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FIRST THESIS:

RESEARCH ON ASTAXANTHIN'S BIOCHEMISTRY

SECOND THESIS:

PROPOSALS GIVEN BY THE SCHOOL

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Mr. CORDIER……President
MENTZER……Examiner
CHOPIN……Examiner
DESSAUX……Examiner

LYON

1958

RESEARCH ON ASTAXANTHIN'S BIOCHEMISTRY

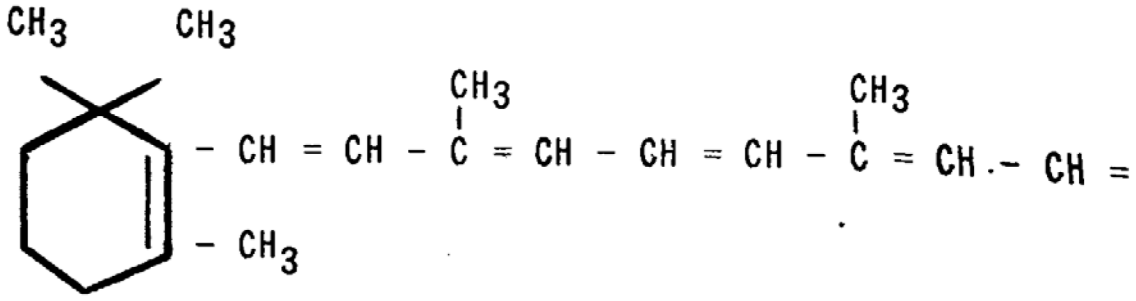
INTRODUCTION

… biological science can often find, by the consideration of the majority, the rigorous nature that it seems at first to lose as a result of individual fluctuation

André DOGNON

The studies on vitamin A deficiency have for a long time determined that among the signs of deficiency, the most constant and most apparent expressions were the arrested weight growth and the damage in the eye and its related organs; the administration of vitamin A or a substance having vitamin A activity results in the deficient animal in the recovery of weight followed by the receding and curing of the eye injuries. This double nature should make vitamin A be referred to as anti-xerophthalmic, growth-promoting vitamin.

When the structure of vitamin A (or vitamin A or retinol) was elucidated in the light of Karrer's work, it appeared that the vitamin activity was very closely related to the chemical composition, and it is a typical notion today that all factors which may prevent or cure the deficiency are chemically related to vitamin A. We can even add that they only have vitamin activity to the extent that the body is capable of converting them into Vitamin A: all substances having in common with retinol the property to restore growth and heal xerophthalmia in the vitamin A deficient animal, have in fact in their molecular structure the axerophthyl group:



that is, a potential vitamin A molecule (*).

Revealing in the Risso Aristaeomorpha foliacea hepatopancreas oils a substance that - at least at certain doses - shows in the vitamin A-deficient white rat a selectively anti-xerophthalmic action, as well as the demonstration that the substance involved is astaxanthin (83), an oxygenated-nucleus carotenoid considered as lacking vitamin activity, should for sure give rise to significant biochemical problems.

(*) Vitamin A of fresh water fish has, however, a double additional bond at 3-4.

Their study has been the objective of research work carried out for over ten years in the biological chemistry laboratory of the Algiers's School of Medicine and Pharmacy. I had the pleasure of joining that research from the beginning and this work integrates into them as a whole. As a matter of fact, there is no doubt it was of great importance to gather in a paper the information, that until now was quite fragmented, on the materials examined, the biological test methods, the results that establish the dissociation between the anti-xerophthalmic activity and the action on growth.

On the other hand, although it is very true that the action on weight growth is nil in the deficient white rat for daily doses from 5 to 10 µg astaxanthin, this is no longer the same when the amount administered is three or four times as big. Then, a normal growth is obtained without the least addition to the vitamin or pro-vitamin A diet. Therefore, it is possible to study a true vitamin A deficiency in the adult animal and to explore in particular the troubles of the reproduction functions.

It was also interesting to specify the localization of the pigment in the body of the treated rat, to conduct the anatomical and pathological study of the animals in the experiment and to investigate, particularly in the liver, whether the administration of astaxanthin causes the build-up and storage of vitamin A. Our results show that this is not at all the reason that leads to attribute to astaxanthin a specific activity.

In addition, astaxanthin, the main shellfish pigment, is plentiful in fish food. In the second part of our work we have studied the effects of pigment administration to a small fresh water Cyprinodontidae, Gambusia holbrooki Grd, We could see the neo-formation of vitamin A from astaxanthin, that appears then as a pro-vitamin A for Gambusia holbrooki.

PART ONE

CHAPTER I

MATERIALS AND METHODS

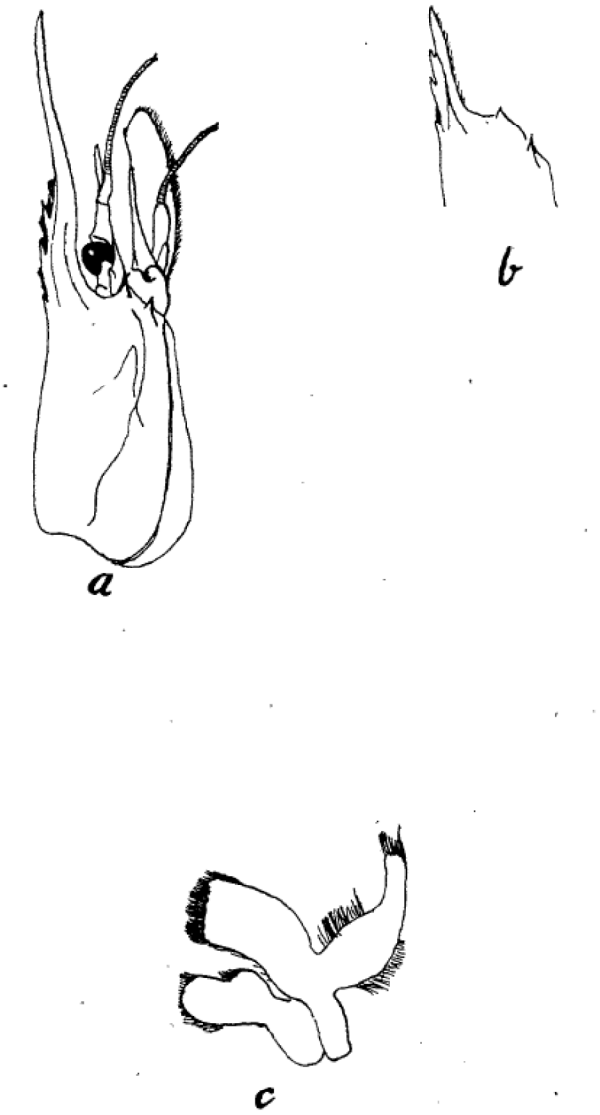


FIG. 2 - Aristeus antennatus - RISSO
a) Carapace of the female
b) Rostrum of the male
c) Maxilla I

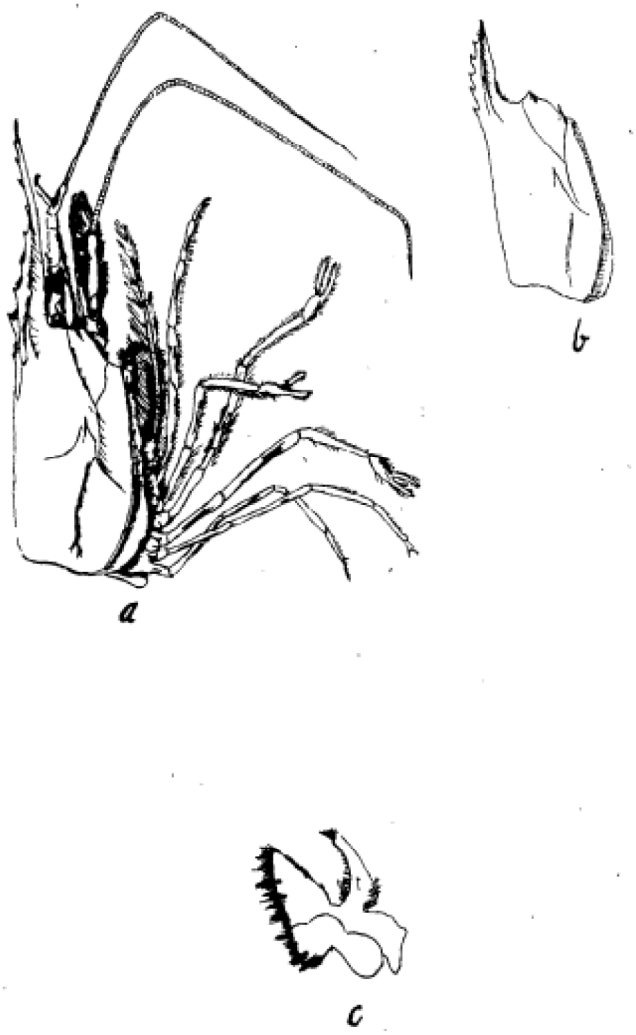


Fig. 1 - Aristaeomorpha foliacea – RISSO
a) Cephalothorax of the female
b) Rostrum of the male
c) Maxilla I

MATERIAL UNDER STUDY

The shellfish species that provided the pigments examined belong to the Decapoda order. They are:

- three Penaeidae:

    Aristaeomorpha foliacea, Risso

    Aristeus antennatus, Risso

    Parapenaus longirostris, H. Lucas;

- one Pandalidae:

    Plesionika edwardsii, Brandt;

- one Scyllaride:

    Scyllarus latus, Latr.

As a comparison, we made some determinations on Squilla Mantis, Latr, that is a stomatopod, and we thought we should report the literature data relating to the vitamin A content of various Euphausiacea species.

A - GEOGRAPHIC DISTRIBUTION ECOLOGICAL DATA ([1])

- Aristaeomorpha foliacea, Risso.

Apart from the Algerian coasts, this shrimp is found in some places of the Mediterranean Sea (Sardinia, Sicily) and in the Atlantic Ocean it is found along the Moroccan coasts. It seems not to migrate beyond 400 meters. The trawlers that bring large amounts of Aristeus antennatus from bottoms of 300, 350 and 400 meters, fish very small amounts of Aristaeomorpha foliacea.

- Aristeus antennatus, Risso

This animal seems quite localized in the Mediterranean Sea, the temperate and subtropical regions of the Atlantic Ocean from Portugal to Cape Verde islands. This is a meso-abyssal species. The trawlers fishing in the Algerian coasts catch plenty of this fish between 300 and 400 meters and only at these depths.

- Parapenaus longirostris, H. Lucas

Very common in the Mediterranean Sea, this species is captured off Algiers and Oran, between 200 and 300 meters. It is plentiful in Morocco between 200 and 400 meters (Gruvel) (96). It can also be found from 70 to 80 meters.

([1]) These short reminders were essentially borrowed from Argilas (2) and Dieuzeide (43).

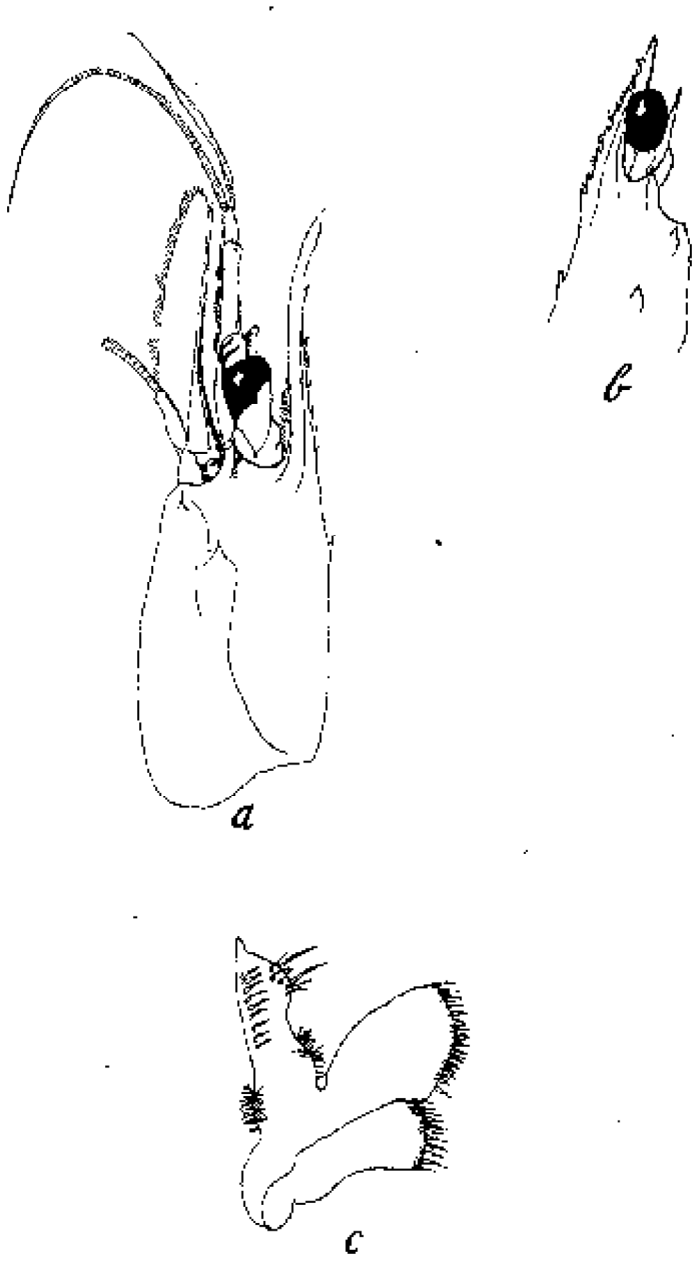


Fig. 3 - Parapenaeus longirostris - H. LUCAS

a) Carapace of the female
b) Rostrum of the male
c) Maxilla I

- Plesionika edwardsii, Brandt;

This species is caught with Aristeus antennatus and Aristaeomorpha foliacea in bottoms between 200 and 500 meters.

- Scyllarus latus, Latr.

This species found in the Mediterranean Sea is also found in the Atlantic Ocean; it lives in the vicinity of bottoms between 4 and 10 meters.

- Squillidae (Squilla mantis, Latr.) lives on sandy but mainly silty bottoms in the vicinity of the littoral and hardly descends beyond 100 meters. It is a carnivore that lives a fossorial life but hunts other shellfish during the night.

B - MORPHOLOGY

The first comprehensive work on the morphology of Penaeidae was conducted by Bouvier (10). Argilas (2), directed by Boutan, specially studied the species of the Algerian coasts. Dieuzeide (43) wrote a monograph on these same species. The data in connection with Pandalidae were borrowed from his research on the fauna of bottoms suitable for trawling in the Castiglione bay (44).

- Aristaeomorpha foliacea is a large-sized shrimp that reaches 20 centimeters in length. It is easy to recognize through the features of the rostrum that has 9 dorsal teeth in the female and 6 in the male (Figure 1). Aristaeomorpha foliacea is dark red with purplish reflections. Unequal pigmentation is stronger in the branchial region and the marginal region of the lower portion of the carapace. The red pigment is localized in the dermis chromatophores the pseudopods of which extend to the outer layer of the carapace or epicuticle. Under the carapace there is an epidermal layer topping the dermis (*); a conjunctival membrane separates the dermis from the central cavity. The stomach pouch (formed by the union of a cardiac pouch and a pyloric pouch) and the intestine are totally enveloped by a strongly pigmented conjunctival sheath. This red pigment is also found in the branchiae and appendices (antennules, antennae, maxillipeds, maxillas).

If the red connective tissue that envelops the stomach pouch is removed by scratching, the stomach pouch can be seen as a blue membrane, verging on mauve. The genital glands that are initially whitish gradually become colored following the development of the eggs; grayish in the first stage, the eggs change to violet upon maturation.

- Aristeus antennatus, Risso: it is generally smaller than Aristaeomorpha in size; however, some specimens that reach 20 centimeters can be found. The rostrum has three teeth on the dorsal edge in both sexes; this clearly differentiates Aristeus antennatus from Aristaeomorpha foliacea (Figure 2).

(*) The whole is improperly referred to as hypodermis.

Aristeus antennatus is washed red in color, with purplish stains in the cephalothorax carapace and the abdomen. In the Aristeus antennatus the eggs pass from cyclamen pink to clear mauve upon maturation.

- Parapenaeus longirostris, H. Lucas: this species is clearly smaller than the two preceding species. Its size is 8 to 10 centimeters in average. Its rostrum has 8 teeth and shows slightly marked sexual differences (Figure 3). It is a pale pink shrimp. Like in the two other Penaeidae, a red-pigmented conjunctival sheath is found that envelops the gastric pouch. The genital glands and their content are blue-greenish in color the intensity of which increases with the development of the eggs.

- Plesionika edwardsii, Brandt (former Pandalus narval H. Milne-Edwards): This is a small-sized, pale-pink colored Pandalidae; it is recognized by the length of its rostrum and the teeth thereof (Figure 4).

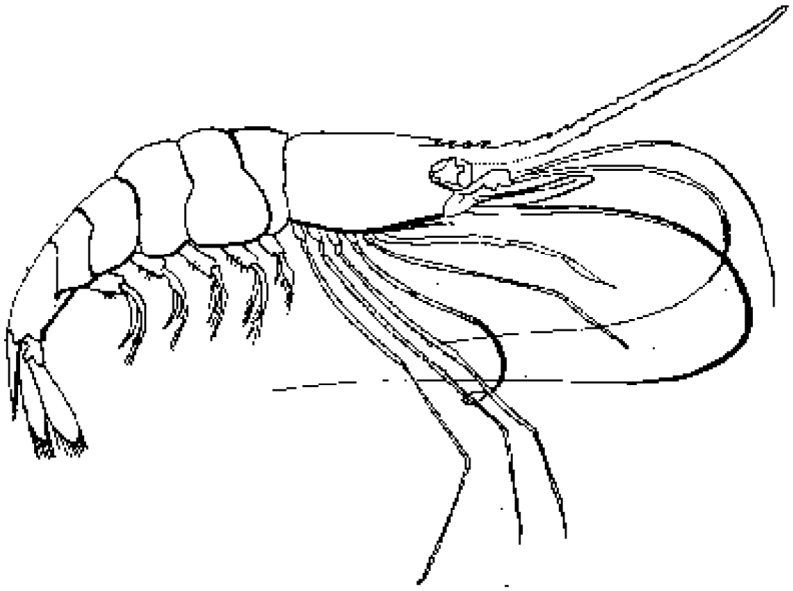


FIG. 4 - PLESIONIKA EDWARDSII, Brandt

Unlike the Penaeidae, Pandalidae carry their eggs; they are in the form of a quite characteristic brilliant blue granular mass. The females are ovigerous from January to May.

- Scyllarus latus, Latr. is a Decapoda species that reaches 30 to 40 centimeters in length. It is brown red in color. The female carries its eggs.
- Squilla mantis, Latr. is a Stomatopod sized from 12 to 15 centimeters. The abdomen is very large in proportion to the cephalothorax; the carapace, relatively short, covers only the first four thoracic segments. The rostrum is comprised of a small articulated plate. The thoracic appendices are quite particular; the second pair of pereiopods is represented by a large-sized biting claw. The orangey red pigmentation is very unequally distributed: certain items are strongly pigmented while other items are colorless. The thoracic segments are also more intensely colored than the abdominal segments. The pigment distribution at the gastric pouch is very different from the Penaeidae: the red conjunctival mass is only present at the pouch base. The eggs, plentiful, are retained by the female in the first abdominal appendices.

All these species have plenty of pigments. The study below will show the close relationship between those pigments.

C - BIOLOGICAL STUDY

The main carotenoid pigment of shellfish was successively described under the names of crustaceo-rubin (117), zooerythrin (165), vitellorubin (150), tetronerythrin (148), haematochrome (104) and finally astacene (or astacin) (131). However, Kuhn and Sorensen (132) show that most of the extraction processes cause an alteration and that astacene is actually a product of the oxidation of the natural pigment that has been named astaxanthin.

The chemical composition of astaxanthin determined by Karrer's works (120) shows that it is the 3,3'-dihydroxy-4,4'-diketo-β-carotene. Tischer (225) was the first to establish the spectral properties (broadband spectrum with a single maximum located at λ = 492 nm in pyridine) (*).

Astaxanthin, which is wide spread in nature, is found in a considerable number of animal and vegetable species (133), (121), (161), (67). Among shellfish, the pigment exists both in a free, esterified form and as differently colored chromoproteins such as the egg ovoverdin (217) and the crustacyanin of the lobster carapace (237). In all the species we have studied, we have identified astaxanthin and evidenced the presence of the three forms. Before reporting their physical and chemical properties (cf. p. 27), it is appropriate to discuss another important matter: the possible presence of carotenes and vitamin A in shellfish.

1. VITAMIN A AND CAROTENE IN SHELLFISH

a) VITAMIN A

History

The issue of the possible presence of vitamin A in shellfish deserves in fact to be examined with the greatest care, because it can allow addressing the general problem of the origin of this vitamin factor in the animal kingdom.

Lederer (136), Euler, Hellstrom and Klussmann (53) did not find any vitamin A in Copepods; Kon and Thompson (127) (128), Fisher, Kon and Thompson (59) obtained the same negative result on three Copepod species, as well as on two Amphipod species and one Cladocera species.

Wald (234) was the first to show the presence of vitamin A in the lobster eyes. Fisher, Kon and Thompson (60) found vitamin A in fourteen Decapoda species.

(*) The three-maximum spectrum (476, 493 and 513 mμ in pyridine) described by Kuhn, Stène and Sorensen (133) is, according to Wald (235), certainly marred by mistakes.

The Euphausiacea, an order close to Decapoda, was thoroughly studied by Kon (124), Fischer et al. (58) (60). In fact, the Euphausiacea are plentiful in Krill that is eaten by whales. Both in the fished species and those taken from the stomach, the vitamin A contents proved considerably higher than in all the other shellfish examined. The Table below (Table I) reproduced according to Kon, emphasizes the fact that, from the standpoint of their vitamin A content, the Euphausiacea are in a quite dominating position.

Insofar as the localization is determined precisely, almost all the vitamin is found in the eye (Fischer Kon and Thompson (58) (60), Batham, Fischer, Henry, Kon and Thompson (5), Fischer, Kon, Plack and Thompson (61)). Tables II, III, IV are illustrative in this regard.

TABLE I

(According to Kon (126))

Vitamin A content of marine shellfish

Species or groups	µg per g	Locations	Common name
Meganyctiphanes norvegica	78	A.M.	Euphausiacea
Thysanoessa raschii	51.3	A	*
Thysanoessa inermis	16.5	A	*
Thysanoessa gregaria	14.1	A	*
Stylocheiron elongatum	3.9	A	*
Stylocheiron maximum	3.9	P	*
Euphausia pacifica	3.3	P	*
Nematoscelis difficilis	3.3	P	*
Thysanoessa spinifera	2.8	P	*
Onisimus plautus	2.8	A	Amphipod
Euphausia superba	2.0	An	Euphausiacea
Syrrhoe crenulata	0.87	A	Amphipod
Caridea (17 species)	0.81	A,P,M	Shrimps
Anomura (7 species)	0.3	A,M	Hermit crabs
Penaeidae	0.15	A,M	Shrimps
Isopoda (3 species)	0.09	A,M	Isopods
Copepod (3 species)	0.09	A	Copepods
Brachyuran (9 species)	0.03	A,P,M	Crabs
Astacura (2 species)	0.02	A	Lobsters
Mysidacea (5 species)	none	A,M	Mysidacea
Branchiopoda	none	A	Branchiopods

An: Antarctica P: Pacific Ocean

A: Atlantic Ocean M: Mediterranean Ocean

TABLE II

(According to BATHAM et al. (5))

Vitamin A distribution in Euphausiacea and Decapoda organs

Species	Average weight of a specimen		Vitamin A contained in a specimen		Vitamin A of eyes per 100
	Whole body	Eyes	Whole body	Eyes	
	mg	mg	µg	µg	
Meganyctiphanes norvegica	60.0	2	0.33	0.3	99
Meganyctiphanes norvegica	280.0	4	1.3	1.26	99
Meganyctiphanes norvegica	400.0	6	4.4	4.3	98
Meganyctiphanes norvegica	500.0	9	7.5	7.35	98
Thysanoessa raschii	60.0	1	1.65	1.6	98
Carcinus maenas	4,100.0	10	0	0.45	100
Eupagurus benhardus	4,100.0	10	0	0.15	100

TABLE III

(According to KON (126))

Vitamin A distribution in Euphausiacea and Decapoda organs

Species and organs	Vitamin A		Total amount of vitamin A in the eyes
	per organ µg	per g organ µg	
Thysanoessa raschii			
Body less the eyes	0.03	0.15	---
Eyes	0.96 pair	363.0	97
Meganyctiphanes norvegica			
Body less the eyes	0.15	0.36	---
Eyes	2.45	429.0	94
Crangon allmani			
Body less the eyes	0.05	0.15	---
Eyes	0.06	36.3	55
Eupagurus bernhardus			
Body less the eyes	0	0	---
Eyes	0.36	20.5	100
Nephrops norvegicus			
Body less the eyes	0.54	0.009	---
Eyes	0.36	0.84	40
Cancer pagurus			
Body less the eyes	0.3	0.03	---
Eyes	0	0	0
Maja squinado			
Body less the eyes	0	0	
Eyes	0.63	21.6	100

TABLE IV

(According to KON (126))

Vitamin A distribution in Meganyctiphanes norvegica (Euphausiacea) organs

Organs	Average weight in mg	Lipids per 100	Vitamin A in g. per organ	Vitamin A in µg per g
Hepatopancreas	22	18	0	0
Stomach	3	8.6	0	0
Eyes (pair)	4	3.6	0.66	166
Rest of body	249	2.1	0.03	0.12
Total	278	3.4	0.69	2.46

The examination of these results shows that, apart from the Euphausiacea characterized by exceptional contents, vitamin A exist in the other crustaceans only as traces (0.15 µg per gram in Penaeidae, cf. Table I) or is completely absent. When it is present, it is substantially concentrated in the eye; vitamin A of the body is almost totally contained in this organ.

Experimental section

Materials and methods

Vitamin A assessment and determination are conducted using the Carr-Price reaction (19) with antimony trichloride in a chloroform solution. The absorption is measured using the Beckman spectrophotometer by monitoring decolorization as a function of time using the Meunier and Raoul (167) method (*). For series measurements, a photo colorimeter with photocell was also used; experimental controls allowed verifying that the sensitiveness and fidelity of the device allowed determinations of sufficient accuracy.

The method was designed for assessing vitamin A in the Aristaeomorpha foliacea hepatopancreas. The operating method that will be described is valid for all the other organs and tissues. If variants should be introduced, they will be quoted with regard to every particular case. However, it is worthy of note now that each extract was controlled using UV spectrophotometry.

(*) The measurement was made at λ = 620 nm ($E^{1\%}_{1\,cm}$ = 5070)

ARISTAEOMORPHA FOLIACEA

Hepatopancreas

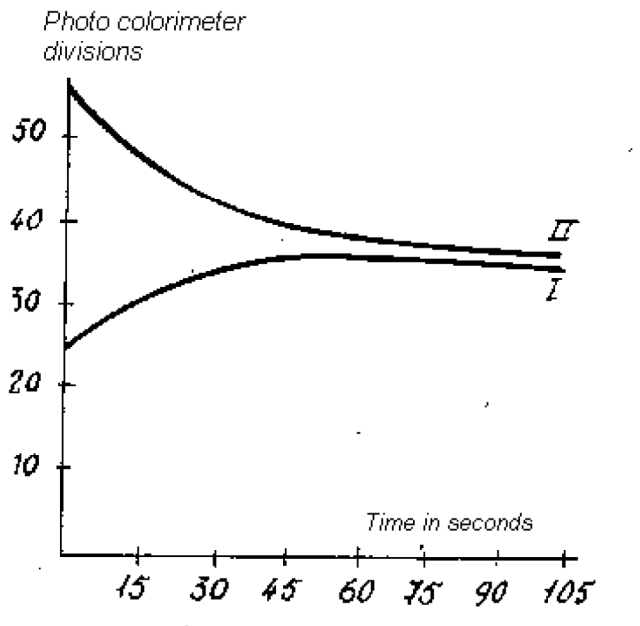
Saponification: 10 g hepatopancreas were saponified using a 60 per 100 (1 mL per gram tissue) aqueous solution of potash according to the Lewis and Bodansky (140) method. Saponification continued in a water bath for 20 minutes under a nitrogen atmosphere. The content of the flask was fast cooled and taken up with an alcohol-water (1 : 2) mixture and extracted three times with petroleum ether. The -petroleum ether solutions gathered, washed with distilled water, then with aqueous potash at 3 per 100 and again with distilled water (50, 50 and 50 mL distilled water), were dehydrated by contact with anhydrous sodium sulfate. The -petroleum ether solution was finally evaporated under partial vacuum under nitrogen atmosphere. The residue was taken up by 1 mL of rectified chloroform and kept on calcium chloride.

Carr-Price reaction: the chloroform solution is added 0.5 mL acetic anhydride, then 5 mL Carr and Price reagent. A color is developed, the absorption of which is measured at the 15th second, then every 30 seconds until 2 minutes. The kinetic curve is built and graphic extrapolation to zero time is performed.

Results: it can be seen right away that neither the color nor the kinetics are typical of vitamin A (168). As a matter of fact, a greenish color is obtained that turns pure green increasing its intensity, and the behavior of the curve alone shows that it cannot be vitamin A (Fig. 5 - curve I).

Experimental control (77). It consists in making the determination on the same oil to which known amounts of vitamin A were added.

30 µg vitamin A is added to 10 g hepatopancreas and the mixture is saponified in the above-described conditions. A clearly blue color is initially developed by the Carr-Price reaction, the downward kinetic curve is characteristic. The interpretation of this curve leads to the following results: at time 120 sec. the curve is no longer downward and shows a plateau (Fig. 5. - curve II).



If the plateau height is deducted (corresponding to base absorption), the absorption value, so corrected, corresponds only to vitamin A; thus, the value found is that of the amount introduced, apart from any experimental errors. This control confirms the results recorded above; vitamin A is absent from hepatopancreas.

Hypodermis

Vitamin A was searched in a 10 g test sample according to the above-described method.

No trace of vitamin A was detected.

Pyloric pouch

The peristomal connective tissue enveloping the pouch was removed by scraping and emptied of any content.

The pouch and the content were treated separately. Work is conducted on 5 g. the result is negative for the two samples.

Peristomal connective tissue

10 g of connective tissue were treated. A negative result was obtained.

Flesh

20 g were taken. The search was conducted on a sample collected from the cephalothorax and a sample from the abdomen. The results were negative.

Carapace

The search was conducted on 30 g. carapace finely ground. A negative result was obtained.

Intestine

A 5 g test sample was used, the Carr - Price reaction was positive, the vitamin A content is 6.0 to 6.6 µg.

However, an important remark must be made: the intestine content could not be emptied before saponification. Therefore it was not possible to determine whether the vitamin found belonged to the intestinal tissue or came from the content.

Eggs

5 g of eggs were treated: the Carr - Price reaction was weakly positive, 1.2 to 1.5 µg per gram of tissue.

Eyes

The above-described method was also used. For verification purposes, we removed the pigment by chromatography and then dosing was made in the usual conditions.

A 2g test sample was used: the Carr - Price reaction was clearly positive; the concentration of vitamin A is higher in the species fished during summer; for winter, the concentrations range from 1.8 to 2.5 µg per gram while for summer they range from 6 to 9.5 µg per gram (cf. Table V).

ARISTEUS ANTENNATUS

Vitamin A examined by the same method could be detected neither in the hepatopancreas nor in the hypodermis, the pyloric pouch (pouch and content treated separately), flesh and carapaces.

It was found in the intestine (not emptied) at 6 to 7.5 µg per gram: in the eggs, 1.2 to 1.3 µg, in the eyes, 2.4 to 4.8 µg (winter Aristeus), 6 to 9.6 µg per gram (summer Aristeus).

PARAPENAEUS LONGIROSTRIS

The results are negative for the hypodermis, the pyloric pouch, flesh, carapaces and eggs. Traces are present in the hepatopancreas; the values found in the intestine are 3 µg per gram (winter fishing), 4.5 µg (summer fishing); in the eyes, 7.5 µg per gram (winter fishing), 11.5 µg per gram (summer fishing)

PLESIONIKA EDWARDSII

The Carr – Price reaction in Plesionika edwardsii was positive only for egg extracts: however, only traces were found.

SCYLLARUS LATUS

Vitamin A was only searched in the eggs; 0.18 µg per gram were found.

All results of these determinations are shown in Table VI.

The examination of this Table shows that the vitamin is present only in the intestines, the eggs and the eyes. The values found in the eggs are very low. In the intestines, it was not possible to measure separately the vitamin in the content and in the organ, due to the small diameter of the latter. Therefore, one cannot presume whether the vitamin is present in the mucosa or in the digestive waste. However, it is worthy of note that it is absent from the content of the pyloric pouch; accordingly, one can think that it will not be present either in the intestine content. Nevertheless, a decisive conclusion could not be made from this argument alone.

It is interesting to report the results per gram of eye lipids. The content of lipids (extracted with the alcohol-ether mixture) is 1.2 to 1.5%, resulting in concentrations of 150 to 750 µg per gram of lipid in the eye tissue.

TABLE VI

VITAMIN A DISTRIBUTION (in µg per gram of fresh organ)

Organs or tissues											
	Month	Eyes		Hepato-pancreas	Pyloric pouch	Intestine and content	OVARIES		Flesh	Carapace	Hypo-dermis
							with eggs	without eggs			
Aristaeomorpha foliacea	Nov. Dec. Feb. May July	2.5 1.8 3.6 6.0 9.0	 7.8 9.5	0 “ “ “ “	0 “ “ “ “	 6.0 6.6	 1.2 1.5	0 0 0 	0 “ “ “ “	0 “ “ “ “	0 “ “ “ “
Aristeus antennatus	Nov. Jan. April June Aug.	1.8 1.8 5.4 6.6 6.0		0 “ “ “ “	0 “ “ “ “	 6.0 7.5 	 1.2 1.3	0 0 	0 “ “ “ “	0 “ “ “ “	0 “ “ “ “
Parapeneus longirostris	Dec. June	7.5 11.4			0 “	3.0 4.5	 “		 “	0 “	0 “
Plesionika edwardsii	---	---		---	---	---	traces		---	0	---
Scyllarus latus	---	---		---	---	---	0.18		---	---	---
Squilla mantis	March	18		---	---	---	---		---	0	---

b) CAROTENES

History

Euler, Hellstrom and Klussman (54) show that among three Calanus finmarchicus pigments isolated, one is from carotene. Lederer (136) found that this pigment is actually present simultaneously with astacine (astaxanthin). He found 0.4 mg per 120 mg of astacine in 500 grams of Copepods.

With regard to Decapoda, carotene traces were reported by Kuhn and Lederer (131), by Kuhn, Lederer and Deutsch (130) (Maja squinado) by Goodwin and Srisukh (127), (128) (Pandalus bonnieri, Crangon vulgaris).

For Euphausiacea, Wagner (232) had thought he could assert the presence of high amounts of carotene. This issue was important because the Euphausiacea constitute most of the Krill eaten by the whales, and it is known that large vitamin A reserves are found in whales. The Wagner's results could then lead to accept that carotene should be considered as the main source of supply of vitamin A for whales. However, the conclusions made by Wagner are disputable due to the identification procedures used. Kon (125) took up this study and, using chromatographic analysis and spectrophotometry, stated the almost total absence of carotenes in the Euphausiacea from the Arctic and Antarctic oceans, both in the material collected from whale stomach and in directly fished animals.

Experimental section

Methods

Identification of carotenes is essentially based on their behavior vis-à-vis the adsorbents and on their spectral features. The extraction method that leads to the dissolution of the pigments in petroleum ether is described above (cf. page 33).

The direct spectrophotometric examination of the -petroleum ether solution of organs or tissues does not reveal the 3-maximum system typical of carotenes (78). However, one can dread that the abundance of astaxanthin that absorbs over a neighboring range of wavelength avoids detecting the pigment if it is present only in small amounts. This is the reason why a chromatographic separation is essential. In fact, as shown by Lederer (136), carotenes are less strongly retained on an alumina column that the oxygenated carotenoid pigments of the astacine (or astaxanthin) type.

As a matter of fact, we have verified that when a -petroleum ether solution containing both astaxanthin (free or esterified) and carotenes is chromatographed on alumina, the carotenes slowly descend the column to finally appear in the filtrate, while astaxanthin is adsorbed in the upper portion of the chromatogram.

Not the least trace of carotenes was found in the extracts examined (Aristaeomorpha foliacea, Aristeus antennatus).

2 - ASTAXANTHIN IN SHELLFISH

As we have just shown, in the species studied, vitamin A is essentially localized in the eyes, while the other organs or tissues do not contain it, apart from mature ovaries where traces have been detected. With regard to carotene, it is totally absent. On the contrary, astaxanthin is found in large amounts, mainly in the Aristaeomorpha foliacea that is the most strongly pigmented species we have examined. It is also plentiful in Aristeus antennatus and, in these two Penaeidae, the pigment is mainly localized in the hypodermis and the peristomal connective tissue.

a) FREE, ESTERIFIED ASTAXANTHIN

Little information is available on the coexistence of the equilibrium relationships which could exist among the various forms in the different tissues or organs. In fact, for small shellfish, the extraction is made on the animal as a whole (Calanus finmarchicus, Lederer (136), Holopedium gibberum, Daphnia magna, Gammarus pulex), Sorensen (215).

With regard to Decapoda, their size generally allows studying the various tissues or organs separately: Kuhn and Lederer (131) show that the pigment extracted from the hypodermis of Homarus vulgaris and Nephrops norvegicus is in the esterified form, while in the carapace, the free and esterified forms coexist. According to Goodwin and Srisukh (69), taking up this study on the same species, the hypodermis pigment is esterified astaxanthin. However, according to them, the free form would be only present in the carapace. Therefore, these data are in part conflicting.

EXPERIMENTAL CONTRIBUTION

Materials and methods

a) dissection - For every species, the carapaces, hypodermis, peristomal connective tissue, hepatopancreas, genital glands are removed separately. The tissues or organs are immediately covered with anhydrous sodium sulfate that dehydrates them and also allows protecting the mass from oxidation.

b) extraction - The tissue coated with sodium sulfate is finely ground and extracted with acetone. Extractions are repeated several times. To the acetone solutions gathered in a decantation glass are added distilled water and petroleum ether. The proportions of water and ether depend upon the amount of tissue water and the lipids present (136). For hypodermis and peristomal connective tissue, the most favorable proportions are as follows: at 1,000 mL acetone solution, 300 mL water and 200 mL petroleum ether are added. For the acetone solution of hepatopancreas or genital glands with a higher lipid load (10 to 11% on average), passing to the -petroleum ether phase is favored by the addition of water and petroleum ether in equal proportions (300 : 300). The extraction of

carapaces needs the addition of hydrochloric acid to the acetone. After maceration, the acetone solution is treated as stated above. After stirring and rest, the -petroleum ether phase is separated, washed several times with distilled water and dehydrated by contact on anhydrous sodium sulfate.

This -petroleum ether solution contains both free astaxanthin and its esters.

DISTRIBUTION BETWEEN SOLVENTS

According to their solubility in petroleum ether and in 90% methanol, the following is separated:

- on the one hand, the pigments that are more soluble in petroleum ether than in methanol (epiphase pigments);

- on the other hand, the pigments that are more soluble in methanol than in petroleum ether (hypophase pigments).

The astaxanthin esters are epiphase esters, free astaxanthin is hypophase in nature.

The -petroleum ether solution of the pigments is stirred with 90% methanol. Two phases are separated. After rest, the alcohol phase is extracted and the petroleum ether is extracted again by stirring one more time with methanol. These extractions are repeated until the alcohol phase is no longer noticeably colored.

The methanol solutions gathered are stirred with petroleum ether after addition of water. Finally, two -petroleum ether solutions are obtained:

- The first one is an ester solution;
- The second one is free astaxanthin.

Measurement of the optical densities allows easily determining the relative concentrations of both solutions.

c) Results

ARISATEOMORPHA FOLIACEA

In the peristomal connective tissue in winter, free astaxanthin and esterified astaxanthin exist in equivalent amounts; in summer, the esters only account for 3/10 of the total pigment.

In the hypodermis, both in summer and winter 2/3 of the pigment is esterified.

In the carapaces, the pigment is always in the free form.

Aristeus antennatus

In the peristomal connective tissue, the free and esterified forms are present in equal proportions in winter; in summer, the esters account for 2/5 of total pigment.

In the hypodermis, 2/3 of the pigment is esterified (winter and summer).

The carapace pigment is in the free form in its entirety. All these results are shown in the Table below.

TABLE VII

Species	Epiphase Hypophase			
	January March	April June	July September	October December
Aristeus antennatus				
Peristomal connective tissue	1/1	2/3	2/3	1/1
Hypodermis	2/1	2/1	2/1	2/1
Carapace	Totally hypophase	Totally hypophase	Totally hypophase	Totally hypophase
Aristaeomorpha foliacea				
Peristomal connective tissue	1/1	3/7	3/7	1/1
Hypodermis	2/1	8/3	8/3	1/1
Carapace	Totally hypophase	Totally hypophase	Totally hypophase	Totally hypophase

We can see that the distribution of free astaxanthin and astaxanthin in the form of esters is substantially the same for the same tissues in the two species. In addition, seasonal changes were recorded and the results are also similar.

CHROMATOGRAPHIC ANALYSIS AND SPECTRAL FEATURES

The chromatographic analysis of each of the two epiphase and hypophase fractions reveals a pigment complexity higher than expected as a result of the distribution between solvents alone.

Materials and methods

The chromatography columns are provided with a device that allows working in an inert atmosphere. The adsorbent is alumina activity II. The control of activity is performed according to Brockmann (14). If a reactivation is necessary, it is easily obtained by heating at 500°C for 5 hours.

Pure, rigorously anhydrous petroleum ether is poured on the alumina column; this allows making sure to obtain a homogeneous settling, then the solution being analyzed is poured slowly. Then, the mixture is washed with petroleum ether (at least 400 mL if the volume of the solution is 200 mL) and the chromatogram is developed with petroleum ether added 1 per 1000 methanol.

After development, each of the zones formed is thoroughly isolated and the pigment is eluted by stirring the colored alumina with petroleum ether containing 5% methanol.

Results: ARISTAEOMORPHA FOLIACEA

I - Peristomal connective tissue extract

The chromatograms of the total extract, the hypophase extract and the epiphase extract showed several zones:

Total extract Solution No. 1	A_1 red zone A_2 orange zone A_3 brick zone A_4 yellow orangey zone A_5 yellow zone	0.5 cm thick 1.0 cm thick 0.6 cm thick 0.3 cm thick 0.1 cm thick
Epiphase solution Solution No. 2	A_1 red zone A_2 salmon zone A_3 orangey red zone A_4 orangey yellow zone A_5 yellow zone	0.8 cm thick 1.5 cm thick 1.0 cm thick 0.8 cm thick 0.3 cm thick
Hypophase solution Solution No. 3	A_1 dark red zone A_2 pink zone A_3 orangey pink zone A_4 orangey yellow zone	1.0 cm thick 2.5 cm thick 0.8 cm thick 0.7 cm thick

II - Hypodermis extract The development also causes several zones to appear.

Epiphase solution Solution No. 4	A_1 red zone A_2 orangey red zone A_3 orangey zone A_4 yellow zone	0.5 cm thick 1.5 cm thick 0.8 cm thick 0.1 cm thick
Hypophase solution Solution No. 5	A_1 red zone A_2 orangey zone A_3 pink zone A_4 orangey yellow zone	0.5 cm thick 1.5 cm thick 0.8 cm thick 0.5 cm thick

ARISTEUS ANTENNATUS

I - Peristomal connective tissue extract

Total extract Solution No. 6	A_1 red pink zone A_2 orangey red zone A_3 old pink zone A_4 yellow orangey zone A_5 yellow zone	0.6 cm thick 1.0 cm thick 2.0 cm thick 0.8 cm thick 0.2 cm thick
Epiphase solution Solution No. 7	A_1 red zone A_2 orangey red zone A_3 clear red zone A_4 orangey yellow zone A_5 yellow zone	0.6 cm thick 1.2 cm thick 0.4 cm thick 0.2 cm thick 0.1 cm thick
Hypophase solution Solution No. 8	A_1 clear red zone A_2 salmon pink zone A_3 orangey red zone A_4 orangey yellow zone	

II - Hypodermis extract The development also causes several zones to appear.

Epiphase solution Solution No. 9	A_1 dark red zone A_2 purplish pink zone A_3 orangey pink zone A_4 orangey yellow zone	0.8 cm thick 0.5 cm thick 1.5 cm thick 0.8 cm thick

III - Carapace extract

Total extract Solution No. 10	A_1 dark red zone A_2 salmon pink zone A_3 clear red zone A_4 orangey zone A_5 yellow zone	0.9 cm thick 1.5 cm thick 0.5 cm thick 2.0 cm thick 0.3 cm thick

The examination of the results shows the heterogeneity of the pigment, that is seemingly homogeneous. In addition, the chromatography of a total extract of Aristaeomorpha foliacea hepatopancreas had revealed such heterogeneity (77), it was also found with total extracts from peristomal connective tissue and carapaces.

The spectrophotometric data in connection with these various fractions are shown in Table VIII.

TABLE VIII

Solution number	Maximum absorption zones in pyridine nm		Solution number	Maximum absorption zones in pyridine nm	
1	A 1	490	6	A 1	490
	A 2	484		A 2	488
	A 3	490		A 3	478
	A 4	482		A 4	484
	A 5	470		A 5	482
2	A 1	490	7	A 1	488-490
	A 2	472		A 2	488
	A 3	488		A 3	488
	A 4	484		A 4	484
	A 5	480		A 5	482
3	A 1	488	8	A 1	488
	A 2	480		A 2	478
	A 3	482		A 3	484
	A 4	482		A 4	480
	A 5	472			
4	A 1	488-490	9	A 1	488
	A 2	488		A 2	480
	A 3	484		A 3	482
	A 4	478		A 4	478
5	A 1	488-490	10	A 1	490
	A 2	484		A 2	480
	A 3	478		A 3	488
	A 4	470		A 4	484
				A 5	482

b) STEREOISOMERS

The chromatographic analysis reveals therefore the existence both in the hypophase and the epiphase or the entire extracts, of five different pigments. These are substances that are chemically identical because saponification transforms them all into astacin. This chemical identity of substances that can only be distinguished by their spectral features had allowed us putting forward, with C. Chechan and R. Grangaud (22), that this should be a new case of cis-trans isomerism of astaxanthin. The fact is already proved for many carotenoids (246), (219), (247), but the existence of stereoisomer forms of astaxanthin had not been reported until now. Recently, R. Grangaud and Mrs. P. Chardenot (75) contributed the demonstration thereof: the epiphase pigment from Aristeus antennatus peristomal connective tissue, showing a narrowband spectrum with a single maximum located at 488 nm in pyridine, was boiled under reflux. After 6 hours, significant changes in the spectral features were found with the occurrence of two peaks at 484 and 492 nm in pyridine. Alumina chromatography also separates 4 clearly identified superimposed zones.

Contact for several days on alumina causes identical transformations: leaving in darkness an - petroleum ether solution of the pigment, after a few weeks the formation of isomers can be seen.

A decisive proof that these are stereoisomer forms is provided by the fact that using the physical means described above, each of these forms, if isolated, is transformed into all the other forms. The same isomerization techniques (action of time, of boiling, of chromatography) applied to astaxanthin solutions led to the same results (21).

The identity of the chromatographic and spectroscopic features of the pigments so prepared and those revealed in Aristaeomorpha foliacea allow concluding that these stereoisomers can be preexistent in the natural media (75).

c) CHROMOPROTEINS

Before their nature was specified by the works of Kuhn and Lederer (131), Kuhn and Sorensen (132), Stern and Salomon (217) and by the physical and chemical studies by Wald (237), the existence of the astaxanthin chromoproteins had been reported by various authors:

Verne (228), (229), (230) insists in a series of papers that “the red pigment of shellfish is located in the carapace and the eggs related to proteins and thus forms water-soluble complexes”.

Lwoff (146), Teissier (22), Brown (15), report similar observations.

Kuhn and Lederer, loc. cit., separate the prosthetic group of lobster egg’s green chromoproteid that they consider first of all as an “astacine ester”; they call it ovoester.

Stern and Salomon, loc. cit., start studying this chromoproteid they call ovoverdin. Kuhn and Sorensen, loc. cit., show that its prosthetic group is not actually an astacine ester but a new pigment for which they propose the name of astaxanthin. In addition, they determine that the blue color of the carapace is also due to the presence of a chromoproteid. The change of color from blue to red that occurs when the carapace is immersed in boiling water results from the release of the prosthetic group caused by protein flocculation. Wald (237) succeeded in extracting this chromoproteid he refers to as crustacyanin.

The techniques used by Stern and Salomon for the ovoverdin, and particularly by Wald for crustacyanin are generally applicable. They have been used for the physical and chemical study below. Also, the description of this study will be preceded by a short analysis of these works.

Physical and chemical properties of ovoverdin (Stern and Salomon (217)).

The lobster eggs are crushed with sand and distilled water.

Ovoverdin is precipitated from its solutions by addition of sodium sulfate, ammonium or magnesium to saturation. In an acetate or phosphate buffer, it is stable between pH 4 and pH 8.

Its visible spectrum shows a maximum at 470 nm and another one at 640 nm. Stern and Salomon show that the pigment is unstable and easily dissociated.

The pyridine, mineral acids and bases entail the breaking of the protein bond: the blue solutions turn red; the reaction is irreversible.

Heat also causes this dissociation, but such dissociation can be reversible if temperature rise does not exceed 60 to 70°C and is not too long.

Physical and chemical properties of crustacyanin (Wald - 237).

In the lobster carapace, the chromoproteid is associated with the calcium salts and does not allow its being extracted with distilled water. It is obtained by maceration in a diluted citric acid solution.

The pigment is precipitated from its solution by 40% ammonium sulfate and dissolved again by dilution or dialysis. The spectrum shows a broadband with a maximum at 625 nm. Its isoelectric point is located at about pH 4.5.

Mineral acids, alcohol, hot acetone make the solutions turn from blue to red, the absorption maximum moves toward 460 nm.

Heat causes the following changes: at 60°C, in a veronal buffer of pH 7, color turns from blue (λ max = 625 nm) to red (λ max = 530 nm). Comparable changes can be seen under the effect of pH variations.

Both for the Wald's crustacyanin and the Stern and Salomon's ovoverdin, the prosthetic group is astaxanthin.

EXPERIMENTAL CONTRIBUTION

- Plesionika edwardsii, Brandt chromoproteins

Egg pigment: mature eggs are in the form of a blue granular mass retained by the abdominal legs from which it can be easily detached.

5 g eggs are ground with sand and taken up by 40 mL distilled water. A strongly colored and limpid solution is obtained by decantation and filtration. Its spectrum shows a broadband with a single maximum at 635 nm in pyridine (cf. Fig. 6).

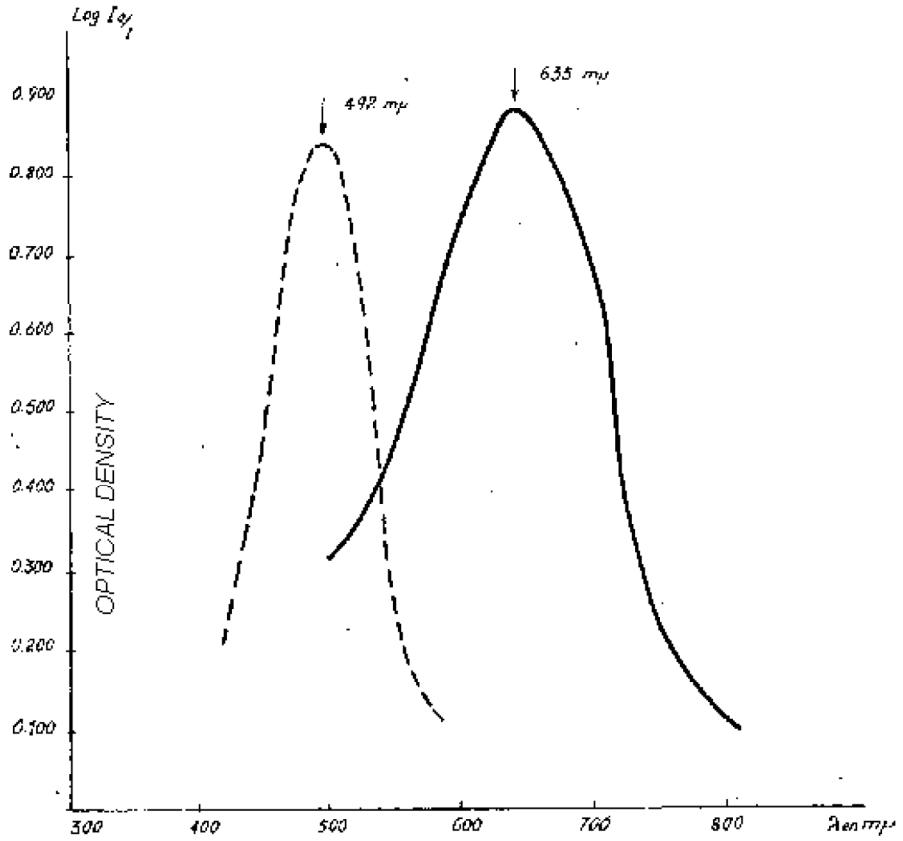


Fig. 6 - Plesionika edwardsii - BRANDT

Absorption spectrum of the chromoproteid and its prosthetic group
——— Chromoproteid
- - - - Prosthetic group

a) **Influence of pH**

When mixing the aqueous solution with acetone-acetic buffers M/15, no dissociation can be seen until pH 4. The color turns from blue to pink below such pH, with this change being irreversible. The mineral acids cause an irreversible dissociation by protein denaturation.

b) **Temperature action**

A test tube containing the solution is immersed in a thermostat set at 70°C. Turning to pink and return to blue by cooling can be seen. If heated at 100°C, the transformation is irreversible.

c) **Organic solvent action**

The aqueous solution is stirred with different organic solvents:

Petroleum ether does not cause any change in color of the aqueous solution.

Pyridine precipitates the orangey red protein, petroleum ether is added; the pigment is dissolved.

The -petroleum ether solution is divided into two parts: one is stirred with 90% methanol, the pigment passes to the methyl phase in its entirety. The other part is evaporated under low pressure in a nitrogen atmosphere. The residue is taken up by pyridine. The spectrum recorded shows a single broadband at 492 nm, typical of astaxanthin (cf. Fig. 6).

The immature eggs have a viscous consistency and their color is paler than the mature eggs. A slightly opalescent solution is obtained from these eggs. The pH variations, the temperature action, the organic solvent action, cause the changes in color recorded with the mature eggs; the prosthetic group is separated by the addition of pyridine or by treatment with acetone and petroleum ether. It shows the spectrum typical of astaxanthin (λ = 492 nm in pyridine).

Pigment extracted from peristomal connective tissue. In the peristomal connective tissue, a part of the pigment can also be extracted in an aqueous solution.

5 g of connective tissue are cooled at - 15°C. The frozen mass is ground with sand and taken up by 40 mL distilled water; a part of the pigment forms an aqueous solution that turns pink. This is still a chromoproteid, the features of which are comparable to those of the preceding cases. The prosthetic group is isolated by treatment with pyridine or by the acetone-ether mixture; it also shows here an absorption maximum at 492 nm in pyridine.

- Aristaeomorpha foliacea chromoproteins

Egg pigment. The eggs are taken from the ovaries (*). 5 g eggs are obtained in an aqueous solution by the above-described process for the Plesionika edwardsii eggs. The amethyst aqueous solution shows a broadband spectrum (γ max = 600 nm in pyridine).

a) **pH action**

The aqueous solution is added buffer solutions of different pH. Turning (reversible) to an orangey brown is visible only below pH 4.

The addition of mineral acids also causes turning, but the reaction is then irreversible.

b) **Temperature action**

The solution is heated in the vicinity of 70°C. The color turns to orangey passing through intermediate colors; by cooling, the initial color reappears. At 100°C turning is irreversible.

c) **Organic solvent actions**

The solution is stirred with pyridine that turns red (protein precipitates); with the acetone-ether mixture, the orangey red pigment forms a -petroleum ether solution. The solution is evaporated; the residue taken up by the pyridine shows a single-broadband absorption spectrum (λ max = 492 nm in pyridine).

(*) The Penaeidae do not carry their eggs.

- Aristeus antennatus chromoproteins

Egg pigment. The aqueous solution obtained by the usual process is cyclamen pink (absorption maximum at 504 nm).

a) **pH action**

Turning can be observed from pH 4. Change is reversible. The addition of mineral acids and acetic acid causes an orangey color. The reaction is then irreversible.

b) **Temperature action**

By heating at 70°C, the solution turns from cyclamen to orangey; the initial color reappears by cooling. At 100°C a brick red coagulum can be seen.

c) **Organic solvent actions**

The action of pyridine, acetone and petroleum ether is successively studied: the protein precipitates with the pyridine and the acetone. The prosthetic group is detached, the solution stirred with petroleum ether transfers its pigment to it. The prosthetic group is characterized by its single-maximum spectrum (492 nm in pyridine).

Peristomal connective tissue pigment. The pigment is extracted by the process described for the Plesionika edwardsii connective tissue. The solution obtained is pink.

The actions of the changes in pH, temperature, organic solvents, lead to the same observations: the prosthetic group, released as a result of the action of pyridine or acetone (that coagulates protein) is characterized by its spectrum: (λ max = 492 nm in pyridine).

Parapenaeus longirostris chromoproteins

Egg pigments. The aqueous solution is clear green. The following is also analyzed here:

- pH action: Turning to orangey can be observed at a pH below 4;
- Temperature action: color passes from green to orange, the reaction is reversible below 70°C (passing through the same intermediate colors); it is irreversible below 70°C;
- Organic solvent actions: the pyridine, the acetone coagulate the protein; the pigment released is astaxanthin (λ max = 492 nm in pyridine).

- Scyllarus latus chromoproteins

Egg pigments. The aqueous solution is orangey yellow; it turns to brick orange by action of the acids, heat, organic solvents. Its prosthetic group is astaxanthin (λ max = 492 nm in pyridine).

- Squilla mantis chromoproteins

Sub-esophageal connective tissue pigment. A few grams of connective tissue are taken and crushed, a part of it is put into an aqueous solution. The addition of acetone forms a precipitate and causes the release of the prosthetic group that is put into a -petroleum ether solution. The -petroleum ether solution shows the spectrum typical of the astaxanthin (λ max = 492 nm in pyridine).

All the spectrophotometric results are reported in Table IX.

This shows that - apart from the very extended color range - all these chromoproteins have quite comparable physical and chemical properties: the changes in pH, the changes in temperature, the action of various solvents, acids and bases cause the same effect: the release, whether or not reversible, of the prosthetic group that in all cases is astaxanthin in its completely “trans” form.

These results can in all points be superimposed to those obtained by Stern and Salomon (217) on the lobster egg ovoverdin and by Wald on crustacyanin. Based on the observations conducted on immature eggs and mature eggs, one can also point out that the prosthetic group remains the same during ontogenesis. This finding can be put side by side with that made by Ball (4) who, in studying the different stages of Lepas fascicularis and Lepas anatifera, found that the eggs are pink while the larvae are blue at eclosion time: in both cases, the prosthetic group is astaxanthin. According to Ball (4), the change in color would express a change in the nature of the bonds between the prosthetic group and protein.

However that may be, this set of experimental facts shows that, through combinations with proteins, astaxanthin can give a great number of very differently colored heteroprotein complexes.

This is indisputably a group of substances the specificity of which is independent from the nature of the prosthetic group (astaxanthin in the completely trans form), and it is logic to suggest the astaxanthin-proteins term to designate the different representatives of this group (73).

d) EXTRACT PREPARATION METHODS

The study of the nature of the physical and chemical properties of the pigment allows specifying the method of preparation of the extracts that are to be used for the biological experimentation.

Table IX

Organs	Absorption maximum in pyridine nm					
	Aristaeomorpha foliacea	**Aristeus antennatus**	**Plesionika edwardsii**	**Squilla mantis**	**Parapenaeus longirostris**	**Scyllarus latus**
EGGS						
mature						
aqueous solution	494-600	504	635	"	"	
prosthetic group ()	492	492	492	492	492	492
immature						
aqueous solution		520	635		"	
prosthetic group	492	492	492		492	492
PERISTOMAL CONNECTIVE TISSUE						
	520	520	"	"	"	"
aqueous solution	492	492	492	492	"	"
prosthetic group						
HYPODERMIS						
aqueous solution	492	492	"	"	"	"
prosthetic group						
STOMACH						
POUCH	600	520	"	"	"	"
	492	492	"	"	"	"
aqueous solution						
prosthetic group						

Two types of extracts were prepared:

1) oily extracts from astaxanthin esters;
2) aqueous extracts from astaxanthin-proteins.

1. Preparation of oily extracts:

The epiphase solution of the peristomal connective tissue or hypodermis is chromatographed. The chromatogram is developed and the astaxanthin esters corresponding to the trans form are eluted by the petroleum ether with the addition of methanol (cf. above, p. 35).

The -petroleum ether solution is added an amount of vegetable (devitaminized) oil calculated according to the optical density of the solution, to obtain an oily extract of the desired pigment concentration. The amount of tocopherol used as anti-oxygen is 28 mg per gram oil. The petroleum ether is removed by distillation under low pressure in an inert atmosphere.

As previously seen, vitamin A and carotenes are absent from the peristomal connective tissue and the hypodermis of the species that have provided the extract.

The two experiments below show that if traces of these factors had escaped from the chemical and spectrophotometric research, the chromatographic method followed for isolating the astaxanthin esters would preclude the possibility of their contamination by the vitamin or carotenes.

Experiment 1: Study of the chromatographic behavior of vitamin A esters (91).

Experiment 2: Study of the chromatographic behavior of vitamin A alcohol.

Experiment 1:

The pigment of 0.5 g of Aristeus antennatus peristomal connective tissue was dissolved in 200 mL petroleum ether. 9 µg of vitamin A is added in the form of a standardized ester solution. The solution is filtered on an alumina column. The pigment is retained in the upper part of the column. After washing with 200 mL petroleum ether, the pigmented zone was removed and the remainder was divided into four sections: the first one of 3 cm and the other three of 5 cm.

The alumina of each of them was stirred with petroleum ether to which 10% methanol was added. The eluates, washed with distilled water, dried on anhydrous sodium sulfate, were evaporated

under low pressure in a nitrogen atmosphere, then taken up by 1 mL chloroform; the same for the ether filtered through the column. In each extract, vitamin A was assessed and dosed using the Carr-Price reaction.

In the section (3 cm) located above the pigmented area, Vitamin A was not found. In the three 5-cm sections, the following was found from top to bottom: 1.5, 2.4 and 3.6 µg, respectively (Fig. 7, a). The filtrate extract produced no coloring by the addition of the Carr-Price reagent. Thus, out of the 9 µg added, 7.5 were found, spread over the lower three fourths of the column and separated from the pigment by a 3-cm zone.

PETROLEUM ETHER SOLUTION (400 mL)
OF ASTAXANTHIN WITH THE ADDITION OF VITAMIN A (18 µg)

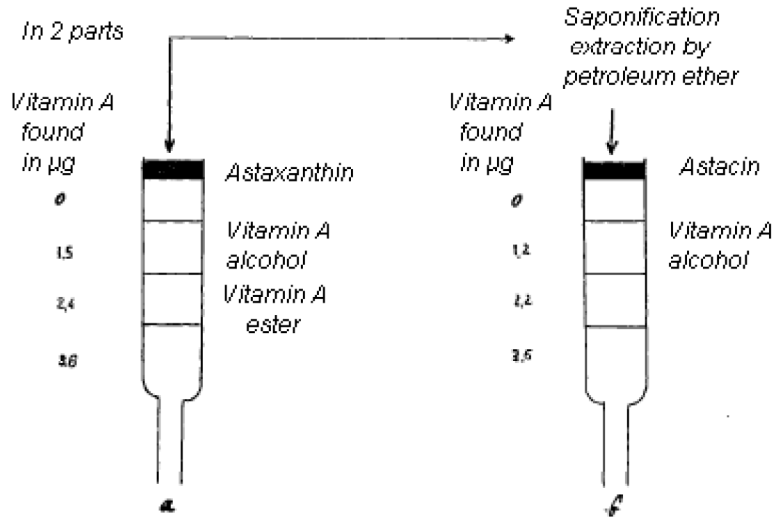


Fig. 7 - Diagram of chromatographic separation of astaxanthin and vitamin A
a - Experience 1
b - Experience 2

Experiment 2:

A petroleum ether solution (200 mL) is prepared as in the preceding experiment, and added 9 µg vitamin A. This solution is saponified; the unsaponifiable fraction extracted with petroleum ether was chromatographed on alumina. According to this method, astaxanthin is transformed into astacine that remains absorbed at the top of the column. The column is divided like in experiment 1, and also as in experiment 1 vitamin A is assessed in each of the sections: the amounts found are from top to bottom: 0, 4.2, 2.2 and 0.6 µg, respectively (Fig. 7 b).

The separation diagrams highlight the different chromatic behaviors of the astaxanthin and esters thereof and vitamin A and esters thereof. These results, in agreement with those from Kon (124) show that alumina chromatography alone allows separating free, esterified astaxanthin from free vitamin and esters thereof. With regard to carotenes, we have already stated above (cf. p. 31) that when they are present, they are filtered slowly through the column. Therefore, one can conclude that, for sure, with regard to the extracts so prepared for the study of the astaxanthin activity, that they can in no event contain the least trace of vitamin A or carotenes.

Preparation of oily extracts of astacin

200 mL of hypophase solution obtained from peristomal connective tissue are saponified by addition of 100 mL 15% alcohol potash. Saponification is continued for 4 hours at room temperature; then 300 ml water and 200 ml petroleum ether are added. Astacine (potassium salt) is precipitated in the form of red flakes that gather the “petroleum ether - alcohol solution” interface.

After filtration, the flakes are washed with 200 ml of 50% alcohol and then with petroleum ether (20 mL), then with distilled water.

The pasty mass is suspended in devitaminized vegetable oil.

Preparation of aqueous extracts of astaxanthin

The aqueous solutions of astaxanthin-proteins are obtained following the above-described method (cf. p. 34); however, the solvent is no longer distilled water but saline (NaCl at 9 per 1,000).

D. BIOLOGICAL TEST METHOD

1. REACTIVE ANIMAL

Many animal species have been used to study the biological properties of substances having vitamin A activity. In fact, for bird and most mammals, vitamin A is essential and the requirement level has been set for most species: chicken, With and Wanscher (242), cat, Guilbert & Hart (97), pork, Nelson (179), monkey, Day (42), Truscott and Van Wagemen (226), dog, Crimm and Short (35), Morgan (174), Guilbert et al. (98), Bradfield and Smith (12); however, as a matter of fact, the only species in which most of the aspects of the vitamin A deficiency problem have been addressed are, with the guinea pig, Mannering (151), Bentley and Morgan (7), Chevallier and Baert (26), the mouse, Mc Carthy and Cerecedo (147), Sherman et al. (207), Paul and Paul (185) and rat. The reactive animal most frequently quoted is the albino rat: Mus norvegicus albinus.

This is the only one we have used in our experiences; we have bred a single variety of a pure Wistar strain, the cell line of which we follow since 1949.

FEEDING DIETS

The feeding diet was chosen to respond to several conditions: it must provide sufficient caloric supply, be well balanced and contain the principles specifically essential for growth and reproduction; particularly the supply of vitamins should be thoroughly controlled, since deficiency conditions entail more or less serious consequences for the offspring. However, as far as vitamin A is concerned, overdose should be avoided so as to limit the size of the liver reserves.

After several tests, wheat was chosen as the sole source of cereal; however, it's phosphorous/ calcium imbalance was offset by adding calcium lactate; as a supplement, casein was added.

Ground wheat is distributed in a mixture with calcium lactate and casein in such a manner that our feeding diet is very close to that proposed by L. Randoin and J. Causeret (192). Its composition is as follows:

Ground wheat:	93.0
Commercially available casein:	5.0
Sodium chloride:	0.5
Sodium lactate:	1.5

The pregnant females receive in addition a wheat germ-based preparation that is included in the diet in a 5% proportion. The diet is completed by supplying lettuce twice a week (E 6 diet).

With this diet, used since many years ago, the animals have been kept in excellent health condition and the offspring ensured satisfactorily.

BREEDING ORGANIZATION

We will limit to general information and some essential details.

The animals stay in a room provided with a ventilation system, where the temperature is kept at about 20°C.

The animals are housed in two kinds of cages:

- Drawer-type individual cages with a grid floor for the subjects of the experiment;
- Cages arranged in batteries where animals intended for reproduction are gathered.

To achieve proper reproduction, the general rules stated below must be followed:

Two females and a male aged from 60 to 90 days (Wistar rats reach their sexual maturity between day 50 and day 60 for both sexes (141)) are placed in the same cage. The females are isolated from day 18 (pregnancy lasts 21 days) and receive pieces of carded cotton and filter paper that allow them to make a nest.

The cages of the pregnant females are placed aside; they are given special care and, particularly, we make sure that they always have water available.

It is accepted (122) (123) that inbreeding is not harmful, that it is only dependent on the genetic inheritance and that, if selecting the subjects intended for reproduction, the cell lines are normal. Our experiments in this field have confirmed this opinion.

All animals are marked, recorded and monitored using individual sheets. Any sterile females or females that have destroyed two consecutive litters are discarded. A female has from four to five litters, the second and the third litters are the largest ones (6 to 8 babies); in the fifth litter, the number of pups falls. The females stop reproducing between month 15 and 18.

2. VITAMIN A DEFICIENCY STUDY CONDITIONS

a) SYNTHETIC FEEDING DIETS

The different types of diets used by the various experimenters present considerably similar compositions. Their total energy value is comparable, the differences only lie in the nature of proteins, carbohydrates and lipids.

L. Randoin and S. Queuille (195), using the muscle peptone, casein or brewers' yeast as a source of proteins, showed that the nature of proteins has no influence on the progress of vitamin A deficiency. Simonnet (212), Penau and Simonnet (186) use the muscle peptone. Coward (30) and Chevallier (24) use casein. These proteins range from 15 to 17%. After several preliminary tests, we made our choice for casein; from the standpoint of its composition, casein is a protein that gives complete satisfaction; commercially available casein in ground form has a constant quality; finally, it is easily used in devitaminizing operations.

With regard to carbohydrates, a comparative study of the influence of their nature on the deficiency process was conducted by L. Randoin and S. Queuille (196). Galactose and lactose are toxic to the young rat if supplied in strong concentrations; fructose and glucose have not a beneficial effect. With maltose, sucrose and dextrin, the development and maintenance of the body are satisfactory (163). The feeding diets proposed by K. Coward are based on rice starch, those of Lewis and Bodansky (140) are potato starch-based, while Chevallier (24) uses dextrin or sucrose. These diets contain from 58 to 73% carbohydrates. Mouriquand and Chaix (178) have, as to them, shown that a change in the proportion of carbohydrates had no influence on the occurrence of xerophthalmia.

We set aside starch (expensive to use) and experimented with dextrin- or sucrose-based diets; we found that the animals having reached a late stage of deficiency showed a certain dislike of the sweet diet. Dextrin was then adopted; however, a sucrose-based diet was distributed among the pups the first days of weaning. In fact, dextrin often forms a starch paste that glues the pup.

With regard to lipids, the research works conducted by Drummond (47), Simonnet (211), Emerique (49), Randoin and Netter (194) established that the different edible oils provide comparable results; peanut oil and olive oil are most frequently used; we have chosen peanut oil.

Minerals are included in the feeding diets as salt mixtures, the most common composition of which is given by the Osborne and Mendel (*) formula.

Finally, we chose a diet having the following composition (diet R 12):

Dextrin	63.5
Casein	17.0
Osborne and Mendel salt mixture	4.0
Dry brewers' yeast	3.5
Peanut oil	12.0

Filter paper at will.

The requirement for B-group vitamins is covered by the brewers' yeast; the vitamin D supply is ensured by food irradiation.

The different feeding diet preparation times are intended to obtain a mixture with a constant composition completely free from A vitamin A factors. Given that these factors are present in casein and peanut oil, those products should undergo a previous purification treatment.

Casein treatment - It consists in removing vitamin A by treating with alcohol at 90°C in a Kumagawa extractor for 5 hours. Casein is then stove dried at 70°C.

Oil treatment - It comprises heating FOLLOWED by several extractions with alcohol at 90°C. The oil, spread in a thin layer in a large crystallizer, is heated for 8 hours at 120°C; during heating, oil is mixed and ventilated. After cooling, oil is extracted by stirring using 3 times its volume of alcohol at 90°C; the procedure is repeated 3 times.

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* Osborne and Mendel salt mixture (183).

Calcium carbonate	134.8
Magnesium carbonate	24.2
Sodium carbonate	34.2
Potassium carbonate	141.3
Phosphoric acid	103.2
Hydrochloric acid	53.4
Sulfuric acid	9.2
Citric acid	111.1
Ferric citrate	6.34
Potassium iodide	0.02
Magnesium sulfate	0.079
Sodium fluoride	0.0248
Potassium aluminum sulfate	0.0245

After settling and separation of the alcohol phase, distillation is conducted under low pressure to remove alcohol. The oil so treated (*) is stored in brown flasks.

Diet preparation - The complete mix is prepared in advance for several meals, except for the addition of oil that is done every morning before distribution of the food intakes. This preparation protocol was adopted after several observations; as a matter of fact, when oil is previously added, it forms with dextrin small pasty granules that the animal eats less willingly. Obtaining sufficient feeding is essential to prevent any nutritional problems from adding to those related to the deficiency and interfere with them.

As soon as the mixture is ready, it is arranged in thin layers and exposed to the light of a UV lamp for thirty minutes. This irradiation is considered as sufficient to produce the sufficient amount of vitamin D.

At the time of weaning, the daily intake is about 10 g. For adults, it reaches 15 or 20 g. The energy requirement (43 calories for the pup, 65 to 85 calories for the adult) is thus met, as well as all essential principles are supplied: amino acids, fatty acids, mineral salts and vitamins, except for vitamin A.

b) EXPERIMENTAL VITAMIN DEFICIENCY

The feeding diet is administered to the rat from weaning. The rats are weaned between day 25 and day 30, at the time when they weigh between 29 and 30 g. It is essential to achieve normal development that these conditions are strictly adhered to.

The administration of the deficient diet has no influence in the beginning on the growth, and the increase in weight is normal. After 4 to 5 weeks, the upward weight curve falls to reach a plateau between day 40 and day 45 of deficiency; after about ten days stabilization, the weight decreases and the animal dies between day 60 and day 70 of deficiency. **With regard to the xerophthalmia signs, they start microscopically by a narrowing of the palpebral fissure; while exophthalmia is regularly observed in the normal Wistar rat, in the deficient rat the bulbus oculi moves within the orbit. Soon, both eyelid edema and cornea drying and opacification occur; opposite to this sign, secretions increase and result in hemorrhage (**). Then considerably large corneal ulcers appear and corneal perforations are not unusual.**

* After extraction, the product is controlled as follows: 20 gr. of product are extracted with (in the case of casein) or dissolved (in the case of oil) in chloroform; the Carr-Price reaction should not yield any color.

** We found that hemorrhagic exudates caused by an accidental trauma before day 20 of deficiency did not accelerate the development of xerophthalmia. The observations by Chaix (20) and Courbières (29) have led to similar remarks.

Although the various authors agree about the consistency of this development - the description provided above represents the typical design -, they differ about the time of occurrence of xerophthalmia. As a matter of fact, the differences lie essentially on the means of observation used, that lead to detect it at an earlier or later stage. **According to Steenbock and Coward (216), the first signs of xerophthalmia are coincident with the arrested growth; to Osborne and Mendel (184), eye injury can occur when the weight curve is still steady**. Chaix (20) could show, using biomicroscopic methods, that the eye signs actually precede by 8 to 10 days the occurrence of macroscopic injuries. This author could so conclude that xerophthalmia represents the earliest sign of vitamin A deficiency.

Under the conditions defined by us, our observations provided this data: xerophthalmia occurs regularly among our animals between the 30th and the 38th day of deficiency, while weight growth stops only from the 40th day **(average female weight: 70 g, average male weight: 75 to 80 g).** This is well shown by the observations below taken from our experimentation (Fig. 8 and 9).

Male rat: weaned at 30 days, weight 30 g:
- 32nd day of deficiency: blinking eyes
- 35th day of deficiency: copious secretions, dull corneas
- 38th day of deficiency: ulcer begins in the left eye, ulcer in the right eye
- 45th day of deficiency: ulcer in both eyes.

Female rat: weaned at 30 days, weight 30 g:
- 32nd day of deficiency: slight exudates in both eyes
- 35th day of deficiency: dull corneas
- 38th day of deficiency: hemorrhagic secretions in the left eye, ulcer begins in the right eye
- 50th day of deficiency: ulcers in both eyes.

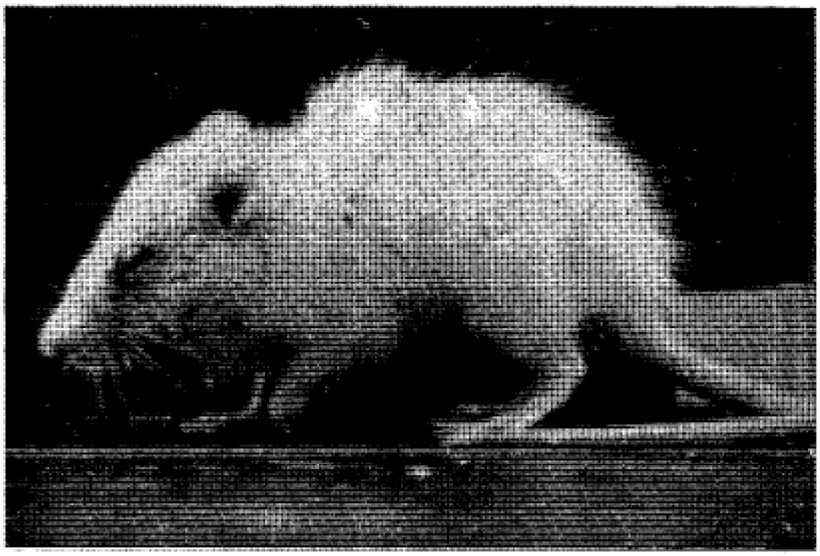


Fig. 8 35th day of deficiency

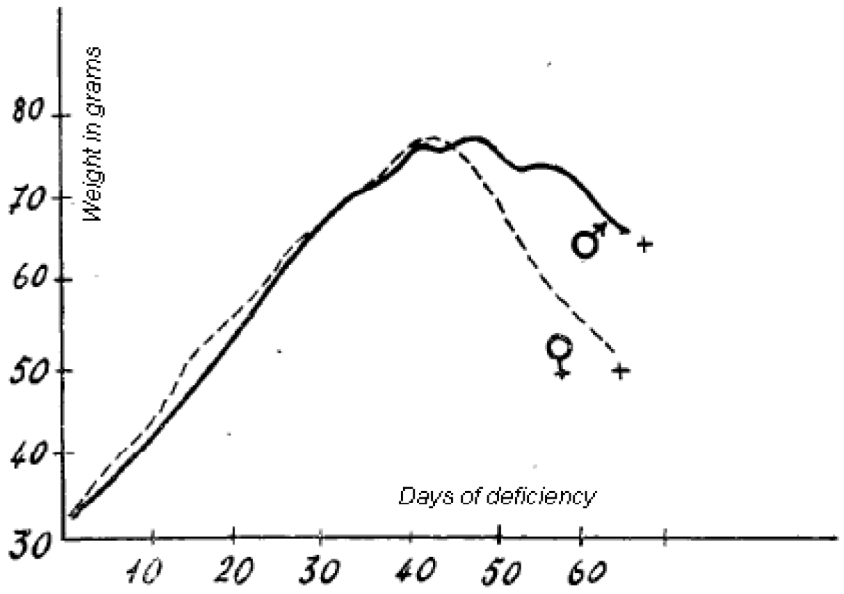


Fig. 9

This vitamin A deficiency Table is typical and we could not insist too much on the consistency of the development described above.

Weight stabilization and xerophthalmia occur along with other troubles such as dentition disorders and various infectious processes confirmed by the results of the autopsy. The particulars of our observations in this field are related to chapter V intended for the anatomical and pathological study of the factor A-deficient animals and the astaxanthin-treated animals.

c) REVERSIBILITY OF DEFICIENCY

If, before the loss of weight is not too marked, a factor having vitamin A activity is administered in a sufficient amount, the animal regains weight and its eyes become gradually healthy. On the contrary, after a certain stage where the loss of weight is too significant and the eye injuries are too severe, the efficacy of the same preparation is nil and the development of the deficiency continues as if the animal had not been treated. It is therefore essential to determine the stage at which the injuries can still recede; however, it is very difficult to identify the exact time at which the injuries have the same severity among the various animals in the experiment, for which reason the authors are far from agreeing on when to start the experiment.

Most of the experimenters refer to the weight of the animals. Sherman and Munsell (208) propose to begin administering the substance being tested after a period of weight stabilization of one week. Javillier and Emerique (116) highlight that the animals are more comparable to each other if action is taken after a loss of weight of 10%.

In our experiment, we adopted this rule very regularly, except, however, in the exceptional cases in which the deficiency develops in an aberrant manner:

1) Sometimes the early occurrence of the deficiency signs can happen with weight stabilization at 50 grams; thus, with the animals not being strong enough, the treatment begins only after a 5 to 6-day plateau.

2) Late deficiencies are also found, attributable without doubt to a slightly higher vitamin A liver reserve (cf. below). Weight stabilizes beyond the 6th week; in this case, the cure test begins after about a twenty-day plateau.

The finding of these aberrant cases - the percentage of which always remained of less than 10% in our experimental design - prompted us to control the liver reserve level in the pups treated and to search the most favorable conditions for such reserve to be limited and to always substantially maintain the same significance.

- Influence of the mother's diet during pregnancy and suckling on the liver reserve level of the rat at weaning.

It is determined that the rat's liver reserve level at birth is higher when the mother's level is high during pregnancy (37) (38) (39) (6) (103). However, there is no proportionality and for mother reserves ranging from 2.7 to 6,000 µg, the changes in vitamin in the pup's liver only range from 0.15 to 0.3 µg.

During suckling, according to Henry et al., the pup stores three times more vitamin when the mother has, at the time of dropping, a reserve of 1,800 to 2,700 µg and is given, while suckling, a factor A-deficient diet. The pup stores six times more if the mother's reserve is low but if the mother receives a very high-vitamin A diet. These results emphasize that the rat's liver reserve at weaning is mainly dependent on the amount received by the mother while feeding the pups.

d) STUDY OF THE CHANGES IN VITAMIN A OF THE LIVER RESERVE

The pup's liver reserve at the time of weaning.

Females subjected to a low-factor A diet were coupled to males under a normal diet; during the entire pregnancy period, the females received the low diet (*). At birth, the mothers with their pups were divided into two batches A and B:

- The mothers from batch B received a high-factor A diet (*);
- The mothers from batch B received a low-factor A diet (**).

The pups were sacrificed when they weigh 30 grams. This weight is reached by day 30. The livers were taken and vitamin A dosed following the above-described method.

The results are shown in the Table below.

TABLE X

Number of rats	Number of suckling days	Sex	Mother's diet during suckling	Rat's weight in g.	Liver's weight in g.	Vitamin A	
						per g. in µg	For liver In µg
7	30	♂	Low factor A	30	2.7	10.8	29
7	30	♀	»	30	2.4	11.1	26.6
7	30	♂	High-factor A	30	2.6	17.0	44.2
7	30	♀	»	30	2.5	17.3	43.3

These results can be compared to those obtained by Henry et al. (103) and emphasize the need to control very thoroughly the mother's diet during suckling. It was important to also specify the time the animal takes, from weaning, to deplete these reserves.

Decrease in the pup's liver reserve from weaning.

Three litters (21 rats) are fed by mothers on the low-factor A diet (diet E 6). They are weaned when they reach 30 g and given the deficient diet (diet R 12).

* Diet E 6

** Diet E 6 supplemented with three carrot intakes per week

7 rats (3 ♀, 4 ♂) were sacrificed the weaning day, their liver reserve was determined.

7 other rats were sacrificed on the 10th day of deficiency; plus 7 other rats on the 18th day. Vitamin A is searched for and dosed in each of the livers.

The results are shown in Table XI.

TABLE XI

Number of rats	Sex	Deficiency days	Rat's weight in g.	Liver's weight	Vitamin A	
					per g. in µg	For whole liver In µg
4	♂	0	30	2.7	10.8	29.0
3	♀	0	30	2.4	11.1	26.6
4	♂	10	42	3.7	4.5	16.6
3	♀	10	40	3.5	5.2	18.2
4	♂	18	50	5.2	0	0
3	♀	18	48	5.0	0	0

These results show that the liver reserve among the males and the females is depleted in less than 20 days if the mother's diet during suckling is strictly controlled. Under these conditions, xerophthalmia and arrested growth, that are in fact the most apparent deficiency signs, are regularly observed according to the experimental design we have described.

3. STUDY OF VITAMIN A ACTIVITY

This study can be conducted following two methods:

- **The curative method that consists in curing the disorders caused by deficiency.**
- **The preventive method in which the substance being tested is administered from weaning: depending on its degree of activity, it can prevent or limit the disorders caused by the vitamin A deficiency.**

In either method, the use of the active principle by the body is dependent on several factors that must be examined before undertaking the study of the two methods itself.

Chemical form of the vitamin A administered.

While Sobel et al. (214), Kagan (119) do not find any difference between the use of the free and the esterified forms, the works of Week and Sevigne (239), Gray (94) and more recently Esh and Sukhamov Bhattacharya (52) showed that maximum absorption is reached when esters are involved.

Nature of the diluents used

Esh and Sukhamov Bhattacharya also studied the influence of the diluents on the absorption. Vitamin A (free and in the form of acetate) is diluted in peanut oil, ethyl oleate or placed in an aqueous medium. Absorption was assessed according to the liver reserve level. The percentage of vitamin A (acetate) absorbed is higher for the peanut oil (34.9% stored in the liver); it reaches 34.6% for the aqueous medium and only 28.6% for the dilution in ethyl oleate. With regard to Vitamin A, the percentages are 30.3% (aqueous medium), 17.1% (oily), 25.0% (ethyl oleate), respectively.

Route of administration:

Teulon Marnay and Gounelle (223) compared the storage of vitamin A in the liver when administered orally and intramuscularly.

With regard to the route of administration by intraperitoneal injection, Chevallier, Augier and Choron (25) found that the vasodilatation phenomena caused by the injection are highly irregular and considerably influence the vitamin absorption process.

Rectal administration was tried out by Radoin, Hugot and Causeret (192) who showed that vitamin A can be absorbed in that way.

Antioxidant protection:

Numerous works have determined that vitamin A oxidizes during the digestive transit. Dubouloz and Gasquy (45) administered the vitamin in the form of palmitate to rats without a liver reserve and found that the percentage stored ranges from 11 to 18% in the absence of antioxidants and from 17 to 19% in the presence of tocopherol. An oxidation process occurs at two times during the digestive transit: in the stomach and the intestine. According to Dubouluz, oxidation in the intestine is negligible (46). However, Hickman shows that for small doses the destruction of vitamin is significant (100). Davies and Moore (41), Moore and Sharman (172) studied the protective effect of tocopherol on the liver reserve. For equivalent doses of vitamin A accompanied by variable amounts of tocopherol, there are considerable changes in the liver reserve level: those variations are shown in the diagram below (Fig. 10).

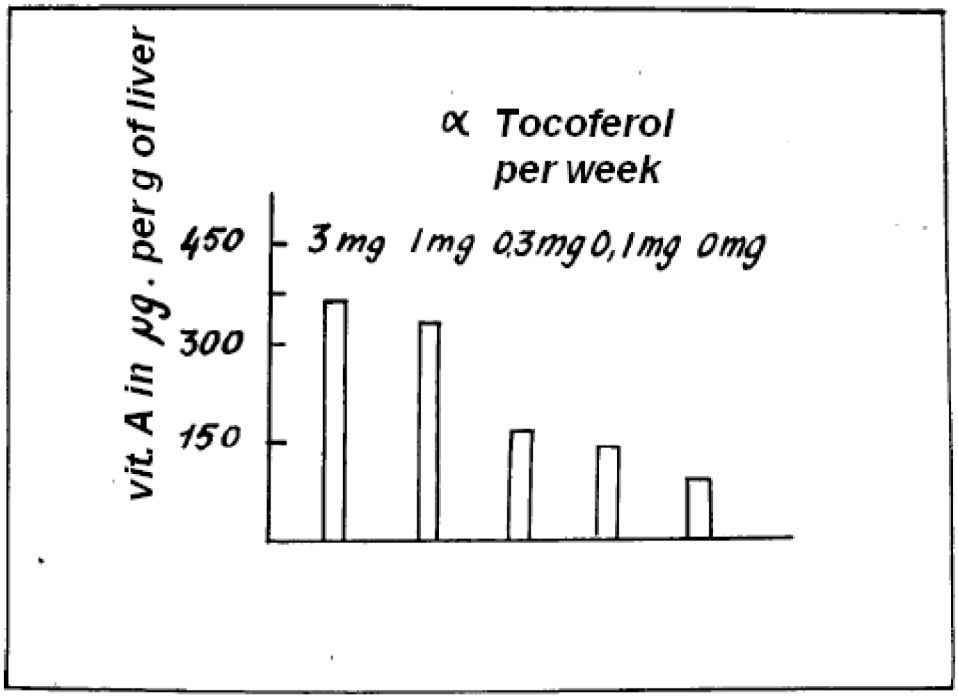


Fig. 10: Vitamin A content of female rat livers after intake of 300 µg vitamin A per week (duration 6 months) and changing doses of tocopherol (172).

Hickman studied the protective effect of tocopherol based on the gain of weight and longevity: the gain (male rats) in 28 days is 20.7, 28.8, 27.6 and 31.7 g. for doses of tocopherol of 0.05, 0.15, 0.5 and 1.5 mg, respectively. Doses exceeding 1.5 mg would be less effective, Hebert and Morgan (100). In fact, the mechanism of action of tocopherol as an antioxidant while contributing to absorption is complex (171), (169), (188), (117), (107), (221), (145). Thus, the data obtained with regard to the optimum dose of tocopherol are quite dissimilar. As a comparison, such data is shown in the Table below:

TABLE XII

Tocopherol	Authors
1 mg per week (delivered in 20 mg oil)	COWARD (30)
2 mg per kg (rat) per day	
3 mg per week	BLAISOT (8)
0.5 mg per day	MOORE and SHARMAN (172)
0.035 to 0.50 mg per day	HEBERT and MORGAN (100)
0.75 mg (M.F.D.) (*)	MASON (153)

(*) Mean Fertility Dose

PERSONAL DATA:

In our experimental designs, the absorption conditions are strictly specified and rigorously identical when two batches of animals for comparison are involved.

1) Vitamin A is used in the form of an ester concentrate (Flétase Spécia);
2) Peanut oil is used as the diluents, devitaminized by the process described above (cf. p. 44);
3) The vitamin or the substance under examination is delivered by mouth in the form of drops or by intraperitoneal injections (daily administration) (**);

(**) According to Coward and Key (34), the results obtained are identical when the same amount of substance is delivered in two doses a week. In our experiment we chose to deliver the dose daily for two reasons:

a) the animals are in our hands all days at the same time, thus the observations regarding each individual allow detecting any anomalous behavior;
b) For freshly prepared extract (astaxanthin esters or chromoproteid solutions), the preparation is made daily from fresh material and delivered immediately.

4) Tocopherol is added when the extract is ready (vitamin A or substance being tested) and protects the active product both during preparation and during the digestive transit.

a) THE CURATIVE METHOD

The curative method design consists in adding to the diet, as soon as the plateau period is reached and xerophthalmia is in full growth, an extract the activity of which will be studied.

If the amount of substance administered matches the maintenance dose, the weight curve will stop falling; it will stabilize at least for some weeks and be therefore parallel to the time axis. If the dose exceeds the maintenance dose, the growth curve will form a certain angle with the parallel to the time axis; this angle is more or less open depending on the dose of the active principle, and the growth curve is substantially a straight line if the observation is limited to a few weeks and if quite young animals have been taken in order not to fall again within the area where growth follows a logarithmic curve. When the most favorable conditions are provided, the maximum angle is about 65°, Javillier (116).

Javillier particularly tried to know the appropriate curve for dosing. He rejects the curve delimiting the angle with the largest amount of opening, the curve corresponding to the optimum, to the highest daily gain of weight (about 2 g); in the area close to the optimum (according to the author), the method lacks accuracy. The method is most accurate in the low "growth angle" (**) area, meaning that for a low vitamin dose, a relatively higher gain is obtained. This data led Javillier to choose the curve providing a growth angle of 30° as the growth curve to obtain when conducting physiological dosing tests. This corresponds to a daily gain of weigh of about 0.55 g.

A 30-day duration is necessary, however sufficient, to obtain a conclusive response of the animal as far as weight is concerned; Coward (30) continued with the test for 5 weeks.

It is accepted that the cure of the xerophthalmia-related lesions is obtained only with doses higher than those restoring normal growth (189), (30). All data found in the literature are perfectly in agreement with this point. However, in absolute value, considerable differences are found; such deviations are attributable to the fact that the experimental designs are not always identical.

Some data found in the literature is presented in the Table below.

(*) The growth angle is the angle delimited by the straight line that, starting from the point of the growth curve corresponding to the beginning of the test, is parallel to the time axis, and by the straight line that, starting from the same point, reaches the point corresponding to the point reached on day 30 by the animal.

TABLE XIII

Curative doses expressed per kg weight and per day	
Vitamin A in µg	Authors
6.0 - 12.0	HUME and CHICK (110)
3.8 - 4.6	GOSS and GUILBERT (70)
3.0	BAUMANN et al. (6)
12.0	HORTON et al. (109)
6.0	LEWIS and BODANSKY (140)

Personal research:

The tests are conducted on batches of rats subjected to the deficient diet from weaning. Upon occurrence of apparent signs of deficiency, with a 10-day weight stabilization and strong xerophthalmia, the animals are distributed in two homogeneous batches comprising the same number of males and females.

The animals then receive daily doses as follows:

Rats from batch I: 0.6 µg of vitamin A diluted in 20 mg devitaminized peanut oil, with the addition of tocopherol (7 mg per gram oil).

Rats from batch II: 1.2 µg of vitamin A diluted in 20 mg of the same oil to which tocopherol was added.

The results of these tests show that a dose of 0.6 µg per day is insufficient to cure xerophthalmia, but that it causes a slight weight recovery. At the 1.2 µg dose, the cure of the xerophthalmia lesions and a significant weight recovery can be observed.

The observations relating to 4 animals (2 ♀, 2 ♂) made in these experiences provide the following results:

Rat ♂ No. 101:

- Maximum weight before treatment: 77 grams
- Plateau duration: 10 days
- Weight at the beginning of treatment: 69 grams
- Daily vitamin A dose: 0.6 µg

Observations:

1st day: ulcer in the left eye, dry right cornea

17th day: ulcers in both eyes

17th day: weight: 72 grams

Rat ♂ No. 111:

- Maximum weight before treatment: 72 grams
- Plateau duration: 12 days
- Weight at the beginning of treatment: 63 grams
- Daily vitamin A dose: 1.2 µg

Observations:

1st day: (stretched) ulcer in the right eye, blood-streaked exudates, dry brown cornea

15th day: the ulcer in the right eye is reduced to a point, cure of left eye is complete

17th day: weight: 72 grams

Rat ♀ No. 112:

- Maximum weight before treatment: 65 grams
- Plateau duration: 7 days
- Weight at the beginning of treatment: 58 grams
- Daily vitamin A dose: 0.6 µg

Observations:

1st day: central ulcer (2 mm in diameter) in the right eye, trouble left cornea, secretions in both eyes

16th day: no improvement

16th day: weight: 63 grams

Rat ♀ No. 108:

- Maximum weight before treatment: 67 grams
- Plateau duration: 11 days
- Weight at the beginning of treatment: 59 grams
- Daily vitamin A dose: 1.2 µg

Observations:

1st day: ulcers in both eyes, (left eye more damaged);

13th day: improvement

17th day: ulcer in the left eye cicatrizing, ulcer in the right eye is reduced to a 2mm point, secretions decreased

Weight: 70 grams.

b) PREVENTIVE METHOD

The active preparation under examination or the natural medium in which the presence of any factors having vitamin activity is searched, is administered from weaning, in addition to the synthetic diet.

In favor of the preventive method we can state that the substance under study is tested on healthy animals not showing, as in the curative method, a pathological condition resulting from the deficiency and, according to Randoin (190), “worsened by badly known secondary affections”.

Javillier (116) draws attention on the fact that the effects of the doses are comparable only if the

dose is related to the same rat weight; what must remain constant “is the proportion of the amount of material tested and the weight of living matter”. Goss and Guibert (70) provided the experimental proof that the rat needs were proportional to its weight (112).

According to Lewis and Bodansky (140), Gosse and Guilbert (70), Hume and Chick (110), 5.4 to 6.6 µg vitamin per kg weight would account for the minimum amount capable of ensuring normal growth and preventing xerophthalmia and the occurrence of keratinized cells. According to Paul and Paul (185), the effect of growth obtained with 6 µg would be below normal and at least 12 µg would be necessary to achieve normal growth and longevity.

Some figures in connection therewith are shown in the Table below.

TABLE XIV

Vitamin A in µg per kg animal	Authors
7.7	BRAUDE et al. (13)
6.0	CALLISON and KNOWLES (17)
6.0	LEWIS and BODANSKY (140)
6.0	GOSS and GUILBERT (70)
6.0	HUME and CHICK (110)
30.0	SHERMAN et al. (206)
30.0	PAU and PAUL (185)

Personal research:

Pregnant rats receive a thoroughly controlled diet (diet E 6); this diet is kept after dropping and during the suckling period.

At weaning, the pups are selected and those whose weight exceeds 30 grams are discarded. Thus, perfectly homogeneous animals having a liver reserve not to exceed a certain level are available.

Each test is conducted on 8 rats: 4 males and 4 females.

The animals are fed the deficient diet and receive the active preparation in the form of drops that are delivered daily to each animal.

Test I - Deficient diet + 20 mg devitaminized oil containing 0.15 µg vitamin A

Test II - Deficient diet + 20 mg devitaminized oil and 0.9 µg vitamin A

Test III - Deficient diet + 20 mg devitaminized oil and 1.5 µg vitamin A

Test IV - Deficient diet + 20 mg devitaminized oil and 4.5 µg vitamin A

Control batch - Deficient diet + 20 mg devitaminized oil

The experiment is continued for 100 days.

The following results were obtained:

- At the 0.15 µg dose, the animals behaved like the control animals with regard to xerophthalmia; the xerophthalmia troubles were observed at the same time and with the same severity.

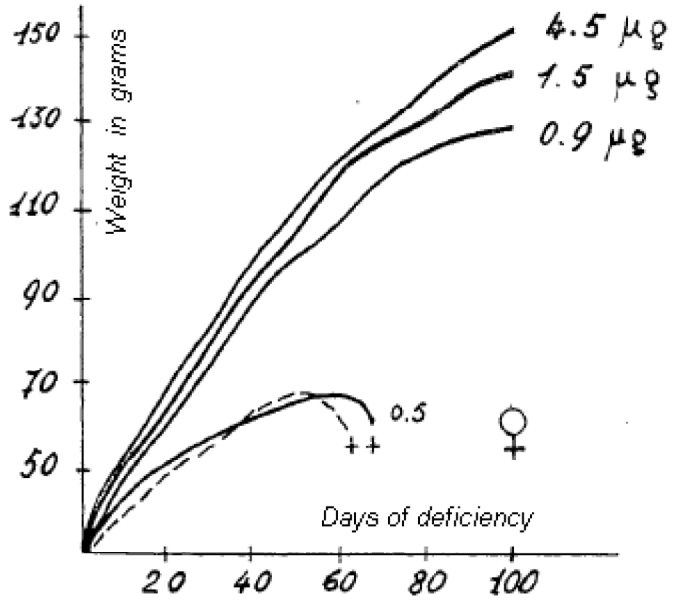
The weight curve shows a higher growth effect than those in the control animals until day 35. Beyond 35 days, the Batch I and the control Batch curves superimpose; however survival of about ten days is observed for the Batch at 0.15 µg vitamin.

- At the 0.9 µg dose, no sign of xerophthalmia was observed. The examination of vaginal smears showed no colpo-keratosis (cf. page 104). Growth was almost normal (female weight at 110 days: 130 grams; male weight: 140 g).

- At the 1.5 µg dose, neither xerophthalmia nor colpo-keratosis was found. The growth effect was marked as compared to the animals receiving 0.9 µg (female weight at 110 days: 145 grams; male weight: 150 g).

- At the 4.5 µg dose, growth and longevity increased considerably.

The results are expressed by the curves below.



c) RELATIONSHIP BETWEEN THE VITAMIN A DOSE ADMINISTERED AND SURVIVAL

According to Coward (30) none of the typical tests - growth recovery, cure of xerophthalmia and colpo-keratosis - is satisfactory because of the difficulties to determine the time of the treatment at which the physiological response can be interpreted with certainty. In the view of this author, the only valid test would be the one involving the death of the animal and its application is excluded only by reason of time. However, many authors have attempted to specify the relationship between the dose of active substance and survival.

Paul and Paul (185) provide detailed figures: below 0.3 g vitamin A per day and per 100g animal, survival does not exceed 80 days (± 2 days); it is 234 days (± 18) with 0.6 µg and 521 days (± 21) with 1.2 µg; by administering 6 µg, survival would reach 643 days.

Sherman et al. (207), (208) add 0.9 µg vitamin A per day to a diet normally containing factors A; survival extends 5% for the females and 10% for the males. With doses four times higher, the survival increases in the same proportions among the females and among the males.

In the view of Lewis and Bodansky (140), above certain doses the growth effect is identical, and for daily doses of 7.5 to 300 µg a day, the weight curves are considerably superimposed.

PERSONAL DATA

a) Curative tests:

Rats that have reached the weight stabilization stage are treated with 0.6 µg vitamin A per day. Survival does not exceed 100 days for the females, 110 days for the males.

Below 0.6 µg, survival among both sexes is always less than 90 days.

b) Preventive tests:

For a dose of 0.15 µg per day, the average days of survival is 80 days for the males, 85 days for the females.

With doses of 0.6 µg the females do not survive beyond six months, the longevity of males is from 7 to 8 months.

Finally, with doses of 0.9 µg, longevity exceeds 18 months; however, the animals stabilize around the 5th month.

DISCUSSION

In the biological tests involving either curative or preventive experiments to study the vitamin A deficiency, the results obtained will be significant only if the experimental design is thoroughly coded and if, to reduce the uncertainty factor contributed by the individual fluctuations (*), the number of animals is sufficiently high.

In all our experiments, during which about 1,000 rats were monitored, we took the greatest care:

1) that every experiment includes a number of animals of at least 12;

2) that every batch comprises the same number of males and females;

(*) Estimated at 25% according to Sherman and Batchelder (204), 30% according to Chevallier (23).

3) to ensure favorable climatic conditions; in fact, the temperature changes that always influence markedly the white rat are particularly harmful for deficient animals (51);

4) to control on a daily basis the animal nutrition. It is typically accepted (Simonnet) that the animals keep normal appetite until the steady weight period is reached during the deficiency. However, we found that for this to be so, it is necessary to flatter the animal's appetite by renewing its intake twice a day, frequently replacing sucrose by dextrin (and vice versa) to change the diet flavor.

By strictly complying with these conditions, the individual factor is reduced to a minimum and the results are obtained with the greatest consistency.

CHAPTER II

ANTI-XEROPHTHALMIC ACTIVITY OF ASTAXANTHIN

The experiments showing the vitamin activity of astaxanthin are presented in this chapter. First of all we will summarize the results obtained by the administration of hepatopancreas oil, peristomal connective tissue and hypoderm; the study of the activity of these extracts was reported in a comprehensive monograph by R. Grangaud (72),

A fortuitous observation allowed determining, in the shellfish oils, the existence of a new vitamin factor. The administration to the vitamin A-deficient white rat of Aristaeomorpha foliacea's hepatopancreas oil at a dose of 20 mg per animal per day caused fast curing of the xerophthalmia lesions while no weigh recovery was recorded.

In these experiments, the absence of growth effect could not be attributed to a toxic action of the oil, because daily 90 mg doses caused a clear weight recovery (81). In addition, these first experiments showed that the activity was proportional to the pigment concentration: only the strongly pigmented summer oils were active (76).

At this stage of the research, the issue of the identification of the active principle came out; one method of approach to determine the identity of this principle consisted in extending the experimentation to other extracts from related species having quite similar physical and chemical features. This is the reason that prompted us to study the oils from Aristeu antennatu, the pigment of which are very close to those of the Aristaeomorpha foliacea.

A - STUDY OF THE ACTIVITY OF ARISTEUS ANTENNATUS HEPATOPANCREAS OILS

The oils were extracted from the hepatopancreas of shrimps fished in February and June.

The extraction was made following the above-described method (cf. p. 27).

Like for the Aristaeomorpha foliacea, June oils are blood red, February oils are pale orangey yellow.

Curative test:

Four batches A, B, C, D of rats fed from weaning with the synthetic diet R 12 (cf. p. 44), also showing a weight stabilization of at least 10 days, signs of xerophthalmia, received:

Batch A: 3 ♀, 3 ♂ : the synthetic diet alone;

Batch B: 3 ♀, 3 ♂ : synthetic diet + 4.5 µg vitamin A;

Batch C: 3 ♀, 3 ♂ : synthetic diet + 20 mg June oil;

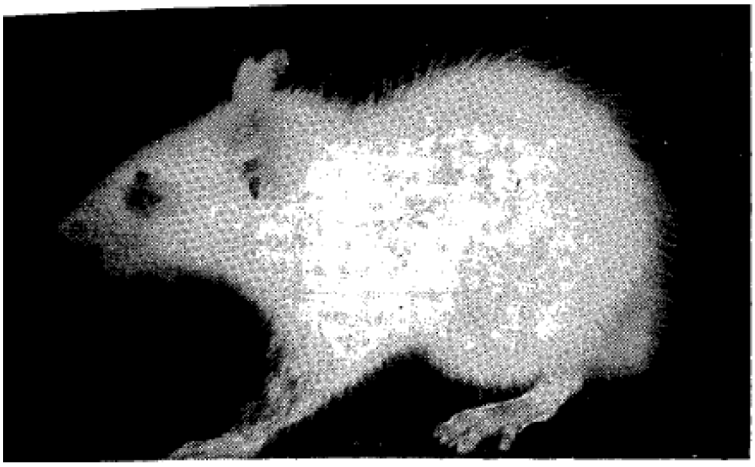


Fig. 12. Summer oil. Beginning of treatment

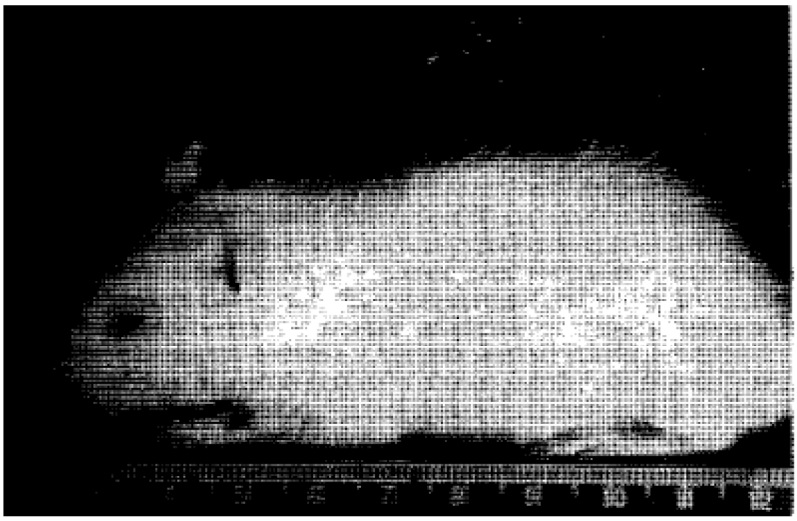


Fig. 13. Summer oil. 20 days of treatment

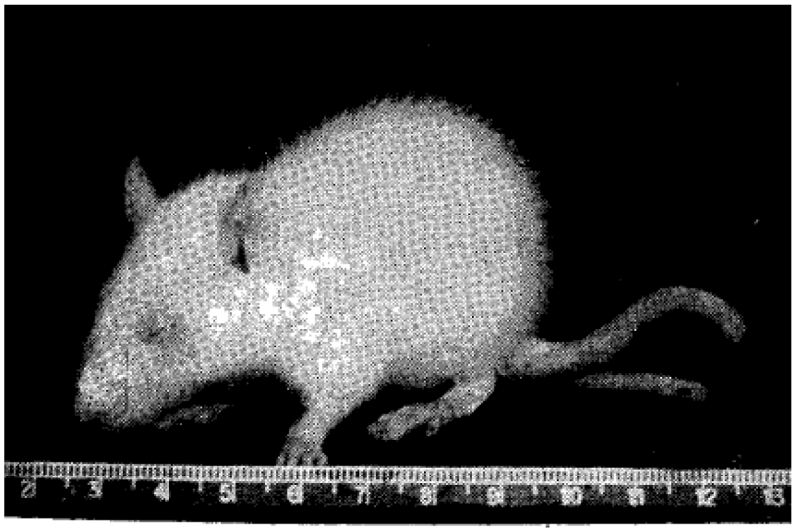


Fig. 14. Winter oil. 20 days of treatment

Batch D: 3 ♀, 3 ♂ : synthetic diet + 20 mg February oil.

At the beginning of the treatment, the animals were from 70 to 80 days old and weighed from 65 to 75 kg.

The animals from batch A (synthetic diet alone) died between the 15th and the 28th day following the occurrence of the first deficiency symptoms. Among the animals of batch B, the xerophthalmia lesions decreased gradually and were cured in 12 to 15 days. **The xerophthalmia in all rats from batch C (2.0 mg June oil** per day**) was cured in less than 12 days, the improvement was already apparent after 4 days of treatment**. However, none of them regained weight considerably and all died with their eyes healed, within the period of 25 days following the first administration of oil (Fig. 12 and 13). Finally, the rats from batch D (2.0 mg February oil per day) behaved like those from batch A in all aspects: no oil activity was recorded (Fig. 14).

In short, these experiments show the anti-xerophthalmic activity of the Aristeus antennatus oil at doses in which, however, the weight recovery is nil. The seasonal changes recorded can be exactly superimposed to those previously observed for the Aristaeomorpha foliacea; they therefore provide an additional argument in favor of the assumption of the pigmentary nature of the anti-xerophthalmic principle present in summer oils.

These experiments were completed with preventive tests. We have emphasized above the advantages of such method, particularly valuable for the study of a new factor.

Preventive test (82)

Thirty Winstar rats 25 to 30 day old weighing from 29 to 30 g were weaned and divided into five batches of six animals each, and received daily:

Batch A: the synthetic diet alone;

Batch B: synthetic diet + 0.15 µg vitamin A;

Batch C: synthetic diet + 0.9 mg vitamin A;

Batch D: synthetic diet + 10 mg Aristeus antennatus oil;

Batch E: synthetic diet + 90 mg Aristeus antennatus oil.

After thirty days, the animals quickly divided into several groups:

- Among the animals from batch A (base diet alone), signs of xerophthalmia appeared and strengthened to death, no rats in this batch survived beyond the 50th day of deficiency.

- For batch B (0.15 µg vitamin A), the eye injury also appeared by the 30th day and the xerophthalmic lesions, less severe than in the animals of batch A, persisted until death that occurred before the 60th day of deficiency.

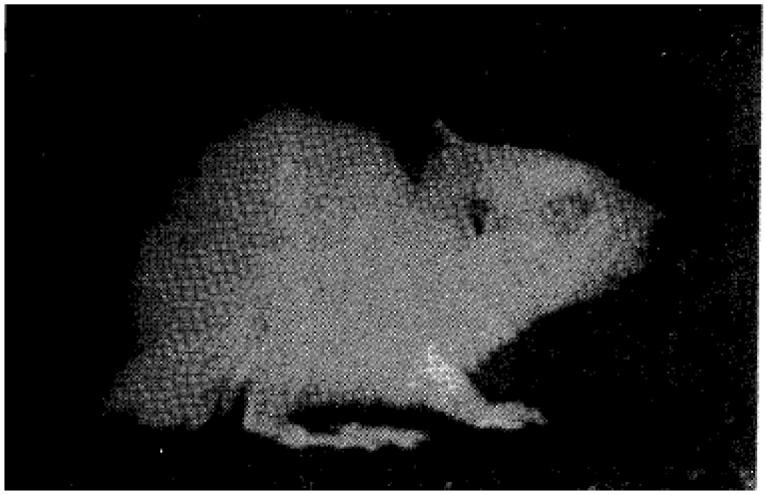


Fig. 15 - Batch A, base diet alone, 38th day of deficiency

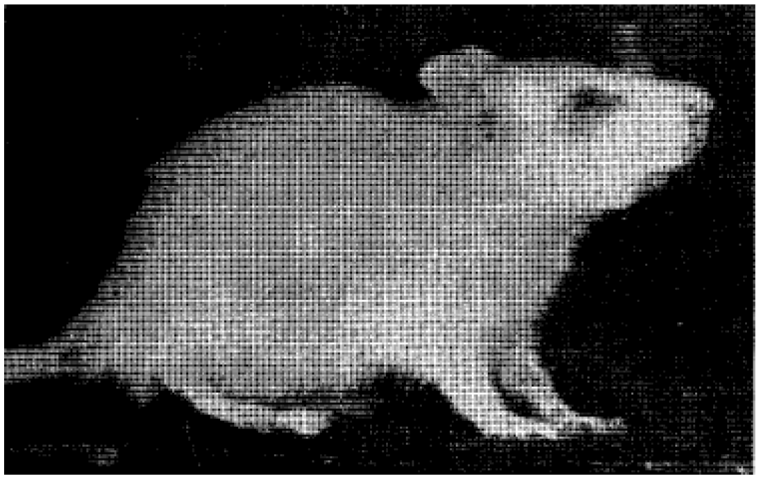


Fig. 16 - Batch B, base diet + 0.15 µg vitamin A, 38th day of deficiency

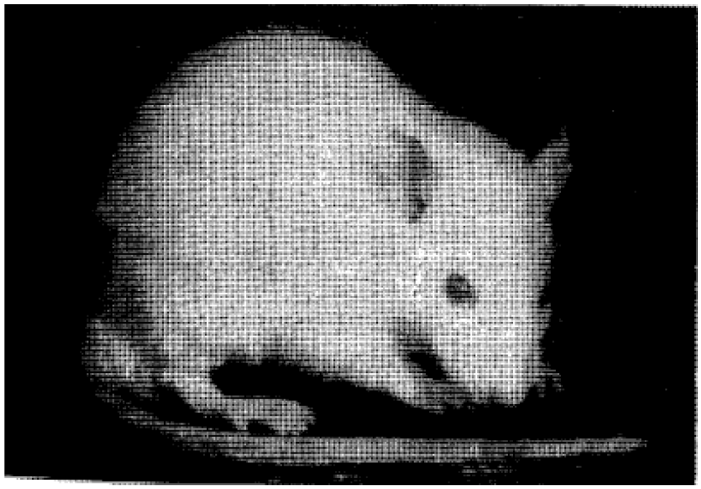


Fig. 17 - Batch C, base diet + 0.9 µg vitamin A, 38th day of deficiency

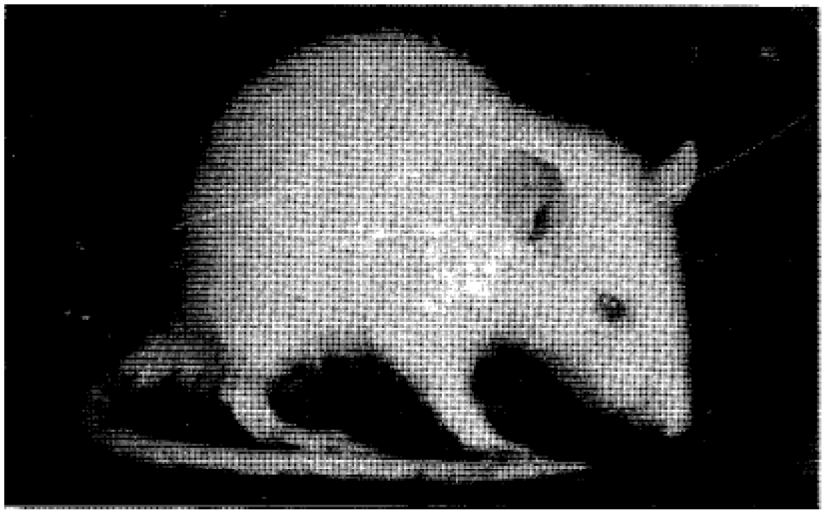


Fig. 18 - Batch D, base diet + 10 mg Aristeus antennatus oil, 38th day of deficiency

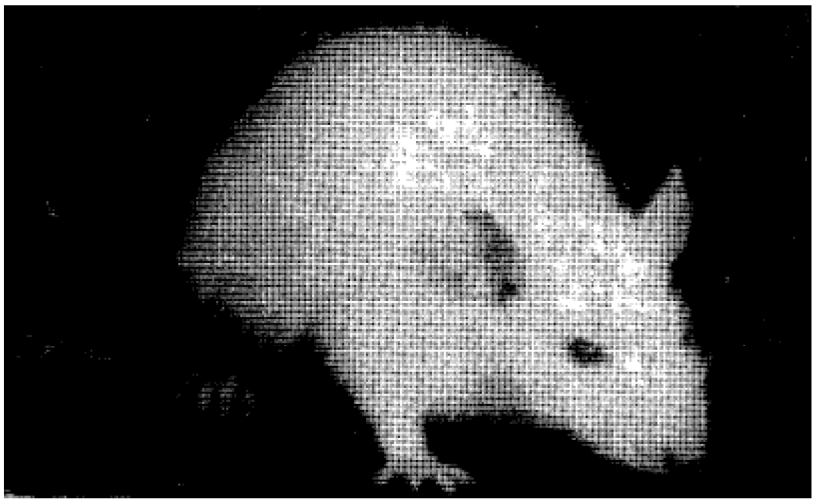


Fig. 19 - Batch E, base diet + 90 mg Aristeus antennatus oil, 38th day of deficiency

- **The rats of batches C (0.9 mg vitamin A) and E (90 mg oil) showed no sign of deficiency; their eyes remained perfectly healthy.**

- Finally, the rats from batch D (10 mg oil) differed on the one hand from the animals in groups A and B and on the other hand from the animals in groups C and E.

As a matter of fact, the animals in this batch behaved, as far as growth and survival are concerned, like the animals of batches A and B, and did not go beyond the 56th of deficiency. **However, they show at no time any signs of xerophthalmia; the Figures herein (Fig. 16, 17, 18, 19) objectify these facts and emphasize that the effects of a small dose of Aristeus antennatus hepatopancreas oil are comparable to those recorded when 0.9 µg vitamin A were administered**. In addition, we will see (cf. Chapter III) the observations relating to these animals' growth and survival; however, it is interesting to make now the following remark: while the administration of 0.15 µg vitamin A per day shows a slight influence on growth, but is not enough to prevent the occurrence and establishing of xerophthalmia**, the Aristeus antennatus hepatopancreas oil, at a dose that has however no influence on the increase in weight, prevents eye injury.**

The conclusion of these experiments in agreement with those conducted in parallel with Aristaeomorpha foliacea, is that the anti-xerophthalmic activity is closely related to the pigment concentration. **In a study with R. Grangaudet and C. Chechan (77), we showed that it is a matter of astaxanthin (3,3'-dihydroxy, 4 4'-diketo - β - carotene).** In the presence of this result that is in disagreement with the typical data (176), it was necessary to check whether or not the astaxanthin oxidation product, the astacine (3,4-3',4'-tetraketo-β-carotene) has vitamin activity.

B - STUDY OF THE EFFECTS OF ASTACINE ADMINISTRATION

Astacine is readily obtained as a sodium salt in the form of red flakes (cf. p. 41). After dissolution in devitaminized oil, a strongly colored red extract is obtained.

Curative test.

The experimental design is the same as previously described.

12 Wistar rats (6 ♀, 6 ♂) fed from weaning the vitamin A-deficient diet and showing apparent signs of deficiency, were divided into 2 batches (A and B) and received per animal, per day, the following:

Batch A (4 ♀, 4 ♂): base diet (R. 12) + 50 mg astacine suspended in 80 mg devitaminized oil.

Batch B (2 ♀, 2 ♂): base diet (R. 12) + 80 mg devitaminized oil.

The treatment lasted 25 days, no improvement of xerophthalmia was recorded (83); in addition, no action on growth was recorded.

The development of xerophthalmia, loss of weight and survival in all animals from batch A were comparable to those in the animals from batch B fed the deficient diet alone.

This experiment therefore confirmed the typical data: astacine has no vitamin A activity.

C - STUDY OF ASTAXANTHIN ESTER ACTIVITY

Technical material.

The chromatographic method of preparation of these esters has been described in Chapter 1 (cf. p. 39). We have seen (cf. p. 40) that alumina chromatography alone is used to separate free, esterified astaxanthin from vitamin A and from its esters if, in spite of the chemical and spectrophotometric controls, we accept that those esters can be present in the oils studied.

After chromatographic separation, the astaxanthin esters were dissolved in the devitaminized peanut oil to which tocopherol was added. **The concentration of astaxanthin in the oil is 20 µg per 100 mg oil with addition of 2.8 mg tocopherol acetate. The activity of these esters was tested in curative and preventive experiments.**

Curative tests (85) - Experimental protocol

Two Wistar rat batches A and B fed from weaning the deficient diet and showing a weight stabilization of at least 10 days and xerophthalmia disorders, received per animal, per day, the following:

Batch A (5 ♀, 3 ♂): base diet + 15 µg astaxanthin esters dissolved in 80 mg devitaminized oil with the addition of tocopherol (28 mg per g oil).

Batch B (3 ♀, 3 ♂): base diet + 80 mg devitaminized oil with the addition of tocopherol (28 mg per g oil).

The oily solution of esters was delivered by mouth using a dropper.

The control animals received oil alone in the same conditions.

Experimental results.

Among the animals from batch B (control animals), the development of the deficiency was evidenced by the increasing severity of the xerophthalmic lesions; loss of weight and death occurred between the 68th and the 87th day of deficiency.

The following observations were made in the animals from batch A:

- 2 (female) animals showed at the beginning of treatment corneal vascularization with a central ulcer (left eye) from 2 to 3 mm in diameter, an ulcer at the beginning (right eye). The eyelids were red and swollen (Fig. 20).

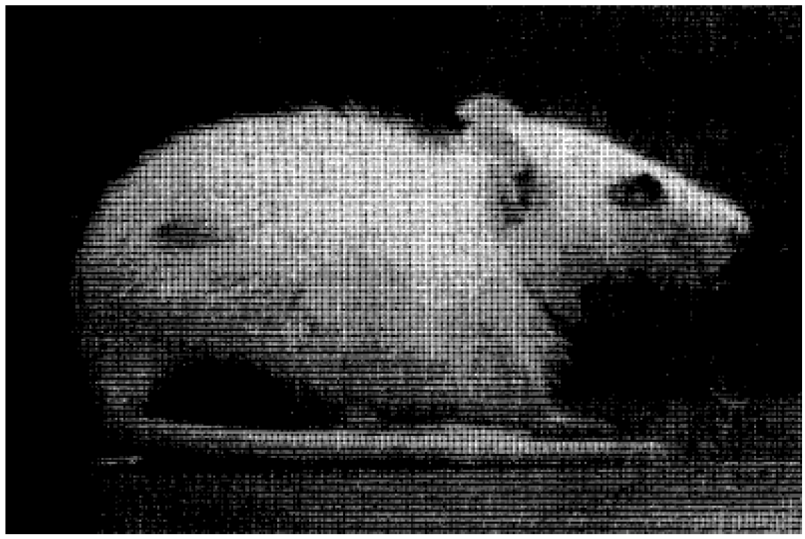


Fig. 20

Improvement was apparent from the third day of treatment, cure was obtained in less than 12 days (Fig. 21).

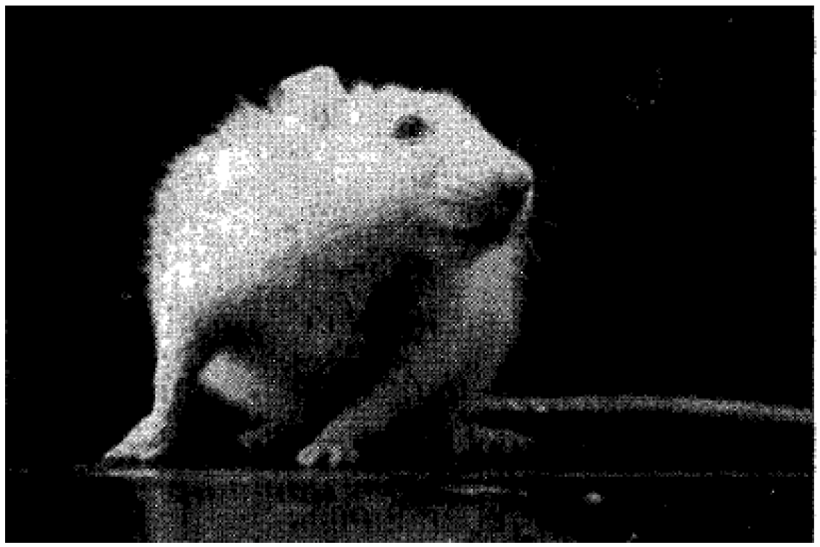


Fig. 21

- 2 rats (1 ♀, 1 ♂) more affected, showed exulceration (right eye and left eye). At the third day of treatment, no improvement was seen (macroscopic examination); on the contrary, worsening is observed (hemorrhage in the right eye).

At the 4th day, a slight improvement is seen: slight decrease of exudates, less purulent cornea.

At days 5 and 6, improvement is apparent: exudates dry.

At day 7, a reduction in the left eye ulcer (in the female) is observed.

At day 15, cicatrization was almost total in both animals.

2 rats showed a central perforation with iris hernia (left eye ♀, right eye ♂) - improvement appeared only at the 8th day, healing is slow but gradual; it is complete at the 25th day of treatment; only a corneal opacity persists. In addition, the infectious processes (scabs and blood-streaked exudates) in the eyelids disappeared completely on the 17th day.

- 1 rat (♀) showed at the beginning of the treatment xerophthalmic disorders less severe than those of the animals referred to above: only a dull cornea, a small palpebral fissure and eyelid exudates could be seen. Cure was obtained in 6 days.

Preventive tests - Experimental protocol.

Twenty-four Wistar rats 25 to 30 day old weighing from 29 to 30 g were weaned and divided into 3 batches A, B and C:

The animals of each batch receive the following per day:

Batch A (3 ♀, 3 ♂): the synthetic diet alone;

Batch B (3 ♀, 3 ♂): synthetic diet + 80 mg oily astaxanthin solution (50 µg) with addition of tocopherol;

Batch C (3 ♀, 3 ♂): synthetic diet + 80 mg oil with addition of tocopherol containing 0.9 µg vitamin A;

All animals received also the same amount of devitaminized oil with addition of tocopherol (20 mg per g oil) delivered daily at the same time, using a dropper. To avoid any lipid overload at the time of weaning where diarrhea sometimes occurs, the oil content of the diet was reduced to 10% (instead of 12%). The animals were thoroughly monitored and weighed on a daily basis for a period of 10 days after weaning, then on a regular basis every five days for the duration of the experiment. Monitoring continued for several months.

Results

Among the animals from the control batch (batch A), the signs of xerophthalmia occurred by the 30th day after weaning and worsened to death, that occurred between the 50th and the 56th day.

Among the animals from batch C, two of them showed moderate signs of xerophthalmia (eyelid blinking - reduction of the palpebral fissure); however, no infectious process was found.

The animals from batch B (50 µg astaxanthin) at no time showed any signs of xerophthalmia. This long-term experiment continued for 135 to 150 days for the females (that died spontaneously) and 210 days for the males (sacrificed). The corneas remained bright, no sign of eyelid irritation was seen.

D - STUDY OF THE ACTIVITY OF CHROMOPROTEINS

The experiments that will be described were intended to check the activity of astaxanthin in a protein complex. The prosthetics group is reversibly detachable and we evidenced this fact on aqueous astaxanthin-protein extracts from eggs of different species.

1. PRELIMINARY TESTS

A first test was conducted by feeding the deficient rats ovaries with mature eggs suspended in devitaminized vegetable oil (84).

- Aristaeomorpha eggs were ground in a mortar: the amount of oil added was measured in such a manner to obtain a final egg concentration of 1 g per 150 mg oil, the astaxanthin level was estimated at 60 µg per g egg (*):

Curative test

16 deficient rats having reached the weight stabilization stage and showing signs of severe xerophthalmia, were divided into two batches A and B that received per animal, per day, the following:

Batch A (4 ♀, 4 ♂): base diet + 500 g eggs (30 µg astaxanthin) ad 80 mg devitaminized vegetable oil

Batch B (4 ♀, 4 ♂): base diet + 80 mg devitaminized oil

After 18 days of treatment, no improvement was recorded; both the animals from batch A and batch B showed increasingly marked signs of deficiency.

To interpret this negative result, apparently opposite to the activity shown by the astaxanthin esters, we suggested that during digestive transit the pigment, quickly released from its protein envelope, should be transformed by oxidation into inactive astacin. This assumption was verified in a second test in which an oily solution of free astaxanthin is added tocopherol that acts as an antioxidant.

2. ORAL ADMINISTRATION OF THE PROSTHETIC GROUP DETACHED FROM ITS PROTEIN ENVELOPE

In this test, the starting material is a chromoproteid in an aqueous solution, extracted from the Aristeus antennatus peristomal connective tissue. The tissue is ground with sand and saline (0.9 % NaCl) - 100 mL aqueous solution was stirred with acetone and petroleum ether (30 mL).

The prosthetic group is dissolved in petroleum ether, the pigment content was estimated by measuring its optical density corresponding to 50 µg for the entire solution. After the addition of devitaminized vegetable oil (400 mg) and tocopherol (12 mg), the mixture was evaporated in a nitrogen atmosphere under low pressure.

Curative test.

14 rats fed the base diet from weaning and showing marked signs of deficiency (severe xerophthalmia and weight stabilization) were divided into two batches A and B that received per animal, per day**, the following:**

(*) For this determination, the eggs were stirred with acetone. After passing the pigment through petroleum ether, the astaxanthin concentration of the solution was calculated by measuring its optical density, taking:

$$E^{1\%}_{1\,cm} = 3{,}300\ (120)$$

Batch A (4 ♀, 4 ♂): base diet + 80 mg oily astaxanthin solution (10 µg)

Batch B (3 ♀, 3 ♂): base diet + 80 mg devitaminized oil

Rats from batch A started to improve from day 4. Cure is achieved in 10 days average (min. 8, max. 12 days). Worsening of eye injuries was seen in animals from the control batch, batch B.

It was therefore natural to study the vitamin properties of chromoproteins by administering them no longer orally but by intraperitoneal injections.

3. ADMINISTRATION OF AQUEOUS EXTRACTS BY INTRAPERITONEAL INJECTIONS

Materials and methods.

Aqueous solutions of eggs from Plesionika edwarsii, Aristeus antennatus and Scyllarus latus and of Aristeus antennatus peristomal connective tissue were prepared.

1.5 grams of eggs or tissue were ground with sand and added saline; **the final astaxanthin concentration was 80 µg per 30 mL solution.**

Each animal was injected 3 mL per day, which corresponds to 150 mg fresh material. These 150 mg contained, in the case of the Aristeus antennatus eggs, 0.2 µg vitamin A (cf. Table VI), less than two hundredths of micrograms for the Scyllarus latus eggs and non-measurable traces in the Plesionika edwarsii eggs.

The solutions were administered at a rate of two 1.5 mL injections per day **to deficient rats showing signs of developing xerophthalmia.**

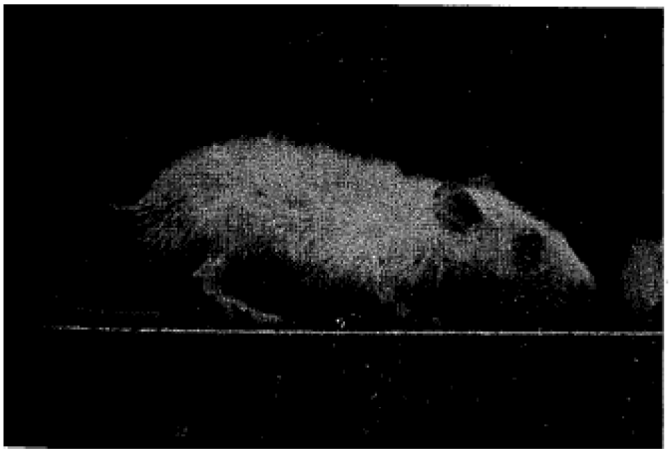
Experimental results

The injected solutions led in a few days and in all cases to the healing of the eye injuries. Figures 22 and 23 objectify the results, all of which are shown in the following Table.

TABLE XVI

Material under study	Number of animals	Xerophthalmia cure
Plesionika edwarsii eggs	3 ♂	in 13 days
Aristeus antennatus eggs	2 ♀, 1 ♂	in 6 days
Scyllarus latus eggs	2 ♀, 1 ♂	in 13 days
Aristeus antennatus peristomal connective tissue	2 ♀, 1 ♂	in 11 days
Human serum	1 ♀, 4 ♂	worsening or stationary condition at the 10th day

At the beginning of the treatment



After 13 days

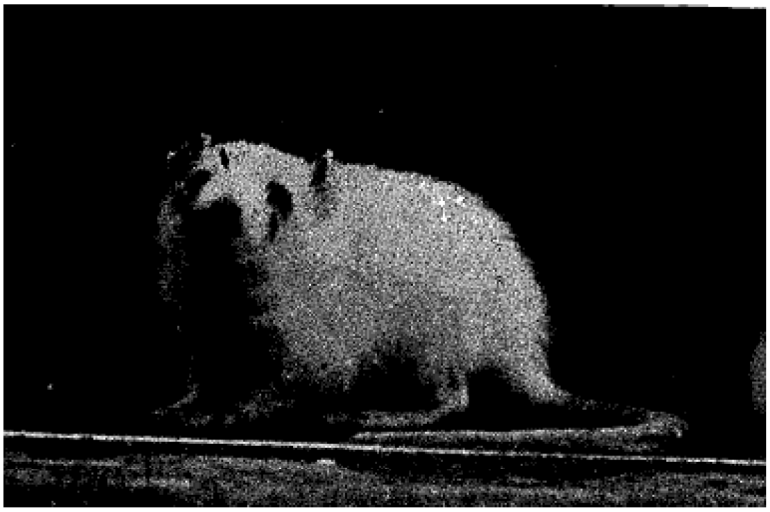
Fig. 22 - Injections of aqueous extract of Plesionika edwarsii eggs

At the beginning of the treatment

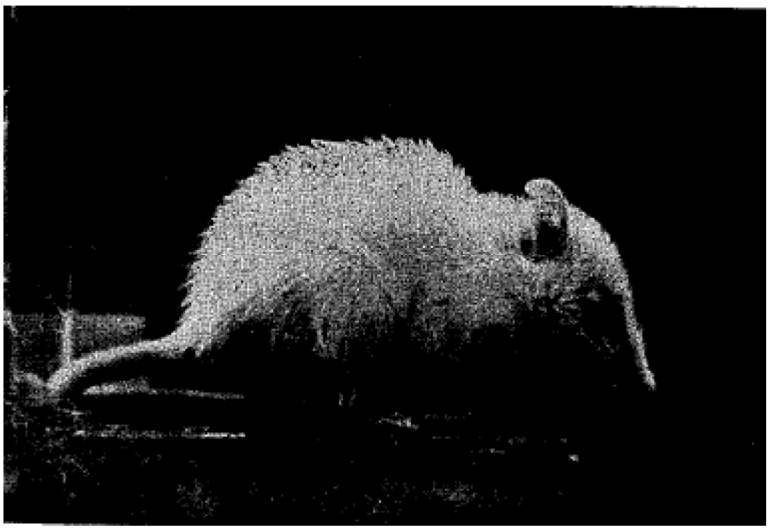
After 6 days

Fig. 23 - Injections of aqueous extract of Aristeus eggs

We found important, for control purposes, to inject to the control rats with vitamin A in a water-soluble form as comparable as possible to that present in the shellfish eggs. We thought that blood serum where the vitamin is present in lipoprotein complexes would be perfectly appropriate for such control. The volumes injected daily to the control rats would correspond to 0.55 µg Vitamin A, that is 2 to 3 times more than in the solution. Among the animals, the xerophthalmia lesions worsened; only one of the treated animals showed a substantially steady state: however, no tendency to improvement was seen (Fig. 24).



At the beginning of the treatment



After 12 days

Fig. 24 - Injections of human serum

In short, the aqueous solutions of chromoproteins show, in the intraperitoneal injections given to the vitamin A-deficient white rat, the same antixerophthalmic activity as the oily solutions of astaxanthin esters administered orally.

Therefore, according to this chapter, it appears that, considered only from the xerophthalmia standpoint, 5 µg astaxanthin show an activity comparable to that obtained with 0.9 µg vitamin A. **Whatever the mode of administration (orally or by intraperitoneal injection), the astaxanthin always leads to the cure of eye injuries in very short periods of time.** When cornea alterations reach an advanced stage resulting in ulcer and even perforation, cure starts earlier than found in similar cases treated with vitamin A.

However, two results, obtained in different experimental conditions from those mentioned above, for which reason they have not been provided, are worthy of being stated because they are particularly illustrative.

1. The astaxanthin (10 µg) test was conducted after failure of a treatment with vitamin A (0.6 µg per day): the reversibility of lesions can be quickly seen.

2. The administration of astaxanthin after healing was replaced by a treatment with vitamin A (0.6 µg per day): the signs of xerophthalmia reappear.

These facts did nothing but confirm the strong xerophthalmic activity of the astaxanthin previously determined.

LOCAL APPLICATION TREATMENT TEST

According to the foregoing, astaxanthin shows a strong anti-xerophthalmic action whatever the route of administration. It was therefore interesting to study the action of the pigment by local application.

It is known that, as far as vitamin A and carotenes are concerned, numerous observations are found in the literature and the experimental results particularly show that vitamin A is involved to the process of repairing a damaged tissue. **The topical application of vitamin A is found in laryngology (63), dermatology (40), (197), (215), ophthalmology. In 1934, Balachoski (3) experimented on the action of carotene in conjunctivitis. Heinsius (102) conducted instillations of vitamin A dissolved in differed oily carriers, on. Injuries caused by conjunctival incisions (in rabbits). Favorable results were obtained in all cases.**

Experimental protocol

The preparation used was an oily solution of astaxanthin esters.

Six deficient rats showing signs of xerophthalmia of different degrees received daily instillations of 4 drops of the preparation on the cornea. The treatment continued for 15 days.

No improvement was recorded, even among the subjects that showed signs of xerophthalmia at the beginning of the treatment.

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CHAPTER III

ACTION OF ASTAXANTHIN ON THE GROWTH OF VITAMIN A-DEFICIENT WHITE RAT

Since the first experiment (80), the vitamin factor revealed in the hepatopancreas oils could be distinguished by its actually anti-xerophthalmic activity, while the action on growth of the deficient rat was weak or nil. It is also known that by administering vitamin A the opposite condition can be seen, the weight curve restarts going upward before any improvement of eye injuries appears.

The possibility of the presence of a toxic substance inhibiting the growth effect was to be precluded because, at strong doses, the hepatopancreas oils have both an anti-xerophthalmic action and an action on growth (81), (82), (155).

The fact that such strong doses of oils were perfectly tolerated naturally ruled out the assumption of toxicity, but did not provide, however, the proof that the pigment administered in a strong dose is sole responsible for the activity recorded. In fact, those strong doses of pigment are necessarily accompanied by strong doses of lipids, and one could ask oneself whether or not they are involved in the action on growth.

The comparison of the biological effects of the summer oils and the winter oils could determine the respective role of the pigment and the lipids.

As a matter of fact, in winter oils the pigment only exists as traces; its concentration in summer oils is from 1 to 1.5 mg per g.

A - COMPARATIVE STUDY OF THE ACTIVITY OF SUMMER OILS AND WINTER OILS EXTRACTED FROM THE ARISTEUS ANTENNATUS HEPATOPANCREAS

Materials and methods

The extracts from Aristeus antennatus hepatopancreas are prepared in summer and winter and kept in small brown glass flasks, carefully stoppered and kept in cold storage.

The biological tests are of the curative type and conducted in parallel so as to make the experimental conditions of the animals treated with winter oils and the animals treated with summer oils strictly similar.

16 Wistar rats are given the deficient diet from weaning. The distribution of the animals into two batches A (summer oil) and B (winter oil) is carefully studied for them to be perfectly homogeneous: when two deficient rats of the same sex reach the same weight, while showing comparable eye injuries, one of them is treated with winter oils and the other one with summer oils. Eventually, two batches (A and B) of comparable animals are available at the time the treatment is administered.

Batch A (4 ♀, 4 ♂): base diet + 20 mg May oil per day (*)
Batch B (4 ♀, 4 ♂): base diet + 20 mg February oil;
Batch C (4 ♀, 4 ♂): base diet alone;

(*) With an astaxanthin concentration of 15 μg

Experimental results

All the control rats (batch C) died between the 50th and the 60th day of deficiency; those from batch B (February oil) behaved like those from the control batch; no oil activity was recorded: the growth curves continued to fall and the animals died within 30 days following the beginning of the treatment.

This experience showed:

1. that the winter oils at a dose of 20 mg per day are totally inactive;

2. that, at the same dose, the pigmented summer oils with a strong anti-xerophthalmic activity, did not show any action on growth.

Nevertheless, the administration of relatively significant doses (90 mg per day) allows restoring an almost normal growth in the deficient animal (81). For a better interpretation of these different results, a preventive test was conducted, accompanied by control tests with vitamin A.

B - STUDY OF THE ACTIVITY OF SUMMER OILS COMPARED TO VITAMIN A ACTIVITY

30 Wistar rats 25 to 30 days old weighing from 29 to 30 g were weaned and divided into five batches of 6 animals each. From weaning ("preventive"-type tests), they received:

Batch A: base diet alone;
Batch B: base diet + 0.15 µg vitamin A;
Batch C: base diet + 0.9 µg vitamin A
Batch D: base diet + 10 mg Aristeus antennatus hepatopancreas' May oil;
Batch E: base diet + 90 mg of the same oil.

Each of the animal received also 2 mg α-tocopherol per week.

The results obtained were as follows:

1. The rats from batches C (0.9 µg vitamin A) and E (90 mg oil) had a normal growth to reach a plateau by the 110th day of treatment, with the average weight being 135 g for batch C and 132 g for batch E.

2. The rats from batches A (control), B (0.15 µg vitamin A) and D (10 mg oil) showed weight curves that fell by the 20th day after weaning, to reach their maximum between the 40th and the 45th day of treatment. At this stage, the average weight was:

- for batch A (control), 59 g;
- for batch B, 63 g;
- for batch D, 60 g.

The animals from batch A (base diet alone) did not survive beyond the 50th day of deficiency, those from batch B (0.15 µg vitamin A) died by the 60th day of deficiency (average weight 64 g). Finally, the rats from batch D (base diet + 10 mg Aristeus antennatus oil) did not survive beyond the 56th day of deficiency.

C - COMPARATIVE STUDY OF THE ACTION OF ASTAXANTHIN AT DIFFERENT CONCENTRATIONS AND IN DIFFERENT LIPID MEDIA

The results obtained by the administration of strong doses of whole oils raise several objections in connection with the role which may be played by the lipids. The purposes of the following experiments were as follows:

1. to dissociate the lipid action from the pigment action by administering the pigment (isolated by chromatography) in a devitaminized vegetable oil;
2. to compare the action of extracts with a higher or lower concentration of astaxanthin, dissolved in the same volume of devitaminized vegetable oil;
3. to parallel the effects of the same doses of pigment administered in different oily carriers.

Preventive tests

These tests were conducted using:

- either extracts with a variable astaxanthin content in the same oily carrier;
- or extracts with the same content of astaxanthin in different oils.

Preparation of extracts

Among these extracts, three were prepared from a pigment isolated by chromatography from hypodermis and peristomal connective tissue solutions (extract No. 1, 2 and 3); extract No. 5 alone was obtained using an epiphase solution then chromatographed.

Extract No. 1 - 200 mL petroleum ether solution (of chromatographed pigment) containing 200 µg of astaxanthin, are added 1g devitaminized vegetable oil with the addition of tocopherol (28 mg per g). The mixture is distillated under low pressure in a nitrogen atmosphere. The astaxanthin concentration is 20 µg per 100 mg oil.

Extract No. 2 - 800 mL of the same solution (800 µg astaxanthin) is evaporated in the presence of 1 g devitaminized vegetable oil with the addition of tocopherol. The astaxanthin concentration is 80 µg per 100 mg oil.

Extract No. 3 - From 200 mL petroleum ether solution with the addition of 1 g winter hepatopancreas oil and 28 mg tocopherol, an extract is obtained in which the concentration of astaxanthin is 5 µg per 100 mg oil.

Extract No. 4 - A petroleum ether solution obtained by stirring an acetone extract of winter hepatopancreas, is added an amount of α-tocopherol and distillated under low pressure in a nitrogen atmosphere. The oily extract obtained contains 28 mg tocopherol per g. oil.

Extract No. 5 - An epiphase solution of peristomal connective tissue is chromatographed. The pigment eluted in a-petroleum ether solution is added devitaminized vegetable oil and tocopherol. The astaxanthin concentration of the extract is 50 µg per 100 mg oil.

Experiments

Wistar rats were weaned and distributed in 6 homogeneous batches, each comprising 4 males and 4 females. Each of the animals was fed the deficient diet (diet R 12) and received orally, on a daily basis:

Batch 1: 5 µg astaxanthin in 25 mL devitaminized vegetable oil.

Batch 2: 20 µg astaxanthin in 25 mL devitaminized vegetable oil.

Batch 3: 5 µg astaxanthin in 100 mL Aristeus antennatus hepatopancreas' winter oil.

Batch 4: 100 mL Aristeus antennatus hepatopancreas' winter oil.

Batch 5: 50 µg astaxanthin in 100 mL devitaminized vegetable oil.

Batch 6: 0.9 µg vitamin A in 100 mL devitaminized vegetable oil.

A batch of control animals, 3 ♀, 3 ♂ received only the base diet. Each extract is added α-tocopherol in an amount calculated on the basis of a daily dose of 0.7 mg per animal. The administration of the extracts begins at weaning and continues for 100 days. In parallel, control batches are maintained: with the survival of the deficient animals not exceeding 60 days, other rats are provided the deficient diet during the experiment.

The results are stated in Figures 25 and 26.

Analysis and discussion of results

1. The comparison of the results obtained by administering 5 µg astaxanthin per day (in 20 mg devitaminized oil) and 20 µg in the same amount of oil shows a similarity of weight in the females from the two batches until the 60th day, in the males until the 70th day. Only by extending the treatment is a weight growth recorded among the animals treated with 20 µg: the average weight of the males at the 80th day of treatment exceeds by about ten grams that of the males receiving 5 µg pigment. The weight of the females receiving 5 µg stabilizes from the 60th day and then falls between the 70th and the 75th day, while with 20 µg they reach the 75th day with an excess weight of 12 to 15 g.

2. The differences recorded between batch 1 at 5 µg in vegetable oil and batch 3 at 5 µg in hepatopancreas oil, are as follows: until the 70th day of treatment, (100 days of age), the growth of batch 3 (♀ and ♂) is higher than that of batch 1; beyond day 70, the weight stabilizes in the animals from the two batches, while the animals from batch 3 maintain an excess weight of about ten grams both in the males and in the females.

3. The comparison of batch 3 (5 µg astaxanthin and 100 mg hepatopancreas oil) and batch 4 with hepatopancreas oil alone (100 mg) shows a parallel weight growth until the 60th day; the influence of the addition of astaxanthin can only be seen from the 60th day. Beyond the 60th day, the animals treated with hepatopancreas oil decline and their survival does not exceed the 80th day among the females and the 55th day among the males. A supplementary test with rats receiving a double dose of hepatopancreas oil (200 mg) confirmed the lack of activity of these oils in relation to growth, with curves that can be considerably superimposed to those for the animals receiving 100 mg oil.

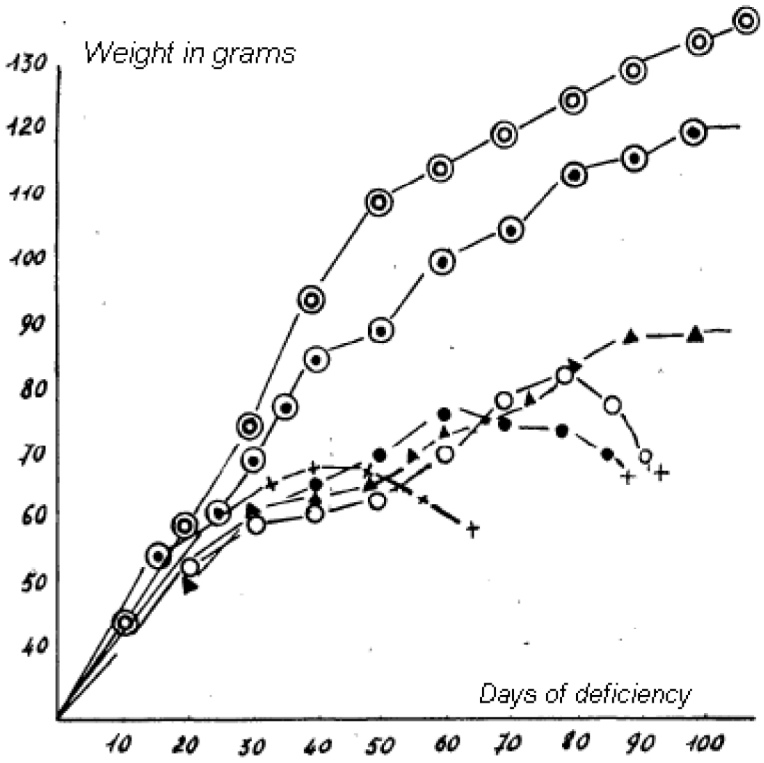

Fig. 25 : Growth curves for ♂ rats with astaxanthin

BATCH 1 :
BATCH 2 :
BATCH 3 :
BATCH 4 :
BATCH 5 :
BATCH 6 :

1

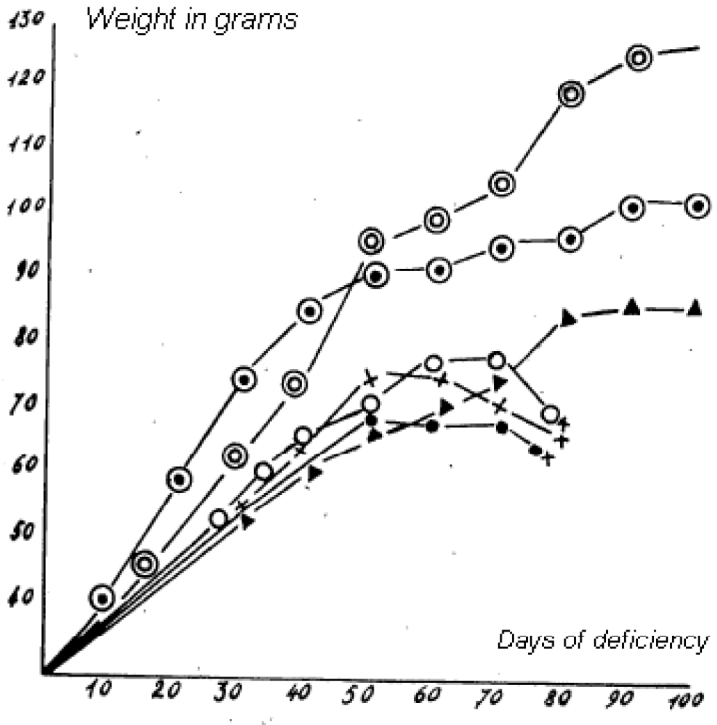

Fig. 26 - Growth curves for ♀ rats with astaxanthin

The growth curves (Fig. 25 and Fig. 26) represent these results and disclose the following facts:

1. Astaxanthin has a slight growth effect only at a dose of 20 µg per animal per day, a dose over 4 times higher than the dose capable of curing the eye injuries.

2. The use of pigment appears slightly better when the oily carrier is a hepatopancreas extract.

3. The hepatopancreas oil administered alone shows no growth effect, and the number of days of survival of the treated animals is substantially equal to that of the control animals.

4. If the growth curves for the animals having received 50 µg astaxanthin (batch 5) are compared to those for the animals having received 0.9 µg vitamin A (batch 6), we can see that they are considerably parallel with, however, a difference of about ten g (below) for batch 5.

Curative tests

In these tests, a dose of 50 µg astaxanthin is administered per animal per day and its effects on the possible weight recovery are compared to the recovery recorded in the animals receiving 0.5 and 1.2 µg vitamin A, also for curative purposes.

The extract administered is prepared from an epiphase pigment chromatographed and dissolved in devitaminized vegetable oil with the addition of tocopherol (20 mg per g).

Experiment

30 rats are given from weaning the base diet (diet R 12) and treated when the signs of deficiency are apparent (weight stabilization since 10 days, severe xerophthalmia lesions). The animals are distributed in 4 batches A, B, C, D. each of the animals receives orally, per day:

Batch A (4 ♀, 4 ♂): base diet + 50 µg astaxanthin in 100 mg oil with addition of tocopherol;

Batch B (4 ♀, 4 ♂): base diet + 0.6 µg vitamin A and 100 mg oil with addition of tocopherol;

Batch C (4 ♀, 4 ♂): base diet + 1.2 µg vitamin A and 100 mg oil with addition of tocopherol.

Control batch: (3 ♀, 3 ♂): base diet + 100 mg oil with addition of tocopherol.

Results

A slight weight recovery was recorded, substantially of the same significance for the rats from batch A (50 µg astaxanthin) and B (0.6 µg vitamin A). The curve representing the growth of batch C (1.2 µg vitamin A) clearly separates from the other two and at the 10th day of treatment it shows an excess weight of about ten gr. over the weight of the animals from batches A and B.

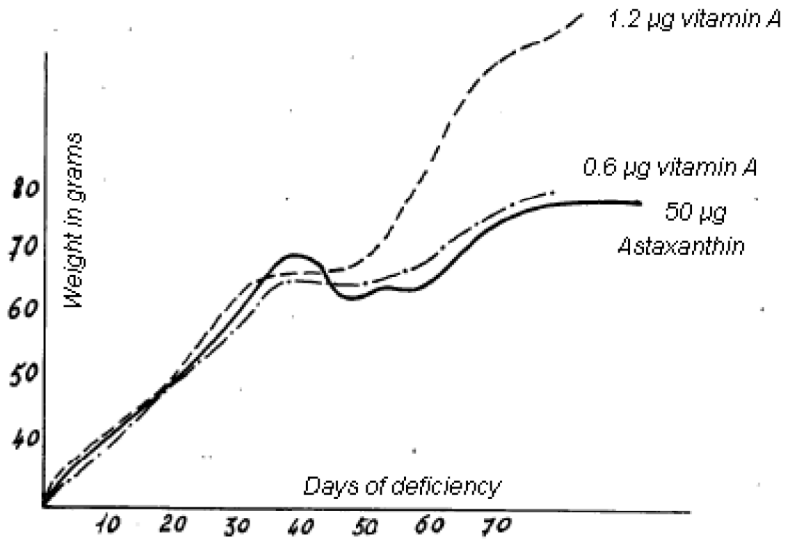

Fig. 27

At the 20th day of treatment, the curves for the animals from batches A and C continue to be parallel and tend to a plateau; on the other hand, the curve for the animals from batch C still climbs upward and at the 20th day the weight differences between curve C and curves A and B reach about twenty grams.

At the 55th day, curves A and B are in a plateau, the animals from the two batches have stabilized at about 65 g; those from batch C have reached 68 g for the females, 75 g for the males.

In short, 50 µg astaxanthin administered for preventive purposes can exert an action on growth of the same significance as 0.9 µg vitamin A. However, a curative treatment with 50 µg causes a weight recovery only comparable to that recorded with 0.6 µg vitamin A.

D - STUDY OF CHROMOPROTEID ACTION

The method of preparation of the extracts and the experiments are described in Chapter II. All tests are curative in nature.

ORALLY ADMINISTERED CHROMOPROTEINS

Aristaeomorpha foliacea eggs - 8 deficient rats (4 ♀, 4 ♂) showing weight curves in a plateau (average weight of males 78 g, of females 68 g) receive 80 mg of oily egg preparation (10 µg astaxanthin) per animal per day.

No growth effect was recorded; except for a subject (female), a fall in weight can be seen in all

animals between the 3rd and the 4th day of treatment; the curve inflection accentuates from the 4th day and the animals die between the 13th and the 20th day of treatment. The average weight of the males is 65 g, 57 g for the females.

Aqueous solution of Aristeus antennatus peristomal connective tissue. In this experiment, the chromoproteid's prosthetic group is delivered in an oily solution. The preparation is administered to 14 (7 ♀, 7 ♂) deficient rats. At the 10th jour of treatment, the growth curves do not show any change, except for a male for which a 3 g growth is recorded.

Between the 10th and the 15th day, a fall in weight can be seen; it is gradual in 4 animals and sudden in the other two. Survival is from 60 to 70 days, except for the above-mentioned male, that dies only at the 80th day.

CHROMOPROTEINS ADMINISTERED BY INJECTION

Aqueous solution of Plesionika edwardsii eggs. 3 mL of aqueous solution are injected to deficient rats (3 ♂) showing weight stabilization since 8, 11 and 12 days, respectively (weights: 78, 80, 83 g).

After three days of treatment, the growth curve ceased to be a straight line, a fall in weight occurs in stages, the animals die between the 21th and the 28th of treatment (weights: 60, 64, 69 g).

Aqueous solution of Aristeus antennatus eggs. Three animals (2 ♀, 1 ♂) are injected.

- female No. 1: initial weight 68 g stabilized since 10 days. At the 6th day of treatment (*) no growth effect was recorded. At the 10th day, the weight declines, the animal dies at the 12th day, weight 58 g;

- female No. 2: initial weight 66 g; between the 1st and the 12th injection - 6 days of treatment - a loss of weight of 2 g can be seen, followed by a 15-day weight stabilization. Thereafter, the animal declines, death occurs at the 29th day of treatment; the animal's weight is 56 g by then;

- male: initial weight 84 g. At the 10th of treatment a loss of weight of 6 g can be seen, followed by a plateau that remains for 30 days at 78 g. The decline is then fast, the animal dies 9 days later, and its weight is 64 g.

(*) 3 ml per day in two 1.5 ml injections, each containing 4 μg astaxanthin

Aqueous solution of Aristeus antennatus peristomal connective tissue.

2 ♀, 2 ♂ are treated by injection, respectively continued for 12, 30 and 35 days. The weight remains stable in all animals for 20 to 25 days. It declines gradually and death occurs between 30 and 35 days, the falls in weight reach from 12 to 20 g.

Human serum

The injected serum contains 0.52 µg vitamin A per mL (**). 6 deficient rats (4 ♂, 2 ♀) having reached the weight stabilization stage are divided into two groups A and B.

Group A receives an injection of 0.3 mL serum (0.15 µg vitamin A) per animal per day.

Group B receives 1 mL in two 0.5 mL injections (0.52 µg vitamin A).

The weight of the animals from batch A remains steady the first 4 days; a slight recovery can be seen from the 5th day (of about 3 to 4 g).

The animals from group B show an increase in weight of 6 to 9 g.

In short, astaxanthin administered for curative purposes only shows a significant growth effect at a dose of 50 µg; when the animals are treated for preventive purposes with doses at least equivalent to 50 µg, the growth effect is enough to lead them to adult age.

(**) The serum used contains 0.52 µg vitamin A per ml. This high content had already been obtained by overload as a result of the massive absorption of vitamin A by the donor subject.

CHAPTER IV

ASTAXANTHIN AND REPRODUCTIVE FUNCTIONS

The experiments reported in the preceding chapter determine that it is possible, with sufficiently high doses of astaxanthin, to obtain normal growth among the vitamin A-deficient animals. The animals so taken to adult age have the weight of a healthy subject and a seemingly excellent health condition. Such subjects therefore allow the study of a true vitamin A deficiency and no longer a sub-deficiency, and it is then possible to determine the extent to which astaxanthin has the property to oppose the signs of the deficiency. The purpose of the experiments that will be described below was to explore the reproductive functions.

Many works focused on the study of the functional reproduction disorders in the vitamin A-deficient rat; however, the difficulty lies on the fact that the animals must reach adult age without having stored too large amounts of vitamin A, what would then result in too late deficiencies. Accordingly, the experimenters attempt to provide from weaning a diet containing only limited amounts of factors A; but, everything else being equal, given that the requirements and, as a consequence, the use and liver storage change with age (118), the animal weight (116), (70), (112) or the sex (9), (173), it is not certain that the liver reserve is absolutely nil at the time when the animal reaches the sexual maturity age and is then given the factor A-deficient diet. For such reason, the experimental conditions achieved are those for a sub-deficiency and not those for a total deficiency.

In the experiments that will be described, the animals fed the vitamin A-deficient diet from weaning are led to adult age by the administration of astaxanthin alone, their liver reserve of Vitamin Ais nil and thus the study of the vitamin A deficiency is undertaken on a new basis.

Reminder of data on the sexual cycle of the rat.

The normal Wistar rat reaches the sexual maturity age between 50 and 60 days, Longs and Evans (141).

During the genital activity phase, the ovary and the female tract host cyclic processes that, as a whole, constitute the sexual cycle. The examination of 2,000 cycles, Evan and Bishop (56), (57) showed that in 71% of the cases, the cycle duration is from 4 to 5 days, in 82% from 4 to 6 days; the average duration is 5.4 days. The study of the cycle is conducted by several methods: the most commonly used method is the one of the vaginal smears. The vaginal secretions are collected using a grooved director previously quenched in a saline solution (0.9 % NaCl). The smear is placed on a slide that is examined directly under the microscope or after fixation and staining with hematin-erythrosine. For the duration of the cycle, changes in the formed elements of the secretions allow distinguishing four phases:

1) The diestrus that lasts about 48 hours, the smear comprises nucleated vaginal cells and numerous leucocytes;

2) The proestrus that lasts about 12 hours; the smear can be distinguished by its uniformity: it only comprises nucleated cells;

3) The estrus lasts 48 hours in average. The smear is comprised of many enucleated cells arranged at the beginning of the estrus in large sheets, then the cells appear isolated, strongly eosinophilic in nature;

4) The meta-estrus lasts from 6 to 10 hours in average; large basophil cells appear. Medium-sized acidophil cells can also be seen. The number of leucocytes is increasingly higher.

A - REPRODUCTIVE FUNCTION DISORDERS IN THE VITAMIN A-DEFICIENT RAT

1. INFLUENCE OF DEFICIENCY ON THE SEXUAL CYCLE

Evans and Bishop (56) were the first to show the influence of the food imbalances and particularly of the factor A-deficiency on the cycle process. The examinations of the vaginal secretions allow detecting an abnormal cycle: while in the normal rat enucleated cells are found arranged in large sheets only at the beginning of the estrus, in the deficient rat these keratinized cells appear for the entire duration of the cycle; we can see a true permanent estrus. This phenomenon is referred to as colpokeratosis, Holweg and Dohrn (108). In the event of vitamin A deficiency, colpokeratosis always appears, at an early stage; this suggested to Coward (32) a biological method for vitamin A determination based on the changes in vaginal secretions: the smear examination conducted during 10 consecutive days allows concluding that a normal or abnormal cycle is involved.

Personal observations

1) Rats are given from weaning a sub-deficiency diet (0.6 µg vitamin A per day). From sexual maturity, the cycle is regularly monitored by the vaginal smear method.

The examination under microscope shows an anarchic cycle process.

2) Rats are treated from weaning with 0.6 µg vitamin A per day. Vitamin administration is interrupted at the sexual maturity age. The signs of deficiency (xerophthalmia - stabilized weight) appear by the 20th day; then the vaginal smear examination is conducted; in all cases the constant presence of keratinized cells was observed.

3) The examination of secretions from rats supplied a diet with the addition of 1.7 µg vitamin A per day shows the normal formed elements for each cycle phase.

2. INFLUENCE OF DEFICIENCY ON REPRODUCTION

a) IN THE FEMALE

Many works were intended to study the troubles caused by the vitamin A deficiency in rats. When the animals are totally deprived of that factor, the sterility of the female (211), (180), (99), (152) can be seen. If the deficiency is not total, the females can be fertilized but gestation is not completed, Evan et al. (57): in the case of a deficiency still allowing embryologic development, a long gestation and a difficult parturition and can be seen. Mason (54).

Depending on the stage at which deficiency appears, dropping can still occur. Giroud (66) showed, for various mammal species and the rat in particular, the occurrence of disorders in the development of the skeleton in the embryo. Anderson (1) stated similar facts and particularly diaphragmatic hernia; Wilson and Warnaky (241) stated urogenital system alterations such as keratinization of urethra at birth; however, malformations due to maternal deficiency are most frequently found in the eye. Warkany and Schraflenberger (238) state eyes lacking anterior chamber, undeveloped iris and ciliary process, an anarchic retinal structure, the animals are born blind.

Personal observations - Experimental protocol

Test I - Three females given the base diet from weaning receive 0.6 µg vitamin A per animal per day: at the 40th day (70-day age) the administration of vitamin is interrupted. A series of vaginal smear examination is conducted: at the 20th day of deficiency, keratinized cells are detected several consecutive days. The females are then coupled to normal males.

Test II - Six females receive in addition to the base diet, 0.9 µg vitamin A from weaning: at the age of 80 days they are coupled to normal males.

Test III - Six females (control batch) fed the base diet receive 1.7 µg vitamin A per animal per day.

They are coupled to normal males.

The females from tests I, II, III also receive 7 mg α-tocopherol per week.

The results are as follows:

In test I (0.6 µg vitamin), two females out of six were fertilized: the gestation seemed to go on normally until the 20th day: the rats then bled copiously and their weights fell abruptly.

In test II, the 6 females had litters of pups born dead.

Test III led to normal litters.

Minimum doses necessary to obtain a normal sexual cycle and restore the reproductive functions.

The data that can be found in the literature show considerable differences among the various authors with regard to the curative doses: in fact, they are essentially dependent on the duration of the deficiency. According to Coward (31), if the animal has not been given the deficient diet more than about twenty days, a weak dose of vitamin is sufficient to restore a normal cycle.

Simonnet (211) resorts to "the physiological age" of the animal related to the age of the various tissues and organs: according to this author, the responses to the same dose administered following deficiencies of the same duration differ from an individual to another.

For a short-duration total deficiency, it is generally accepted that a dose of 1.2 µg per day would account for a curative dose (110), (185), (30).

Much more data is found in connection with the preventive dose to meet the animal requirements under normal circumstances, and for the particular physiological condition that gestation constitutes. According to most experimenters (185), (70), (31), a daily dose of 0.7 to 0.8 µg delivered from weaning would prevent colpokeratosis: reproduction would require doses two and three times higher (185) and at higher doses the reproduction time would be increased (205).

The results recorded by us in experiments of preventive nature led us to similar conclusions:

- at least 1.5 µg per day is necessary and sufficient to obtain not only viable but normal and robust litters (from 5 to 6 pups of average weight at birth).

Below such minimum doses, the cycle is no longer normal, the reproductive functions are disturbed and the disorders appear along with anatomical-pathological lesions that, moreover, are not only localized in the genital system.

The complete study will be presented in Chapter V. Therefore, we will limit ourselves for the moment to a description of the genital system lesions.

Anatomical pathology of the genital system of deficient females

In 1931, Green (95) stated that pregnant rats fed a deficient diet show fast an infection of the genital tracts. According to McIlamby (164), over 40% of the deficient rats show vaginal and uterine bleeding. Richards (198) states similar facts and the frequency of uterine cysts.

Personal observations

15 rats are fed from weaning a factor A-deficient diet for 40 to 45 days: with the liver reserve being nil (cf. p. 61), minimum (less than 0.6 µg) daily doses are administered per day, corresponding in total to the maintenance dose.

The animals are sacrificed; the autopsy shows between the 55th and the 75th day of deficiency:

- vaginal bleeding (in 2 females);
- congestion of uterus (in 1 female);
- uterine cysts (in 2 females);
- vaginal infection (in 2 females);
- uterine abscess (in 1 female).

In 3 other females, only the congestive appearance of the uterine horns could be seen. Four rats showed nothing abnormal in their genital system.

b) IN THE MALE

Everything else being equal, it is known that the liver reserve of the female is larger than the male's, Moore, Sharman and Ward (178), the male requirements should be larger, since the storage corresponds in fact to what is not used by the body. This must be brought together to the observations by Simonnet (211) who shows that, in the same deficiency conditions, the male is more sensitive to the deficiency than the female. For the same duration of the deficiency, he sees that the females are still fertile while the males are sterile.

In the male, the factor A deficiency results in an atrophy of testes, where the histological examination reveals a reduction of the diameter of the seminiferous tubules and the disappearance of the elements of the germinal cell line.

Spermatogenesis arrest can be temporary if the deficiency is not too long; but a long-term total deficiency causes the final gland atrophy. Evans (55) showed that a male fed a deficient diet and receiving tocopherol becomes sterile in three months; on the other hand, if its diet contains factor A, the animal is fertile, even in the absence of factor E.

Anatomical pathology of the genital system of deficient males

Moore and Mark (170) showed prostate alterations; the reduction in weight of the endocrine glands caused by the deficiency was underlined by Sure (220) (154); Simonnet, by stating precisely the changes in weight of various organs of the normal animal and the deficient animal, revealed the atrophy of testes that leads to a loss of weight that reached 35 to 43%.

Personal observations

The autopsy of the males of our experiment, that by the way are unsuited for fertilizing, allowed us to make similar observations:

The testes of the three males with sub-deficiency (0.6 µg vitamin A per day from weaning), sacrificed at 7 months, weighed 0.810 g, 0.900 and 0.950 g, respectively. Among the males of the

same age, given a daily dose of 1.8 µg vitamin and fed the same base diet, the weights of the testes were: 1.320 g, 1.400 g and 1.510 g. Moreover, the macroscopic examination did not reveal any apparent anomaly or lesions, apart from atrophy.

The lessons learnt from the works reported above relating to the signs of the vitamin A deficiency and our own experiences have allowed us to establish an experimental protocol for exploration of the reproductive functions in the vitamin A-deficient animals receiving high astaxanthin doses.

B - STUDY OF THE REPRODUCTIVE FUNCTIONS IN THE VITAMIN A-DEFICIENT RAT TREATED WITH ASTAXANTHIN

As it has just been reported, the study of the deficiency in the adult and its reversibility by the administration of an active factor brings out several issues in connection with the adjustment of the experimental designs. In particular, with regard to the preliminary period of the tests (*), a compromise should be found between the dose of vitamin A enough to lead the animal to the adult age and the dose that could entail a liver overload.

By the administration of astaxanthin from weaning, this issue is simplified and the study of any possible action of astaxanthin on the reproductive functions is therefore advantageously undertaken in the following experimental conditions:

- the rat reaches adult age showing a normal weight and a seemingly excellent general condition;

- its liver reserve of factor A is absolutely nil (**).

Accordingly, it is possible to determine precisely the extent to which astaxanthin has the property to oppose the signs of vitamin A deficiency.

Two types of experiments were undertaken:

- in the first one, the animals receive oils extracted from the hepatopancreas (summer oils) of Aristeus antennatus;
-
- in the second one, astaxanthin esters in an oily solution are administered.

(*) Period starting at weaning and continuing to sexual maturity

(**) It is depleted before the 20th day of deficiency (cf. p. 61)

1. ADMINISTRATION OF WHOLE OILS

Preliminary test:

- A batch of 3 females (batch A) receives, from weaning, in addition to the deficient diet, 90 mg Aristeus antennatus hepatopancreas oil. They are coupled at the 100th day after weaning to males of the same age given the same base diet, but receiving 0.9 µg vitamin A per day per animal.

- Parallel to this experiment, a batch of three males (batch B) receives from weaning, for 100 days, in addition to the deficient diet, 90 mg Aristeus antennatus hepatopancreas oil (July extraction). Then they were coupled to three females of the same age having received from weaning, in addition to the deficient diet, 0.9 µg vitamin A.

- A batch of 3 males and 3 females (batch C) received from weaning 0.9 µg vitamin A. the results were as follows:

The three females from batch A delivered litters of pups born dead (*). The animals were sacrificed at the 5th month: among the females, vitamin A could not be detected in liver tissue; Vitamin Atraces were present in the liver of the males.

None of the females from batch B was fertilized. The animals were sacrificed at the 5th month. No trace of vitamin A was present. Among the females, a reserve of 6 µg per gram of liver tissue was detected.

TABLE XVII

Batches	Standard diet	Litters	Vitamin A liver reserve
A: 3 males 3 females	0.9 µg vitamin A Aristeus antennatus hepatopancreas oil	abortions	traces none
B: 3 males 3 females	Aristeus antennatus hepatopancreas oil - 0.9 µg vitamin A	none	6 µg per g
C: 3 males 3 females	0.9 µg vitamin A 0.9 µg vitamin A	none	6 µg per g 6 µg per g

These preliminary experiments therefore show that it is impossible to obtain in the deficient white rat a normal reproduction by administration of whole oil containing astaxanthin at doses that, moreover, can not only prevent and cure the xerophthalmia but also ensure normal growth. However, given

(*) In addition, the vaginal smear tests previously conducted showed an apparent colpokeratosis.

the animals from the batch fed 0.9 μg vitamin per animal per day delivered no litters, one could ask oneself whether, in these preliminary experiments, the amount of 0.9 μg vitamin A per day administered to the males from batch A and the females from batch B was sufficient to allow the reproductive functions being performed normally; one could also think that the addition of vitamin E to the diet would have led to different results.

Test I. - To answer those two questions, experiments were undertaken on 5 new batches A, B, C, D, E each comprising three males and three females. Each of these batches received the deficient base diet and 1 mg tocopherol per week (*) per animal. In addition, the following was administered:

Batch A: males and females receive 90 mg Aristeus antennatus hepatopancreas (summer) oil.

Batch B: females: preparation of Aristeus antennatus peristomal connective tissue with the same concentration of astaxanthin as the preparation intended for batch A.
males: 4.5 μg vitamin A per day.

Batch C: females: 4.5 μg vitamin A per day.
males: preparation of peristomal connective tissue (cf. batch B).

Batch D: males and females: 1.5 μg vitamin A per day.

Batch E: males and females: 4.5 μg vitamin A per day.

The results can be summarized as follows:

Batch A delivered no litters.

Batch B delivered litters of pups born dead.

Batch C delivered no litters.

Batches D and E delivered normal litters.

TABLE XVIII

Batches	Standard diet 1 mg α-tocopherol per week	Litters	Vitamin A liver reserve
A: 3 males 3 females	hepatopancreas oil hepatopancreas oil	none	none none
B: 3 males 3 females	4.5 μg Vitamin A peristomal connective tissue oil	abortions	75 μg per g none
C: 3 males 3 females	peristomal connective tissue oil 4.5 μg Vitamin A	none	60 μg per g
D: 3 males 3 females	4.5 μg Vitamin A 4.5 μg Vitamin A	normal litters	6 μg per g 5.4 μg per g
E: 3 males 3 females	4.5 μg Vitamin A 4.5 μg Vitamin A	normal litters	60 μg per g 60 μg per g

(*) Dose proposed by Coward (30).

2. ADMINISTRATION OF ASTAXANTHIN ESTERS (159), (160), (160 a).

Materials and methods

The astaxanthin esters are prepared following the above-described method, suspended in devitaminized vegetable oil with the addition of tocopherol: the pigment concentration is 200 µg per gram of oil added 25 mg tocopherol.

12 animals (6 ♀, 6 ♂) are fed from weaning the deficient diet and receive in addition 80 mg oily suspension of astaxanthin esters (50 µg) per animal, per day.

This dose is delivered for 6 months without interruption. During these 6 months, two types of experiments were undertaken:

In the first one, the 12 animals were divided into 6 couples.

In the second one, two batches were formed: the 6 females from the first experiment, still receiving the astaxanthin ester preparation, were coupled to normal males (*) (batch A). As to the males, treated with astaxanthin, they were coupled to normal females (*) (batch B).

In parallel, experiments were undertaken with a control batch (batch C) comprising 2 females and 2 males. These control animals received, in addition to the base diet, 4.5 µg vitamin A and 2.5 mg α-tocopherol per animal per day.

First experiment: males and females receiving the astaxanthin ester preparation:

5 females out of 6 were not fertilized. The sixth one delivered at the age of 85 days a three-pup viable litter. These three pups fed the deficient diet from weaning showed signs of xerophthalmia from the 5th day. The administration of astaxanthin esters (at a dose of 50 µg per animal per day) made the eye injuries to recede in a few days. However, their general condition remained precarious and they died at 45, 110 and 120 days, respectively. The autopsy confirmed the external signs of vitamin A deficiency. The three animals showed abscesses, one in the anal region, the other one in the duodenal loop, the third one in the submandibular glands. Bladder bleeding was found in one of them and intestinal bleeding was found in another one.

Second experiment:

Batch A (females treated with astaxanthin, males receiving vitamin A). all females were fertilized; while their weight was previously in a plateau, from the 10th day after copulation a weight growth was recorded that reached 30 g (curves of Fig. 28, 29, 30 and 31).

(*) the animals receive from weaning, in addition to the deficient diet, 80 mg devitaminized vegetable added 4.5 µg vitamin A and 2.5 mg α-tocopherol.

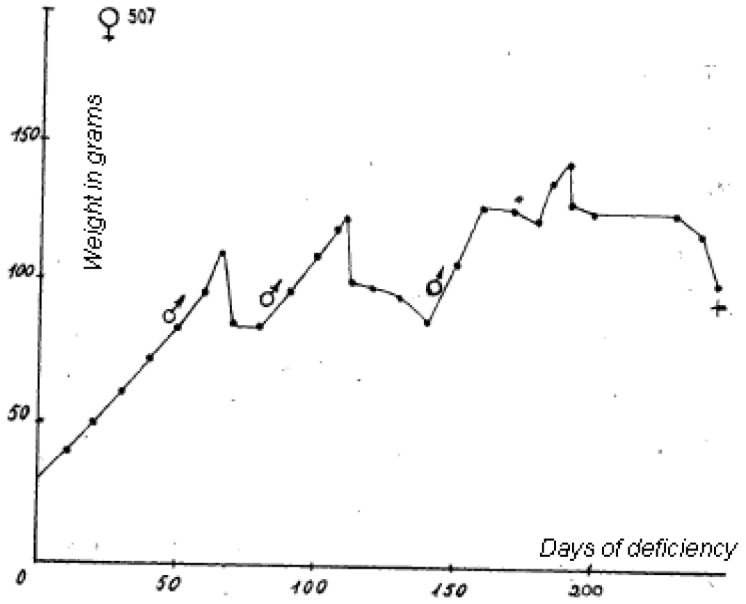
♀ 507
Weight in grams
150
100
50
0
50
100
150
200
Days of deficiency

Fig. 28

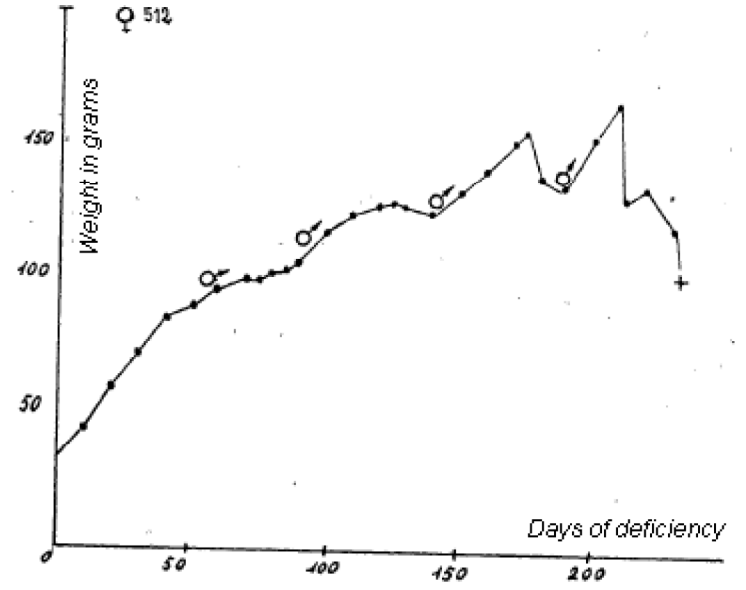
♀ 512
Weight in grams
150
100
50
0
50
100
150
200
Days of deficiency

Fig. 29

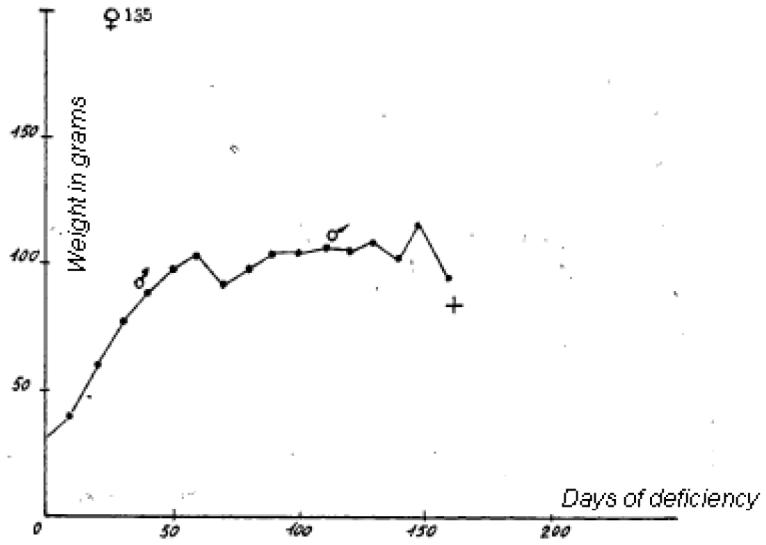
♀ 135
Weight in grams
150
100
50
Days of deficiency
0
50
100
150
200

Fig. 30

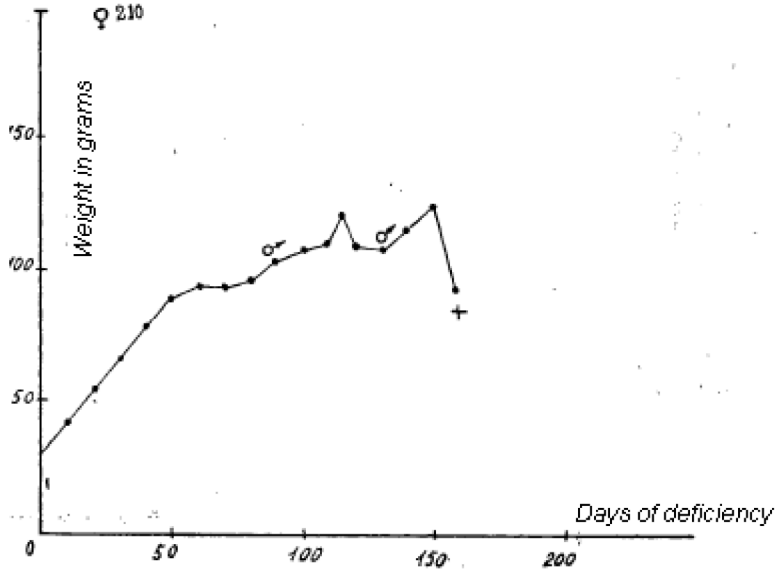
♀ 210
Weight in grams
150
100
50
Days of deficiency
0
50
100
150
200

Fig. 31

Widening of the abdominal wall also appeared, thus revealing gestation. However, between the 20th and the 25th day all pregnant females had an abortion or delivered litters with pups born dead, the fall in weight reached 35 g in 24 hours, abortion was accompanied by strong bleeding; the female that had been the only one to be fertilized in the first experiment behaved as its congeners in the subsequent experiments.

Mating was repeated three times (*) and the same phenomena were seen. Five females died at the age of 5 months and a half and one at 6 months. The sixth one that had reached an early sexual maturity survived 2 months after the last abortion.

The males were sacrificed at the 9th month.

Batch B (males treated with astaxanthin, females receiving vitamin A). None of the females was fertilized. The males were sacrificed at the 9th month. Their weight had stabilized from the 7th month, four of them declined slowly; at the 9th month the losses of weight recorded were from 20 to 30 g.

3. ANATOMICAL-PATHOLOGICAL EXAMINATIONS

The autopsy of the animals that died spontaneously or were sacrificed led to the following observations:

1) Males: 18 males were examined; two of them died spontaneously, 16 were sacrificed between the 8th and the 9th month. The autopsy revealed a degeneration of the testes in 14 of them, with the gland weight being less than 0.9 g.

2) Females: out of 18 subjects examined, 13 died spontaneously between the 5th and the 6th month of treatment (6 and 7 months of age); 4 females were sacrificed at the 9th month, one female ill at the 7th month. The results of the autopsy were as follows: 4 of them showed uterine bleeding; two of them, uterine cysts; one of them vaginal bleeding simultaneously with suppurating uterine cysts; vaginal infection was seen in other three.

In addition, other infectious processes were also seen among the females (vesical calculus in three of them, a bulky abscess in the neck region).

4. DISCUSSION

These experimental results show that the administration of both whole oils extracted from hepatopancreas and astaxanthin esters does not allow ensuring normal reproductive functions.

The fact that during the first experiment (*) a female delivered three pups alive can be attributed to a precocious puberty (45 days).

(*) The females were left 2 weeks at rest after each gestation.

(*) First experiment with astaxanthin esters

Accordingly, it is logic to accept that this female, at the time of fertilizing and during gestation, still had a small vitamin A liver reserve. In fact, we showed above that generally among the rats of our breeding the liver reserve is depleted before the 20th day of weaning (age of animals: 50 days).

These experiments determine therefore that astaxanthin, that when administered at strong doses is capable of maintaining a normal growth that allows leading the animals to adult age, is not however capable of being substituted for vitamin A as far as the reproductive functions are concerned.

CHAPTER V

LOCALIZATION OF ASTAXANTHIN IN THE BODY OF THE TREATED RAT

Given the results recorded above, meaning essentially the dissociation of the anti-xerophthalmic action and the action on growth and on the reproduction functions, it was interesting to study the localization of the pigment in the body of the treated rat, in order to attempt to address the general problem of the biochemical mechanism of the vitamin activity of the astaxanthin.

R. Grangaud (70) determined that the pigment administered to the vitamin A-deficient rat did not build up in the liver. Whatever the mode of administration (orally or by intraperitoneal injection) it did not build up in the liver but is regularly found in the retina. Its presence was investigated in other organs or tissues by R. Grangaud and Th. Douard (89) and, in order to determine the specificity of its localization and compare its behavior to that of another carotenoid with an oxygenated beta-ionone nucleus, the same research was conducted in rat organs that had received zeaxanthin.

These experiments were completed with new tests by increasing the astaxanthin concentration and extending the duration.

Finally, we tried to determine whether the preferential localization in certain places was related to the chemical form of the pigment by comparing the results found in the **animals treated with free, esterified astaxanthin or chromoproteins (administered orally or by injection).**

A - LOCALIZATION OF ASTAXANTHIN AND ZEAXANTHIN (89)

MATERIALS AND METHODS

Preparations administered:

Astaxanthin: This is an oily solution of pigment, isolated by Aristeus antennatus peristomal connective tissue chromatography following the above-described method, containing 7 mg tocopherol per g.

Zeaxanthin: corn meal is macerated in acetone with addition of methyl alcohol (500 : 50). The solution, diluted with water, is recovered with the petroleum ether. After dehydration through contact on anhydrous sodium sulfate, the petroleum ether solution is vacuum-distillated. A pasty residue is obtained (8.3 g per 225 g corn).

The residue, placed again in a petroleum ether solution, is chromatographed on alumina. The pigment is retained at 5 to 6 mm from the top, forming a yellow area of 12 mm in height. Developing with petroleum ether with addition of methanol (2%), a yellow fraction is obtained that passes to the filtrate. After washing the chromatogram with petroleum ether, the pigmented area that remained on the column is isolated with petroleum ether with the addition of 5% methanol. After vacuum distillation, an oily residue of 0.340 g is obtained, that is added 2.5 mg tocopherol.

Pigment administration conditions and duration.

The tests were conducted both on animals given the vitamin A-deficient diet from weaning and normal subjects fed wheat and lettuce (diet E 6).

Tests are preventive in nature.

Sixty animals received from weaning, in addition to their diet, 20 to 30 µg astaxanthin in the form of an oily extract or changing doses of a zeaxanthin solution in the same oil volume. Tests continued for several weeks.

Pigment research - Experimental protocol.

At the end of the experiment, the animals were sacrificed and the organs removed (eyes, pituitary gland, thyroid, suprarenals, ovaries). From removal, they were subjected to extraction using the usual method (cf. p. 27). For such purpose, they were finely ground with anhydrous soda sulfate and extracted with acetone. The acetone solutions are added petroleum ether and water. The petroleum ether phase was separated, dried on anhydrous sodium sulfate, filtered and chromatographed on alumina. A column of 5 mm in diameter was used: in these conditions, the presence of astaxanthin in the solution examined resulted in the occurrence of a very clear ring in the upper part of the column, orangey pink in the first case, yellow in the second.

After the chromatogram was developed with petroleum ether, the orangey pink pigment retained at the top of the column was acidified using acetic acid: the color turned cyclamen pink. The pigment was identified by its spectral features and by its transformation into astacin.

Results

a) **With regard to astaxanthin, like in the previous experiments, the pigment was found in the eyes in the retina** and in the form of traces in the liver tissue. In addition, it was possible to detect it in the pituitary glands, thyroids, suprarenals and ovaries. The pigment was detected neither in the kidneys nor in the testis.

b) Regarding the zeaxanthin, traces of carotenoid were detected in the pituitary glands, thyroids, suprarenals and ovaries. It was not found in the eye and not any more in the liver, kidney and testis (Table XIX).

These results lead to some comments:

For astaxanthin, localization is without doubt most apparent in the eye; upon dissection, the rat retinas having received the pigment showed most often a salmon color that already reveals the presence of the carotenoid before any extraction, and in many cases the extract from a single pair of eyes gives a chromatogram that is still perfectly readable.

Pigment was regularly found in the suprarenals, but in very small amounts. However, an animal showed salmon-colored markedly hypertrophic glands, the carotenoid content was much higher than in the other subjects and we can give no explanation of this exceptional case.

The astaxanthin content in the ovaries was extremely low; in the first experiments in which the animal received only very small pigment doses, astaxanthin was completely absent; however, among 8 females that received from 20 to 30 µg astaxanthin, it was found in 4 of them.

Among the animals treated with zeaxanthin, carotenoid was assessed at the 45th day of treatment. The rats were then under full vitamin A-deficiency; particularly, the eyes showed enormous ulcers. It is possible that the carotenoid could not be detected for such reason. At a less advanced stage of deficiency, the result could have been different.

A last remark: astaxanthin has no tendency to build up in the liver tissue. The body of the treated rat makes no storage, what explains that the interruption of the treatment quickly leads, often within two or three days, to a strong development of xerophthalmia.

TABLE XIX

Organs	Astaxanthin				Zeaxanthin			
	Normal diet		Deficient diet		Normal diet		Deficient diet	
	♂	♀	♂	♀	♀	♂	♀	♂
Eyes	+	+	+	+	---	---	---	---
Pituitary	+	+	+	+	---	---	---	---
glands	+	+	+	+	+	+	+	+
Thyroids	+	+	+	+	+	+	+	+
Suprarenals	---	---			---	---	---	---
Kidney	traces	traces	traces	traces	---	---	---	---
Liver		+		---	+		+	
Ovaries	---		---			---		---
Testes								

B - LOCALIZATION OF ASTAXANTHIN AFTER ADMINISTRATION OF THE ESTER FORM

In this second series of experiments, the rats examined received astaxanthin (curative test) in the form of esters at doses 3 and 4 times higher than those administered in the first tests.

The examinations also involved animals treated with these same extracts at equivalent doses, during preventive, long-term experiments.

Materials and methods

Administered preparations

The method of preparation of the esters is the same as previously described (cf. pp. 39 and 27); the esters are delivered in an oily solution with addition of tocopherol.

Pigment administration conditions and duration:

a) Curative test: the oily extract was administered to 12 deficient rats (6 ♀, 6 ♂) showing clear signs of deficiency, each animal received 100 mg oil containing 50 µg astaxanthin (extract No. 5) per day**. The treatment continued for 5 weeks (*).**
b) Preventive test: 14 animals (5 ♀, 9 ♂) received from weaning an ester extract (extract No. 5). It was administered orally at a dose of 100 mg (containing 50 µg astaxanthin) per day**, per animal.**

Treatment continued for the 5 females until their (spontaneous) death that occurred in the 5th month, 5- ½, 6th and 6th month of treatment.

The administration of this extract continued among all males, sacrificed after 7 months of treatment without interruption.

Pigment research

The experimental protocol is as described in experiment 1. Among the spontaneously dead or sacrificed animals, search for pigments was conducted as previously done in the eyes, livers, thyroids, pituitary glands, suprarenals and ovaries; it was completed with examinations involving the spleen, kidney, lungs, **central nervous system**, blood, aponeurosis.

The extraction was conducted on the organ in its entirety, to the exception of liver, where an aliquot (5 g) was treated.

The pituitary glands (**) and the thyroids (**) were extracted per 3 (males and females separately). The suprarenals were extracted per pairs. For the blood collected by cardiac puncture, 1 mL was used. The chromatographic method is the same as previously described.

Results

Animals treated using the curative method:

Astaxanthin was regularly found (with no exception) in the retina, pituitary glands, thyroids, suprarenals and ovaries.

Both among the males and the females, minuscule traces were found in the liver tissue. In the

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(*) Eye injuries were healed before the 2nd week of treatment.

(**) Average weight of 3 pituitary glands ..... 10 to 11 mg
Average weight of 3 thyroids..... 20 to 30 mg
Average weight of suprarenals (pair).... 22 to 28 mg

pancreas, kidney, spleen, lung, blood, **central nervous system**, no trace of pigment was detected.

Animals treated using the preventive method:

The same results were obtained with regard to the eyes (***), pituitary gland, thyroids, suprarenals and ovaries.

The pigment was not found in the kidney, except for a positive result in a female. The identity of the pigment after chromatography was confirmed by the spectral features (the absorption spectrum with a single broadband feature, max. 490 nm in pyridine).

In the liver, minuscule traces were found; the searches conducted on the pancreas, spleen, lung, **central nervous system** and blood were negative.

All the results are shown in the following Table (Table XX).

TABLE XX

	Curative test		Preventive test	
Organs	6 ♀ duration 5 to 7 months	♂ duration 7 months	5 ♀ duration 30 to 38 days	9 ♂ duration 35 days
Eyes	+	+	+	+
Pituitary glands	+	+	+	+
Thyroids	+	+	+	+
Suprarenals	+	+	+	+
Kidney	none (*)	none	none	none
Ovaries	+		+	
Testes		none		none
Seminal vesicles		none		none
Liver	traces	none	traces	traces
Pancreas	none	none	none	none
Spleen	none	none	none	none
Lung	none	none	none	none
Blood	none	none	none	none
Central nervous system	**none**	**none**	**none**	**none**
Aponeurosis	none (*)	none	none	none

(***) The sacrificed or spontaneously dead animals showed perfectly healthy eyes.

(*) Positive result in a female

C - LOCALIZATION OF ASTAXANTHIN AFTER TREATMENT WITH AQUEOUS SOLUTIONS ADMINISTERED BY INTRAPERITONEAL INJECTION

Preparation for administration:

In this third series of experiments, the examined rats received chromoproteins in an aqueous solution, prepared from eggs of different species (**Aristeus antennatus, Plesionika edwarsii, Scyllarus latus**) and from Aristeus antennatus' peristomal connective tissue.

Pigment administration conditions and duration

Curative tests were conducted. Generally, the duration of treatment did not exceed 18 days. However, for 3 animals, the treatment by injection of peristomal connective tissue continued for 35 days after cure; the doses administered correspond, for each of the tests, to amounts of astaxanthin of less than 10 µg.

The animals were sacrificed 24 hours after the last injection.

Results.

The results can be superimposed to those recorded in the previous experiments: in the eyes, pituitary glands and suprarenals, astaxanthin was detected and identified following the procedure already described.

The pigment could not be searched in the thyroids; in fact, 3 animals died showing abscesses in the thyroid region. Among the other animals treated, the number of surviving males or females from the same group did not reach 3; the thyroid examination could not be conducted.

No pigment was found in the ovaries. The pigment was present in the testis of a male that had received aqueous injections of peristomal connective tissue (35 days of treatment).

All the results are shown in the Table below.

TABLE XXI

CURATIVE TEST						
Organs	**Pandalus egg pigment**	**Aristeus egg pigment**		**Scyllarus egg pigment**	**Aristeus antennatus peristomal connective tissue pigment**	
	duration: 18 days	**Duration: 8 days**		**duration: 18 days**	**Duration: 35 days**	
	2 ♂	2 ♀	1 ♂	2 ♀	2 ♀	2 ♂
Eyes	+	+	+	+	+	+
Pituitary	+	+	+	+	+	+
glands	---	---	---	---	---	---
Thyroids	+	+	+	traces	traces	traces
Suprarenals	none	none	none	none	none	+
Kidney		none			none	
Ovaries	none		none	none		+
Testes	none	none	none	none	none	traces
Liver	none	none	none	none	none	traces
Spleen	none	none	none	none	none	none
Blood						
Central nervous system	**none**	**none**	**none**	**none**	**none**	**none**

D - DISCUSSION

The results obtained in the three series of experiments show that, in the different conditions adopted, where the main variables are on the one hand the amount and the form of the astaxanthin administered, and on the other hand the route of administration, the pigment is always localized in the retina, the pituitary glands and the thyroids.

With regard to the retina, the pigment is plentiful therein since a single pair of eyes gives a colored solution that is sufficient to allow its identification by chemical or by spectral means.

In the other organs where astaxanthin was found, the concentration in proportion to the gram of organ is extremely low. In the liver in particular, the pigment was only detected as traces or was totally absent. In this regard, the astaxanthin did not behave as vitamin A that builds reserves.

It was then interesting to investigate whether the liver reserve level of rats that were fed the normal diet or the deficient diet was modified by the administration of the pigment.

E - RESEARCH AND DOSAGE OF VITAMIN A FROM THE LIVER OF ASTAXANTHIN-TREATED RATS

Vitamin A is analyzed through different types of experiments, to determine whether the administration of pigment can modify the reserve level of the liver.

The livers of rats from 4 batches were examined:

Batch I: rats fed the normal feeding diet (diet E 6, low factor A)

Batch II: rats given the same diet, with the addition of 90 mg oily solution of astaxanthin esters per animal per day.

Batch III: rats fed the vitamin A-deficient diet (diet R 12) and receiving 90 mg 90 mg oily solution of astaxanthin esters per animal per day.

Batch IV: rats fed the vitamin A-deficient diet (diet R 12) and receiving 0.9 µg vitamin A per animal per day.

According to the method already described, the livers are removed and the vitamin A dosage is conducted on 5 g of tissue.

The results are shown in Table XXII below.

TABLE XXII

Batch number	Number of rats		Diet	Treatment	Amount administered in µg	Duration of experiment in days	Vitamin A of liver in µg / g	
	♀	♂					♀	♂
I	6	7	E 6	O	0	30	23.9	22.6
II	7	7	E 6	As.	50	30	21.9	21.6
III	6	6	R 12	As.	50	35	0	0
IV	6	6	R 12	V. A.	0.9	35	15	14.3

E 6: wheat + lettuce diet twice a week

R 12: factor A-deficient diet

As: astaxanthin

V.A.: vitamin A

CHAPTER VI

ANATOMICAL-PATHOLOGICAL EXAMINATIONS

This chapter describes the study of the anatomic lesions seen during vitamin A deficiency and those recorded in the deficient animals treated with astaxanthin.

Much data can already be found in the literature relating to the anatomical and pathological observations during vitamin A deficiency. The first part of this chapter will therefore be intended to summarize the works in connection with that matter and their comparison with our own observations on our control animals.

The results of the autopsy of our animals treated with astaxanthin shall be examined in detail in the second part.

A - ANATOMICAL-PATHOLOGICAL EXAMINATIONS OF VITAMIN A DEFICIENT RATS (*)

Wolbach and Howe (244), focusing their attention on the injuries occurred at the beginning of the deficiency, show that those injuries mainly consist in a transformation of epitheliums that gradually stratify. This finding extends to all epithelial tissues in the respiratory, digestive and genitourinary systems; the cuboidal epithelium is transformed into squamous epithelium with keratosis lesions.

Generally, all tissues are affected by deficiency, but Wolbach (243) remarked that these epithelial metaplasia phenomena generally appear in the same order: the salivary, submandibular and parotid glands are the first to be affected, then the tongue, the pharynx, the respiratory tract with the trachea, the genital and urinary systems, the eye and its related Harderian gland, meibomian gland, the skin.

Keratinization is unusual in the liver, kidneys, suprarenals and pituitary gland.

This development of deficiency in the different systems or organs relates to the growing animals. In adults, keratinization is less general and ulceration more frequent, Richards (198). Recent works by Irving and Richards (114) confirmed that the sensitiveness of the different tissues to the deficiency varies with age.

Richards and Simpson (198) mainly focused on the lesions caused by deficiency in the adult: no infectious process was seen before the 25th day; cecum inflammations appear first (83 per cent of the cases), they extend to the small intestine and the duodenum; stomach keratosis appears later and from the 6th week on the frequency of tongue abscesses, bleeding points and ulcerations of gastric mucosa can be noticed.

(*) A detailed study of this issue has been recently conducted by Wolbach (243) and Moore (169 a).

Planel (187) highlights in the young rat the frequency of the buccopharyingeal accidents and the ulcerations of the small intestine mucosa; no histological alteration was found in the large intestine; he also underlines the frequency of keratosis of the esophageal and stomach mucosa.

With regard to the respiratory tract, Wolbach and Howe (248) report nasal catarrh, pulmonary and pleural infections, Bradford (11), Coward, Key, Dyer and Morgan (33), lung abscesses.

Urinary tract alterations are often found: keratinization of the vesical epithelium, ureters, Hedenberg (101), bladder bleeding, Busson (16).

The keratinized cell desquamation result in calculus, Wolbach, Osborne and Mendel (182) point out the presence of calculus in 10% of the deficient animals. Van Leersum (227) found in recent experiments 197 cases of bladder stone in 645 deficient rats (*). Clausen (27) attributes the formation of calculus both to the joint action with vitamin A and D deficiency and to a mineral imbalance. In addition, changes in the calcium metabolism were reported since 1933 by Emerique (50) who stated that the overall calcium content in the factor A-deficient rat is higher than in the normal animal.

Smith and Lantz (213) report on the contrary a reduction in the calcium content in the teeth. Apart from this structure alterations, Orten, Burn and Smith (181), Paul and Paul (185) point out changes in pigmentation: while orangey translucent incisors can be seen in the normal rat, they are opaque, chalk-white in the deficient rat (245), (200).

With regard to bones, vitamin A deficiency only causes serious trouble in the young rat. Giroud and Martinet (66) underlined the frequency of skeleton malformations in the young and the embryo.

The troubles caused by the deficiency in the genital tract were mentioned in a previous chapter: precocious keratinization of the vagina mucous membrane, uterine and vaginal infections; atrophy of glands and related organs in the male. The influence of deficiency on the weight of the endocrine glands was highlighted by Simonnet (211), Sure (220), Serfaty and Olivereau (201).

Personal research work

The observations on live subjects and the autopsy results relate to:
1) Control rats fed the synthetic factor A-deficient diet from weaning that died spontaneously between the 60th and the 80th day of deficiency.

2) Rats fed the same diet but having received from weaning doses of vitamin A of about 0.6 µg sufficient to lead them to adult age.

(*) Calcium oxalate and magnesium phosphate or lime phosphate calculus.

Control animals:

a) observations of the live subject.

Out of about one hundred control animals, over sixty show abscesses in the submandibular glands and the sublingual glands. In about thirty animals abscesses in the abdomen can be seen.

b) autopsy results

Upon sacrifice of the animals or upon their spontaneous death, they are weighed and their organs immediately examined.

The following was found:

- in most cases, both in males and females, extreme thinness;

- in the females, bleeding and suppuration phenomena (in 20 to 30% of the subjects) in the vagina, abscesses in full development in the sternum or the axillae.

Adult animals:

Observations of the live subjects.

The abscesses in the trachea, the size of which frequently reaches the size of a hazelnut, are found in over one half of the animals. The females show vaginal infections characterized by a tensed, purplish, wet abdominal wall. Hemorrhagic oozing can be frequently seen.

Autopsy results

In the males and females, the cecum (always bulky in the normal animal) seems still dilated; hemorrhagic points can be macroscopically distinguished.

Stomach ulcers were recorded in 6 adults (males and females) belonging to a batch of 12 animals, dead or sacrificed the 65th day of deficiency.

The autopsy reveals in the females copious vaginal bleeding, suppurating or hardened uterus cysts, abscesses that dilate the uterine horns.

The liver is generally healthy; however, the existence of small whitey nodules spread in the liver tissue was found in several subjects.

Vesical calculus can be often seen, more frequently in the females than in the males.

The lung and the brain show abscesses more seldom (4 lung abscesses, 2 brain abscesses in about sixty adults).

B - ANATOMICAL-PATHOLOGICAL EXAMINATIONS OF DEFICIENT RATS TREATED WITH ASTAXANTHIN

The examinations were conducted on all animals of the experiments described in the preceding chapters that died spontaneously or were sacrificed.

In certain cases, the animal is sacrificed within the same time as the death of a control animal is recorded, in order to compare the attacks in two animals of the same age. Autopsy is carried out in the animal that died or was sacrificed; the organs are removed and then weighed and examined.

1. RATS TREATED WITH ARISTEUS ANTENNATUS HEPATOPANCREAS WHOLE OILS

Preventive test:

Number of animals: 9 ♀, 9 ♂
Duration of treatment: 178 days.
Age animals at autopsy: 7 months

Females (9):

- died spontaneously (2) at the 120th and 140th day.
  vaginal infection (in the 2)
  abscess in the intestine

- sacrificed at the 178th day.
  abscess in the trachea (2)
  abscess in belly (abdominal wall) (1)
  cecum inflammation (3)
  bladder stone (1).

Males (9):

Two subjects had shown abscesses during treatment: one of them in the sternum (between the axillae); the other one in the ear region. These abscesses had healed spontaneously; however, several abscess recurrences were seen in the zygomatic arch.

Autopsy results:

- ulceration of stomach (2).
  abscess in the submandibular gland region (2).
  lung abscess (1).

Curative test:

Number of animals: 26
Duration of treatment: 15 to 20 days
Age of animals: 90 to 95 days

In the live animals, only neck or axilla abscesses were noticed; head alopecia, dry and standing hair on the back.

The autopsy revealed in two females an abscess in the duodenal loop, hemorrhagic points in the intestine, abscesses in the mandibular glands (apparent in the live animal), one brain abscess (1 ♂); one stomach ulcer (pyloric region in a female); the liver (median lobe) of a male showed grayish stains.

2. RATS TREATED WITH ASTAXANTHIN ESTERS

Preventive test:

Number of animals: 12 (6 ♀, 6 ♂)
Duration of treatment: 161, 163, 165, 230, 260, 262 days for the females.
210, 215, 220, 240, 260 days for the males.

Observations:

Females

- died at the 161th and 163th day of treatment;
  vaginal infection in both;
  bladder dilated by calculus (1);
- died at the 165th day;
  uterine bleeding;
  abscess in the ear region;
- died at the 230th day;
  thin animal;
  bladder totally filled with calculus;
  uterus cysts;
  abscess under the neck;
  cecum inflammation;
- died at the 260th day;
  bladder dilated by calculus (2 to 6 mm in thickness);
  uterine bleeding;
  abscess in the submandibular region;
  brain tumor;
- died at the 262nd day;
  some calculus in the bladder.

Males

The 6 males were sacrificed (the astaxanthin esters had been administered from weaning for 210, 215, 220, 240, 260 days).

The males sacrificed at the 210th, 215th and 220th day of treatment showed a downward weight curve, the other three showed weight stabilization since the sixth month of treatment.

Observations:

- male sacrificed at the 210th day of treatment;
  atrophied testes;
  abscess in the neck;
  cecum inflammation;
  thin animal;
- male sacrificed at the 210th day of treatment;
  atrophied testes;
  stomach ulcer;
  thin animal;

- male sacrificed at the 220th day of treatment;
  atrophied testes;
  abscess in the duodenal loop;
  thin animal;

- male sacrificed at the 260th day of treatment;
  atrophied testes (in the 3 of them);
  abscess in the ear region (1 animal);
  inflamed cecum (in the 3 of them);

Curative test:

Number of animals: 27
Duration of treatment: 20 to 30 days.
Age of animals: 90 to 100 days.

19 animals show abscesses in the submandibular region or in the ear region or under the eye - a single animal shows a brain tumor, liver cysts (6 to 10 small cysts, more in the median lobe) in 4 females and 2 males; cecum inflammation more or less marked (in 10 subjects), stomach ulcers (in 4 animals, 3 ♀, 1 ♂), vesical calculus (in 1 male - duration of treatment 40 days).

3. RATS TREATED BY CHROMOPROTEID INJECTIONS

Curative test:

Plesionika edwardsii egg pigment
Number of animals: 2 males
Duration of treatment: 18 days.
Age of animals: 110 days.

- atrophied testes (in the 2);
  Big abscess in the neck (in 1 subject)

Aristeus antennatus egg pigment
Number of animals: 3 (2 ♀, 1 ♂)
Duration of treatment: 8 days.

Females:

- vaginal infection (1);
  uterine cyst (in the same one);
  abscess in the submandibular gland.

Male:

1 abscess in the thyroid.

Scyllarus latus egg pigment
Number of animals: 2 females
Duration of treatment: 18 days.

Observation: (big) abscess under the neck (1).

Aristeus antennatus peristomal connective tissue pigment
Number of animals: 4 (2 ♀, 2 ♂)
Duration of treatment: 35 days.

Females:
- neck abscess (1);
  hypertrophied suprarenals (1).

Males:
- atrophied testes (2);
  stomach ulcer (1);
  pharynx abscess (1).

4. DISCUSSION

The comparison of the two series of examinations, deficient control animals and deficient animals treated with astaxanthin, leads to the following comments:

1. The systematic study of the alterations caused by vitamin A deficiency shows that the anatomical-pathological lesions are those typically described by all experimenters, both in the live animal and the autopsied animal: precocious colpokeratosis, submandibular abscesses affecting 60 to 70% of the young subjects from the 25th day of deficiency, physical distress expressed in thinness, prostration, hindquarter paralysis; in the adult animal, neck abscesses, vaginal infections and testicular atrophy hit a strong majority of subjects. The stomach ulcers, the cases of bladder stone, less general, can however be seen frequently.

2. The comparison of the results among the deficient control rats and the deficient animals treated with astaxanthin shows the same infectious processes when the pigment is administered at a small dose, for curative purposes, and the physical distress is comparable to that in the control animals. The abscesses in the submandibular regions, colpokeratosis, and vaginal infections can be seen as frequently. Except for the eye and related organs, in which the administration of astaxanthin restored the integrity of tissues and made the infectious processes to recede; the autopsy results are totally comparable in both cases.

When astaxanthin is administered (also for curative purposes) at high doses (of at least 50 µg), slowing down of the development of infectious processes can be seen in the live animal. Although colpokeratosis is determined in all cases, the autopsy seldom reveals genital tract suppuration phenomena; the same findings are made in the digestive system where the lesions are less apparent. In addition, the cachectic conditions are more unusual.

In the experiments of preventive nature the results confirm that the administration of strong doses delays the occurrence of lesions: in the females treated with doses of 50 µg per day, the growth of which went almost normally, neck abscesses were found only by the 5th month of treatment; colpokeratosis is detected precociously, and vaginal infections and vesical calculus can be seen

after the 6th month of deficiency (with treatment). In all males treated with 50 µg astaxanthin for preventive purposes, infectious processes could not be found until the 7th month, and the survival of the animals was the same for all subjects (longer than the 8th or 9th month). However, the genital system was affected and testicular atrophy was found in most of the cases (*).

3. With regard to the observations on the animals that were treated with chromoproteid injections, the results are parallel to those recorded for the rats treated orally with small astaxanthin doses or for the control rats fed the base diet alone; here again marked physical distress conditions can be seen and the autopsy shows lesions or infectious processes that prove that astaxanthin injected in a small dose has exerted no protection effect outside the ocular system.

In short, the disturbances found in the animals treated with astaxanthin are similar to those from a vitamin A deficiency or sub-deficiency: the infectious processes, the development and the locus of which are identical, are always found and hit in the same order the tissues of the same age. However, the eye and related organs are perfectly healthy; this confirms that astaxanthin plays in this region, like vitamin A, an essential role in nutrition of the conjunctiva and the cornea. **In addition, the localization of the pigment in the retina allows considering the possibility of its involvement in the formation of retinal pigments** (236), (72). Without being able to state precisely the time at which an enzymatic mechanism could become involved, it appears that astaxanthin could act in the form of a derivative close to and without oxidative cleavage of the molecule. However, this assumption will only be discussed at the end of part two in the light of the data obtained about the biogenesis of vitamin A in fish and about the experiments that have allowed examining and interpreting the transformation of astaxanthin into vitamin A in Gambusia nolbrooki (eastern mosquito fish).

(*) Degeneration of testes and related glands was mentioned by Giroud and Desclaux (64) as a consequence of malnutrition. In our long-term experiments of preventive nature, testicular atrophy was found without any signs of malnutrition being detected.
Testicular atrophy was also stated after the administration of fish liver oils and attributed to the fat acids in these oils (28), (168). We saw this dystrophy in experiments where the pigment isolated by chromatography was no longer delivered in oils extracted from Penaeoidea hepatopancreas but in peanut oil.

PART TWO

RESEARCH ON BIOGENESIS OF VITAMIN A IN FISH

The origin of vitamin A in fish lies among the general biology issues that, in spite of a considerable number of research works, have not yet been given a totally satisfactory solution.

As a matter of fact, it is not currently proved that food provides, either naturally or in the form of carotene, the Vitamin Apresent, sometimes in large amounts, in the viscera of certain fish. According to biological data, fish feed themselves with crustaceans and the latter with Diatoms; now then, Diatoms contain carotene and do not have any vitamin A (115) and only vitamin A is found in fish. As stated by Lederer, in Copepods, that constitute the intermediate link of the "diatoms - crustaceans - fish" chain, carotene has almost disappeared and vitamin A "has not yet been formed therein", what suggests to the author the assumption of the possibility of a vitamin A synthesis by fish, regardless of carotene (137).

Accordingly, it was logical to ask oneself whether fish are capable of using, as vitamin A precursors, carotenoid substances present in their food, and in particular astaxanthin that is so widely spread in the aquatic fauna (136), (132), (121) and particularly plentiful in shellfish.

A - ACTUAL DATA ON THE ORIGIN OF VITAMIN A IN FISH

It is presently well established (Lederer, Kon), opposite to Wagner's opinion (cf. p. 26) that, in a shellfish body, carotene is most often absent or is only present in the form of traces. On the other hand, we have seen that vitamin A was characterized therein unequivocally: Wald (234), Kon and Thomson (127), Fisher, Kon and Thomson (62) proved its presence in most of the species they examined; we have ourselves characterized it in Penaeidae and Pandalidae and showed that vitamin A, that cannot be detected elsewhere in the body, is however regularly present in the eyes (cf. Table VI) although in small amounts.

Therefore, it is indisputable that shellfish can provide a vitamin A food supply, while certain species are relatively rich in this factor: such is the case of the Euphausiacea that constitute the krill eaten by whales. Whales, as proved by Kon et al., therefore draw from their food the amounts of preformed vitamin A that compose their large liver reserves; however, if one refers to Table I (cf. p. 19) it is easy to convince oneself that, in this regard, the various crustacean species are not quantitatively equivalent; from this standpoint, Euphausiacea occupy a privileged position and, among them, Meganyctiphanes norvegica, Thysanoessa raschii and Tysanoessa inermis, species particularly plentiful in the krill, show the highest vitamin contents. On the contrary, in other shellfish vitamin A is essentially localized in the eyes and its concentration in proportion to the body as a whole is always low (Tables II, III, IV). The works by Kon et al. are in this regard perfectly in agreement with our own
observations on Penaeidae and Pandalidae (cf. Table I). Therefore, the whale case could not be

generalized, it is not absolutely certain that fish are in a comparable condition and that they always find in their food vitamin A in sufficient amounts to allow it alone to ensure meeting their requirements and forming reserves. For example, Merluccius vulgaris (90) shows particularly suggestive vitamin concentrations and differences of 390 to 1,500 µg per g of unsaponifiable matter for the liver and 750 to 15,000 µg for the intestine can be seen. The vitamin content per gram tissue in the same subject is in half the cases higher in the intestine than in liver: it even happens that the amount of vitamin in the intestine as a whole, the weight of which is four to five times lower than the liver, exceeds by much the amount of vitamin stored in this organ in its entirety (*).

The Merluccius case is not an exception and the works conducted by Lovern, Edisbury and Morton (on halibut (142)) show that the vitamin A content in the viscera is higher than in the liver. These facts recall for vitamin A in the intestine a significance other than just a reserve. The lack of correlation between the contents of vitamin in the liver and the intestine, the extent of the differences in concentration recorded for intestine vitamin and its localization in the place where pro-vitamins are transformed into vitamin A (68), (209), (224), (240), (162) suggest the assumption that it accounts for a product subject to the fluctuations of the supply of pro-vitamins from food.

Now then, Merluccius vulgaris chase shrimp banks on which they feed; shrimps contain substantially no carotene (cf. p. 36) and little vitamin A, the Mediterranean species (in particular Aristaeomorpha foliacea and Aristeus antennatus) are strongly pigmented and we have seen that the main pigment is astaxanthin.

Nevertheless, this carotenoid does not build up in Merluccius as in many fish. Moreover, it is not unusual to find partially-digested and completely decolorized shrimps in the digestive tract. Certainly, one can imagine that the pigment is simply destroyed in the course of digestive transit, but a priori one cannot preclude that it may come to another end. In this regard, we can summarize the previous experiments conducted by Mc Walter and Drummond (149) that prove that, in young

(*) Lovern recorded similar facts and underlined that in halibut (May fishing) the vitamin A content in viscera is higher than in liver (144).

fish issued from eggs containing a carotenoid pigment (probably astaxanthin), the pigment content falls during development while the vitamin A content increases at the same time. And it is also interesting to recall the experiments of Steven (218) that show the use of astaxanthin by the trout: the main carotenoid pigments in the trout in natural state are lutein and astaxanthin. The animals bred in an aquarium maintain their normal pigmentation on condition that their food diet is of the same nature; but if the diet comprises earthworms and ground meat, only lutein survives, while astaxanthin disappears completely (**); although no information is available about its end, there is therefore no doubt about its use.

However that may be, the observations reported above suggest to follow the end of astaxanthin in the fish body by studying the vitamin A concentration in the liver, the eyes and the intestine (86), (87).

B - EXPERIMENTAL CONTRIBUTION

1. MATERIAL UNDER STUDY

Selection of the reactive animal

We focused on Gambusia holbrooki Grd, a small oviparous cyprinodontiforme native to Texas (at the latitude of North Africa) that was imported by Edm. and Et. Sergent (202), (203) in 1926. The Algeria's Pasteur Institute breeds it and widely promotes its dissemination in the Algerian watercourses and marshes because, by making the anopheles larvae disappear, Gambusia constitutes an efficient means of biological prophylaxis in the fight against malaria. Gambusia is carnivore, but extremely voracious; they accept the most diverse food and make perfectly do with a diet composed entirely of semolina.

The females are oviparous, the eggs hatch at the time of laying; there proliferation is fast and abundant; they give 6 to 7 generations for one season and the number of young reaches up to one hundred in a single litter. The females are isolated at laying in an enclosure provided with a wire mesh, immersed in the fish tank, because Gambusia willingly practices cannibalism.

The size of the adults hardly exceeds 40 mm in average for the females and 30 mm for the males. No doubt that selecting a larger-size fish could have been more rational, but the Gambusia offered

(**) When food contains astaxanthin in an overabundant amount, "salmonization" of the animals occurs by storage of the pigment in the muscular tissue. With R. Grangaud, R. Dieuzeide and Th. Douard, we have conducted artificially this salmonification by feeding the rainbow trout with Aristaeomorpha foliacea's peristomal connective tissue (79).

several kinds of advantages for the type of experiment undertaken.

- their proliferation is fast and plentiful (*) and it is possible to have available a considerable number of animals as needed;

- they easily accept vitamin A deficiency.

These two features are essentials for the study of the problem set. The first tests showed us all the advantages we could take from breeding Gambusia and above all the results proved encouraging beyond our expectations; the same reactive animal was used in the following experimental designs.

Astaxanthin sources and preparation of extracts

The extracts (astaxanthin esters) are prepared from Aristeus antennatus peristomal connective tissue following the method already described (cf. p. 39). The astaxanthin esters obtained in an oily solution with the addition of tocopherol (2 mg α-tocopherol - as anti-oxygen - per gram oil) are added to the diet for Gambusia.

Diets

In the first tests the base diet was composed of semolina in its entirety. To avoid the effects of multiple deficiencies (of essential amino acids and vitamin factors), we administered the Gambusia a complex diet having the following composition:

- semolina or grilled, crushed and sieved breadcrumbs. 85%

- devitaminized casein. 10%

- dried, irradiated brewers' yeast. 10%

- vitamin D (crystallized calciferol): 100 U.I./g diet.

0.5 g of oily extract of pigment is added per gram of this mixture for the animals treated.

Experimental protocols

We found that the vitamin A deficiency can be rapidly obtained in Gambusia holbrooki: after 30 days of a Vitamin A-less diet, vitamin A is no longer detected in its body. This point being established, the experimental protocol was defined as follows:

The batches of animals being examined are for 35 days fed the deficient base diet. At the end of that period the control animals are sacrificed and the deficiency is controlled by the assessment of vitamin A: the animal is saponified in its entirety or the organs separately (eyes, livers, intestines, eggs) and the Carr - Price reaction is carried out according to the usual method.

(*) On condition that water is in the vicinity of 25°C.

Then either fragments of peristomal connective tissue or the complex base diet with the addition of oily pigment extract is supplied to the animals. At the end of the experiment, the animals are sacrificed and vitamin A is assessed following the same technique as for the control animals.

2. EXPERIMENTAL RESULTS

Preliminary tests:

First test: five Gambusia holbrooki specimens (4 ♀, 1 ♂) were fed semolina for 35 days. At the end of this period, two males were sacrificed. The Carr - Price reaction conducted on the unsaponifiable matter of the entire animal revealed no trace of vitamin A. The three remaining fish received during the 26 following days fragments of peristomal connective tissue. They were then sacrificed. The Carr - Price reaction conducted on the unsaponifiable matter revealed the presence of vitamin A at a concentration of 3.6 µg per gram fresh tissue.

Second test: this test was conducted on 10 animals (five treated: 2 ♂, 3 ♀ and five control animals: 3 ♂, 2 ♀) like the preceding test. Vitamin A was not detected in the control animals; it was present at a rate of 3.6 µg per g of fresh tissue in the treated G. holbrooki.

A third and a fourth test (4 animals and 14 animals) gave identical results: no vitamin A in the control animals; 3 µg vitamin A per gram in the animals from the third experiment and 1.8 µg in the animals from the fourth experiment, respectively.

Final experiments

First experiment:

11 animals (4 ♂ and 7 ♀) receive the base diet for 35 days. Deficiency is found to be total (negative Carr - Price reaction) in 4 animals at the end of this period.

The remaining 7 animals (2 ♂ and 5 ♀) are then treated; the oily solution of pigment extracted from the peristomal connective tissue is added to the base diet. The vitamin A content found is 1.5 µg per g.

Second experiment:

The animals (25 ♀ and 9 ♂) received the complex base diet. After 35 days the deficiency was found in eleven control animals (9 ♀ and 2 ♂); the remaining animals were treated for 26 days like those from the first experiment (base diet with the addition of oily extract of pigment from the peristomal connective tissue), then sacrificed and desiccated separating the males and the females. Among the males, vitamin A was present in the eyes at a rate of 10.2 µg per g of fresh organ; it was detected neither in the liver nor in the intestine. Among the females, vitamin A was also present in the eyes at a rate of 10.5 µg and as traces in the intestine. It was absent in the liver and the eggs.

Third experiment:

The animals from this batch (37 ♂ and 20 ♀) received the complex base diet for 35 days, at the end of which total deficiency was found in eleven control animals (3 ♂, 8 ♀). The remaining animals

received the base diet for 25 days with the addition of an oily extract of hypodermis pigment.

Among the males, the vitamin A content found was 8.4 µg per g for the eyes. Vitamin traces were present in the liver and the intestine; among the females, 12 µg vitamin A per gram were found in the eyes. No trace was detected in the liver and the intestine.

The results were gathered in the following Tables:

TABLE XXIII
Preliminary tests

Number of animals	Duration of deficiency	Duration of treatment	Astaxanthin source	Vitamin A per gram in µg
Control animals: 2	35 days	---	---	none
Treated animals: 3	"	26 days	C*	3.6 µg**
Control animals: 5	"	---	---	none
Treated animals: 5	"	26 days	C*	3.6 µg**
Control animals: 2	"	---	---	none
Treated animals: 2	"	26 days	C*	3.0 µg**
Control animals: 5	"	---	---	none
Treated animals: 9	"	26 days	C*	1.8 µg**

(*) C: fragments of peristomal connective tissue
(**) The determination was made on the animals in their entirety.

TABLE XXIV
Conversion of astaxanthin into vitamin A in Gambusia Holbrooki Grd.

Number of animals	Duration of deficiency	Duration of treatment	Astaxanthin source	Vitamin A per gram in µg		
Control animals: 4	35 days	---	---	none		
Treated animals: 7	"	26 days	E.C.*	1.5 µg**		
				eyes	liver	intestine
Control animals: 11	"	---	---	none	none	none
Treated animals: 7♂	"	26 days	E.C.*	10.2	none	none
16 ♀	"	26 days	E.C.*	10.5	none	traces
Control animals: 11	"	---	---	none	none	none
Treated animals: 34 ♂	"	26 days	E.H.*	8.4	traces	traces
12 ♀	"	26 days	E.H.*	12.0	none	none

(*) E.C.: oily extract of peristomal connective tissue
E.H.: oily extract of hypodermis
(**) The determination was made on the animals in their entirety.

3. DISCUSSION OF RESULTS

a) DEMONSTRATION OF THE TRANSFORMATION OF ASTAXANTHIN IN VITAMIN A

The preliminary tests showed that, in Gambusia fed semolina, vitamin A is regularly no longer detectable after less than 35 days of diet. When the deficiency is extended, the animals decline and die generally in less than three months after having shown signs of physical distress: the fins are perforated, the caudal fin, normally fan-shaped, shuts itself up in a thin brush, the body curls up in a circular arc that gives the animal a typical attitude. If, after 35 days, the base diet is replaced by fragments of Aristeus antennatus peristomal connective tissue, none of these deficiency symptoms appear and, after 25 days, vitamin A is detectable in a remarkable amount in the body of the treated animal. This means therefore that in the Aristeus' peristomal connective tissue - where neither preformed vitamin A nor carotene is found - there is a factor that has pro-vitamin A properties for Gambusia holbrooki Grd. The pro-vitamin revealed was likely to be nothing but astaxanthin, given that Aristeus peristomal connective tissue is rich in such factor.

The administration of this pigment separated by chromatography to the deficient animals allows proving that it is in fact like that: the method of preparation of the active extract indeed excludes the possibility of a substance other than astaxanthin being involved to explain the formation of vitamin A as was observed.

The method of identification and determination used (Carr - Price reaction, kinetic method) allows asserting without doubt that the substance that originated in the treated fish is indeed vitamin A.
These first experimental results allowed concluding that astaxanthin is a pro-vitamin for Gambusia holbrooki Grd., but did not determine precisely if it is only vitamin A or a mixture of vitamins A_1 and A_2.

The purposes of the following experiments were as follows:

1. to find out whether the synthesized vitamin A was only vitamin A_1 (retinol) or whether vitamin A_2 (hydro-retinol) was also present;

2. to determine precisely the kinetics and the stages of the transformation of astaxanthin into vitamin A.

b) STUDY OF THE NATURE OF NEWLY FORMED VITAMIN A (92)

It is usual that vitamins A_1 (retinol) and A_2 (hydro-retinol) (235) are simultaneously found in freshwater fish. Morton and Creed (175), by administering to perches (perca fluviatilis) carotene-rich diet, recorded a considerable increase in vitamin A_1 and A_2 reserves and could conclude that

carotene can play in freshwater fish both the role of pro-vitamin A_1 and pro-vitamin A_2. Inhoffen and Pommer (113) consider - without giving any experimental argument to support this assumption - that the same must happen with other pro-vitamins A although the conversion of vitamin A_2 into vitamin A_1 has to date never been observed in vivo (210).

Materials and methods:

The Gambusia used in the first tests were males and females without distinction, and the development of the deficiency, in particular the anomalies in the caudal fin, was found in both sexes; in addition, the adult female reached a size almost two times larger and dissection was much easier. The gestating females were discarded: although we made no observation in this connection, one could dread gestation disturbing metabolism of vitamin A and carotenoid pigments and, as a consequence, a cause of error being introduced.

Diets:

The composition of the synthetic diet used in the first experiments was slightly modified, with yellow dextrin being substituted for white bread crumb. The formula is then as follows:

Diet No. 1:	Devitaminized casein	20
	Yellow dextrin	72
	Irradiated brewers' yeast	4
	Devitaminized vegetable oil (at 1% tocopherol).	4

Casein is mixed with dextrin and brewers' yeast. By the addition of water and kneading, a dough piece is obtained that is stove dried. The desiccated mass is crushed and added with tocopherol oil.

Diet No. 2: This diet is prepared from the base formula No. 1 and its preparation is identical until the addition of oil. Before incorporating the devitaminized oil with the addition of tocopherol, an estimated amount of astaxanthin ester solution is added, in such a manner to obtain an oily solution of pigment of a known titer. For such purpose, 4 g. oil are mixed with 1,000 mL of apetroleum ether solution containing 5 mg astaxanthin esters (concentration estimated by chromatography at λ = 470 nm), obtained according to the above-described operating method (cf. p. 39); then the solvent is removed by evaporation under low pressure in an inert atmosphere.

Detection and determination of vitamin A factors

As soon as Gambusia is fished, it is kept at 0°C. The organs immediately removed are immersed in 3 mL absolute alcohol, placed in a volumetric flask and submerged throughout the dissection in a cooling mixture composed of ice and salt. It was found that operating in these conditions the alcohol does not evaporate and that organ weighing is significant at ± 0.01 g. After weighing, the organs are

saponified following the Lewis and Bodansky method (cf. p. 22). The saponification residue is taken up by 2.5 mL anhydrous rectified chloroform and vitamins A_1 and A_2 were characterized in choloroform solution aliquots by the Carr-Price reaction (kinetic method of Meunier and Raoul). The measurements are made in the Beckham spectrophotometer at

γ = 620 nm ($E^{1\,\%}_{1\,cm}$ = 5070) for vitamin A_1 and at

λ = 693 nm ($E^{1\,\%}_{1\,cm}$ = 4100) for vitamin A_2.

Experimental protocols and results:

a) First experiment: was intended to characterize vitamins A_1 and A_2 which may have been newly formed after administration of astaxanthin.

807 Gambusia specimens (♀ and ♂) were fed for 12 days the vitamin A-deficient base diet (diet No. 1). At the end of that period, 300 subjects (150 ♀ and 150 ♂) taken as control animals (batch I) were sacrificed and vitamins A_1 and A_2 were looked for and determined in the intestines, livers and eyes.

The remaining 507 Gambusia specimens (batch II) received then for the following 15 days the base diet with the addition of astaxanthin (diet No. 2). They were then sacrificed and the same examinations as on the control animals were conducted. All the results of this first experiment are reported in Table XXV.

TABLE XXV

		Vitamin A_1 in µg per g of fresh tissue	Vitamin A_2 in µg per g of fresh tissue
Batch I 300 control animals	Intestines Liver Eyes	none 4.0 7.6	none 1.8 2.2
Batch II 507 subjects	Intestines Liver Eyes	4.3 8.2 12.5	2.0 5.6 traces

These results show that vitamins A_1 and A_2 quickly disappear from the intestinal mucosa of the deficient animals, and that the administration of astaxanthin causes the neo-formation of the two factors.

Second experiment: the purpose of this experiment was to determine precisely the kinetics of the transformation of astaxanthin after administration to the deficient Gambusia specimens a single meal of the diet to which astaxanthin was added.

600 Gambusia ♂ specimens (*) were fed for eight hours the deficient diet. After this period, 150 animals used as control animals were sacrificed and it was found that vitamin A_1 had totally disappeared from the intestine; traces (non measurable) of A_2 still remained.

The remaining 450 Gambusia specimens were then distributed in three equal batches and received a single plentiful meal of diet No. 2. They were then sacrificed 2, 3 and 7 hours later, respectively, and vitamins A_1 and A_2 were looked for and determined in the intestine.

The following results were recorded (Table XXVI).

TABLE XXVI

	Vitamin A_1 in µg per g of fresh tissue	**Vitamin A_2 in µg per g of fresh tissue**
Batch I (150 animals)	none	none
Batch II (150 animals)	6.6	none
Batch III (150 animals)	1.65	traces

c) KINETICS AND STAGE OF THE TRANSFORMATION OF ASTAXANTHIN INTO VITAMIN A

This experiment therefore shows that vitamin A_1 appears in a significant amount from the 3rd hour after the administration of astaxanthin. At the 7th hour, the concentration of retinol in the intestinal mucosa is still noticeable, but already strongly decreasing. With regard to vitamin A_2, small amounts of which were detected in the intestines of the animals from batch III (sacrificed 7 hours after the single meal), it is not possible to assert that a neo-formation is involved because traces of this vitamin were also present in the intestinal mucosa of the control animals.

During this experiment, 2 mL of the petroleum ether solution (accounting for 1/5th of the extract of the intestines of batch III) were chromatographed on an alumina column of 0.5 cm in diameter. After washing with petroleum ether (2 mL), the chromatogram was developed with petroleum ether with the addition of 0.05% methanol. While astacine (produced during saponification by astaxanthin oxidation) was highly absorbed in the upper part of the column, an orange ring detached and then descended slowly. This operation therefore allowed revealing in the extract the presence of a carotenoid pigment, the behavior of which under chromatography showed that it could be carotene. To attempt its identification, a third experiment was undertaken.

(*) Since this experiment was conducted in June at the time when most females were in gestation, females were discarded.

Third experiment: in this experiment the females were preferably selected to the males because of their relatively large size, but the pregnant females were discarded (*) and the duration of the deficiency was set at 23 days.

630 subjects were used:

150 animals were used as control animals: the absence of vitamins A_1 and A_2 was found after 23 days.

480 Gambusia specimens are divided into three equal batches. Each batch is given, with a one-hour difference, a single meal of a diet overloaded with astaxanthin, then the animals are sacrificed 3 hours later. Dissection is immediately performed (on the cooled animals) and the intestines are immersed, as the dissection progresses, in cooled alcohol to prevent the enzymatic phenomena to extend beyond the three-hour period set.

The intestines of the three batches were gathered. The whole accounting for 5.150 g of fresh tissue was saponified (**) and the unsaponifiable matter put in a-petroleum ether solution. The petroleum ether solution, washed and then dehydrated by contact on anhydrous sodium sulfate, was chromatographed. The chromatogram developed by petroleum ether with the addition of 0.05% methanol allowed isolating a pigment migrating slowly in the same manner as β-carotene. It was eluted by stirring the colored alumina with petroleum ether with the addition of 1% methanol; the eluate, washed and dehydrated, is chromatographed again. After elution, spectral measurement is conducted: it is found that the spectrum is comparable to that of β-carotene, with a slight difference toward the short wavelengths of the main maximum (448 nm instead of 452 nm for the entirely trans carotene) that can be attributed to the presence of stereoisomers.

A supplementary experiment completed the identification: the petroleum ether solution of the pigment was added an equal volume of β-carotene solution in the same solvent and of the same concentration: when developed, a single zone was formed confirming that the pigment present in the extract was actually β-carotene.

d) DISCUSSION:

The results of the experiments with Gambusia holbrooki Grd. as a whole show that:

1. the Gambusia specimens can become quickly vitamin A deficient (in less than 35 days) when they are fed a vitamin A-less diet.

2. the symptoms of deficiency do not appear and survival is normal if fragments of Aristeus antennatus peristomal connective tissue or hypodermis are given to the animal or if the deficient diet is added an oily extract of astaxanthin;

(*) The pregnant females show a typical abdominal black stain that allows distinguishing them from the other females.

(**) Saponification was intended to allow removal of most of untransformed astaxanthin.

3. noticeable amounts of vitamin A can be detected in the animals so treated for 25 days; the vitamin mainly builds up in the eyes;

4. astaxanthin behaves both as a pro-vitamin A and as a pro-vitamin A_2;

5. vitamin A_1 is detected in the intestinal mucosa a short while after ingestion of the pigment, and the concentration is maximum at the 3th hour. Vitamin A_2 appears after, what leads to consider its formation by dehydrogenation of vitamin A_2 in C_3;

6. the presence of β-carotene is found, what seems to indicate that it represents the intermediate stage of the transformation of astaxanthin into vitamin A, given that the reduction of the oxygenated functions precedes the oxidative cleavage of the molecule; however, it was not determined that this is the main way and additional experiments should be attempted in this field.

GENERAL SUMMARY AND CONCLUSIONS

Throughout this work intended for the study of the vitamin properties of astaxanthin, we have in the first place focused our attention on the material under study.

We have provided a detailed description of the crustaceans that supplied us the extracts; we have mainly studied with the greatest of care their carotenoid pigments and looked for the presence of vitamin A and carotenes. Therefore, we could show that both in Aristaeomorpha foliacea and Aristeus antennatus that vitamin A can be detected neither in the hepatopancreas nor the hypodermis or the peristomal connective tissue. It is localized almost in its entirety in the eyes, where its concentration is about 3 to 11 µg per gram of fresh organ. The involvement of this factor could not be put forward to explain in whole or in part the effects obtained in the vitamin A-deficient white rat by the administration of hepatopancreas oil and hypodermis or peristomal connective tissue extracts.

Also, in the technical section concerned with the separation of astaxanthin esters, we show that the method of preparation would completely remove the carotene and vitamin A if these factors are present.

Another part of our material under examination was also thoroughly studied: this refers to chromoproteins of various colors from eggs, tissues and membranes from six crustacean species. By using a proteid denaturalization agent, releasing the prosthetic group by protein flocculation, we were able to show that in all cases trans astaxanthin is involved (γ maximum = 492 nm). Vitamin A was assessed in this material and the results show that, per gram of fresh substance, the vitamin A content does not exceed 1.2 µg for mature ovaries of Aristaeomorpha foliacea and Aristeus antennatus; for Plesionika edwardsii and Scyllarus latus eggs, only traces were detected.

These chromoproteins could be obtained in an aqueous solution and we determined that here too, the method of preparation of the injected water-soluble solutions involves the removal of vitamin A and carotenes.

Therefore, these are essentially:

- astaxanthin esters isolated by chromatography and dissolved again in devitaminized vegetable oil;

- astaxanthin-proteins in their water-soluble form, which were used in our biological tests.

We previously studied with great care the development of the deficiency in the Wistar rat, stating in particular the importance of the liver reserves monitoring the time these reserves disappeared. Two types of tests allowed us to study and compare the vitamin-related features of astaxanthin: curative-type tests and preventive-type tests.

We could also find and state precisely a result previously obtained with whole oils extracted from hepatopancreas: the anti-xerophthalmic effect is already noticeable for doses not entailing any weight recovery.

A comparable result was recorded when the pigment, in the form of an aqueous solution of astaxanthin-proteins, is injected in the peritoneum: eye injuries recede in a few days while the weight of the animal remains steady or falls.

At higher doses, an action on weight growth appears; previous experiments had underlined that hepatopancreas' whole oils, if administered at preventive doses (90 mg per animal per day), had a growth effect. We systematically studied to which extent the action of hepatopancreas lipids could be concurrent with the action of astaxanthin. By administering extracts with different astaxanthin contents and extracts with the same content delivered in different oily carriers (oil extracted from hepatopancreas or devitaminized vegetable oil), we could show:

- that the astaxanthin esters, administered at strong doses (50 µg per day) are sole responsible for the normal weight growth of the young rat treated from weaning and also fed a factor A-deficient diet;

- that, even at a dose of 200 mg per animal per day, the lipids extracted from hepatopancreas exerted no action on growth.

The animals so led to adult age by the sole administration of astaxanthin have the weight of a normal subject and a seemingly excellent health condition. The exploration of the reproductive functions in these subjects not having received the least trace of vitamin A or carotene from their weaning was undertaken. Experiments conducted during several months on the animals treated with hepatopancreas oils or with astaxanthin esters led to the following observations:

- the females show permanent estrus marked by the presence of horn cells in the vaginal smears; however, they can be fertilized by being coupled to normal males but premature abortion or stillborn litters show a pathological development of gestation.

- the males are hit by sterility;

- these functional disturbances in the males and the females can be seen whatever the dose of astaxanthin administered.

In short, the results of the biological tests as a whole provides the confirmation of the vitamin activity of astaxanthin previously determined and establish precisely the following points:

1. the anti-xerophthalmic action is found whatever the route of administration of the pigment: orally or by intraperitoneal injection.

2. the growth effect is obtained only using doses about ten times higher than those preventing xerophthalmia;

3. excess astaxanthin administered from weaning prevents neither colpokeratosis nor reproductive function disorders.

These results were completed by an anatomical-pathological examination of the treated rats. The results of the autopsy of animals that died spontaneously or were sacrificed revealed in all cases disorders and lesions typical of vitamin A deficiency. However, the administration of pigment from weaning opposes to a certain extent the accidents caused by the factor A deficiency that, accordingly, appears later and is less severe. In addition, the survival time of the males treated with astaxanthin from weaning is substantially normal: this is not the same in the females, which do not survive beyond six months and show in most cases vaginal infections.

The study of the localization of the pigment in the body of the treated rat also drew our attention. We conducted a systematic research of astaxanthin in the organs and the tissues of the treated animals.

- With regard to retina, we found the results previously obtained with the hepatopancreas oils and hypodermis extracts: in the treated rat, astaxanthin or any of its transformation products is found in the eye.

- In the liver, the pigment is absent or presents as traces, which confirms the impossibility of storage in this organ.

With regard to the pituitary gland, the thyroid, the suprarenals, the results are always positive.

- The localization in the ovaries and testes was not observed on a regular basis.

- After administration of zeaxanthin, this carotenoid was never found in the retina; however, traces were detected in other organs, which proves that the absence of pigment in the eye cannot be attributed to its inability to cross the intestinal barrier.

In fact, **the prevailing localization of astaxanthin in the retina allows us to understand the selective action of astaxanthin in the eye and related organs.** The first results had suggested that astaxanthin, in natural form or as a close derivative, can be involved in the formation of retinal pigments taking the place normally reserved for Vitamin A.

Nevertheless, the results obtained in the second part of this work allow us to consider another assumption. By experimenting on Gambusia holbrooki we could determine the

transformation of astaxanthin into vitamin A. Astaxanthin appears therefore as a pro-vitamin A for fish.

In the light of these results, we tried to determine precisely whether the newly formed vitamin A was only vitamin A_1 or whether vitamin A_2 was also present; we also tried to determine specifically the kinetics and the stages of the transformation of astaxanthin into vitamin A, and we found:

1. that (for Gambusia holbrooki Grd.) astaxanthin behaves simultaneously as a pro-vitamin A_1 and as a pro-vitamin A_2; vitamin A_2 appeared in the intestine after vitamin A_1, what speaks in favor of its formation from A_1.

- astaxanthin to vitamin A_1 to vitamin A_2 and not by a direct way.

2. that β-carotene is formed on a temporary basis, which seems to indicate that the reduction in the oxygenated functions of astaxanthin must precede the oxidative cleavage of the molecule.

The results just reported highlight the general biochemical importance of astaxanthin and reveal its privileged position in the series of carotenoids with oxygenated β-ionone nucleus. As with every experiment, new unknowns are revealed which dictate new research whose goal will be to gather into a synthetic whole the fragmented data that they established.

I hereby certify to the best of my knowledge and ability that the translation referenced below is true and accurate translations from French to English of the French language original and was performed by a professional translator with over 10 years translation experience.

The above facts are personally known to me or are based on representations made to me by the translator.

Date final translated version approved	*Author, Title and Journal*	*Our file name*
May 29, 2013	**THESES** PRESENTED at the **UNIVERSITY OF LYON** **FACULTY OF SCIENCES** TO OBTAIN THE DOCTORATE OF NATURAL SCIENCES BY Renée MASSONET Assistant at the Algiers' School of Medicine **FIRST THESIS:** RESEARCH ON ASTAXANTHIN'S BIOCHEMISTRY Presented orally on April 22, 1958 before the Examination Jury	*Massonet thesis -EngENTIRE final 24May2013*

5/29/2013

Ricardo Reyes Date
Project Coordinator
The Language Link of Connecticut

May 29 2013

Notary Public Date

ANDREAS F. WERNER
NOTARY PUBLIC
MY COMMISSION EXPIRES NOV. 30, 2016

Translators footnote:
1) Axerophthol as a term is no longer used in scientific English and as a general term in this context is a synonym for Vitimin A. All references to axerophthol have been translated as Vitamin A.

2) Massonet makes a distinction between "cerveau" and "encephalon". In this translation, "cerveau" is translated as brain and "encephalon" as central nervous system. The distiction being the ecephalon is that part of the central nervous system that includes all higher nervous centers enclosed within the skull and continuous with the spinal cord. The brain is simply the part contained in the skull.