system and water drain, were used for culturing fish (Fig-1).

The water was jetted in each tank by nozzle with a flow rate of 2 gallons per minute to give sufficient swirl which could clean the tank continuously by swirling down the waste materials (excess feed, feces of fish etc.) to the center of the bottom of the tank and the materials could be carried out by water through the holes of center pipe. Furthermore, this jet water could carry in some amount of oxygen. Rainbow trout like to live in swift turbulent water. The temperature of the water was approximately $11 \pm 2^{\circ}\text{C}$.

The aeration system which is composed of air compressor, air line and air stone could supply an sufficient oxygen level of approximately 8ppm for fish.

The water drain is composed of two concentric pipes. In the outer pipe, there were holes in the top and bottom. As mentioned above, water and waste materials were drained out through the bottom holes then up to the inner pipe to materials, the water could be drained out through the top holes. Thus the water level in the tank would be kept lower than outer concentric pipe and the fish would be prevented from being flushed out.

A = water nozzle, B = aeration system, C = drain pipes,
D = water level, E = overflow holes

Figure 1. Laboratory aquaria

- Al-Shafiq, A. and Tinsley, K. In preparation, 1982.
- Arar, A.S., Phaff, H.J. & Starr, M.B. (1961) Carotenoids of *Phaffia rhodospirillum*, a red-pigmented fermenting yeast. *Phytochem.* 10, 1001-1007.
- Arar, B. (1981) Hydrolytic capacity of the trout *Salmo gairdneri* intestinal mucus with respect to dietary carotenoid digestion. *Comp. Biochem. Physiol.* 88B, 173-176.
- Barua, A., and Day, R. C., 1976. Occurrence and Conversion of xanthophylls into dehydroxanthin in a fresh water fish. *British Journal of Nutrition* 37 p. 319-327.

APPENDIX C

BIBLIOGRAPHY

- Barua, A., Senapati, B., 1977. Formation of vitamin A in a fresh water fish. *Biochem. J.* v. 164 p. 133-136.
- Barua, A.B., Singh, H.L., and Day, R.C. 1973. Conversion of carotenoids into vitamin A in a fresh water fish *Salmo gairdneri*. *Possible* *Am. J. N.* 33 p. 12.
- Barua, A., Senapati, B., 1977. Formation of vitamin A in a fresh water fish. *Biochem. J.* v. 164, p. 131-136.
- Benayahu, A. & Zil, Arie, H.J.S. Targui, J.B. (1978). An electron microscopic study of lipid absorption in the pyloric caeca of rainbow trout (*Salmo gairdneri*) fed with water-rich zooplankton. *Zell Tissue Res.* 202, 475-486.
- Fawcett, J.C. (1978) Carotenoids: a pigment for salmonids *Prog. Fish. Cult.* 38, 189-191.

- Al-Khalifa A. and Simpson, K. In preparation, 1986.
- Andrewes, A.G., Phaff, H.J. & Starr, M.P. (1976) Carotenoids of Phaffia rhodozyma, a red-pigmented fermenting yeast. Phytochem. 15, 1003-1007.
- Ash, R. (1980). Hydorlytic capacity of the trout (Salmo gairdnerii) intestinal mucosa with respect to three specific dipeptides. Comp. Bio. Chem. Physiol. 65B, 173-176.
- Barua, A., and Das, R. C., 1975. Occurrence and Conversion of anhydrolutein into dehydroretinol in a fresh water fish., British Journal of Nutrition 33 p. 319-327.
- Barua, A., DAS, R., and Verma, K., (1977) Biochemical Journal 168, 557-564.
- Barua, A., Goswami, U., 1977, Formation of vitamin A in a fresh water fish, Biochem. J. v. 164 p. 133-136.
- Barua, A.B., Singh, H.T., and Das, R.C. 1973, Conversion of lutein into dehydro lutein by fresh water fish saccobranhus fossilis Br. J. N. 30-P-12.
- Barua, A., Goswami, U., 1977, formation of vitamin A in fresh water fish, Biochem. J. v. 164, p. 133-136.
- Bauermeister, A. & EM, Pirie, B.J.S. Tsargent, J.R. (1979). An electron microscopic study of lipid absorption in the pyloric caeca of rainbow trout (Salmo gairdnerii) fed wax ester-rich zooplankton. Cell Tissue, Res. 200, 475-486.
- Bauernfeind, J.C. (1976) Canthaxanthin: a pigmenter for salmonids Prog. Fish. Cult. 38, 180-183.

- Bergot, P., Solari, A. and Luquet, P. (1975). Comparison des surfaces absorbantes des caeca pyloriques et de intestin. Chez La Truite arc-en-ciel (Salmo gairdnerii). Ann Hydrobiol. 6, 27-43.
- Bieri, J.G., Tolliver, T.J., and Catignani, G.L., 1979, Simultaneous determination of -tocopherol and retinol in plasma or red blood cell by high pressure liquid chromatography. Am. J. Clin. Nut. 32:2143.
- Buchecker R. (1981) Carotenoid Chemistry and Biochemistry (Edited by Britton G. and Goodwin T.W.), pp. 175-181, Pergamon Press, Oxford.
- Burnstock, G. 1959. The morphology of the gut of the brown trout (Salmo trutta). Quart. J. Micor. Sci. 100(2) 183-198.
- Choubert, G. Jr. & Luquet, P. (1975) Feeding test of capelin oil, (Mallotus villosus) on rainbow troupe: Amount and qualities of carotenoid content in the muscle skin. Ann. Hydrobiol. 6, 123-130.
- Choubert, G. and Luquet, P., 1975. Nature des carotenoides fixes au niveau de la peau et du muscle de la truite arc-en-ciel ayant ingere de l'huile rouge de capelan. Ann. Hydro-biol., 6:123-130.
- Choubert, G. and Luquet, P., 1982. Fixation et retention musculaire de la canthaxanthine par la truite arc-en-ciel. Ann. Zootech., 31:1-10.

- Choubert, G. and Luquet, P. 1983, Utilization of shrimp meal for rainbow trout (Salmo gairdneri Rich.) pigmentation. Influence of fat content of the diet. *Aquaculture*, 32:19-26.
- Crane, R., and Wilson, H., 1958. In vitro method for the study of the rate of intestinal absorption of sugar. *J. App. Physiol.* 12, 154-156.
- Davies, B.H. (1976) Carotenoids. In Chemistry and Biochemistry of Plant Pigments (Edited by Goodwin, T.W.) Vol. 2, pp. 38-165. Academic Press, N.Y. and London.
- Davis, B.H., 1976, Chemistry and Biochemistry of plant pigments (Goodwin, Ed.) Vol. 2, pp. 38-165. Academic Press, N.Y.
- Duefel, J. (1965) Pigmentierungsversuche mit-Canthaxanthin bei Regenbogenforellen. Arch. Fish. Wiss. 16, 125-132.
- Egger, K. (1965). Die Ketocarotenoide in Adonis annua L. Phytochem. 4, 609-618.
- El-Gorab, M., Underwood, B. and Loerch, J. 1975. The roles of bile salts in the uptake of α -carotene and retinol by rat everted gut sac.
- Ezensor, D.N. and Stokoe W.M. (1981). Light and electron microscopic studies of the absorption cells of the intestine, caeca and rectum of the adult rainbow trout, salmo gairdnerii, *J. Fish Biol.* 18, 527-544.

- Foss, P. Storebakken, T., Schiedt, K., Liaaen-Hensen, S., Austreng, E. & Streiff, K. (1984) Carotenoids in diets for salmonids. 1. Pigmentation of rainbow trout with individual optical isomers of astaxanthin on comparison with canthaxanthin. Aquaculture 41, 213-226.
- Fox, D.L. (1957). The pigments of fish. In Physiology of fishes (Edited by Brown, M.E.) Vol. 2, pp. 367-385. Academic Press N.Y. London.
- Fujita T., Satake M., Hikichi S., Takeda M., Shimeno S., Kuwabara H., Miki W., Yamaguchi K. and Konosu S. (1983a) Pigmentation of cultured yellowtail with krill oil. Bull. Jap. Soc. scient. Fish. 49, 1595-1600.
- Fujita, T., Satake, M., Watanabe, T., Kitajima, C., Miki, W., Yamaguchi, K. and Konosu, S. (1983b). Pigmentation of cultured red sea bream with astaxanthin diester purified from krill oil. Bull. Jap. Soc. Scient. Fish. 49, 1855-1861.
- Georgiev, G.S., 1971, Carotenoids and vitamin A content in *Salmo irideus* eggs and their significance in the initial periods of the embryogenesis, *Folia Balcanica*, VII (a) p. 1-10.
- Goodwin, T.W. (1950) Carotenoids and reproduction. Biol. Rev. Cambridge 25, 391-413.
- Goodwin, T.W. (1971). Carotenoids (Edited by Isler), pp. 641-656.
- Goswami, B. et al. 1984 (Abst.). Studies on the metabolism of astaxanthin in the fresh water fish, Heteropneustes fossilis, 7th International Sym. on Carotenoids.

- Goswami, U. and Barua, A., 1981, Origin of Retinol in freshwater fish, current science V. 50 (3)^P 150-151.
- Goswami, U. and Bhattacharjee, S., 1982 Biosynthesis of 3-Dehydroretinol:Metabolism of Lutein (BE-Carotene-3,3 Diol). in *clarias batrachus* and *ompok pabo*, Biochemistry international, V. 5 (4) p. 545-552.
- Goswami, V., (1983). Indian Journal of Animal Science 53, 50-52.
- Goswami, V., 1984, Metabolism of cryptoxanthin in freshwater fish, British Journal of Nutrition V. 52, 575-581.
- Granguard, R., Vignais, P., Massonet, R. and Moutti, J. (1957) Biol. Soc. Chem, Biol. Paris 39, 1271.
- Granguard, R. 1962. Conversion in vitro de l'astaxanthine en vitamin A par l'intestin de *Gambusia holbrooki* Grd. C.R. Acad. Sci. 254, 579-581.
- Gross, J. and Budowski, P. 1966, Conversion of carotenoids in to vitamin A₁ and A₂ in two species of fresh water fish. Biochem. J. 101 747-754.
- Habibi, H. and Bernard, W. (1984). Intestinal transport of levcine in intact and gonadectomized under yearling rainbow trout, salmo gairdnerii, Comp. Bioch. Phy. Vol. 79A, No. 3, pp. 349-352.
- Hata, M. and Hata, M. (1971) Carotenoid pigments in goldfish (Carassius avratus). I. Composition and distribution of carotenoids. Int. J. Biochem. 2, 11-19.

- Hata, M., Hata, M. and Onishi, T., 1973, Conversion of β -carotene and retinol₁ to retinol₂ in freshwater fish, Tohoku Journal of Agri. Research V. 24 (4)P 197-204.
- Hata, M. and Hata M. (1972a) Carotenoid pigments in goldfish-IV. Carotenoid metabolism Bull. Jap. Soc. scient. Fish, 38, 331-338.
- Hata M., and Hata M. (1972b) Carotenoid pigments in goldfish-V. Conversion of zeaxanthin to astaxanthin. Bull. Jap. Soc. scient. Fish, 38, 339-343.
- Hata M. and Hata M (1975) Carotenoid metabolism in fancy red carp, *Cyprinus carpio*-I. Administration of carotenoids. Bull. Jap. Soc. scient. Fish 41, 653-655.
- Hata M. and Hata M. (1976) Carotenoid metabolism in fancy red carp, *Cyprinus carpio*-II. Metabolism of C-zeaxanthin. Bull. Jap. Soc. scient. Fish. 42, 203-205.
- Hokazano, S. 1979. Biochemical studies on amino acid metabolism in fish, M.S. Thesis, Kagoshima. University of Japan.
- Hollander, D., and Muralldhara, K.S. 1977. Vitamin A₁ intestinal absorption in vivo: influence of liminal factors on transport. Am. J. Physiol. 232(5) E471-477.
- Hollander, D., 1981. Intestinal absorption of Vitamins A, E, D, and K. The J. of Laboratory and Clinical Medicine, V. 97-4-449.
- HSU, W.J. Rodriguez, D.B., and Chichester, C.O. 1972. The biosynthesis of astaxanthin VI. The conversion of ¹⁴C lutein and ¹⁴C- β -carotene in goldfish. Int. J. Biochem, 3, 333-338.

- Johnson, E.A., Villa, T.G. and Lewis, M.J. 1980. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. *Aquaculture*, 20:123-134.
- Kamata, T., Neamtu, G.G. & Simpson, K.L. (1977) The pigmentation of rainbow trout (Salmo gairdneri) with Hyppophae rhamnoides oil. Rev. roum. Biochem. 14, 253-258.
- Kamata, T. (1976) The pigmentation of rainbow trout, Salmo gairdneri. Master's Thesis, University of Rhode Island, Department of Food Science and Nutrition.
- Kamata, T., Neamtu, G.G. & Simpson, K.L. 1986. Manuscript II. Utilization of Adonis aestivalis as a dietary pigment source for rainbow trout, Salmo gairdneri Ph.D. Diss. University of Rhode Island.
- Karrer, P., Loewe, L. & Hubner, H. (1935) Konstitution des Astacins. Helv. Chim. Acta 18, 96-100.
- Karrer, P. & Benz, F. (1934) Pflanzenfarbstoffe über ein neues Vorkommen des Astacins ein Beitrag zu dessen Konstitution. Helv. Chim. Acta 17, 412-416.
- Katayama, T., Kamata, T., Shimaya, M., Deshimaru, O. and Chichester, C.O. (1972). The biosynthesis of astaxanthin VIII. The conversion of labelled β -carotene - $^3\text{H}_2$ into astaxanthin in prawn, Penaeus japonicus. *Bull. Japan, Soc. Sci. Fish.* 38(10):1171-1175.

- Katayama, T., Kunisaki, Y., Shimaya, M., Simpson, K.L. and Chichester, C.O. (1974). The biosynthesis of astaxanthin XIV. The conversion of labelled β -carotene $15, 15, ^3H_2$ into astaxanthin in the crab portunum trituberculatus, Comp. Biochem. Physiol. 46b(2):269-272.
- Katayama, T., Miyahara, T., Tanaka, Y., Sameshima, M. and Chichester, C.O. (1974). The biosynthesis of astaxanthin XV. The carotenoid in chidai, red sea bream, Erynnis japonica, and the incorporation of labelled astaxanthin from the diet of red sea bream to their body. Bull. Jap. Soc. Sci. Fish. 40(1):97-103.
- Katayama, T., Yokoyama, H., and Chichester, C.O. (1970a). The biosynthesis of astaxanthin II. The carotenoids in Benibuna, Carassius auratus, Bull. Japan. Soc. Sci. Fish. 36:702-708.
- Katayama, T., Shimaya, M., Sameshima, M. and Chichester, C.O. (1973). The biosynthesis of astaxanthin XII. The conversion of labelled β -carotene 3H_2 into body astaxanthin in the lobster, panulirus japonicus, Int. J. Bio. Chim. 4(21), 223-226.
- Katayama, T., Yokoyama, H. and Chichester, C.O. 1970^b. The biosynthesis of astaxanthin I. The structure of β -doradexanthin and β -doradexanthin. Int. J. Biochem 1(4) 438-444.
- Kithara T. (1983) Behavior of carotenoids in the chum salmon (*O. keta*) during anadromous migration. Comp. Biochem. Physiol. 76B, 97-101.

- Kitahara T. (1984) Behavior of carotenoids in the chum salmon (*O. keta*) during development. *Bull. Jap. Soc. scient. Fish.* 50, 531-536.
- Krause, M.V. and Mahan, L.K. (1979). *Food Nutrition and Diet Therapy*. 6th ed. W.R. Saunders Company, Philadelphia, London, Toronto.
- Kuhn, R. & Lederer, E. (1933) Über der Forbstoffe des Hummers Astacus gammarus L. und ihre Stammsubstanz das Astacim. Ber. 66, 488-495.
- Kuhn, R., Stene, J. & Sorensen, N.A. (1938) Über Astaxanthin und Ovoverdin. Ber. 71, 1879-1888.
- Kuhn, R. & Sorensen, N.A. (1938) Über die Farbetoffe des Hummers (Astacus gammarus L) Z. Angew. Chim. 51, 465-466.
- Kuo, H.C., Lee, T-C., Kamata, T. & Simpson, K.L. (1976) Red crab processing waste as carotenoid source for rainbow trout. Alimenta 15, 47-51.
- Lambertsen, G. & Braekkan, O.R. (1971) Method of analysis of astaxanthin and its occurrence in some marine products. J. Sci. Food Agric. 22, 99-101.
- Lambertsen, G. and Olaf, R. 1969. In vivo conversion of vitamin A₁ to vitamin A₂ *Acta Chem. Scand.* 23(3) 1063-1064.
- Lee, R.G., Neamtu, G.G., Lee, T-C. & Simpson, K.L. (1978) Pigmentation of rainbow trout with extracts of floral parts from Tagetes erecta and Cucubita maxima. Rev. roum. Biochem. 15, 287- 293.

- Liaaen-Jense, S., 1978. Marine carotenoids in : P. Scheuer (Editor), Marine natural products, chemical and biological perspectives, Vo. 2. Academic Press, New York. pp. 2-73.
- Little, T., Hills, F., 1978, Agricultural Experimentation Design and analysis, John Wiley and Sons, N.Y.
- Louis, T., Baum, N., Bordner, C., Conklin, D., (1983). Carotenoids as source of pigmentation in juvenile lobsters fed a purified diet. *Cand. J. Fish. Aquat. Sci.*, 40: pp. 699-704.
- Marcotte, G. and Nove LA (1984). In vitro intestinal absorption of glycine and l-alanine by rainbow trout, salmo gairdnerii, *Comp. Biochem. Physiol.* Vol. 79A No. 2, pp. 209-213.
- Matsuno, T., Katsuama, M., Maokao, H., and Komori, T., (1985). Reductive metabolic pathways of carotenoids in fish (3S, 3S). Astaxanthin to tunaxanthin, A, B and C. *Comp. Biochem. Physiol.* 80B, 4:779-789.
- Matsuno, T., Maoka, T., Katsuyama, M., Ookubo, M., Katagiri, K. & Jimura, H. (1984) The occurrence of enantiomeric and meso-astaxanthin in aquatic animals. Bull. Jap. Soc. Sci. Fish. 50(9), 1589-1592.
- Matsuno, T., Matsutaka, H., Katsuyama, M., and Nagata, S., 1980. Occurance of 3-epilutein from fish. *Bull. Jap. Soc. Scient. Fish.* 46 337-340.
- Matsuno T., Nagata S., Iwahashi M., Koike T. and Okada M. (1979) Intensification of color of fancy red carp with zeaxanthin and mysoxanthophyll, major carotenoid constituents of Spirulina. *Bull. Jap. Soc. scient. Fish*, 45, 627-632.

- Matsuno T., Matsutaka H. and Nagata S. (1981) Metabolism of lutein and zeaxanthin to ketocarotenoids in goldfish *Carassius auratus*. Bull. Jap. Soc. scient. Fish, 47, 605-611.
- Matsuno T. and Nagata S. (1980) Biosynthesis of characteristic, principal carotenoids of Japanese common catfish parasiloxanthin (7', 8'-dihydro-zeaxanthin) and 7,8-dihydro-parasiloxanthin. Bull. Jap. Soc. scient. Fish, 46, 1363-1367.
- Matsuno T., Katsuyama M., Iwashi M., Koike T. and Okada M. (1980a) Intensification of color of red Tilapia with lutein rhodoxanthin and spirulina. Bull. Jap. Soc. scient. Fish. 46, 479-482.
- Miki, W., Yamaguchi, K., Konosu, S., Takane, T., Satake, M., Fujita, T., Kuwabara, H., Shimeno, S., and Taked, M., 1985. Origin of tunaxanthin in the integument of yellowtail (*Seriola quinqueradiata*), Comp. Biochem. Physiol, Vol. 80B No. 2 pp. 195-201.
- Miki W., Yamaguchi K., Konosu S. and Watanabe T. (1984) Metabolism of dietary carotenoids in eggs of red sea bream. Comp. Biochem. Physiol. 77B, 665-668.
- Moore, T., (1957) "Vitamin A". Amsterdam, Elsevier N.Y.
- Morton, R.A. and Greed, R.H. 1939 Biochem. J. 33, 318-324.
- Morton, R.A. (1953). Spectroscopic properties of vitamin A₂, analyst vol. 78 pp. 74-79.
- Neamtu, G., Tamas, V. & Bodea, C. (1966) Die carotenoids aus Einigen Adonis asten. Rev. roum. Biochem. 3, 305-310.

- Olson, J. 1964a, The biosynthesis and metabolism of carotenoids and retinol (vitamin A), *J. of Lipid Res.* [5], p. 281-299.
- Olson, J. 1964b, The effect of bile and bile salts on the uptake and cleavage of β -carotene into retinol ester (vitamin A ester) by intestinal slices, *J. of Lipid Res.* [5], p. 402-408.
- Peterson, D.H., Jager, H.K., Savage, G.M., Washburn, G.N. & Westers, H. (1966) Natural coloration of trout using xanthophylls. *Trans. Am. Fish Soc.* 95(4), 408-414.
- Quackenbush, F., Firch, J., Rabourn, W., McQuistan, M., Petzold, E., and Kargi, T., 1961, Analysis of Carotenoids in Corn Grain, *Agricultural and Food Chemistry*, V. 9(2) pp. 132-135.
- Saito, A. & Regier, L.W. (1971) Pigmentation of rainbow trout by feeding dried crustacean waste. *J. Fish., Res. Bd. Canada* 29, 509-512.
- Schachter, D., Dowdie, E., and Schenker, H., 1960, Accumulation of ^{45}Ca by slices of small intestine. *Am. J. Physiol* 198, 275-279.
- Schiedt, K., Vecchi, M., and Blinz, E., 1986. Astaxanthin and its metabolites in wild rainbow trout (*Salmo gairdneri*, Comp. Biochem. physiol. v. 83b No. 1 pp. 9-12.
- Schiedt, K., Leuenberger, F.J., Vecchi, M. & Glinz, E. (1985) Absorption, retention and metabolic transformation of carotenoids in rainbow trout, salmon and chicken. *Pure & Appl. Chem.* 57, 685-692.

- Schmidt, P.J. & Baker, E.G. (1969) Indirect pigmentation of salmon and trout flesh with canthaxanthin. J. Fish. Res. Bd. Canada 26, 357-360.
- Seybold, A. & Goodwin, T.W. (1958) Occurrence of astaxanthin in the flower petal Adonis annua L. Nature 184, 1714-1715.
- Simpson, K.L., Katayama, T. & Chichester, C.O. (1981) Carotenoids in Fish Feeds. In Carotenoids as Colorants and Vitamin A Precursors. (Edited by Bauernfeind, J.C.) pp. 463-538. Academic Press, N.Y., London.
- Sire, M.F., Lutton C. and Vernier, J.M. (1981). New views on intestinal absorption of lipids in teleostreans fishes: an ultra structural and biochemical study in the rainbow trout. J. Lipid Res. 22 (81-94).
- Smith, R., Lane, C., (1971). Amino acid transport by the fish intestine. Exp. in Physiol. and Biochem. 4, 93-103.
- Spinelli, J., Lehman, L. & Weig, D. (1974) Composition, processing and utilization of red crab (Pleuroncodes planipes) as an aquacultureal feed ingredient. J. Fish. Res. Bd. Canada 31, 1025-1029.
- Sporn, M., Roberts, A., Goodman, D., 1984, The Retinoids, Academic Press, N.Y.
- Steel, R.E. (1971) Shrimp processing waste as a pigment source for rainbow trout, Salmo gairdneri. Master's Thesis. Oregon State University. Department of Food Science and Technology.
- Steven, D.M. (1947) Carotenoid pigmentation of trout. Nature 160, 540-541.

- Storebakker, T., Foss, P., Austreng, E. & Liaaen-Jensen, S. (1985)
Carotenoids in diets for salmonids II. Epimerization studies
with astaxanthin in Atlantic salmon. Aquaculture 44, 259-269.
- Straub, O. (1976) Key to Carotenoids. List of Natural
Carotenoids Birkhauser, Basel.
- Tanaka, Y., (1978). Comparative biochemical studies on carotenoids
in aquatic animals Mem. Fac. Fish. Kagoshima Uni. 27, 355-
422.
- Tanaka, Y., Katayama, T., Simpson, K., 1981. Stability of
carotenoids on silica gel and other adsorbents, Bulletin of
the Japanese Soc. of Sci. Fisheries 47(6) pp. 799-811.
- Tanaka, Y., Simpson, K., and Chichester, C.O. (1976b) The
biosynthesis of astaxanthin-XX. The carotenoids in marine
red fish and metabolism of the carotenoids in red sea bream,
Chrysophyrus major Temminch and Schlegel. Bull. Jap. Soc.
scient. Fish, 12, 1177-1182.
- Tanaka T., Katayama T., Simpson K.L. and Chichester C.O. (1976a)
The biosynthesis of astaxanthin-XIX. The distribution of -
doradexanthin and the metabolism of the carotenoids in
goldfish. Bull. Jap. Soc. scient. Fish. 42, 885-891.
- Thommen H. (1971) Carotenoids (Edited by Isler O.), pp. 637-656.
Birkhauser Verlag. Basel und Stuttgart.
- Tischer, J. (1938) Über die Carotinoides von Haematococcus
pluvialis II. (Carotinoide der Subwasseralgen V. Teil) Z.
Physiol. Chim. 252, 225-233.

- Tischer, J. (1941) Über die Caroteinoide von "Euglenarhodon" mit Astacin (Carotinoide der Subwasseralgen VIII. Teil) A. Physiol. Chim. 267, 281-284.
- Torrissen, O. and Braekkan, O.R., 1979. The utilization of astaxanthin forms by rainbow trout (Salmo gairdneri) Proc. World Symp. Finfish Nutr. Fishfeed Technol., Hamburg, 20-23 June 1978. Vol. II. Heenemann, Berlin, pp. 377-382.
- Torrissen O., Tidemann, E. Hansen, F. and Raa, J., 1981. Ensiling in acid - method to stabilize astaxanthin in shrimp processing byproducts and improve uptake of this pigment by rainbow trout (Salmo gairdneri). Aquaculture, 26:77-83.
- Torrissen, O. & Braekkan, O.R. (1979) The utilization of asntaxanthin forms by rainbow trout (Salmo gairdneri). Proc. World Symp. Finfish Nutr. Fishfeed Technol. Hamburg, vol. II, pp. 377-383. Heememan, Berlin.
- Ward, W.E. 1968. The uptake of amino acids by intestinal segment of the trout, salmo gairdnerii. Ph.D. Thesis of College of Arts and Sciences, Kent State University. Ohio, U.S.A.
- Weinreb, E.L., and N.M. Bilstad (1955). Histology of digestive tract and adjacent structures of the rainbow trout (salmo gairdnerii). Copeia (3): 194-204.
- Wilson, T.H. and Wiseman G. (1954). The use of Sacs of everted small intestine for study of the transference of substance from the mucosal to serosal surface. J. Physiol, Lond. 123, 116-125.

- Weedon, B. C. L (1971). In "Carotenoids" (Isler, ed.) pp. 29-59.
Birkhaeuser, Basel.
- Williams, S.R., 1977. Nutrition and Diet Therapy. 3rd Ed. The
C.V. Mosby Company, Saint Louis.
- Yamamoto, T. (1966). An electron microscopic study of the
columnar epithelial cell in the intestine of fresh water
teleosts; gold fish (*carascius auratus*) and rainbow trout
(salmo irrideus). Z. Zellforsch, Mikrosk Anat. 72, 66-87.
- Yamamoto. H.Y. Chichester, C.O. and Nakayama, T. 1962.
Biosynthetic origin of oxygen in the leaf xanthophylls Arch.
Biochem. Biophys. 96:645.