ELECTRON MICROSCOPIC STUDIES OF MITOSIS IN AMEBAE

I · *Amoeba proteus*

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ABSTRACT

Individual organisms of *Amoeba proteus* have been fixed in buffered osmium tetroxide in either 0.9 per cent NaCl or 0.01 per cent $CaCl_2$, sectioned, and studied in the electron microscope in interphase and in several stages of mitosis. The helices typical of interphase nuclei do not coexist with condensed chromatin and thus either represent a DNA configuration unique to interphase or are not DNA at all. The membranes of the complex nuclear envelope are present in all stages observed but are discontinuous in metaphase. The inner, thick, honeycomb layer of the nuclear envelope disappears during prophase, reappearing after telophase when nuclear reconstruction is in progress. Nucleoli decrease in size and number during prophase and re-form during telophase in association with the chromatin network. In the early reconstruction nucleus, the nucleolar material forms into thin, sheet-like configurations which are closely associated with small amounts of chromatin and are closely applied to the inner, partially formed layer of the nuclear envelope. It is proposed that nucleolar material is implicated in the formation of the inner layer of the envelope and that there is a configuration of nucleolar material peculiar to this time. The plasmalemma is partially denuded of its fringe-like material during division.

INTRODUCTION

The amebae have been choice material for several types of cytological investigations for many decades. In contrast to the various nuclear division processes of protozoa, the amebae exhibit a nearly typical sequence of mitotic events although the chromosomes are small, spheroidal, and numerous. In electron microscope studies, two special features have again drawn attention to amebae: first, a helical structure observed in the interphase nucleus (11) and second, a complex nuclear envelope (1, 6, 7, 8, 10, and 12).

The amebae, two multinucleate giant forms as well as the uninucleate, *A. proteus*, were originally chosen by one of us (Roth) to study mitosis. The processes of food taking, digestion, and pinocytosis were also studied and a report written on these subjects (15). The present report is limited to *A. proteus* and to the events of division as compared to interphase morphology. It gives information particularly on the mitotic changes of helices, the layers of the nuclear envelope, and the nucleolar material.

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MATERIALS AND METHODS

Culture and Selection of Organisms: Organisms were obtained from the General Biological Supply House, Inc., Chicago, in about 1957 and cultures were established in 5-inch finger bowls. The fluid was replaced about once each week with water (double-distilled in glass), and at the same time food organisms were added. Initially, axenically grown *Tetrahymena* were used as food but it was found later that division stages could be selected more accurately if a mixture of *Paramecium caudatum* and *Chilomonas paramecium* was used.

Organisms were selected under a dissecting microscope at a magnification of about 100 and five to ten organisms were processed together through the fixation-embedding procedure. The mulberry-like appearance of dividing amebae (3) was the major identifying feature, but other features must be utilized if a high percentage of organisms with mitotic nuclei are to be obtained; *e.g.*, dividing organisms are not usually attached to the substrate, they send out no large pseudopodia, they are found in highest number several hours after feeding, and they are completely covered by small, knobby pseudopodia in the size range of 20 to 40 μ. Careful selection at this stage of preparation is essential since a great amount of time is involved in the preparation of each organism for electron microscopy. Determination of the division stage was made by correlating the electron microscopic images with the descriptions from light microscopic studies (3, 5).

Fixation and Embedding: Organisms were fixed in 1 per cent osmium tetroxide which was buffered and had other substances added. The earliest work utilized an osmium solution buffered with veronal acetate to pH 8.0 and with 0.9 per cent sodium chloride added; it was found, however, that this or lower concentrations, or the absence of sodium chloride, caused the formation of a large space between the plasmalemma and cytoplasm during the first few minutes in the fixing solution. Whether this space resulted from a shrinkage of cytoplasm, a stretching of the plasmalemma, or a combination of the two was not obvious. The organisms pictured in Figs. 1 to 7 and Figs. 12 to 13 were fixed in this way; of these, Figs. 1 to 5 are from interphase and show structures essentially identical with those previously observed and reported. The addition of a low concentration of calcium (0.01 per cent $CaCl_2$) and the omission of sodium chloride were found to correct this difficulty; this is essentially the same fixation used by Pappas (13) except that the pH is lower, the temperature higher (22°C.), and the time longer (30 to 50 minutes).

Dehydration was carried out in ethanol at 10 to 20 minute intervals: 2 changes in 50 per cent, 1 in 75 per cent, 1 in 95 per cent, and 2 in absolute. Three infiltration steps of 20 minutes each were used; the methacrylate mixture consisted of 2 parts ethyl methacrylate and 3 parts *n*-butyl methacrylate with 1 per cent luperco CDB. Polymerization took place at 60°C. in gelatin capsules each containing only one or two organisms.

Sectioning and Microscopy: Sections of single organisms were cut in the range of 50 to 200 mμ, but usually at 100 to 150 mμ settings of the Porter-Blum microtome; the appropriate interference colors were regularly observed on almost all sections above 75 mμ. Sections were placed on carbon membranes and covered with methacrylate membranes which were silver to gold colored (14); the latter procedure reduces sublimation of methacrylate from the section and results in less distortion of the tissue under bombardment in the microscope.

In some cases phosphotungstic acid staining was beneficial and was performed before covering with methacrylate by inverting the grid, section side downward, on a drop of 40 per cent aqueous solution of the 24-tungsten compound for about 1 hour. A short rinse in distilled water was usually sufficient to remove excess stain.

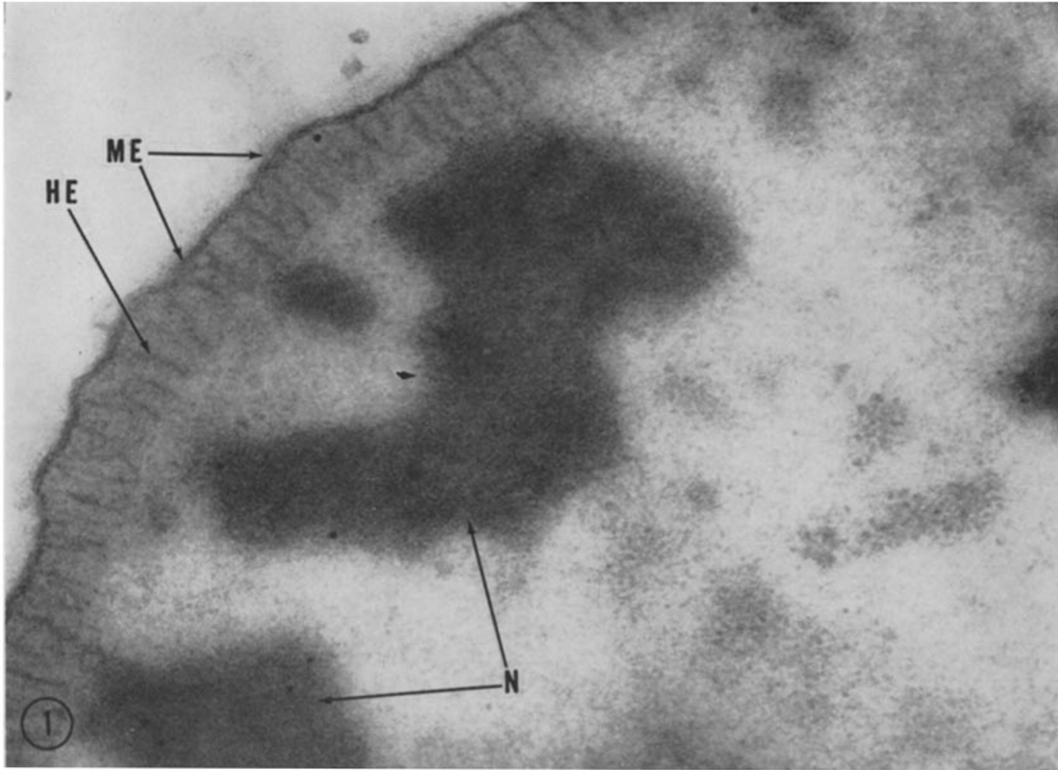
The electron microscope used was an RCA EMU 3A or 3E instrument operated at 100 kv.

FIGURE 1

Interphase: a section perpendicular to the complex nuclear envelope which is composed of an inner, thick, honeycomb-like layer (*HE*) and an outer layer of membranes (*ME*). The numerous nucleoli (*N*) are peripheral in a large, biconcave, disc-shaped nucleus. × 27,000.

FIGURE 2

Interphase: a view nearly tangential to the membranes of the envelope. The walls of the honeycomb layer (*HE*) are sometimes seen to be layered; annuli (*A*) facing into the openings of the honeycomb structure below are visible in the outer envelope layer. Part of a nucleolus (*N*) is also included. × 68,000.


ME
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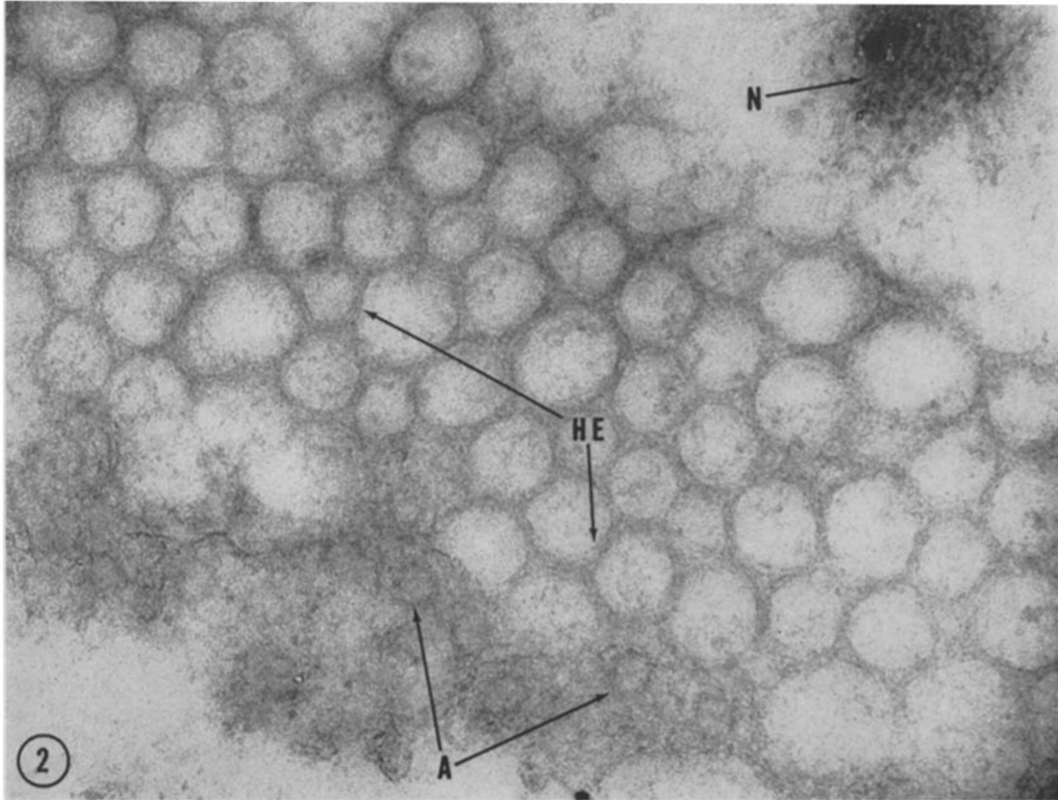
N
1


N
HE
A
2

with a 25 μ objective aperature. Photographs were taken using Kodak medium-contrast lantern slide plates.

Technical Limitations: Eight dividing organisms were studied, one at each of the stages described plus two in late anaphase. It is evident, therefore, that the observations of mitosis are not exhaustive of the subject and it is possible that an observation at a given stage may represent an anomalous condition or an abnormal organism. However, the continuity of one stage with the preceding and following ones and the close correlation with light microscopic studies (3, 5) and with the few electron microscopic observations of Cohen (4) all argue for the validity of the series.

OBSERVATIONS

Morphology in Interphase: Organisms in interphase typically possess one flattened, slightly biconcave nucleus. In some cases, however, two or three nuclei may be present, presumably owing to a lack of plasmotomy following some previous nuclear division. The structure of nuclear envelope observed here conforms in its essential features to the descriptions by Bairati and Lehmann (1), Harris and James (8), Greider, Kostir, and Frajola (6, 7), Pappas (12), and Mercer (10). It is composed of outer membranes (Fig. 1, *ME*) and an inner, thicker layer that resembles a honeycomb in appearance (Figs. 1 and 2, *HE*). The annular structures (Fig. 2, *A*) of the membranes face into the lumina of the inner layer (Fig. 2, *HE*). At high magnification, cross-sections of the walls of the inner layer may have an appearance of three dark layers separated by two light layers (Fig. 2, *A*). Nucleoli[1] are numerous and are peripherally arranged (Figs. 1 and 2, *N*) just inside the nuclear envelope. The more central portions of the nucleus have granular materials of several types, but the most striking structures are the helices first described by Pappas (11). Several helical strands usually appear to be attached to each other at a common center and protrude outward (Figs. 3 and 4, *H*) and a few sections obtained suggest that this is the cross-sectional image of long cords having this form (Fig. 21, *H*).

Regarding the cytoplasm, striking changes have been observed during division in this study only in the plasmalemma; for this reason other cytoplasmic components will not be described. In interphase the plasmalemma according to previous observations (10–12, 17) is composed of a dense

[1] The term "nucleoli" is applied here to the numerous, peripheral bodies of interphase nuclei. The early literature states that these bodies typically contain small amounts of chromatin, but Chalkley (2) and Heller and Kopac (9) have shown that they are Feulgen-negative (except for a short time during nuclear reconstruction). They do contain ribose nucleic acid (9). Thus, the evidence is mounting that this use of the term is proper.

FIGURES 3 and 4

Interphase: helices (*H*) are present and appear to be attached at one end to a common center or axis. × 47,000 and × 50,000 respectively.

FIGURE 5

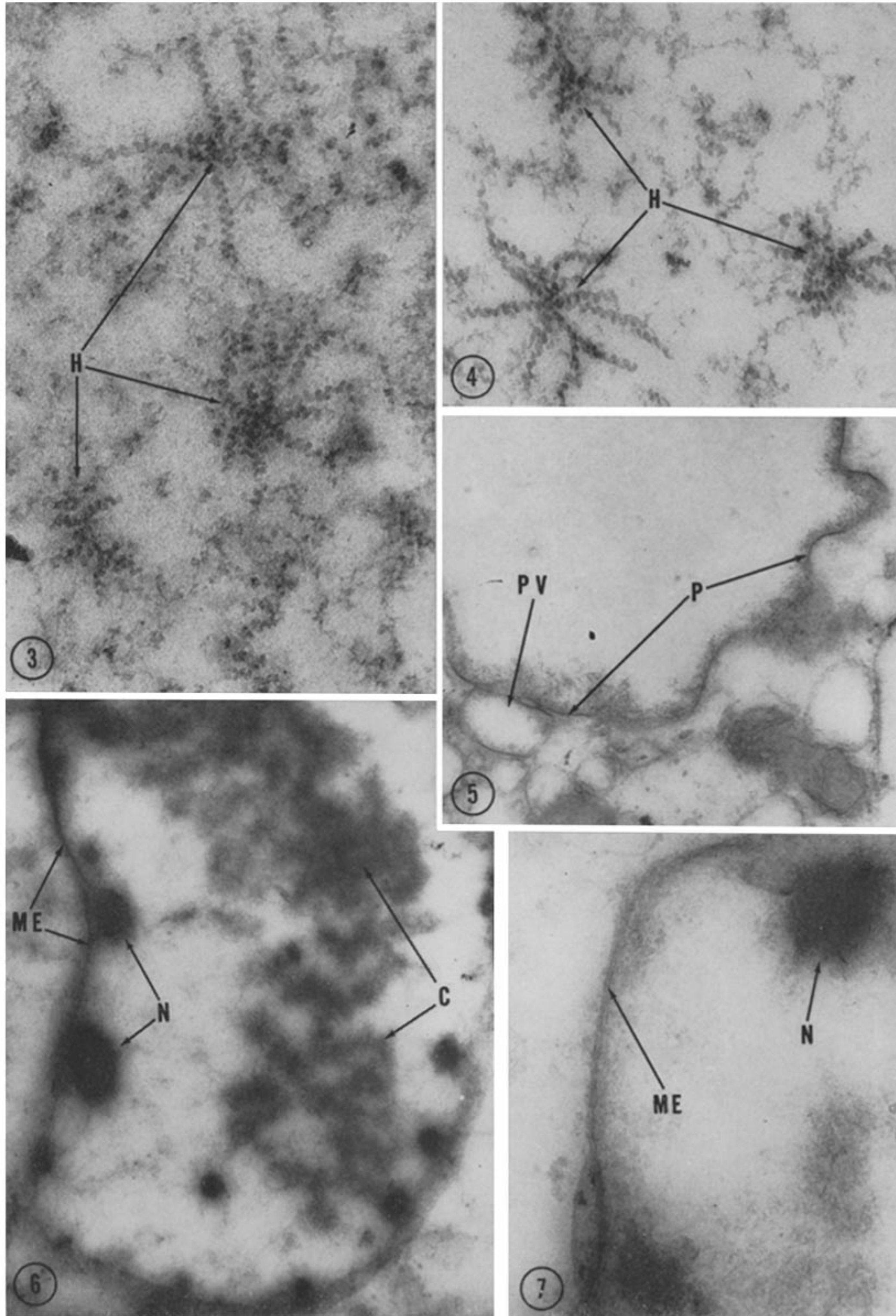
Interphase: the plasmalemma (*P*) is composed of a membrane which has a "fringe" on its outer surface. Pinocytic vesicles (*PV*) show this fringe on their inner surface after they are formed. × 18,000.

FIGURE 6

Late prophase: the inner layer of the nuclear envelope is now missing, leaving an envelope composed only of membranes which are still continuous (*ME*). The chromatin has now condensed into a central network (*C*) and nucleoli (*N*) have diminished in size and number. Spindle fibrils are not yet present. The total volume of the nucleus has enlarged so that the shape is nearly spherical. × 14,300.

FIGURE 7

Late prophase: although the membranes of the nuclear envelope (*ME*) are uninterupted, the inner layer has disappeared, so that only a thin, relatively unorganized material remains. Some nucleoli (*N*) still remain peripherally placed. × 42,000.


H
H
PV
P
ME
N
C
ME
N
3
4
5
6
7

membrane with an exteriorly directed "fringe" (Fig. 5).[2]

Late Prophase: The nucleus at this time has swollen to a nearly spherical shape and has radically changed its appearance. The nuclear envelope is now composed only of the membranes (Figs. 6 and 7, *ME*) which are now clearly parallel and evenly separated; the inner layer has disappeared. Nucleoli are less frequent, since they are now reduced both in size and in number (Figs. 6 and 7, *N*). Centrally, no helices are present as in interphase, but chromatin has begun to appear in a condensed form in the shape of a rather irregular network (Fig. 6, *C*). No spindle fibrils are observed. According to Chalkley and Daniel (3) this stage lasts for about 10 minutes of the 33 minutes involved in nuclear division.

Metaphase: Although the membranes of the nuclear envelope are still present (Fig. 8, *ME*), large openings or gaps are present (Figs. 8 and 10, *O*); the honeycomb, inner layer is absent. The small chromosomes which are usually rod-shaped and about 0.5 to 0.8 μ long and 0.2 μ in diameter (Figs. 8 and 9, *C*) are arranged in a metaphase plate and are attached to spindle fibrils (Fig. 9, *S*). No darkening or density increase has been observed here in the kinetochore region as has been reported in other material. Spindle fibrils measure about 15 mμ in diameter, have a tubular appearance, and possess no periodic structure. A few nucleoli remain and a fine, filamentous material occupies the remainder of the nucleoplasm. Prominent granules (Fig. 11, *G*) are present in large numbers in the cytoplasm at all stages and in the spindle area during metaphase (Fig. 9, *G*); they are not to be confused with preparation artifacts (Fig. 8, *R* and *GR*).

The plasmalemma (Fig. 11, *P*) is continuous but largely denuded of its external material. The change in shape of the organism to a sphere studded with many small pseudopodia (3) probably indicates rather marked physiological changes, but no other morphological changes were observed.[3] The cytoplasmic contents of these small pseudopodia are not unusual except that many recently formed pinocytic vesicles, stainable with phosphotungstic acid, are present (15).

[2] Mercer (10) has shown a double-layered structure by high resolution microscopy, but this morphology is not resolved at the magnifications usually employed here.

[3] Further evidence of physiological change is given by Chalkley and Daniel (3), who maintain that the contractile vacuole ceases to function in anaphase and telophase.

Metaphase: Figs. 8 to 11 are from a single organism.

FIGURE 8

The nucleus is now large and nearly spherical. The chromosomes (*C*) are arranged on a metaphase plate and spindle fibrils (*S*) are present extending from the chromosomes. Several gaps (*O*) are shown in the nuclear envelope (*ME*). Rare nucleoli (*N*) persist. Granules (*GR*) and other materials (*R*) pictured are artifacts. × 11,000.

FIGURE 9

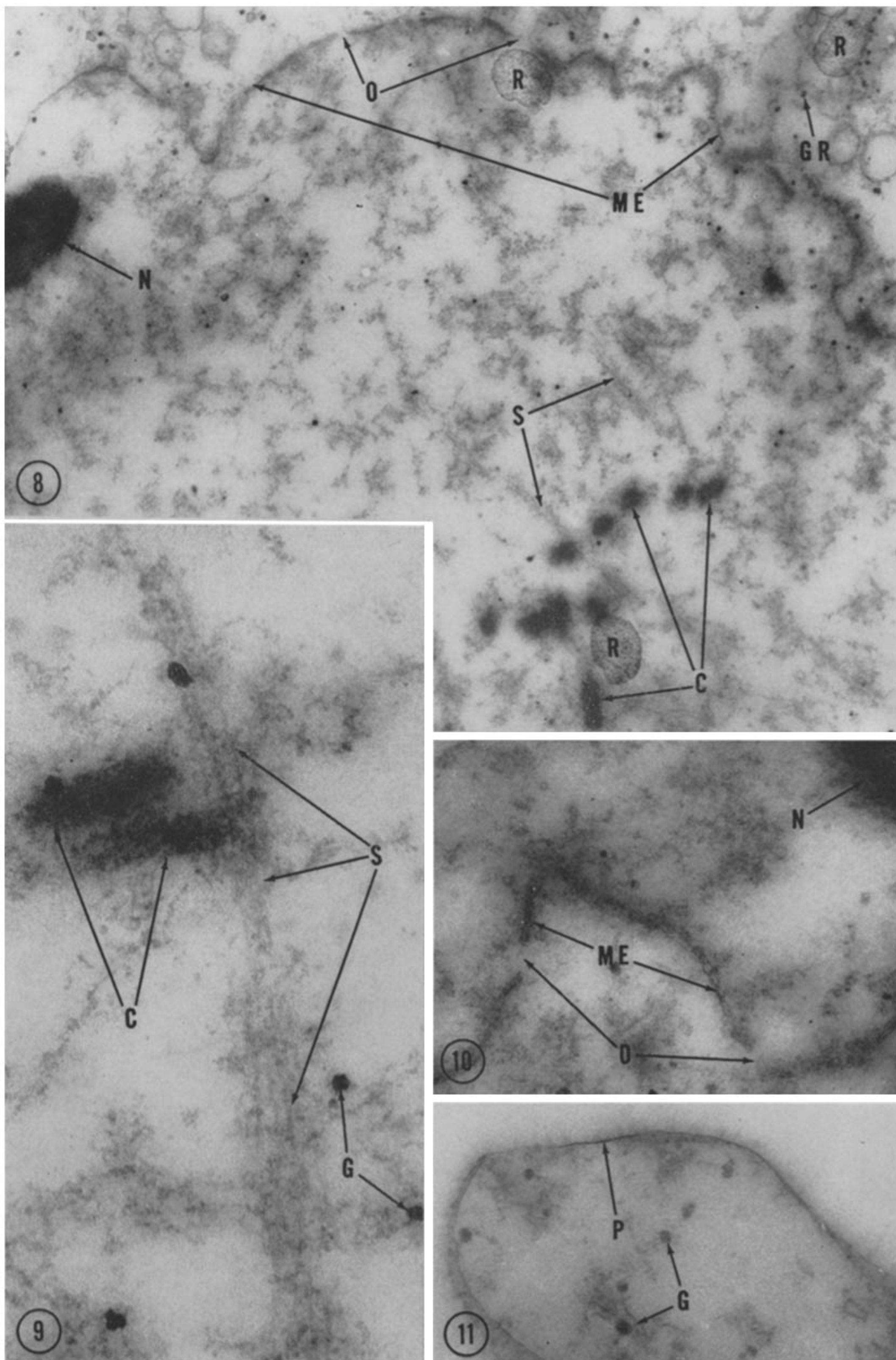
Spindle fibrils (*S*) are shown in small bundles attached to two chromosomes (*C*) which show only slight internal regularity. No high density region has been detected at the kinetochores. Granules (*G*) occur in the spindle in large numbers; they are morphologically similar to those seen in the cytoplasm at all stages. × 42,000.

FIGURE 10

The membranes of the nuclear envelope (*ME*) are now interrupted by numerous gaps (*O*) which probably allow the mixing of cytoplasm with nucleoplasm. Part of a nucleolus is included. × 25,000.

FIGURE 11

The plasmalemma (*P*) is now mostly denuded of its "fringe" material. Granules (*G*) which are frequent and typical in the cytoplasm are much smaller than the artifact granules (*GR*) in Fig. 8. × 34,000.


O
R
R
ME
GR
N
S
8
C
C
C
S
9
S
G
N
ME
O
10
P
G
11

Late Anaphase: The nucleus now has a concave-convex shape (Fig. 12) and is enclosed by membranes which are probably continuous. The nuclear volume is very small as compared to that in other stages. Chromatin (Fig. 13, *C*) is not as condensed as it appeared in metaphase and nucleoli have not yet begun to form.

Telophase: The membranes of the nuclear envelope are probably continuous again (Figs. 14 and 15, *ME*), but the inner layer is still missing. The chromosomes are now arranged in a chromatin network (Fig. 14, *C*) with dense nucleoli of various sizes arranged on its periphery. The nucleoli always appear denser than chromatin and show an homogeneous granulation (Fig. 15, *N*).

Two such nuclei were present in this organism, hence cytoplasmic division (plasmotomy) had not yet occurred.

Reconstruction Stage: The nucleus is now single (division of the organism has occurred), has the shape of a flattened, slightly biconcave disc (Fig. 16 is a cross-section), and has re-formed the honeycomb-like, inner layer of its envelope. In these respects, the nucleus has an interphase appearance. However, there is a strikingly different appearance caused by the configuration of the nucleolar material, of which a large amount now exists in both sheets and granules (Fig. 16, *N*). Like the granular nucleoli of interphase, this material is at the periphery of the nucleus; in fact, some sheets of nucleolar material are in contact with the inner layer of the envelope (Figs. 16 to 18). In the preceding 10 to 20 minutes, the inner layer has accumulated a thickness of about 200 mμ, which is at least two-thirds of its maximum measurement at interphase. Thus, the rapid deposition of envelope material is correlated with a peculiar configuration of nucleolar material.

Small amounts of chromatin (Fig. 17, *C*) are still associated with nucleolar material, which is identified by its structure, position, and density. Helices have again appeared (Fig. 18, *H*), but are often near nucleoli in a peripheral position rather than in their more central interphase location. The plasmalemma remains almost denuded of its "fringe" (Fig. 19, *P*).

This peculiar nucleolar morphology, which was observed in the light microscope by Dawson *et al.* (5), occurs 10 to 30 minutes after division of the nucleus in the organisms which they studied.

At a later stage of reconstruction, probably about 2 to 3 hours after its division, the nucleus shows only slight differences from the interphase morphology. Nucleoli (Figs. 20 and 21, *N*) are not as closely applied to the envelope as they are later and, in a few cases, they are associated with material that is probably chromatin (Fig. 20, *C*). Helical material (Figs. 20 and 21, *H*) is present more frequently than earlier and is often located within 2 or 3 micra of the envelope (Figs. 20 and 21, *HE*) and closely associated with nucleoli.

A summary chart (Table I) which lists the

Figure 12

Late anaphase: a complete cross-section of the thin, disc-shaped nucleus. × 3200.

Figure 13

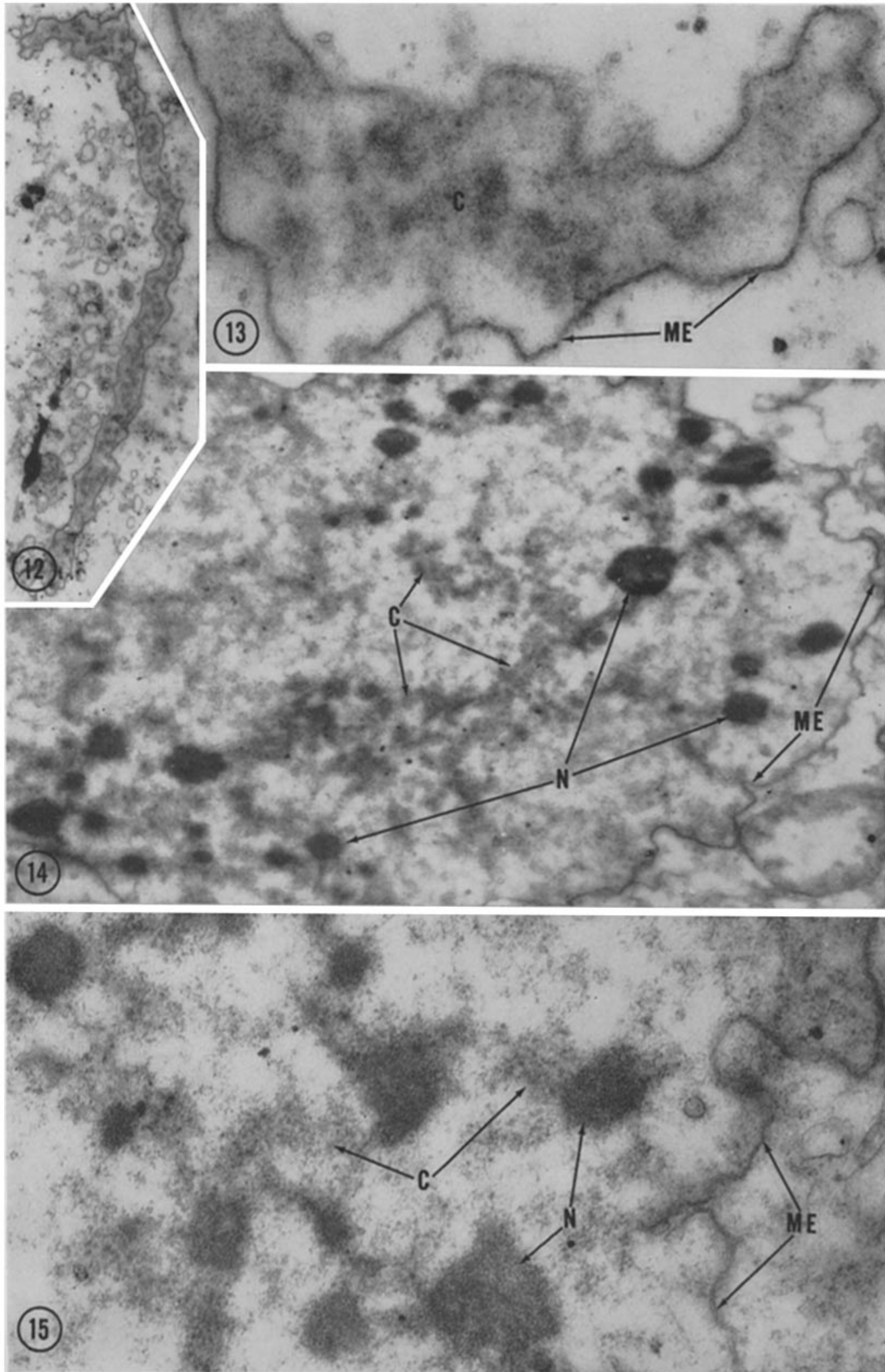
Late anaphase: higher magnification of a portion of the nucleus of Fig. 12 showing the membranes of the envelope (*ME*) and the chromatin (*C*) which is not as much condensed as in metaphase. × 22,000.

Figure 14

Telophase: the chromatin (*C*) is again arranged in a network which shows the formation of nucleoli (*N*) at its periphery. The contour of the nucleus is quite irregular, but the membrane appears to be continuous. × 11,000.

Figure 15

Telophase: nucleoli (*N*) are dense and granular while the chromatin (*C*) is now semi-dispersed and of lower density. The nuclear envelope (*ME*) is almost continuous again but still lacks the inner layer. No helices were observed, but a low density homogeneous component also exists in the nucleoplasm. × 26,000.


13
C
ME
12
14
C
N
ME
15
C
N
ME

presence and absence of components at the division stages observed is included. It is interesting to notice that the honeycomb, inner layer of the nuclear envelope, most of the nucleolar material, and the helical material disappear at about the same time and reappear again at approximately the same time. The stages in which these materials are absent are correlated with the period of chromatin condensation with the exception that short overlapping periods exist. One observes also that the nuclear envelope membranes are present at all times, but have discontinuities during metaphase and, according to a statement by Cohen (4), also at anaphase.

DISCUSSION

Helices and Chromosomes: The helices typical of interphase nuclei of amebae do not coexist with condensed chromatin or chromosomes in the mitotic time sequence. They are therefore composed of materials which dissolve or otherwise disappear during mitosis; we do not know whether they are composed of proteins or of deoxyribose nucleoprotein in a configuration which is unique to interphase. We can be sure, however, that such helices are not chromosomes or chromosome fragments "left over" from mitosis, because their morphology is obviously different from that of metaphase chromosomes. Thus, helices represent either a DNA configuration unique to interphase or are not DNA at all. The close association of helices with nucleoli in the reconstruction nucleus suggests a nucleolar origin of helices.

Chromosomes in this organism are unusually small and numerous. This size might suggest a somewhat simpler organization which would be favorable for electron microscopy; but that this is not the case can be seen by close examination of Fig. 9. This study has not included a careful examination of a great number of chromosomes sectioned in many planes, because this has been done in a concurrent study of metaphase chromosomes in the giant amebae (16).

The Nuclear Envelope and Nucleoli: The reappearance of the inner, thick layer of the nuclear envelope occurs during a rather short period immediately following the re-formation of the nuclear membranes. Correlated with this in time is the appearance of an unusual nucleolar configuration which brings nucleolar material into close association with a large part of the surface of the inner nuclear membrane. The coincidence in time of occurrence and the juxtaposition of this nucleolar configuration and the newly formed honeycomb layer suggest a functional, perhaps cytochemical interrelationship. Nucleolar material may cause, participate in, or mediate the synthesis. A cytochemical study of the honeycomb would be helpful and should be undertaken.

Early reconstruction stage: Figs. 16 to 19 are from the same organism which still contained two nuclei.

FIGURE 16

Survey micrograph of a cross-section of the disc-shaped nucleus. The most striking feature is the dense nucleolar material (*N*) which is peripherally located in granules and folded sheets. × 3000.

FIGURE 17

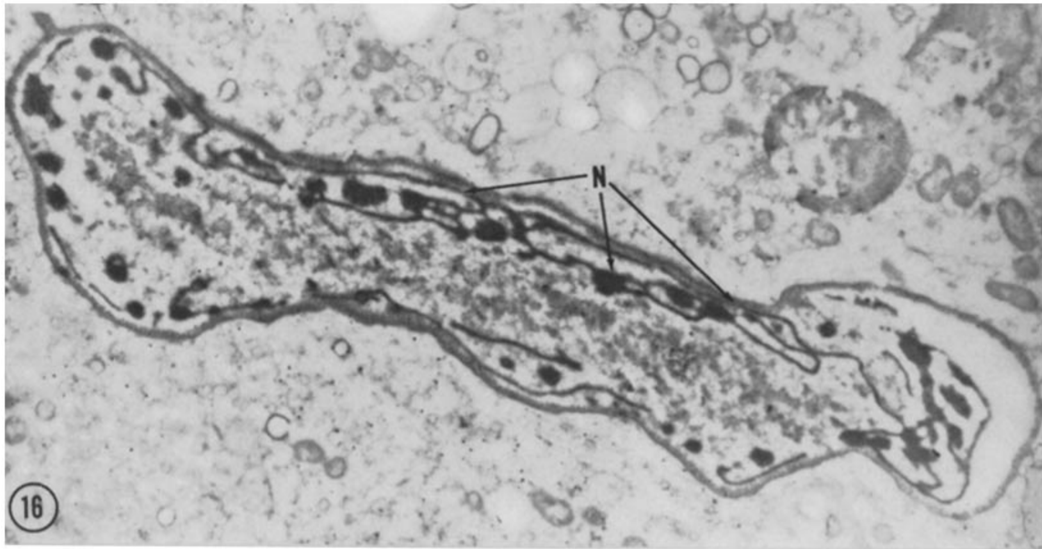
The nucleolar material (*N*) is folded on itself and closely applied to the inner envelope layer (*HE*) which is now about two-thirds as thick as in interphase. Small amounts of chromatin (*C*) are still associated with denser nucleolar material (*N*). × 32,000.

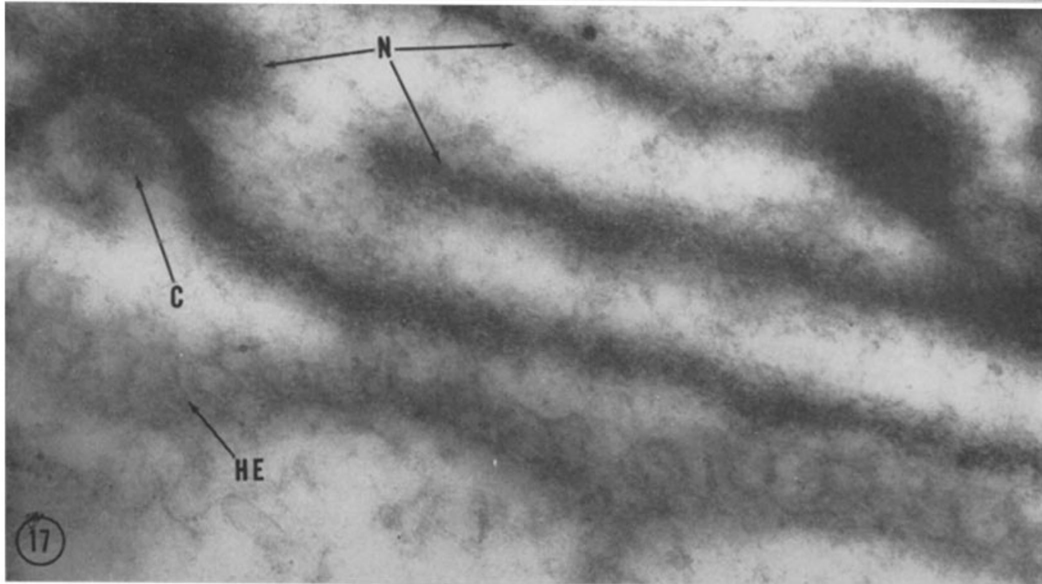
FIGURE 18

Helices (*H*) are again present but are located near nucleolar material (*N*) and near the nuclear envelope (*HE*). × 26,000.

FIGURE 19

The plasmalemma (*P*) remains nearly denuded of its "fringe". × 10,000.


N
16


N
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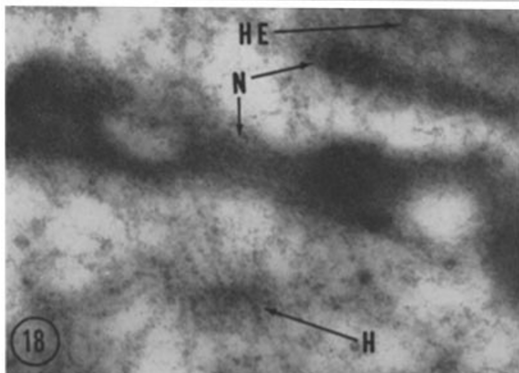
17


HE
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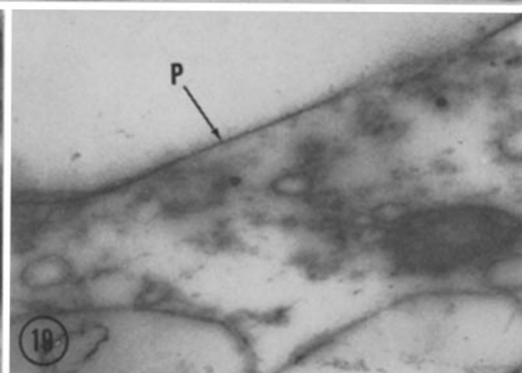
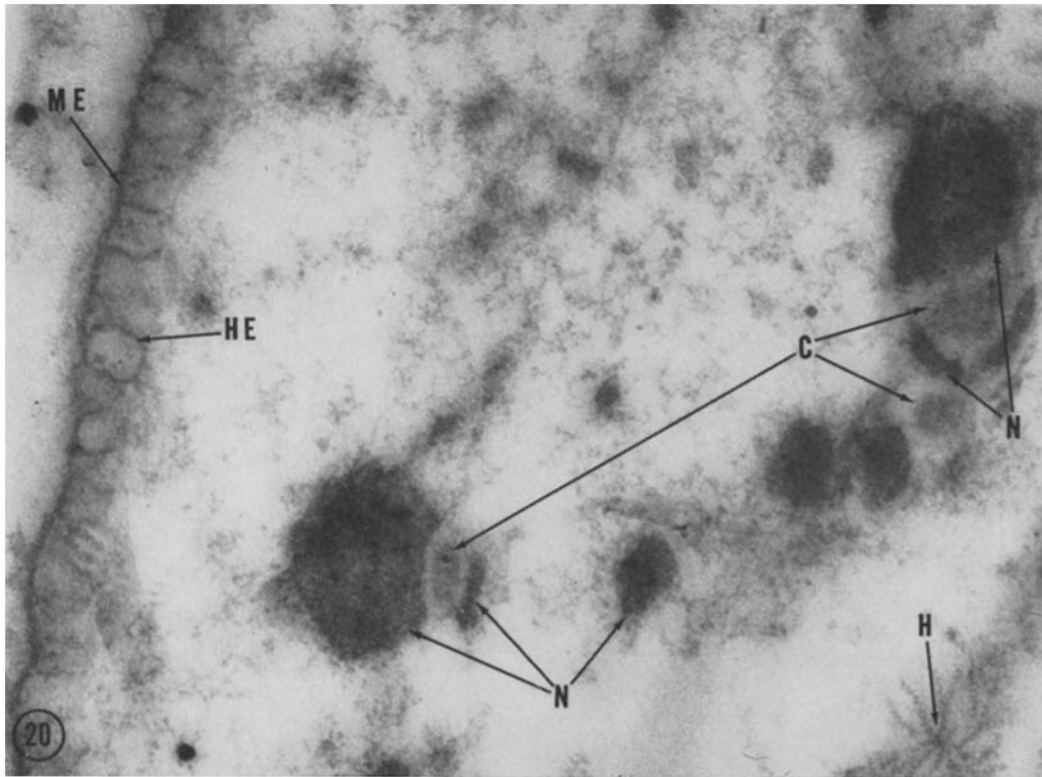
18


P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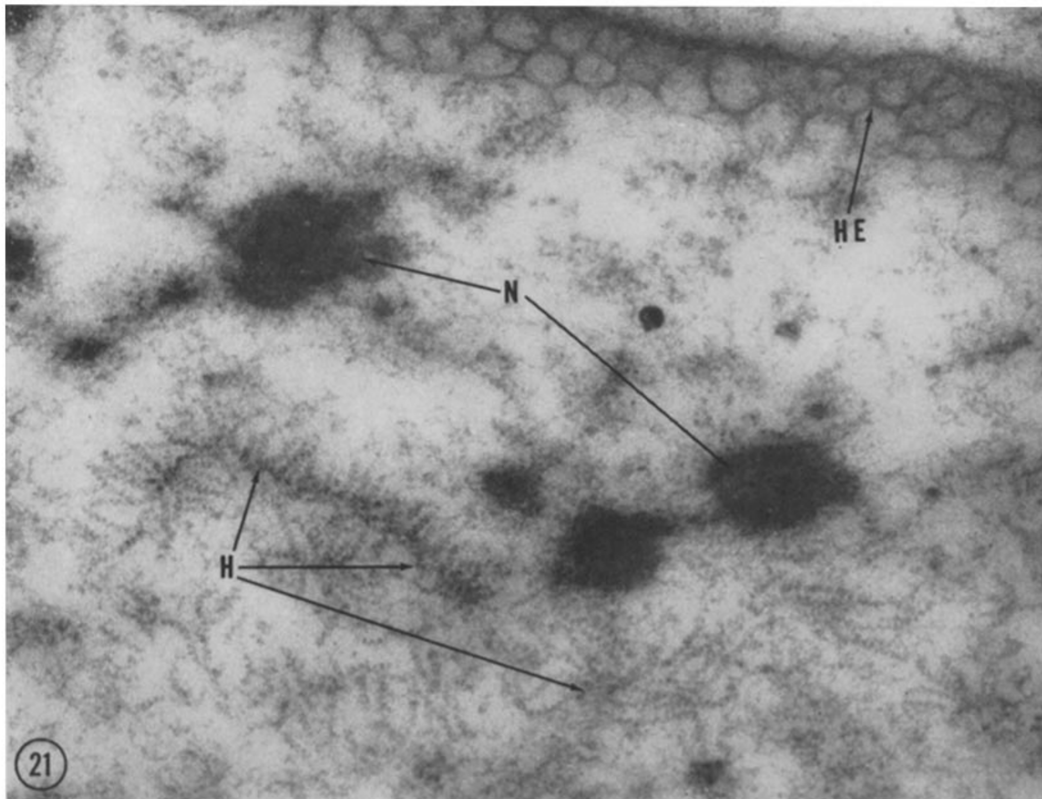
HE
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H
21

TABLE I

Time of Occurrence of Components during Mitosis in Amoeba proteus

	Interphase	Prophase Early*	Prophase Late	Metaphase	Anaphase	Telophase	Reconstruction Early	Reconstruction Late
Chromatin condensed	−	+	++	++	++	++	+	+
Nucleoli	++	+	+	−	−	+	+	+
Helices	++	?	−	−	−	−	+	++
Inner layer of envelope	++	++	−	−	−	−	+	+
Membranes of envelope	++	++	++	+	++	++	++	++
Plasmalemma "fringe"	++	?	?	+	+	+	+	?

++, present, fully formed; +, present, but incomplete or reduced; −, absent; ?, undetermined.
* From Cohen (4).

The method of origin for the sheet-like, nucleolar configuration is unknown and, similarly, the origin of honeycomb material is not adequately explained. However, though questions remain, we are perhaps provided here with an illustration of a rather direct involvement of nucleoli in the formation of structure.

As Cohen (4) has described, the thick, inner layer of the nuclear envelope is missing at metaphase; he has suggested that the reaccumulation of material may give an indication of the state of nuclear maturation. Measurements in this study show that most of this layer has already accumulated in the first 30 minutes or so of the 4 to 5 hour process of nuclear maturation. After another 2 hours the thickness has not greatly increased though it has still not quite reached the interphase measurement. Thus, it seems probable that the accumulation is indeed correlated with nuclear maturation although, if increasing thickness were plotted on a time scale, it would probably not give a simple linear result. Rather, it seems that the maximum rate of accumulation would be correlated with the greatest dispersion or surface exposure of nucleolar material, which occurs in nuclear reconstruction. Thus, we implicate nucleolar material with the formation of the inner layer of the nuclear envelope and furthermore state that a peculiar nucleolar configuration is associated with the formation of most of this layer.

It is significant that the membranes of the nuclear envelope are interrupted by large openings at metaphase. This breaking of the nuclear membranes evidently corresponds to the dissolving of the membranes in metazoan mitoses. Although the membrane changes here are less, the consequences are the same; a "mixoplasm" (18) is

Late reconstruction stage: Figs. 20 and 21 are from the same organism.

FIGURE 20

Only spherical nucleoli are present (*N*), with which small amounts of chromatin (*C*) are still associated; later in nuclear reconstruction and in interphase, nucleoli will become situated still more peripherally so that they nearly contact the envelope. The envelope now consists, as in interphase, of outer membranes (*ME*) and an inner layer (*HE*) which has not yet reached its maximum thickness. Helices (*H*) are present. × 29,000.

FIGURE 21

At this stage, helices are present (*H*) in a peripheral location rather than central as in the interphase nucleus. This large cord (*H*) which is composed of many helical strands is estimated to be 2 μ from the nuclear envelope (*HE*). Nucleoli (*N*) are also present and are closely associated with the helical material. × 31,000.

formed from both cytoplasm and nucleoplasm and spindle fibrils appear thereafter. The development of discontinuities in the envelope and their duration are not accurately known, but they have not yet appeared at late prophase and are no longer observed in late anaphase.

Chalkley (2) described a faint Feulgen reaction in newly formed nucleoli and attributed this to their origin from chromatin. The late reconstruction nucleus observed here shows that the cause of this phenomenon is the small amount of chromatin still present near or within nucleoli at this stage (Figs. 17 and 20).

The Plasmalemma: The changes observed here in the plasmalemma "fringe" were unexpected. The implication of the partial or nearly complete denudation of the plasmalemma membrane is either that membrane is being formed faster than fringe material or that fringe material is being lost or utilized during division.

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