Title: Transformation of astaxanthin to vitamin A by ocular tissue of the rat in vitro.
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During the course of previous research, it has been shown that fish are capable of utilizing astaxanthin as a precursor of vitamin A. In effect, the administration of this carotenoid to *Gambusia holbrooki* Grd leads, in a deficient animal, to the neoformation of detectable retinol in the intestinal mucosa, liver, and eyes (1,2).

(1) R.Grangaud and R. Massonet, C.R.Acad.Sc., 1955, v.241, p. 1087.
(2) J.P.Moatti, State Doctoral Thesis (Pharmacy), Algiers 1959.

Mammals do not have the same ability as fish with regards to astaxanthin which is not a real provitamin for them: administered to the vitamin A deficient white rat, astaxanthin limits its action to the ocular apparatus, manifesting properties that we can consider to be selectively antixerophthalmic (3,4,5). This topographically-limited activity is, in all aspects, comparable to that which vitamin A exercises in the eye and its appendages.

It was therefore logical to try to interpret, on the biochemical level, the yet unknown mechanism of the astaxanthin action among rats, to wonder if the latter would be limited to the ocular tissue, as in the conversion ability witnessed among fish.

A short note (6) summarizes results of experiments which were conducted in vivo and in vitro and which support the verification of that hypothesis.

The objective of the present work is to describe, in detail, the *in vitro* experimental techniques showing the reality of the conversion of astaxanthin into vitamin A by the rat's ocular tissues.

The concept of these experiments was to incubate, in a complex medium of vitamin A deficient rat eyes in the presence or absence of astaxanthin diacetate. The respective concentrations of vitamin A of the eyes placed in contact with the carotenoid and the control eyes were then determined.

EXTRACTION OF THE ASTAXANTHIN AND PREPARATION OF DIACETATE. -- In order to avoid the possible interference of natural provitamin A or pre-formed retinol, the following technique was used for the preparation of the astaxanthin diacetate: carotenoid was extracted from the inner wall of stomach pockets of two large red penaeid shrimp (*Aristeomorpha faliacea* and *Aristeus antennatas,* Risso) where there is, in the form of a blue chromoprotein whose physico-chemical characteristics closely belong to those of the crustacyanin studied by Wald (7). This chromoprotein is, in effect, soluble in water and it is easy to detach it from the prosthetic group. The spectral characteristics of the latter (λmax = 492 mμ in pyridine) show that it is completely transformed astaxanthin. After dissection, the sampled pockets of 5 kg of shrimp were emptied of their contents, agitated with 100 ml of distilled water; the limpid solution was diluted with 4 times its volume of acetone; under these conditions, the astaxanthin was detached from its protein copula and the color of the solution immediately turned orange-red. By an addition of 50 ml of petrol ether and 100 ml of water, the pigment was extracted in the light phase which was separated by decantation and dried on anhydrous sodium sulfate. The solution was then filtered in a magnesium oxide column (200 mm in length, 20 mm in diameter). Astaxanthin was adsorbed in the upper part of the column (over a 2 cm length)

(3) R.Grangaud and R. Massonet, C.R.Acad.Sc., 1948, v.227, p.568.
(4) R.Grangaud and R. Mnssonet, C. R. Acad. Sc., 1950, v.230, p.1319.
(5) R.Grangaud, Doctoral Thesis in Physical Sciences, Lyon, 1950.
(6) R.Grangaud, R.Massonet, Th.Conquy, and J.Ridolfo, C. R. Acad. Sc., 1961, v.252, p.1854.
(7) G.Wald, N.Nathanson, W.P.Jencks and E.Tarr, Biol. Bull., 1948, v.249, p.95.

which was washed with 50 ml of hexane diluted with 1 ml of acetone. The purpose of this washing was to eliminate the vitamin A carotenes and esters, if they were present. A second washing was performed using a mix of 92 ml of hexane and 8 ml of ethanol to eliminate possible traces of retinol (8). The column was then sectioned: the pigmented zone was immersed in 20 ml of pyridine which immediately induced elution.

The red solution was concentrated under reduced pressure and in an inert atmosphere to a volume of 2 ml. XL drops of acetic anhydride were added and the entire mix was left for 9 hours at ambient temperature. 20 ml of petrol ether and 40 ml of water was then added. The light phase was separated by decantation, washed with distilled water then dried on anhydrous sodium sulfate. The solution was again chromatographed but this time on aluminum oxide (column of 200 mm in length, 20 mm in diameter) which was processed the same as on magnesium oxide by successively passing the hexane-acetone mix. The pigment was eluted in pyridine and, finally, treated with 3 ml of hot pyridine. After addition of 1 ml of water and 24 hours of rest at 0°C, a crystalline precipitate was obtained which was then subjected to two re-crystallizations. The crystallized diacetate was dispersed in Tween 80 at a concentration of 1 mg/ml (9).

EXPERIMENTAL PROTOCOLS AND RESULTS. – 12 Wistar rats, weighing exactly 32 grams, were weaned and subjected to the previously-described synthetic regime (10). After 40 days, the signs of deficiency were being manifested (weight gain termination, beginning of xerophthalmia), the animals were decapitated and the eyes were immediately removed: the right eyes of 6 of the subjects and the left eyes of the other 5 (Lot 1) were placed in a small colloidion bag which already contained 1 ml of fresh blood serum and 1 milli-mole of α-tocopherol dispersed in 1 ml of water. The 12 remaining eyes (Lot II) were placed in a second bag containing the same mix but diluted with 1 ml of astaxanthin dispersion. The two bags were immersed in a Krebs-Ringer buffered solution with a pH of 7 and maintained in an oven at 37°C for 12 hours. At the end of this time, the eyes of each lot were saponified in 1 ml of alcoholic potassium hydroxide at 60 pp 100, at 80°C for 15 minutes. The unsaponifiable [components] were treated by petrol ether, washed, evaporated using chloroform and, in each chloroformic extract, the search for and dosing of vitamin A was performed using the Carr and Price reaction, the Meunier and Raoul kinetic technique (11), and measurements were made using a photocolorimeter. The following results were obtained: the Lot I eyes

(8) S.Y.Thompson, J.Ganguly and S.K.Kon, Brit. J. Nutr., 1949, v.3, p57.
(9) The technique of preparation is identical to that described by J.G.Bieri (J.Nutrition, 1951, v.44, p.2) for obtaining a dispersion of β–carotene.
(10) R. Massonet, Doctoral Thesis in Natural Sciences, Lyon, 1958.
(11) P.Meunier and Y.Raoul, Diagnostic chemistry of Vitamin Deficiencies. Masson and Co., Paris 1942.

(control) contained 0.75 µg of vitamin A, the Lot II eyes (incubation in the presence of astaxanthin) contained 1.65 µg.

In parallel to this experiment, in order to verify that the prepared astaxanthin diacetate as it was described did not contain carotene or vitamin A, the following control was again performed: two lots of deficient rat intestines were incubated in identical conditions to those of the aforementioned experiment. 1 mg of β-carotene, dispersed in 1 ml of Tween 80, was added to one of the incubation liquids. To the other was added 1 mg of astaxanthin diacetate in the same state of dispersion. Later analysis performed on the unsaponifiable [components], using a general technique, revealed the presence of vitamin A, neo-formed in the lot into which the β-carotene had been added; no trace of retinol was detected in the lot incubated in the presence of astaxanthin diacetate.

Conclusion. – The conclusion which we gain from these experiments is, therefore, that the neo-formed vitamin in the eye can only be attributed to a transformation of astaxanthin. It is likewise concluded that it is the retinal tissue which is the base of the reaction. Additional experiments were undertaken to obtain more detailed information.

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