Ultrastructural Study of *Haematococcus lacustris* (Girod.) Rostafinski (Volvocales)

I. Some aspects of carotenogenesis

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Received July 13, 1982

Under certain conditions, several green algae are able to synthesize and accumulate large quantities of carotenoids which, masking the chlorophylls, end by giving to those organisms a more or less intense reddish colour.

These secondary carotenoids, which are different from those which are integrated in the photosynthetic lamellae of chloroplasts (primary carotenoids), have been named, since a long time, haematochrome (Fritsch 1935). Chemically, the secondary carotenoids include several oxo-derivatives of β -carotene (Czigan 1968), among which, for the alga studied here (*H. lacustris*), the 3–3' dioxi-4, 4' dihydroxi-carotene (astaxanthin) is the most important (Goodwin and Jamikorn 1954).

The determining factors of accumulation of astaxanthin and other secondary carotenoids have been extensively studied, sometimes with contradictory results, namely in respect of the light conditions (Goodwin and Jamikorn 1954, Droop 1955). In the meantime Czigan (1968), after a detailed study of several species of Chlorococcales and Volvocales, defined the essential factors controlling the biogenesis of these pigments.

On the other hand, the structural modifications of the cells associated with the genesis of the astaxanthin in the genus *Haematococcus* is a controversial subject, specially in what respects the sites where the pigment appears, and the organelles eventually intervening in their synthesis. The first electron microscope studies are not conclusive, either because the fixation method was not the most convenient (Bowen 1965), or because the first stages of the accumulation of the pigment were not sufficiently analysed (Wygash 1963, 1965).

Later on, two more detailed studies on this subject were carried out, but the respective results are discordant. So, according to the observations of Lang (1968), the pigment is not synthesized inside any organelle but actually in the groundplasm (cytosol). However, Sprey (1970) considers that the secondary carotenoids initially appear in the plastoglobuli of the chloroplasts which evolve in chromoplasts through a process similar to that has been often described in higher plants (for bibliography, see Mesquita *et al.* 1980b).

With the aim to obtain some more data on this problem, cells of *H. lacustris* were periodically studied in the electron microscope, during the aging of the cultures, the colour of which progressively changes from green to orange-green and finally to red. The results of this study are hereby presented.

Material and methods

The alga studied here is the species *Haematococcus lacustris* (Girod.) Rostafinski which was isolated from a harvest carried out in the surroundings of Oporto.

The cultures, in liquid or solid medium (with agar at 1.2%), were maintained at 16–18°C with a photoperiod of 12 hrs fluorescent light (≈ 4000 lux) alternating with 12 hrs dark.

For the study of the process of accumulation of the haematochrome, the cells were initially transferred to fresh medium. As from this moment (zero time), samples were harvested after 4, 6, 8, 11, 15 and 20 days, each of these being studied in the optical and electron microscopes.

These observations were carried out simultaneously in cells cultivated in liquid and solid media.

For the study in light microscope, cells were observed either in natural conditions or after “*in toto*” staining with black Sudan. This same stain was used on one-micron sections according to the technique of McGee Russel and Smale (1963).

For electron microscopy, the cells dispersed in the liquid medium were slightly centrifuged (3000 rot/min.) for 2–3 minutes.

The pellet thus obtained was fixed with 2% glutaraldehyde in 0.025 M Sorensen's phosphate buffer at pH 6, 8 for 90 minutes at room temperature and, after washings in the buffer, post-fixed with 1% osmium tetroxide, in same conditions.

After pre-embedding in agar, the material was dehydrated through alcohol series and embedded in Spurr's resina (Spurr 1969).

The ultrathin sections obtained with a diamond knife in an ultramicrotome LKB (Ultratome III) were picked up on copper grids, stained with uranyl acetate and lead citrate (Reynolds 1963) and observed in a Siemens Elmiskop 101 electron microscope.

Results

For the general study of the ultrastructure, young cells of 4-days aged cultures (in liquid or solid medium) were selected.

In liquid medium the biflagellated cells (zoospores) show a relatively thin wall, separated from the plasmalemma by a large space, this being crossed by fine protoplasmic strands which can be seen sectioned longitudinal, transversal or obliquely

Figs. 1–3. 1, (motile-cells, 4-days). The very thin wall (arrow) is separated from the protoplast by a wide space (*) and the chloroplast (c) presents a complicate morphology. Note that, excepting the stigma (s), only small and very rare osmiophilic globules (arrowhead) can be seen near the nucleus. $\times 8,800$. 2, *idem*. Detail of a young zoospore. The periplasmic space (*) is crossed by protoplasmic strands where the mitochondria (m) are the only visible organelles. The chloroplast (c) appears with a reticulate profile. $\times 13,200$. 3, *idem*. Detail concerning the ultrastructure of the chloroplast: the photosynthetic apparatus is differentiated in grana (2–6 thylakoids) and intergrana and the pyrenoid shows the typical structure of that of the Chlorophyceae. Note the almost complete absence of plastoglobuli. $\times 40,000$.

Note. In the captions, the age of the cultures (or of the cells) is counted since when the material is transferred to fresh medium.

Abbreviations used in Figs. 1–25: a-astaxanthin (pigment), c-chloroplast, ch-chromatin (chromosome), cv-contractile vacuole, d-dictyosome, er-endoplasmic reticulum, m-mitochondria, N-nucleus, ne-nuclear envelope, nu-nucleolus, p-pyrenoid, s-starch, v-vacuole, w-cell wall.

Figs. 4-8. 4, *idem*. The proximal face of the dictyosomes (d) appears systematically associated to e.r. (arrows). $\times 13,200$. 5, *idem*. Contractile vacuole region. The vacuole itself is surrounded by many vesicular profiles some of which seem to fuse with each other (arrow). $\times 28,600$. 6, *idem*. The tonoplast (membrane of the vacuole) is continuous with E.R. membrane (arrow). Inside the vacuole, a great and very osmiophilic inclusion (*) is seen. $\times 35,200$. 7, *idem*. Components

(Fig. 1). Mitochondria are the only organelles which appear in these extensions (Figs. 1 and 2).

In view of its complex morphology (reticulate or lobed) the parietal chloroplast appears, in the ultrathin sections, under the form of numerous fragments apparently isolated from each other (Fig. 1). Apart from a photosynthetic apparatus which is differentiated into grana (with 2–6 thylakoids) and inter-grana, pyrenoids with the typical structure of the Chlorophyceae (Dodge 1973), extrapyrenoidal starch grains and small and very rare plastoglobuli can be seen in the stroma of the chloroplasts (Figs. 1, 2 and 3). We should emphasize that this last characteristic of the chloroplasts (the scarcity and small dimensions of the plastoglobuli) is maintained until the last stages of the aging process of the cultures. The only really evident osmophilic globules are those that constitute the intraplastidal stigma (eyespot), which is formed by only one layer of granules located under the plastidal envelope (Figs. 1 and 8).

The dyciosomes always have their proximal face associated to a E. R. cisterna (Fig. 2), contrasting with what we have observed in other algae, where that correlation is systematically established with the nuclear envelope (Mesquita and Fátima Santos 1976a, b; Mesquita *et al.* 1980a).

The vacuoles, the reticular origin of which is sometimes evident, frequently contain very osmiophilic inclusions (Fig. 6) which might correspond to the phosphated reserve currently named volutine. Apart from these vacuoles, contractile vacuoles can also appear; these (one by cell) are localized near the cell surface and surrounded by numerous vesicles some of which seem to fuse with the membrane of the contractile vacuole (Fig. 5).

The central interphase nucleus contains most of the chromatin in the dispersed stage and a voluminous and structurally well differentiated nucleolus (Figs. 1, 9 and 14).

The non-motile cells, from cultures in solid medium with the same age (4 days), show a thicker wall; under this wall, a small periplasmic space, with a variable electrodensity and internally limited by a rather sinuous plasmalemma is observed (Fig. 9). The remain ultrastructural characteristics of the protoplasm are similar to those of the motile cells (cultures in liquid medium), but there is no stigma nor contractile vacuoles.

As to the essential aim of this work, that is, the research of the cytological modifications associated to the accumulation of the astaxanthin, this study was carried out simultaneously in motile cells (zoospores) cultivated in liquid medium and in non-motile cells (cysts) cultivated in solid medium. The pigment (astaxanthin) was identified through its intense affinity to the osmium and black Sudan. (Figs. 19a–d). The ages of the cultures are referred to the moment when the cells are transferred to a fresh medium.

of the cell endomembranous system: correlation of the nuclear envelope (ne) with the endoplasmic reticulum (e.r.) and a dictyosome (d). $\times 44,000$. 8, *idem*. Stigma (eyespot). The osmiophilic globules forming this structure are arranged along one layer only, localized immediately under the plastidal envelope (arrow). The periplasmic space (*) is internally limited by the plasmalemma (arrowhead). $\times 35,200$.

Figs. 9-13. 9, non-motile cells (akinetes) (4-5 days). The cell has a wall which is not still very thick (arrow), and it is covered inside with very sinuous plasmalemma (p). The profile of the nuclear envelope is also rather sinuous. Note the scarcity of osmiophilic inclusions in the cytoplasm (arrowhead) and the absence of plastoglobuli. $\times 11,000$. 10, *idem*. Detail of Fig. 9. One of the rare osmiophilic inclusions (astaxanthin) present in this cell shows a "filamentous" aspect (arrow).

Although the accumulation of pigment is not rigorously synchronous in all the cells of a culture, we found that in the 4-days old green cultures the majority of the cells do not yet contain astaxanthin or this is only deposited in very small quantities. These stages are therefore the most adequate to study the sites where the pigment appears, and the organelles eventually intervening in their synthesis.

In effect, in some of these cells, near the nuclear envelope, small osmiophilic globules, sometimes in like-rosary associations, are seen in the groundplasm (hyaloplasm) (Fig. 1). Although the small Golgi-vesicles are occasionally similar (in their dimensions and electrodensity) to the pigment granules, the systematic association of these granules with the E. R. profiles (Figs. 14–17) seems to be functionally more significant. This correlation is much more evident in the cultures in solid medium where the cells are transformed into cysts. In these cells, the inclusions of astaxanthin, although with the same perinuclear localization (Fig. 9), appear with a more varied morphology including spherical, rod-shaped "filamentous" and reticulated forms (Figs. 9–13).

At first sight, the profiles of these polymorphic inclusions remember us of more or less dilated cisternae full of osmiophilic material (astaxanthin) (Figs. 10 and 11). However, a more extensive study (at great magnification) has shown us that these inclusions are limited by an electrodense line only, which is very different from the typical tri-lamellar structure (unit-membrane) clearly visible in some E. R. profiles (Fig. 18).

The accumulation of pigment during the aging of the cultures was then studied in the optical and electron microscopes. In 6-days old cultures the inclusions of astaxanthin form more or less extensive perinuclear caps (Fig. 20), which later (in 8–10 days old orange-green cultures) end by completely involving the nucleus (Fig. 21).

The quantity of pigment increases and progresses towards the periphery of the cell; the result is a protoplast practically full of great astaxanthin inclusions localized between the nucleus and the parietal chloroplast (Fig. 22).

Up to the age of 10–12 days the above referred morphological difference of the deposits of pigment between cells cultivated in liquid or solid medium are maintained.

In final stages, however, that difference is masked through the fusion of the inclusions, which, in the meantime, does not easily take place (Fig. 24).

It is most important to stress that during this process, the typical structure of the chloroplast is maintained, and astaxanthin deposits inside of this organelle were never found (Figs. 23–25).

The granules of the stigma and, eventually rare plastoglobuli are the only osmiophilic inclusions observed in the stroma of the chloroplasts (Figs. 1, 3 and 8). The number and dimensions of the plastoglobuli, as well as the ultrastucture of the chloro-

×48,000. 11, *idem*. The pigment deposits (arrow) are morphologically similar to E.R. cisternae (e.r.) and to the nuclear envelope (ne). ×39,600. 12, *idem*. In this cell, the nucleus appears surrounded by astaxanthin deposits (a) with a varied morphology where the reticulated form is predominant. ×8,800. 13, *idem*. Detail of Fig. 4. The reticulate-shaped pigment deposit (a) does not appear clearly limited by a biomembrane. ×35,200.

Figs. 14–18. 14, motile cell (4 days). The pigment deposits, which in the motile cells normally present a globular form, are still rare and dispersed in the perinuclear cytoplasm (arrows). $\times 13,200$. 15, idem. Detail of the Fig. 14. Note that the pigment globules are localized in the groundplasm and they are not limited by a biomembrane. They appear preferentially associated to the E.R. (e.r.). $\times 48,000$. 16–17, idem. Details of other cells showing the same correlation pig-

plast are not significantly modified through the process of accumulation of secondary carotenoids.

In effect, even in old red cultures (11–20 days), where the chloroplast is pressed against the cell wall due to the great quantity of astaxanthin, the thylakoids and the plastidal envelope are maintained intact, that is, without any signs of degradation (Fig. 25).

Whenever the cells of old cultures (sometimes more than two months old) are changed to fresh medium, a progressive disappearance of secondary carotenoids is observed, this leading the cultures to the initial green colour.

Discussion

The problem of localization of the secondary carotenoids at the ultrastructural level is controversial and the data available in the literature (Wygasch 1965, Lang 1968, Sprey 1970) are not concordant.

In effect, the observations of Wygasch (1965) and mainly those of Lang (1968) point out very clearly to the extraplastidal localization of the astaxanthin. On the other hand, according to Sprey (1970), the secondary carotenoids appear in the chloroplastidal plastoglobuli, the increase in dimension and number of which takes place simultaneously with the lipophanerototic degeneration of the thylakoids.

Lang (1968) emphasizes that: "the pigment granules form in the ground substance and not within any organelle or vesicle". This author describes the perinuclear region where this pigment begins to appear as being a dense zone, rich in ribosomes and E. R. However, he concludes that neither this organelle nor the dyctiosomes, in spite of their topographic localization, are directly involved in the synthesis of the pigment.

The small globules of astaxanthin always appear outside the E. R. cisternae, they increase in number and coalesce to form greater inclusions. These inclusions, after surrounding the nucleus, end by taking up all the space among the lobes of chloroplast but they never appear inside this organelle (Lang 1968).

The results of Sprey (1970) are completely contradictory, as, in accordance with them, the accumulation of secondary carotenoids in *H. lacustris* leads to the transformation of the chloroplasts in chromoplasts following a mechanism very similar to that which has been many times described in flowers and fruits of higher plants (for bibliography see Mesquita *et al.* 1980b). This mechanism would include the following: lipophanerosis of the chloroplastidal thylakoids; increase of the plastoglobuli with secondary carotenoids, at the expense of materials coming from that lipophanerosis; passage of these plastoglobuli to the cytoplasm, after rupture of the plastidal envelope (Sprey 1970).

Our results do not support this interpretation and they are more in accordance with the point of view of Lang (1968). In effect, from the first to the last stages of

ment globules-E.R. (arrows) $\times 48,000$ (Fig. 16); $\times 3,000$ (Fig. 17). 18, non-motile cell (akinetete) (4 days). Partial view of the protoplasm of an akinete. Note the difference between the dense line which limits the rod-shaped pigment deposit (arrow) and the unit-membrane of an E.R. cisterna (arrowhead). $\times 64,000$.

Figs. 19–22. 19–a–c. Motile cells respectively aged with 4, 6 and 8 days. The astaxanthin globules (arrows) increase and progressively surround the nucleus (N) (1 μ m sections stained with black Sudan B). $\times 1,600$. 19–d. Non-motile cell (cyst) aged with 11 days. Besides the parietal chloroplast with many starch grains (s), the cytoplasm is almost integrally full of astaxanthin deposits (a). (Staining with black Sudan B). $\times 1,600$. 20, motile-cell (6 days). The pigment globules

the process of accumulation of the hematochrome, images which in some way could have been interpreted as a transformation of chloroplasts in chromoplasts, were never observed. What happens is that, due to the complicate morphology of the chloroplast and to the deformation of this organelle, in cells with a great quantity of pigment (15–20 days old cultures), we have sometimes the impression that the inclusions of astaxanthin are localized inside the chloroplast.

However, carefully following the outline of the plastidal envelope, we have always found that this localization is illusory; the pigment deposits are invariably sited in the cytoplasm, whereas the lamellae of the chloroplasts are maintained intact (Fig. 25).

Some biochemical studies carried out in *H. lacustris* seem to support this interpretation as, although the chloroplastidal β -carotene can also be used in the biosynthesis of the carotenoids, the synthesis “*de novo*” from an exogenous carbohydrate source is the fundamental pathway to the production of the secondary carotenoids in this alga (Donkin 1976).

As Lang (1968) suggested, in respect to cell organelles involved in this synthesis, our observations point out to the E. R. and do not support the intervention of the dictyosomes (Galli *et al.* 1966).

Actually, although in the initial stages of deposition of the pigment, the granules of astaxanthin are eventually similar (in dimensions and electrodensity) to Golgi vesicles, their relationship with E. R. is the most constant and significant event during all this process (Figs. 14–17).

Particularly in the cysts (akinetes), where the deposits of secondary carotenoids show a much more marked polymorphism (we can not find, now, any explication for this), that spatial correlation is outstanding (Fig. 11).

We should however stress that the profiles which were fully identified as E. R. cisternae do not contain pigment in their lumen (Fig. 18). On the other hand, while the membranes of E. R. show the typical trilamellar structure of the unit-membrane, the pigment deposits, even when they are morphologically similar to E. R. cisternae, appear outlined by an electrodense line (Fig. 18) similar to the hemi-membrane described in the spherosomes of several plant tissues (Schwarzenbach 1971, Jatsu and Jacks 1972, Wanner and Theimer 1978). However, we did not observe any images confirming the mechanism which has been proposed to explain the ontogeny of that hemi-membrane (Schwarzenbach 1971, Wanner and Theimer 1978).

According to Kleinig *et al.* (1978), the oleosomes (spherosomes) isolated from *Daucus carota* cells are not bordered by “a true phospholipid containing membranous structure. . . a relatively stable phase boundary around the oleosomes prevents the confluence of these droplets”.

We should not exclude the hypothesis that the outlining of the pigment inclusions

(a) begin to accumulate in the perinuclear cytoplasm. Compare with Fig. 19-a-b. $\times 5,300$. 21, *idem.* (8 days). The pigment globules (a), in larger quantities, now surround the nucleus completely. Compare with Fig. 19-c. $\times 5,300$. 22, *idem.* (11 days). The astaxanthin globules (a), in larger quantities and with larger dimensions, progress towards the cell periphery invading the cytoplasmic trabeculae which separate numerous vacuoles (v). Many starch grains (s) can also be seen. $\times 6,600$.

Figs. 23-25. 23, non-motile cell (cyst) (11 days). The cell wall is now already rather thick (arrow). The cytoplasm between the nucleus (N) and the parietal chloroplast (c) is almost completely full of large and polymorphic astaxanthin deposits (a). Plastoglobuli or any other osmiophilic material are not seen in the stroma of the chloroplast (c). The starch (s) is abundant. Compare with the Fig. 19-d. $\times 6,600$. 24, idem. Partial view of the protoplast: four large pigment in-

in *H. lacustris* may be of the type proposed by Kleinig *et al.* (1978). This could explain because the fusion of these inclusions does not easily take place.

Anyway, in discordance with Sprey (1970) and in support of the observations of Lang (1968), our results lead us to consider that the accumulation of the secondary carotenoids takes place in the hyaloplasm, that is outside the lumen of the E. R. cisternae. This, obviously, does not mean the exclusion of this organelle from the synthesis of those pigments.

Abstract

The process of accumulation of secondary carotenoids (astaxanthin) in *Haematococcus lacustris* was analysed in the optical and electron microscopes. The more relevant results of this cytological study are the following: *a.* In motile cells, the lipochromes are spherical inclusions, initially of small size, and located in the perinuclear cytoplasm apparently in preferential correlation with E. R. Although without a true limiting biomembrane, these sudanophilic and osmiophilic inclusions do not easily fuse when contacting. *b.* In the cysts, the pigment deposits, although being identical to the ones of the motile cells in what concerns their location and affinity for lipids "stains", show a variety of shapes, including "long filaments", rods and more or less complex reticulate forms. *c.* Cells of the older cultures (11–20 days) appear almost uniformly "stained" deep red. This process is reversible, i.e., these cultures again turn green when successively subcultured to fresh media. *d.* Apart from the stigma globules, the haematochrome is always localized outside the chloroplast, the photosynthetic apparatus of which remains practically intact through this process. Then, no sign of plastidal transformation, (chloroplast in chromoplast) comparable to the changes which take place in various organs of higher plants was observed.

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clusions (a) contact with each other (without fusion) and with the plastidal envelope. Note that the grana of the chloroplast remain intact. $\times 22,500$. 25, idem. The broken line marks the outline of a part of a chloroplastal profile, in contact with an astaxanthin inclusion (a). This figure shows that the pigment inclusion is restricted to the groundplasm. In the stroma of the chloroplast, starch grains (s) are seen but no pigment deposits. The photosynthetic apparatus remains practically intact. $\times 35,200$.

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