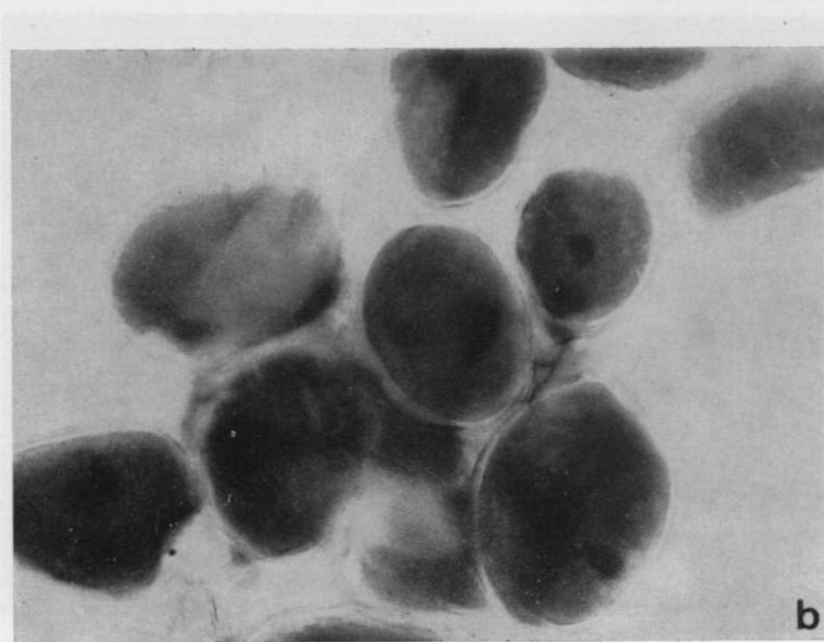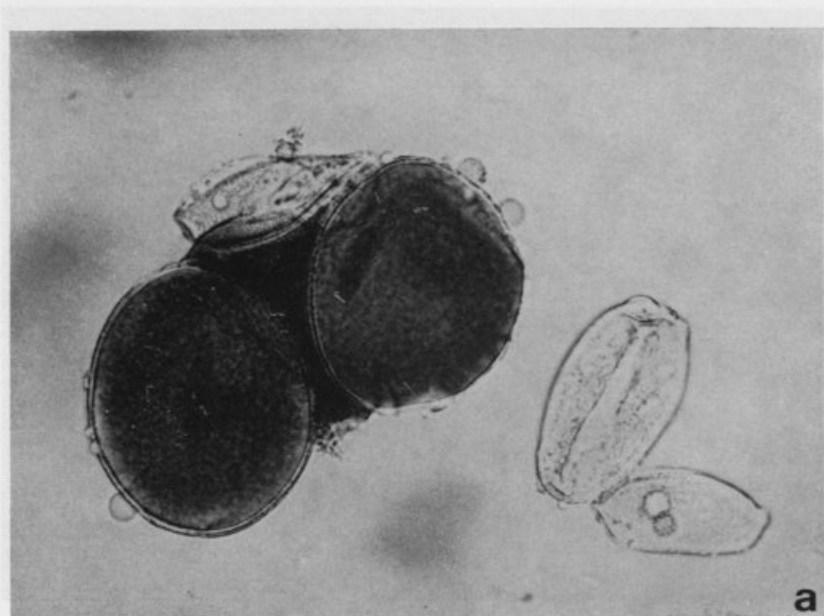Population IV — Route Guarda-Vilar Formoso,
près du croisement vers Cerdeira.

Plante C, fleur B (jaune)

a — Grains à n = 7 et à n = 14. × 900.
b — Grain à n = 21. × 900.

Population IV — Route Guarda-Vilar Formoso, près
du croisement de la route vers Cerdeira.

Plante C, fleur B (jaune)

a — Triade à grains géants et grains stériles. × 900.
b — Grains de plusieurs dimensions (7, 14 et 21). × 900.

ULTRASTRUCTURAL AND STEREOLOGIC STUDY OF *CRICOSPHAERA CARTERAE* (PRYMNESIOPHYCEAE) FOLLOWING EXPOSURE TO MONENSIN

I. TREATMENT OF 3-4 WEEKS OLD CULTURES

by

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ABSTRACT

3-4 weeks old cultures of *Cricosphaera carterae* (a marine coccolithophorid), maintained in a climatized chamber (12/12 light/dark cycle, 3000 lux of cool white fluorescent light, 16-18° C) were aditioned with monensin (10^{-5}M). After the treatment (24 h) these cells and those of the control were fixed in glut./OsO4 and prepared for E. M. by the current technique. The aim of this study was to compare the relative volume of different intracellular compartments of treated cells with the control, for what the Weibel method and Student t test were used. At the level of the nucleus, mitochondria, chloroplasts and E. R. significant volumetric differences were not found, although some structural alterations of the photosynthetic apparatus could be rarely seen. In this alga, the secretory system responsible for the scales and coccolith production is very complex, including Golgi cisternae and numerous vesicles, vacuoles and intracellular coccolith precursors (ICP). So, his delimitation for morphometric study is very difficult. However, in our experimental conditions, the effects on Golgi area are not evident too and, generally, we can say that the secretory system of this alga is insensible or very little sensible to a monensin treatment.

INTRODUCTION

MONENSIN is a lipophilic carrier (ionophore) for monovalent cations, specially Na+ (GEISOW and BURGOYNE, 1982; BOSO and col., 1984) which has been frequently used for analysis of secretory processes in animal (LEDGER and col., 1980; TARTAKOFF

and VASSALLI, 1978; MORRÉ and col., 1985) and plant cells (MOLLENHAUER and col., 1982, 1983; MORRÉ and col., 1983; BOSS and col., 1984; SHANNON and STEER, 1984; DOMORZYCH and col., 1985), because their effects upon the Golgi apparatus.

The most frequent structural alteration consists of a swelling of Golgi vesicles and cisternae namely at the maturing face of the dictyosomes (MOLLENHAUER and col., 1982, 1983; MORRÉ and col., 1983, 1985; ROBINSON, 1981) through a mechanism envolving the maintenance of a proton gradient (GEISOW and BURGOYNE, 1982; BOSS and col., 1984; MORRÉ and col., 1985). Furthermore, SCHNEPF (1983), in caulonema cells of *Funaria hygrometrica,* has also observed a slight dilation of the mitochondrial cristae and swelling of thylakoids, the last depending on exposure to the light. Since proton pumps operate at different levels in the cell, we have thought, according to the suggestion of other Authors (GEISOW and BURGOYNE, 1982; SCHNEPF, 1983) that will be interesting to quantify changes of the volume of intracellular compartments in plant cells following a monensin treatment.

Up to date, these experimental studies have been carried out on typical secretory systems, such us, the root cape of maize (MOLLENHAUER and col., 1982; SHANNON and STEER, 1984), on tissue cultures (MORRÉ and col., 1983, 1985), and more rarely, on other materials (SCHNEPF, 1983).

As to the Algae, similar studies are confined to an Euglenoid (MOLLENHAUER and col., 1983) and a green alga (DOMORZICH and col., 1985), according to we know in the literature.

So, we have shoosen *Cricosphaera carterae* (golden alga) for these experimental studies, because this marine coccolithophorid has a complex secretory system which is responsible for the production of the components of his cell covering (scales and coccoliths) (PIENAAR, 1969).

MATERIAL AND METHODS

The culture of *Cricosphaera carterae* was obtained from the culture collection of algae, University of Texas at Austin (culture L. B. 1014) and maintained in the following conditions: 12/12 light/dark/cycle; 3000 lux of cool white fluorescent light; temperature of 16-18° C; composition of culture medium, as indicated in the Table I.

Stock solutions of monensin (Sigma) were prepared by dissolving an adequate amount of this substance in 1 c. c. of 100 % ethanol and diluting with destilled water. The experiences were carried out with cultures growing in the above referred conditions and aditioned with the monensin solution in order to obtain the final concentration of $10^{-5}M$. As controls, we have used cultures containing or not ethyl alcool (never exceeding 1%) and growing in the same conditions.

Samples were periodically harvested (from 10 min. to 72 hour) but, for this quantitative study, we have shoosen a treatment of 24 h.

TABLE I

Composition of the culture medium
(Erdschreiber solution)

Filtred seawater	1000 ml
Soil extract	50 ml
NaNO₃	0.2 g
Na₂HPO₄12H₂O	0.03 g
Vitamin B₁₂	1 ml (15 μg/100 ml)

Following a slight centrifugation to concentrate the cells, these were fixed at room temperature for 1-2 h, through the substitution of the culture medium by the fixative solution (3 % glutaraldehyde in 0.2M sodium cacodylate buffer at pH 7.5 with 0.01M CaCl2 and 0.25M saccharose). After several washings in the same buffer and posd-fixation in 1% osmium tetroxide, the cells were treated according the current technique for electron microscopy (MESQUITA and FÁTIMA SANTOS, 1976a, b).

For quantitative studies which concern 5 cultures, ultrathin sections from two blocks per culture were obtained and 12 micrographs were taken at random on them. So, 60 micropraphs with the final magnification of × 18 000 from monensin treated cells and the same number from controls were analysed. The relative volumes of organelles and other intracellular compartments were calculated by the Weibel method (WEIBEL, 1973; STEER, 1981), for what a test area 14 × 13.5 cm was used. This grid contains 112 1 cm lines doing 224 points and the total line lenght of 112 cm.

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The comparison of the average values obtained both in the monensin treated cells and controls was accomplished by employing the Student *t* test.

RESULTS AND DISCUSSION

On suitable cell profiles two peripheric chloroplasts the E. R. of which (chloroplast E. R.) is continuous with nuclear envelope can be seen (Pl. I, II and Pl. III, figs. 1 and 2). Sometimes, this relationship between chloroplast E. R. and nuclear envelope seems to be simple (Pl. II, arrow), but, frequently, this connexion is very complexe (Pl. II, figs. 1 and 2). Then, an intricated endoplasmic reticulum network puts in continuity the perinuclear cisternae with the chloroplast E. R. (Pl. III, fig. 2, arrows). The photosynthetic apparatus of the chloroplasts is normally constituted by bands of three thylakoids, and there is no girdle band (Pls. I, II and III). Although, this number can be higher, the voluminous pyrenoids, one for each chloroplast are always crossed by paired thylakoids (Pls. I, II and III).

The mitochondria show an accentuated polymorphism (Pl. I, II, III and IV), very long profiles enveloping other organelles have been frequently seen (Pls. II, III, fig. 1, Pl. IV, fig. 1). However, we have never found light swellings in the matrix (PIENAAR, 1969) and the most evident associations which we have observed were those mitochondria-chloroplast (Pl. III, fig. 1 and Pl. IV, fig. 2) and/or mitochondria-nucleus (Pl. II and Pl. IV, fig. 1) and not mitochondria-endoplasmic reticulum (PIENAAR, 1969).

The single and polarized Golgi body is composed by apressed plane or more or less curled cisternae (Pl. V and Pl. VI, figs. 1 and 2) some of which display swellings in the central region (Pl. V, figs. 1 and 2; Pl. VI, figs. 1 and 2, arrowheads).

Our observations concerning the structure of this organelle and that of secretory system are in accordance to the description of PIENAAR (1969) in *Hymenomonas carterae*. So, this system, where scales and coccoliths are produced, includes, apart the Golgi body, numerous vesicles, vacuoles and intracellular coccolith precursors (ICP), this is, organelles bounded by a unit-membrane with a heterogenous, very dense and amorphous content (PIENAAR, 1969). Frequently, profiles of scales in different stages of development can be seen into these ICP (Pl. VI, fig. 3).

The ultrastructure we have just described is identical both in controls and monensin treated cells. Rarely, we have seen some chloroplasts the thylakoids of which were swollen and/or vesiculated. However, we are not sure that this alteration is an effect of the monensin because exceptionally it was also observed in control cells (artifact?).

Some uncommon ultrastructural characteristics, such as, the pronounced polymorphism of the mitochondrial profiles (including «aberrant forms») can be seen in treated (Pl. II, Pl. IV, fig. 1) or in non-treated cells (Pl. I, Pl. III, fig. 1).

As we have already referred (see Introduction), one of the most frequent alterations which have been described in monensin treated cells, is the curling, swelling and, sometimes fragmentation, of dictyosome cisternae, namely those of the *trans*-face (MOL-LENHAUER and col., 1982, 1983; DOMOZYCH, 1985). According to the experiences of MOLLENHAUER and col. (1983), on *Euglena gracilis,* there will be a «fixation effect», this is, the alteration is mediated by glutaraldehyde: the monensin weakens a structural element which maintains the flattened form of the cisternae... and these swell when exposed to glutaraldehyde (MOLLENHAUER and col., 1983).

In *Cricosphaera carterae,* it was not possible to detect alterations of the morphology of the dictyosomes which could be considered an effect of monensin. As a matter of fact, plane and apressed or strongly curled cisternae can be observed in Golgi bodies of both treated and control cells (compare Pl. IV, fig. 1 with Pl. V and Pl. VI, figs. 1, 2).

As to the swelling and/or shrinking of organelles or other cell compartments only a quantitative study can to clarify eventual volume variations, particularly whenever these are not very evident.

So, we have realized this study (see Material and Methods) and the results we have obtained showed that the relative volume of chloroplasts, mitochondria and endoplasmic reticulum in monensin treated cells does not undergo significant variations when compared with controls (see Table II).

Concerning the complex secretory system of this alga (Golgi body, vesicles and ICP), his delimitation for morphometric study is very difficult. Neverthless, in our treatment conditions (see Material and Methods), monensin does not seem to change signi-

TABLE II

Volumetric density (%) of organelles and cell compartments in monensin-treated cells and controls (not significant differences).

	NUCLEUS	MITOCHONDRIA	CHLOROPLASTS	E. R.	GOLGI AREA	I. C. P.
Controls	12.61 ± 0.66	5.04 ± 0.26	27.11 ± 1.92	2.48 ± 0.29	14.69 ± 1.68	7.36 ± 1.68
Monensin treated cells (10^{-5}M-24 h)	12.13 ± 0.89	4.62 ± 0.36	27.94 ± 0.98	2.80 ± 0.41	13.71 ± 1.17	9.80 ± 1.19

ficatively the relative volume of this cellular compartment (Table II).

About the action mechanism of monensin, it was suggested (BOSS and col., 1984), from studies with dissipator agents of chemical gradients (i. e., proton pump inhibitors), that it is necessary to maintain a proton gradient (acid, inside cell compartment) for swelling occur. Then, the monensin mediated influx of Na+ is concomitant with the issue of H+. So, the concentration of osmotically active cations inside may reach sufficiently elevated levels to determine influx of water and the correspondant swelling of the cisterna.

Furthermore, only the most mature cisternae of the Golgi body would be capable of generating a significant proton gradient (BOSS and col., 1984). In summary, according to these Authors, the swelling of Golgi apparatus induced by monensin depends on the existence of active proton pumps.

Meanwhile, SANDEAUX and col. (1988), studying the transmembrane flux of radioactive Na mediated by monensin, in different experimental conditions, have concluded that the transport is affected by the concentrations of Na+ and pH of the solutions on both sides of the membrane.

So, the composition of the culture medium (see Table I) and/or natural habitat (high concentration of Na+), in some way, can be related with the weak reaction of the secretory system of this alga to the usual monensin treatment.

Excluding the swelling of Golgi compartment, other effects can be observed in *Cricosphaera carterae,* in different treatment conditions (SANTOS DIAS and MESQUITA, 1987). So, we think that the hypothesis of impenetrability of monensin in the cells must be ruled out.

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PLATE 1

All figures concern cells of *Cricosphaera carterae*. Culture conditions are generally described in the text (see Material and Methods) and specific treatments are referred in the correspondent captions.

ABBREVIATIONS

c   — chloroplast
er — endoplasmic reticulum
g   — Golgi apparatus
ICP — intracellular coccolith precursor
m   — mitochondria
N   — nucleus
nu — nucleolus
p   — pyrenoid
v   — vacuole

General view of a control cell.
Two peripheric chloroplasts (c) each with a voluminous pyrenoid (p),
a lobuled nucleus (N), the Golgi apparatus (g) and polymorphic
mitochondrial profiles (m) stand out.

× 15 000.
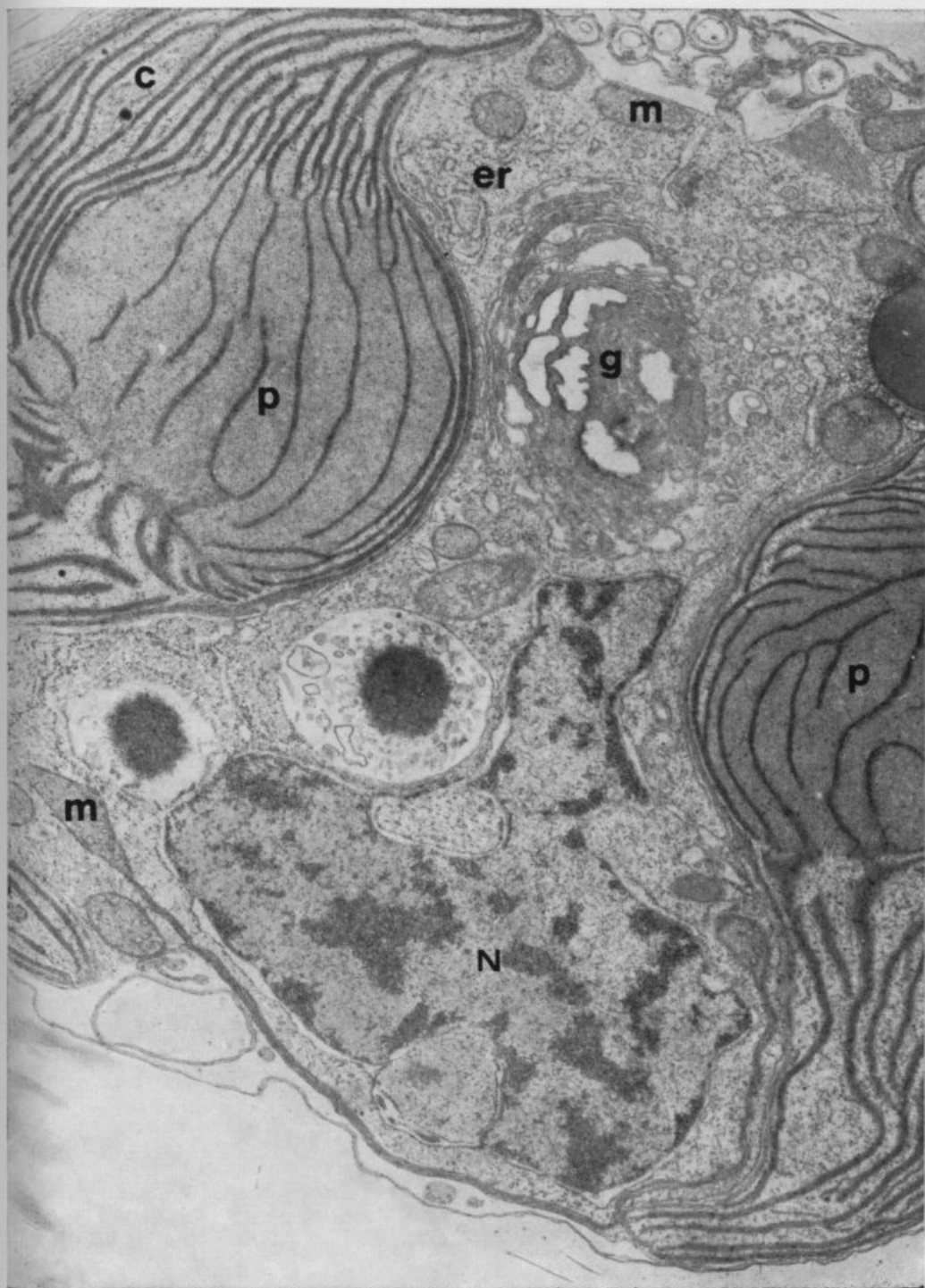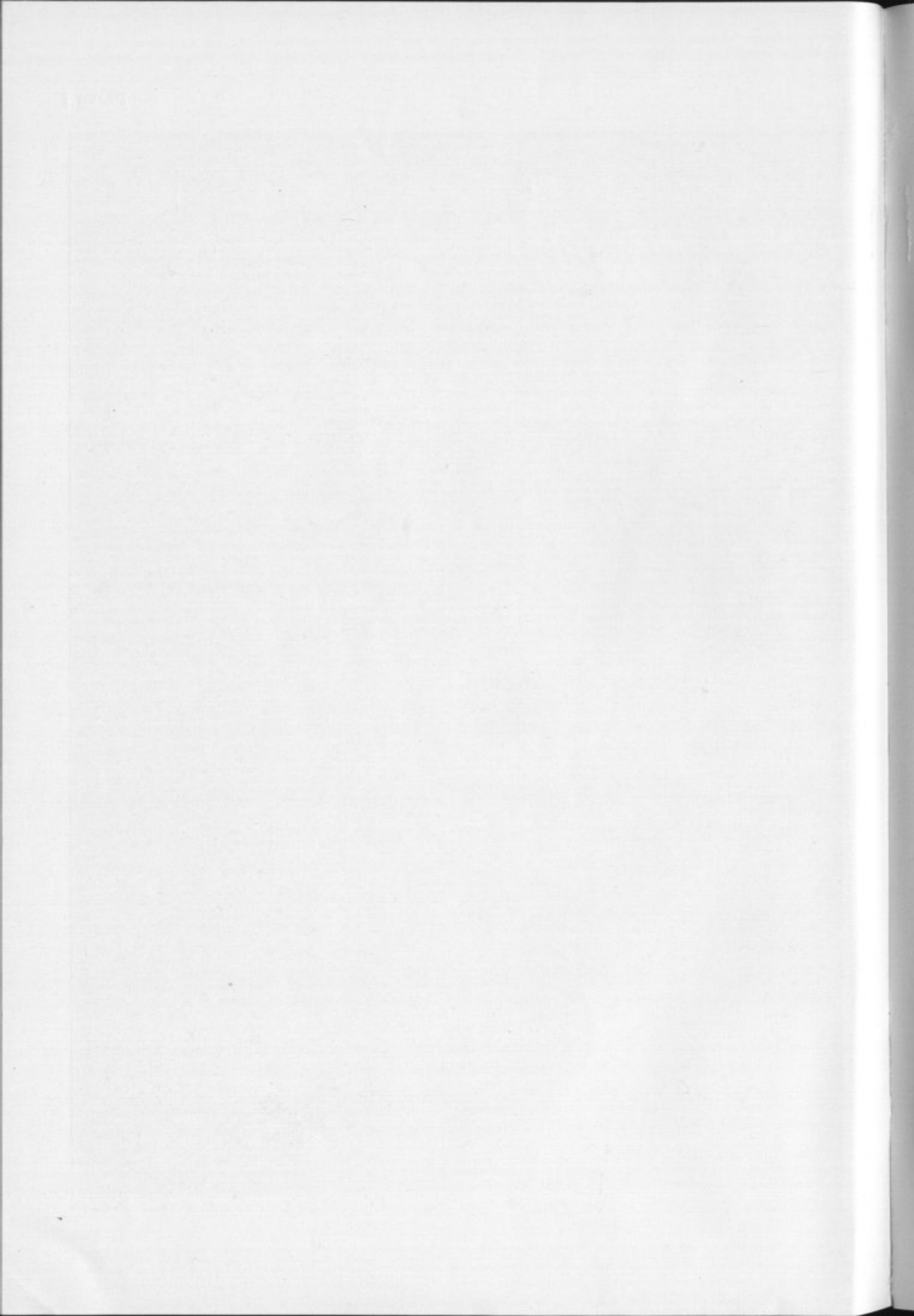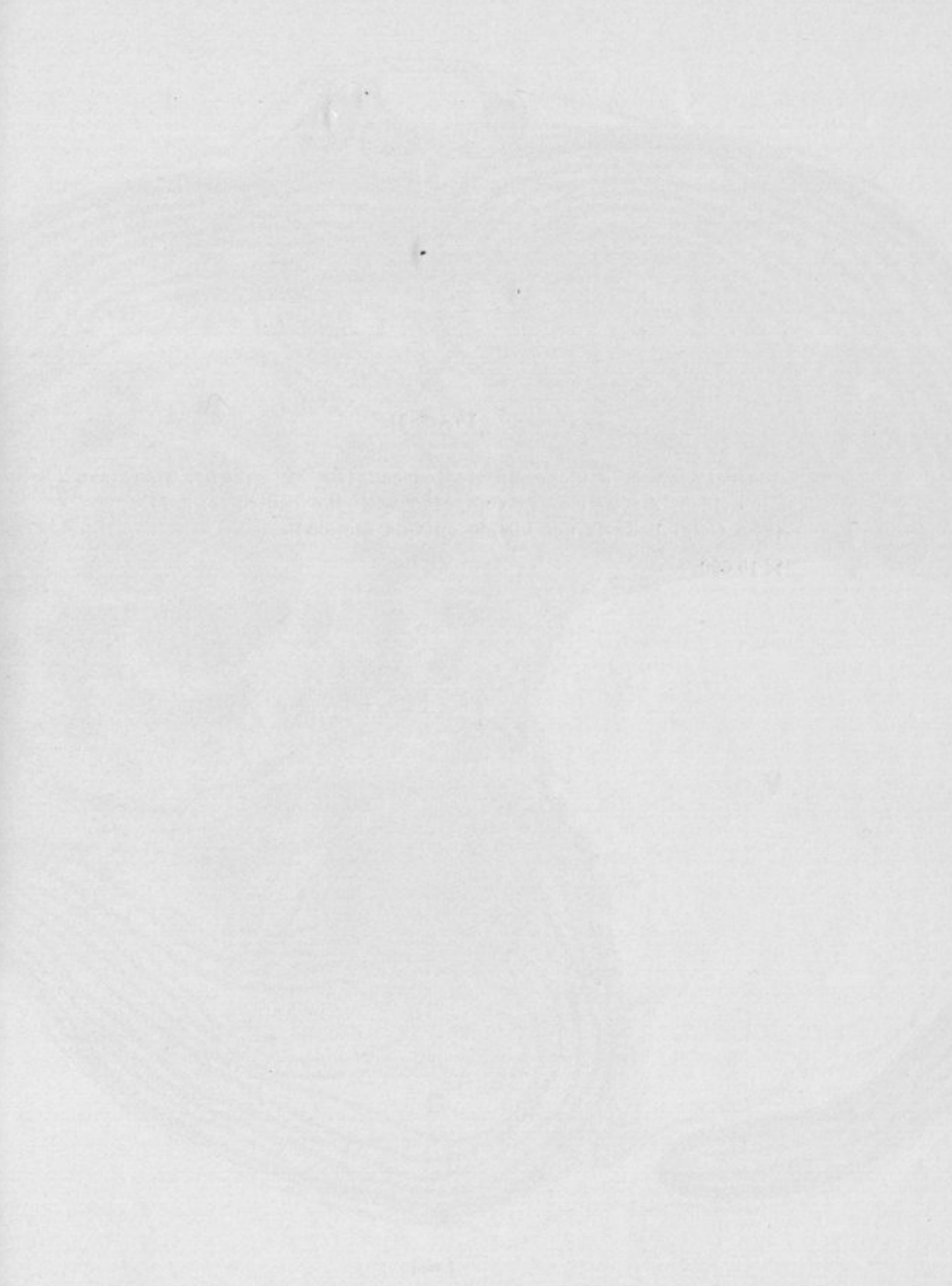
PLATE I

Plate II

PLATE II

General view of a monensin treated cell (10^{-5}M — 24 h). There are not evident differences as compared with the control (Pl. I). (The Golgi body is not visible on this section).

$\times$ 15 000.

PLATE II

PLATE III

PLATE III

Fig. 1. — Partial view of a control cell, where all organelles are represented. Remark the morphological diversity of mitochondrial profiles (m), its close association with chloroplasts (c) and the complex endoplasmic reticulum (e. r.).

× 13 200.

Fig. 2. — Detail of a complicated network of endoplasmic reticulum which puts in direct continuity the perinuclear cisterna with the chloroplast E. R. (arrows). The structure of the photosynthetic apparatus and paired thylakoids crossing the pyrenoid can also be observed.
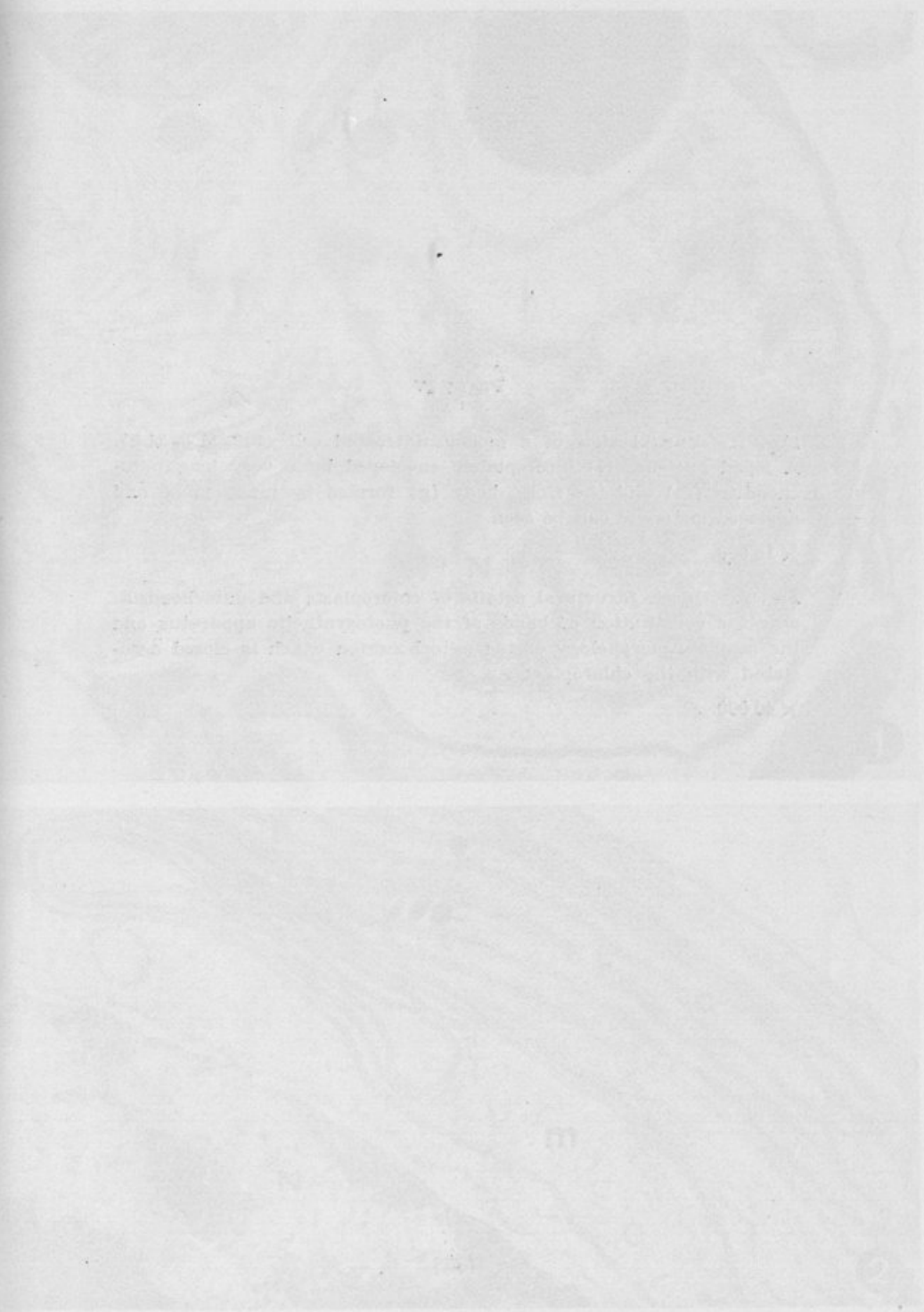
× 26 400.
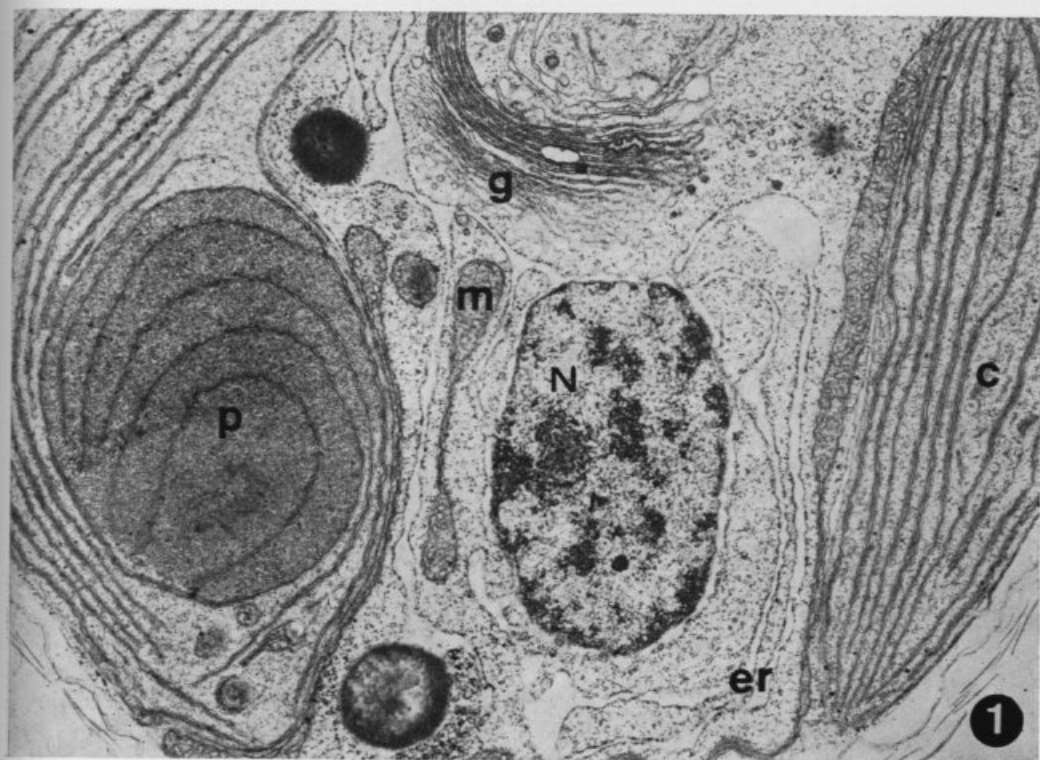
PLATE III

PLATE IV

Fig. 1. — Partial view of a monensin treated cell (10^{-5}M — 24 h). A lobed nucleus (N) incompletely enveloped by a very long mito-chondria (m) and the Golgi body (g) formed by many plane and apressed cisternae can be seen.

$\times$ 15 000.

Fig. 2. — Idem. Structural details of chloroplasts and mitochondria: note the constitution of bands of the photosynthetic apparatus and the complex morphology of the mitochondrion which is closed asso-ciated with the chloroplast.
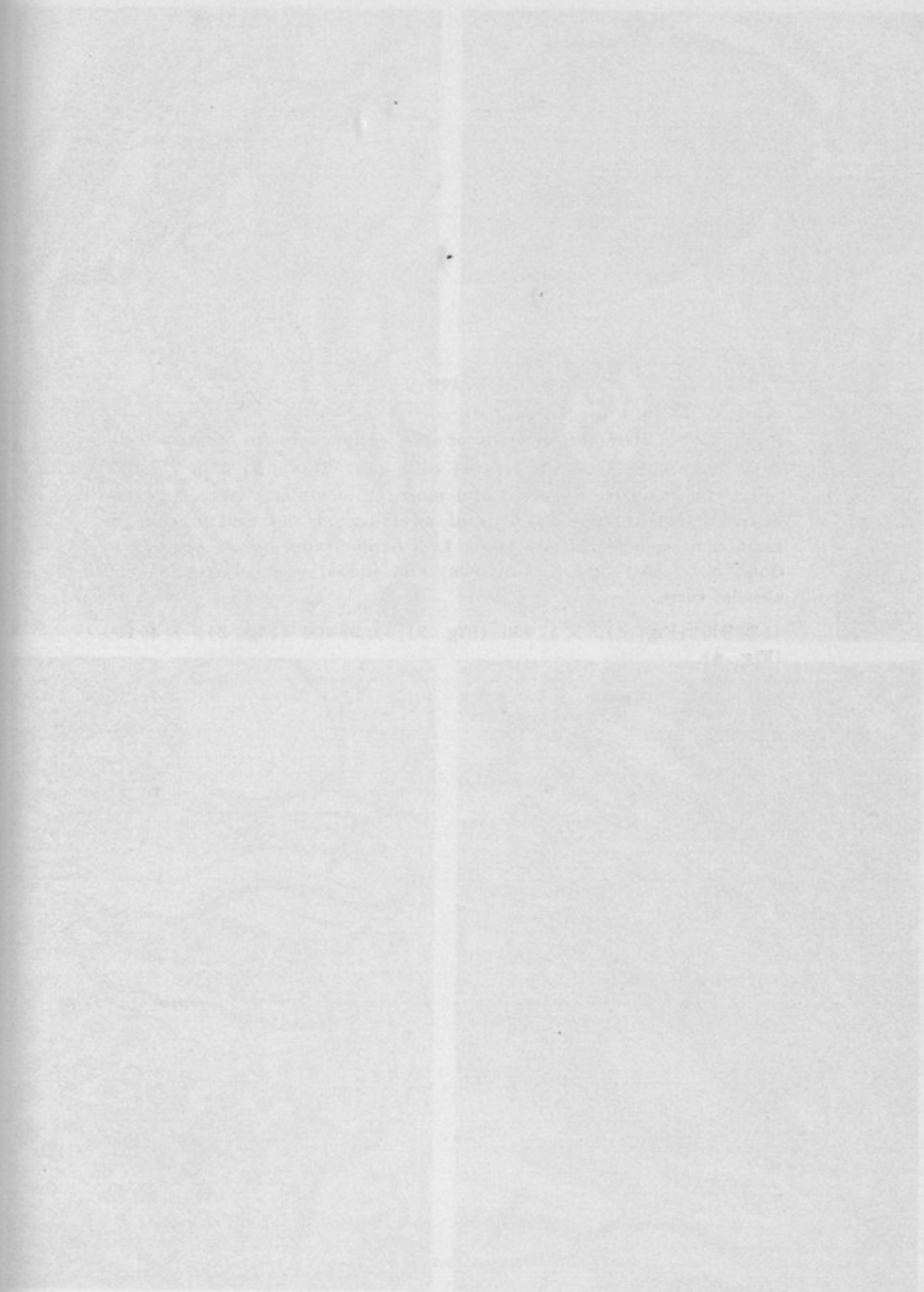
$\times$ 40 000.

PLATE IV
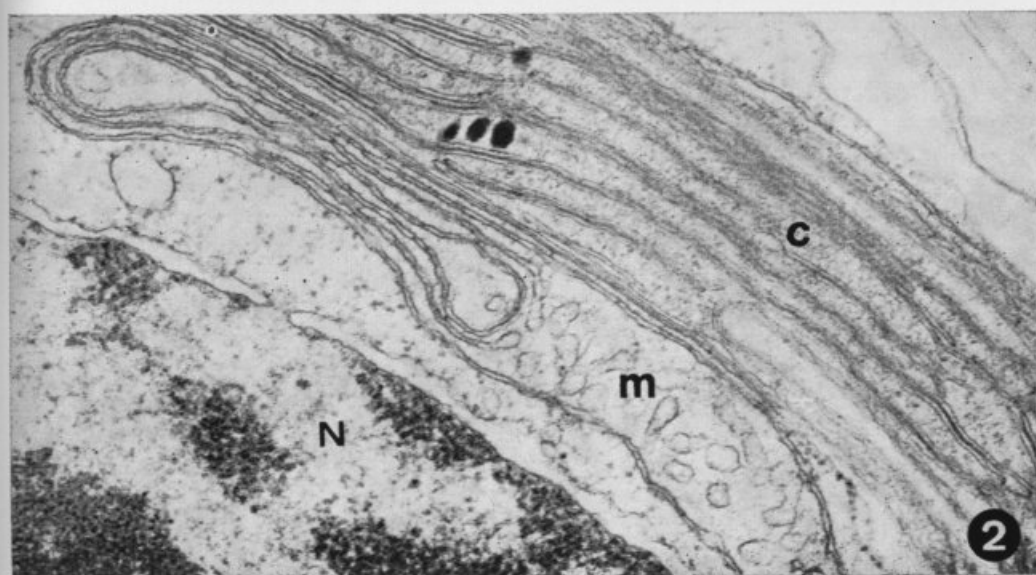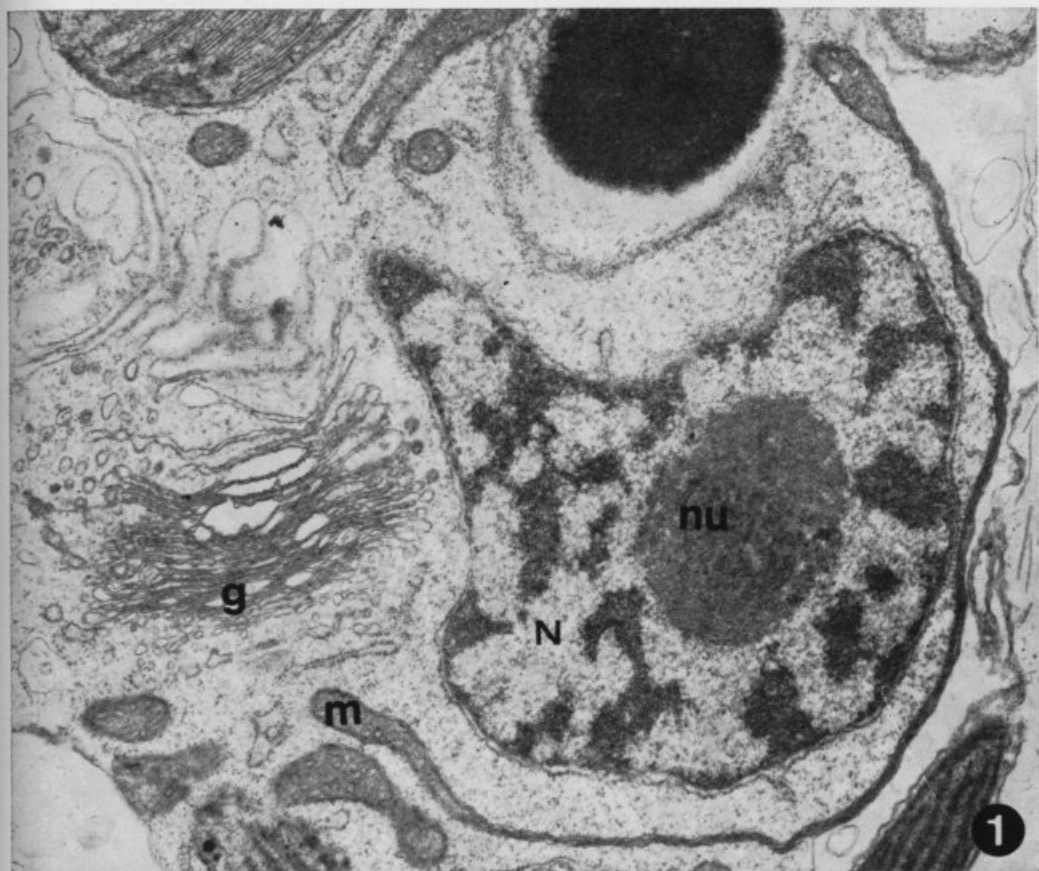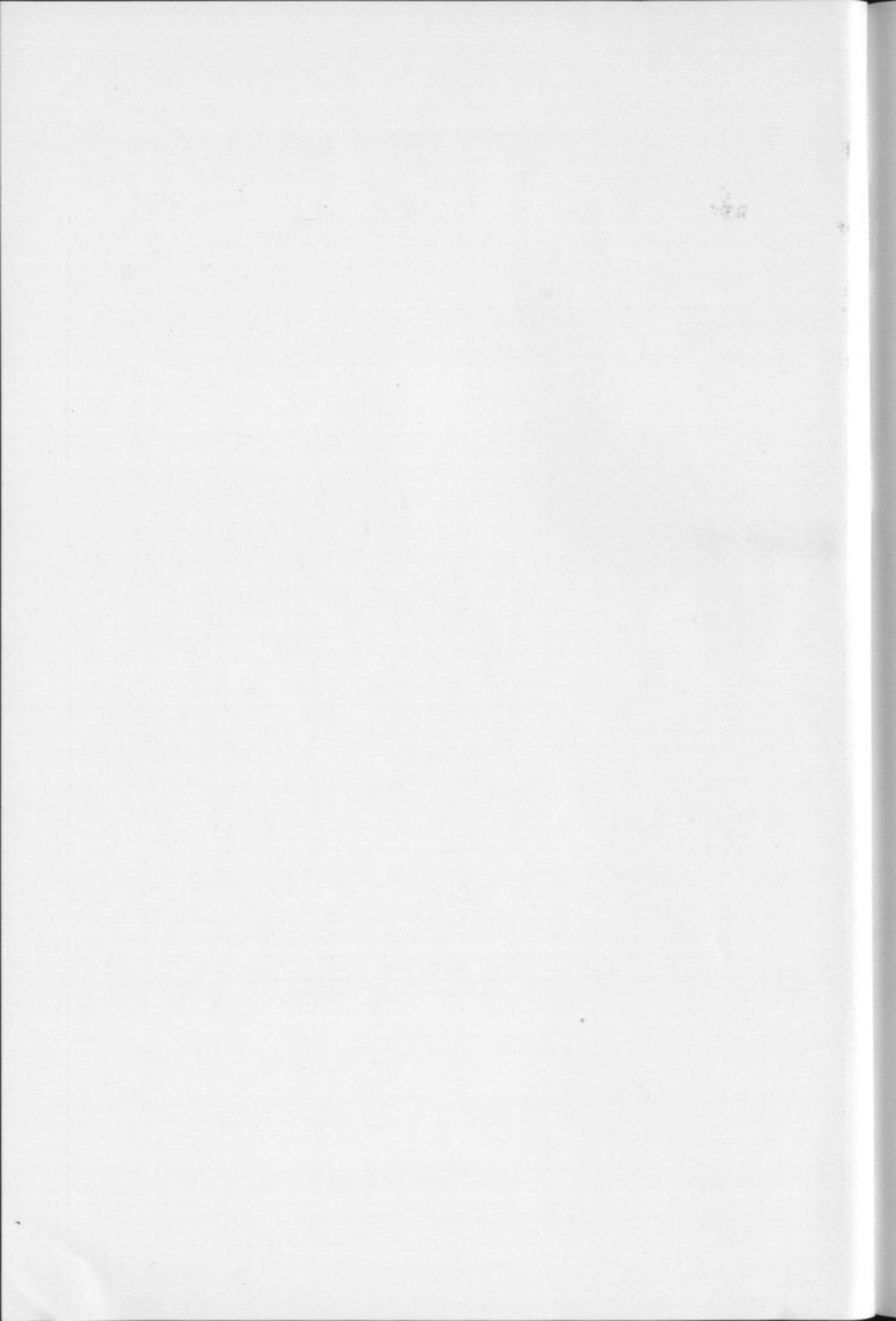
PLATE V

Figs. 1-4. — Different aspects of the Golgi area in control cells (figs. 1-3) and monensin treated cells ($10^{-5}M$ — 1 h).
Golgi cisternae are apressed and more or less plane (figs. 1, 4) or intensely curled (fig. 2). Typical swellings of the central zone of some dictyosome saccules (figs. 1, 2 arrowheads), scale profiles in Golgi cisternae (figs. 3, 4-arrows) and coated vesicles (fig. 3) can also be seen.

$\times$ 30 800 (Fig. 1); $\times$ 17 600 (Fig. 2); $\times$ 26 400 (Fig. 3); $\times$ 35 200 (Fig. 4).
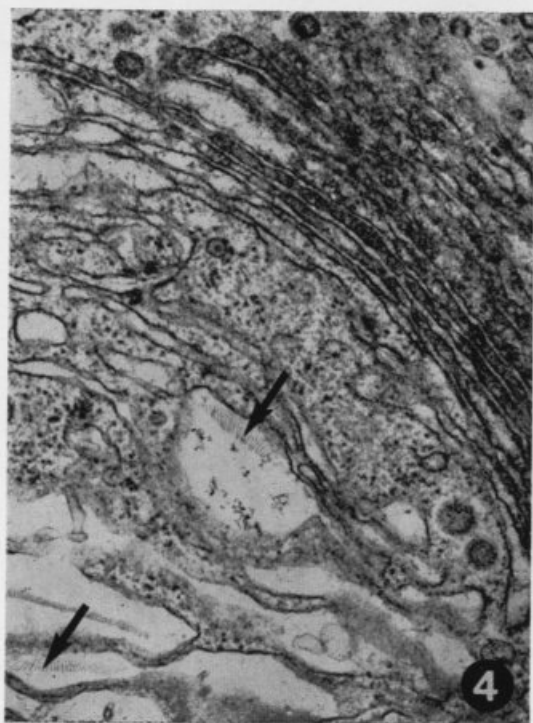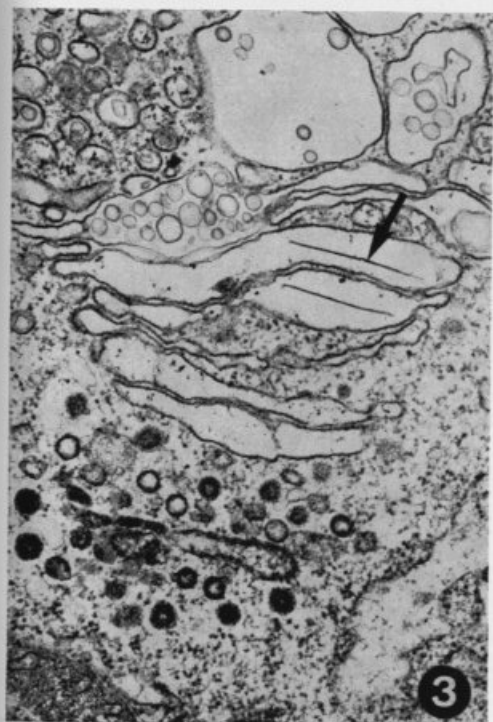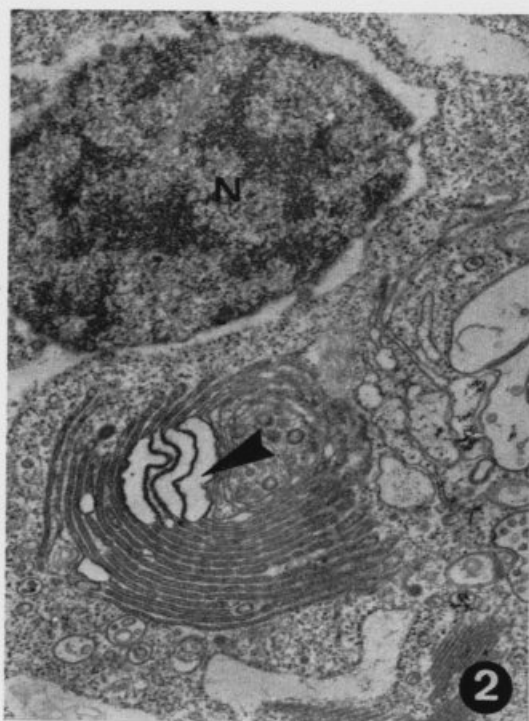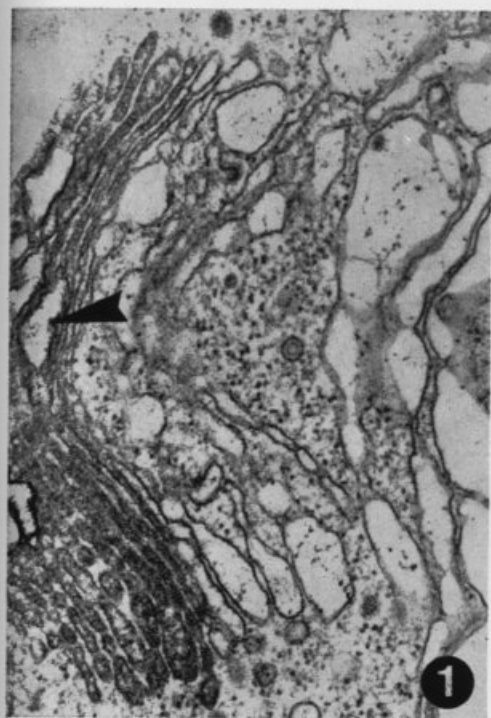
PLATE V

PLATE VI

[34]
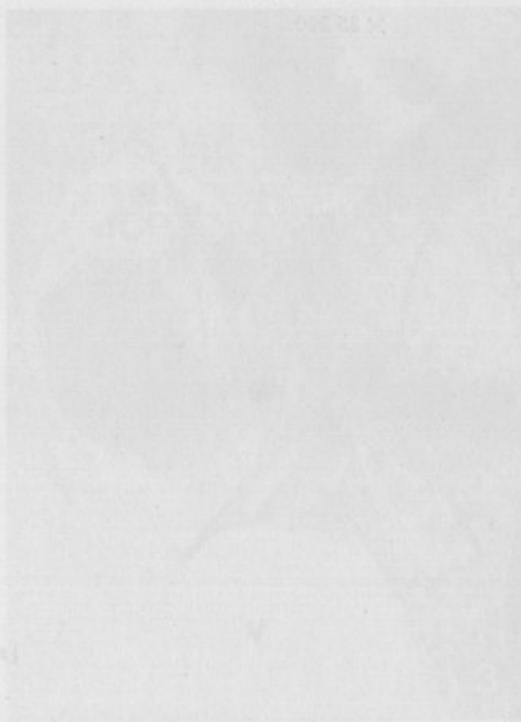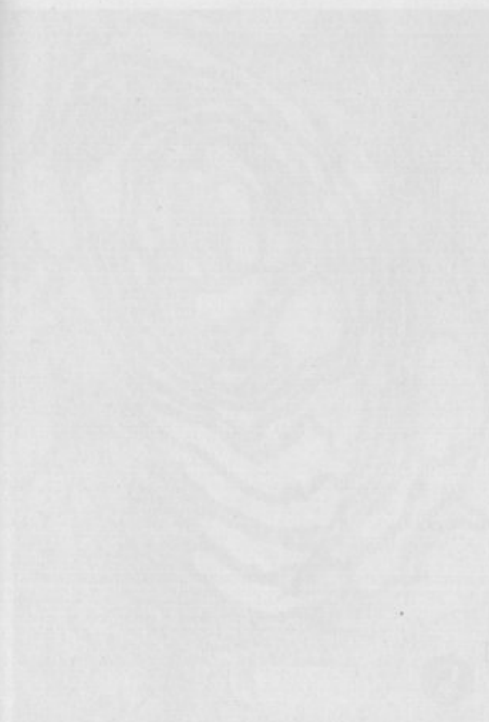
PLATE VI

Fig. 1 — Golgi body of a monensin treated cell $(10^{-5}M - 72 \, h)$: apart the typical swellings of the central zone of some cisternae, these are not tumefied (Compare with Pl. III, fig. 1 and Pl. IV, fig 1).

$\times$ 30 800.

Fig. 2. — Idem. Many cisternae of the Golgi body are strongly curled, but the same aspect can be seen in the control (Compare with Pl. V, fig. 2).

$\times$ 30 800.

Fig. 3. — Intracellular coccolith precursors (ICP) containing scales and/or coccoliths (arrows) in different stages of development.

$\times$ 35 200.
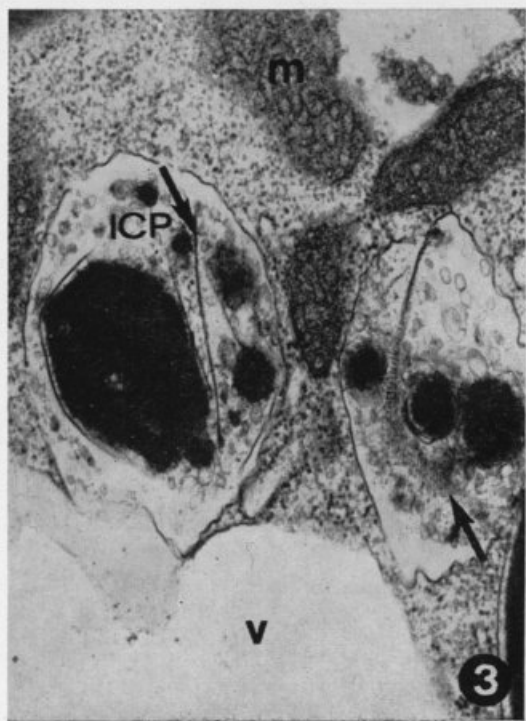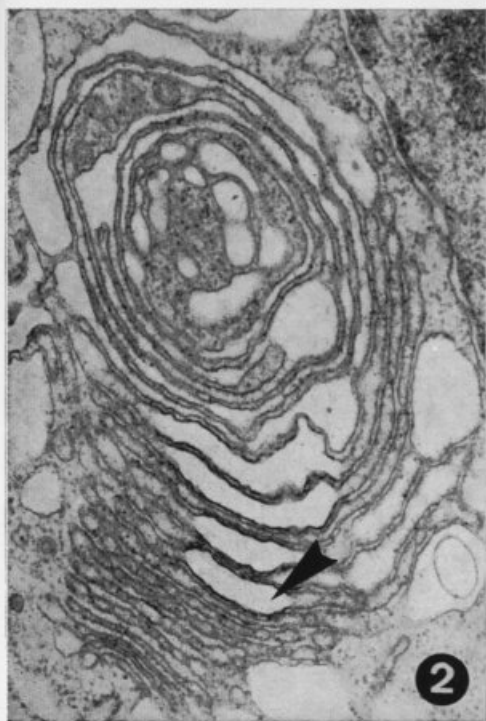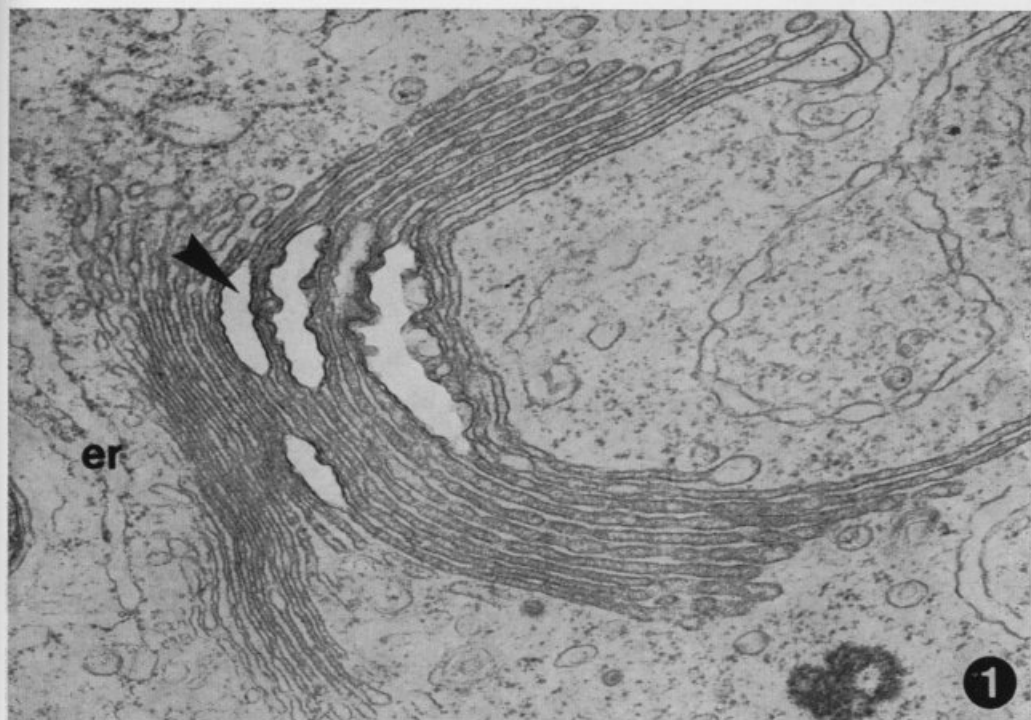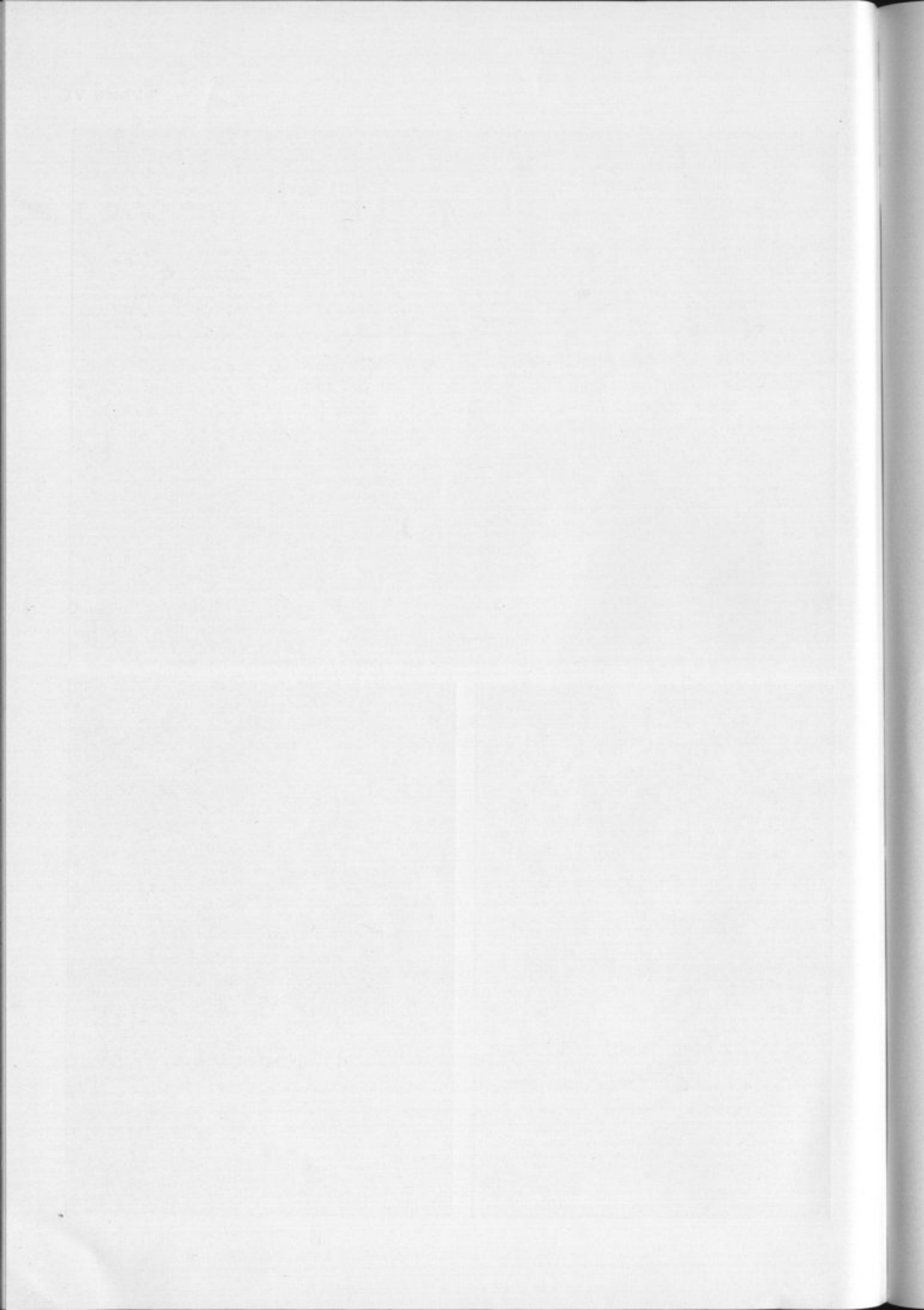
PLATE VI

ULTRASTRUCTURAL AND STEREOLOGIC STUDY OF *CRICOSPHAERA CARTERAE (PRYMNESIOPHYCEAE)* FOLLOWING EXPOSURE TO MONENSIN

II. CONTINUOUS TREATMENT OF GROWING CULTURES

by

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ABSTRACT

In a previous work (MESQUITA and SANTOS DIAS, 1987) we have studied the effects induced by monensin on *Cricosphaera carterae*, namely on its secretory system which is responsible for scales and coccoliths production. Then, 3-4 weeks-old cultures were aditioned with a stock solution of this ionophore in order to obtain the final concentration of 10^{-5}M, and samples were harvested periodically from 10 min. to 72 h. Generally, the alga has shown not to be significantly sensible to the treatment in these conditions.

The results presented now concern new experiences in which a small inoculum was transferred to the standard medium (Erdschreiber's solution) containing monensin at the concentrations of 10^{-5}M and 2.10^{-5}M.

So, the cultures have grown for 3-4 weeks under the continuous action of the drug, all the other conditions being maintained unchanged. The relative volumes of organelles and other intracellular compartments and the comparison of the average values obtained both in treated and untreated cells were achieved by employing methods which have been described in another article (MESQUITA and SANTOS DIAS, 1987). In these new conditions, some effects stand out significantly such as, an accentuated increase of the number and dimensions of lipid globules, enlargement of the vacuolar space and reduction of plastidal volume. Curiously, some changes (i. e., those concerning the lipids) which can be clearly observed in 10^{-5}M monensin treated cells, are not evident when the concentration of the drug is doubled $(2.10^{-5}$M).

INTRODUCTION

Electron microscope studies of animal and plant cells following exposure to monensin are frequently found in the literature the last years (MOLLENHAUER and col., 1982, 1983; MORRÉ and col., 1983, 1985; SHANNON and STEER, 1984; DOMOZYCH and col., 1985).

The swelling of Golgi cisternae, which has been described manytimes in these papers seems to be related with active proton pumps (BOSS and col., 1984; MORRÉ and col., 1985).

In a previous work we have carried out a similar study with *Cricosphaera carterae* (MESQUITA and SANTOS DIAS, 1987). We have selected this alga because, on one hand, there was not any study of *Chromophyta* under this point of view and, on the other hand, this *Prymensiophyceae* has an interesting secretory system which produces the scales and coccoliths of its cell covering (PIENNAR, 1969).

So, we have investigated the eventual changes of the volume of organelles and other intracellular compartments induced by monensin in this marine alga (MESQUITA and SANTOS DIAS, 1987).

3-4 weeks old cultures were treated with this ionophore at the concentration of 10—5M for 10 min. to 72 h. Generally, the alga has shown to be almost insensible to the treatment in these conditions (MESQUITA and SANTOS DIAS, 1987).

So, the results presented now concern new experiences which were performed with a different methodology for the monensin treatment (see Material and Methods).

MATERIAL AND METHODS

Cultures of *Cricosphaera carterae* were grown in the algoteca of the Department of Botany (University of Coimbra) in conditions similar to those mentioned in the first article (MESQUITA and SANTOS DIAS, 1987).

However, the treatment of the cells with monensin was carried out in a different way: the inoculum was transferred to 200 c. c. Erlenmeyers, each containing 50 c. c. of culture medium (sol. Erdschreiber) aditioned with stock solution of monensin (Sigma Chemical Co. Ltd.) to yield the final concentrations of 10—5M and 2.10—5M. So, these cultures have grown during 3-4 weeks

under the continuous action of monensin, all the other conditions being maintained unchanged (MESQUITA and SANTOS DIAS, 1987).

As controls, cultures without monensin and growing in the same conditions were used.

Harvest of samples, their preparation for electron microscope study and quantitative analysis methods were as previously described (MESQUITA and SANTOS DIAS, 1987).

RESULTS AND DISCUSSION

Ultrastructure of control cells of *Cricosphaera carterae* was described in a previous work (MESQUITA and SANTOS DIAS, 1987). However, for comparison with monensin treated cells the following characteristics must be recalled and emphasized: two parietal chloroplasts, the photosynthetic apparatus and pyrenoid of which are well developed; a spherical or more or less lobbed nucleus localized in the anterior pole of the cell, the outer membrane of which is continuous with chloroplast E. R., as is typical in the *Prymnesiophyceae* and other *Chromophyta;* a Golgi body composed by many plane or curled cisternae, those of the maturing face being frequently swollen and forming, jointly with intracellular coccolith precursors (PIENAAR, 1969), the secretory system of this alga; several and polymorphic mitochondrial profiles; a moderately developed vacuolar apparatus located at the posterior region of the cell (for these characteristics, see figures of Pl. II and III).

In cells growing for 4 weeks in presence of $10-5M$ monensin, alterations of the secretory system are note evident.

However, some modifications stand out when compared with control cells. As a matter of fact, in monensin treated cells the vacuoles are much more developed, sometimes only one great vacuolar profile can be seen (compare Pl. IV, fig. 2 and Pl. V, fig. 1 with Pls. II and III). Then, the surrounding cytoplasm is shown as a thin layer where some organelles seem to experiment an accentuated decrease of their volume. This is very clear at the level of the pyrenoids some of which apparently protrude in the vacuole (Pl. I, figs. 3, 4; Pl. IV, fig. 2).

Apart from these modifications, other fact becomes prominent in cells exposed to $10-5M$ monensin: it is the appearance of numerous and voluminous globules which, manytimes, similarly

to what happens with the pyrenoids, seem to bulge into the vacuoles (Pl. I, figs. 3, 4; Pl. IV, fig. 2; Pl. V, fig. 1).

These globules are stained black with black Sudan B (Pl. I, figs. 3 and 4), show a great affinity to the osmium and are not bordered by a biomembrane (Pl. IV, figs. 1, 2; Pl. V, fig. 1). So, they are considered as lipid inclusions.

Curiously, these globules are almost inexistent in cells exposed to $2.10-5M$ (Pl. I, fig. 2; Pl. VI, figs. 1, 2) which, under this point of view, are comparable to control cells (Pl. I, fig. 1; Pl. II, figs. 1, 2; Pl. III, figs. 1, 2).

This morphological and cytochemical study was complemented with a quantitative analysis performed according to the formulas of Weibel (see MESQUITA and SANTOS DIAS, 1987).

Stereological and statistical studies have confirmed or revealed quantitative changes at the level of cellular components (Tables I and II) as we have above referred.

As a matter of fact, in monensin treated cells $(10-5M)$, although relative volumes of nucleus, endoplasmic reticulum and Golgi area (represented as percentages of cellular volume) do not show expressive variations as compared with controls, the same is not true at the level of lipid globules, vacuoles, chloroplast and mitochondria, to which highly significant differences were found (Table I).

When the drug concentration is doubled $(2.10-5M)$, the other conditions being maintained, only the volumetric variations of vacuoles and chloroplasts are significant (Table II).

As to the secretory process, these results confirm those we have obtained in the first experiences (MESQUITA and SANTOS DIAS, 1987), this is: in comparison with other cell types (MOLLENHAUER and col., 1982, 1983; MORRÉ and col., 1983; SHANNON and STEER, 1984; DOMOZYCH and col., 1985), the secretory system of *Cricosphaera carterae* has shown very little sensible to monensin, even when the treatment conditions are diversified, namely the duration of exposure to the drug and its concentration.

The hypothesis according to the peculiar comportment of this alga (comparatively with other algae or other cell types) can be correlated with the elevated $Na+$ contents of the culture medium and/or natural habitat must be considered.

It would be interesting to analyse the effects of monensin on *Cricosphaera carterae* growing in a poorer sodium chloride medium. Meanwhile we have not been able to cultivate it in these conditions.

The accumulations of ... polysaccharides are frequently considered as ... these treatment conditions, the ... the ... observed in *Cricosphaera carterae* ...

TABLE I

Comparison of relative volumes (%) of different cellular components of *Cricosphaera carterae*, in monensin treated cells (10^{-5} M) with the correspondant controls (student t test); the difference between average values is significant for lipid globules, vacuoles, chloroplasts and mitochondria.

	NUCLEUS	MITOCHONDRIA	CHLOROPLASTS	SECRETORY SYSTEM			LIPID GLOBULES	VACUOLES
				E. R.	GOLGI AREA	I. C. P.		
Controls	14.39 ± 1.27	4.57 ± 0.36	20.87 ± 1.48	1.88 ± 0.18	4.73 ± 0.43	4.70 ± 1.21	0.54 ± 0.09	22.75 ± 2.23
Monensin treated cells (10^{-5} M)	14.39 ± 1.27	1.97 ± 0.20	6.98 ± 0.71	1.21 ± 0.17	4.77 ± 0.62	3.93 ± 0.20	8.84 ± 4.42	39.77 ± 3.50
P values	—	< 0.001	< 0.001	—	—	—	< 0.001	< 0.01

Dute, W. C. MacKenzie
1981 Monensin-mediated ... *Cricosphaera carterae* mediated by a ...

Domozych, D. S. & ...
1985 The distribution ... and secretory activity in the ... coccolithophorid alga *Cricosphaera* ... *Journal of ...*

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1987 Ultrastructural study ... *Cricosphaera carterae* (Prymnesiophyceae) exposed to monensin. I. Treatment of ... 2-A ...

Mollenhauer ...
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Mollenhauer, H. H. ...
1983 Monensin ... *Protoplasma*, 114: ...

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1983 Kinetics ... following monensin treatment ... *Cell Biol.*, 68, ...

Nassar, D. ...
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TABLE II

When the concentration of monensin is doubled (2.10⁻⁵M), the changes of relative volumes are significant only at the level of vacuoles and chloroplasts

	NUCLEUS	MITOCHONDRIA	CHLOROPLASTS	SECRETORY SYSTEM			LIPID GLOBULES	VACUOLES
				E. R.	GOLGI AREA	I. C. P.		
Controls	14.39 ± 1.27	4.57 ± 0.36	20.87 ± 1.48	1.88 ± 0.18	4.73 ± 0.43	4.70 ± 1.21	0.54 ± 0.09	22.75 ± 2.23
Monensin treated cells (2.10⁻⁵M)	13.32 ± 0.86	3.55 ± 0.30	11.95 ± 0.64	2.15 ± 0.15	5.20 ± 0.75	5.99 ± 0.82	0.58 ± 0.10	29.91 ± 1.77
P values	—	—	< 0.001	—	—	—	—	< 0.05

It would be interesting to investigate the effects of monensin on *Cricosphaera carterae,* growing in a deficient sodium chloride medium. Meanwhile, we have not achieved to cultivate it in these conditions.

The accumulation of lipids and vacuolation are frequently considered as symptomes of cell aging. So, in our treatment conditions, the monensin seems to accelerate this process in *Cricosphaera carterae.*

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It would be interesting to investigate the effects of monensin on Chroogphaera cariense, growing in a deficient sodium chloride medium. Meanwhile, we have not achieved to cultivate it in these conditions.

The accumulations of lipids and vacuolation are frequently considered as symptoms of cell aging. So, in our treatment conditions, the monensin, seems to accentuate this process in Chroogphaera cariense.

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All plates concern cells of *Cricosphaera carterae*. For culture and monensin treatment conditions, to see Material and Methods and MESQUITA and SANTOS DIAS (1987).

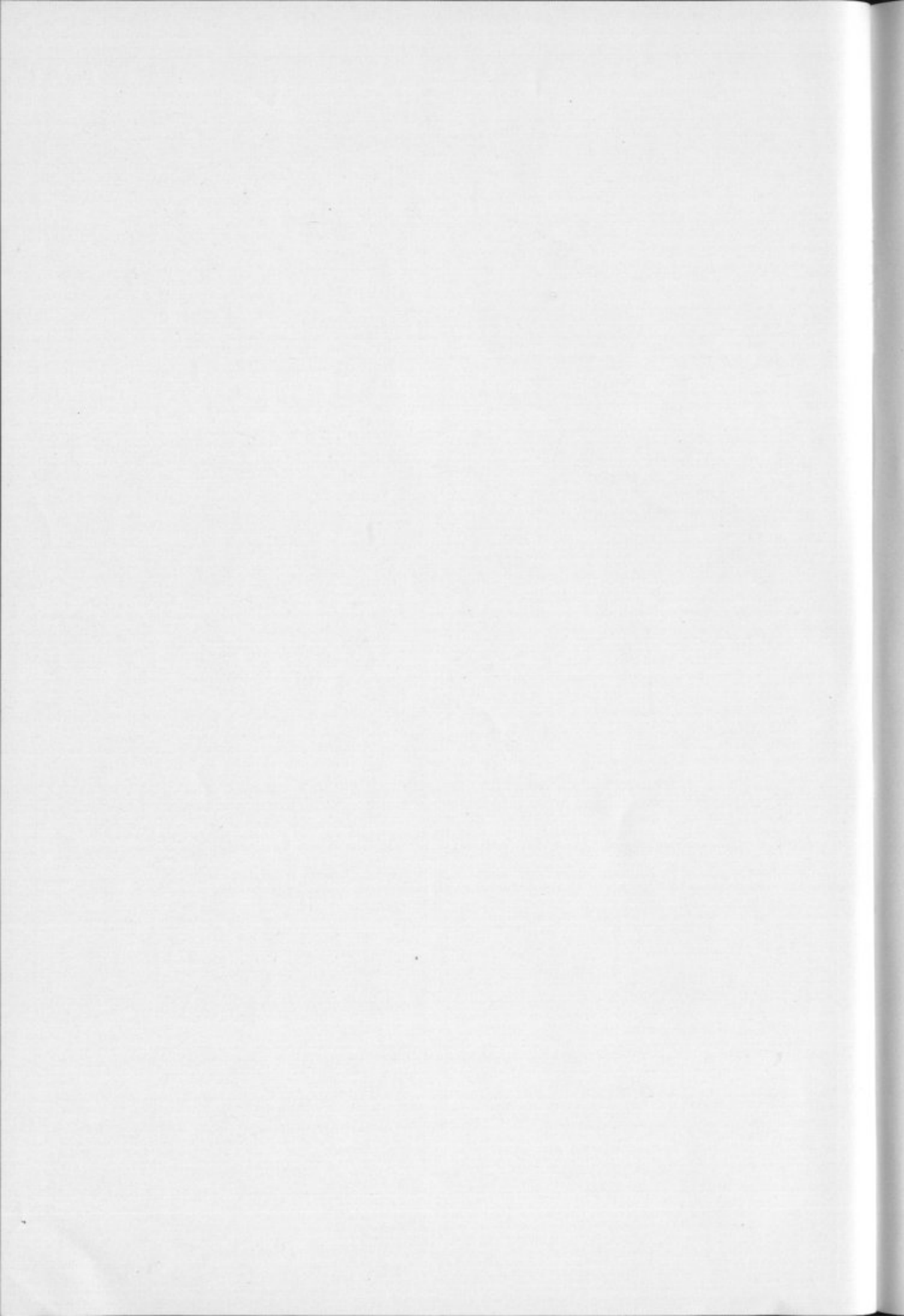The concentrations of monensin used in the experiences are mentioned in the captions.

ABBREVIATIONS

c   — chloroplast
er — endoplasmic reticulum
g   — Golgi apparatus
ICP — intracellular coccolith precursor
l   — lipidic globules
m   — mitochondria
N   — nucleus
nu — nucleolus
p   — pyrenoid
v   — vacuole

PLATE I

1 µm sections stained with black Sudan B. Control cells (fig. 1) and 10^{-5}M (figs. 3 and 4) or 2.10^{-5}M (fig. 2) monensin treated cells. In comparison with control (fig. 1), all treated cells (figs. 2, 3, 4) are much more vacuolated (v) and present thinner chloroplasts (c). Apart from this, numerous, voluminous, spherical and sudanophyll inclusions (lipidic globules) can be seen in 10^{-5} monensin treated cells (figs. 3 and 4) but not in the control (fig. 1) or in 2.10^{-5}M monensin treated cells (fig. 2).

× 2200.

PLATE I

PLATE II

[PLATE II]

Figs. 1 and 2. ...

PLATE II

Figs. 1 and 2. — General views of untreated cells. All typical ultra-structural characteristics of this alga can be observed: peripheric and relatively thick chloroplastidal profiles (c), polymorphic mitochondria (m), a single Golgi body (g) near the nucleus (N) both occurring toward the anterior pole of the cell and the vacuole (v) at the opposite pole.

$\times$ 11 000.
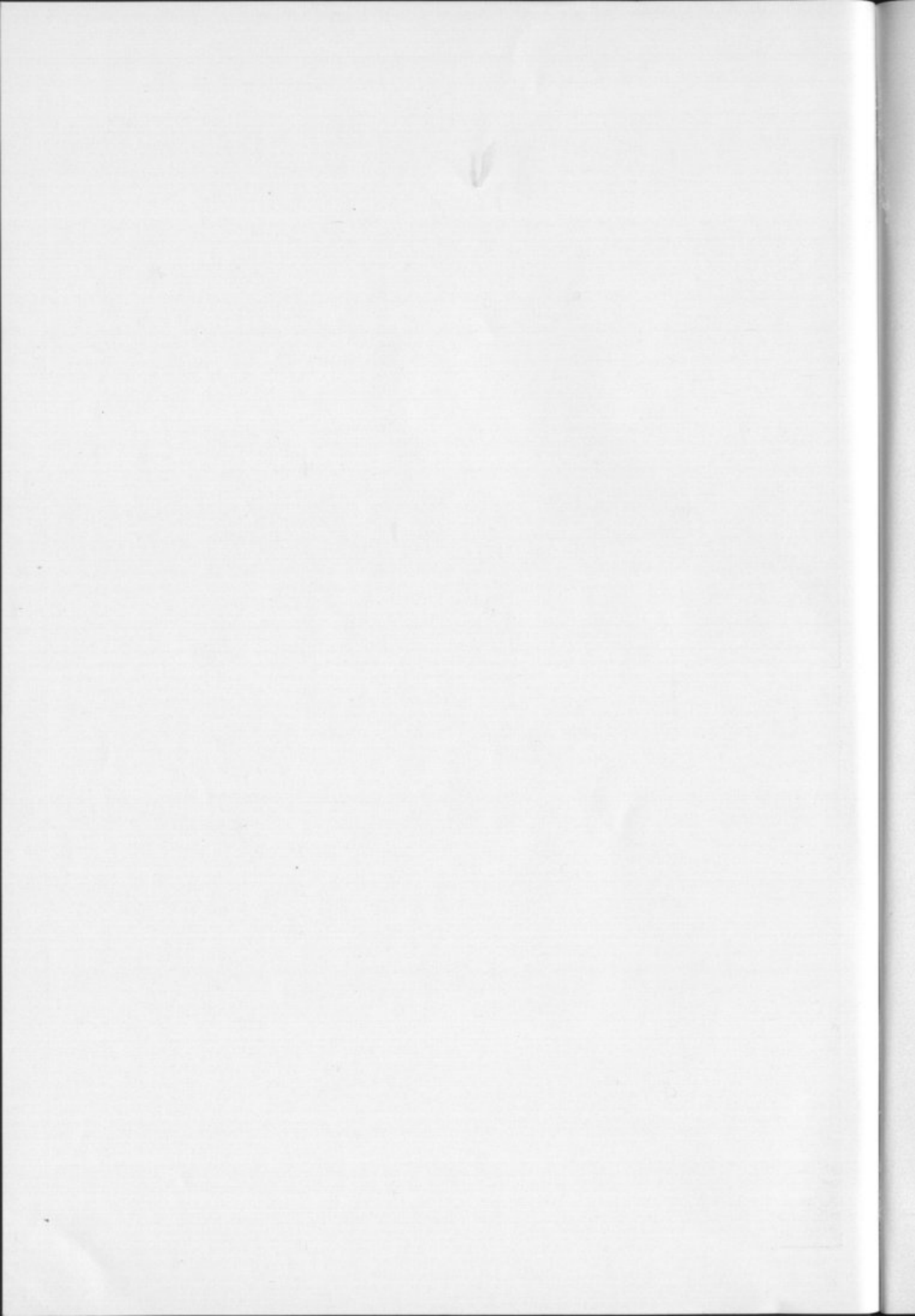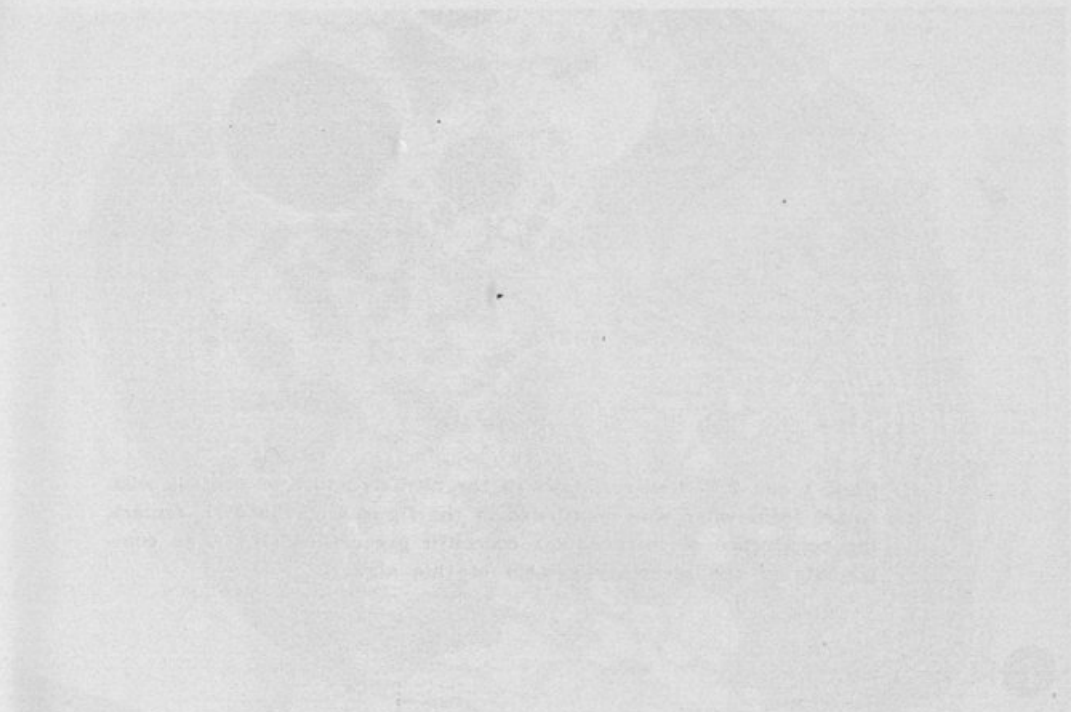
PLATE II

PLATE III

PLATE III

Figs. 1 and 2. — General views of the ultrastructure of control cells. Apart from what was mentioned in the figures of Plate II, remark the occurrence of intracellular coccolith precursors (ICP), as components of the secretory system of this alga.

$\times$ 11 000.
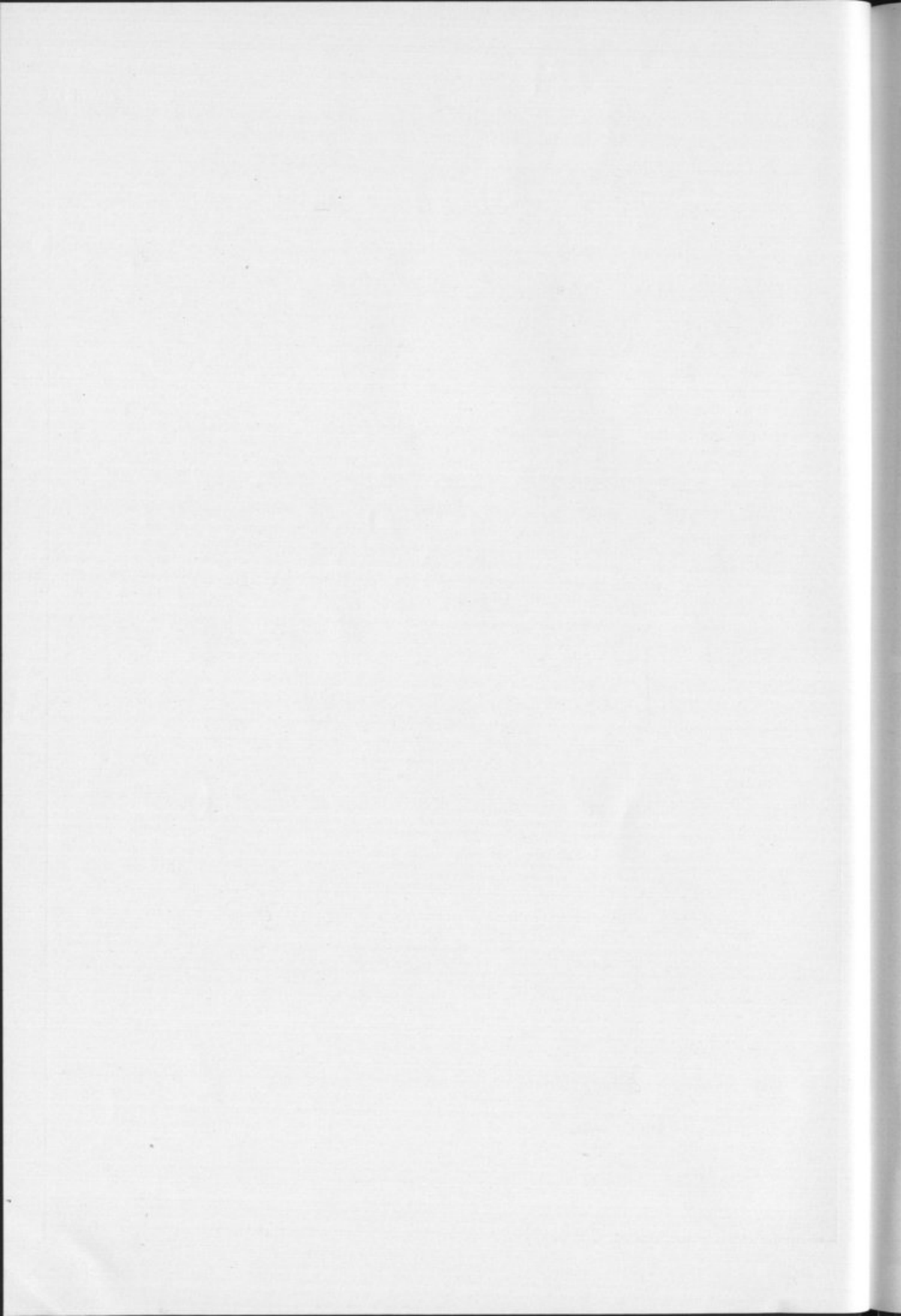
PLATE III

PLATE IV

Figs. 1 and 2. — These cells were harvested from a culture the growth of which was realized in the continuous presence of monensin at the concentrations of 10^{-5}M for 3 weeks.

When compared with untreated cells (Plates II and III), the vacuoles (v) are much more developed (fig. 2), the chloroplastidal profiles (c) are thinner and the lipid globules are numerous and voluminous (figs. 1 and 2).

Sometimes, these globules seem to protrude into the vacuoles (fig. 2).

$\times$ 11 000.
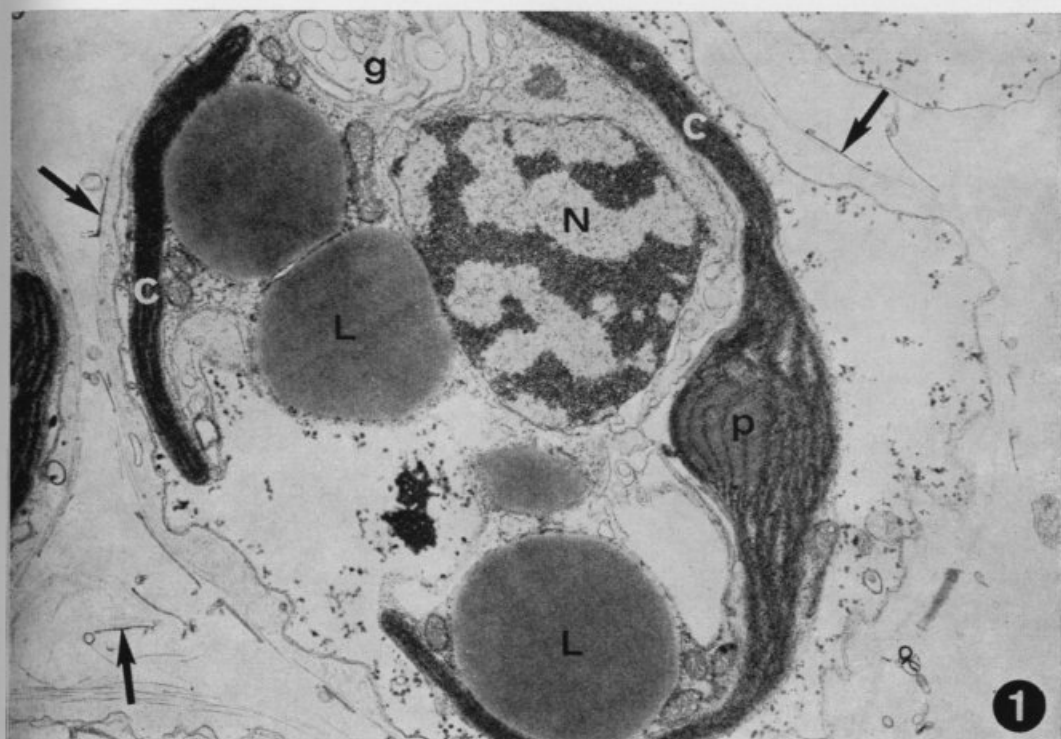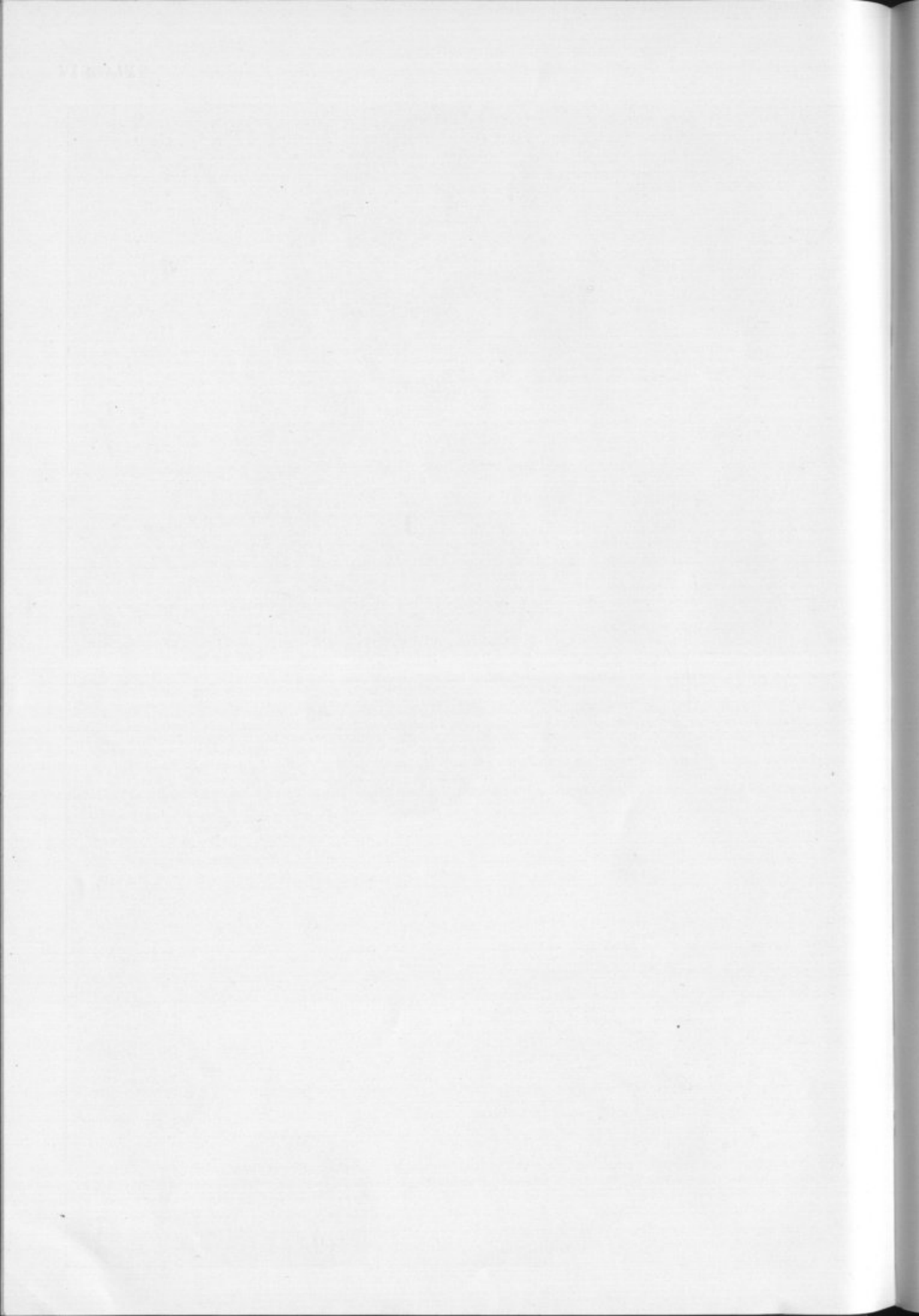
PLATE IV

PLATE V

Fig. 1. — General aspect of the ultrastructure of a monensin treated cell (10^{-5}M): both the nucleus (N) and Golgi body (g) are localized in the anterior pole of the cell, the rest being almost completely ocupied by the vacuoles (v). Note the thin chloroplastidal profiles (c) and the great lipid globules.

$\times$ 11 000.

Fig. 2. — Detail of a monensin treated cells (10^{-5}M) showing the Golgi body some mature cisternae of which contain scales and/or coccoliths (arrows).

$\times$ 30 800.
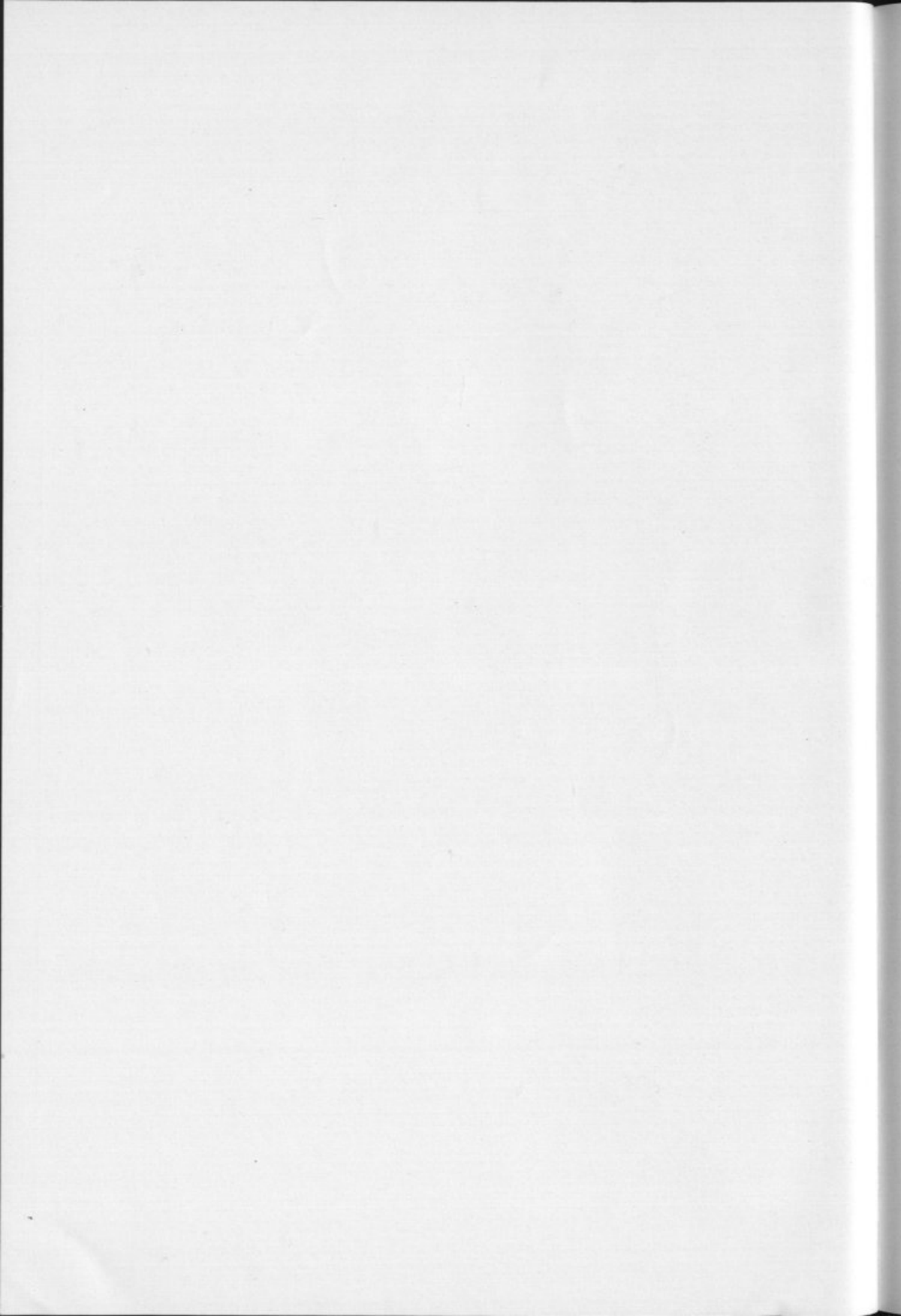
PLATE V

PLATE VI

PLATE VI

Figs. 1 and 2. — General views of cells which were harvested in the same conditions of those referred in Plate IV, except the concentration of monensin $(2.10^{-5}M)$. In comparison with that Plate, the scarcity of lipid globules in these cells is evident.

$\times$ 11 000.

PLATE VI
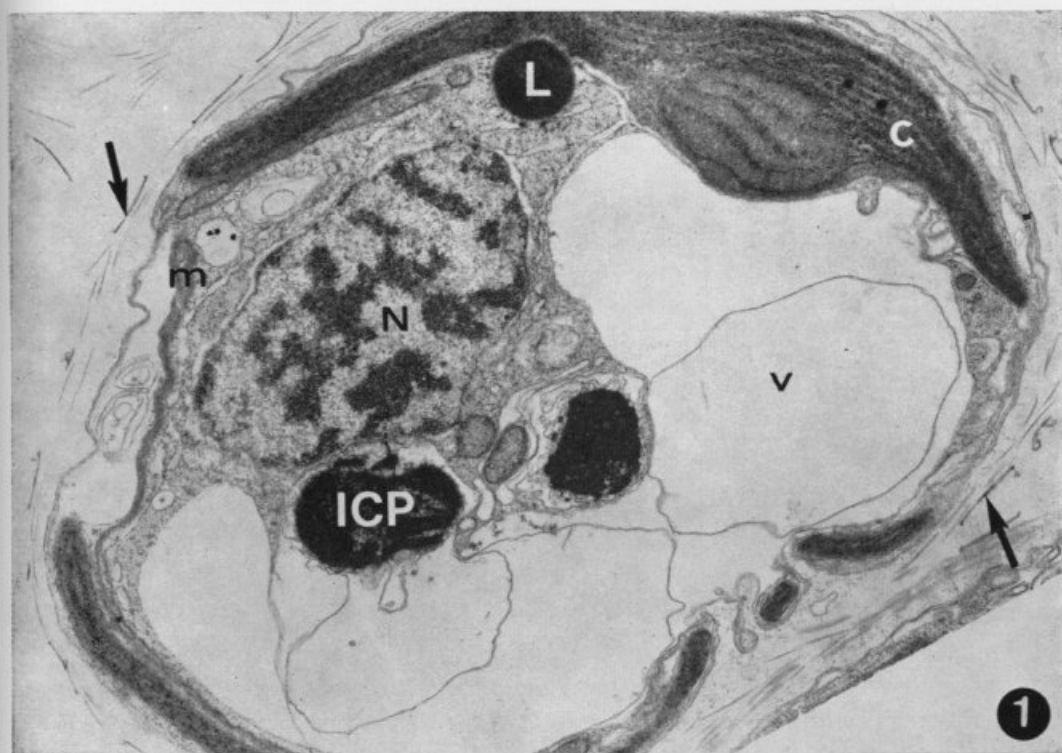
IN VITRO CULTURE
OF *URGINEA MARITIMA* (L.) BAKER, FOR
RAPID CLONAL PROPAGATION

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ABSTRACT

The *in vitro* clonal propagation of *Urginea maritima*, a liliaceous species with some medicinal interest, was studied. Using seedlings hypocotyl explants, the organogenesis, as controled by two plant regulators BAP and NAA, supplemented to the MURASHIGE & SKOOG basal medium was investigated. NAA alone can induce callus, roots and occasionally shoots. BAP in combination with NAA has a potent synergistic effect on the induction of shoots and a clear antagonistic effect on callus and root initiation. The most suitable medium for keeping production of shoots continuously without morphological abnormalities was the one with 1 mg/l BAP + 0.1 mg/l NAA. An efficient rooting of shoots was obtained in a medium with 0.5 mg/l NAA. Repeated subculturing results in a increased incidence of tetraploid plants.

INTRODUCTION

THE bulbs of *Urginea maritima* (L.) Baker, a member of *Liliaceae,* of wide occurrence in the portuguese flora, has for long been known to be a source of cardiac glycosides of scillaren type. We found it interesting to study the possibility, by using *in vitro* techniques, to achieve rapid clonal micropropagation of this species, as a method that can lead in future, to the isolation of variant lines with some medicinal importance. Although the *in vitro* responses of several species of the liliaceous family have been studied (see revisions by HUSSEY, 1975; 1982) only recently the genus *Urginea* have been examined for *in vitro*

propagation (JHA *et al.,* 1984 on *U. indica* and EL GRARI and
BACKHAUS, 1987 on *U. maritima*). However their results are not
directly comparable to the ones presented in this paper as they
have used bulb-scale explants instead of seedlings hypocotyl
explants.

MATERIALS AND METHODS

Seeds of *Urginea maritima* (L.) Baker were surface sterilized
in 70 % ethanol for 2 min., followed by immersion in 7 % calcium
hypochlorite on a giratory shaker (150 r. p. m.) for 20 min., and
subsequently rinsed three times with sterile distilled water. The
seeds were germinated aseptically on solid (MS) MURASHIGE and
SKOOG (1962) basal medium in the dark at $27 \pm 1°$ C. When the
seedlings were 10-20 days old, hypocotyl segments (0.5 cm long)
were dissected and placed in culture tubes containing 25 ml MS
medium supplemented with various concentrations and combina-
tions of NAA and BAP and 3 % sucrose. The pH of all media
was adjusted to 5.8, and 0.8 % (w/v) Difco Bacto agar was added
prior to autoclaving. All the cultures were maintained in the
dark at $25 \pm 1°$ C for 6 weeks, and then transferred to a 12 h
photoperiod.

For chromosome counts, root tips were pretreated with
0.05 % colchicine at 25° C for 3 hours, fixed in ethanol-acetic
acid 3:1 (v/v) and prepared as acetic orcein squashes.

RESULTS

Organogenesis

The earliest visible response ocurred within three weeks and
consisted in callus, root and shoot formation on whole surface or
on the cut ends of the explants. Table I summarizes the morpho-
genetic responses of hypocotyl explants to various concentrations
and combinations of BAP and NAA after 10 weeks of culture.
The results obtained show (Table I) that NAA alone induces
shoot and root formation in concentrations ranging from 0.1 to
1 mg/l (Fig. 1), but at higher concentrations (Fig. 2) greatly
increases callus growth. BAP alone induced multiple shoots
(Fig. 3) in concentrations ranging from 0.5 to 10 mg/l. Increasing
the BAP level enhanced the number of shoots but shoot elon-

TABLE I

Effect of NAA and BAP on organogenesis in hypocotyl segments
of *Urginea maritima*

(Data scored after 10 weeks of culture; 15-30 explants per treatment)

GROWTH REGULATORS		FORMATION OF		
BAP (mg/l)	NAA (mg/l)	MULTIPLE SHOOTS (% Explants with shoots)	ROOTS (% Explants with roots)	CALLI (Growth) a
0	0	0	0	—
	0.1	8.3	20.8	+
	0.5	12.5	33.3	++
	1.0	18.1	100	++
	5.0	5.3	10.6	+++
0.1	0	0	0	—
	0.1	8.3	16.6	+
	0.5	9.5	33.3	++
	1.0	16.7	14.2	++
0.5	0	13.3	0	—
	0.1	23	15.3	+
	0.5	42	17.6	+ b
	1.0	23.5	12.9	++ b
	5.0	13.2	6.7	++ b
1.0	0	25	0	—
	0.1	50	0	—
	0.5	64.5	19.3	+
	1.0	90	10	+ b
	5.0	16.6	0	++ b
5.0	0	30.8	0	—
	0.1	62.5	12.5	—
	0.5	88	14.2	+ b
	1.0	57.2	0	+
	5.0	40	0	+
	0	33.3	0	—
	0.1	50	0	—
	1.0	61.5	15.3	+ b
	10.0	11.7	5.8	+

a — nil; + low; ++ moderate; +++ high.
b Callus with small shoots.

gation was progressively reduced and at 10 mg/l shoots were
greatly stunted and swollen (Fig. 4). In addition, BAP completely
inhibited callus and root formation. Multiple shoot regeneration
occurred over a wide range of BAP and NAA concentrations and
each increase in BAP level promoted a progressive inhibition
of the rhizogenic and callogenic effects of NAA. Inhibitory effects
of cytokinin on NAA-induced root formation was clear. The number
of explants producing roots formed by treatment with 1 mg/l NAA
was reduced by BAP even at a low concentration (0.1 mg/l).
Root formation induced by the same concentration of NAA was
completely inhibited by BAP when applied at 5 mg/l.

Clonal micropropagation

In order to obtain an efficient continuous shoot multiplication
(Fig. 5), clusters of 3-4 shoots from primary explants where
subcultured in fresh media (clonal micropropagation media). The
best results were obtained in a medium containing BAP (1 mg/l)
in combination with low NAA concentration (0.1 mg/l). After
8 weeks in this medium each subculture gives 15-20 new shoots.
Media with a lower BAP concentration (0.25 and 0.5 mg/l) pro-
moted elongated shoots but very poor shoot multiplication,
whereas higher BAP concentrations (5 and 10 mg/l) produce
abnormally shaped shoots.

Rooting of shoots

Shoots with 1 cm or longer were transferred individually to
three rooting media: 1) MS without growth regulators; 2) MS with
0.1 mg/l NAA; 3) MS with 0.5 mg/l NAA. It was found that
rooting capacity depends on the BAP concentration of the micro-
propagation media where the shoots had previously been deve-
loped (Table II). Shoots coming from a medium with 0.5 or
1 mg/l BAP rooted at high percentage in about two weeks
but those previously cultured on 5 mg/l BAP did not root so
easily and the roots were frequently abnormal (Figs. 6-8).
Although the shoots developed roots even on auxin free medium
the percentage of rooted shoots could be increased by adding
NAA to the medium. An efficient rooting was obtained in
presence of 0.5 mg/l NAA (Table II). Fig. 9 shows a plantlet
ready for potting, after 4 weeks on rooting medium.

TABLE II

The effect of BAP (in the micropropagation media) in decreasing
the rooting capacity of shoots (in the rooting media). Data
represent an average of 100-150 shoots per treatment
recorded after 4 weeks

Clonal micropropa-gation media	$^0/_0$ of shoots forming roots on rooting media		
	NAA (mg/l)		
BAP (mg/l)	0	0.1	0.5
0.5	43.7	81.5	90.9
1.0	54.2	59.0	80.0
5.0	22.2	32.4	58.3

Transfer to soil and characters of regenerated plants

The plantlets after washing in tap water were directly trans-
ferred to potting soil and maintained in greenhouse for two
months. Morphologically the plant leaves appeared normal
(Fig. 10). Afterwards the plants were transferred to the field and
most of them (about 90 %) have survived. All plants formed in
culture, directly from hypocotyl explants, were diploid (2n = 60,
Fig. 11). However examination of plants (20 individuals) obtained
after one year of subculture on the clonal micropropagation
medium containing 1 mg/l BAP + 0.1 mg/l NAA showed appro-
ximately 15 % tetraploid plants (Fig. 12).

DISCUSSION

In the present investigation contrary to results in other
Liliaceae (KATO, 1975; HUSSEY, 1975, 1976) the supply of exoge-
nous cytokinin-auxin was essential for organogenesis induction on
hypocotyl explants of *Urginea maritima*. The effects of NAA,
BAP and NAA-BAP combinations show quite distinct morpho-
genetic responses. The auxin NAA was the only necessary hormone
for callus and root induction. It is interesting to note that in
Urginea maritima (JHA *et al.,* 1984) auxins alone could not induce
callusing. Although low NAA concentrations induce, occasionally,

shoot differentiation, BAP is very effective in shoot induction without an intervenient callus. That cytokinins are causative agents of *in vitro* bud differentiation is well established (SKOOG and MILLER, 1957; NARAYANASWAMY, 1977; REINERT *et al.*, 1977; EVANS *et al.*, 1981). The synergistic effect of BAP in inducing shoots in *Urginea* hypocotyl expants cultured in BAP-NAA combinations is clear (see Table I). High concentrations of BAP tend to supress NAA induced callus and root differentiation. This antagonistic effect of BAP on these morphogenetic aspects is similar to that described for *Hyacinthus* (SANIEWSKY *et al.*, 1974) and contrary to results in *Bellevalia* (LUPI *et al.*, 1985). The most suitable medium for keeping production of shoots continuously without morphological abnormalities as swollen and very short leaves, was the one with 1 mg/l BAP + 0.1 mg/l NAA. Finally, it was observed that repeated subculturing results in a increased proportion of tetraploid plants. This may be explained by chromosome endoreduplication as a general phenomenon occurring in *in vitro* tissue culture (D'AMATO, 1978).

ACKNOWLEDGMENTS

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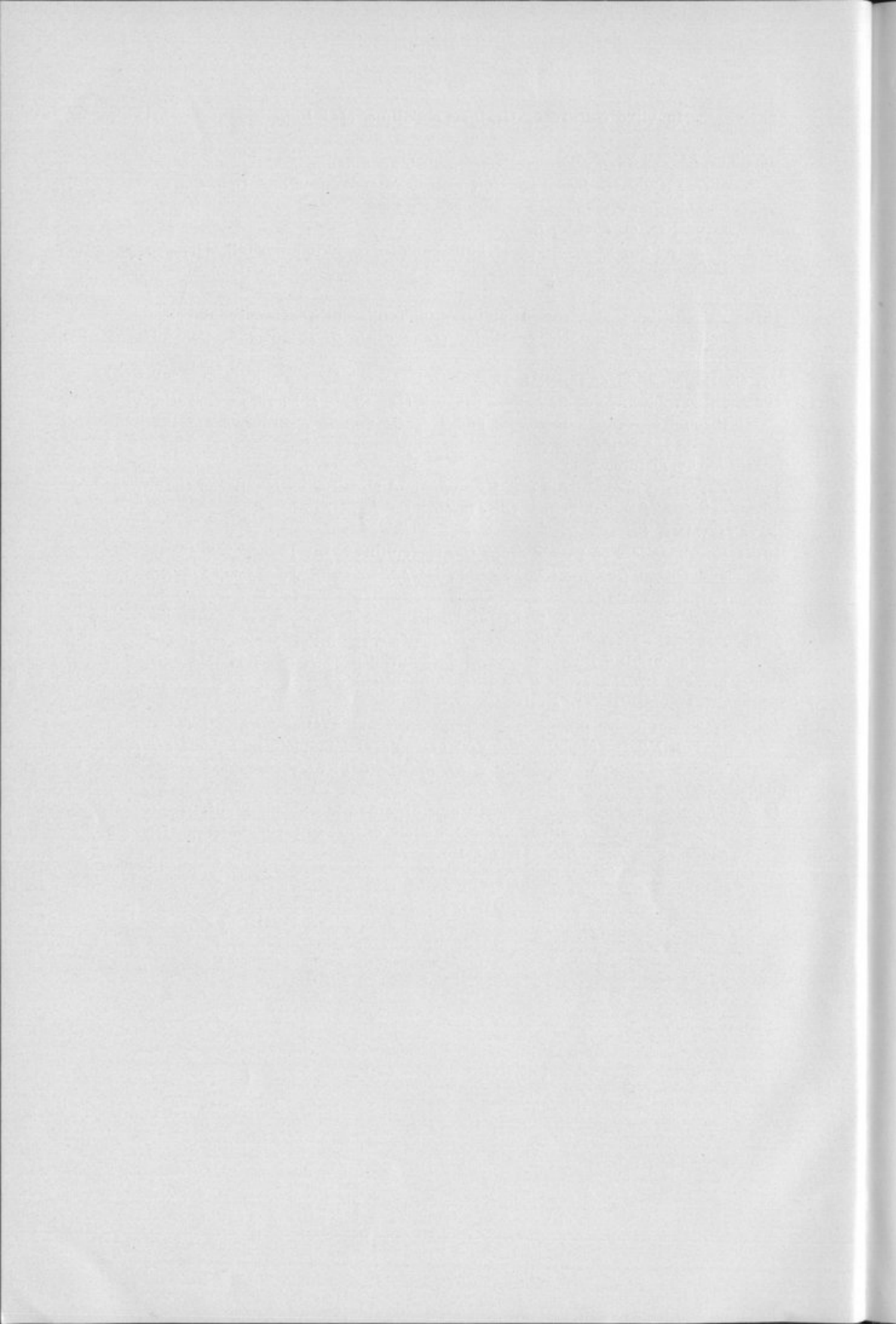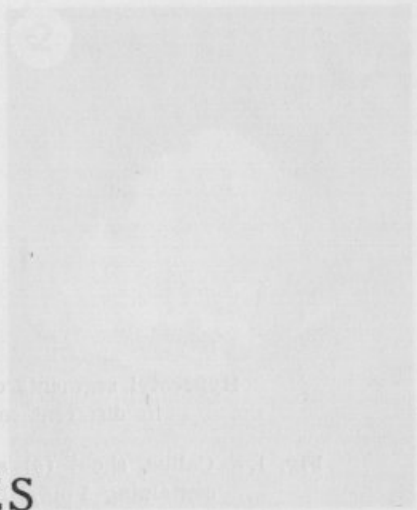
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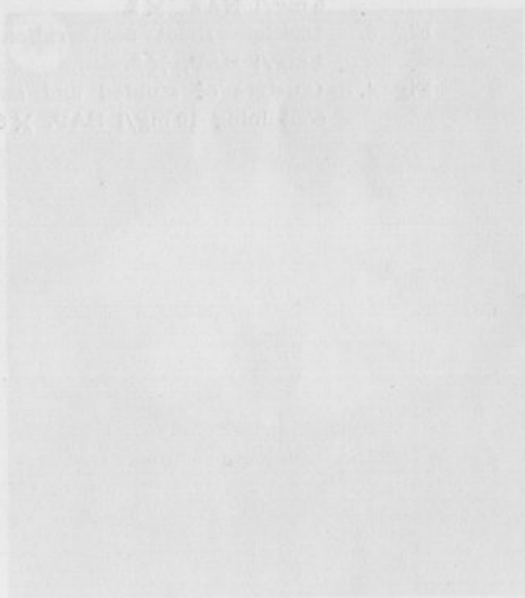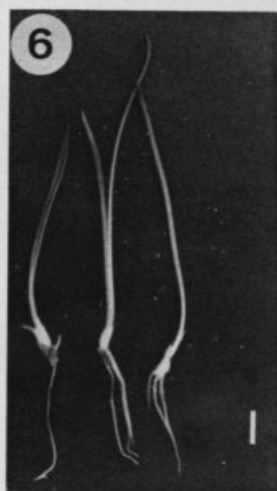
PLATE II

PLATE II

Clonal micropropagation of *Urginea maritima* (L.) Baker

Fig. 5. — A 3 month old culture in the shoot multiplication phase
    on MS medium containing 1 mg/l BAP + 0.1 mg/l NAA.
    Bar = 1 cm.
Figs. 6-8. — Different morphological aspects of rooted shoots coming
    from media with different BAP concentrations: 0.5 mg/l
    (Fig. 6); 1 mg/l (Fig. 7) and 5 mg/l (Fig. 8). Bar = 1 cm.
Fig. 9. — A rooted regenerated plantlet ready for potting.
    Bar = 1 cm.

In vitro regenerated plants of *Urginea maritima* (L.) Baker
and their karyotypes

Fig. 10. — Plants after 2 months transfer to potted soil. Note the
developing bulb.
Fig. 11. — A metaphase plate from root tip of regenerated plant
showing diploid chromosome number (2n = 60). × 1250.
Fig. 12. — A metaphase plate from root tip of regenerated plant
showing tetraploid chromosome number (4n = 120). × 1250.

PLATE III
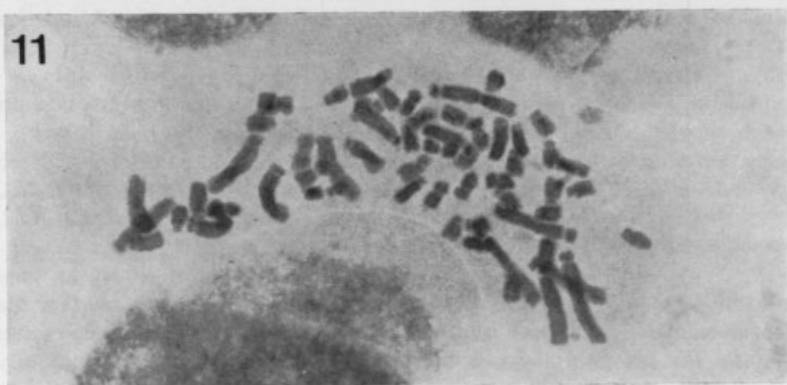
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Iconographia Palynologica Pteridophytorum Italiae

por E. Ferrarini, F. Ciampolini, R. E. G. Pichi Sermolli
& D. Marchetti. *Webbia* 40 (1): 1-202 (1986). Firenze.

Reconhecida pelo próprio autor a artificialidade do seu Sistema Sexual, Lineu pensou na elaboração de um Método Natural, de que, como refere Stearn na sua introdução ao facsimile da edição de Species Plantarum de 1753, publicou um esquema intitulado Fragmenta methodi naturalis, nas suas *Classes Plantarum* (1738). Stearn acrescenta que Lineu fez depois duas séries de lições sobre o assunto, a primeira, em 1764, aos seus estudantes Ferber, Fabricius, Zoega, Meyer e Kuhn e a segunda, em 1771, a Giseke, Vahl, Edinger e Tislef. Das suas próprias notas e das de Fabricius, Giseke publicou, em 1792, já depois da morte de Lineu, as *Praelectiones in Ordines naturales Plantarum.*

Este método de classificação, que deveria traduzir as relações naturais das plantas, congregou as escolas que, criticando Lineu, se ocupavam da elaboração da classificação natural, em especial a escola francesa em que se destacam os nomes de Adanson, dos Jussieu, dos De Candolle, de Corrêa da Serra e outros. O problema da classificação natural mostrou-se logo de início extremamente complexo, pois que, de harmonia com Adanson, a sua elaboração deveria ser baseada sobre o conjunto dos caracteres. Deste modo, as características da morfologia externa de todos os órgãos começaram a ser estudados com a maior minúcia, a fim de se descortinarem as afinidades e as diferenças das plantas. Em breve, porém, se verificou que outros caracteres poderiam ser importantes para os fins desejados, entre os quais os anatómicos. Sendo assim, iniciaram-se os mais diversos estudos sobre a anatomia de todos os órgãos, procurando, como se procedera com a morfologia externa, descortinar as analogias e as diferenças entre os grupos que iam sendo estabelecidos. Perante os problemas cada vez mais difíceis que se apresentavam aos investigadores, estes procuravam novas fontes de informação, passando da anatomia para a citologia. Desde a segunda metade do século XIX que os investigadores tinham notado que, quando as células se dividiam, no núcleo surgiam certos corpos apelidados por Waldeyer

cromossomas que se apresentavam em regra constantes no número e também na forma. Tendo-se depois demonstrado que estes corpos desempenhavam a importante função de serem os transmissores dos factores hereditários, compreende-se que os seus caracteres tenham sido considerados da maior importância para o estabelecimento de classificações naturais. Deste modo, surgiram numerosos cito-taxonomistas cujos estudos reuniram uma massa muito importante de dados que têm sido usados e continuarão a sê-lo por todos os que se ocuparem da classificação dos vegetais.

Um outro campo em que as observações dos estudiosos têm insistido é o exame das superfícies, cuja observação pelo microscópio óptico mostrava pormenores que poderiam ter importância na classificação. Da observação de particularidades das epidermes de caules e folhas, passou-se ao estudo das superfícies dos esporos de Criptogâmicas e grãos de pólen das Espermatófitas.

A medida que os estudos das superfícies se revelavam interessantes, as técnicas para a sua observação iam progredindo até que se deu um passo muito importante com a invenção do «Scanning Electron Microscope», que nos últimos tempos tem sofrido melhoramentos consideráveis. Simultaneamente, têm-se aperfeiçoado também as técnicas de preparação, de modo que hoje o exame da superfície dos esporos e dos grãos de pólen tornou-se uma importante fonte de dados para serem usados no estabelecimento das classificações naturais.

É evidente que este objectivo se não pode conseguir somente com o estudo das plantas que povoam actualmente a Terra e das conservadas em herbário. No processo evolutivo, as espécies dão origem a outras e muitas desaparecem, constituindo estas elos que os taxonomistas necessitam conhecer para o estabelecimento das relações entre as hoje existentes. Sabendo-se que, em certas condieçõs, os esporos e os grãos de pólen são conservados no estado fóssil em condições de poderem ser estudados pelas técnicas modernas, facilmente se compreende a importância que eles apresentam para os taxonomistas.

Tudo isto vem a propósito do notável trabalho em epígrafe publicado em *Webbia* **40** (1): 1-202 (1986) pelos 4 investigadores italianos acima mencionados, e do qual se pode ficar com uma noção exacta pelo resumo apresentado pelos autores, e cuja tradução livre é a seguinte:

«O principal objectivo deste trabalho é a ilustração, pelo 'scanning electron microscope', dos esporos das *Pteridophyta* nativas de Itália. Estas elevam-se a 124 taxa específicos e infra-específicos, de cujos esporos se dão imagens em 550 microfotografias (3-8 para cada taxon) reunidas em 71 estampas.

O trabalho consiste de um prefácio, uma introdução e quatro partes de texto propriamente dito. O prefácio é principalmente destinado a explicar o objectivo do trabalho e a agradecer aos amigos e colegas que amavelmente prestaram a sua ajuda nas nossas investigações.

Na introdução, tenta-se dar uma informação geral e pormenores sobre o arranjo taxonómico das *Pteridophyta* italianas (das quais se apresenta uma lista), bem como a indicação dos materiais e métodos usados na pesquisa e das investigações feitas para obter os dados citológicos e geobotânicos necessários.

A primeira parte fornece os dados pertinentes a cada espécie e subespécie, ocasionalmente a alguma variedade, estudadas no trabalho. Estes dados consistem de informação variada subdividida nos seguintes parágrafos: a. b, c) o nome da espécie ou subespécie, antecedido pelo número do género e da espécie e seguido pelos sinónimos; d) o espécime-testemunho; e, f, g) legendas das figuras e a descrição e tamanho dos esporos; h) o tipo de reprodução, o nível de ploidia, números de cromossomas (n e $2n$) e fórmula genómica; i, l) a distribuição total, a distribuição na Itália e a citação dos mapas de distribuição; e, m) algumas notas concisas sobre o habitat e distribuição altitudinal na Itália. Notas sobre a taxonomia, nomenclatura, citologia, geobotânica e outros assuntos são apresentados para algumas famílias, géneros e espécies.

A segunda parte consiste de uma chave dos géneros, espécies e subespécies baseada sobre os caracteres dos esporos. A chave trata exclusivamente das *Pteridophyta italianas* e é principalmente destinada para a identificação dos mencionados taxa. No entanto, representa um bom meio para indicar os caracteres distintivos de géneros, espécies e subespécies e para avaliar as suas afinidades.

A terceira parte do trabalho é um glossário dos termos empregados em pteridologia. Consiste de mais de 142 termos referentes somente aos esporos das *Pteridophyta*. Os nomes correspondentes em latim ou inglês são dados para alguns termos. São apresentados 37 desenhos, mostrando os caracteres gerais dos esporos das *Pteridophyta* e os tipos de ornamentação mais comuns. A lista das publicações mencionadas no texto é dada no fim do trabalho, consistindo de quase 150 referências bibliográficas, tratando dos mais variados assuntos...».

Este importante artigo resultou da colaboração dos 4 autores mencionados no início, mas houve uma certa divisão de trabalho entre eles, o qual é devidamente mencionado. Assim, a PICHI SERMOLLI são devidos: 1) o número que identifica a planta na base de um sistema que se encontra explicado no parágrafo sobre o enquadramento taxonómico das *Pteridophyta* italianas; 2) o nome das entidades estudadas com citação dos nomes do autor ou autores; 3) principais sinónimos limitados aos nomes adoptados nas floras italianas; 4) as indicações relativas ao tipo de gamia, nível e tipo de poliploidia, número cromossómico e eventual fórmula genómica. FERRARINI ocupou-se: 1) das indicações relativas ao exemplar-testemunho, isto é, dados fornecidos nas etiquetas do exemplar escolhido para a fotografia pelo SEM e a medição dos esporos; e 2) as dimensões mínima, média e máxima resultantes da medição de 25 esporos colhidos no exemplar referido. Por sua vez, a CIAMPOLINI ficou-se devendo: 1) a indicação do número das estampas e figuras com a respectiva explicação, incluso a medição das ampliações; e 2) descrição dos esporos ou eventualmente dos microsporos e macrosporos. Finalmente, a MARCHETTI deve-se: 1) a distribuição geral na Itália baseada em pesquisas bibliográficas, no herbário e no campo; 2) o elenco das cartas indicativas da distribuição geográfica, dispostas segundo a ordem cronológica de publicação; e 3) a indicação sumária sobre o habitat e a distribuição altimétrica. Finalmente, existem notas de natureza diversa que levam o nome do autor ou autores que as redigiram.

Desta colaboração resultou, como dissemos, uma obra importante, que vai certamente servir de modelo a muitas outras que serão efectuadas noutros países e das quais resultará um conhecimento muito mais perfeito das *Pteridophyta* de todo o mundo.

A parte taxonómica pode considerar-se perfeita, o que era de esperar em virtude do facto de ela ser essencialmnte tratada por PICHI-SERMOLLI, que poderemos considerar entre os mais elevados expoentes da pteridologia mundial.

As descrições dos esporos são concisas, claras, não muito sobrecarregadas com a terminologia por vezes complexa usada pelos palinologistas. O glossário, acompanhado de muitas figuras elucidativas, presta um valioso auxílio aos não especialistas, que não têm dificuldade em obterem uma ideia clara da forma e da ornamentação dos esporos. Embora os autores (PICHI-SERMOLLI & FERRARINI) não tenham ficado inteiramente satisfeitos com a tentativa que fizeram em elaborar uma chave baseada unicamente sobre os caracteres dos esporos para a determinação dos géneros, e outra para as das espécies e subespécies, porquanto esse objectivo se torna difícil de atingir para o caso dos géneros com muitas espécies, essa tentativa não deixa de mostrar o valor diagnóstico dos caracteres dos esporos. Os autores pensam que essas chaves poderão ser aperfeiçoadas, permitindo que, só por si, sejam suficientes para se atingir o objectivo. De qualquer modo, não há dúvida que o valor diagnóstico dos caracteres dos esporos é grande e que poderão ser usados juntamente com os provenientes de outras fontes de informação, já que o grande objectivo a alcançar é o estabelecimento de uma classificação baseada no conjunto dos caracteres.

CIAMPOLINI merece felicitações pelo trabalho de preparação do material para o SEM e pela quantidade e alta qualidade das fotografias que obteve. É um verdadeiro prazer percorrer as 71 estampas que ilustram esta *Iconographia* e calcula-se o elevado número de fotografias que foi necessário executar para se obterem as imagens para mostrar com clareza a forma (bases, partes apicais, faces, etc.) de tantos esporos, bem como as particularidades das suas ornamentações.

Estão, pois, de parabéns os 4 autores italianos que em boa hora reuniram os seus conhecimentos e aptidões em vários domínios para produzirem uma obra que, como tivemos ocasião de dizer, será considerada um modelo, que será seguido noutros países e de onde resultarão grandes progressos para o conhecimento das *Pteridophyta* e para o estabelecimento da sua classificação natural.

A. Fernandes

Med-Checklist. Inventaire critique des plantes vasculaires des pays circumméditerranéens (1984; 1986)

Dans la notice bibliographique de l'oeuvre en épigraphe, publiée la dernière année dans le vol. LIX, pag. 381, de ce *Boletim*, nous avons fait une lamentable confusion, mentionnant le décès de M. le Prof. H. M. BOURDET, quand, en réalité, nous voulions nous référer à M. le Prof. GILBERT BOCQUET, Directeur du Conservatoire et Jardin Botaniques de la Ville de Genève. Cette confusion a certainement résultée du fait que les deux noms commencent par la même lettre et elle nous semble étrange, puisque nous avons adressé nos condoléances non seulement a l'Institution de laquelle M. le Prof. BOCQUET était le prestigieux Directeur, mais aussi à Madame BOCQUET.

A M. le Prof. H. M. BOURDET, à qui nous souhaitons une longue et heureuse vie pleine de succès scientifiques, nous lui prions de bien vouloir nous excuser de cette confusion que nous regrettons profondément.

A. Fernandes

ÍNDICE

INSTRUÇÕES AOS COLABORADORES

1. O *Boletim da Sociedade Broteriana* é uma revista destinada à publicação de artigos originais em todos os domínios da Botânica. No entanto, artigos muito extensos sobre florística, fitogeografia e fitossociologia são publicados geralmente nas *Memórias,* enquanto que os trabalhos de divulgação científica e os referentes à história da Botânica são reservados para o *Anuário* — as duas outras revistas da Sociedade.

2. Destinado principalmente à publicação dos artigos elaborados pelo pessoal científico do Instituto Botânico de Coimbra, nele se inserem todavia trabalhos da autoria de membros da Sociedade, bem como os de outros investigadores, quer portugueses, quer de outras nacionalidades. A publicação de qualquer artigo, porém, está na dependência de aprovação pela Comissão Redactorial.

3. Os originais entregues para publicação devem ser dactilografados a dois espaços e possuir uma margem da largura habitual. Poderão ser redigidos em português, inglês, francês, alemão, italiano ou espanhol. O nome do autor (ou autores) deverá figurar na primeira página, bem como o endereço da Instituição em que trabalha(m). Um resumo não excedendo aproximadamente 300 palavras, preferivelmente em inglês, deverá iniciar o artigo.

4. Os nomes latinos dos géneros, espécies e categorias infraspecíficas que figurarem no texto devem ser sublinhados uma só vez, enquanto que os nomes dos autores, quando não escritos em maiúsculas, devem ser sublinhados com um traço ondulado. As palavras em negro devem ser sublinhadas duas vezes. Os nomes dos autores citados no texto devem ser seguidos pela data da publicação entre parênteses.

5. No que respeita à ordenação e disposição da bibliografia, seguir as normas utilizadas em um dos volumes recentes desta publicação.

6. As figuras a intercalar no texto, geralmente reproduzidas em zincogravura, não deverão exceder a mancha tipográfica. As estampas *hors-texte* (em regra fotogravuras) serão impressas em papel *couché* e não deverão ultrapassar 13 × 18 cm. Sempre que as figuras sejam de pequenas dimensões, aconselha-se a sua reunião em estampas com as dimensões acima indicadas.

7. Cada autor (ou grupo de autores) receberá 50 separatas grátis, sendo as excedentes que pretender fornecidas ao preço do custo e pagas directamente à Tipografia.

INSTRUCTIONS AUX COLLABORATEURS

1. Le *Boletim da Sociedade Broteriana* est un périodique destiné à la publication d'articles originaux concernant tous les domaines de la Botanique. Cependant, des articles très longs sur floristique, phytogéographie et phytosociologie sont en général publiés dans les *Memórias,* tandis que les travaux de divulgation scientifique et ceux concernant l'histoire de la Botanique sont réservés au *Anuário* — les deux autres revues de la Société.

2. Ayant particulièrement pour but la publication des articles élaborés par le personnel scientifique de l'Institut Botanique de Coimbra, ce périodique publie aussi les travaux des membres de la Société, ainsi que ceux d'autres botanistes, soit portugais, soit de quelque autre nationalité. Toutefois, la publication des articles est sous la dépendance de l'avis de la Commission de Rédaction.

3. Les manuscrits doivent être dactylographiés à deux espaces et avoir une marge. Ils peuvent être rédigés en portugais, anglais, français, allemand, italien ou espagnol. Le nom de l'auteur (ou des auteurs) devra figurer à la première page après le titre du travail, ainsi que l'adresse de l'Institution où il(s) travaille(nt). Un résumé ne dépassant pas 300 mots, de préférence en anglais, devra ouvrir l'article.

4. Les noms latins des genres, des espèces et des catégories infraspécifiques devront être soulignés une fois, tandis que les noms des auteurs, quand non dactylographiés en lettres majuscules, doivent être soulignés par une ligne ondulée. Les noms des auteurs cités dans le texte doivent être suivis de la date de la publication mise entre parenthèses.

5. En ce qui concerne la bibliographie, voir un des volumes récents du *Boletim*.

6. Les figures du texte, en général des dessins à l'encre de Chine, ne doivent pas, avec les légendes, dépasser 10,5 × 18 cm. Les planches hors-texte ne devront pas dépasser 13 × 18 cm. Les figures à petites dimensions doivent être réunies dans des planches aux dimensions ci-dessus mentionnées.

7. Chaque auteur (ou groupe d'auteurs) recevra 50 tirages à part gratuits, tandis que ceux excédant ce nombre lui seront fournis au prix du coût et devront être payés par l'auteur directement à l'Imprimerie.