A GREEN ALGA WITH MINIMAL EUKARYOTIC FEATURES: *NANOCHLORUM EUCARYOTUM*

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1. Introduction

Recently we discovered a new, very small eukaryotic alga *Nanochlorum eucaryotum* (*N.e.*) (Wilhelm *et al.*, 1982) in our sea water aquarium in the institute at Mainz. Growth and photosynthesis of *Nanochlorum* have been described (Wilhelm and Wild, 1982). The organism grows autotrophically in our laboratory in continuous culture in logarithmic phase at more than twice the salt concentration of sea water with a generation time of 27 hr.

The known properties of *N. e.* so far reported are given in Table I. Strangely, for more than 300 generations meiosis or mitosis never have been observed. Before division starts the nucleus enlarges without any visible change in the appearence of the nuclear content, the inner leaflet of the nuclear double membrane grows a circular fold, enlarging towards the center and finally pinching the otherwise unaltered nuclear content into halves.

Prior to nuclear fission however, the chloroplast and the single mitochondrion divide, a Golgi apparatus appears which doubles, and while the cell membrane invaginates, new wall material is formed, resulting in a doubled cell wall between the daughter cells, and finally divides the whole into two entities.

These findings raise some principle questions concerning the eukaryotic status of *N. e.* which has been discussed in the literature under different aspects (Margulis, 1981). In comparison to most 'fully eukaryotic' eukaryotes, *N. e.* shows deviations (Table II) and the question may be asked: What are the adequate and sufficient conditions respectively and their combinations characterizing the eukaryotic status?

We are aware of our actual unability to answer this question.

In this paper we show that *N. e.* apparently does not contain tubulin-like proteins that are susceptible to common mitotic spindle inhibitors, that it does not contain histones in concentrations found in most eukaryotes and that nucleosome-like features are absent from all of its subcellular particles.

The investigation of *N. e.* however may disclose in a model like fashion what the state

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of a 'marginal eukaryote' is and how far deviations can go without disabling biological fitness.

In this context it seems of importance to report here of an organism resembling *N. e.* in all respects that does not stem from an artifical biotope since it has been isolated from a natural biotope in the Northern Adriatic.

TABLE I

Some characteristics of *Nanochlorum eucaryotum*

(1)	Cell volume:	$1.5 \pm 0.6\,\mu^3$ (under conditions of unbalanced growth: $4.1 \pm 0.6\,\mu^3$);
(2)	Cell wall:	10–30 nm thick, even, smooth;
(3)	Subcellular elements:	1 dictyosome;
		1 mitochondrion ($12 \pm 5\%$ of the cell volume), spherical or ellipsoidal;
		1 chloroplast ($60 \pm 10\%$ of the cell volume), with chlorophyll a, b and with smooth bands of three to fivefold thylakoid lamellae, with plastoglobuli, without pyrenoids;
		1 nucleus ($21 \pm 5\%$ of the cell volume), with variable number of nucleopores;
		cytoplasm ($8 \pm 6\%$ of the cell volume, may increase considerably, e.g. with unbalanced growth), with few tubuli, that are covered with 16 ($\pm$ 4) nm thick particles;
(4)	Shape:	spherical, rarely slightly oval, sometimes double forms, otherwise single;
(5)	Nuclear division:	nuclear pores disappear; the nuclear membrane invaginates and pinches the nuclear content into two equal parts; the appearance of the nuclear content doesn't show alterations;
(6)	DNA content:	$6.1 \pm 0.8 \cdot 10^{-14}$ g/cell;
(7)	RNA content:	$2 \pm 1 \cdot 10^{-13}$ g/cell;
(8)	Protein content:	very variable , depending on growth conditions: $1 \cdot 10^{-13} - 1 \cdot 10^{-12}$ g/cell;
(9)	Energy requirement:	cell division and growth: 100–2500 lux; inhibition: 10^4 lx;
(10)	pH tolerance for growth:	pH 3–9;
(11)	Salt tolerance:	0–12% NaCl equivalents;

TABLE II

Features in *N. e.* deviating from typical eukaryotic state

1.	Size and DNA content are at the lowest ones found in typical eukaryotes.
2.	These single-celled organisms only contain of one single subcellular organelle each: chloroplast, mitochondrion, dictyosome.
3.	Nuclear division occurs without formation of condensed chromosomes.
4.	Only binary fission has been observed.
5.	Spindle apparatus, phragmoplast, fibrillar structures within the nucleus or outside of it never have been observed.
6.	The nuclear membrane never dissolves or even loosens up.

2. Material and Methods

2.1. Search for *N. e.* in Natural Biotopes

38 samples were taken in the Northern Adriatic from the shores up to 5 nautical miles from the Center for Marine Research at Rovinj (Istria, Yugoslavia) at all the places where we during the previous 2 years had taken samples, since ultimately from one of them the *N. e.* detected in our sea water aquarium in Mainz should stem.

13 samples were water samples from the surface and 13 from the bottom at 24–32 m. They were filtered by gravity through a 3 μm filter. The filtrate was then put through a 25 mm .42 μm filter (Millipore, Neu-Isenburg, F.R.G.). These filters were cut into quarters and put into sterile flasks. To two of them, 10 ml of sterile Adria water was added, to the two others 10 ml of sterile solution according to Table III. They were plugged and put into a roller apparatus (30 rpm) under a 12 hr 2000 lx daylight, 12 hr dark cycle, at 22 °C.

9 samples were rinsed off from macro-algae, shells and sponges with sterile Adriatic water with 250 ml each.

3 samples came from stones. One of them, from 'Colona', the source of the successful preparation, was in a fine crack going 4 cm deep into an otherwise indisturbed, about 12 kg, rock of white calcium carbonate. When a piece had been knocked off with a hammer a 3 cm broad coloured band became visible (Figure 1).

The blue green zone showed single-celled organisms considerably larger than *Nanochlorum* without nuclear structures under the microscope, whereas in the bright green area among others, small green organisms of the expected diameter of around 1 μm could be discerned. The organisms of the different layers could be brushed off with sterilized instruments. They were suspended in 250 ml sterile artificial or natural sea water, that had been replenished either by 'terra rossa' extract (vide infra) or by a trace

TABLE III

Composition of the artificial culture medium used for isolation and propagation of *Nanochlorum e.*

No Component	mole/l	Concentration factor compared to sea water	*No* Component	mole/l	Concentration factor compared to sea water
1 NaCl	1.1	2.3	25 $NiSO_4 \cdot 6H_2O$	$5 \cdot 10^{-7}$	4.2
2 $MgCl_2$	$1.5 \cdot 10^{-1}$	2.8	26 $BaCl_2$	$5 \cdot 10^{-7}$	3.3
3 Na_2SO_4	$8.8 \cdot 10^{-2}$	3.1	27 KI	$5 \cdot 10^{-7}$	1.1
4 KNO_3	$3.3 \cdot 10^{-2}$	6.9	28 SeO_2	$1 \cdot 10^{-7}$	$9.1 \cdot 10$
5 KCl	$2.2 \cdot 10^{-2}$	2.2	29 $TiOSO_4 \cdot H_2O$	$1 \cdot 10^{-7}$	5.0
6 $CaCl_2$	$2.2 \cdot 10^{-2}$	2.2	30 $ZrOCl_2 \cdot 8H_2O$	$1 \cdot 10^{-7}$	3.0
7 KH_2PO_4	$1.1 \cdot 10^{-3}$	$3.8 \cdot 10^2$	31 As_2O_2	$1 \cdot 10^{-7}$	2.9
8 $NaHCO_3$	$1.1 \cdot 10^{-3}$	2.4	32 V_2O_5	$1 \cdot 10^{-7}$	2.6
9 H_3BO_3	$1.0 \cdot 10^{-3}$	2.4	33 $Ce(SO_4)_2 \cdot 4H_2O$	$5 \cdot 10^{-8}$	$7.1 \cdot 10^3$
10 KBr	$8 \cdot 10^{-4}$	0.95	34 $Y(NO_3)_3$	$5 \cdot 10^{-8}$	$4.6 \cdot 10^3$
11 LiCl	$5.0 \cdot 10^{-4}$	$2.0 \cdot 10$	35 $La(NO_3)_3$	$5 \cdot 10^{-8}$	$2.3 \cdot 10^3$
12 $Sr(NO_3)_2$	$5.0 \cdot 10^{-4}$	5.6	36 $Be(NO_3)_2 \cdot 4H_2O$	$5 \cdot 10^{-8}$	$7.1 \cdot 10^2$
13 Humic Subst.	$1.0 \cdot 10^{-4}$	$1.5 \cdot 10^2$	37 $Sc(NO_3)_3$	$5 \cdot 10^{-8}$	$5.6 \cdot 10^2$
14 NaF	$1.0 \cdot 10^{-4}$	1.5	38 $BiONO_3$	$5 \cdot 10^{-8}$	$5.0 \cdot 10^2$
15 Rb_2SO_4	$5.0 \cdot 10^{-5}$	$3.6 \cdot 10$	39 $TlNO_3$	$5 \cdot 10^{-8}$	$1.0 \cdot 10^2$
16 EDTA	$5 \cdot 10^{-5}$	—	40 $WO_3 \cdot H_2O$	$5 \cdot 10^{-8}$	$9.3 \cdot 10$
17 $FeSO_4$	$1.5 \cdot 10^{-5}$	$2.8 \cdot 10^2$	41 $HgCl_2$	$5 \cdot 10^{-8}$	$5.0 \cdot 10$
18 $MnCl_2 \cdot 4H_2O$	$5 \cdot 10^{-6}$	$1.4 \cdot 10^2$	42 PbOAc	$5 \cdot 10^{-8}$	$3.4 \cdot 10$
19 Ga_2O_3	$5 \cdot 10^{-6}$	1.2	43 CsCl	$5 \cdot 10^{-8}$	$2.5 \cdot 10$
20 $ZnSO_4$	$1 \cdot 10^{-6}$	6.7	44 $CdSO_4$	$5 \cdot 10^{-8}$	$2.2 \cdot 10$
21 MoO_3	$1 \cdot 10^{-6}$	$1 \cdot 10$	45 $SbCl_3$	$5 \cdot 10^{-8}$	$2.0 \cdot 10$
22 $Al_2(SO_4)_3$	$5 \cdot 10^{-7}$	$1.3 \cdot 10$	46 $AgNO_3$	$5 \cdot 10^{-8}$	$1.8 \cdot 10$
23 $Co(NO_3)_2 \cdot 6H_2O$	$5 \cdot 10^{-7}$	$7.4 \cdot 10$	47 UO_2Ac	$5 \cdot 10^{-8}$	3.9
24 $CuSO_4 \cdot 5H_2O$	$5 \cdot 10^{-7}$	$1.1 \cdot 10$	48 $KTeO(OH)_5H_2O$	$5 \cdot 10^{-8}$	–

element mixture that approximated its elementary composition to yield the solution of Table III.

The 250 ml suspension, in 500 ml Erlenmeyer flasks were shaken with 2 strokes per second, at an illumination of 800 lx, 22 °C for 6 weeks.

2.2. Preparation of culture fluid

The mixture in its final form (Table III) was composed of several stock solutions. Stock a: components 1–5, Stock b: component 6, Stock c: 7–12. Stock d: component 13, for which 1 kg of dry peat moss (Floratorf) + 30 g Kieselgel 'Silica' (Woelm, Eschwege, F.R.G.) were suspended in 3 l of distilled water and kept at 37 °C for 24 hr. It was then centrifuged and to the clear supernatant ethylene-diamine-tetraacetate-disodium (EDTA) was added. It was divided into two portions. In one of them the components 26–48 were dissolved respectively, the components, 14, 15 and 17–24 in the other, each one at 1000 fold concentration of the final culture medium. Component 25 was added to the final solution separately. The culture medium in its final composition is heated in

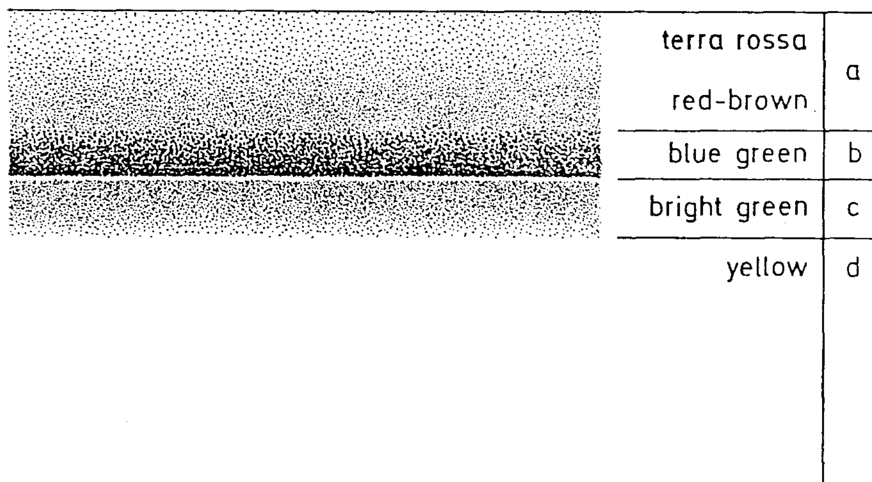


Fig. 1. Location of *Nanochlorum eucaryotum*. The sketch gives a view onto the wall of a crack of calcium carbonate rock from Colona. The upper heavy line corresponds to the rock's surface, from there on spreads a 1 cm wide red brown zone (a) coloured by fine soil particles (terra rossa) followed by a dark blue green band (b) inhabitated by cyanobacteria. Below this follows a bright green, 4 mm wide band, from which *Nanochlorum e.* could be isolated. Below this follows a faint yellow band, about 1.3 cm wide and then fresh fracture faces, emerge.

an autoclave to 110 °C for 30 min. On cooling turbidity arises, which however soon disappears when inoculated with more than 10^4 cells per ml.

For the first successful isolation experiments a 'terra rossa' extract has been used to supplement the artificial sea water made of the components 1–11, 13, and 14. For this purpose 1 kg of dried deposit from the mouth of a spring time fresh water well was extracted with 1 l of 6 M HCl in a roller bottle over night at 25 °C. It was centrifuged until clear and the supernatant evaporated under vacuum at 50 °C until a dry residue was formed. This was suspended as a fine slurry in 1 l of solution of components 12 and 15 and kept on a roller for a day, while it almost dissolved. Upon standing over night the supernatant was decanted and used instead of components 17–46 in a concentration of 1 ml per 1 l of solution with components 1–11, 13, and 14.

2.3. Continuous culture

5 l flasks with conical bottoms containing 4 l of solution (Table III) were inoculated with the content of a test tube, containing a cell clone and gassed with compressed air at 400 l per hr at 22 °C. The vessel was illuminated at 1000 lx and sterile solution (Table III) was pumped continuously into the 5 l flask at 2 l per day in such a way that the volume surpassing 4 l is driven out into a receiving bottle and harvested.

Whenever large amounts of cells are needed, air gassing is avoided and the cultures are gassed with natural gas from the city line (Table IV). Under these conditions the cells grow at their highest doubling rate and density. Further implications of this are still under investigation.

TABLE IV

Analysis of the natural gas used[a]

	Volume per cent
CH_4	84.70
N_2	5.60
C_2H_6	5.50
CO_2	1.86
C_3H_8	1.66
C_4H_{10}	0.53
C_5H_{12}	0.10
C_6H_{14}	0.03
C_7H_{16}	0.01
C_8H_{18}	0.01
C_6H_6	0.01

[a] We are grateful to the Stadtwerke Mainz AG for their analysis.

2.4. Immunological identification

For this white, male NMRI mice were injected with 50 mg or about 10^{10} of *Nanochlorum* cells, washed in 0.2 ml of saline solution.

Ten days later the mice were killed, their spleens removed, and the immunocytes liberated by gentle homogenization. The putative *Nanochlorum* cells, were added in some assays, and authentical *Nanochlorum eucaryotum (Mainz II)* cells, or *Chlorella* cells in others. After 2 hr incubation at 4 °C the rosettes were counted at a multiplicity of 1 spleen cell each for more than 10 *Nanochlorum* cells (Nowotny, 1979). Using this method *Nanochlorum* can be distinguished from other algae, e.g. different *Chlorella* (Deutsche Algensammlung, Göttingen, F.R.G.) species, even in small volumes.

2.5. Inhibitor assays

In these experiments cells were grown in 10 ml cultures in sets of ten parallel samples in roller tubes, at 30 rpm, 1000 lx, 22 °C in 12 hr light, 12 hr dark cycles with daily changes of culture fluid. Cell numbers were evaluated as turbidity difference of the growing culture and its supernatant after centrifugation at 1000 g for 10 min at 22 °C. The instrument was calibrated weekly for cell number correlation. Statistical significance evaluation was with the non-parametric X-test (Van der Waerden and Nievergelt, 1976).

Agents of poor water solubility were dissolved in 10 μl of dimethylsulfoxide (DMSO), which then was added to the 10 ml culture, the control receiving the additive without the inhibitor. The solutions were freshly prepared.

The experiments lasted up to 96 hr with positive values of generation times, otherwise they were stopped at 48 hr.

The cells were inspected by light microscopy after 48 hr for crude morphological changes, for contamination with alien organisms, and for precipitation.

Whenever feasible the highest possible concentration has been tested, as far solubility and the price allowed for. The active concentrations were diluted and tested until the effect has disappeared.

For the determination of intracellular inhibitor concentration, inhibition of L5178Y cell propagation has been used.

At the end of the experiment the *N. e.* cells were sedimented at 10 000 g for 20 min at 3 °C, washed with 1 volume of culture fluid (Table III), the dry sediment was suspended in 1 volume of 50% aqueous DMSO and subjected to a series of 5 cycles of freeze-thawing on dry ice and warming to 40 °C. It was then centrifuged at 15 000 g for 40 min at 25 °C. The clear supernatant was added as a concentration series to 5 roller tubes each with 3000 L5178Y mouse lymphoma cells per ml growing in suspension culture in Fischer medium with 10% horse serum. They were run in parallel to a concentration series of inhibitor added to 50% DMSO extracts of control *N.e.* cells. After an incubation time of 72 hr the cell numbers and their volume distribution were evaluated in a Coulter counter with size distribution plotter (Coulter Electronics, Hialeah, Fla., U.S.A.).

This procedure allowed for determination of intracellular inhibitor concentrations which we judge to be exact within one order of magnitude. With colchicine additional methods were applied.

^{3}H-Colchicine, spec. act. 2.5 Ci/mmole (Amersham-Buchler, Braunschweig, F.R.G.) was used. At low colchicine concentrations the intracellular level was estimated by radioactivity measurements of chloroform extracts of *N.e.* cells, where quenching is more than 90%. At $8 \cdot 10^{-6}$ mole colchicine/l in the growth medium the drug is accumulated by a factor of at least of 10 in 48 hr.

With the higher concentration at $8 \cdot 10^{-3}$ mole/l after 48 hr of incubation the *N.e.* cells were ground and extracted into chloroform, evaporated to dryness and chromatographed on HPTLC Kieselgel 60, F 254 nm plates (E. Merck, Darmstadt, F.R.G.), using the solvent system ethylacetate-isopropanol-aqueous ammonia (25%) (50 : 30 : 2 by Vol). Colchicine under these conditions appears as a spot, strongly quenching the fluorescence caused by 254 nm excitation. The colchicine quenching spot stays clear of the *N.e.* pigments. Its quenching intensity was evaluated using a TLC scanner.

2.6. Analytical methods

2.6.1. For DNA isolation *N. e.* sediment was suspended and frozen into micropellets as described recently (Zahn, 1983). These are shaken with 2 volumes of 1 mm glass beads in a tissue disintegrator (Braun, Melsungen, F.R.G.) with CO_2-cooling. The DNA was isolated and treated further (Zahn, 1983).

2.6.2. Further analytical methods were as described (Wilhelm *et al.*, 1981; Zahn, 1983).

2.6.3. For nucleosomal preparations and DNA periodicities caused by nucleosome beading we followed the recipe of Bodansky *et al.* (1979).

2.6.4. *Histones.* For histone preparations different procedures have been followed: Sonnenbichler, 1975; Monahan and Hall, 1975; Johns, 1977; Bidney and Reek, 1977; Yamada *et al.*, 1978.

For their characterization we tried several procedures, described by Luck *et al.*, 1958; von Holt and Brandt, 1977; Spring and Cole, 1977; Kays *et al.*, 1979; Certa and von Ehrenstein, 1981 (among others).

2.7. Electron microscopy

2.7.1 Preparation for spreading was as described (Zahn, 1983). In some cases squash osmotic shock preparations of *N.e.* were achieved by treatment with an enzyme inhibitor mix: EDTA 2 g/l, phenylmethylsulfonylfluoride 800 mg/l, Na-tetrathionate 100 mg/l, Na-iodoacetamide 100 mg/l, Na-di-sulfite 100 mg/l, pH 8.

The cells were sedimented (1000 g, 10 min, 22 °C) and the sediment taken up with 3–10 volumes of inhibitor mix in some cases 1 : 100 diluted with water. Some cells were disrupted by osmotic shock, exposing the nuclear content for spreading on activated carbon film. After rotary shadowing with Pt/Pd at an angle of 7 degrees and/or contrasting with uranylacetate, the specimens were ready for inspection.

In some cases spreading was successful with salt-free cytochrome solution (Kleinschmidt and Zahn, 1959). Electronmicroscopic evaluation was with an Elmiskop 101 with image amplifier (Siemens, Berlin, F.R.G.).

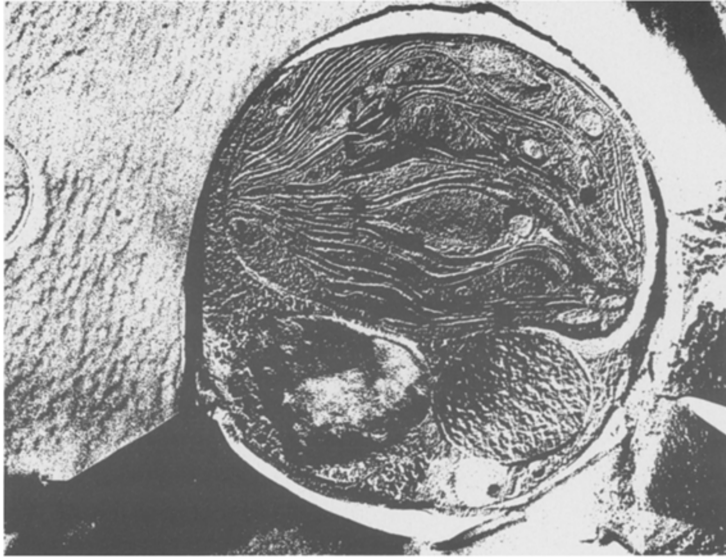
2.7.2. *Freeze fracture.* We essentially followed the procedures as described by Moor (1964; 1981). For this, *N. e.* cells were pelleted in capped Eppendorf tubes, and the dry pellet was taken up with a minimum of water, so that droplets of 50 μl could be placed on gold supports. (No glycerol was used). This was then frozen by submersion in liquid Freon 22 (Balzers, Liechtenstein) and placed into the thermostatted manipulation head of a freeze fracture apparatus, equipped with an electron gun evaporator (Balzers). After cutting with the wedge at a specimen temperature of −100 °C the specimen was etched in generally for 1 min. This was then coated at an angle of 45° with 2 nm of platinum, followed by a coating at 90° with 15 nm of carbon under the control of a quartz oscillator (Balzers).After disintegration of the adherent biological material by concentrated sulfuric acid, the carbon replicas were inspected with an electron microscopie at magnifications of 10 000–30 000 times.

3. Results and Discussion

A brief summary of our actual knowledge on *Nanochlorum eucaryotum*, stemming from experiments reported in this paper is given in Figure 2 and Table V.

3.1. Identity

Figure 2 gives a picture of freeze fracture through *N.e.* towards the end of its logarithmic growth phase, which shows the organism in its 'fattest' aspect. The round cell is bordered by a thin cell wall, which is partially split showing a slit. The cytoplasm can be recognized as the continuous phase between three large round compartments, showing coarse granulation and vacuoles. A Golgi apparatus can be discerned. The cellular organelles are surrounded by double membrane systems that show at some parts of their circumference. The largest body is the chloroplast with thylakoid bands,



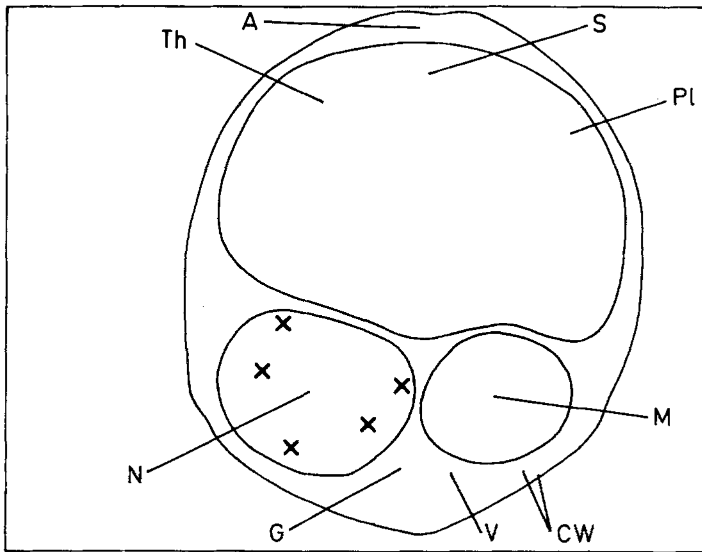


Fig. 2. Freeze fracture of *Nanochlorum eucaryotum* giving essential details of a cell of 1.4 µm × 1.6 µm. There is an artifactual gap (A) around the cell splitting the thin cell wall (CW) (30 nm). The largest subcellular body (about 60% of total cell volume (TCV)) is the plastid with thylakoid bands (Th). Between the thylakoids starch grana (S) and plastoglobuli (P1) are found in cells approaching the end of the logarithmic phase. The second largest body is the always nearly round nucleus (N, 21% TCV) with some nuclear pores (x), which partially has been stripped down to and across its nuclear membranes. The mitochondrion (M, 12% TCV) which suffered fracture across its inner structure, is always nearly round. The Golgi region (G), mostly with vacuoles (v), is sometimes visible, (Shadowing was from lower left corner).

TABLE V

Additional features of *Nanochlorum eucaryotum*

1.	Re-isolation from nature:	a morphologically, physiologically and immunologically similar organism;
2.	DNA base ratio:	$\frac{G + C}{(A + T) + (G + C)} \times 100 = 47.6 \pm 0.03\%$;
3.	5-methyl-cytosine content:	1 mole/100 mole bases;
4.	DNA superstructures:	only smooth and sometimes intertwined, filamentous structures have been isolated; nucleosomes are absent; no periodicities have been observed upon nuclease digestion;
5.	NO_3-reduction:	+
6.	Fe^{+++}-utilization:	+
7.	Inhibition of cell division:	(7.1) Colchicine[a,b] ($8 \cdot 10^{-6} - 8 \cdot 10^{-3}$ mole/l): no inhibition;
		(7.2) Colcemid ($8 \cdot 10^{-8} - 10^{-5}$ mole/l): no inhibition[b];
		(7.3) Trifluralin ($10^{-5} - 3 \cdot 10^{-4}$ mole/l): no inhibition[b];
		(7.4) Cytochalasin B ($2 \cdot 10^{-7} - 2 \cdot 10^{-5}$ mole/l): no inhibition[b]:
		(7.5) Azide ($1.5 \cdot 10^{-5} - 4.5 \cdot 10^{-4}$ mole/1): inhibition;
		(7.6) Diethylpyrocarbonate ($2 \cdot 10^{-6} - 6 \cdot 10^{-8}$ mole/l): inhibition:
		(7.7) Chloramphenicol ($3 \cdot 10^{-5} - 3 \cdot 10^{-3}$ mole/l): inhibition;
		(7.8) Cycloheximide (10^{-7}–10^{-4} mole/l): inhibition;

[a] Intracellular concentration determined by chromatography.
[b] Intracellular concentration determined by testing cell culture inhibition.

starch granules and plastoglobuli. A pyrenoid is lacking. The mitochondrion has fractured near its equator, exposing a coarse inner granulation. The etching procedure makes its envelope structure, and especially its close relationship to the chloroplast visible. This has been seen in all cases, whereas a close neighbourhood to the nucleus is not always observed. The nucleus, in most cases round, seems to be deformed. This is

caused by incomplete ablation of surrounding material and of its outer membrane system. Five large nucleopores are visible.

The *N. e.* cells are tough, with their small radius of curvature hard to fracture. They are not staying in continuity with the embedding matrix.

From the morphological aspect the cells isolated from the Colona stone do not show any deviation from *N. e.* from Mainz. They have to be considered as morphologically identical.

The same holds for the immunological assay. The *N. e.* from Mainz and from Colona gave clear 'rosetting' (Nowotny, 1979) with relatively few large immunocytes, each one of them surrounded by a string of *N.e.* of more than 10 in one plane, while with *Chlorella pyrenoidosa*, *Chlorella fusca*, *Chlorella ellipsoidea* and *Chlorella vulgaris* no such aggregation can be seen. Some caution must be used however, when this technique is applied to cells as small as *N. e.* Since they do not adhere as strongly as larger animal cells do, vigorous shaking has to be avoided.

3.2. Histones

Since we suspected *N.e.* belongs to a group of organisms that never during their evolution had acquired all the features of full eukaryotism, we were interested in studying proteins which, throughout all taxa of eukaryotesare considered to be highly conserved i.e. histones (Isenberg, 1979). The enormous wealth of literature is rather scarce when it comes to small eukaryotes, the eukaryotic microbes (Horgen and Silver, 1978). Quite clearly, dinoflagellates like *Gymnodinium* do not have histones. Their basic nuclear, acid-extractable proteins differ substantially from histones; One consequence of this is that upon spreading of the DNA containing structures no 'beading' or DNA molecular weight clustering, typical for the higher eukaryotes, can be detected (Rizzo and Burghardt, 1982). In *Chlorophytes*, like *Chlorella ellipsoidea* and *Volvox*, the 'histones', in the form of acid-soluble proteins, proved to be quite different from calf-thymus histones. The same apparently holds for members of the order *Rhodymeniales* (Horgen and Silver, 1978).

This is perfectly in line with our intensive, fruitless searches for histones in *N.e.*. (Table V) Other eukaryotes considered to represent lower taxa, e.g. *Saccharomyces*, contain the highly conserved histones however (Isenberg, 1979). This absence of histones as in *N.e.* may thus become an important point in our understanding of eukaryotic evolution.

3.3. Inhibitors (Table V)

Azide was tested, since it is used as a sterilizing agent. It is a strong inhibitor of ATPase and a series of other enzymes at a range comparable to the one used for *N.e.* (Jain, 1982).

Diethylpyrocarbonate, another sterilizing agent, known to form amide bonds in proteins between free amino and carboxyl groups, is inhibitory in a range to be expected from the literature (Jain, 1982).

For the two drugs chloramphenicol and cycloheximide reciprocal inhibition was

shown. The inhibition of protein synthesis by chloramphenicol in bacteria, 'blue-green algae', and in mitochondria and chloroplasts has been documented, and was absent in the cytoplasm of eukaryotes such as fungi, green algae, higher plants and mammals. The reverse has been reported to be true for cycloheximide. This is the consequence of their action on ribosomes, the chloramphenicol-sensitive 70 S procaryotic ribosomes being insensitive to cycloheximide, the 80 S cytoplasmic eukaryotic ribosomes being cycloheximide-sensitive and chloramphenicol-insensitive (Smillie and Scott, 1969; Jain, 1982).

In *N.e.* the amount of cytoplasm is relatively small, so that not many reserve substances and precursors seem to be present, making the macromolecular syntheses dependent on the metabolic status of the cellular organelles, especially of the large chloroplast. Thus is, besides a high sensitivity towards cycloheximide acting on the eukaryotic ribosomes in the cytoplasm, there is an appreciable inhibition by chloramphenicol, acting presumably on the 70 S ribosomes of the chloroplast (Table V). Thus while chloramphenicol is a 20–100 times better inhibitor of typical prokaryotic division at concentrations of $3 \cdot 10^{-6} - 3 \cdot 10^{-5}$ mole/l than on eukaryotes (Otten *et al.*, 1975) the higher value is overlapping with the minimal range in *N.e.* (Jain, 1982). In this context it will be interesting to find out, what the sensitivity will be under heterotrophic conditions.

Cytochalasin B, a drug that is known to inhibit intracellular motility phenomena (Dustin, 1978) at concentrations around or below $3 \cdot 10^{-5}$ mole/l (Jain, 1982) does not show measurable effects. Since determination of intracellular concentration may be inexact, not too much weight can be given to this observation however. For future experiments this introduces the interesting question of how an apparently marginal eukaryote solves its problems of shifting of subcellular structures.

3.4. Mitotic apparatus

This is considered as a leading marker in taxonomic evolution and has been incorporated as such into working hypotheses (e.g. Heath, 1980; Margulis, 1981). A frequently used poison for the synthesis of the mitotic apparatus and its function is colchicine. By reaction with one binding site (Bryan, 1972) of the tubulin subunits colchicine prevents their assembly into microtubules. This is the basis of its poisonous action, and leads to mitotic arrest (Dustin, 1978).

In *N.e.*, under conditions of apparently normal growth and propagation, colchicine is without significant effect, even at high concentrations. Therefore the question may be asked whether colchicine is able to enter the cells under the given conditions. At low concentrations in the culture fluid ($8 \cdot 10^{-6}$ mole/l) the drug is accumulated by a factor of at least 10 in the course of 48 hr, as measured with radiolabelled alkaloid. At the higher concentrations ($8 \cdot 10^{-2}$ mole/l) with the chromatographic method it could be shown that the outside concentration had been reached. Upon longer incubation periods, cells die, while thin flat, featherylike crystals appear occasionally at the outer cellular circumference, below the cell wall (Bauer and Zahn, unpublished). This affords based on the insensitivity of the 'mitotic' apparatus towards colchicine, colcemid and

trifluralin (Table V) at the concentrations tested, and which had been found active in other taxa (Dustin, 1978; De Rosa *et al.*, 1978; Jain, 1982), this can signify that the colchicine binding site is not accessible in an otherwise functional protein or that such a protein may not be present. At the moment we cannot decide which of the two alternatives is present in *N. e.*. Investigations to clear up the matter are under way. As a working hypothesis we assume that a colchicine binding protein is present in *N. e.* giving rise to crystal formation. Whether such crystals are caused by a microtubular derivative which is not in the doublet form, corresponding to those of the marginal bundle of thrombocytes and of nucleated erythrocytes (Behnke and Forer, 1967; Behnke, 1970) is at the moment an open question.

We think it to be unlikely that a mitotic apparatus with typically active proteins like the tubulins of higher eukaryotes exists, since in such a case they ought to be inhibited by the colchicine treatment. As already shown in Table II and as substantiated by our further observations chromosome formation, dissolution of nuclear envelope, nor formation of fibrillar structures within, nor outside of the nucleus never have been observed. The deviations from the fully eukaryotic state in the mitotic apparatus and its function in *N. e.* establishes that this organism belongs to a taxon with marginal eukaryotic features, features that deserve further experimental efforts in order to be understood.

In this context the apparent absence of typical histones along with the missing nucleosome architecture may lead to the anticipation of possible alterations in genomic distribution and reshuffling in mitosis. Here the term 'mitosis' is to be considered in its wider sense (Dustin, 1978). This among other considerations raises the question about the fidelity of marker transmission in such organisms, e.g. if the replicated genomes 'attached to the nuclear envelope at a differentiated site" (Pickett-Heaps, 1974).

4. Conclusion

The possible total lack of histones, along with the apparent deviations in the protein properties and morphology of the mitotic apparatus indicate that *Nanochlorum eucaryotum* should be classified as a marginal eukaryote. An organism, closely resembling or identical with *N. e.* has been isolated from the Northern Adriatic Sea at Istria, Yugoslavia.

Investigations of marginal eukaryotic state with *N. e.* as model organism are expected to yield meaningful results with explanatory potential for our understanding of eukaryotic phylogeny.

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