Protoplasma 126, 130—139 (1985)

PROTOPLASMA

Ultrastructure of Mitosis in *Amoeba proteus*

D. B. GROMOV*

Institute of Cytology of the USSR Academy of Sciences, Leningrad

Received September 17, 1984
Accepted November 13, 1984

Summary

The fine structure of *Amoeba proteus* nuclei has been studied during interphase and mitosis. The interphase nucleus is discoidal, the nuclear envelope is provided with a honeycomb layer on the inside. There are numerous nucleoli at the periphery and many chromatin filaments and nuclear helices in the central part of nucleus.
In prophase the nucleus becomes spherical, the numerous chromosomes are condensed, and the number of nucleoli decreases. The mitotic apparatus forms inside the nucleus in form of an acentric spindle. In metaphase the nuclear envelope loses its pore complexes and transforms into a system of rough endoplasmic reticulum cisternae (ERC) which separates the mitotic apparatus from the surrounding cytoplasm; the nucleoli and the honeycomb layer disappear completely. In anaphase the half-spindles become conical, and the system of ERC around the mitotic spindle persists. Electron dense material (possibly microtubule organizing centers—MTOCs) appears at the spindle pole regions during this stage. The spindle includes kinetochore microtubules attached to the chromosomes, and non-kinetochore ones which pierce the anaphase plate. In telophase the spindle disappears, the chromosomes decondense, and the nuclear envelope becomes reconstructed from the ERC. At this stage, nucleoli can already be revealed with the light microscope by silver staining; they are visible in ultrathin sections as numerous electron dense bodies at the periphery of the nucleus.
The mitotic chromosomes consist of 10 nm fibers and have three-layered kinetochores. Single nuclear helices still occur at early stages of mitosis in the spindle region.

Keywords: Mitosis; Nuclear envelope; Endoplasmic reticulum; *Amoeba proteus*.

1. Introduction

The fine structure of the nucleus and cytoplasm of large freshwater amoebae has been studied extensively by now. Not only the ultrastructure of normal interphasic amoeban cells (DANIELS 1973, FLICKINGER 1973, 1974, PAGE and KALININA 1984) but also the morphological changes caused by different damaging factors have been investigated (ORD 1979 a). The dependence of the cytoplasmic organelles upon the nucleus has been explored in micrurgical experiments (FLICKINGER 1973). However, still little is known of the ultrastructure of mitosis in amoebae. There is only one paper describing mitosis in *Amoeba proteus* at the ultrastructural level (ROTH *et al.* 1960). At that time the electron microscopy had merely begun to develop and modern techniques for fixation and embedding had not been known yet, so it was not possible to reveal a number of morphological peculiarities of mitosis in amoebae. Some fragmentary data concerning mitosis in *A. proteus* were presented in several subsequent papers dealing mainly with other problems (GOLDSTEIN and KO 1978, ORD 1979 b). Nevertheless, there is still no sufficiently detailed description of mitosis in the large amoebae, especially in *A. proteus*.
The ultrastructure of mitosis being a taxonomic character of the amoebae, its investigation in *A. proteus* is of great interest in the comparative aspects as well. A great diversity of mitotic types is characteristic of unicellular organisms (HEATH 1980, RAIKOV 1982), thus allowing to discuss the problems of evolution of mitosis. Therefore the investigation of the fine structure of mitosis is rather interesting in any group of protozoans which have not been sufficiently studied yet in this respect.

2. Materials and Methods

The strain "B" of *Amoeba proteus* from the amoeban strain collection of the Laboratory of Cytology of Unicellular Organisms of the Institute of Cytology, USSR Academy of Sciences, was used in the

* Correspondence and Reprints: Institute of Cytology, Tikhoretsky Avenue 4, 194064 Leningrad, USSR.

present work. The amoebae were cultured in a mineral medium, with addition of *Tetrahymena pyriformis* as food (PRESCOTT and CARRIER 1964). For fixation, dividing amoebae were selected from a mass culture, since they can easily be distinguished from the interphase cells by their appearance (CHALKLEY and DANIEL 1933).

In order to reveal the nucleoli with the light microscop, Ag-NOR staining was used. The amoebae were fixed in 45% acetic acid on a slide while heating it slightly over a spirit-lamp for 0.5–1 minutes. After that the slide was air dried. The dry preparations were rinsed in distilled water, placed in a 0.5% solution of $Na_2B_4O_7$ for 20 minutes, and rinsed again. Then the preparations were air dried and stained with silver nitrate (HOWELL and BLACK 1980): 4 drops of 2% gelatin solution and 2 drops of 50% silver nitrate were mixed on the slide, covered with a cover-slip and heated in a thermostat at 60 °C for 1–2 minutes. The cover-slip and the silver nitrate solution were washed away with distilled water, and the preparation was dehydrated via alcohol and xylene and mounted in balsam.

For electron microscopy two techniques of fixation were used:

A. Fixation with a mixture of glutaraldehyde and osmium tetroxide in modification of ROTH *et al.* (1970). To a small volume of culture medium (2–3 drops) containing one or several amoebae, an equal volume of 12% glutaraldehyde in 0.03 M phosphate buffer (pH 7.4) containing $2 \cdot 10^{-5}$ M $MgSO_4$ and $2 \cdot 10^{-3}$ M saccharose was added at room temperature. Thirty seconds later, an equal volume of ice-cold 1% OsO_4 in the same buffer was added to the mixture, and the amoebae were fixed in an ice bath for 20–30 minutes. After that the material was washed 2 times in distilled water (each time for 5 minutes) at room temperature.

B. Fixation with a mixture of glutaraldehyde, tannic acid, and saponin. To a small volume (2–3 drops) of culture medium containing one or several amoebae, an equal volume of 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.2% tannic acid and 0.05% saponin was added. The material was fixed for 40 minutes at room temperature and rinsed 3 times in 0.05 M cacodylate buffer (each time for 5 minutes). Post-fixation was carried out in 1% OsO_4 in 0.05 M cacodylate buffer. Then the material was rinsed in 2 changes of distilled water. The concentrations of tannic acid and saponin in the glutaraldehyde solution was taken from MAUPIN and POLLARD (1983), though the final concentrations of these substances in the fixative were half as high due to its dilution with culture medium.

After fixation, the amoebae were pre-embedded in agar blocks (1.5% agar), dehydrated through ethanol solutions of increasing concentration and acetone, and embedded in a mixture of Epon and Araldite (MOLLENHAUER 1964). Semi-thin sections were stained with 0.5% methylene blue, containing 0.5% $Na_2B_4O_7$. Ultrathin sections were stained with saturated aqueous uranyl acetate (3 hours) and lead citrate (10 minutes).

3. Results

3.1. Interphase

The interphase nucleus of *A. proteus* is discoidal (Figs. 1 and 2). There are numerous nucleoli at the periphery of the nucleus; they can be visualized under the light microscope by silver staining (Fig. 2). The size of the nucleoli strongly varies, the larger ones being irregular in shape. Fibrogranular and granular regions are visible in the nucleoli at the ultrastructural level (Fig. 3). The nuclear envelope shows the so-called honeycomb layer on the inside, consisting of numerous "cells". The chromatin is located in the central part of the nucleus in the form of dispersed 10 nm thick filaments; dense heterochromatin bodies never occur. Moreover, bundles of microfilaments and the so-called nuclear helices specific for the amoebae are revealed in the nucleus (Figs. 4 and 5). All these structures had been described in some previous papers (DANIELS 1973, FLICKINGER 1973—general characteristics, LESSON and BHATNAGAR 1975—filaments in the nucleus, MINASSIAN and BELL 1976 a—nuclear helices).

3.2. Prophase

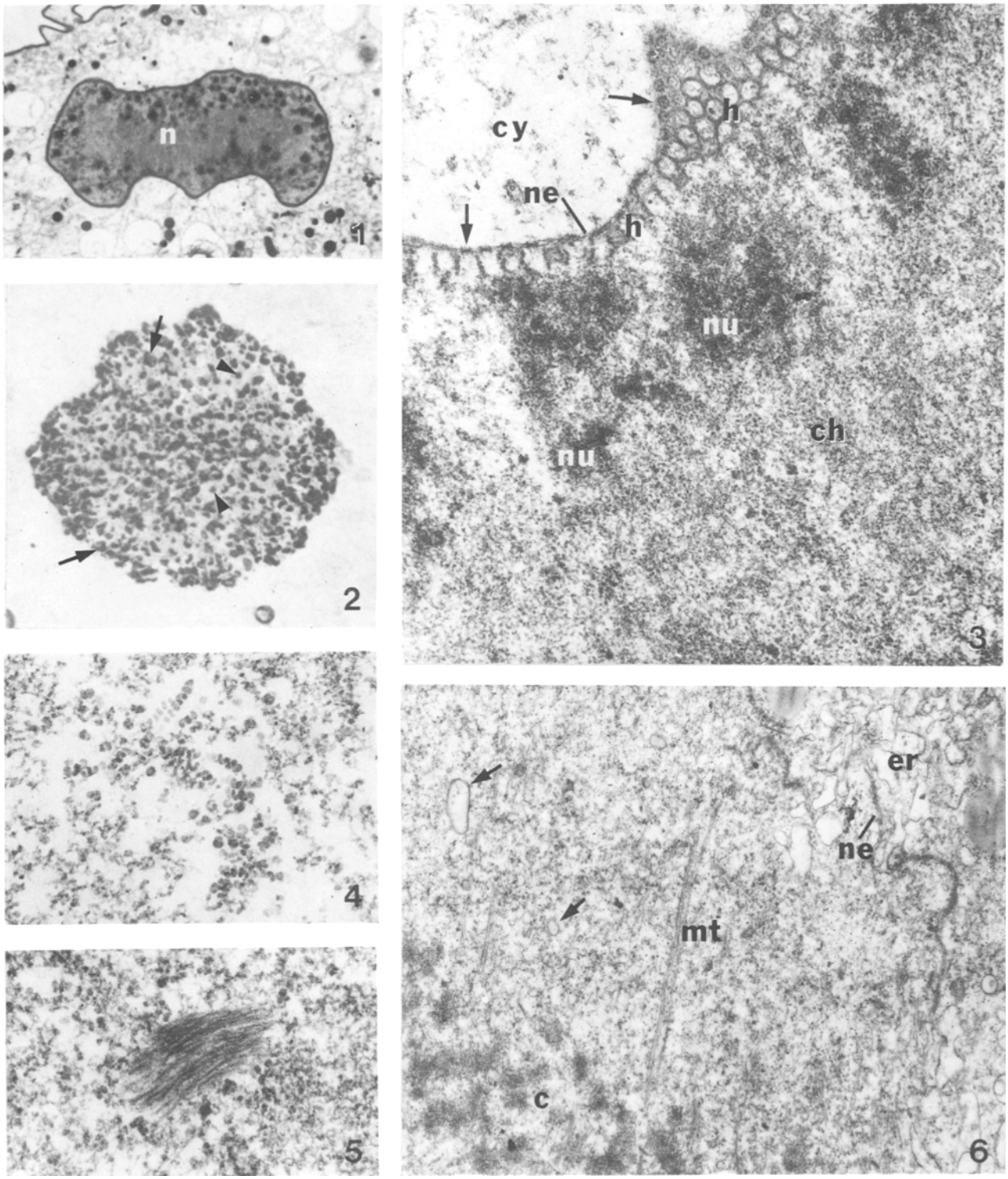
In prophase (Fig. 7) the nucleus becomes spherical, and the numerous chromosomes condense. Due to the flatness of the interphase nucleus, the chromosomes are from the very beginning arranged into an equatorial plate. The nucleoli become vacuolized, and their number decreases. The honeycomb layer loses its "cellulated" aspect, but it is still present as an amorphous layer of fibrillar material under the nuclear envelope. At many places, especially in the future pole regions, the nuclear envelope membranes become continuous with the membranes of the rough endoplasmic reticulum that are numerous around the nucleus; small openings appear in the nuclear envelope. A spindle consisting of parallel bundles of microtubules is formed inside the nucleus in an acentric arrangement.

3.3. Metaphase

One can hardly distinguish between the prophase and the metaphase in *A. proteus* mitosis, because the chromosomes are located at the equator of the nucleus from the very beginning of the nuclear division. In metaphase (Fig. 6) the nuclear envelope disintegrates greatly, especially in the mitotic pole regions. The honeycomb layer disappears completely; the spindle remains acentric just as before, while a lot of membrane vesicles appears inside it. The nuclear envelope fragments are connected with the cisternae of the rough endoplasmic reticulum.

3.4. Anaphase

In the early anaphase the margins of the anaphase plates begin to curve towards the mitotic poles, so that by the end of the anaphase (Fig. 9) each plate becomes cup-shaped. In anaphase, remnants of the nuclear envelope are present in the form of numerous fragments scattered among the chromosomes (Fig. 8). Many fragments of the nuclear envelope are continuous with



Figs. 1–5. Interphase nuclei. Figs. 1 and 2 are light micrographs, Figs. 3–5, electron micrographs

Fig. 1. Semi-thin section (*n*). × 1,300. Fig. 2. Ag-NOR staining. Small (arrowheads) and large (arrows) nucleoli can be seen. × 1,300. Fig. 3. A part of an nucleus. *cy* cytoplasm, *nu* nucleoli, *ch* chromatin, *h* honeycomb layer of nuclear envelope (*ne*); arrows indicate pore complexes. Fixation B; × 34,000. Fig. 4. Group of nuclear helices (*he*). Fixation A; × 54,000. Fig. 5. Microfilaments (*mf*) in the caryoplasm. Fixation B; × 60,000

Fig. 6. Metaphase. *c* chromosomes, *mt* spindle microtubules, *er* rough endoplasmic reticulum, *ne* nuclear envelope; arrows indicate membranous vesicles in the spindle region. Fixation A; × 27,000

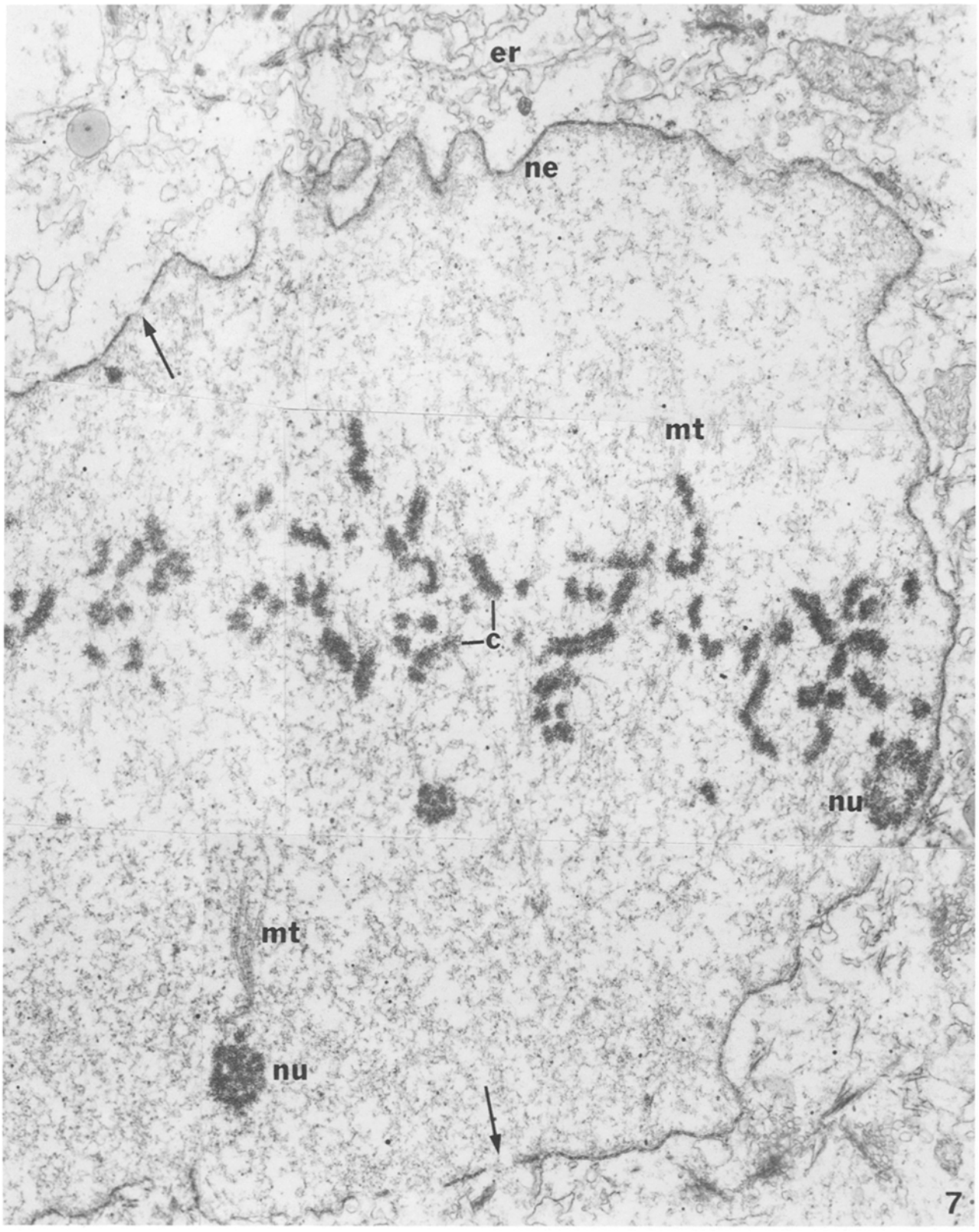
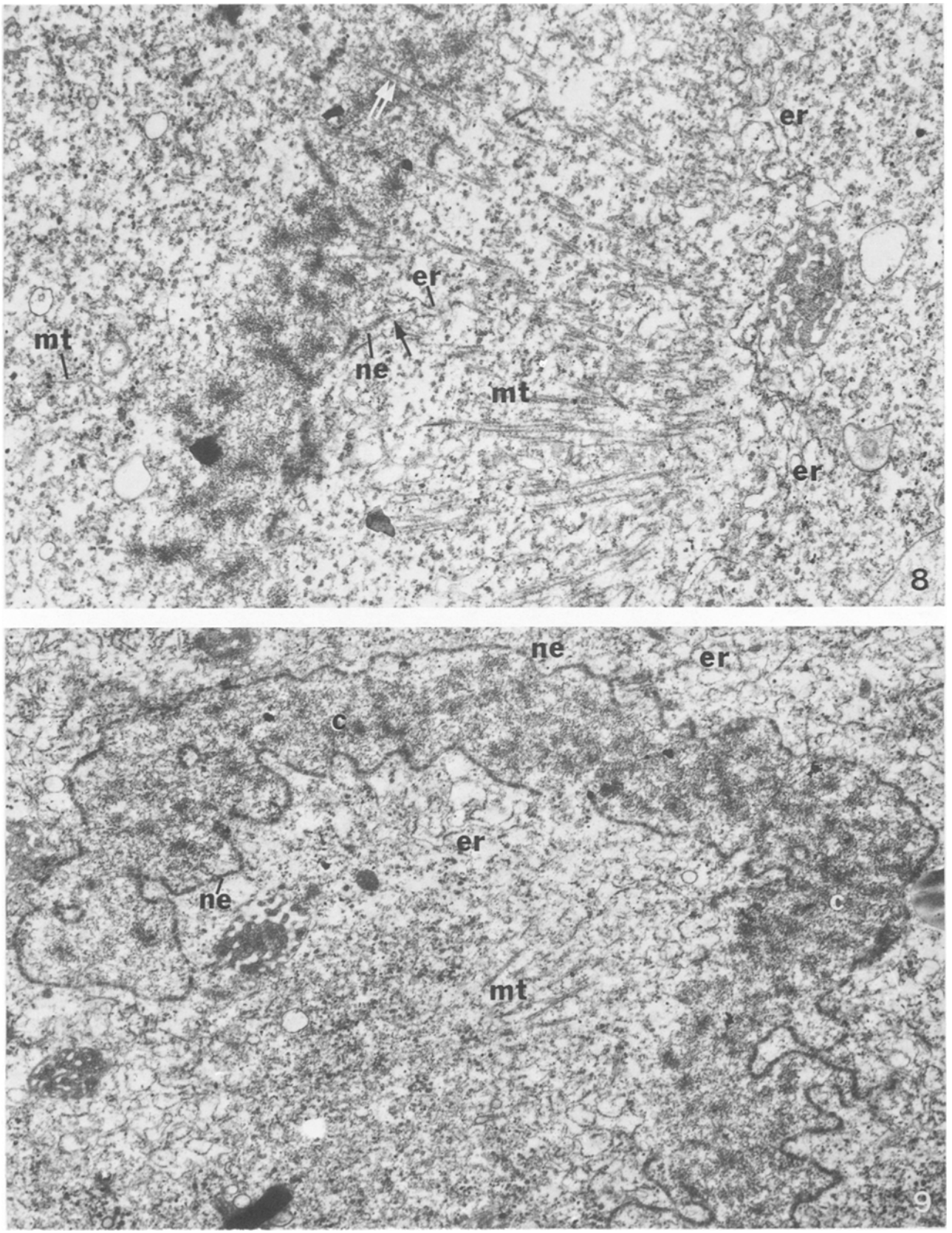
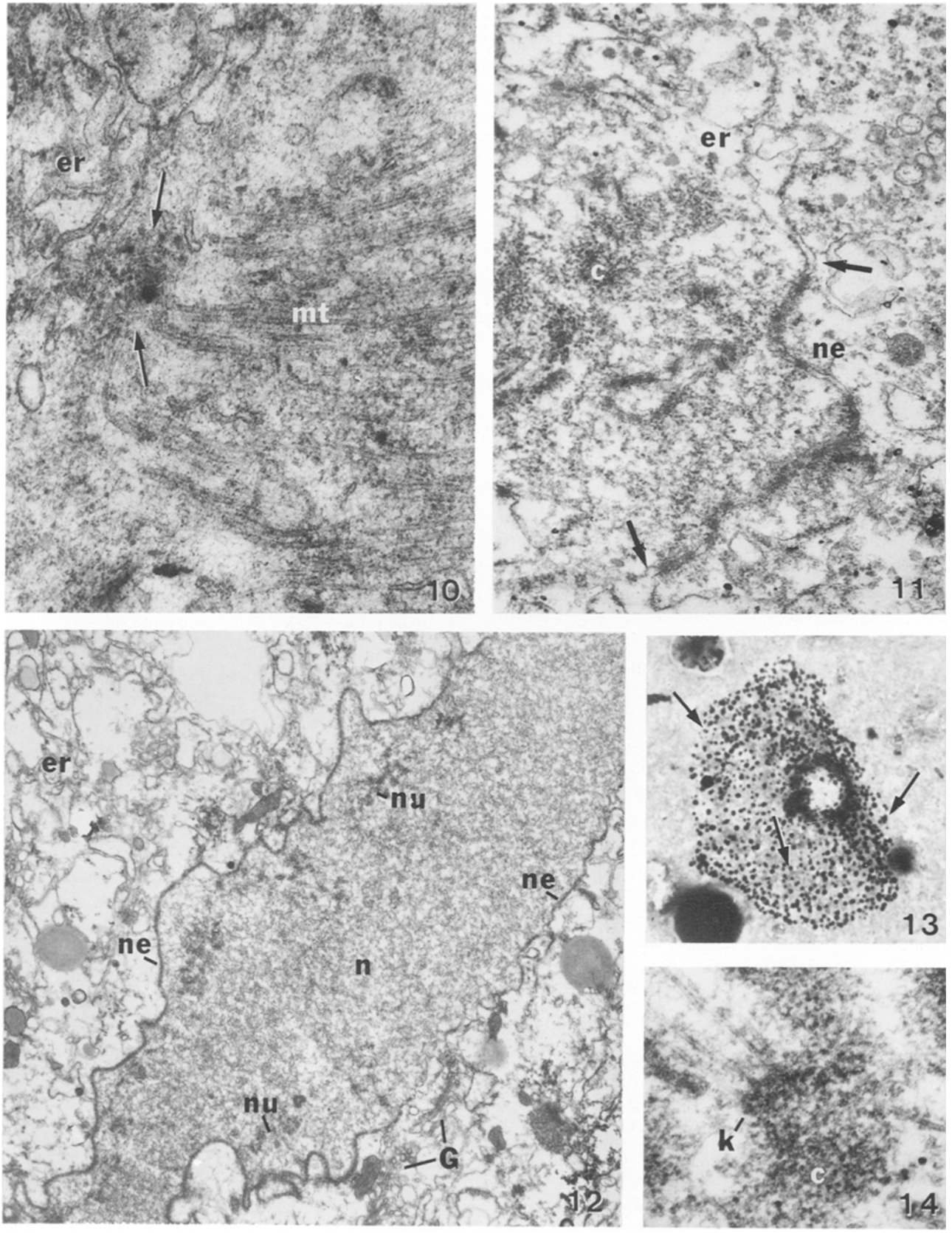


Fig. 7. Prophase. *c* chromosomes, *nu* nucleoli, *ne* nuclear envelope, *mt* microtubules of the mitotic spindle, *er* rough endoplasmic reticulum cisternae; arrows indicate openings in the nuclear envelope. Fixation A; × 13,000



Figs. 8–9

Fig. 8. Part of the spindle during early anaphase: arrow indicates connection between endoplasmic reticulum (*er*) and nuclear envelope fragments (*ne*), double arrows—microtubules which pierce the anaphase plate. *c* chromosomes, *mt* spindle microtubules. Fixation A; × 31,500.
Fig. 9. Daughter nucleus during late anaphase. Symbols same as on Fig. 8. Fixation A; × 21,500



Figs.10–14

Fig. 10. Spindle pole region during anaphase: arrows indicate electron dense material and granules at the focus where spindle microtubules (*mt*) converge. Fixation A; × 64,500. Fig. 11. "Cap" of nuclear envelope on the margin of the anaphase plate; arrows indicate connections between nuclear envelope and endoplasmic reticulum. Fixation A; × 43,000. Fig. 12. Nucleus (*n*) during telophase. Fixation B; × 18,000. Fig. 13. Light micrograph of telophase nucleus after Ag-NOR staining: numerous nucleoli are visible (arrows). × 1,300. Fig. 14. Chromosome with kinetochore (*k*). Fixation A; × 77,000. Other symbols: *nu*—nucleoli, *c*—chromosomes, *ne*—nuclear envelope, *er*—rough endoplasmic reticulum cisternae, *G*—Golgi apparatus

the endoplasmic reticulum cisternae which occur both inside and around the spindle. The nuclear envelope also forms a ring around the margin of each anaphase plate; this is visible in sections as caps at the margins of the plates (Fig. 11). Both the ring and the nuclear envelope fragments in the anaphase plates are connected with the endoplasmic reticulum cisternae; the latter surround the entire spindle forming a kind of envelope around it (Fig. 8). This envelope has ribosomes on both sides and carries no pore complexes yet. The envelope is not complete, but perforated with numerous small openings. The complete envelope begins to form around each chromosome plate in the late anaphase, but its formation being completed by the end of telophase only.

In anaphase the spindle microtubules converge at the poles, forming cone-shaped half-spindles. Alongside with the kinetochore microtubules attached to the chromosomes, there are some microtubules in the spindle which pierce the anaphase plate (Fig. 8). However, the number of microtubules between the anaphase plates is significantly less than between the chromosomes and the spindle poles. In the mitotic pole regions, the microtubules end in immediate proximity to some endoplasmic reticulum cisternae surrounding the spindle (Fig. 10). Accumulations of diffuse electron dense material and groups of granules similar in size to ribosomes are now revealed at the foci where bundles of microtubules converge. According to previous papers (ROTH *et al.* 1960, RAIKOV 1982) no centrioles, procentrioles, or any other special structures have been revealed at the spindle poles.

In the late anaphase (Fig. 9) the number of microtubules greatly decreases; apparently, the microtubules disappear between the anaphase plates, the distance between the two plates exceeding 100 μm by that time.

3.5. Telophase

The telophase stage of karyokinesis coincides in *A. proteus* with the cytokinesis. At this stage (Fig. 12) the nuclear envelope proper becomes restored almost completely; however, it retains at many places the connection with cisternae of the endoplasmic reticulum. There is no honeycomb layer yet.

The chromosomes can not be discerned any more at this stage. There are numerous electron dense fibrillar bodies on the periphery of the nucleus. These seem to be developing nucleoli, since they can be revealed by Ag-NOR staining even at this stage (Fig. 13).

3.6. Kinetochores and Nuclear Helices

The mitotic chromosomes, as one can see in sections, consist of fibrils 10 nm thick; at the places where the spindle microtubules are attached to the chromosome there are three-layered kinetochores (Fig. 14). A bundle of several microtubules is attached to each kinetochore. The groups of nuclear helices, which are abundant in nondividing nuclei, apparently disappear at the very beginning of mitosis. However single helices still occur in prophase, metaphase and early anaphase at the spindle region. There are no nuclear helices in late anaphase and telophase.

4. Discussion

The mitosis in large freshwater amoebae is usually considered an example of open mitosis with acentric type spindle (RAIKOV 1982). However, in the present work the nuclear envelope is shown not to disintegrate completely during mitosis in *Amoeba proteus* (Fig. 15). It only loses its pore complexes and transforms into a system of rough endoplasmic reticulum cisternae which separates the mitotic apparatus from the surrounding cytoplasm. The spindle is thus located within the transformed envelope up to the early anaphase inclusive. The nuclei separate by the end of anaphase; at this stage there are complete nuclear envelopes bordering the daughter nuclei at the rear sides of the chromosomes plates (which respect to the direction of their anaphase movement).

It is not clear when the microtubules between the anaphase plates are disassembled—before the separation of the nuclei or after it. The properties of the endoplasmic reticulum cisternae surrounding the dividing nucleus differ from those of the normal nuclear envelope. The former lack pore complexes and have small openings; the entrance into the nucleus of the colloid gold particles injected into the dividing amoeba (FELDHERR 1966) might be possibly explained by the existence of these openings. Our data allow to relate the mitosis in *A. proteus* to semiopen mitosis with an intranuclear spindle rather than to open mitosis. There is also some analogy in the behaviour of the nuclear envelope during mitosis between *A. proteus* and the gregarine *Stylocephalus* (DESPORTES 1970). In this protozoan, the nuclear envelope is well preserved during mitosis, being represented by fragments surrounding the chromosomes. The fragments lack pore complexes. The difference from *A. proteus* is that a complete envelope is formed around each chromosome in *Stylocephalus*, while the spindle is not surrounded by

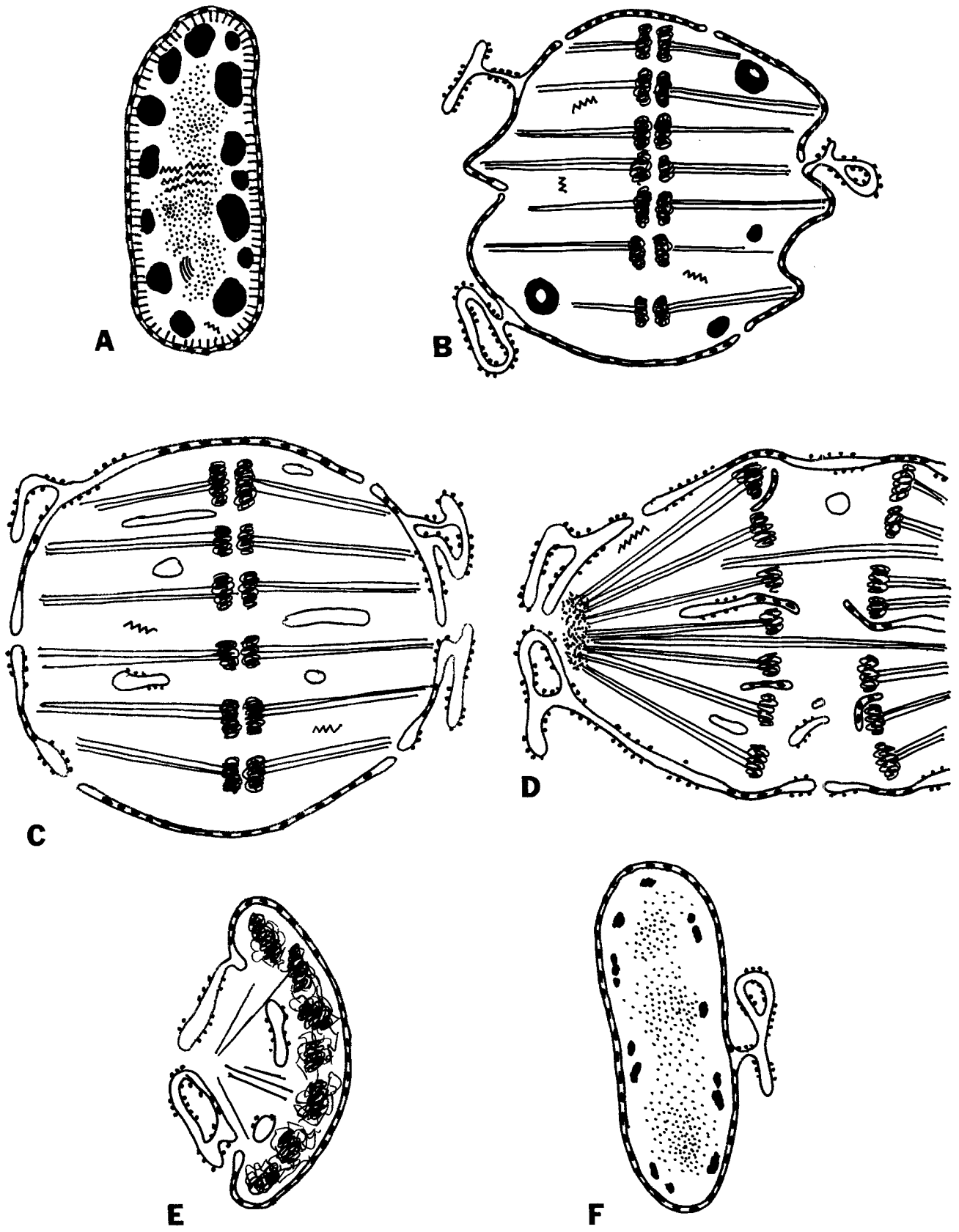


Fig. 15. Schematical drawing of nuclear division phases. *A* interphase, *B* prophase, *C* metaphase, *D* early anaphase, *E* late anaphase, *F* telophase. Commentaries see in Discussion

an envelope of its own. Therefore the spindle of this gregarine cannot be considered intranuclear. During mitosis in the plasmodia of *Plasmodiophora brassicae* there are endoplasmic reticulum cisternae surrounding the entire dividing nucleus, but here they do not appear to be transformed nuclear envelope fragments (GARBER and AIST 1979).

It is known that after warming of amoebae which were cooled at early stages of division in order to destroy the spindle microtubules, the new microtubules begin to assemble at the kinetochores (ROTH 1967). Owing to these data and the fact that the microtubules do not converge at the spindle poles (ROTH 1960), the current opinion is that the kinetochore regions play the part of microtubule organizing centres (MTOCs) in amoeban mitosis (RAIKOV 1982). It appears however that there are microtubules piercing through the chromosomal plates in the amoeban anaphase spindle, and that in anaphase the microtubules do converge forming cones at the spindle poles. The assembly of at least some category of microtubules is therefore likely to be induced at the spindle poles, the diffuse electron dense material revealed in the foci of microtubule termination then representing another kind of MTOC.

It is most probable that in early mitosis (prophase to metaphase) the spindle is formed as an acentric one, owing to the assembly of microtubules at the kinetochores (Fig. 15). The growing microtubules cause the swelling of the nucleus from a discoidal to a spherical form. The fact that the polar regions of the nucleus form extensions at that time favors this supposition. In early anaphase however, the half-spindles become conical, the assembly of microtubules now begins at the spindle poles and the chromosomes start moving. The curving of the anaphase plate is probably the result of convergence of the distal ends of the microtubules which occurs at the spindle poles. It is not clear how the microtubule ends are drawn together to sertain regions of the division poles.

The distance between each spindle pole and the chromosomes practically remains constant and never exceeds 30 μm at any mitotic stage, while the chromosome plates drift apart from one another for more than 200 μm. Therefore a decrease of the length of the chromosomal microtubules and, as a consequence, an approach of the chromosomes to the spindle poles can not be the mechanism of the anaphase movement in amoeban mitosis. It is likely that the chromosomal microtubules only connect the chromosomes with the spindle poles while the anaphasic separation of the chromosomes occurs due to the fact that the poles themselves move apart. Such interpretation of mitosis in amoebae do not contradict to recent conceptions of mechanisms of formation and functioning of the mitotic apparatus (INOUÉ 1981).

It is interesting that 10 nm thick filaments are revealed in the mitotic chromosomes of the amoebae. Such filaments are usually considered to correspond to DNA-histone nucleosomal fibers, while filaments 25 nm thick are likely to correspond to the supernucleosome level of chromatin packing (CHAMBRON 1978, GALL 1981). Filaments of 25 nm diameter occur neither in interphase nor in mitosis in the amoebae. This fact may suggest the absence of the supernucleosome level of chromatin packing in the amoebae. Undoubtedly, further special investigations are necessary to confirm this possibility.

The great number of nucleoli in *A. proteus* nuclei can be explained either by their polyploidy (ORD 1979 b) or by ribosomal gene amplification (MAKHLIN *et al.* 1979, RAIKOV 1982). The high number (several hundred) of chromosomes and the big size of the cell provide evidence in favour of the former hypothesis, while the excessive DNA synthesis during interphase, the incorporation of ^{3}H-thymidine into the nucleoli during the G_2-phase of the cell cycle (MINASSIAN and BELL 1976 b), and the absence of connections between nucleoli and condenced chromosomes during prophase rather favour the latter one. Both hypotheses thus seem to be equally likely. So, the great number of nucleoli in *A. proteus* nuclei can be explained both by their polyploidy and by ribosomal gene amplification.

5. Acknowledgements

The author is indebted to Dr. I. B. RAIKOV for valuable advices during discussion of the results and for the revision of the manuscript. Thanks are due also to E. V. SABANEYEVA for the English translation of the paper.

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