ANNAL. BIOL. TIHANY	33	37—44	HUNGARIA 1966

HISTOLOGICAL AND CHEMICAL STUDIES ON THE YELLOW PIGMENT PRESENT IN THE NERVE- AND OTHER TISSUES OF *ANODONTA CYGNEA L.*

E. LÁBOS, I. ZS.-NAGY and L. HIRIPI

Biological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary
Received: March 30th 1966

It is known from literature (ref. GOODWIN 1952) that various carotenoids exist in the organs of several marine lamellibranchiates. The data refer chiefly to *Mytilus californiatus*, nevertheless, investigations were performed also on the tissues of other species as: *Pectenculus gylcimeris*, *Pecten maximus*, *Pecten Jacobaeus*, *Volsella nodiolus*, *Lima excavata* and other species belonging to the *Pleurobranchus* genus. The analyses show that beta-carotene and lutein are present in highest quantity in the tissues examined. Besides these, however, other carotenoids as glycimerin, pectenoxanthin, mytiloxanthin, hopkinsiaxanthin, zeaxanthin and astaxanthin were also demonstrable. The amount of these carotenoids in the tissues of the molluscs depend on feeding conditions and are presumably related to certain metabolic processes and even to sexual cycles (GOODWIN 1952).

It is known long ago that the central nervous system (SCHULTZE 1879) and also several other tissues of fresh-water *Anodonta* and *Unio* species contain a surprising quantity of yellow pigments. No chemical data were found in literature with regard to the analysis of this pigment. It was suggested by NAGY (1962) on basis of certain positive histochemical reactions that the yellow pigment present in the nerve cells of *Unio pictorum* is identical with lipofuscin and this author observed a parallelism between accumulation of this pigment and the age of the animal. She did not find carotenoids in the nerve cells (NAGY 1962). It was thought necessary to investigate this problem not only because of the above contradiction existing between fresh-water and marine molluscs but also because it is suggested that a relationship exists between extensive pigmentation and the function of cells. The objective of these investigations was the histological and qualitative chemical analysis of these pigments.

Material and methods

Investigations were performed on 13—20 cm long specimens of *Anodonta cygnea L.* The animals were kept in aquaria containing Balaton-water. Pigment analyses were conducted on the tissues of ganglia and feet in different seasons of the year.

The histological localization of the pigments is well observable in native cryostat-sections. For this purpose 8 μ thick sections were made. The sections were treated with concentrated acids (HCl, H_2SO_4, TCA) for the histochemical demonstration of carotenoids (PEARSE 1960). Staining with alcoholic Sudan-black was used (PEARSE 1960) for the demonstration of lipoids in general.

To avoid emulsion formation the pigments were extracted from the tissues in the presence of anhydrous Na_2SO_4 by various organic solvents. Benzene, ethanol, acetone, carbon tetrachloride, carbon disulphide, n-heptane, n-hexane, cyclo-hexane and petroleum ether were used. The absorption of UV and visible light by the extracts was measured by BECKMAN Model G-2400 spectrophotometer at 365 mμ wave-length, whereas the visible emission of the samples with the same spectrophotometer using its fluorescence device (DU/DK).

The CARR-PRICE reaction was also performed for the demonstration of the carotenoids.

A synthetic (95%) beta-carotene (Light) solution was used as standard.

Results

Differences in pigmentation were observed in the individuals examined and also in the various tissues. In some individuals the colour of the tissues were paler yellow with a shade of brown in it and the tissues of other specimens were of bright yellow colour often with a red tint. No relationship between degree of visible pigmentation and size of animals was demonstrable. Because a relationship exists between size and age of the molluscs, consequently no parallelism was observable between increased pigmentation and the age of animals. Young, small sized mussels may also be strongly and adult large ones less pigmented. Uniform pigmentation was observed in general in mussels originating from the same habitat.

In the cryostat-sections the localization of the bright yellow pigment is readily seen. The cytoplasm of nerve cells contains very much golden yellow pigment granules of 1—5 μ size. These granules occur sometimes in such a great number that they are fused along their bordering lines and are forming a mass of pigment which fills in the whole cytoplasm *(Fig. 1 A)*. Yellow granules of the same appearance are observable in the glia cells, in the epithelial cells of the foot, in the cells of connective tissue, in the epithelial cells of the duct of sexual organs and in the most varying cell formations of gametogenesis. In all these areas the yellow colour turns blue on the effect of concentrated acids and this colour is maintained for longer or shorter periods. Destruction of sections results soon after a treatment with cc. H_2SO_4, therefore cc. HCl is more suitable for examination purposes *(Fig. 1B)*. In the ganglion the bright yellow colour which is observable also by the naked eye changes blue also in Susa fixative for instance and disappears later in the dehydrating alcoholic bath series.

After 1 minute long staining with Sudan-black an intensive black colour reaction is observable in areas where the yellow pigment granules are localized *(Fig. 1/C)*.

The yellow pigment is not soluble in water, it may be extracted, however, from the tissues by any of the above mentioned solvents. The sections are also indicative of this. A yellow substance of a considerable light absorption may

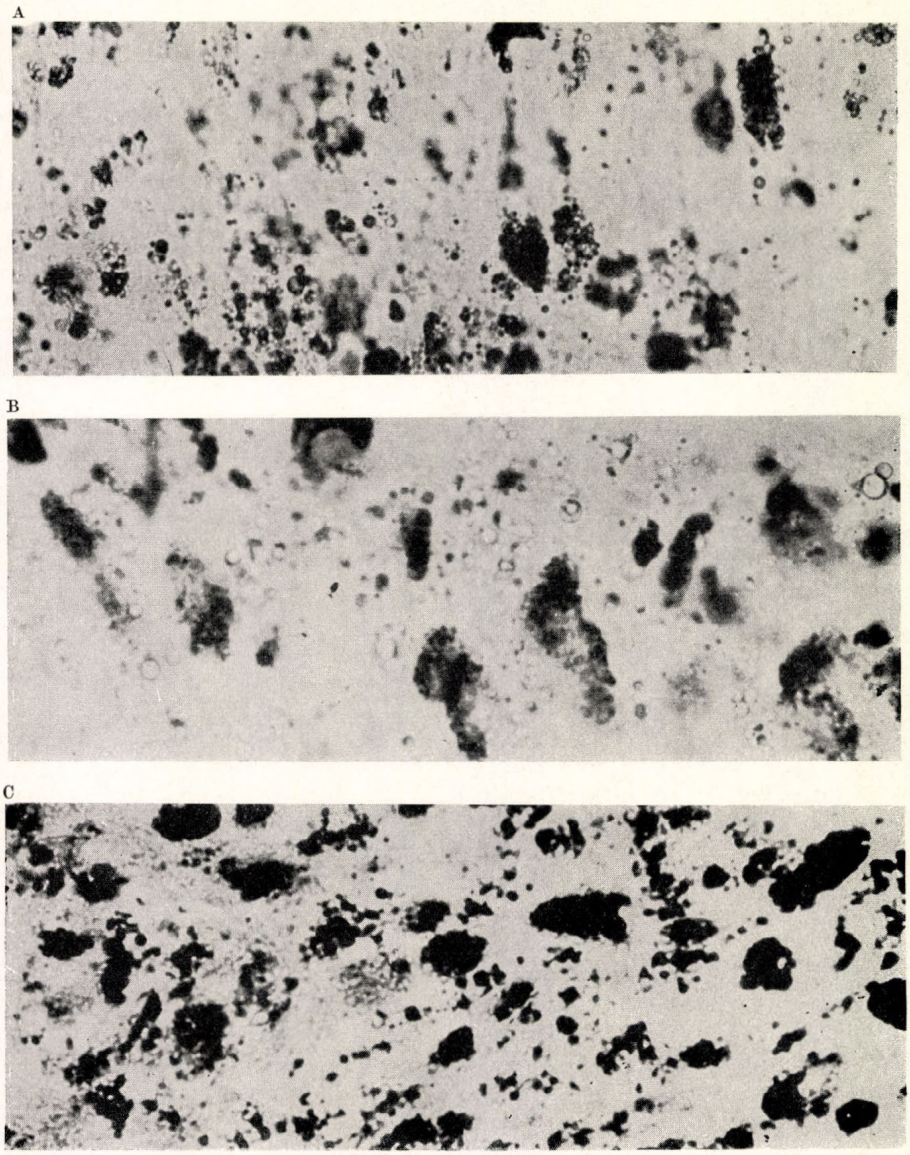

Fig. 1. A) A part of the section of visceral ganglion. Native condition. The substance observable in the cells corresponds to the yellow pigment. × 500
B) A part of the section of visceral ganglion. On the influence of cc. HCl the yellow colour of the pigment changes for blue and its granular localization becomes more distinct. × 500. C) Sudan-black staining of visceral ganglion. The localization of the intensive staining corresponds in general to the localization of the pigment. × 500

1 A. ábra Viscerális ganglion metszetének részlete. Natív állapot. A sejtekben látható anyag a sárga pigmentnek felel meg. Nagyítás: 500 ×.

B. ábra Viscerális ganglion metszetének részlete. cc. HCl hatására a sárga pigment kék színű lesz, szemcsézettsége még kifejezettebb. Nagyítás: 500 ×.

C. ábra Viscerális ganglion Sudan-fekete festődése. Az erős festődés lokalizációja nagyrészt megfelel a pigment lokalizációjának. Nagyítás: 500 ×.

be extracted by any of the above solvents. The degree of extraction by ethanol was examined also after fixation in BOUIN and in 4% formalin respectively. The time needed for complete extraction was different for the various individuals also in sections of equal thickness. In many cases, however, the amount of extracted pigments was not proportional to the time of extraction.

The absorption spectrum of the extracts has at least three maxima or inflexion points between 400—500 mμ, namely at 425—430, 450—460 and

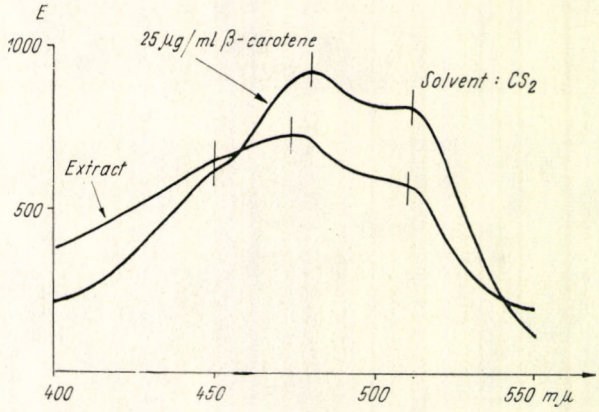

Fig. 2. The absorption of the pigment extracted from the foot by CS_2 and that of the standard beta-carotene in visible light. The amount of pigment expressed in 0.02—0.1 mg/g wet weight beta-carotene equivalents

2. ábra A talpból CS_2-vel extrahált pigment és standard beta-karotin látható fényben mért abszorpciója. A pigment-mennyiség 0,02—0,1 mg/g nedves súly beta-karotinnal ekvivalens.

470—480 mμ wavelength. The absorption measured in carbon-tetrachloride shows a considerable redward shift in comparison to other extracts *(Fig. 2)*.

Absorption maxima at 428, 448 and 476 mμ wavelengths were measured in n-hexane (Fig. 3). The synthetic beta-carotene solution used as control has absorption maxima at 428, 449 and 477 mμ. The absorption at 428 mμ of the ganglion extract seems to be an inflexion point and not a maximum, thus the absorption curve was normalized to that of the carotene solution on the basis of the most distinct maximum observed at 476 mμ and the differential curve obtained was examined. It was demonstrable by this procedure that also another chromophor exists here which is pale yellow and has an absorption maximum at about 420 mμ. This component is present also in ethanol, benzene and acetone extracts.

A light-sensitivity of similar degree was experienced after UV irradiation in the control beta carotene and in the hexane extract *(Fig. 3)*.

The activity produced by CARR—PRICE reaction is definitely characteristic of the carotenoids. Serotonine did not produce colour-changes either in the pigment extract or in the control beta-carotene solution, thus under the given conditions charge-transfer complex was not produced.

The petroleum ether extracts show considerable emission at 365 mμ excitation in the spectrum from 490 mμ to 650 mμ and at maxima of 535 and 580 mμ (corrected spectrum) *(Fig. 4)*.

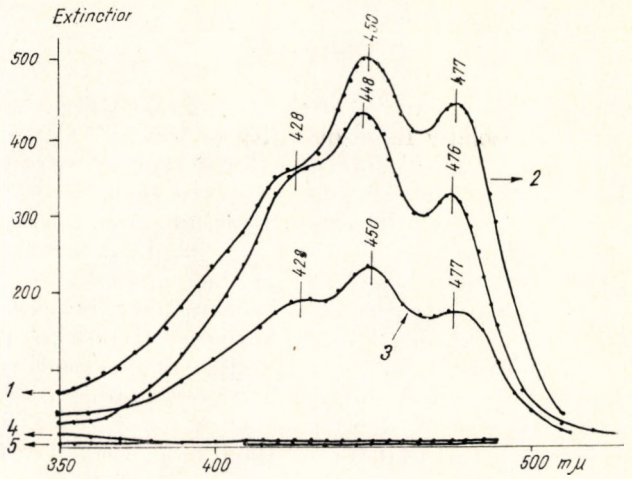

Fig. 3. The absorption spectra of the pigment extracted from the central nervous system (cerebral, visceral and pedal ganglia) (1), from the foot (3), and that of 2.5 μg/ml beta-carotene solution (2). The curves 4 and 5 were drawn after 1 hour UV-irradiation of the extract and of the standard respectively (250 watt, 100 mm, 90° incidence, mercury-arc, in quartz test tube)

3. ábra A központi idegrendszerből (1) cerebrális, viscerális és pedális ggl) és a talpból (3) extrahált pigment, valamint 2,5 μg/ml beta-karotin (2) abszorpciós spektruma.
A 4. és 5. görbe az extraktum ill. a standard 1 órás UV-besugárzás után készültek (250 watt, 100 mm, 90° beesési szög, higany-ív, kvarc-kémcsőben).

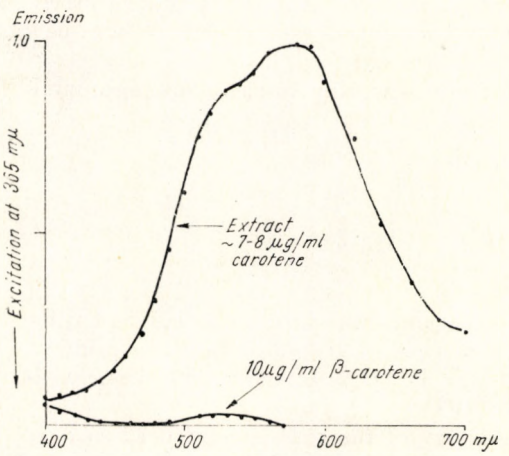

Fig. 4. The emission of petroleum ether extract and of standard between 350 and 700 mμ Corrected spectra. Excitation at 365 mμ

4. ábra Petroléteres extraktum és standard emissziója 350—700 mμ között. Korrigált spektrumok. Gerjesztés 365 mμ-nal.

Carotene content of ganglia was estimated also quantitatively on basis of hexane extracts. The average pigment-content in the total ganglia of 50 mussels computed on basis of the maximum at 449 mμ was 0,1 mg/g wet weight expressed as beta-carotene equivalent.

Discussion

The yellow pigment occurring everywhere in the tissues examined, and which is present in especially large quantity in the nerve cells changes blue with concentrated acids, and this indicates the presence of carotenoids (PEARSE 1960). The strong Sudan-black staining observed in a localization identical with the pigment proves that the carotenoids and lipoids exist together. It is inferred on basis of this observation that the pigment belongs to the group of lipochromes (PEARSE 1960). The fact that this pigment is soluble in organic solvents also supports this statement. The observation that occasionally longer time is necessary after fixation for the alcoholic extraction of the pigment from the sections may be explained by the nature of the lipochromes, for these being bound to proteins ("chromoproteid") are less soluble in organic solvents after fixation and may exist in such condition in paraffin embedded material (PEARSE 1960). It is probable that NAGY (1962) has observed in the nerve cells of *Unio pictorum* the lipochrome in this condition and took it for lipofuscin. None of the histochemical reactions performed by NAGY (1962) is specific on lipofuscin and thus the suggestion that the pigment is exclusively of lipofuscin nature is not considered correct. The negative results on the carotenoids obtained by NAGY (1965) can be explained by the fact that $SbCl_3$-reaction was performed on material embedded in paraffin.

Considering the solvent dependency of carotenoids of identified structure and that of the standard beta-carotene (KARRER and JUCKER 1949; LIAAEN, JENSEN AND JENSEN 1965) it is inferred on basis of the analytical data on the carotene-component of the pigment extracted from the tissues of *Anodonta cygnea L.* that this component stands nearest to beta-carotene. This suggestion is confirmed by the followings:

1. In hexane the absorption maxima of pigment and of standard beta-carotene was identical:

428—428 mμ
448—449 mμ
476—477 mμ

2. In carbon disulphide the kind and degree of the redward shift is similar with that observed in case of standard beta-carotene.

3. With $SbCl_3$ and H_2SO_4 a bluish-green colouring is observable in chloroform.

4. Light sensitivity of the extract and beta-carotene are similar.

In view of these considerations it seems that there is no essential difference in the chief pigments between sea-shell (GOODWIN 1952) and fresh-water mussel. In case of fresh-water mussel the pigment belongs to the group of carotenoids and its chief component is identical with beta-carotene. Its absorption

curve differs from that of the human *nerve cells lipofuscin.* HYDÉN and LINDSTRÖM 1950).

In the tissues of *Anodonta cygnea* besides beta-carotene a pale yellow chromophor is also present with an absorption maximum at 420 mμ. This substance is presumably identical with the component with an absorption at about 418—425 mμ which is present in the nerve cells of *Aplysia* species and is considered by CHALAZONITIS (1961) as belonging to the haem-protein. This may, however, as well be a carotene derivative. This phenomenon further the smaller resolving power of CS_2-extract and its smaller redward shift than that in case of beta-carotene suggest that various oxidation products may be present. This difference, however, is not sufficient for the more detailed definition of this component.

A considerable yellowish-green emission *(Fig. 4)* was also observed in the extract at an exitation of 365 mμ. This was not observed in the case of beta-carotene. The autofluorescence of nerve cells is most probably due to the presence of this component (DAHL et al. 1962; ZS.-NAGY 1967).

There are only hypotheses as regards the role of the carotenoid present in the tissues examined, but existing in greatest amount in the nerve cells. Due to its special electron configuration (PULLMAN and PULLMAN 1963) beta-carotene may be an important factor in oxidative metabolism. This is confirmed also by the observation of CHALAZONITIS and GOLA (1964) that in the nerve cells of *Helix pomatia* the respiratory ferments are concentrated exactly on areas containing carotene pigments.

Summary

Authors investigated the yellow pigment present in the nerve- and other tissues of *Anodonta cygnea L.* by histological and spectrophotometrical methods. Histochemically this pigment belongs to the group of lipochromes, where the carotene component is predominantly identical with beta-carotene.

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HISZTOLÓGIAI ÉS KÉMIAI VIZSGÁLATOK AZ *ANODONTA CYGNEA L.* IDEG- ÉS EGYÉB SZÖVETEINEK SÁRGA PIGMENTJÉN

Lábos Elemér, Zs.-Nagy Imre és *Hiripi László*

Összefoglalás

Szerzők *Anodonta cygnea L.* ideg- és egyéb szöveteiben található sárga pigmentet vizsgálták hisztológiai módszerekkel és spektrofotometriával. A pigment hisztokémiailag a lipochrom csoportba tartozik, amelyben a karotin-komponens túlnyomóan β-karotinnal azonos.

ГИСТОЛОГИЧЕСКОЕ И ХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ ЖЕЛТОГО ПИГМЕНТА НЕРВНОЙ И ДРУГИХ ТКАНЕЙ БЕЗЗУБКИ

Элэмер Лабош, Имре Ж.-Надь и Ласло Хирипи

Изучали гистологически и спектрофотометрически желтый пигмент нервной и других тканей беззубки. По гистохимическим характеристикам пигмент соответствует липохромам. Его каротиновая составляющая большей частью относится к β-каротину.