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Structural and biochemical studies of the primary nucleus of two green algal species, Acetabularia mediterranea and Acetabularia major

Strukturelle und biochemische Untersuchungen des Primärkerns der Grünalgen Acetabularia mediterranea und Acetabularia major

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Abstract

Nucleolus – electron microscopy – Acetabularia – transcription – gene activity – ribosomes

Primary (giant) nuclei of the green algae *Acetabularia mediterranea* and *A. major* were studied by light and electron microscopy using *in situ* fixed material as well as manually isolated nuclear components. In addition, cytochemical reactions of nuclear structures and biochemical determinations of nuclear and cytoplasmic RNA and of genome DNA content were performed. The data obtained and the structures observed are interpreted as demonstra-

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[1]) Prof. Dr. Werner W. Franke, Division of Membrane Biology and Biochemistry, Institute of Experimental Pathology, German Cancer Research Center, D-6900 Heidelberg, Im Neuenheimer Feld 280, Bundesrepublik Deutschland. – Some results obtained in the course of this study have been presented in preliminary notes (W. W. Franke, E.-D. Jarasch, J. Kartenbeck, U. Scheer, J. Stadler, M. F. Trendelenburg, and H. Zentgraf): Zelldifferenzierung und Kern-Cytoplasma-Interaktion. Jahresbericht 1973 des Sonderforschungsbereiches "Molekulare Grundlagen der Entwicklung", SFB 46 der Deutschen Forschungsgemeinschaft, Freiburg i. Br., pp. 54–62; M. F. Trendelenburg, H. Spring, U. Scheer and W. W. Franke: Morphology of nucleolar cistrons in a plant cell, *Acetabularia mediterranea*. Proc. Nat. Acad. Sci. USA **71** (1974) 3626; and at the "Third meeting of the international research group on Acetabularia" held at Paris, July 11th to 12th 1974 (M. F. Trendelenburg, H. Spring, U. Scheer and W. W. Franke: Demonstration of active nucleolar cistrons of the *Acetabularia* primary nucleus with the spreading technique; W. W. Franke, S. Berger, U. Scheer and M. F. Trendelenburg: The nuclear envelope and the perinuclear lacunar labyrinthum); and at the 9th FEBS meeting in Budapest, 1974, August 25th to 30th (M. F. Trendelenburg, H. Spring, U. Scheer, and W. W. Franke: Visualization of the nucleolar cistrons in plant cells Abstr. Commun. S3 C5, p. 128).

tions of transcriptional activities of different gene classes. The most prominent class is the nucleolar cistrons of precursors of ribosomal RNA which occur highly repeated in clusters in the form of regularly alternating intercepts on deoxyribonucleoprotein axes of transcribed rDNA, the fibril-covered matrix units, and the fibril-free "spacer" segments. A description and a classification of the various structural complexes which seem to represent transcriptional activities is given. Quantitative evaluations of these arrangements are presented. The morphology and the dimensions of such structures are compared with the RNA molecular weight determinations and with the corresponding data reported from various animal cell systems.

It is suggested that the formation of the giant nucleus is correlated with, and probably due to, an enormous amplification of transcriptionally active rDNA and packing of the extrachromosomal copies into the large nucleolar aggregate bodies.

Introduction

In the plant kingdom some Dasycladacean green algae, the most prominent genus being *Acetabularia*, exhibit, upon germination of the zygote, a dramatic growth of the nucleus (for reviews see [53, 69, 74, 145]). This process results in the formation of a giant nucleus (up to 100 µm in diameter or even larger) which is associated with a unique perinuclear membrane complex ([32, 53, 177, 182]; see these articles for further references). This increase in nuclear size is concomitant with the development of a large aggregate of nucleolus-like structures, corresponding to an increase in total apparent nucleolar volume by about 100-fold (c. f. [8, 164]), an increase in the total number as well as in the package density of nuclear pore complexes [53, 189], the appearance of small distinct intranuclear structures perhaps of chromosomal nature ([4]; see also below), and the formation of numerous (several thousand) perinuclear dense bodies [13, 14, 186]. It is obvious that this giant primary nucleus has to fulfill special functional requirements since it has to provide most of the protein synthetic machinery necessary to form the extremely large and morphologically complex plant [69]. Several earlier articles have dealt with structural aspects of this giant primary nucleus. The present study presents results of an attempt to describe comprehensively the nuclear components by combined structural, cytochemical and biochemical methods, with special emphasis on the appearance of nuclear contents in spread and positively stained preparations.

Material and methods

Materials

Acetabularia mediterranea cells were germinated and cultured in a 12:12 hours darklight rhythm in "Erdschreiber" medium according to the procedures described by Hämmerling [72], Beth [7], Lateur [99] and Lateur and Bonotto [100]. In most experiments the algae were used after attaining a total stalk length of about 3 cm. At this length, the algae were shortly before or at the onset of cap formation. Almost fully grown *Acetabularia major* cells (4 to 8 cm in total stalk length; for a general cytological description of this species see [155]) were kindly provided by Drs. S. Berger and H. G. Schweiger (Max-Planck-Institute for Cell Biology, Wilhelmshaven, Federal Republic of Germany). In addition, 20 to 30 mm large cells of *A. mediterranea* grown in the laboratories of Drs. J. Brachet (Université Libre de Bruxelles, Belgium) and H. G. Schweiger (Wilhelmshaven) were used in a variety of experiments. "Regenerating" plants were obtained according to the methods of Hämmerling [70, 71, 73] and Shepard [158]. In some special experiments "aged" plants (i. e. at the time of

"bleaching" of the rhizoid and lower stalk part) were used for preparation of "old" primary nuclei (c. f. also [4]). Preencystment caps containing secondary nuclei were fixed with the procedure previously described [4, 50, 53]. Cysts were collected as described elsewhere [81]. Swarming gametes as well as the negatively phototactic zygotes were obtained and collected by light-trapping as described by SHEPARD [158].

Chemicals and radiochemicals

All chemicals used were of reagent grade and purchased from Merck (Darmstadt, Federal Republic of Germany) or Serva Feinbiochemica (Heidelberg, Federal Republic of Germany), if not otherwise indicated. Pancreatic ribonuclease and pronase (free of nucleases, Calbiochem,

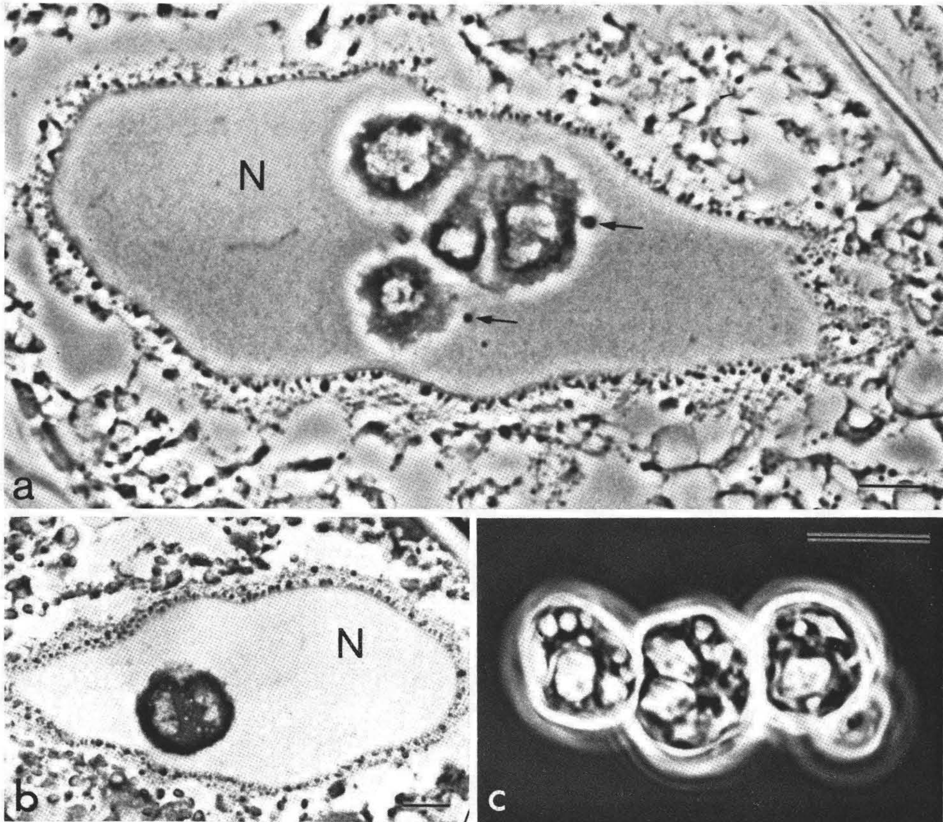


Fig. 1 a to c. Survey light micrographs of *Acetabularia mediterranea* nuclei (N) fixed *in situ* (**a** *and* **b**) and of isolated nucleolar aggregates (**c**). Figures **a** and **c** are in phase contrast, **b** shows the appearance after staining with gallocyanine. Note the large aggregates of nucleolar material in the relatively homogeneous nucleoplasm and the numerous perinuclear bodies. Both these structures, the nucleoli and the perinuclear bodies, react positively with gallocyanine, with or without (**b**) a preceeding hydrolysis (for details see Materials and methods). Figure **c** shows aggregates of isolated spherical nucleolar subunits as they were used in the spread preparations. Note the cavern system within the interior of the nucleolar subunits. – The scale indicates, in all three micrographs, 10 μm.

San Diego, California, U.S.A.) were prepared as described earlier (c. f. [49]). ^{3}H-uridine (26 Ci/mmol) and ^{32}P-phosphate (50 to 100 Ci/mg P) was from the Radiochemical Centre (Amersham, England), and ^{3}H-thymidine (20 Ci/mmol) was from NEN (Boston, Massachusetts, U.S.A.).

Isolation of nuclei and nuclear components

The rhizoids of 30 to 40 mm large cells were dissected from the stalk and the nuclei were squeezed from the rhizoids in a small Petri dish containing the specific isolation medium (see below). The isolation and "nuclear wash" procedures were monitored with a dissecting microscope at a magnification of 50 x. The released nuclei were washed several times by sucking up and down in a micropipette in isolation medium. Isolation media were either the sucrose and Mg^{++}-containing medium recommended by Brändle and Zetsche [15] or, for spread preparations, media containing 0.083 M KCl and 0.017 M NaCl ("5:1 medium" after Callan and Lloyd, [23]; see also [175]), or 0.1 M KCl (see [125, 127, 128]), or 0.1 M NaCl, or 0.1 M CsCl. The best results were obtained, in our hands, with the "5:1 medium". For isolation of total nuclear contents and nucleoli, the nuclei were transferred with a micropipette onto a silicone-coated slide. Under observation with a dissection microscope the nuclear envelopes were disrupted with microneedles, and the whole content or the individual nucleolar components were collected with the aid of a micropipette.

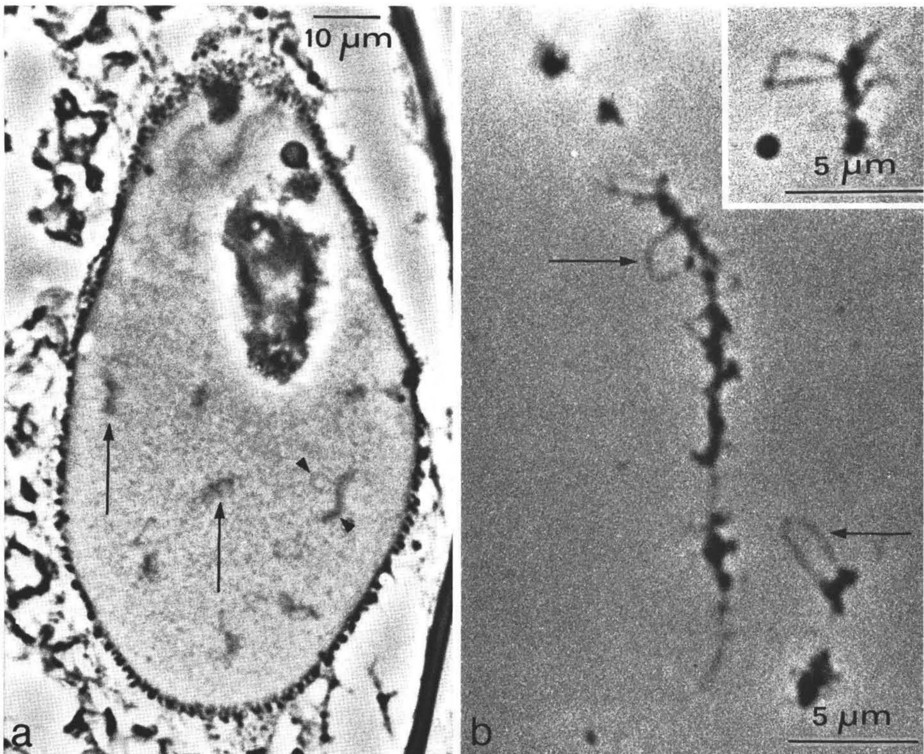


Fig. 2 a and b. Phase contrast light micrographs showing the "lampbrush-chromosome-like" structures *in situ* (**a**, some are denoted by arrows), and after isolation (**b**, for details of preparation see Methods). Characteristic loops are recognized in Figure **b**, especially in the insert. – The scale in **a** indicates 10 µm, the two scales in **b** indicate 5 µm.

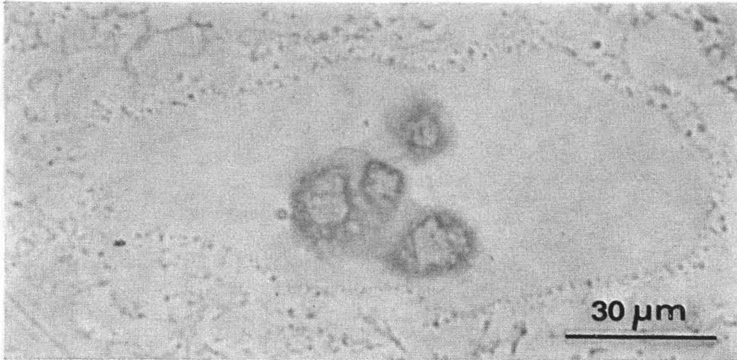


Fig. 3. Bright field micrograph of a 1 µm thick section through an *Acetabularia mediterranea* primary nucleus fixed *in situ* and stained by the Feulgen reaction as described in Methods. The nucleolar aggregates are clearly positively stained, especially in their cortical regions; the nucleoplasm reveals only a very faint colorization, which, however, is lost in this color print. Sometimes one gains the impression of a faintly positive reaction in the perinuclear dense bodies. The scale indicates 30 µm.

Nuclei from regenerating plants were collected in the same manner at different times after removal of the greater part of the stalk.

Light microscopic preparations of nuclear components

Liberated nuclear components were observed, using phase contrast optics (Zeiss photomicroscope III) in flat microchambers, in hanging drop arrangements, and in squash preparations. Permanent mounts of spread nuclear contents were prepared according to the technique described by GALL [54] for visualization of amphibian lampbrush chromosomes.

Spread preparations for electron microscopy

Whole nuclear contents or the liberated nucleoli and chromosome like material, respectively, or isolated nuclear envelope "ghosts" were transferred into a drop of distilled water adjusted to *p*H 9 with sodium-borate buffer at 12° C. Usually the resulting buffer concentration was 0.1 mM sodium borate. The incubation times were between 5 and 15 minutes, times between 10 and 15 minutes resulting in a more favorable dispersion of the nucleoprotein structures. The drop with the dispersed material was then processed and stained as described by MILLER and BEATTY ([127, 128]; see also [125, 150, 175]).

Thin section preparations

Fixation, dehydration and embedding procedures were as described earlier [53]. Ultrathin sections for electron microscopy were prepared using diamond knives from Rondikn (Honolulu, Hawaii, U.S.A.) with a Reichert ultramicrotome OMU3 (Vienna, Austria). For light microscopic studies and for orientation during search of the nuclei 1 to 2 µm thick sections were made with glass knives. The sectioning was routinely monitored in the light microscope using phase contrast optics (Zeiss photomicroscope III, Carl Zeiss, Oberkochen, Germany).

Electron microscopic observation and evaluation of micrographs

Electron micrographs were made with a Siemens Elmiskop IA or 101 or with a Zeiss EM 10 at 80 kV (with sections) or at 60 kV (with spread preparations). For quantitative work and screening, use was made of the precisely controlled and quick specimen stage transport system of the Zeiss EM 10. Length and contour length measurements were performed as

described previously [37, 49, 150, 173 to 175]. Relative ratios of total nuclear and total nucleolar volumes were estimated in randomly chosen, non-oriented sections from twenty different primary nuclei by using point-lattice methods [161, 179, 180]. Nucleolar volume was defined by virtually drawing the contour lines circumscribing "zone C" structures (for definition and explanations see Results).

Cytochemicals reactions in thin sections

For cytochemical observations we used dissected rhizoids, whole cells, cysts, gametes or zygotes which were fixed solely in 2.5 % glutaraldehyde in 0.05 M sodium cacodylate buffer, *p*H 7.2, for 2 hours at room temperature. After dehydration in a series of graded ethanol solutions and finally in propylene oxide the objects were embedded in Epon (c. f. [53]). 1 to 2 μm thick sections through the nuclei and the perinuclear zone were transferred onto glass slides. Prior to some reactions such as the staining with acridine orange, ethidium bromide, and gallocyanine-chloride, the epon resin was dissolved from the sections with sodium methoxide according to MAYOR *et al.* [119].

Acridine orange. A 0.01 % solution of acridine orange was prepared in 0.1 M Michaelis veronal acetate buffer (*p*H 7) or in 0.06 M Sørensen phosphate buffer (*p*H 6). The de-eponized sections (see above) were stained for 5 minutes with the dye, rinsed with distilled water, and the retained stain was differentiated for 1 to 5 minutes with 0.1 M $CaCl_2$ made up in distilled water. Observations were made in the light microscope using fluorescence illumination.

Ethidium bromide. For staining with the ethidium bromide the de-eponized sections were first hydrolyzed for 15 minutes with 1 N HCl at 60° C. After rinsing with cold distilled water the slides bearing the sections were immersed in a Tris-HCl buffer solution (0.18 M, *p*H 7.5) containing 40 μg/ml ethidium bromide (Sigma, St. Louis, U.S.A.). After 20 minutes of incubation the slides were rinsed again with distilled water and observed in the fluorescence microscope.

Gallocyanine-chloride. Staining with gallocyanine-chloride [39] of de-eponized sections with or without a preceeding hydrolysis (described below) was performed as outlined by RUTHMANN [147].

Feulgen-reaction. For the DNA-specific staining reaction according to FEULGEN and ROSSENBECK [41], 1 to 2 μm thick sections were used with or without previous removal of the epon resin. Both methods gave essentially identical results. The sections were hydrolyzed with 1 N HCl at 60° C for different times in the range of 5 to 60 minutes, and the staining conditions were as described by GRAUMANN ([63], see also [146]). The maximum differential hydrolysis was obtained between 15 and 20 minutes. Attempts to perform the Feulgen-reaction with smears and squashes of rhizoidal contents containing the nucleus after fixation in ethanol:acetic acid (90:10 v/v) failed.

Bernhard's procedure. For electron microscopic identification of deoxyribonucleoprotein we applied Bernhard's differential bleaching method [5] using material fixed only in glutaraldehyde (see above). Staining was performed with 4 % aqueous uranyl acetate for 10 minutes. The time range for the incubation of the sections in the neutral EDTA-solution (0.2 M) was varied between 10 and 35 minutes (see also [45, 53]). After the differential bleaching and an intermediate rinse with distilled water the sections were briefly poststained with lead citrate [141].

Preparation of microsomal RNA

For each experiment 200 plants at the onset of cap formation were used, i. e. under our laboratory conditions at an average of about 85 days after zygote germination. The plants were immersed in about 100 ml of ice-cold isolation medium (0.1 M Tris-HCl, *p*H 7.4, 5 mM $MgCl_2$, 0.4 M sucrose) and were immediately cut into about 2 mm long pieces with fine scissors. Care was taken in all preparative steps that the temperature did not rise above 5° C. The suspended mince was then vigorously homogenized first with a Dounce-type homogenizer and then by ten strokes with a tightly fitting glass-Teflon motor driven Potter-Elvejhem homogenizer. The homogenate was filtered through a double layer of nylon

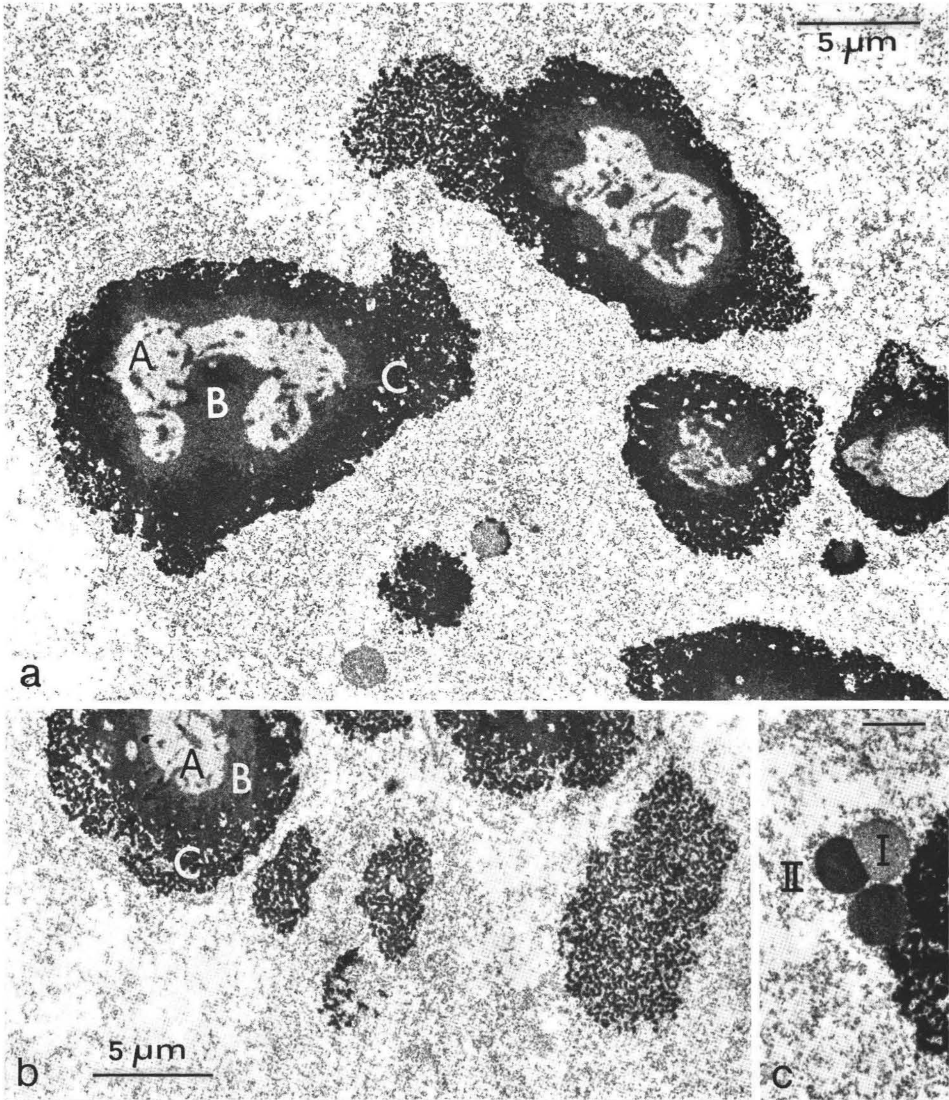


Fig. 4 a to c. Details of the nucleolar aggregates as revealed in ultrathin sections of *in situ* fixed primary nuclei of *Acetabularia mediterranea*. Note the composition of the individual nucleolar subunits in a relatively electron translucent core (A), which is surrounded, penetrated and traversed by dense material of a shell zone (B), which again is ensheathed by a very dense cortex of granulofibrillar material (C). **b** Shows various grazing sections through these nucleolar cortices. Associated with the nucleolar periphery are sometimes small spherical structures up to 1 µm in diameter, which are either relatively weakly stained (I) or electron dense (II). Note the relatively homogeneous distribution of fibrillar material, perhaps representing dispersed chromatin, in the nucleoplasm. – The scales in **a** and **b** indicate 5 µm, the scale in **c** indicates 1 µm.

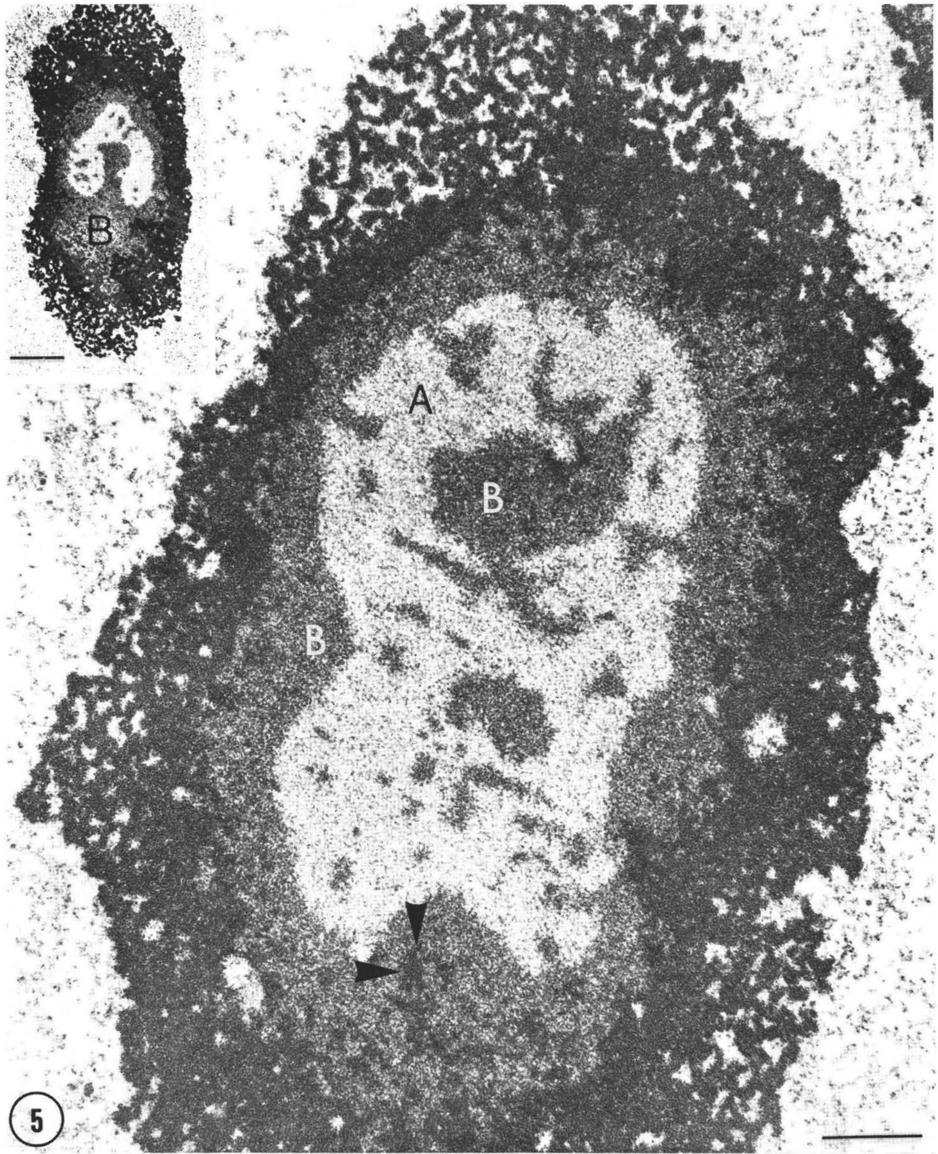


Fig. 5. Ultrastructural details of the composition of the nucleolar subunits (see the previous Figure) as revealed at higher magnification. Note the finely fibrillar texture in the internal ("light") zone A, and the dense package of small granulofibrillar structures in zone B, extensions from which deeply penetrate zone A (see also the *insert*), and the very dense package of granules in the cortical zone C, from which also ramifications project into zone B (some of which are denoted by the two black arrowheads in the bottom part). – The scale indicates 1 μm, the scale in the *insert* denotes 2.5 μm.

cloth (Schweizer Seidengazefabrik, Zürich, Switzerland; mesh size 25 to 65 μm), and the filtrate was adjusted with 70 % (w/v) sucrose to a final concentration of 1 M. The pellet obtained upon 10 minutes centrifugation at 5000 x g in a cooling centrifuge (Minifuge, Christ-Heraeus, Osterode/Harz, Federal Republic of Germany) was rehomogenized in isolation medium containing 1 M sucrose and again centrifuged under the same conditions. The final pellet, consisting mainly of plastids, wall fragments, nuclei, mitochondria and debris, was stored frozen or immediately precipitated (see below). The combined supernatants of this and the previous centrifugation were then spun for 6 hours at 110 000 x g in an ultracentrifuge (P 65, Weinkauf-Hitachi, Brandau b. Darmstadt; or L 2, Beckman-Spinco, München, Federal Republic of Germany). The pellet obtained was designated "total microsomal pellet". The "final supernatant" was dialysed against 10 mM Tris-HCl, 3 mM $MgCl_2$, *p*H 7.0, and was processed for analysis. All fractions were either directly used for extraction of nucleic acids (see below)

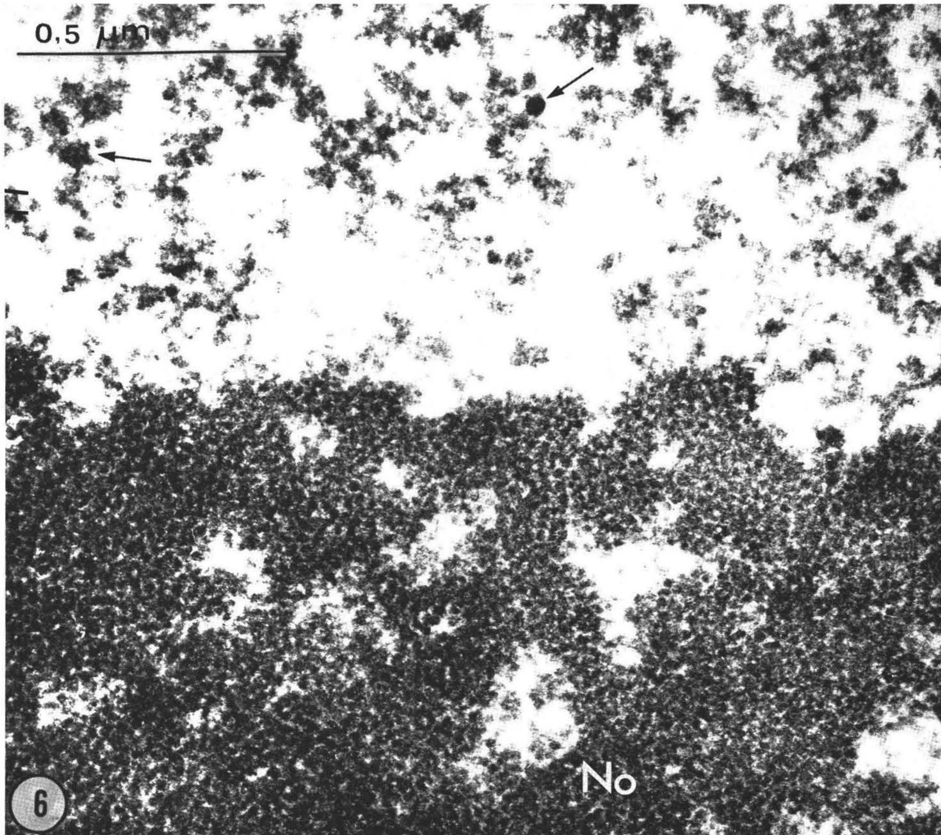


Fig. 6. Details of the nucleolar (No) cortex region (zone C) at higher magnification. The individual densely packed granules constituting this zone are identified. Note that these nucleolar cortex granules are by far smaller than the previously described (ref. [53], see also the following Figure) electron dense granules which occur in the nucleoplasm and are especially frequent in association with the nuclear pore complexes. Some of such granules are denoted by small arrows in the upper part. – The scale indicates 0.5 μm.

or were precipitated in cold 5 % trichloracetic acid (TCA) and washed three times in the same acid solution, each time being collected by repeated centrifugation in the cooling centrifuge.

Isolation and analysis of nucleic acids

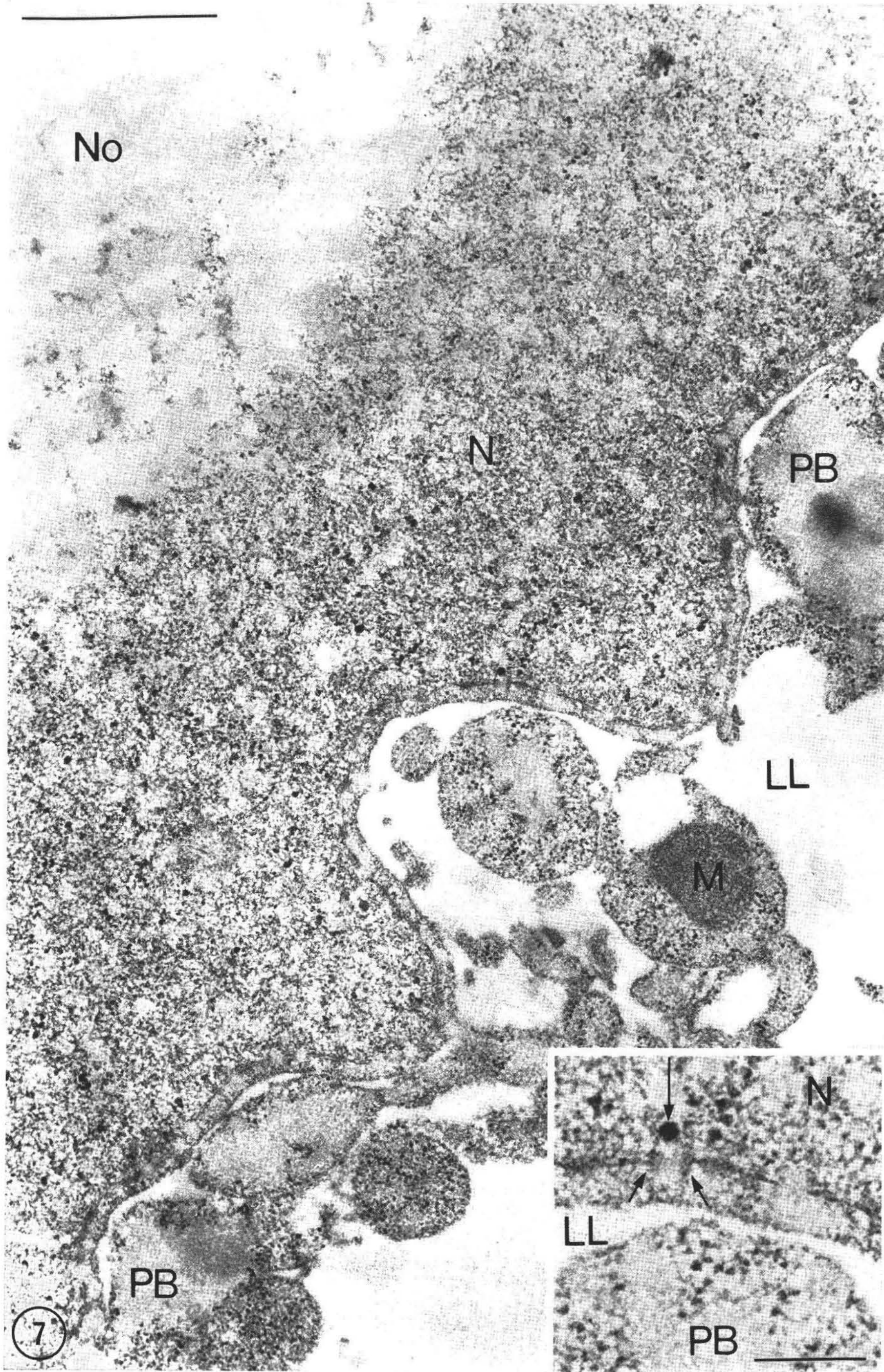
RNA and DNA were isolated from cell fractions, from whole cells, or from isolated primary nuclei. In the latter case the nuclei were collected and stored in ice-cold 70 % ethanol-acetic acid (3:1). DNA isolation followed the modified procedure of Marmur [117] as described elsewhere [49] or the Sarkosyl-CsCl method outlined by Gall et al. ([56]; see also [57]). For RNA extraction the cell fractions were homogenized at 4° C in Tris-HCl (0.05 M, *p*H 7.6) containing 1 % NaCl and 2 % (w/v) sodium tri-isopropyl naphthalene sulphonate with a motor driven Potter-Elvejhem homogenizer and shaken with an equal volume of phenol/cresol/8-hydroxyquinoline. Further processing was as described by Loening [107]. To the nuclear pellet, drained of ethanol, 0.5 ml of 0.02 M Tris-HCl (*p*H 7.4) containing 0.5 % SDS and 1 mg/ml self-digested pronase (RNAse free; CalBiochem) was added. After 10 minutes at room temperature 20 µg of *E. coli* RNA was added and the RNA was precipitated by adding NaCl, to a final concentration of 0.1 M, and 2.5 vol absolute ethanol. After storage at − 20° C the pellet was resuspended in 50 mM Tris-HCl buffer (*p*H 7.4) containing 2 mM Mg-acetat and 10 µg/ml DNAse (RNAse-free, Worthington, Freehold, New Jersey, USA) and incubated for 10 minutes at room temperature. RNA was again precipitated by adding 2.5 vol of ethanol and storage at − 20° C. The pellet was then homogenized in a small Potter-Elvejhem homogenizer in a total volume of 1 ml triisopropyl naphthalene sulphonate solution and extracted with phenol/cresol as described above.

Gel electrophoretic analyses were carried out under nondenaturing conditions either in 2.25 % acrylamide-0.5 % agarose composite gel slabs [143, 150] or in tubes containing 2.4 % acrylamide gels [107] with the buffers indicated by these authors. Gel electrophoresis under denaturing conditions was performed in formamide [163] according to the modified procedure of Gould and Hamlyn [62]. In all cases *Escherichia coli* rRNA was run on the same gel as reference for molecular weight determinations. The gels were scanned at 260 nm in a Gilford Spectrophotometer 2400 S (Oberlin, Ohio, U.S.A.) equipped with a linear gel transport. The formamide gels had to be washed for several hours in distilled water before scanning of UV absorption.

Determination of haploid and diploid DNA contents

Gametes or zygotes, after immobilization of the cells by a brief temperature shock at 55° C, were counted in a Neubauer cell counting chamber or in a Coulter electronic particle counter. An aliquot was then analyzed for phospholipids, protein, DNA, and RNA as described in the following section. The contents determined were corrected for mononuclear and binuclear (and/or diploid) algae from the ratio of bi-to-tetraflagellate swarmers which was determined in an aliquot sample.

Fig. 7. Appearance of the primary nucleus of *Acetabularia mediterranea* and the perinuclear region as revealed after application of Bernhard's differential staining and bleaching procedure (for details see Methods). Note the disappearance of stain in the nucleolus (No) and the inner part of the perinuclear bodies (PB), in contrast to the retention of uranyl stain in other structures, such as the granulofibrillar nucleoplasmic ground substances (N), the extensions of such granulofibrillar strands into the nucleolar (No) interior, the dense large nucleoplasmic granules (shown in detail at partial magnification in the insert, denoted by an arrowhead), with the nuclear pore complexes, and in the cytoplasmic ribosomes. – Scale indicates 1 µm in the large picture and 0.25 µm in the insert.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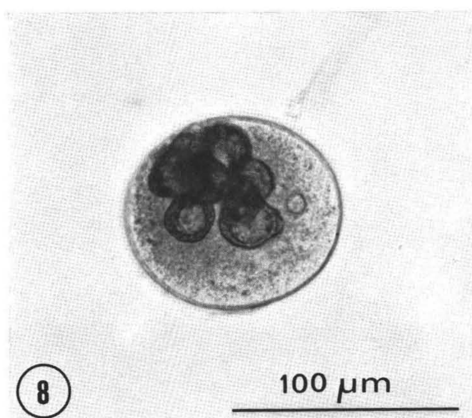


Fig. 8. Typical appearance of a primary nucleus of *Acetabularia mediterranea* isolated from the rhizoid and visualized by phase contrast light microscopy. Note the predominance of three structures, the nuclear envelope, the nucleoplasm, and the nucleolar aggregates with their lighter core regions. – Scale indicates 100 μm.

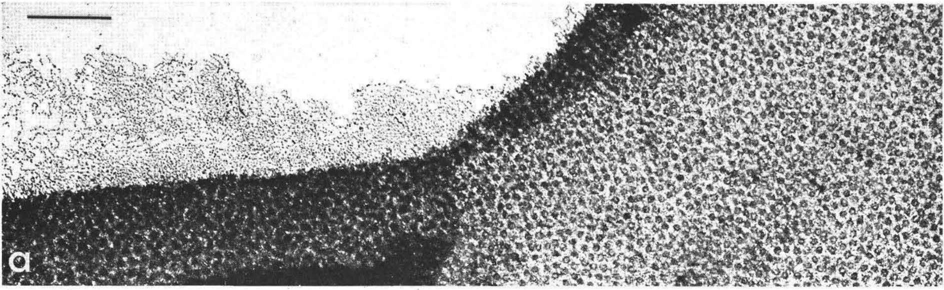
Chemical determinations

Nucleic acids were determined by their UV absorbance at 230, 260 and 280 nm wavelengths, using either the isolated nuclei acids purified as described above or the nucleoside-containing acidic hydrolysate (see below), by colorimetry using the orcinol [120] and the diphenylamine [20] reaction, respectively, and by phosphorus determination after differential alkaline hydrolysis with 0.4 M KOH for 20 hours at 37° C according to the method of SCHMIDT-THANNHAUSER [153]. Phosphorus was determined according to GERLACH and DEUTICKE [59]. For protein determinations the Nessler reagent [165] was found to be more reliable for this material than the procedure of LOWRY *et al.* [111]. Phospholipids were estimated from the determination of methanol-chloroform extractable phosphorus using a mean molecular weight of 770 D. Sequential determinations of phospholipids, RNA, DNA and protein were performed as described in previous publications [38, 47].

Incorporation of radioactive nucleotides

Zygotes, germlings of 1 mm length and almost fully grown plants (ca. 30 mm in *A. mediterranea*) were incubated in "Erdschreiber" medium containing tritiated uridine or thymidine or ^{32}P-phosphate (see "Chemicals and Radiochemicals"). In order to obtain high incorporation the radioactive compounds had previously been concentrated with a rotary evaporator to a total volume of about 0.02 ml and diluted in "Erdschreiber" medium resulting in a final volume of 3 ml. The effective precursor radioactivities then were 1.6 mCi in 3 ml for ^{3}H-uridine and 6 mCi in 3 ml for ^{3}H-thymidine. Incubation times were either 30 minutes or 6 hours. After incubation the plants were intensively washed with "Erdschreiber" medium and either fixed for autoradiography as described above or were used for isolation of primary nuclei or microsomes and nucleic acid extraction.

Fig. 9 a and b. Manually isolated nuclear envelope of *Acetabularia mediterranea* which shows a sequence of overfoldings (I to IV, in **b**). Note the high package density of nuclear pore complexes and the association with some fibrillar material as identified in the upper region of **a.** – The scale in (a) denotes 1 μm, the scale in (b) denotes 10 μm.


a
10 μm
II
IV
I
III
b

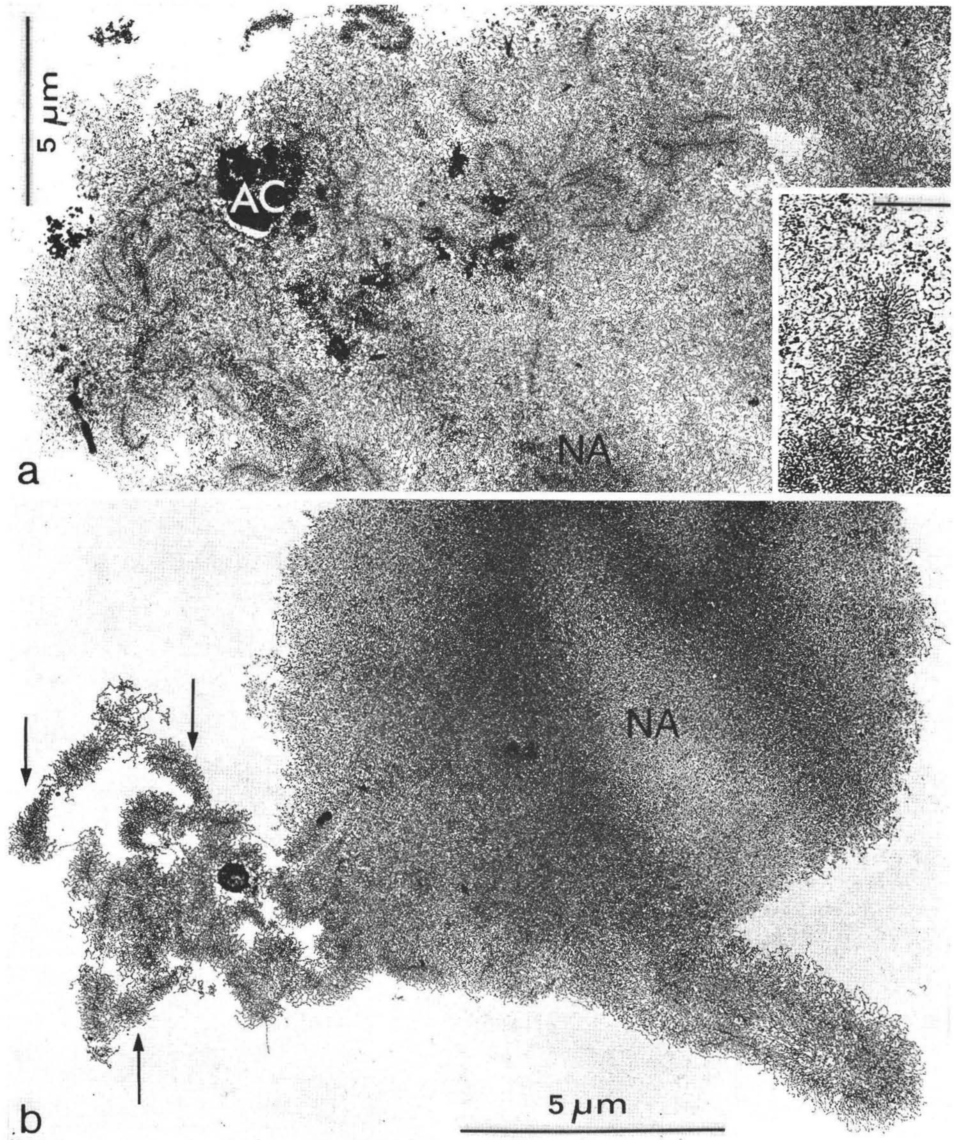


Fig. 10 a und b. Moderately spread and positively stained nucleolar aggregate (NA) subunits isolated from *Acetabularia major*. The generally finely fibrillar subarchitecture of these aggregates is evident. In some regions one notes very densely stained aggregate clumps (AC) of variable diameters and the fibril covered intercepts on the DNA-containing core strands (for detailed interpretation see text and the following Figures). This situation is illustrated in greater clarity at partial magnification in the *insert* of **a**. The composition of long axial fibrils with intercepts associated with lateral fibrils of increasing length is frequently revealed in more detail in the peripheral regions of these aggregates as denoted by the arrows in the left part of both Figures. – The scales indicate 5 µm; the scale in the *insert* of **a** denotes 1 µm.

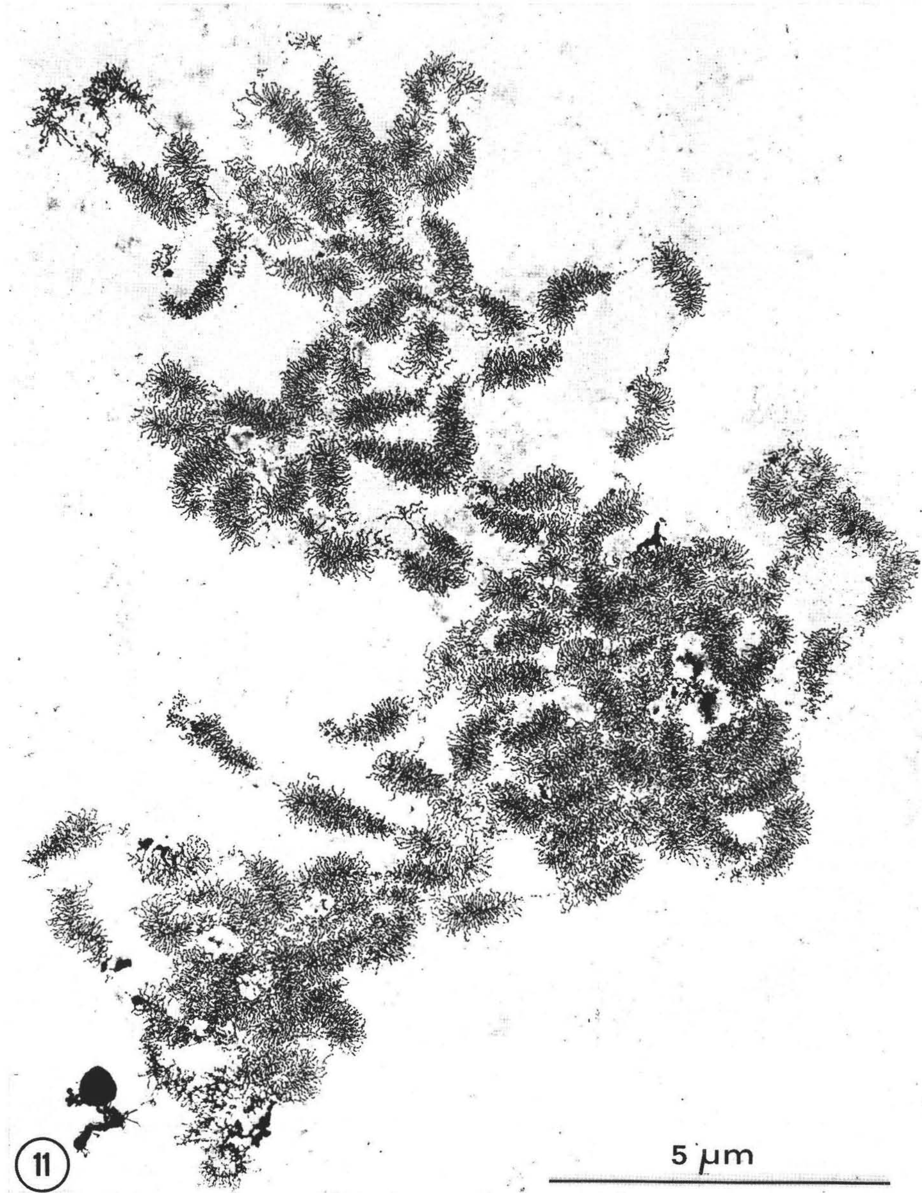


Fig. 11. More intensively spread complete nucleolar aggregate subunit of *Acetabularia mediterranea* (for details of preparation see Methods). Note the numerous fibril-covered regions ("matrix units") which occur in one such unit. – The scale indicates 5 µm.

Autoradiography

For autoradiography 1 to 2 μm thick sections were coated with the Kodak emulsion NTB-3 (Kodak, Rochester, N. Y., U.S.A.). After different times of incubation the silver grains were developed with D-19.

Results

Light microscopic observations

In the later stages of its development, the primary nucleus of both species of *Acetabularia* studied is characterized by its large size (Figs. 1, 2, 3, 8; c. f. [53, 69, 154, 155]), a rather homogeneous appearance of the nucleoplasm ("nuclear sap"), and the appearance of up to ca 20,000 perinuclear bodies (see Figs. 1 a, 1 b, 7; refs. [13, 32, 53, 177]). Its most prominent structures are the nucleolus-like globules, which are often aggregated into large (up to 80 μm in diameter) bodies, and which in later stages of nuclear maturation even tend to fuse into one condensed giant ball (for detailed description and discussion of such later stages of nuclear maturation see [4]). Each of these nucleolar components is characterized by a central, relatively light core region, which sometimes appears to be subdivided in a system of cavities ("vacuolization") as is especially apparent after isolation of the nucleoli and their subunits (Fig. 1 c; ref. [175]), and a denser shell region ("cortex"). The appearance of the nucleoli, especially that of the isolated nucleolar subunits, is highly reminiscent of nucleolar descriptions in insect oocytes [2, 26] and, in particular, in certain stages of amphibian oogenesis (e. g. [22, 97, 123]; see in particular Fig. 2 of ref. [129]). In some sections the very periphery of the nucleolar bodies seemed to be constituted by another, less dense and more diffuse sheath, in which occasionally smaller spherical structures are visualized (Fig. 1 a; for further details of nucleolar fine structure see [4]). In some but not all primary nuclei we noted small (about 1 μm broad) individual strands of dense material, which frequently revealed looplike lateral protuberances (Fig. 2 a). These are more clearly recognized in the squash and spread preparations of nuclear materials (Fig. 2 b) and look like typical lampbrush chromosomes. The overall shape of the giant nucleolar aggregates was highly variable in both species in different primary nuclei. Two major forms, the sausage-shaped coil configurations (see e. g. [175], see also [182]) and more compact globules (Fig. 8), could be distinguished. Upon disruption of the nuclei during the isolation procedure the nucleolar aggregates tend to progressively fragment into smaller spherical subunits (Fig. 1 c; see also [175]). However, it was difficult to determine exact numbers of the ultimate constituent nucleolar subunits. In some nuclei we were able to count up to 130 (in *A. mediterranea*) and 160 (in *A. major*) of such spherical subunits of diameters in the range of 5 to 10 μm. Other authors (e. g. [164]) have reported the existence of up to 30 subunits. In other nuclei, the larger

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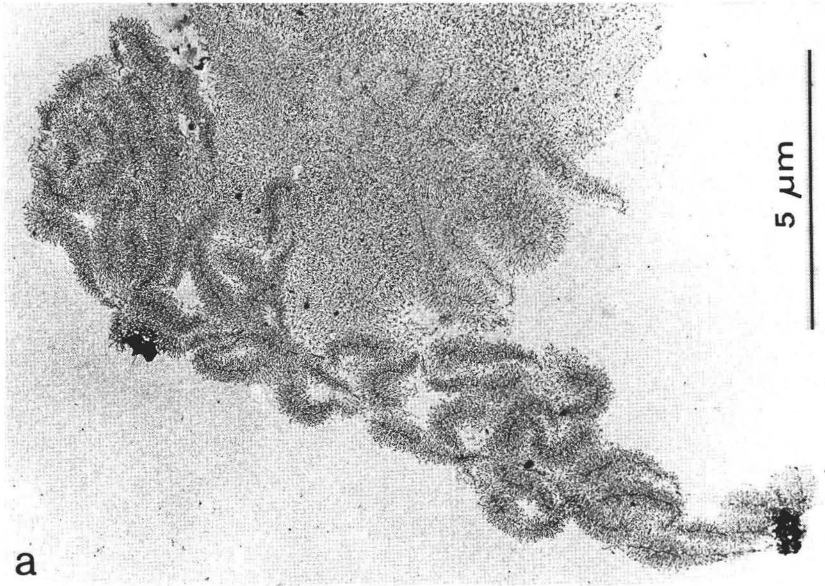
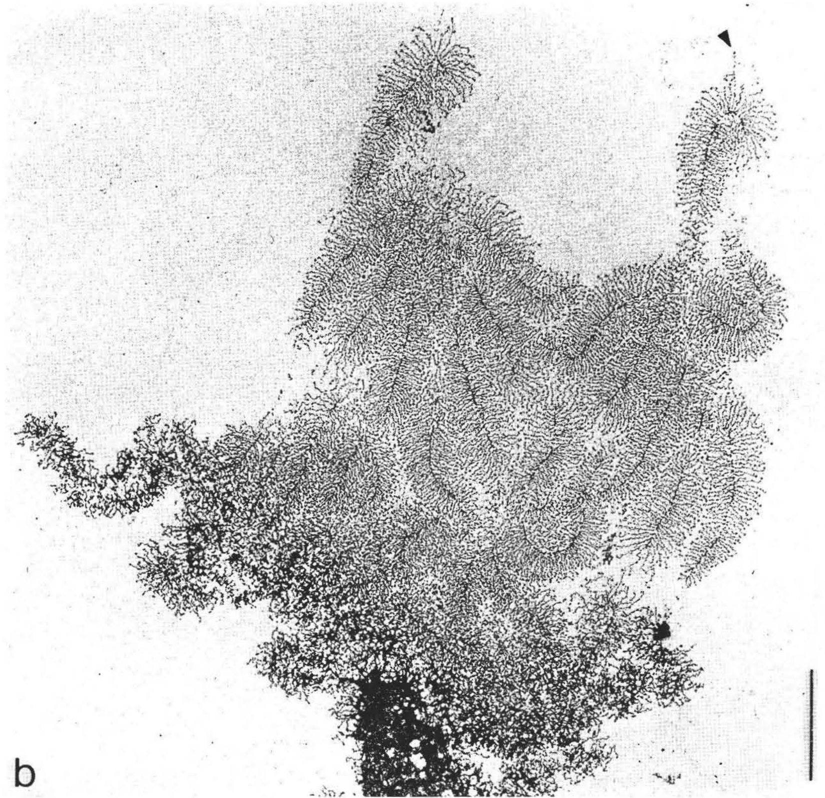
Fig. 12 a and b. Moderately spread nucleolar aggregate material of *Acetabularia mediterranea* (for details of preparation see Methods). Note the numerous fibril-covered regions **b** reveals, within one such aggregate subunit, a gradient of spreading intensity (from bottom to top) which might be artifical but might also represent *in vivo* differences. Note, in the upper right corner of **b**, an example of an unusually unravelled lateral fibril (arrowhead). – The scale in **a** indicates 5 μm, the scale in **b** 1 μm.


a
5 μm


b

nucleolar aggregates were, for unknown reasons, much more stable and fewer but larger subunit corpuscules were found. We gained the impression that the "sausage-shaped" nucleolar aggregates were more readily fragmented into their subunit corpuscules than the large spherical nucleolar bodies. The occurrence of numerous nucleolar bodies in a large nucleus is not without parallels in other cell systems. It is common, for instance, in oocytes of various amphibia and insects (e. g. [9, 22, 40, 95, 96, 113, 123, 178, 185]), in which they occur either individualized and dispersed, or aggregated into one or a few larger bodies [2, 3, 9, 103, 105]. Since it is known that dynamic changes in the state of aggregation of nucleolar structures can take place in the course of developmental processes and/or in response to specific physiological conditions (e. g. [22, 82, 97, 114, 148]), much significance cannot be ascribed to countings of nucleolar units identifiable at a certain stage as correlating to the numbers of nucleolar (rRNA) genes present (see, however, [18]). All three structures, the nucleoli, the nucleoplasmic loop containing strands and the perinuclear bodies are stained with toluidine and methylene blue [4], thus indicating the presence of acidic macromolecules. These structures, in particular the nucleoli and the perinuclear bodies, also react positively with gallocyanine-chloride (Fig. 1 b) and the staining was reduced in intensity after hydrolysis but did not completely disappear. After application of acridine orange dye to thin sections (without a preceeding hydrolytic treatment) the nucleoplasm revealed a homogeneously greenish fluorescence, the perinuclear bodies were markedly green fluorescent (for details see [162]), and the nucleolus exhibited an orange color. Further studies, however, in particular such involving differentiation of the reaction by various hydrolytic pretreatments and postreatment washes as well as investigations of unembedded material have to be conducted before a clear interpretation of the effects with this fluorescence dye can be given. Staining with ethidium bromide showed the characteristic yellow fluorescence in the nucleolus, in the perinuclear bodies, and in the cytoplasmic organelles; again, however, more detailed work has to be done before the DNA content of these structures, which is suggested by this reaction, can be taken as proven. The Feulgen reaction, which was not succesful in our hands when using whole isolated nuclei, yielded a clear result in thin sections. The nucleoli were strongly positive, especially in their cortical zones, whereas the nucleoplasm showed only a very faint pink color (Fig. 3). This is indicative of the presence of considerable amounts of DNA in the nucleolar bodies, a finding which is in contrast to observations in normal ("small") nucleoli but has precedents in observations of Feulgen-positive nucleoli and nucleolus-equivalent DNA-containing aggregates in a variety of animal cells (see in particular the classic demonstration by Bauer, [2]; see also [26, 42, 102, 112, 133, 176]). It is interesting to note that it is especially extrachromosomal nucleolar aggregates which frequently show a pronounced "vacuolization" of their interior, similar to that seen in *Acetabularia* primary nuclei (see also [2]).

Electron microscopic observations in ultrathin sections

Descriptions of the fine structure of both *Acetabularia* species used in the present study have already been communicated [13, 14, 32, 53, 155, 177, 182]. Pertinent to the questions dealt with specifically in this article are the following ultrastructural details. The basic nucleoplasm is occupied by a loose, finely granulofibrillar meshwork (diameter of the constituting filaments about 20 nm). An ultrastructural component frequently recognized in the nucleoplasm, in particular in the peripheral zone (in association with the nuclear pore complexes), are the 30 to 50 nm large, very densely stained

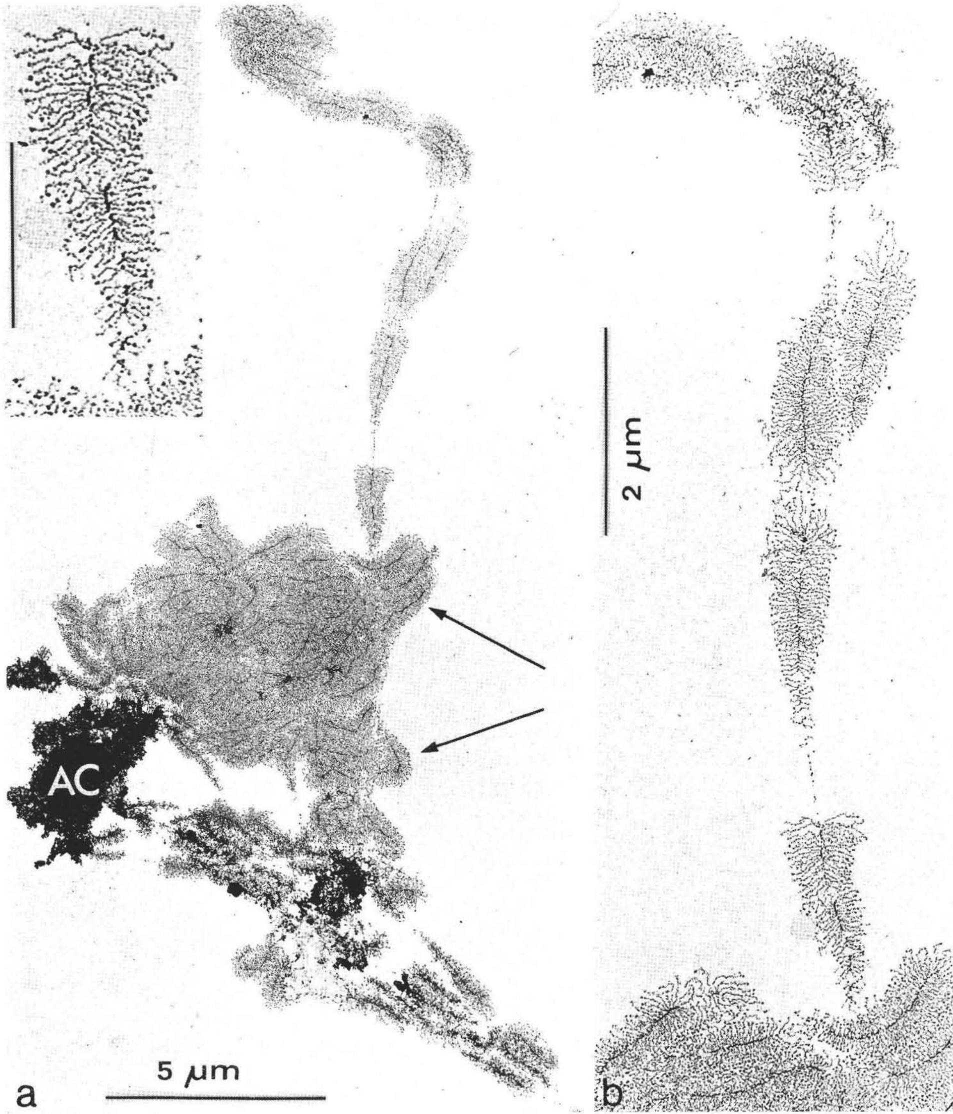


Fig. 13 a and b. Similar preparations as shown in the previous Figure showing in (**a**) the various parts of an incompletely spread *Acetabularia mediterranea* nucleolar subunit, the aggregate clump (AC) regions with relatively contracted lateral fibrils (in the lower part of **a**), regions with more extended lateral fibrils but with the individual matrix units still adhering to each other (denoted by the pairs of arrows in the central region of **a**), and fully extended strands (in the upper part of **a**, and, at higher magnification, in **b**). Note, on the extended axis, the sequence of fibril covered (matrix units) and fibril free (spacer) intercepts. Note also the appearance of terminal dense knobs on the lateral fibrils (e. g. in the *insert* of **a**), and the close spacing of the knobs representing the insertion points of the lateral fibrils on the axis within the matrix units. – The scales denote 5 μm in **a**, 2 μm in **b** and 1 μm in the *insert* of **a**.

granules (e. g. Fig. 6, 7; see also [53]). Only occasionally we encountered strandlike thickenings of dense material (see also [4]) which might correspond to the loop containing strands observed by light microscopy.

The nucleolus reveals three major zones. One distinguishes an inner (core) region characterized by rather homogeneously distributed, loosely packed and finely filamentous material (zone A; Fig. 4, 5). Zone A might correspond in structural organization to the "clear zone", the "fibrillar contours", or the "light lacunar area" in the nucleoli of other cells as described by various authors (e. g. [30, 61, 139, 140, 187, 188]; for observations in green algae see also the articles by Godward [60], Jordan and Godward [91], and the discussion of the "nucleolus organizer" nature of such intranucleolar regions by these authors). This zone A, which fills the "cavities" observed in the light microscope, is surrounded and/or penetrated by the more densely packed granulofibrillar material (filamentous widths of the same order of magnitude; Figs 4, 5) of zone B, which resembles in several structural aspects the "*pars fibrosa*" of other nucleoli (for reviews see [6, 21, 30]). Not frequently a dorsoventrality of the nucleolar bodies was indicated by the appearance of one predominant columella of zone B material penetrating into zone A (e. g. the left nucleolar body in Fig. 4 a and the insert in Fig. 5). Zone B again is surrounded by heavily stained, very densely packed material which appears to be composed by about 50 nm large granules as the predominant structural constituents (zone C; Figs. 4, 5, 6). In its periphery this zone C frequently forms an intricately branched system of coiled strands interdigitating with the nucleoplasmic filamentous matrix as is especially clearly seen in grazing sections (Fig. 4 b). Figure 4 c illustrates the two types, the light and the dense one, of spherical bodies measuring up to 1 μm in diameter (see also [4, 53]) which are characteristically found at the nucleolar periphery.

Morphometric determinations of the relative nuclear volume occupied by nucleolar structures showed a pronounced variability, even in primary nuclei of similar size and from plants with similar size. The range of values for nucleolar volumes was from 70×10^3 to 150×10^3 μm^3 and these figures correspond to partial volumes of 22 to 49 % of the total volumes of the specific nuclei (c. f. also the light microscopic approximations by Stich [164]). It is obvious that in the early stages of *Acetabularia* cell growth the number of nucleolar subunits and the total nucleolar mass increases dramatically [164]. As to the later stages of cell growth of the *Acetabularia* vegetative phase we gained the impression that neither the number of distinct spheroidal nucleolar subunits nor the nucleolar volume is correlated with nuclear size or with cell age.

When Bernhard's [5] staining procedure was applied to *Acetabularia* nuclei [53] a bleaching indicative of DNA-containing structures was observed in the nucleoli and

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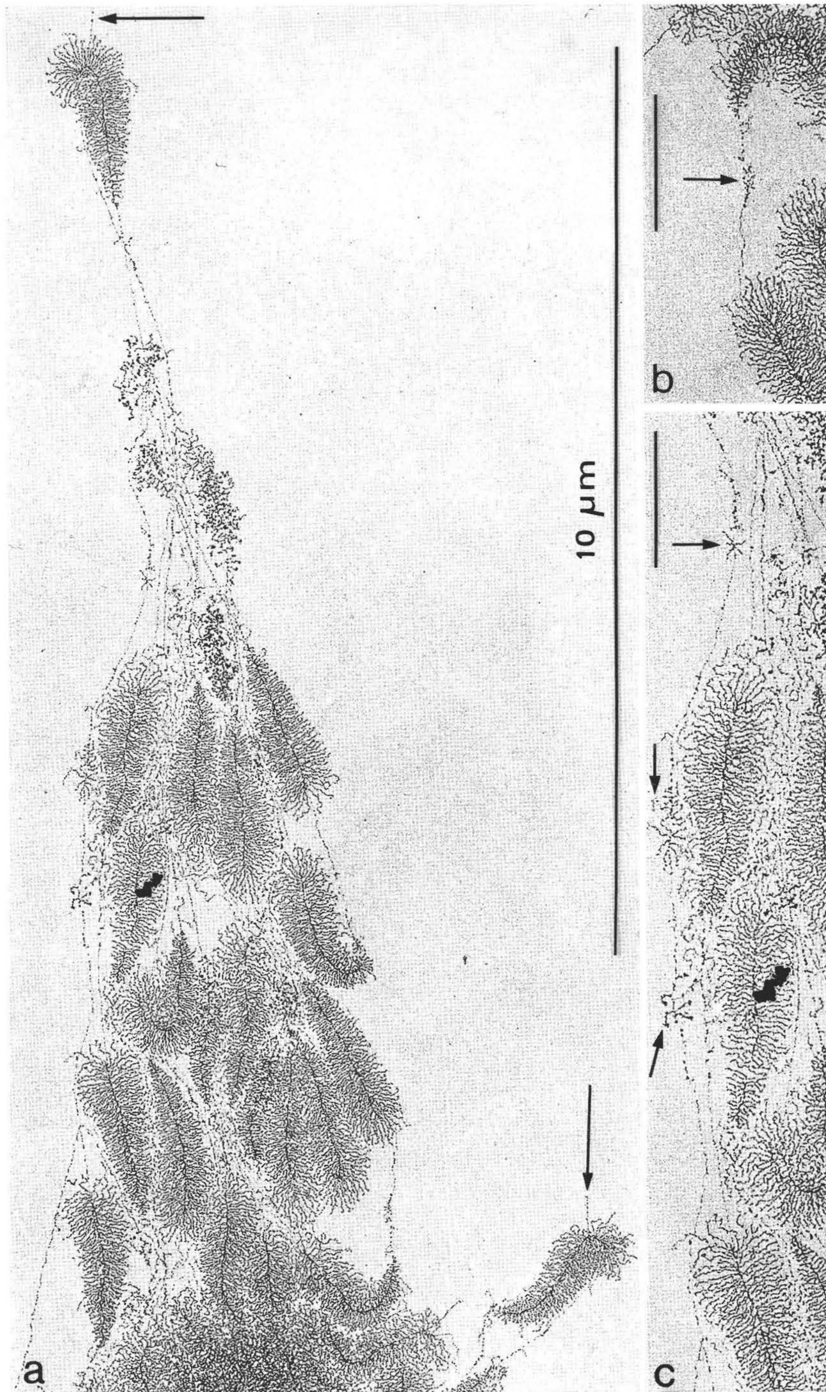
Fig. 14 a to c. Thoroughly spread nucleolar material isolated from *Acetabularia major*. Note the sequence of spacer and matrix intercepts on the extended axes. The arrows in **a** denote individual, stretched ("relaxed") lateral fibrils in the terminal region of the specific matrix units. In some of the axes small groups consisting of only a few lateral fibrils are revealed (shown in **b** and **c** at higher magnification), which could either represent so called "spacer transcript" fibrils **b**; for details see text and ref. [150]), not fully covered intercepts corresponding to matrix units, or transcripts of DNA which neither codes for pre-rRNA nor contains normal rDNA spacer intercepts (see e. g. **c**). Note that some of these fibrils also show terminal, densely stained knobs (see lower arrow in **c**). – Scale indicates *10* μm in **a** and 1 μm in **b** and **c**.


10 μm
a
b
c

the perinuclear bodies (Fig. 7), in contrast to the retention of uranyl stain in most of the granulofibrillar material of the nucleoplasm and the dense granules described above, in the nuclear pore complex structures, and in the cytoplasmic ribosomes. This (unusual) result is in accord with the above mentioned Feulgen reaction-positive character of the nucleolar aggregate of the *Acetabularia* primary nucleus.

Electron microscopic observations of isolated nuclear components

The large size and the defined intracellular position of the primary nucleus allows its manual isolation [53, 70, 175, 183]. Usually the intermediate zone and some "perinuclear labyrinthum" material tends to remain attached at the nuclear envelope, and special care must be taken in washing the nuclei to remove these adhering cytoplasmic contaminants [32, 182]. An isolated primary nucleus of *A. mediterranea* as used for preparation of nuclear subcomponents, is presented in Fig. 8. One identifies the nuclear envelope, the relatively light nucleoplasmic ground substance and the dense nucleolar aggregates (for examples of sausage-shaped nucleolar aggregates present in isolated nuclei see [175, 182, 184]).

The nuclear envelope. A survey electron micrograph of the isolated, washed and positively stained nuclear envelope is presented in Fig. 9 (see also [53]). Possibly as a consequence of the collapsing of the nuclear envelope during its disruption and during preparation on the grid, it usually shows many foldings (Fig. 9 b). Quantitative evaluations confirm our previously reported figure of 70 to 85 pore complexes per square micron of relaxed nuclear envelope (for a detailed discussion of the possible artifacts in determinations of nuclear pore densities see [43, 44, 46, 53, 92]). At higher magnification the pore complexes frequently showed defined substructures, such as the presence of central and annular granules (see [53]). It was repeatedly noted that tangles of filaments remained in association with the nuclear envelope fragments during isolation (Fig. 9 a). This is similar to observations with isolated amphibian and insect oocyte nuclear envelopes (e. g. [44, 173]), which sometimes showed attachments of typical "Christmas-tree" fibril formations (for explanation see below).

Spread nuclear and nucleolar contents. When nuclear contents or isolated nucleolar material were prepared using moderate spreading conditions we frequently observed large dense aggregates up to 20 µm in diameter with either sharply defined circumscriptions (Fig. 10 b) or less regular confines (Fig. 10 a). On closer inspection, these aggregates revealed a composition on finely fibrillar structures with a few interspersed, very densely stained aggregate clumps (Figs. 10, 12, 13, 15, 25). Most likely these large fibrillar aggregates represent the nucleolar subunit bodies flattened onto the grid (see also [175]).

Fig. 15. Total survey of moderately spread nucleolar subunit aggregate of *Acetabularia major* made up either by one extraordinarily large nucleolar subunit or by two closely adjacent subunits. This micrograph shows both more aggregated (in the upper right) and more dispersed (in the lower left) axial fibrils and some heavily stained aggregate clumps (AC). Note the high number of matrix units in one such subunit. The *insert* shows especially extended lateral fibrils in terminal (upper arrowhead in the left insert) or start regions (denoted by the vertical arrow in the upper part of the left insert) as well as situations suggesting local detachment of lateral fibrils from the matrix unit complex (e. g. denoted by the two arrowheads in the right *insert* and the lower arrowheads in the left *insert*). This micrograph represents a total of about 230 individual cistrons. – The bar indicates 10 µm; the scales in the *inserts* denote 1 µm.

In certain situations one can identify within these fibrillar masses, in particular in their peripheral regions, the characteristic "Christmas-tree-like" assemblies (matrix units, see below) of lateral fibrils attached to axial core fibers (Fig. 10 a and b); that is structures which have been described as characteristic for nucleoli active in transcription in a variety of animal cell systems (compare [37, 75 to 78, 80, 121, 125 to 132, 150, 173, 174]). This composition was more clearly identified upon more intensive spreading, and the individual transcriptional units contained in one such putative nucleolar subunit became

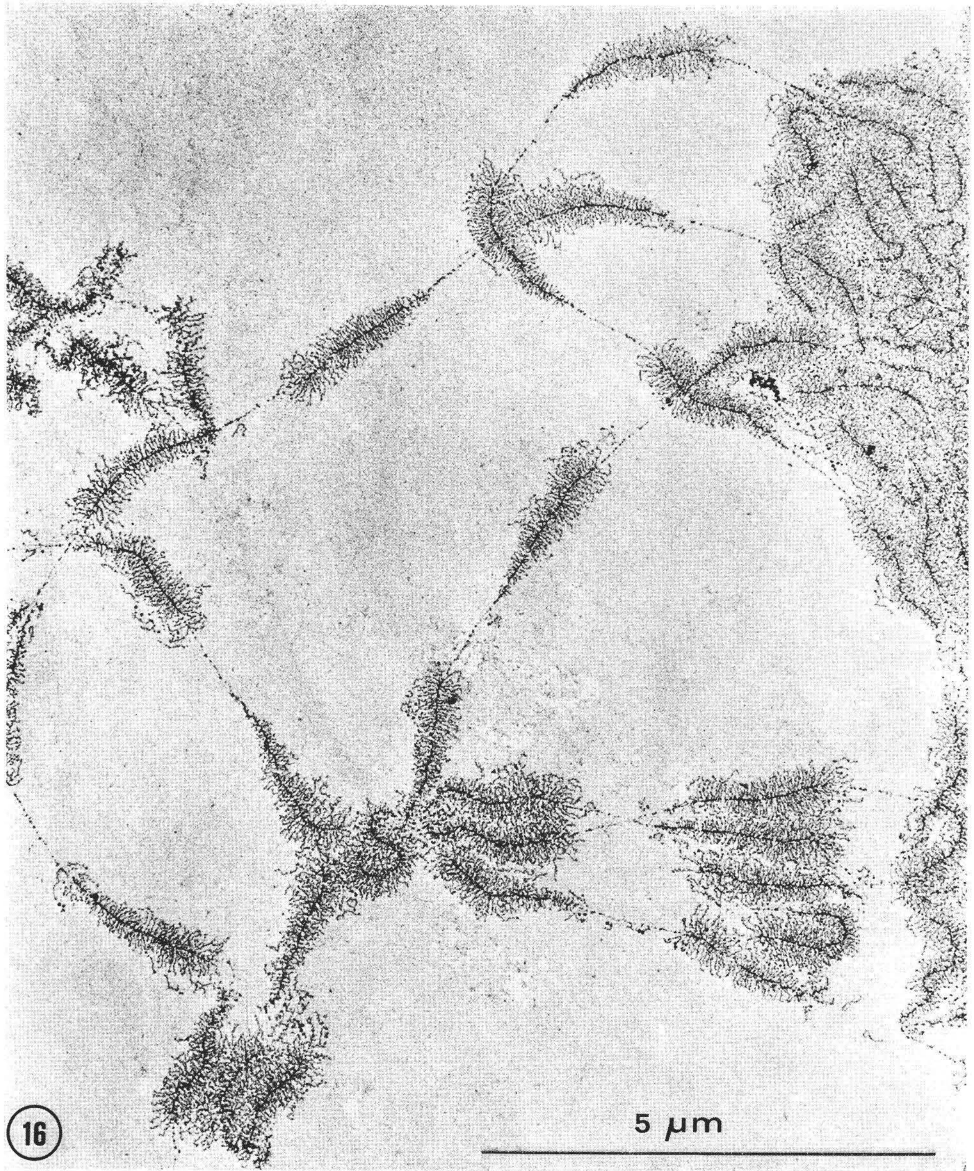


Fig. 16. Higher magnification of the more extended part of the nucleolar subunit shown in the survey of the previous figure. The regular arrangement of fibril-covered ("matrix") and fibril-free ("spacer") regions on the axes is clearly recognized as well as a tendency of matrix regions to laterally aggregate, thus sometimes producing parallel orientations of adjacent axial stretches. – The bar indicates 5 µm.

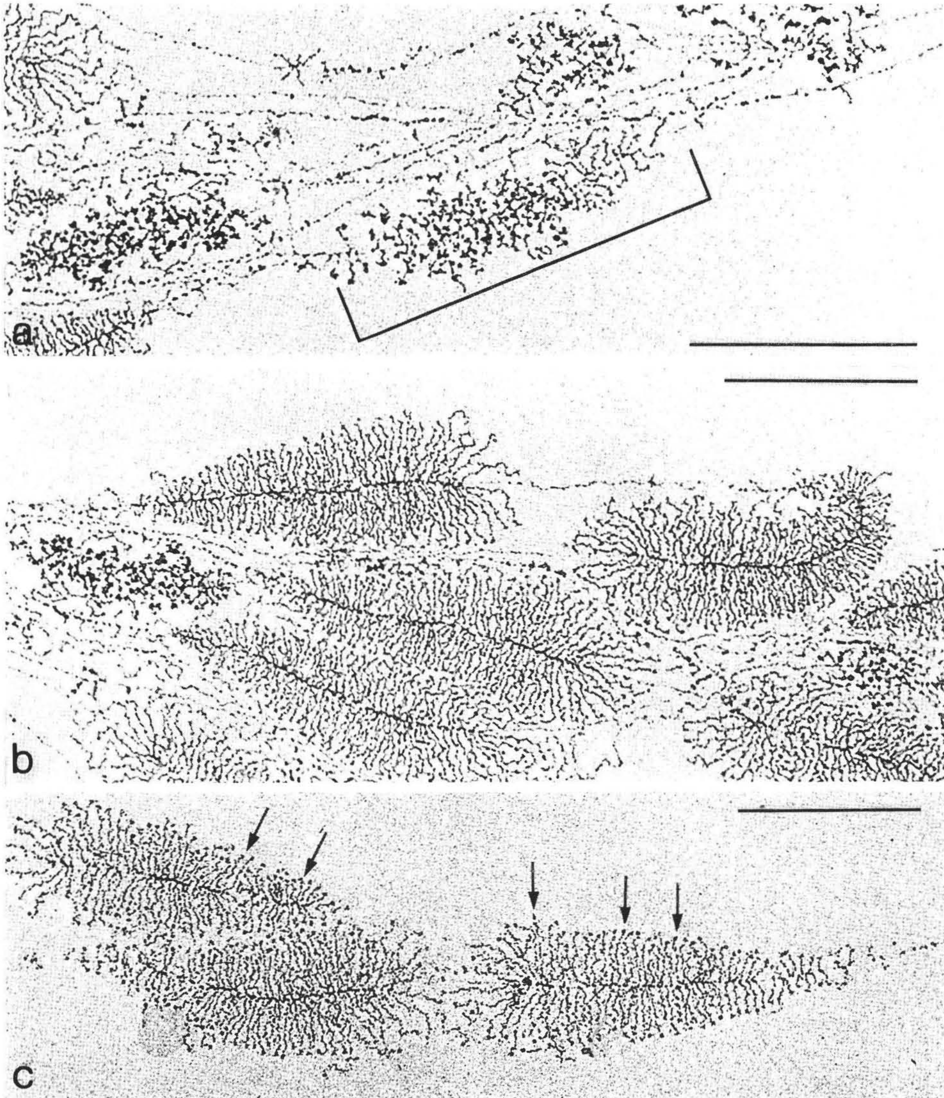


Fig. 17 a to c. Details of matrix unit morphology as revealed after intensive spreading. Some matrix units appear to be diluted in their lateral fibril density (indicated by the brackets in **a** which is a partial magnification from Fig. 14 a). – **b** and **c** show the normal, extremely dense packing of insertion points of the lateral fibrils, which in some preparations show terminal dense knobs (indicated by the arrowheads in **c**). **b** shows a preparation from *Acetabularia major* and **c** gives an example from *Acetabularia mediterranea*. Matrix units of the type presented in **b** and **c** contain a total of about 100 to 115 individual lateral fibrils attached, which is equivalent to 100 to 115 active RNA polymerase A molecules (according to the interpretation of MILLER and coworkers, see References). All scales indicate 1 μm.

distinct and could be counted (e. g. Figs. 11, 14, 15, 16). Usually this disentanglement and spreading out of the fibrillar material appeared to begin in the periphery of such aggregates (Figs. 12 a and b, 13). It should be noted that the spreading result frequently was not homogeneous in a given aggregate, and relatively clumped fibrils in one part of an aggregate were seen adjacent to fairly extended fibrils (Figs. 12 b, 13, 15). The typical morphology of such extended fibrils is shown in Figures 13 to 19. One distinguishes long axial fibrils of about 10 to 16 nm in width, which are set in regular intervals by series of closely spaced shorter lateral fibrils (7 to 12 nm in width). In each series of lateral fibrils (i. e. in matrix units *sensu* Miller and coworkers) one notes a gradual increase in length, the longest fibrils identified in the terminal region are of about 0.7 μm. Somewhat different from the situations observed in most of the animal cell systems studied, in particular from those of the extrachromosomal nucleoli of some insects (for references see [173, 174]), the gradual length increase of the lateral fibrils continues for almost the whole matrix unit (see e. g. Figs. 12 to 20). At the free ends of the lateral fibrils, especially in the terminal regions of the matrix units, one frequently but not always recognizes densely stained knobs of a diameter of 17 to 30 nm. The insertion points of the lateral fibrils on the axes are also usually identified by the presence of a dense small knob, which according to the general interpretations of Miller and Beatty [127] might represent the RNA polymerase A molecules attached to both the template and the nascent RNA-containing fibrils. The close package of these basal knobs along the axis, which is characterized by a mean center-to-center distance of about 15 to 25 nm (that is somewhat less than the distance reported for various animal cells; compare [125, 127, 129, 150, 173, 174]), results in the appearance of a thickened, intensively stained axial intercept within the matrices (Figs. 13, 14, 17). The axial intercepts free from such lateral fibrils are also relatively identical in length (for quantitative data see below) and occupy about 25 to 35 % of the axis, similar to what has been described in spread nucleoli from some amphibia and insects [37, 77, 78, 80, 121, 125, 127 to 130, 150]. These spacer intercepts are often characterized by the presence of densely stained knobs which, however, appear somewhat larger in size and show larger spacings between knobs (Fig. 19). The measurement of the width of the axial and the lateral fibrils cannot be done with high accuracy due to the local variations and the superimposed granularity (Figs. 13 to 19). This granularity might result from precipitation and staining artifacts during the treatment with ethanolic PTA or might represent local coiling of the ribonucleoprotein (RNP) *in vivo*. It should be emphasized, however, that in our spread preparations of *Acetabularia* primary nucleus contents, there was no consistent and significant difference in width between the axial and the lateral fibrils (for different observations c. f. [150]). In various cistrons we encountered lateral fibrils which were considerably longer than their neighboring fibrils. Such exceedingly long fibrils were noted in start (Figs. 15, 18, 22) as well as in terminal (Figs. 12, 14 to 16) regions of the matrix units. The existence of these disproportionally long lateral fibrils

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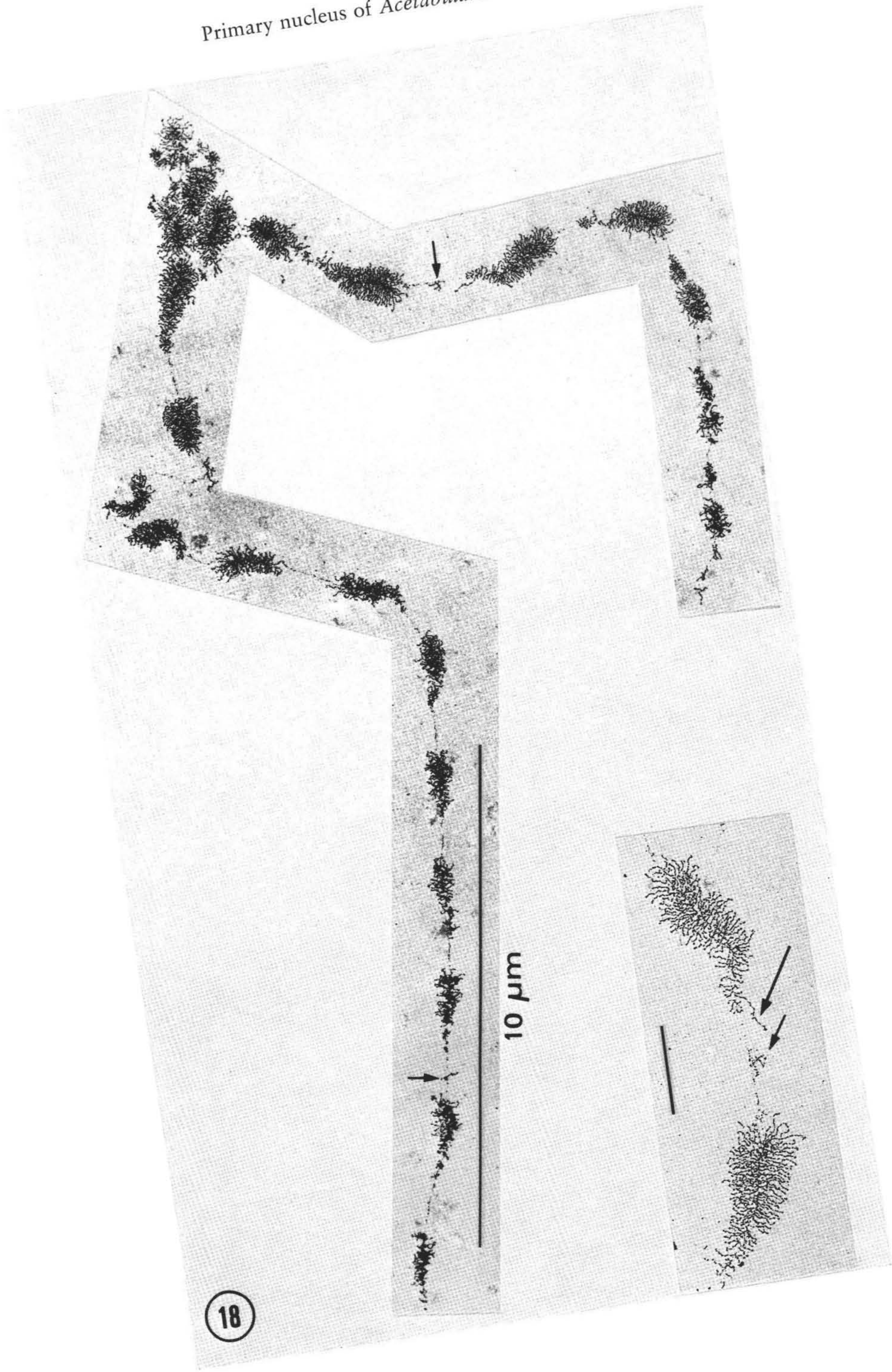
Fig. 18. Survey electron micrograph of a spread preparation of contents of primary nuclei of *Acetabularia mediterranea* illustrating the regular arrangement of 23 repeats, each consisting of a matrix unit and a spacer intercept. Occasionally, isolated individual fibrils or small groups of fibrils are seen in the spacer regions (some of which are denoted by the arrowheads; for higher magnification see *insert*). Note also in the insert another example of an extraordinarily long, probably unravelled, lateral fibril in the starting region of a matrix unit (denoted by the upper arrow). – The scale denotes 10 μm, that in the *insert* 1 μm.


10 μm
18

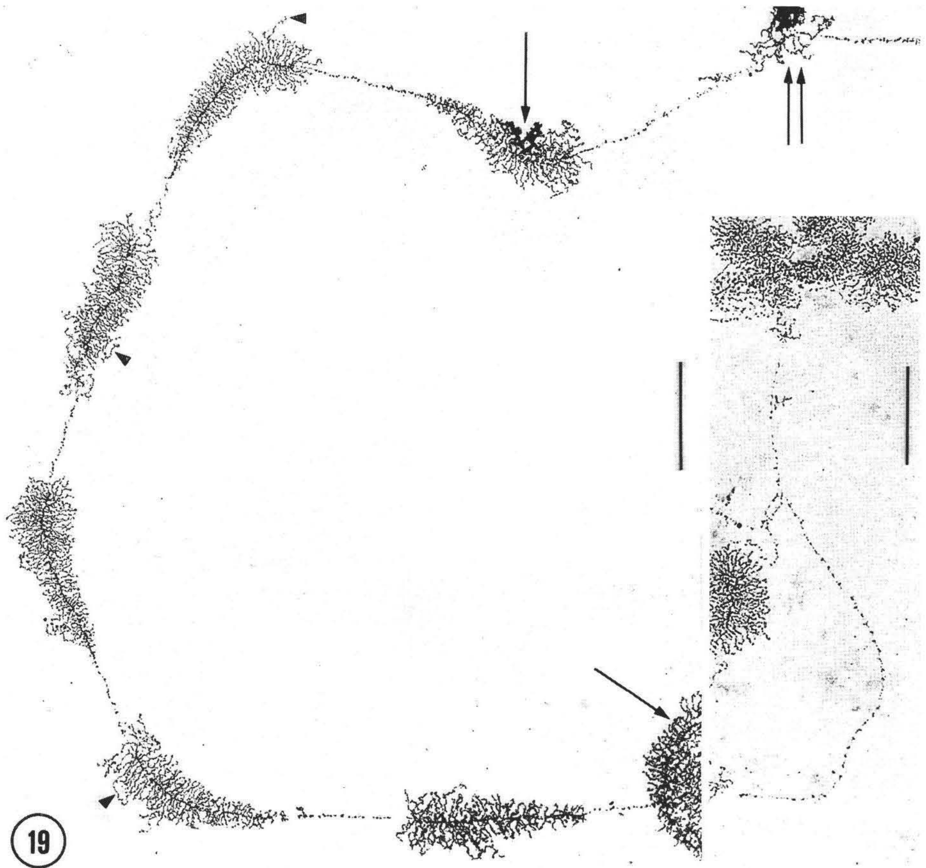
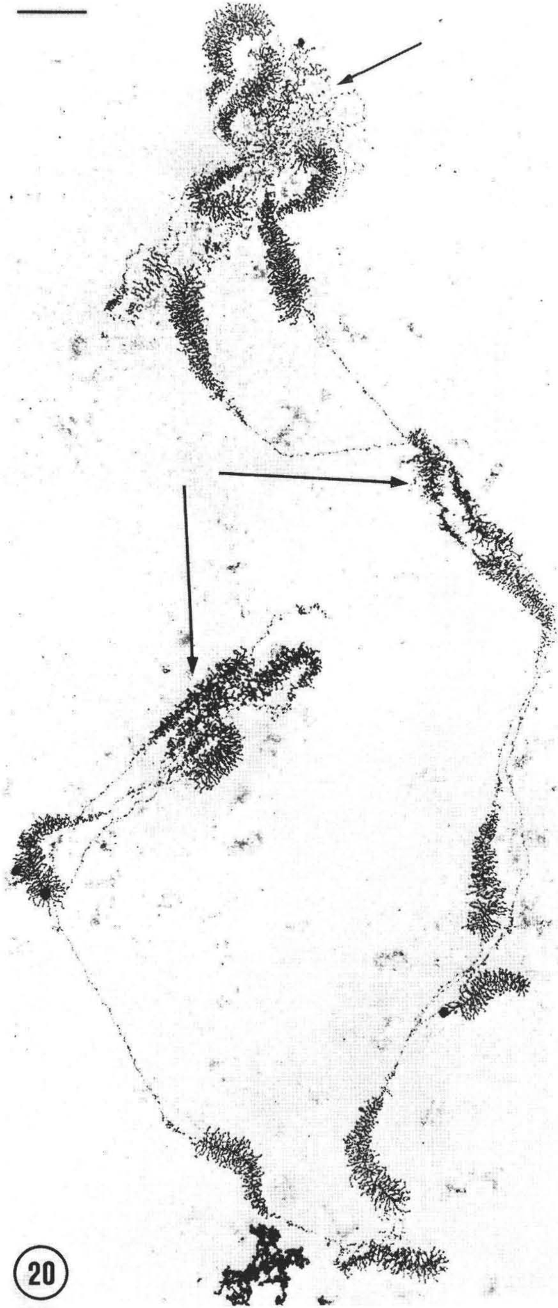


Fig. 19. Spread preparation of nucleolar material from *Acetabularia major* showing several instances of unusually long and "detached" lateral fibrils indicated by the arrowheads. Note that in the matrix unit indicated by the vertical arrow in the upper part a local dense clumping of lateral fibrils is revealed. Note also the occurrence of a poorly fibril-covered matrix unit in this sequence (denoted by the pair of arrows in the upper right) and an example of snailshell-like intramatrical coil configuration (denoted by the arrow in the lower right). The *insert* illustrates the somewhat regular distribution of small dense dots in a fibril-free axial intercept of *Acetabularia mediterranea*. – Scales indicate 1 μm.

Fig. 20. Intensely spread preparation of *Acetabularia mediterranea* nuclear contents showing the occurrence of unusually long fibril free axial intercepts, which exceed in length the usual spacer intercepts (for details see text). Note also the local heterogeneity in the degree of fibril extension among the individual matrix units (the arrows in the center denote two examples of incompletely spread matrix units). The arrow in the top part denotes a tangle of fibrils in this spread material aggregate, the character of which cannot be discerned. – The scale indicates 1 μm.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could be interpreted in several ways; for example, it might indicate a higher degree of unravelling of the protein-covered pre-rRNA molecules. Alternatively, it might reflect an early cleavage step in the processing of pre-rRNA and/or a conformational change of the whole RNP-fibril which does not take place in such individual fibrils (for related speculations on pre-rRNA processing in the growing molecule see [31, 66, 68, 110, 150, 174]). In addition we frequently observed isolated fibrils of up to 0.5 μm in length and similar in overall appearance to the lateral fibrils in the immediate proximity of matrix units (Figs. 15, 16, 19), a localization suggestive of detachment, perhaps at preformed sites, of terminal parts of lateral fibrils (perhaps induced or favored by the

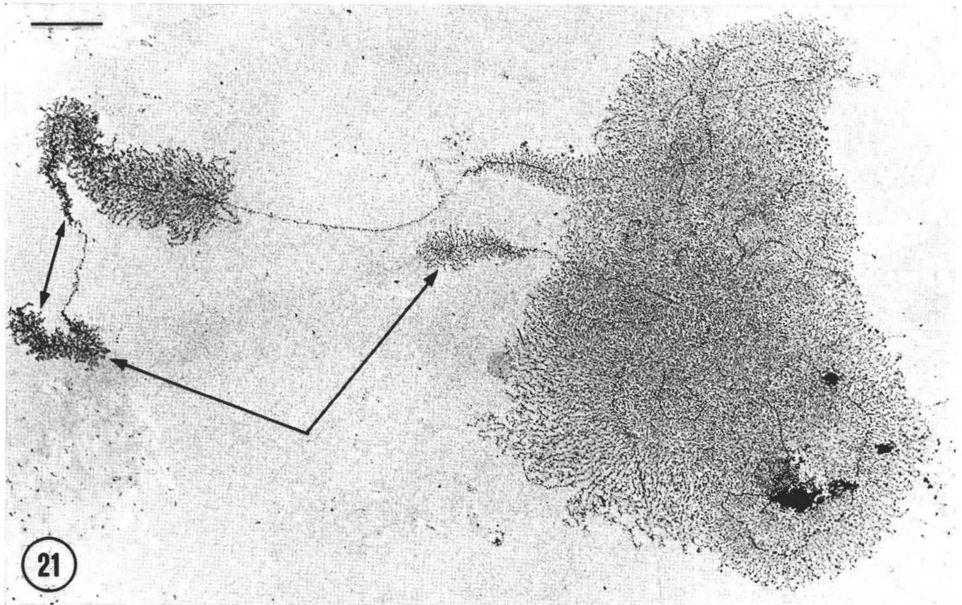
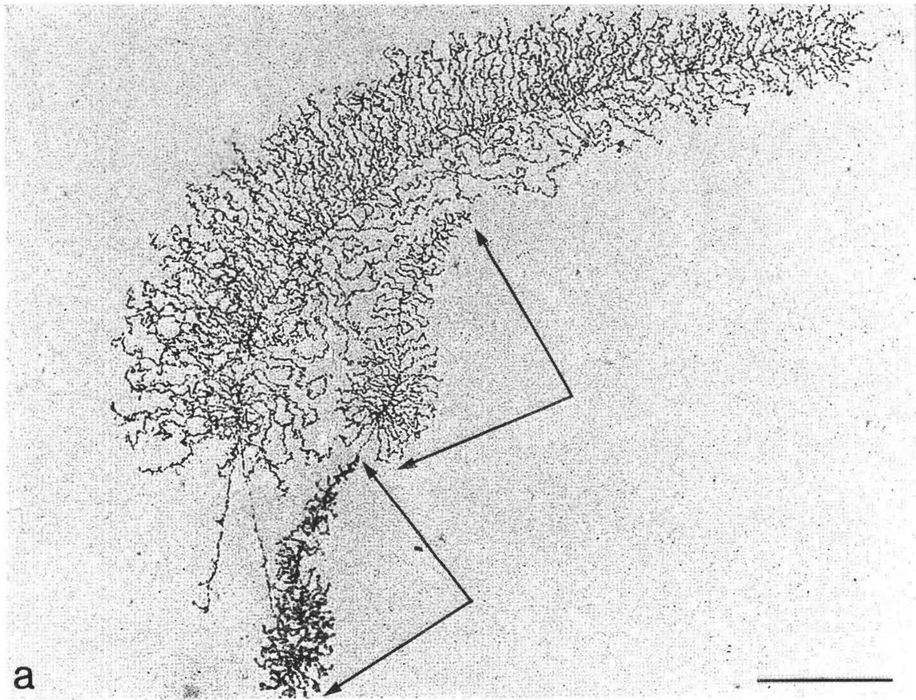


Fig. 21. Electron micrograph of a spread preparation of *Acetabularia mediterranea* primary nuclei. This unit contains two classes of matrix units, short ones (as revealed for example in the more favorably spread peripheral parts (two of them are denoted by the pair of arrows in the lower part) and much longer ones that can be recognized in spread form (e. g. in the upper left corner) and within the aggregate shown in the right half. Note the example of an inverse orientation at the two matrix units shown in the left (illustrated by the double-headed arrow). – The scale denotes 1 μm.

Fig. 22 a and b. Comparison of the three major size classes in spread preparations found in *Acetabularia major*, the about 2 μm long one (**b**, and the two denoted by the two pairs of arrows in **a**), the about 3.5 to 5 μm long ones (an example is seen in the upper right corner of **b**), and the giant matrix units of up to 12 μm length, an example of which is shown in **a** (in direct comparison with the short matrix units). The arrow in **b** denotes an unusually short matrix unit intercept, which, however, might well represent the first part of a "normal" (i. e. 2 μm long) matrix unit in which the fibrils are lacking in the terminal region. – The scales indicate 1 μm.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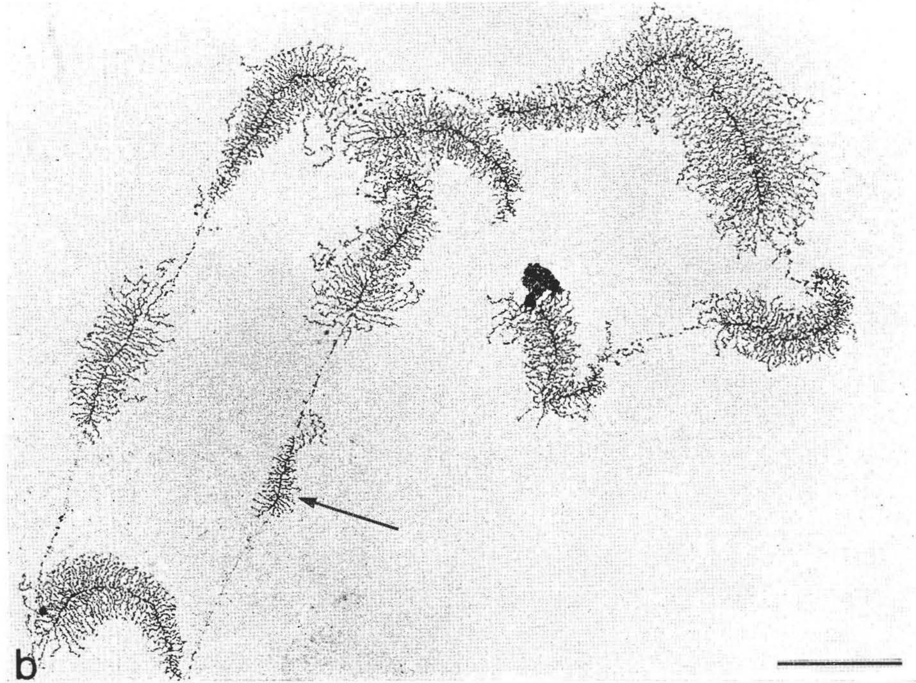
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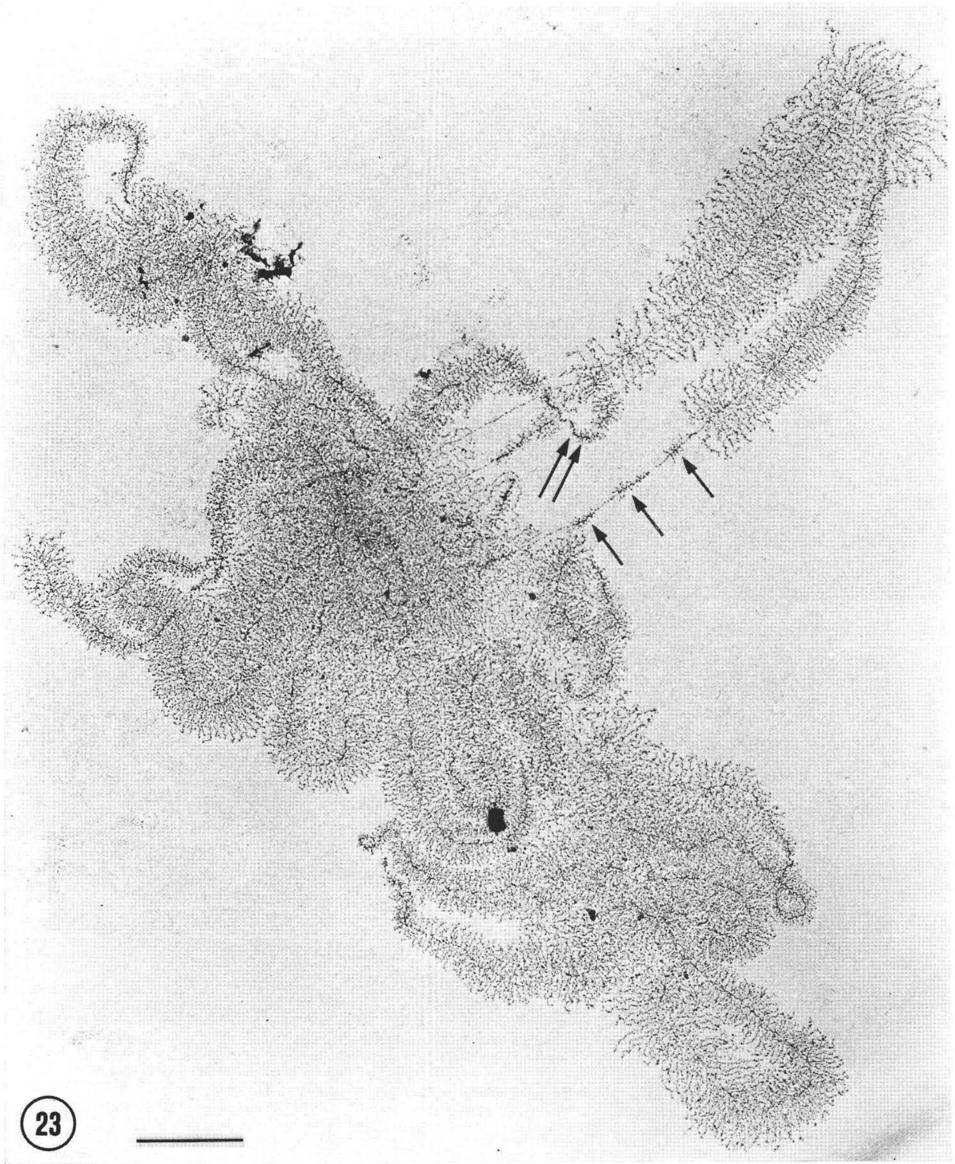

Fig. 23. Spread preparation of nuclear contents isolated from *Acetabularia mediterranea* showing various long matrix units. The arrowheads denote a series of three regularly spread small groups of fibrils adjacent to the terminal region of a long matrix unit. The double arrow points to a situation which might represent the inverse situation. Note, in some of the lateral fibrils in these long matrix units, the terminal electron opaque knobs (e. g. in the matrix unit in the upper right). The scale denotes 1 μm.

mechanical stresses exerted during the preparation). In this connection, attention should be called to the abundance of similar fibrils in extremely spread preparations of isolated nucleoli (e. g. Fig. 36).

The lateral fibrils of the matrix units are not the only lateral fibrils on matrix unit bearing axes. Similar to observations in spread extrachromosomal nucleoli from oocytes of various amphibian species and the water beetle *Dytiscus marginalis* [150, 173], we

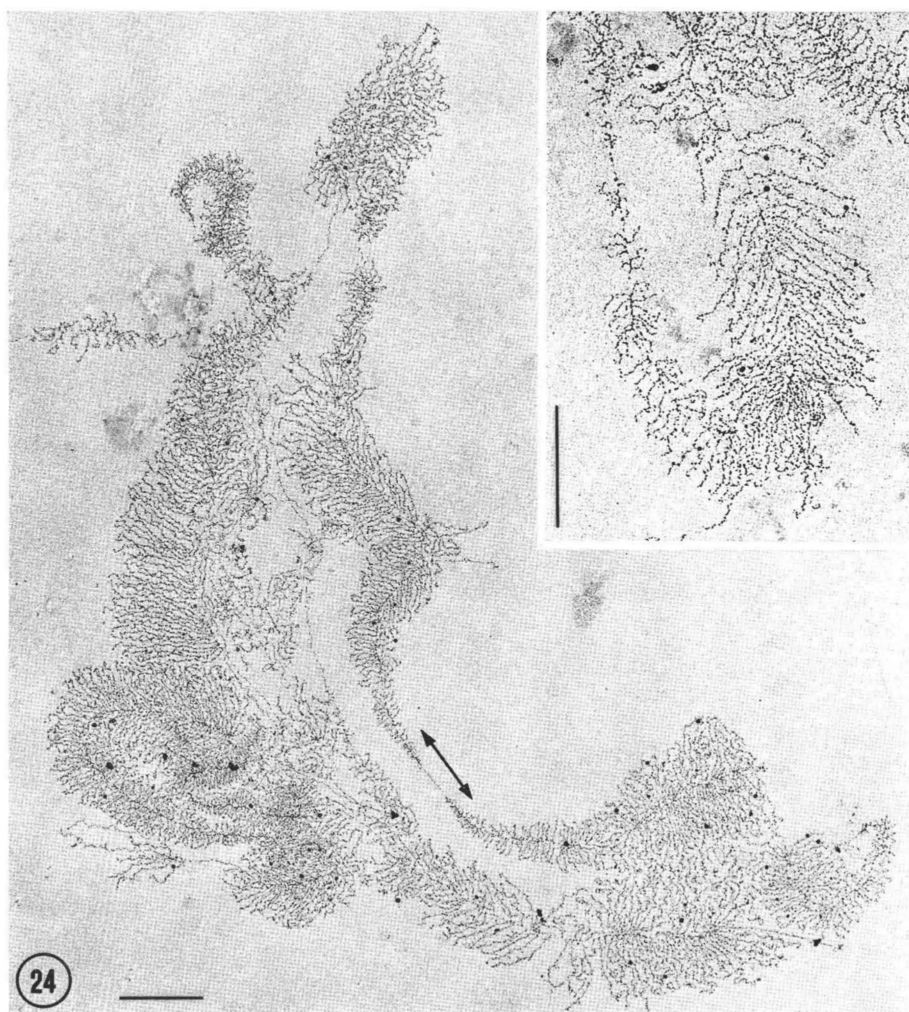

Fig. 24. Spread and positively stained preparation of nuclear contents of *Acetabularia mediterranea* showing an aggregate containing predominantly long and extremely long ("superlong") matrix units. The doubleheaded arrow denotes an inverse orientation of two adjacent matrix units with only a very short spacer intercept between. Note the great length of the lateral fibrils in these long matrix units (for example up to 1 μm in the matrix unit shown in the *insert*). – The scales denote 1 μm.

noted in *Acetabularia* primary nuclei the occurrence of small units consisting of up to 10 individual fibrils (with increasing length within each group) in "spacer" intercepts. These so-called "spacer transcript units" occur either at the start of the matrix units in the form of "prelude pieces" (c. f. [150]) or in other, more central portions of the spacer intercepts. The existence of such small groups of fibro-granular material in

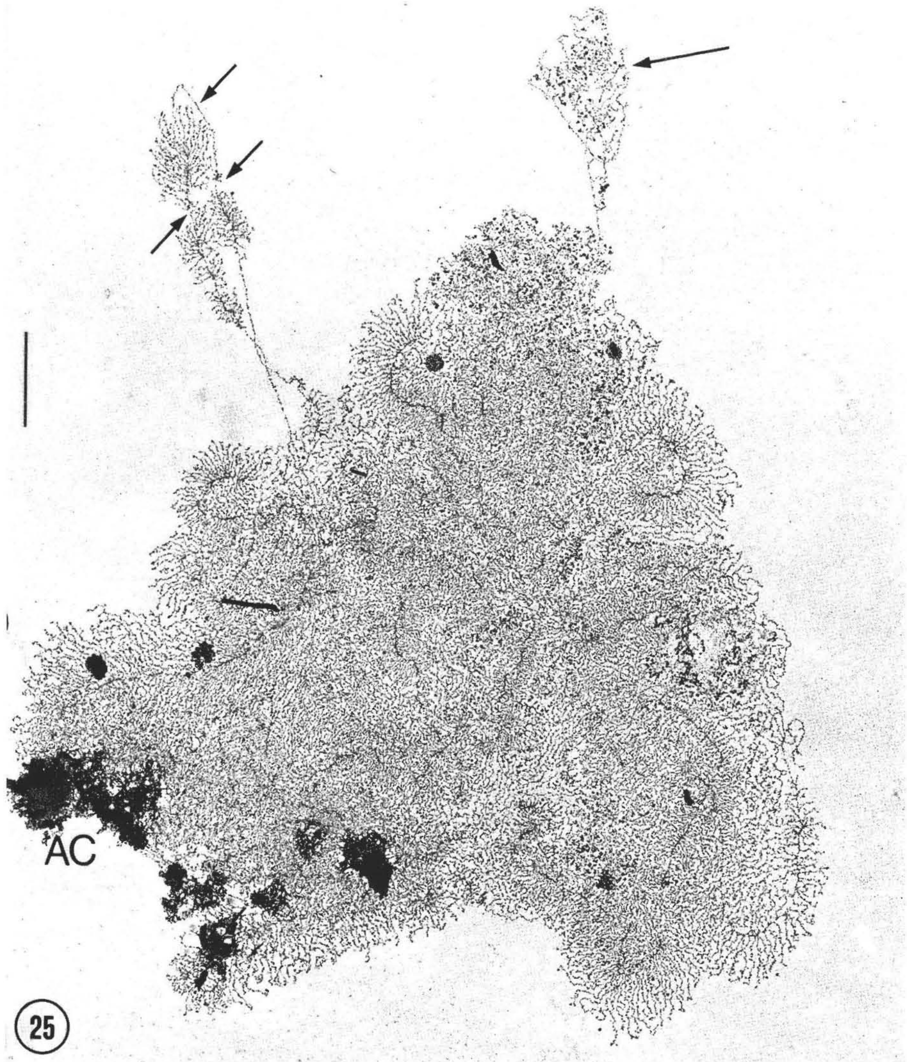

Fig. 25. An incompletely spread aggregate from *Acetabularia major* primary nuclei that mainly contains matrix units of the larger size classes. The arrowheads in the upper left denote regions that apparently represent stretches free of fibrils, which might demonstrate intramatrical axial intercepts. The arrow in the upper right denotes an unidentified granulo-fibrillar tangle. AC, aggregate clump. – The scale indicates 1 μm.

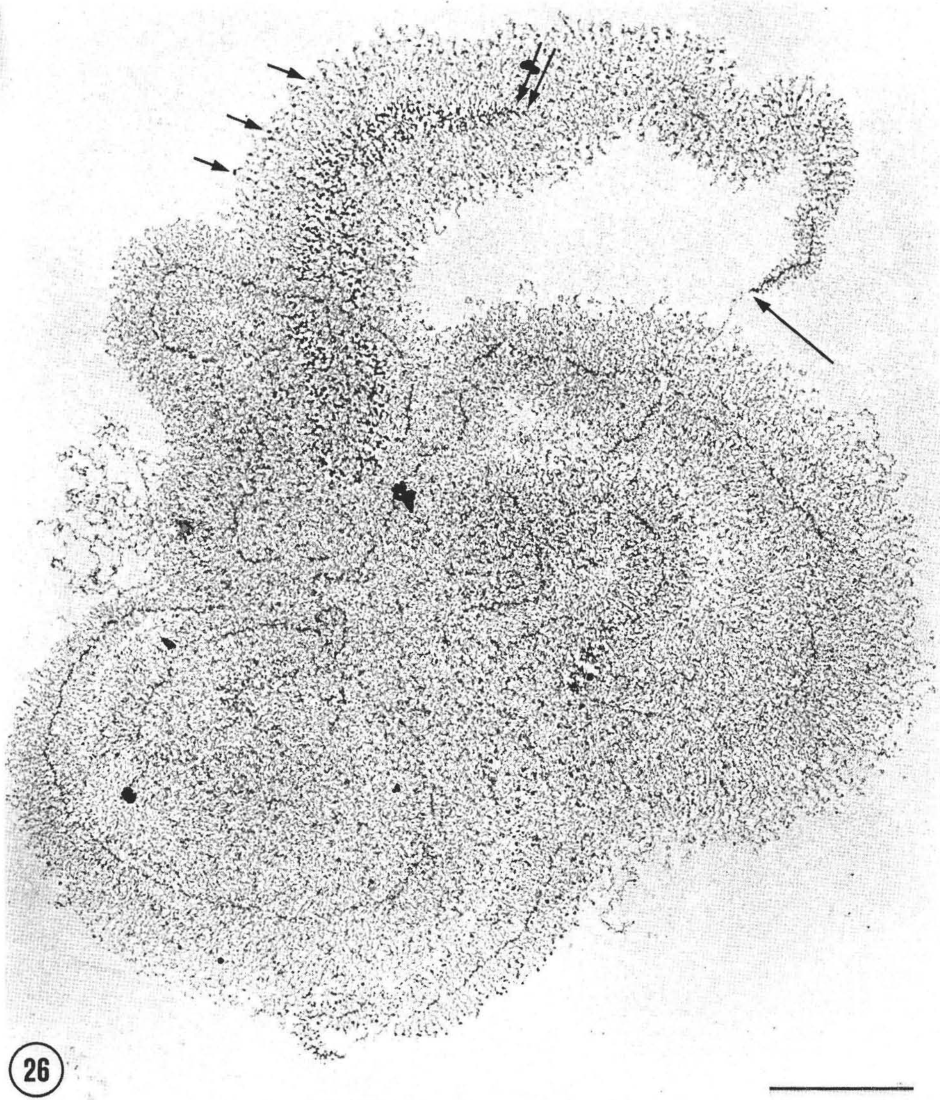

Fig. 26. Spread preparation showing an aggregate unit from the nuclear content of *Acetabularia mediterranea* which contains extremely long matrix units (the start of one matrix unit is denoted by an arrow). Note the occurrence of terminal knobs on some of the lateral fibrils (arrowheads in the upper left). Note also that in some of these matrix units the basal parts of the lateral fibrils are more intensely stained (the beginning of this zone is denoted by the double arrow). – The scale indicates 1 μm.

spacer intercepts has recently also been noted [126, 131] in spread preparations of actinomycin D-treated *Triturus* oocytes and after storage of female *Triturus* at 12° C for several months. The character of the putative RNA contained in these spacer transcripts is unclear. As to the specific case of the "prelude pieces" one could envisage that they represent the presence of a "read-through" transcription process [150] or a specifically defined conformational coiling after pre-rRNA of a certain length has been formed. In the light of the latter hypothesis this would mean that the lateral fibrils of the "prelude pieces" do contain RNA which is a covalently linked part of the primary transcript pre-rRNA molecule.

Occasionally matrix units were seen which revealed a looser package of lateral fibrils (e. g. Fig. 17 a). From such isolated observations, however, it cannot be decided to what extent this reflects localized preparation artifacts or the reduced transcription of individual cistrons. Preliminary experiments in which the nucleolar material of "aged" or actinomycin D-treated primary nuclei was used suggested that at least the induced inactivation of rRNA transcription results in a random removal of groups of neighbored lateral fibrils, similar to the situation described for actinomycin-treated *Triturus alpestris* oocytes [151].

In certain preparations it was possible to count large numbers of cistrons on individual axes (up to 40; Fig. 18 presents an example of 23 matrix units; see also [173]). When we determined numbers of matrix units identified in the individual nucleolar aggregate subunits (for definition see above) we noted that there seems to be a range of maximal numbers of 120 to 150 matrix units. Exceptional situations in which larger numbers were determined (Fig. 15 presents an example of 230 cistrons within one aggregate morphological unit) are open to the argument that they represent multiple aggregates of nucleolar units (see, e. g., Fig. 15).

In our spreading experiments of *Acetabularia* primary nuclei contents we noted a tendency of the matrix units to laterally aggregate or to exhibit intramatrical (snail-house-like) coil configurations (Figs. 14, 16, 19, 20). This phenomenon, which was also observed in amphibian oocyte nuclei [150] and was pronounced in the larger matrix units present in the oocyte nuclei of the house cricket and the water beetle [173, 174], is probably due to the stickiness of the swollen RNP-fibrils. Sometimes we also observed limited aggregation of some lateral fibrils resulting in the formation of a dense clot (e. g. Fig. 19). As we [150] and others [121] have emphazised in studies of amphibian oocytes and *Drosophila* spermatocytes, respectively, the degree of dispersion and spreading usually is not homogeneous in a specific preparation but might vary even between adjacent matrix units or within one and the same matrix unit (Figs. 19, 20, 22 a). Although the dimensions of spacer and matrix unit intercepts in both species appear to be relatively homogeneous and regularly distributed (see below and Fig. 37) we occasionally found, particularly in *A. mediterranea,* axes with "normal" matrix units

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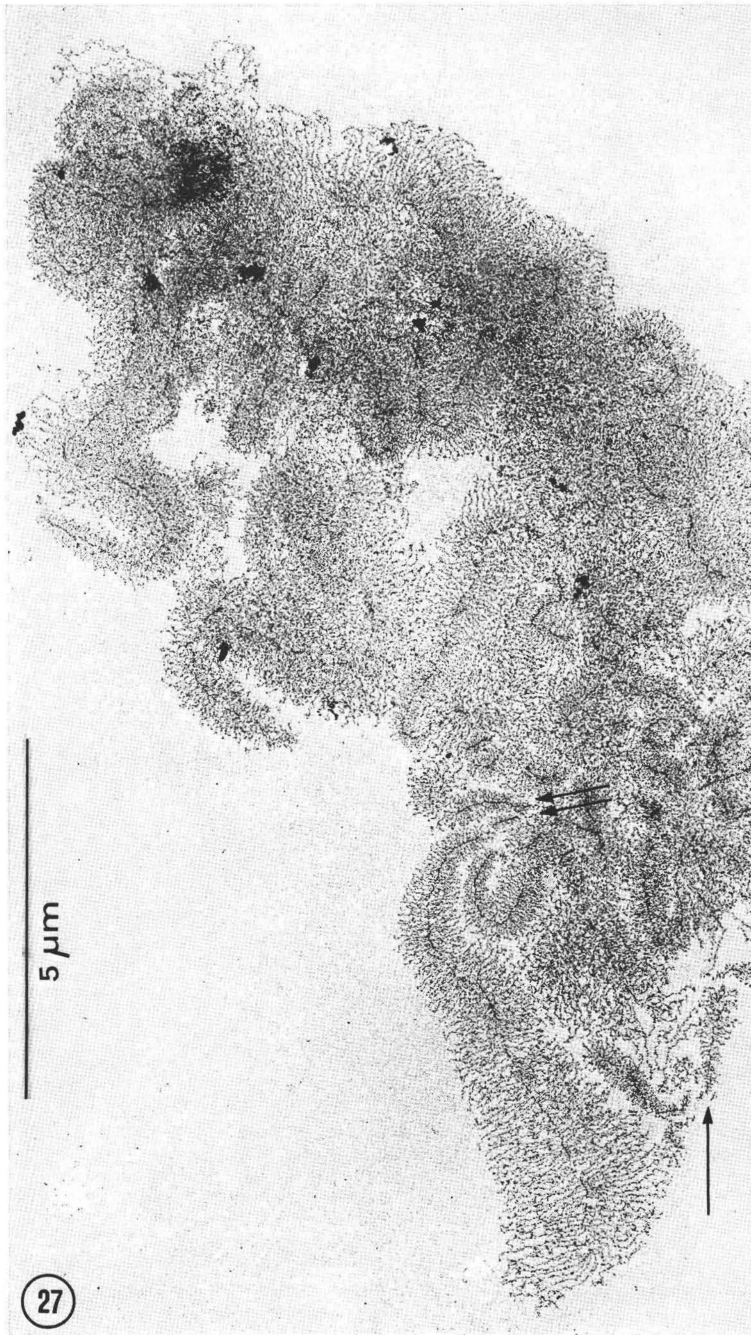
Fig. 27. An aggregate body isolated from the nuclear interior of an *Acetabularia mediterranea* primary nucleus as revealed after spreading and positive staining with PTA (for details see Methods). In this frequent type of nuclear body the coincident presence of matrix units of the various size classes is revealed. The arrow in the lower right denotes matrix units of the short type left from which one giant matrix unit is recognized. The two arrows point to the start regions of two adjacent matrix units, the upper one belonging to the short class and that oriented to the bottom part belonging to the giant matrix units. – The scale indicates 5 μm.


5 μm
27

but extraordinarily long "spacer intercepts" (Fig. 20). The specific spacer lengths seemed to exclude, at least in situations of spacer lengths of about 2 to 5 μm (c. f. Figs. 20 and 37), that the appearance of long spacers was due to the existence of single "silent" (i. e. non-transcribed) cistrons (for interpretations in favor of silent cistrons see [113, 121, 150]).

Fig. 28. A moderately spread aggregate from a primary nucleus of *Acetabularia major* characterized by the presence of giant matrix units; the start region of one matrix unit is denoted by an arrow. Note the extremely dense packing of lateral fibrils within these matrices which, in addition, are entangled so that the individual lateral fibrils can hardly be traced. – The scale indicates 1 μm.

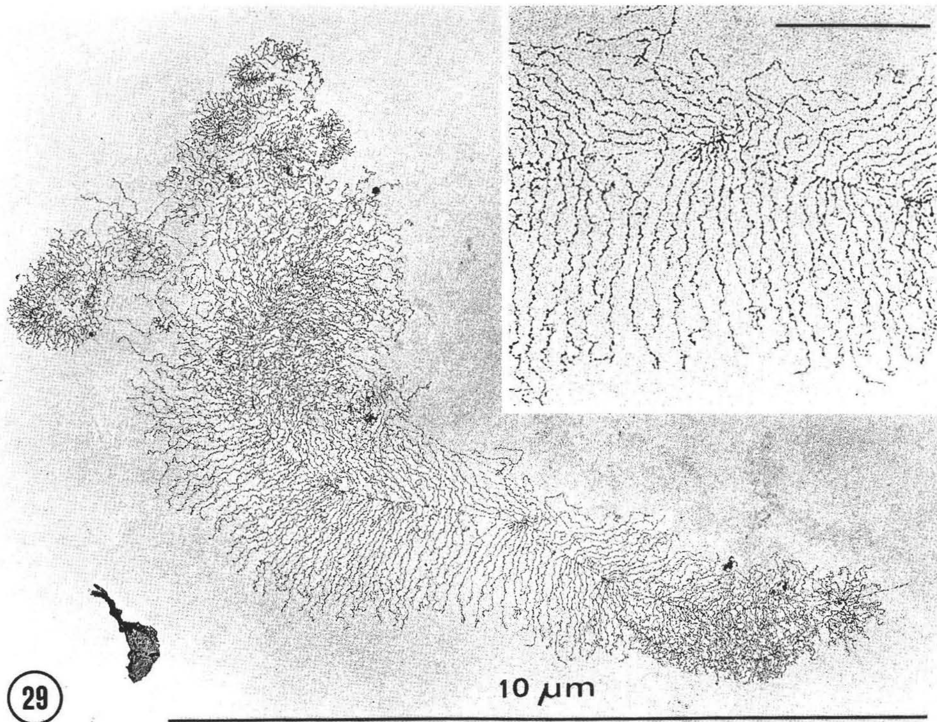


Fig. 29. Another type of spread matrix unit of the giant size class from *Acetabularia major*. The lateral fibrils are more extended, compared with the appearance shown in the previous figure, and consequently can be traced in their entire length (for example, up to 2 μm as demonstrated in the *insert*). – The bar denotes 10 μm, the scale in the *insert* represents 1 μm.

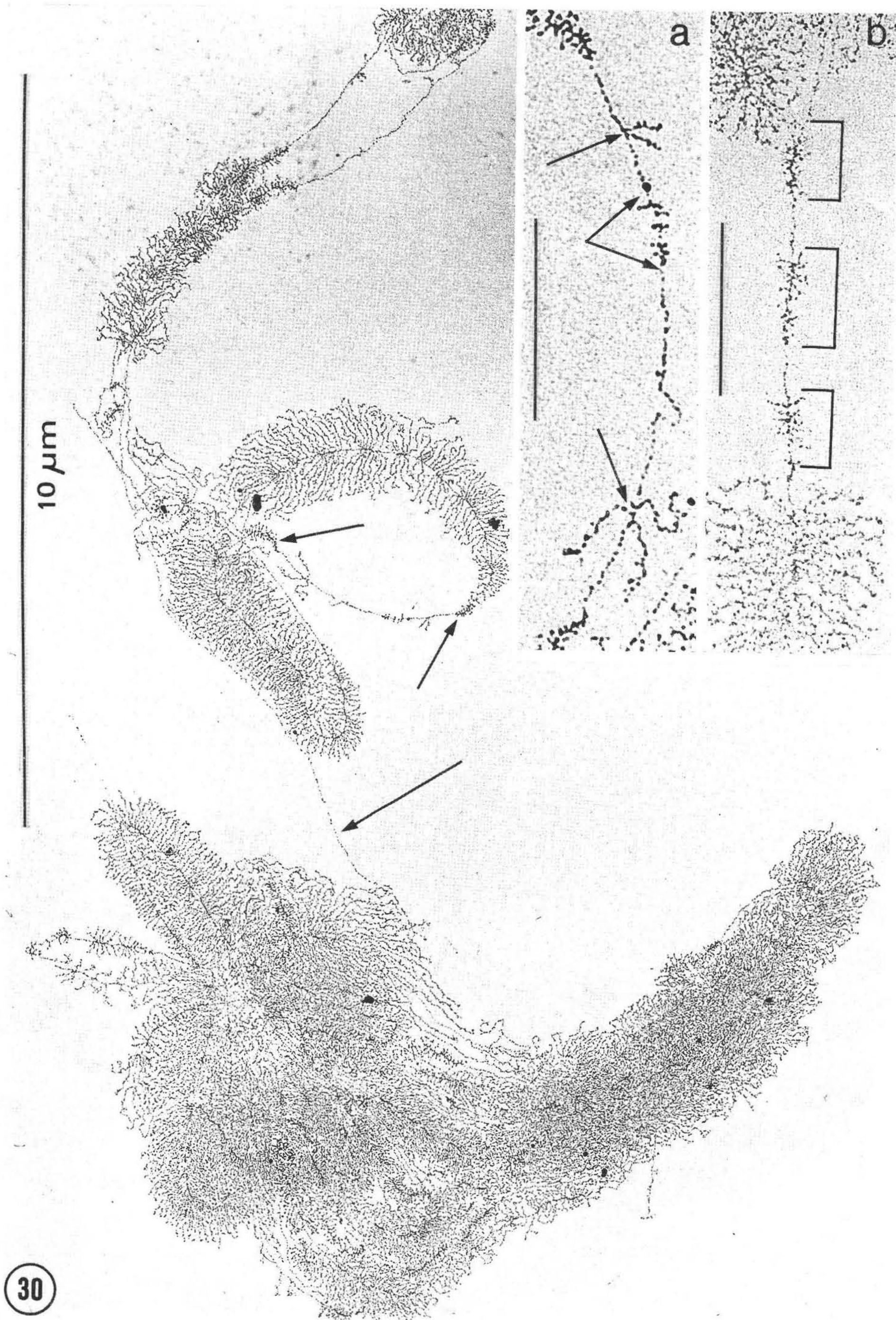
Besides the type of matrix unit hitherto described, i. e. those which are about 1.6 to 2.2 μm long, separated by regular spacer intercepts and occur in repeats (c. f. Figs. 37 and 38), there are other types of matrix units which are considerably longer (Figs. 21 to 38). These larger matrix units can be roughly subdivided into two major size classes (for details see Figs. 28 and 37) which, however, are not distinctly separated from each other. Such longer matrix units occur, in isolated nucleoli and nuclear contents in both species investigated, sometimes interspersed with matrix units of the above described shorter (1.6 to 2.2 μm) size class within the same aggregate and frequently on the same axis (Figs. 21, 22, 27, 31, 33). On the other hand, it appeared that there were aggregates which exclusively contained larger matrix units (e. g. Figs. 23, 24, 25, 26, 28, 30). Regarding their subarchitecture, the longer matrix units revealed identical arrangements as described for the shorter ones, except for the greater fibril lengths in their terminal regions (Figs. 22 a, 24, 28, 29). In the longer subclass of these matrix units, in which total lengths of up to 12 μm were measured (see Figs. 26, 27, 29, 31, 32), lateral fibrils of up 3 μm length were traced in particularly well spread preparations (Figs. 29 and 32). With both subclasses of longer matrix units we repeatedly found situations in which the start→terminus vectors of matrix units adjacent in the same axis were

running in opposite directions (e. g. Figs. 21, 23, 24, 30, 33) including cases of "tail-to-tail" (i. e. start-to-start; see e. g. Figs. 21, 23, 24, 30, 33) as well as of "head-to-head" (terminus-to-terminus; e. g. Fig. 33) configurations. Such situations are somewhat similar to the "head-to-head" association of nucleolar cistrons described by Miller and Beatty [129] in oocytes of *Triturus*. In such situations of vectorially opposite arrangements of adjacent cistrons individual matrix units of the short (i. e. about 2 µm long) size class could be involved (e. g. Fig. 21; c. f. ref. [175] for an example in *A. mediterranea*). We never observed such inverse orientations in homogeneous axes containing only the short matrix units (see Figs. 10 to 20). In some but not all preparations, terminal knobs were noted on the lateral fibrils (e. g. Fig. 26), sometimes even over almost the entire matrix unit length. In addition, we occasionally observed, in more terminal regions of the giant matrix units, very densely stained (and perhaps less extended) knobs in basal parts of the lateral fibrils (e. g. at the double arrow in Fig. 26).

Fully extended axial fibrils bearing such giant ("superlong") matrix units were very rarely observed. It appears as if they are less readily extended and spread during the preparation than the axes in which the short matrix units predominate (see above). In general, the larger matrix units showed a greater tendency to remain in an aggregate form and to inter- and intramaterial adherence (Figs. 26, 27, 28, 31, 32). This is probably a consequence of the increased ratio of lateral versus axial fibril mass and the greater stickiness of the RNP-fibrils (see above). Spacer intercepts in between the long and the giant matrix units occurred, but could only in rare instances be traced (e. g. Fig. 30). Again we found, in such spacers next to long matrix units, instances showing isolated small groups of lateral fibrils ("spacer transcripts") which were either arranged in regularly spaced clusters (e. g. Fig. 23 and insert b in Fig. 30) or occurred as isolated fibrils (e. g. Fig. 30, insert a). Situations suggesting the occurrence of either irregular, relatively short matrix units or incomplete (thinned) "normal" matrix units were noted in axes containing long matrix units as well as in those containing short units (e. g. Figs. 25 and 34).

The long and extremely long (giant) matrix units in the *Acetabularia* primary nucleus strongly resemble the configurations which have been described by Miller and coworkers [75, 76, 122, 124, 125, 129, 131, 132] in nuclei and "nucleolar cores" of amphibian oocytes ([129]; see their Fig. 8) and have been tentatively interpreted as representing the extended and spread chromosomal lampbrush loops *in statu transcribendi* or as portions of *nucleolar* axes "where abnormally long (lateral) fibers apparently have been synthesized" [129], respectively. A similar interpretation of the

Fig. 30. Intensively spread aggregate from a primary nucleus of *Acetabularia major* containing mainly matrix units of the large size classes (long and extremely long). The two short arrows (in the center) indicate the start regions of two cistrons on the same axis, which obviously run in opposite direction. The long arrow denotes one of the extended axial fibrils, which are not associated with lateral filaments for a long distance. Note the lateral fibrils of various lengths that are associated with the axial intercept in between the matrix units denoted by the short arrows. Such small groups of lateral fibrils (including spacer transcript units) can be classified into those which occur in an alternating somewhat regular pattern of a defined length (0.5 µm) spaced by well defined, fibril-free axial intercepts (*insert* **b**) and others which occur isolated as groups of only two to five identifiable, individual lateral fibrils with no marked length gradient (*insert* **a**). Note, in **a**, the difference in lateral fibril length among three groups of lateral fibrils present (denoted by arrows). – The scale indicates 10 µm, in the inserts 1 µm.


10 µm
a
b
30

long and very long matrix units as transcribed cistrons of non-pre-rRNA character might be constructed for our demonstration in *Acetabularia* primary nuclei. However, there also exist in other organisms examples of relatively long matrix units apparently involved in rDNA transcription [150, 173, 174]. Also the basis for interpreting such giant matrix unit structures as expressions of transcriptional activity is based merely on their structural homology to defined rDNA nucleolar cistrons (see also [75, 76, 124, 125, 131, 132]). There appears to be no reason to *a priori* exclude the occurrence of cistrons coding for non-ribosomal RNA in nucleoli and in DNA molecules which contain rDNA (see the debate as to the existence for genes coding for heterogeneous non-pre-rRNA in nucleoli in chapter V of ref. [21]).

From preparations of isolated and "washed" nucleolar aggregates from *Acetabularia* primary nuclei there is no basis for the argument that short cistrons are exclusive to the "true" nucleolar material, in contrast to a chromosomal, non-nucleolar origin of the larger and the giant matrix units. We observed long matrix units also in purified nucleolar material, even after intensive and repeated washing in the incubation medium (e. g. Fig. 36).

Separated from as well as associated with the matrix unit containing aggregates, tangles of filaments without any clearly identifiable lateral fibrils were frequently observed (see e. g. Figs. 31 and 32 a). Their structural appearance suggested that such strands may represent transcriptionally inactive axial fibrils; this would be also compatible with their morphological similarity to the "naked" axial fibrils described by MILLER and BEATTY [128, 129] from *Triturus viridescens* nucleolar cores after removal of the lateral fibrils by digestion with ribonuclease. It cannot be decided if these fibrils contain rDNA or are potentially transcribed at all. Occasionally, another sort of matrix-unit free and tangled fibril was observed associated with some densely stained granules (e. g. Fig. 25) which could not be resolved in detail and characterized by transcriptional products.

In a few instances a type of fibrils different from all the above described fibrillar structures was observed (e. g. Fig. 32). These were groups of somewhat aligned, densely stained, but less tortuous, ca. 5 nm broad filaments. While their nature remains unknown, it might be noteworthy that fibrillar tangles of this type have been found in spread contents of oocyte nuclei of *Dytiscus marginalis* (TRENDELENBURG, unpublished).

No free, distinctly granular or globular regular structural component was observed in spread preparations of *Acetabularia* primary nuclei. MILLER and associates [130] have reported that even spread nucleolar *pars granulosa* material from amphibian oocytes appears in extended, thin fibrils upon swelling and spreading. However, we sometimes observed very heavily stained granular particles in association with matrix unit-containing aggregates which at first view were suggestive of staining artifacts, but

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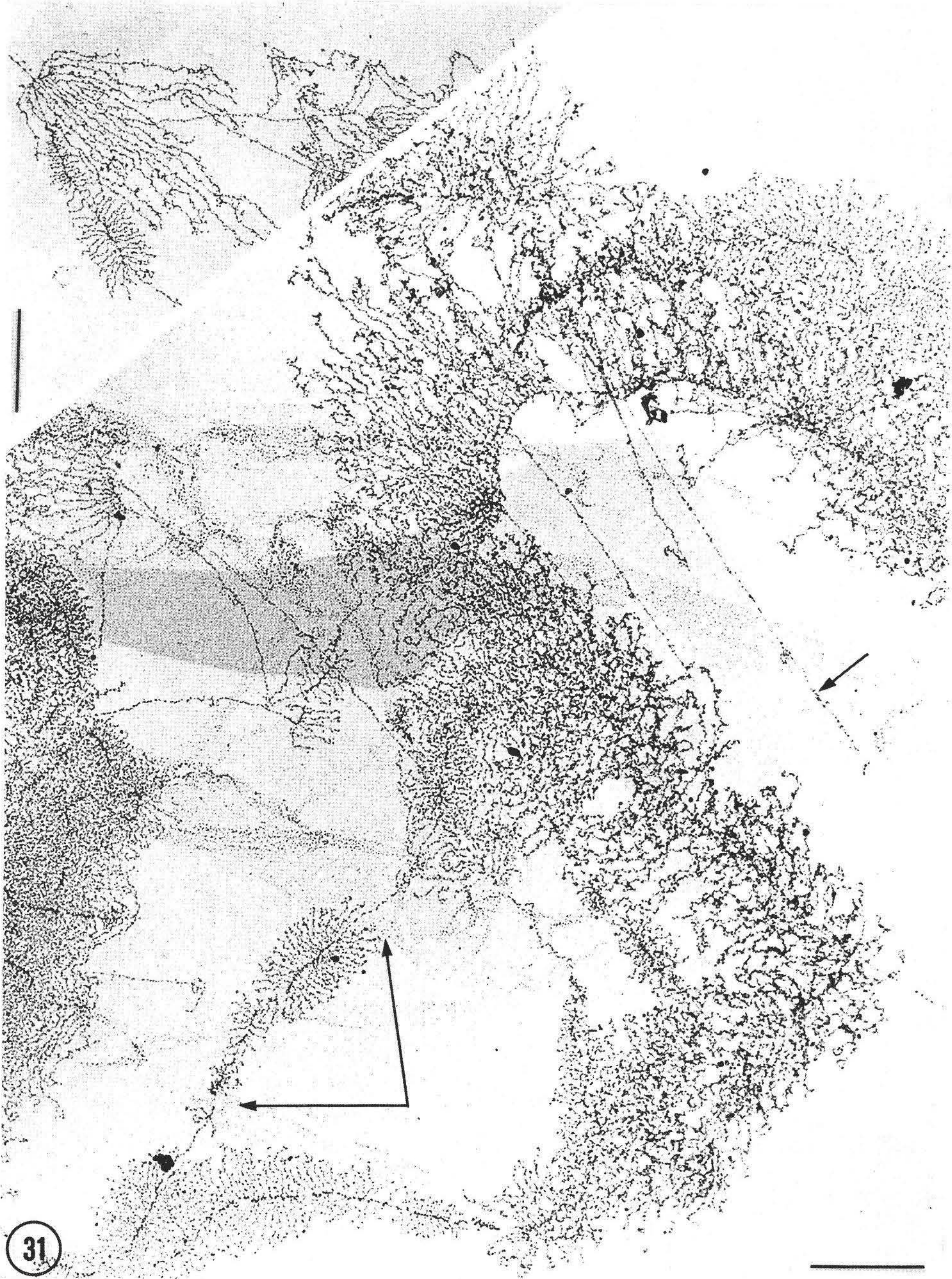
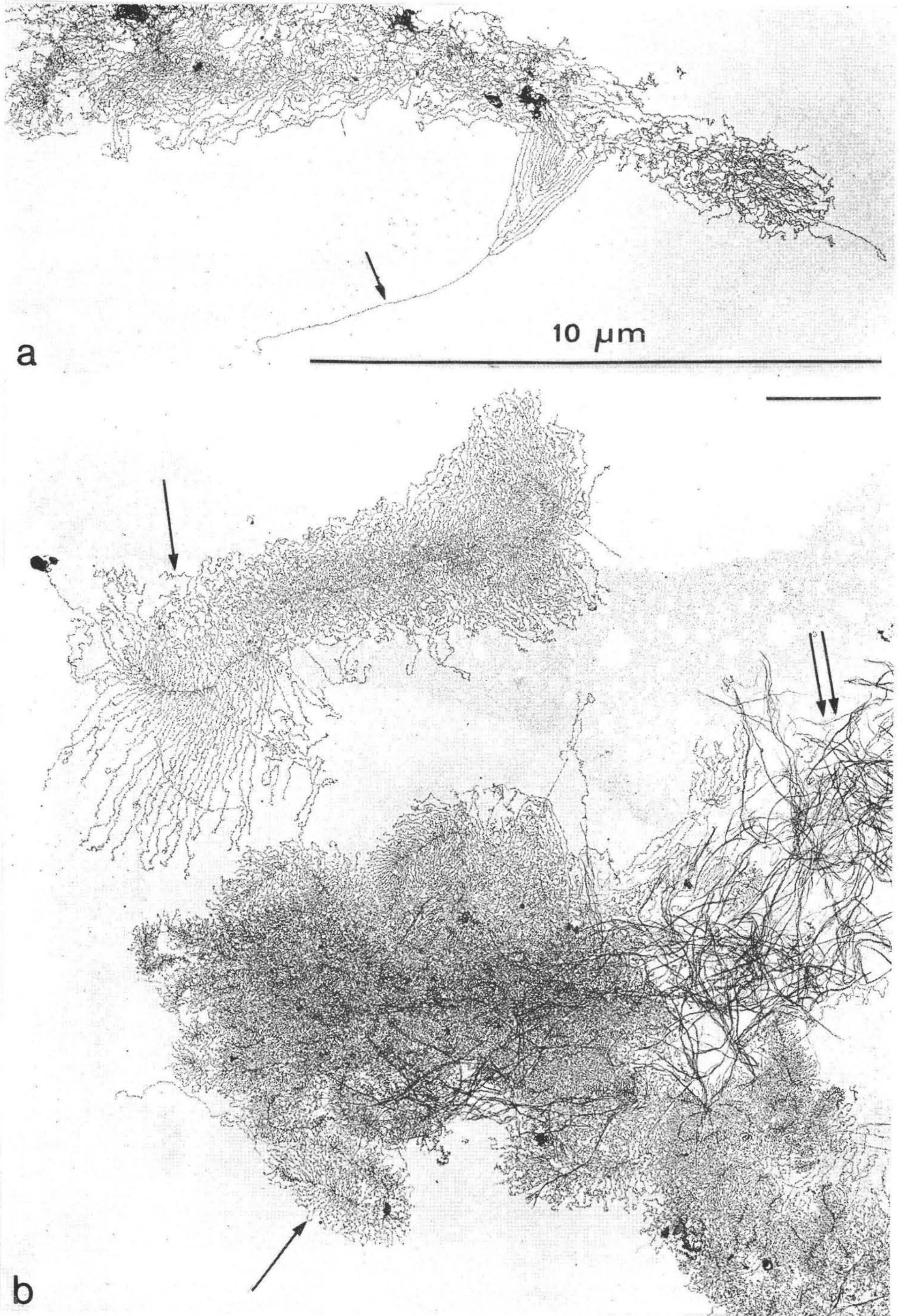
Fig. 31. Intricate meshwork of axes that bear matrix units of different size classes in spread nuclear contents of *Acetabularia mediterranea*. One identifies matrix units of the about 2 µm length class (one especially clear example is indicated by the pair of arrows in the lower left) and, besides those of the 3.5 to 5 µm length class (one example is seen in the left bottom part), giant transcriptional units (with no clearly discernible ends), as well as axial strands without any associated lateral fibrils (see, e. g. the arrow in the right). In most situations, the relationship of adjacent cistrons (especially in the neighborhood of giant matrix units) is not clearly resolved, which is partially due to the nearly identical width of axial and lateral fibrils in the extremely long ("superlong") matrix unit (see in particular in the insert.) – The scales indicate 1 µm.


31


10 µm
a
b

on closer inspection might as well be tentatively interpreted as true granules or densely packed coils, since structures apparently intermediate in staining and/or package density between a compact granular and a more unravelled fibrillar state could be observed (e. g. Fig. 35).

Quantitative evaluation. In a preliminary communication on rDNA morphology in *A. mediterranea* some measurements of the lengths of matrix units, spacer intercepts and whole repeat units have been presented [175]. In the present article we show a comparison of such values in both species (Fig. 37). In the primary nucleus of both species the matrix units of the short type (illustrated by the open blocks in the intermediate panel b and b′ of Fig. 37), i. e. the putative cistrons for pre-rRNA, were distributed in a rather sharply limited mode with a mean peak value of 1.84 µm (*A. mediterranea*) and 1.77 µm (*A. major*), respectively, which would fit into a Gaussian distribution curve. Measurements of the corresponding spacers indicated a somewhat higher variability, including examples of extremely short [175] as well as of rather long spacer intercepts (the upper panel in Fig. 37). A suggested mean (peak) length was approximately 0.96 µm (*A. mediterranea*) and 1.05 µm (*A. major*), respectively. The longer matrix units were also measured and arbitrarily classified into two size classes

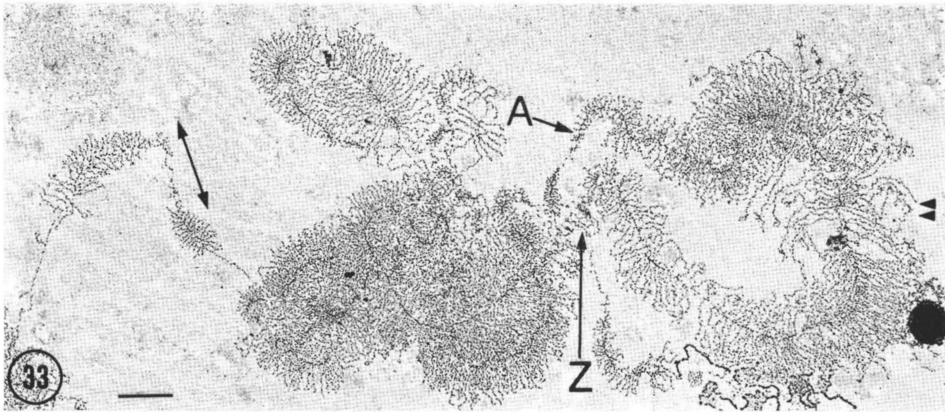


Fig. 33. A spread nuclear aggregate from *Acetabularia mediterranea* containing matrix units of diverse size classes revealing a situation suggestive of a "head-to-head" orientation in a giant matrix unit (in the very right). A denotes the one start in this symmetry loop, Z the other; the arrowhead in the very right points to the region where the two termini meet. The double-headed arrow at the left indicates another example of an apparent "tail-to-tail" relationship of adjacent cistronic units (i. e. start regions of matrix units running in opposite directions). – The scale indicates 1 µm.

Fig. 32 a. The figure represents an entangled fibrillar complex in a spread preparation from the primary nucleus of *Acetabularia major* in which a clear distinction of long lateral fibrils and "naked" (fibril-free) axial fibrils (arrow) is not possible. – **b.** Shows the coincident occurrence of aggregates of fibrils that appear to be heavily and more rigid in contour (double arrow on the right) with transcriptionally active material containing matrix units of the different size classes (compare the two units denoted by the arrows in the left). The nature of these relatively dense and rigid fibrils is not known. – The scales indicate 10 µm (in **a**) and 1 µm (in **b**).

(see panel b and b′ in Fig. 37) but the observed number of corresponding and clearly traced spacer intercepts was too low to be included in this graphical presentation. From these measurements one obtains ratios of spacer unit: matrix unit lengths of about 0.5 in both species, a value which is within the range of values reported for the chromosomal and extrachromosomal nucleoli of a variety of amphibia and insects

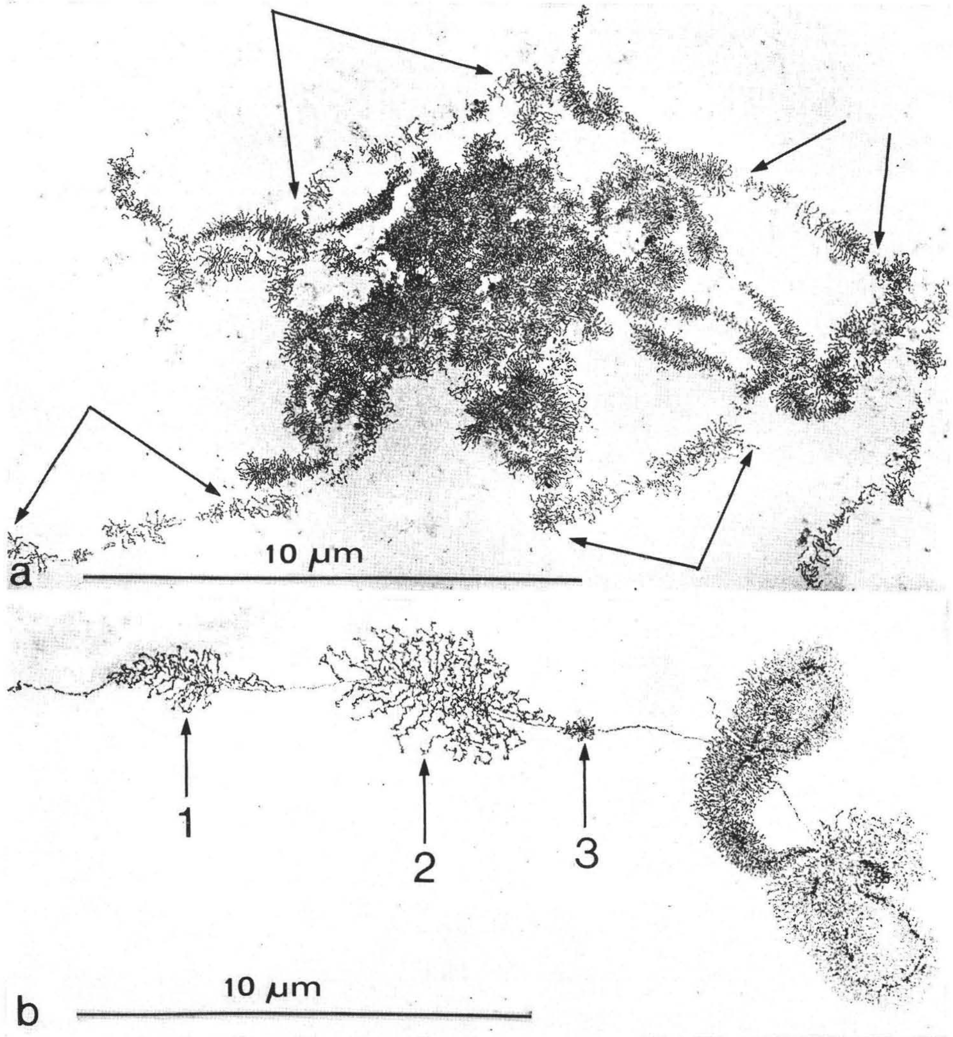


Fig. 34. Matrix unit-like groups of lateral fibrils with lower package density and irregular spacings (examples are denoted by the pairs of arrows in the spread preparation of an aggregate containing short matrix units in **a**) and without clearly defined start and terminal regions (see, e. g., 1, 2 and 3 in **b**) on axes that bear short and long matrix units. It cannot be decided whether these unusual and incomplete matrix units are due to preparation artifacts or represent the specific *in vivo* state. – The scales indicate 10 µm.

[37, 77, 78, 80, 121, 125, 127, to 130, 150] but which is well below the values determined in the extrachromosomal rDNA in the oocytes of *Acheta* and *Dytiscus* and those observed in HeLa cell nucleoli [75 to 78, 124, 125, 173, 174]. A compilation of most of these values has recently been made [174]. As to discussions and speculations of true variability among pre-rRNA cistrons the reader is referred to SCHEER *et al.* [150].

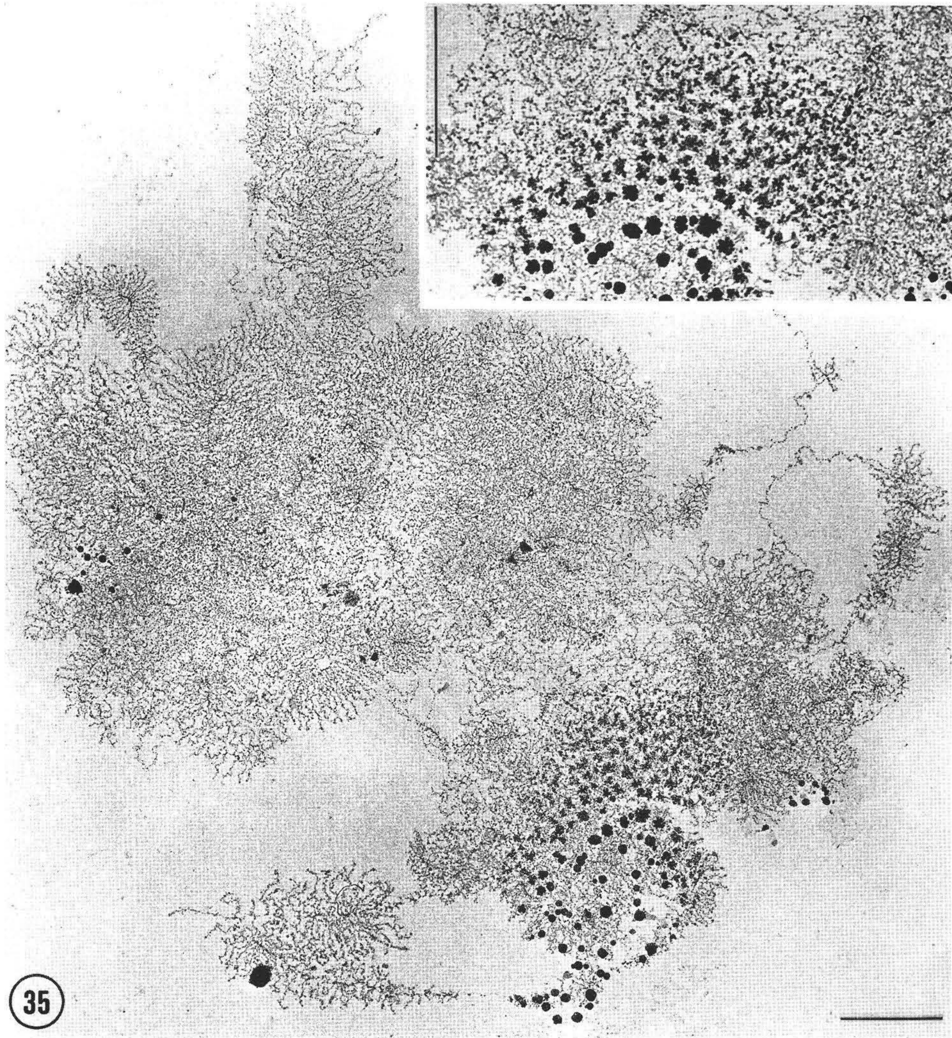


Fig. 35. Moderately spread nuclear aggregate of *Acetabularia mediterranea* in which dense sharply or less distinctly defined granules appear in association with the matrix unit material (in the lower part of the Figure and, at higher magnification, in the *insert*). These "dots" may represent PTA staining artifacts or contamination but could as well reflect granular *in vivo* structures not unravelled during the preparation. – The scale indicates 1 μm.

It is obvious that differences in the degree of fibril extension and spreading of the preservation of the natural fibrillar associations in the preparation, and of *in vivo* transcriptional activity can greatly affect the validity of quantitative evaluations [12, 150]. Therefore only fully transcribed axes and well and uniformly spread preparations should be selected for such determinations. The marked differences between the nucleolar matrix units of, for example, the primary nucleus of *Acetabularia* (ca. 1.8 μm), of the oocytes of various urodelan and anuran amphibia (2.5 to 2.9 μm), of spermatocytes and somatic cells of a dipteran insect, *Drosophila* (2.4 to 2.6 μm), and of the oocytes of some coleopteran and orthopteran insects (3.8 and 5.6 μm, respectively) are obviously significant and cannot be explained by differences in fibril dispersion and preservation.

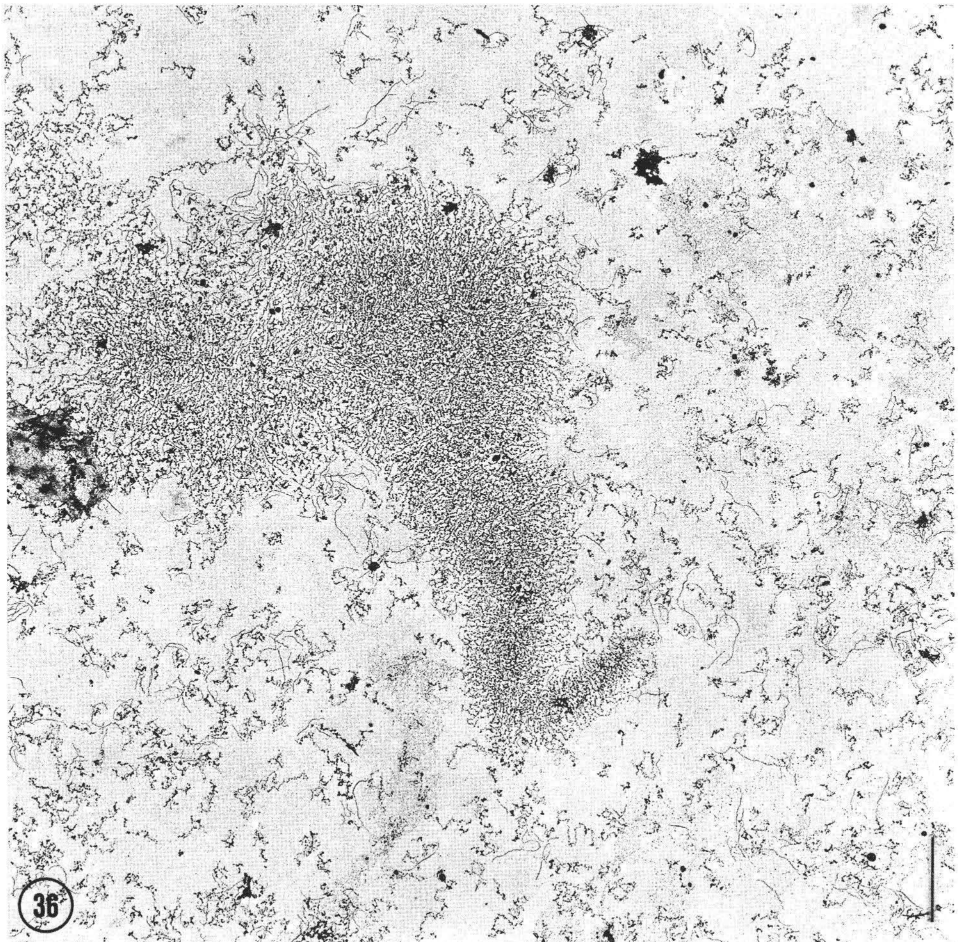


Fig. 36. Extensively spread and positively stained preparation of manually isolated and purified nucleolar material from *Acetabularia mediterranea* illustrating the occasional occurrence of giant matrix units in defined nucleolar structures and the predominance, in such preparations, of rather homogeneously distributed small isolated fibrils which sometimes appear to be in a partially granular (coil) configuration. – The scale indicates 1 μm.

It is interesting that the nucleolar matrix units of the primary nucleus of *Acetabularia*, despite their lower length, contain as many as 110 lateral fibrils, i. e. synthetically active RNA polymerase molecules. This seems to be achieved by a closer packing of the transcriptional complexes. However, the number of "working" RNA-polymerase molecules per matrix unit (cistron) cannot be regarded as a constant figure in eukaryotic nucleoli in general, since the matrix units in the extrachromosomal bodies of the insects *Acheta* and *Dytiscus* contain as many as 250 to 280 and 200 to 250 attached fibrils, respectively (TRENDELENBURG, unpublished). The latter observation in *Dytiscus* and *Acheta* and *Dytiscus* contain as many as 250 to 280 and 200 to 250 attached fibrils, RNA polymerases per pre-rRNA cistron if one assumes that a terminated mature fibril becomes translocated along the axis for a certain distance with its RNA polymerase molecule attached, i. e. by a "running out" process.

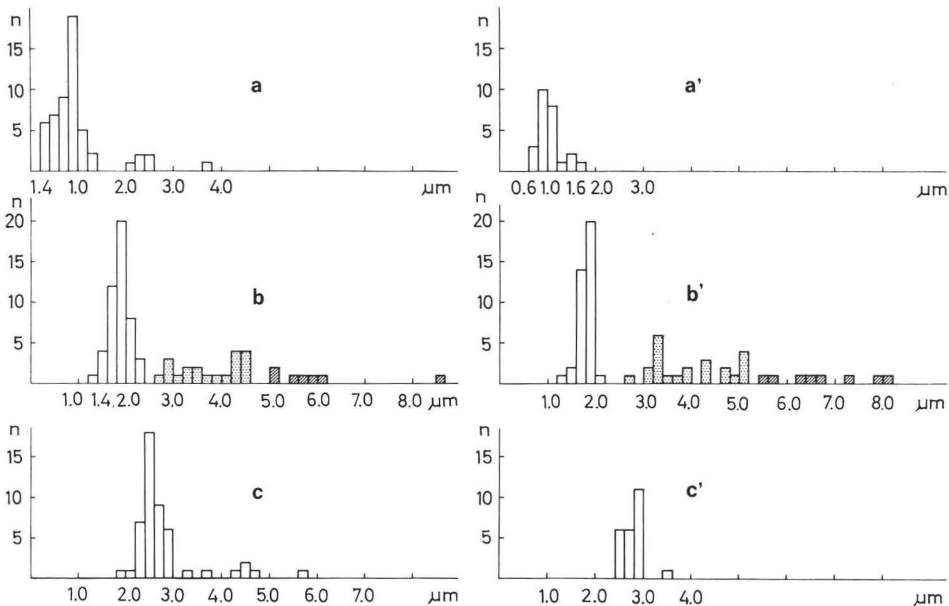


Fig. 37. Length distributions of matrix units revealed in spread preparations of nuclear material from *Acetabularia mediterranea* (**a, b, c**) and *Acetabularia major* (**a′, b′, c′**). The number of individual measurements (n) is indicated on the ordinate. The similar distributions of lengths of spacer intercepts (upper panel, **a** and **a′**), matrix units (medium panel, **b** and **b′**), and total repeat units (**c** and **c′**) in the primary nuclei of both species is apparent. In the measurements of spacer intercepts only those observed in connection with the short matrix units have been regarded; consequently, only this class of cistronic units has been considered for the determinations of total repeating units (**c** and **c′**). Note that three groups of matrix units can be established (**b** and **b′**) according to their lengths, the 1.4 to 2.4 µm long ones (white blocks), the longer ones (from about 2.8 to 4.6 µm in the spreadings of material from *Acetabularia mediterranea*; up to 5.2 µm in *Acetabularia major*) and the extremely long, giant ones ("superlong") (5.0 to ca. 12 µm in *Acetabularia mediterranea*, 5.4 to ca. 12 µm in *Acetabularia major*).

RNA determinations

The material recovered in the microsomal fraction from *A. mediterranea* was characterized by its weight ratios of RNA: DNA (< 0.01), RNA: protein (0.30), and phospholipids: protein (0.6), indicative of a high content of ribosomes but also of smooth surfaced endomembranes. The presence of a considerable amount of ribosome-free membranes in this fraction was also demonstrable by electron microscopy of sections through the fixed and embedded pellets. Determinations of total microsomal RNA resulted in an average figure of 520 nanograms per cell. Recoveries on acrylamide gels indicated that 85 % of this was undegraded cytoplasmic ribosomal RNA with molecular weights of 1.35×10^6 and 0.68×10^6 Daltons, as determined by non-denaturing conditions. Determinations in formamide containing gels were less clear

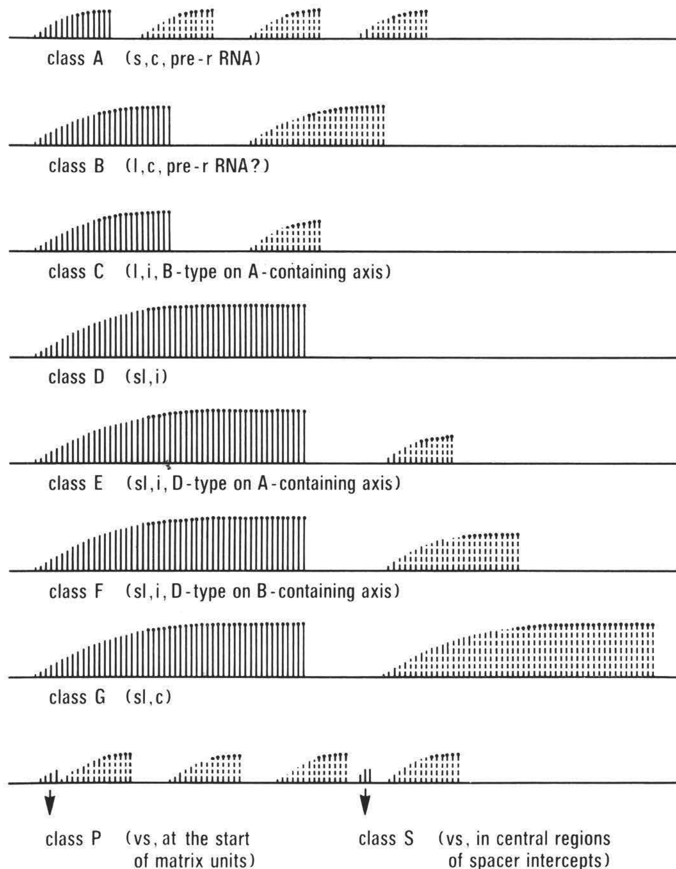


Fig. 38. Classification of matrix units (transcribed cistronic intercepts) according to morphological criteria. – s short. – pre-rRNA containing DNA sequences coding for pre-rRNA. – c clustered. – l long. – i isolated. – sl extremely long ("superlong"). – vs very short. – Class P illustrates the "prelude pieces" *sensu* SCHEER *et al.* [150]. – Class S includes all other forms of "spacer transcript" fibril groups.

and revealed the presence of some hidden, probably induced molecular breaks. Preliminary estimates of molecular weights from the identified peak components indicated values of 1.19×10^6 and 0.59×10^6 Daltons, respectively. Assuming that the degradation of ribosomal RNA in the cytoplasm of *Acetabularia* is negligibly low [93] one can calculate that a minimum of 440 nanograms of cytoplasmic rRNA is formed at an average per cell during the 85 days after germination, corresponding to a mean ribosome formation rate of 17 000 ribosomes per second.

Molecular weight determination of ^{3}H-uridine – and ^{32}P-labeled nuclear RNA (Fig. 39) indicated the presence of pre-rRNA components with molecular weights of 2.2 and 2.0×10^6 D, as determined under non-denaturing conditions. A considerable degree of conformational folding of this component was suggested by a shift in electrophoretic mobility under denaturing conditions (SCHEER et al., in preparation). While molecular weight determinations of cytoplasmic rRNAs in diverse plant cells are in fair agreement (e. g. [16, 25, 27, 28, 31, 65 to 68, 83, 101, 108, 109, 136, 142, 145]) the values reported for the size of their nuclear precursors vary considerably (e. g. [16, 27,

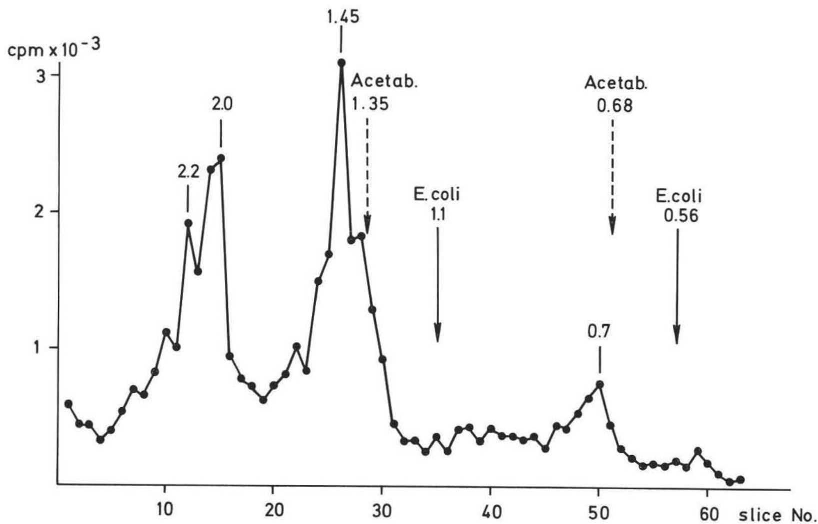


Fig. 39. Gel-electrophoretic separation of radioactive nuclear RNA of *Acetabularia mediterranea*. Fifty almost fully grown plants (with a total length of 30 mm) were incubated for 2 hours in 5 ml Erdschreiber medium without the usual phosphate content but with 200 μCi/ml ^{32}P at 20° C. The algae were then washed five times with a total of one liter of non-radioactive medium. They were then either used directly or were further incubated for 2 hours (as shown in this figure). Subsequently, 30 nuclei were manually isolated which took a total of 80 minutes. Each nucleus was immediately transferred to ice-cold ethanol-acetic acid. The collected nuclei were washed two times in 70 % ethanol. RNA extraction and DNAse treatment were performed as described in Materials and methods. The RNA was analyzed on slabs of 2.25 % acrylamide–0.5% agarose gels. The positions of the coelectrophoresed *E. coli* rRNA are denoted by the arrows (solid lines), the positions of the cytoplasmic rRNAs from *Acetabularia mediterranea* are shown by the arrows with the broken lines. The apparent molecular weights have been calculated, assuming a semilogarithmic correlation between molecular weight and electrophoretic mobility, and are indicated for the specific components in million Daltons.

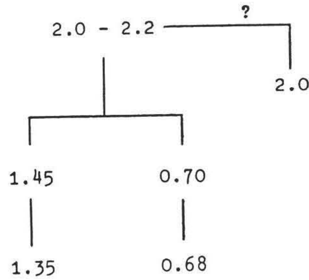


Fig. 40. Proposed scheme for nuclear-cytoplasmic pre-rRNA processing in *Acetabularia mediterranea*. The components are indicated by their molecular weights in million daltons. The transition denoted by the question mark indicates a possible cleavage to a (perhaps anomalous) stable high molecular intermediate.

28, 31, 66 to 68, 90, 94, 101, 109, 110, 136, 142, 145]). From our determinations and the demonstrated morphology of the pre-rRNA cistrons, it is clear that in *Acetabularia* only very little, if any, pre-rRNA material is lost during the processing (see also Discussion). An existence of pre-rRNA molecules larger than about 2.5 million Daltons would not be compatible with both the observed morphology of nucleolar cistrons (see Fig. 37) and the determined molecular weights of the nuclear RNAs (Fig. 39). We consistently noted in gel electrophoreses of nuclear RNAs a pronounced component with a mean peak molecular weight of 1.45×10^6 D and very little, if any, RNA with a mobility identical to that of the cytoplasmic large rRNA (Fig. 39). The best fit of our data into a preliminary rRNA processing scheme is presented in Figure 40 (for references in other plant systems see above). However, it is obvious that detailed determination of the molecular weights of pre-rRNA molecules under denaturing conditions must be accomplished before such a scheme can be regarded as proven.

DNA determinations

The DNA content of the haploid nucleus of *A. mediterranea* was determined as 1.29 picogram (9, 35, and 42 million swarmers were used in three determinations). Values obtained with the diphenylamine method were usually somewhat lower than those obtained by phosphorus determinations after alkaline hydrolysis of nucleic acids according to Schmidt-Thannhauser [153]. This figure for *Acetabularia* is considerably higher than that reported by Sueoka *et al.* [166] for *Chlamydomonas reinhardii*. However, we also found, in parallel experiments, slightly higher nuclear DNA contents in various species of *Chlamydomonas* than did Sueoka et al.

Discussion

Results obtained in this study demonstrate that the primary nucleus of *Acetabularia* is a suitable object for studies of nuclear structures and functions, in particular with respect to the morphology of transcriptional activities. The primary nucleus of *Acetabularia* is the first plant material in which direct morphological demonstrations of gene transcription have been achieved [51, 175]. In general, one can state that the structures observed closely resemble those described in a variety of animal cells, in

particular those demonstrated in oocytes [124 to 132, 150, 173, 174]. The vegetative cell growth of these algae shows a great many similarities to some animal cell systems that are likewise characterized by giant growth and the development of a giant nucleus. Table 1 presents some of the structural and functional similarities in a comparison of *Acetabularia* with "classic" representatives of giant growth in animal oogenic systems, on the one hand, and with a typical non-dividing "steady state" gland cell, the mammalian hepatocyte, on the other hand. It is suggested from this comparison that many of the striking morphological changes that occur during giant growth are related to the increased activity in ribosome formation (see also below and refs. [52, 175]). From the determined average rate of ribosome formation of about 17 000 ribosomes per second

Tab. 1. Some cell efficiency characteristics associated with giant nuclear growth.

	giant cells			An example of a non-dividing, non-growing cell
	Xenopus laevis, oocyte	*Acheta domesticus*, oocyte	*Acetabularia mediterranea*, primary nucleus	Rat (adult), average hepatocyte
max. cell diameter (μm)	1,200	2,100 × 510	ca. 1,000 × 45,000	ca. 15
max. nuclear diameter (μm)	500	130	100	8
max. number of nuclear pores	38×10^6	2.6×10^6	3×10^6	$3\text{–}7 \times 10^3$ (4)
max. number of cytoplasmic ribosomes	10^{12}	n.d.	1.3×10^{11}	8×10^6
ratio rDNA: 1C DNA (pg:pg)	30:3	3.48:2.0	0.2:1.29 (2)	0.01:3.4
mean rate of nuclear volume increase (μm^3/h)	65,700 (1)	526	360	0.0
mean rate of ribosome formation (per sec)	313,000 (1)	n.d.	17,000	25
mean rate of net nuclear pore formation (per min)	480 (1)	18.9	23.0	0.0
estimated total number of (active) pre-rRNA cistrons	2×10^6 (1)	> 1,600	> 23,000 (> 17,000) (3)	480–1,100
nuclear pore flow rate (ribosome RNA equiv./pore/min)	2.6 (1)	n.d.	0.3	0.23–0.57 (4)
references evaluated	11,149	35,104, 106,134	53,73, this study	11,48,138

(1) lampbrush phase
(2) estimated minimum (see text)
(3) depending on the specific mode of calculation (see text)
(4) depending on the electron microscopic procedure used for determination (ref. [48])

during the postgermination period up to the onset of cap development, one can calculate, assuming the simultaneous production of a mean of 110 pre-rRNA molecules per cistron, an average molecular weight of 5.0 million Daltons per rDNA repeating units (see Fig. 37; for molecular weight estimations of double stranded DNA see, e. g. refs. [12, 98, 144, 168]), and a mean chain elongation rate of 40 nucleotides per second as in various other eukaryotes ([16, 24, 64, 79, 149]; for rates in prokaryotes see ref. [36]), that a minimum of 23 000 pre-rRNA cistrons are required to allow such a production. This would correspond to a total of 0.2 picogram of DNA, i. e. about 16 % of the haploid nuclear genome content. The real figures might even be higher since it is likely that ribosome production is not continuous during the whole period [93] and that the chain elongation rate might also be reduced during some time intervals. This estimation fits with a different approximation of the minimum figure of rDNA copies present made by multiplication of the number of matrix units per individual aggregate and the number of distinct individual nucleolar subunits identified per nucleus (see Results) which results in a total figure of about 17 000 in *Acetabularia mediterranea* and 21 000 in *Acetabularia major*. (This slight difference, however, cannot be regarded as significant and characteristic for the species.) The high amount of rDNA in the *Acetabularia* primary nucleus suggests either an enormous degree of polyploidy or, more likely, a high degree of rDNA amplification.

The literature contains some reports of high redundancies (up to several thousands) of pre-rRNA cistrons in the "normal" diploid genome of a variety of plant cells (e. g. [11, 33, 84, 88, 89, 116, 118, 137, 159, 167 to 171]). However, the dramatic increase in nucleolar mass in *Acetabularia* upon zygote germination, the formation of a large DNA-containing aggregate nucleolar body similar to those in various animal oocytes, the relatively moderate redundancies of rDNA copies reported in the normally sized nuclei from other algae including instances among the Euglenophyta and Chlorophyta [87, 156], and the observation that the aggregates which bear the matrix units frequently occur as distinct, isolated morphological entities and are apparently not all attached to chromatin masses, leads us to hypothesize that, during early phases of nuclear growth, rDNA copies are selectively replicated and amplified into extrachromosomal units (see also ref. [53]). This amplification might perhaps proceed by a similar "rolling circle" mechanism as recently described in the oocytes of the clawed toad, *Xenopus laevis* [10, 85, 86]. Despite the lack of identifiable circular configurations, which bear matrix units, in spread preparations of *Acetabularia* nuclear contents we would not exclude the possibility that these axes are circular *in vivo* as has been demonstrated, at least with smaller circles, in the rDNA of *Xenopus* and *Dytiscus* oocytes [10, 57, 85, 86, 173], and in the macronucleus of *Tetrahymena pyriformis* [55].

Various authors have claimed rDNA amplification in plant cells during special parts of the cell cycle, under special physiological conditions, or in special phases of cell developmental processes (e. g. refs. [1, 29, 170]) the examples including one green alga, *Chlamydomonas reinhardii* [87]. However, in all the systems studied the extent of amplification which was concluded from molecular hybridization of isolated DNA or *in situ* was rather moderate, if determined at all, and the data reported are far from conclusive. Our studies in *Acetabularia* suggest that the giant growth of this alga in its vegetative phase might represent a special example of large rDNA amplification. Further analyses of primary nucleus DNA by hybridization techniques should now be made.

Our observations in spread preparations of nuclear components from the primary nucleus of *Acetabularia mediterranea* and *Acetabularia major* have led to more detailed

classification of matrix units, the putative transcriptionally active regions in deoxyribonucleoprotein (DNP) fibrils, simply based on morphological criteria (Fig. 38). The most highly repeated and uniform class of matrix units which quantitatively dominated in our preparations is the type designated as "class A". This class closely resembles in size and pattern of arrangement the pre-rRNA producing matrix units in a variety of animal cell systems [37, 75 to 78, 80, 121, 125 to 132, 150]. Therefore, and because of its occurrence in isolated nucleoli, we interpret this class as a relatively homogeneous population of transcribed cistrons of pre-rRNA. Our observations further indicate that in *Acetabularia* primary nuclei the majority, if not all, of these cistrons are actively engaged in transcription. As already mentioned, the class A matrix units of *Acetabularia* are much shorter than those described in animal cell systems [75 to 78, 80, 121, 124 to 130, 132, 150]. When one compares the matrix unit length of this class with the molecular weights calculated for the corresponding cytoplasmic rRNAs and the putative pre-rRNA by gel electrophoresis (Fig. 39) it becomes obvious that little, if any, loss of RNA can occur during the rRNA processing steps (see also ref. [175]). On the basis of the concept that length of the matrix units represents the length of double stranded DNA in B-conformation one strand of which is transcribed into a RNA molecule with an equivalent number of nucleotides, the data for *Acetabularia* would hardly be compatible with the existence of considerably longer pre-rRNA molecules such as those described in a variety of plant (e. g. [16, 31, 67, 90, 101, 142, 145]) and animal (for reviews see, e. g., [19, 21, 109, 135, 160]) cells. Another alternative, namely that there exist rDNA transcripts in the nucleoli of *Acetabularia* primary nucleus which do not contain the sequences of both the 26 S and 18 S rRNA molecules (see also ref. [172]) seems very unlikely in view of the present knowledge of rRNA formation in eukaryotes in general.

Matrix units of class B and C (Fig. 38) which might occur in clusters (B) or in mixed arrangements with class A type matrix units (C) are distinguished by their greater lengths (Fig. 37). Since we were unable to find them in long extended and well spread axes we cannot decide whether they might occur as highly repeated and regularly arranged. Therefore, more detailed analyses must be made before a hypothesis as to their possible character can be made, especially as to whether they also represent transcription products of rDNA and include rRNA sequences or not. The lengths of some of the matrix units of this size class would well allow for the interpretation as demonstrations of "read through" transcripts containing sequences homologous to the DNA of two adjacent class A matrix units and the enclosed spacer intercept. In this context it is also worth noting that matrix units of similar lengths have been described in the transcriptionally active extrachromosomal rDNA in the oocytes of *Dytiscus marginalis* and *Acheta domesticus* [173, 174]. Therefore, we would not *a priori* regard this longer class of matrix units in *Acetabularia* as representing transcriptional events in non-rDNA.

The very long matrix units ("superlong", sl, class D to G in Fig. 38) strongly resemble the structures which Miller and associates have described in amphibian oocytes as images of lampbrush chromosome loop transcription [75, 76, 122, 124, 125, 129, 132] and because of their giant size are widely interpreted as demonstrations of the formation of precursors of messenger RNA molecules (for reviews of the recent discussions of the existence of giant pre-mRNA molecules see e. g. [34, 58, 115, 152, 160, 181]). However, there is only indirect and poor evidence for this speculation, and one should be aware of the reported occurrence of such matrix units in nucleolar material aggregates as reported by Miller and Beatty [129] in *Triturus viridescens* oocytes and, in the present article, in the primary nuclei of two *Acetabularia* species. Again we must

admit that a clear demonstration and distinction of the arrangement of such giant matrix units in class E, F or G patterns was not possible, probably due to the "stickiness" of these giant matrix units. There is, however, a strong indication that mixed arrangements of "very long", long and short matrix units on the same axis are common (see Results).

Class P matrix units ("prelude pieces") have been described in animal systems as well [150]. Such structures might either represent a proleptic ("wrong") initiation of a transcriptional event within a "spacer" region followed by a reinitiation at the "right" cistronic begin or might indicate an abrupt conformational change in RNP packing in the nascent fibril. They could also represent the transcription of a distinct class of non-pre-rRNA cistrons located in "spacer" intercepts of rDNA containing molecules. These two latter considerations would also be conceivable in explaining the class S matrix units which include all small groups of lateral fibrils attached to spacer intercepts at some distance from start regions of the three larger matrix unit size classes defined above.

Since Brown *et al.* [17] have reported a difference in the relative lengths of the spacer intercepts in *Xenopus laevis* and *Xenopus muelleri,* we should perhaps add that we do not ascribe significance to the minor differences observed in spacer and matrix unit lengths between *Acetabularia mediterranea* and *Acetabularia major* but rather emphasize the far-reaching similarity of the nuclear structures in both species.

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Note added in proof: We attract the reader's attention to a detailed analysis of the nucleoli of *Acetabularia major* by S. Berger and H. G. Schweiger (80 S Ribosomes in *Acetabularia major.* Redundancy of rRNA cistrons. Protoplasma, in press). In a recent article, M. J. Miller and D. McMahon (Synthesis and maturation of cytoplasmic ribosomal RNA in *Chlamydomonas reinhardi.* Biochim. Biophys. Acta **366,** 35–44 [1974]) have published a processing scheme for the maturation of cytoplasmic ribosomal RNA in the green alga *Chlamydomonas reinhardii* which is very similar to that presented in this study for *Acetabularia.* A series of recent studies have again emphasized the variations in pre-rRNA gene multiplicity in a variety of plant species, including examples of marked variability among different varieties within one species (e. g. R. B. Flavell and D. B. Smith: Variation in nucleolar organiser rRNA gene multiplicity in wheat and rye. Chromosoma **47,** 327–334 [1974] and J. Mohan and R. B. Flavell: Ribosomal RNA cistron multiplicity and nucleolar organisers in hexaploid wheat. Genetics **76,** 33–44 [1974]; c. f. refs. [137, 170, 171]). In a reevaluation of the literature we became aware of the article by J. H. Sinclair (Buoyant density of ribosomal genes in *Chlamydomonas reinhardi.* Exptl. Cell Res. **74,** 569–571 [1972]) which contains some data indicative of a selective amplification of nucleolar cistrons during the meiosis of *Chlamydomonas reinhardii* (see also ref. [166]). As to the possible interpretations of the class P and S matrix units (see Fig. 38) it is interesting to note the demonstration by G. M. Rubin and J. E. Sulston (Physical linkage of the 5 S cistrons to the 18 S and 28 S ribosomal RNA cistrons in *Saccharomyces cerevisiae.* J. Mol. Biol. **79,** 521–530 [1973]). This situation is in contrast to that described for various animal cells.

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