1. LM INVESTIGATIONS OF DIFFERENT STAINED FOSSIL BOTRYOCOCCUS COLONIES

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Abstract

Colonies of the *Botryococcus braunii* KÜTZ. algue extracted from the Upper Tertiary oil shale of Pula (Hungary) were investigated with the light-microscope. Stained, non-stained and solvated colonies were the subjects of these researches. The stain acceptance is suitable for researches of this kind, too, to establish the degrees of maturity of the organic components of the colonies.

Key words: Botryococcus braunii, oil shale, Neogene, stain acceptance, Pula, Hungary.

Introduction

The basic morphology of the genus of the *Botryococcus* KÜTZ. is extremely peculiar and isolated in the Plant Kingdom. This is the reason why the taxonomic position has been widely discussed and changed. This genus was ranged with the *Heterocontae* (KISS, 1939) respectively within the the *Chlorophyceae* WILLE (in ENGLER and PRANTL, 1910). Morphological statements by FREMY and DANGEARD (1938) and CHADEFAUD and EMBERGER (1960), COMBAZ (in DURAND, 1980) and GLIKSON, LINDSAY and SAXBY (1989). Resuming the basic morphology is as follows (Plate 1.1.).

1. The colonies are enveloped in mucilage (= Schleimmasse = mucilagineuse = une enveloppe gelatineuse hyaline = hydrocarbon matrix).
2. The differentiations of the cellular components of the colonies are: pedunculus, cupula. The individuum are in pair in the cupula and are forming one "polipier" sensu CHADEFAUD and EMBERGER (1960).
3. COMBAZ (1980) (Cf. CHADEFAUD and EMBERGER, 1960), published a scheme about the organelles of the protoplasm (nucleus, amidon, oil drops, plastis). An important component is the cuticle cellular. The cuticle of the mother cell is a part of the basal region of the "polipier" (Plate 1.1.). The morphology of the pedunculus is very characteristic. (Plate 1.1., 1.2.)

TEM data about fossil *Botryococcus* colonies from the oil shale were published by KEDVES (1983) and GLIKSON, LINDSAY and SAXBY (1989). The oil reservoir function of the holes in mucilage and in the more or less compact wall substance of the fossil colonies was pointed out (KEDVES, 1983). Biopolymer organization (KEDVES, 1988)

and thin layer chromatography was first described in two papers from the locality of Pula (KEDVES, 1986a, b).

Concerning the ecology of the *Botryococcus braunii* KÜTZ. algue the following can be pointed out:

i. Occurence in eutrophic fresh water or in humid soil (WILLE, in ENGLER and PRANTL, 1910) in Europe, North America and Africa. These colonies can also be present in salt lakes or in the water of marine lagoons.

ii. Stenotherm species after the paper of KISS (1939). The geological distribution of this kind of algue is extremely large. JARZEN (1978) wrote the following, p. 32: *"Botryococcus* KÜTZING (Pl. 1, fig. 3) is a colonial green algae, whose colonies form irregular globose masses encasted in a heavy, often dark, cohered mucilage. TRAVERSE (1955) have reviewed the fossil occurences of the genus and notes that the fossil record probably extends back at least to the Ordovician." (Cf. NARAYANA RAO and MISRA, 1949).

"The hydrocarbon secreting alga *Botryococcus* has been identified in organic remains of sediments ranging from Precambrian to Recent," (GLIKSON et al., 1989, p. 595).

Taking into consideration several results the chemical compositions of the fossil forms is determined by the following factors:

1. The basic composition of the different cellular elements of the colonie.
2. The ontogenetical state of the algue.
3. The molecular alterations in the wall also during the life.
4. The fossilization of the algue.
5. Taphonomical processes.
6. The preservation in the sediments.

The aim of this research is complex. By the LM method and the stain acceptance to get information, about the maturity of the different part of the colonies (Cf. POTONIÉ and REHNELT, 1971). The advantage of the light-microscopy combined with cytochemical methods is that a mass of data can be obtained. The statistical evaluation of the great number of information is important from the point of view of the Ontogenesis, Ecology, and Taphonomy.

Materials and Methods

The material of investigation is an average sample from the oil shale deposits of Pula (Hungary, Transdanubia). The oil shale of Transdanubia (Hungary) was first discussed by JÁMBOR and SOLTI (1975). Following JÁMBOR (1980) there are peculiar geological and paleoenvironmental conditions during the formation of these volcanic lakes. The water of the lakes is oligohaline water. The Geology (Cf. JÁMBOR, 1980, HETÉNYI, 1985, JÁMBOR and SOLTI, 1975, SOLTI, 1981), Ecology (Cf. NAGY, 1976, HAJÓS, 1976) and Geochemistry (HETÉNYI, 1985, 1987–1988, HETÉNYI and PÁPAY, 1986, ARATÓ and BELLA, 1976) of the oil shale was the subject of several investigations. The most important statements are as follows:

1. The investigated oil shale is a volcanic lake type. (JÁMBOR and SOLTI, 1975, JÁMBOR, 1980). 2. There was a geysirite activity during the sedimentation of the

alginite (SOLTI, 1981). 3. The water was oligohaline 0.3%, indicated by the diatoms published by HAJÓS (1976). 4. The clima was warm-temperate (annual average temperature: 10–12 °C), following HAJÓS (1976). E. NAGY (1976) described the pollen grains of Mediterranean conifers. 5. The water depth was some meters only based on the benthonical algues (Cf. HAJÓS, 1976). 6. The riparian Woodland resulted a peculiar local clima (E. NAGY, 1976). 7. The above mentioned conditions resulted the water flowering with the *Botryococcus braunii* forming the alginite.

The pulverized material was mixed with water. After stirring the inorganic components settled down and the organic fraction was separated, and newly dried. The investigation was prepared as follows. 1. Intact colonies (after separations mounted in glycerin-jelly hydrated at 39.6%). 2. Colonies partially dissolved with methanol. 3. To get information about the alterations or maturity of the colonies the following stains were used: 3. 1. BISMARCK Brown (vesuvin) 1% dissolved in ethanol 30%. 3. 2. BISMARCK Brown (vesuvin) 1% dissolved in ethanol 70%. 3. 3. Chrysoidin 2% dissolved in ethanol 40%. 3. 4. Safranine T 2% dissolved in ethanol 50%. 3. 5. Eosin 1% dissolved in distilled water. 3. 6. Fe-Haematoxylin (after EHRLICH). 3. 7. Malachite Green 1% dissolved in distilled water. 3. 8. Methylene Blue 1% dissolved in distilled water. 3. 9. Toluidine Blue 0.2% dissolved in distilled water.

The pictures were taken with an objective of oil immersion; Carl Zeiss Jena, GF Planachromat HI 100x/1.25∞/0.17–A, except, Plate 1.1.

Results

The characteristic morphology with the different cellular organelles is illustrated in Plate 1.1. The pictures were taken from colonies stained with Toluidine Blue, with an objective Carl Zeiss Jena, GF Planachromat 40x/0.65∞/0.17–A. Plate 1.2. illustrates examples about the degradation and the kerogen accumulation. The description of the detailed results is given in the order under the title Materials and Methods.

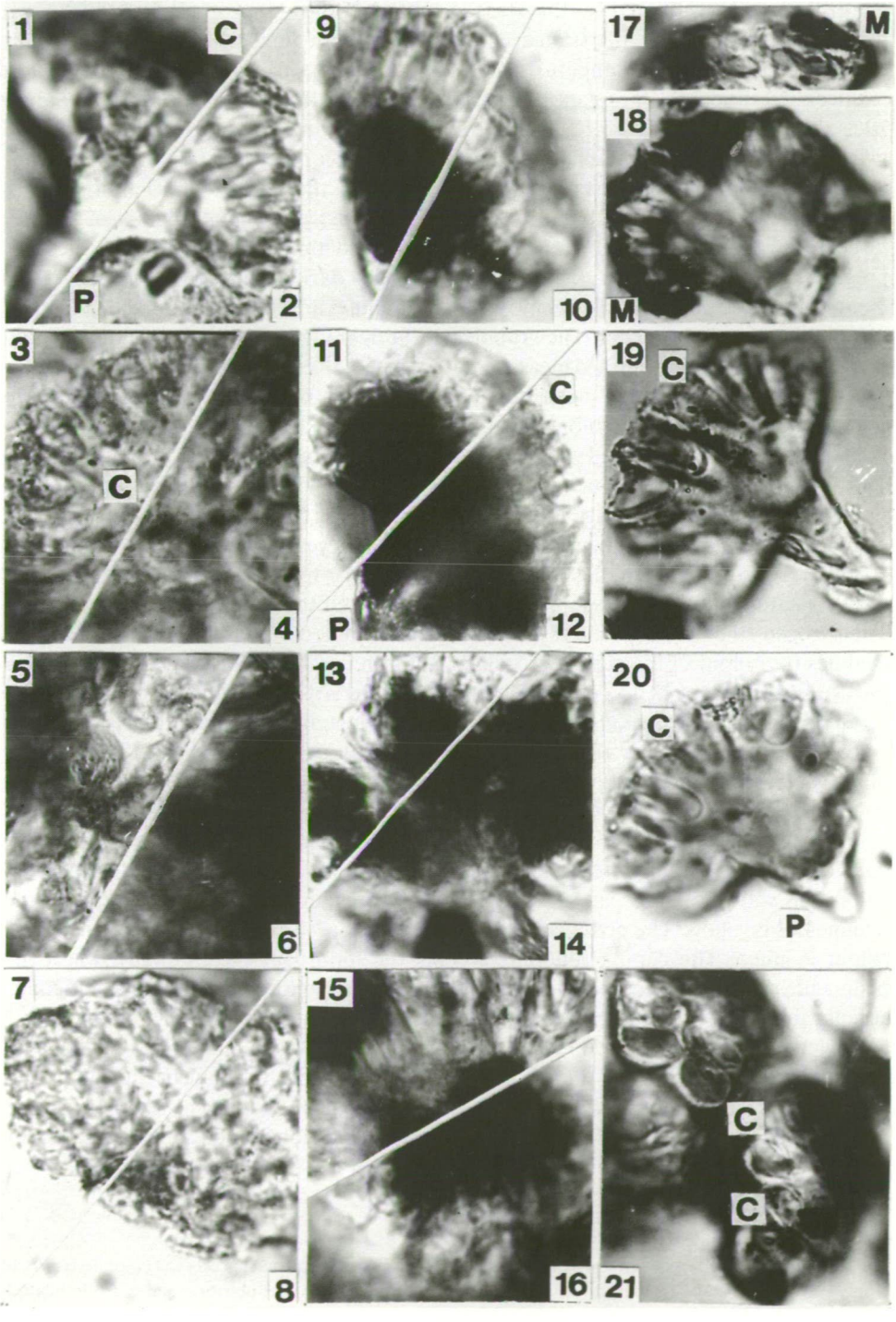
1. Intact colonies (Plate 1.2., figs. 3,4, and 13,14). It is a relationship between the preservation stage and geochemical maturity of the colonies. The accumulation of kerogen may be indicator of the "maturity level." In the case of kerogen accumulation, the colonies are at least partially degraded (Plate 1.2., figs. 13,14). The ontogenetically primitive colonies are less degraded in contrast to the completely developed ones. The presence of the green granules is also an indicator of the maturity level. On the basis of the data of the material of Pula the following can be distinguished. 1. 1. Clear greenish-yellow, semi-transparent colonies without kerogen accumulation and/or green granules. 1. 2. Clear greenish-yellow to brownish-yellow colonies with green granules. Colonies of this kind may be degraded to some level. 1. 3. Partially degraded colonies containing kerogens (brown particles), and green granules. 1. 4. The advanced degradation process is well shown with relatively high quantity of kerogens and green granules. 2. Colonies partially dissolved with methanol (Plate 1.2., figs. 7,8, and 9,10). The preliminary investigations did not result in characteristic alterations. The colonies are more transparent in contrast to the intact ones only. Further experiments are necessary with this solvent. 3. Stained colonies (Plate 1.1., 1.2., figs. 1,2,5,6, 11,12, 15–21). In comparison to the intact colonies the following characteristic

CUTICLE
OF THE MOTHER CELL
CUPULA
MUCILAGE
CUTICLE
CUPULA
PEDUNCULUS
CUPULA

features were investigated by the stained material: the green granules; the brown particles of the kerogen; the stain acceptance of the mucilage; the different particles of the cupula and the pedunculus. Particular attention was paid to the degradation surfaces. 3.1. and 3.2. BISMARCK Brown (vesuvin) 1% dissolved in ethanol 30% and 70% (Plate 1.2., figs. 1,2,11,12). No significant differences were found between the two kinds of dissolved stains. The colonies have not accepted this stain but the solvent effect of the ethanol was observed which is similar to those of the methanol (2.) 3.3. Chrysoidin 2% dissolved in ethanol 40% (Plate 1.2., fig. 20). The inner regions of the wall respectively the cupula and the pedunculus accepted this stain but only at the well preserved colonies. The colour is orange occasionally dark orange, or yellow. Worth of mentioning is the unstained part of the wall. The granules are stained at the well preserved colonies in a middle per cent. The granules of the degraded colonies have not accepted the stain or only to a very limited measure. The colour of the completely damaged colonies is yellow. No colour change was observed at the brown particles and the mucilage, in contrast to the non-stained colonies. 3.4. Safranine T 2% dissolved in ethanol 50% (not illustrated). The not degraded wall have accepted the stain only in a very poor measure. The degradations surfaces much better accepted the stain. The granules accepted occasionally the stain. The mucilage well accepted the stain in this way, its colour is red. The pedunculus is in general yellow, in this way this is the original colour. But the cupula have accepted partially the stain, the colour may be yellow or red. 3.5. Eosin 1% dissolved in distilled water (Plate 1.2., figs. 15,16). The wall accepted the stain, the colour is orange. The kerogen (the brown particles) is as at the non stained material. At some degraded colonies the colour of the cupula is orange. But in general it seems that the cupula and the pedunculus have not accepted the stain. The colour is as originally. The mucilage also accepted this stain. 3.6. Fe-Haematoxylin (after EHRLICH) (not illustrated). The slide colour changed; after a certain time to yellow. The colour of the wall is grey or greyish-blue. The cupula accepts the stain much more than the pedunculus. The colour of the pedunculus is originally yellow. The granules are green or occasionally bluish-violet. The colour of the kerogen has not changed; brown. 3.7. Malachite Green 1% dissolved in distilled water (not illustrated). The colour of the pedunculus at the well preserved colonies is yellow or transparent. At the partially degraded colonies the colour of the pedunculus is yellow. The pedunculus accepts the stain at the degraded specimens so the colour is green. The cupula generally accepts the stain. But exceptionally, when the pedunculus has not accepted the stain, the colour is yellowish-green. The mucilage accepts the stain very well, its colour is dark green. Kerogen accepts the stain. 3.8. Methylene Blue 1% dissolved in distilled water (Plate 1.2., figs. 17,18). The wall does not accept the stain in contrast to the granules. The outer part of the cupula is blue, the inner part is yellow. The pedunculus is yellow only rarely accepts the stain. The kerogen accepts the stain. 3.9. Toluidine Blue 0.2% dissolved in distilled water (Plate 1.1., 1.2., figs. 19,21).

◂ Plate 1.1

Botryococcus braunii KÜTZING from Alginite of Pula. General aspect from well preserved colonies from the Upper Neogene of Pula. Coloured with Toluidine Blue. The different cellular organelles are indicated. 1200 x.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The colonies accept the stain, the colour is green. This indicates the accumulation of the aromatic lignin derivates. The granules rarely accept the stain, at the well preserved colonies. At the degraded specimens the granules have been colored. The mucilage accepted the stain, colour: violet. The cupula is at the well preserved colonies green or greenish-yellow. The pedunculus is mostly violet. The colour of the degraded colonies and the kerogen is also violet.

Discussion and Conclusions

In consequence of the polycondensation and polymerisation during the diagenesis the kerogen is three dimensional network of organic molecules. The chemical composition has been investigated by several laboratories. From the very extensive data of a mass of publications the following citations will be pointed out.

DOUGLAS, EGLINGTON and MAXWELL (1969), p. 569: "The hydrocarbon content of coorongite, a Recent rubbery deposit derived from the alga *Botryococcus braunii,* has been investigated by infrared spectrometry, gas chromatography and combined gas chromatography-mass spectrometry."

ANDERS and ROBINSON (1971), p. 661: "Fifty-two cyclic alkanes, isolated from the bitumen of Green River shale, were analyzed by mass spectrometry."

The acceptance of the stains well indicates the heterogenity of the colonies. Namely the colonies of different "maturity" and degradation level differentially accept the stains. The differences in the degree of the maturity well shown at the coloration with the Chrysoidin. The differences in the degradation level were demonstrated with the coloration of the Toluidine Blue, Safranine T and Malachite Green. Less expressed are the following stains: Chrysoidin and Eosin. Kerogen well accepted Safranine T, Malachite Green, Methylene Blue and Toluidine Blue. This fact indicates the presence of the aromatic derivates. The pedunculus accept less the stains in contrast

◂ Plate 1.2.

1–21. *Botryococcus braunii* KÜTZING from Alginite of Pula. 1000 x.
1,2. Well preserved colony coloured with Vesuvin.
3,4. Unstained slightly degraded colony with green granules.
5,6. Partially degraded colony stained with Eosin.
7,8. Completely degraded unstained colony, dissolved with methanol.
9,10. Kerogen accumulation at the basical part of the colony. Unstained material dissolved with methanol.
11,12. High measure of kerogen accumulation stained with Vesuvin (dissolved in ethanol 30%).
13,14. Unstained colony. The accumulation of the kerogen extends near to all parts of the colony. Unstained material.
15,16. The kerogen accumulation is at basis of the colonies. Stained with Eosin.
17,18. Well shown is the mucilage coloured with Methylene Blue.
19. The peculiar morphology of the pedunculus. The granules are coloured with Toluidine Blue.
20. Well preserved colony stained with Chrysoidin.
21. The granules coloured with Toluidine Blue.

C = cupula, M = mucilage, P = pedunculus.

to the cupula. Chrysoidin and Toluidine Blue coloured in an important measure the pedunculus. Malachite Green and Methylene Blue at the degraded colonies have coloured the pedunculus. It is necessary to point out that Toluidine Blue coloured the colonies in a different way. The colour of the cupula at well preserved colonies is green. The colour of the degraded cupules is violet. Methylene Blue coloured the outer part of the cupula. (Cf. CHADEFAUD and EMBERGER, 1960). The green granules have not totally accepted the stain. The stain acceptance was moderated at Chrysoidin, Toluidine Blue and Methylene Blue. In a very poor measure Safranine T, and Fe-Haematoxylin have coloured the green granules.

Resuming this was useful for several points of views. But it is necessary to develop this methodical concept, too. For this it is necessary to investigate the mechanism of the different coloration.

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Appendix

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