THE ROLE OF ENDOPLASMIC RETICULUM IN
THE REPAIR OF AMOEBA NUCLEAR
ENVELOPES DAMAGED MICROSURGICALLY

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SUMMARY

The nuclear envelopes of amoebae were damaged microsurgically, and the fate of the lesions was studied with the electron microscope. Amoebae were placed on the surface of an agar-coated slide. Using a glass probe, the nucleus was pushed from an amoeba, damaged with a chopping motion of the probe, and reinserted into the amoeba. Cells were prepared for electron microscopy at intervals of between 10 min and 4 days after the manipulation. Nuclear envelopes studied between 10 min and 1 h after the injury displayed extensive damage, including numerous holes in the nuclear membranes. Beginning 15 min after the manipulation, pieces of rough endoplasmic reticulum intruded into the holes in the nuclear membranes. These pieces of rough endoplasmic reticulum subsequently appeared to become connected to the nuclear membranes at the margins of the holes. By 1 day following the injury, many cells had died, but the nuclear membranes were intact in those cells that survived. The elaborate fibrous lamina or honeycomb layer characteristic of the amoeba nuclear envelope was resistant to early changes after the manipulation. Patches of disorganization of the fibrous lamina were present 5 h to 1 day after injury, but the altered parts showed evidence of progress toward a return to normal configuration by 4 days after the injury. It is proposed that the rough endoplasmic reticulum participates in the repair of injury to the nuclear membranes. The similarity of this repair process to reconstitution of the nuclear envelope in telophase of mitosis is noted, and the relationship between the nuclear envelope and the rough endoplasmic reticulum is discussed.

INTRODUCTION

Much information has accumulated concerning the responses of cells to a variety of injurious agents, including microorganisms, radiation, and chemicals. In recent years, emphasis has been placed upon studying the effects of various agents on cell fine structure and on biochemical processes. In the case of some chemicals and antibiotics, it has been possible selectively to alter one organelle or to inhibit a particular metabolic reaction. Such selective inhibition has proved helpful in deducing normal functions and relations of cell components. For example, the use of actinomycin D to inhibit RNA synthesis has been important in studying nuclear structure and function, particularly with respect to the nucleolus (Busch & Smetana, 1970), while cycloheximide and chloramphenicol, which inhibit cytoplasmic and mitochondrial protein synthesis respectively, have been tools in studying the metabolic relation between mitochondria and the remainder of the cell (Roodyn & Wilkie, 1968; Sager, 1972). In some cases, lasers have also proved useful in selectively damaging particular organelles such as mitochondria (Amy & Storb, 1965) or other defined parts of cells.
Similarly, information on the properties of parts of cells and their relation to other cell organelles can be obtained by studying the effects of controlled mechanical injury to cells. For example, properties of the cell surface have been deduced from examination of its response to mechanical injury in amoebae and several different kinds of eggs (Heilbrunn, 1930; Holtfreter, 1943; Chambers & Chambers, 1961; Gingell, 1970; Luckenbill, 1971; Szubinska, 1971; Bluemink, 1972). Direct mechanical injury to cells or their parts has not been widely used in studies of this sort, however, because of the difficulty of manipulating most kinds of cells microsurgically. The uninucleate free-living amoebae such as *Amoeba proteus*, however, lend themselves particularly well to investigation of the effects of mechanical injury, since microsurgical manipulations are readily performed and have been employed in numerous studies on nucleocytoplasmic interactions (Jeon & Danielli, 1971).

During the course of studies on the dependence of the Golgi apparatus and other cytoplasmic organelles on the nucleus (Flickinger, 1969, 1971, 1972), we occasionally observed ultrastructural changes in transplanted nuclei that were thought to represent evidence of repair of the nuclear envelope following minor damage sustained during the operation (Flickinger, 1970). These observations suggested that the rough endoplasmic reticulum was involved in repair, but the incidence of damage and the number of observations of the repair process were too small to form definite conclusions. Since confirmation and study of this phenomenon would furnish information on the relation between the nuclear envelope and cytoplasmic membranes during interphase, in the present study we intentionally damaged the nuclear envelopes of amoebae by microsurgical manipulation and systematically studied the fate of the lesions.

**MATERIALS AND METHODS**

**Cultures**

Stock cultures of *Amoeba proteus* were maintained in Prescott’s amoeba medium (Prescott & Carrier, 1964). They were fed daily with washed *Tetrahymena* grown in proteose peptone. Cultures and experimental animals were maintained at 21 °C.

**Microsurgery**

Operations were performed while the amoebae were flattened on the surface of an agar-coated glass microscope slide, as in the technique for nuclear transplantation described by Jeon (Jeon & Lorch, 1968; Jeon, 1970). Using a braking pipette, individual amoebae were placed in a small drop of medium on the agar surface. The medium was then withdrawn, flattening each amoeba on the surface of the agar. The nucleus was then manipulated using a glass probe approximately 2–10 μm wide at the tip, controlled with a de Fonbrune micromanipulator. In the initial experiments, the probe was inserted into the amoeba and the nucleus was prodded about 10–15 times with the tip of the probe. It was discovered that insufficient damage was obtained in this way, however, and the following method was adopted. The nucleus was pushed out of an amoeba and was placed on the agar surface alongside the cell. The nucleus was damaged by striking it with the probe, using a vertical chopping motion. Eight strokes were utilized for most of the samples. The nucleus was lifted on the tip of the probe and reinserted into the amoeba. Small drops of medium were added to each of the amoebae and they were transferred to a Syracuse watch glass for recovery. The amoebae were maintained at 21 °C in watch glasses.
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until they were fixed for electron microscopy. In one experiment, amoebae were maintained individually in separate depressions in a plastic dish and observed at daily intervals to study the length of time the cells survived and to determine if cell division took place. Cells maintained for one day or less were not fed. Amoebae maintained for more than one day were fed small amounts of *Tetrahymena* beginning on the second day.

**Preparation for electron microscopy**

Samples were prepared for electron microscopy at intervals of 10, 15 and 20 min; 1 and 5 h; and 1 and 4 days after manipulation. Each sample consisted of approximately 50–100 amoebae. The amoebae were placed in a Syracuse watch glass containing Karnovsky's fixative (Karnovsky, 1965), consisting of 5% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer at pH 7.3. The cells were rinsed overnight in 0.05 M buffer in a watch glass, and the next day the cells were embedded in a small cube of agar to prevent their subsequent dispersion (Flickinger, 1969). The amoebae within the agar cube were postfixed for 1 h in 1% OsO₄ in 0.1 M cacodylate buffer, dehydrated, and embedded in Araldite.

Sections 1 µm thick were cut with glass knives, mounted on slides, and stained with 0.5% toluidine blue in 0.5% sodium borate for light microscopy. Thin sections for electron microscopy were cut with a diamond knife on a Porter-Blum MT-1 microtome, stained with lead citrate (Reynolds, 1963), and examined in a Philips EM-300 electron microscope.

**RESULTS**

**General observations**

A difficult problem in this experiment was to administer a sufficient but not an excessive amount of damage to the nuclear envelope. Initially, we attempted to injure the nucleus by inserting a glass needle into an amoeba and prodding the nucleus about 10–15 times. When the cells were subsequently examined in the electron microscope, however, holes in the nuclear envelope were encountered only rarely. Perhaps this may be explained by the low probability of encountering small punctate holes in random sections through the nucleus. Alternatively, perhaps small lesions were repaired so rapidly that they were not detected. In any event, in an attempt to produce greater damage, we discovered that the nucleus could be pushed from the amoeba on to the agar surface alongside the cell, damaged severely by a vertical chopping motion of the glass probe, and reinserted into the amoeba. Surprisingly, over half of the amoebae showed 'reactivation' (Lorch & Danielli, 1953; Jeon, 1968), consisting of the formation of pseudopods, resumption of motility and the ability to adhere to the substrate, even after the nucleus had been subjected to up to 10 or 12 strokes of the probe.

Clearly it is possible with this technique to damage the nucleus so severely that detection of damaged areas would not be a problem, but the amoebae would show no evidence of repair and recovery. On the other hand, as demonstrated in our initial experiments by simply prodding the nucleus, milder trauma maximizes the possibilities for subsequent long-term survival of the amoebae, but detection of nuclear envelope damage requires a long and tedious search and the number of observations is too small to be of significance. Thus we compromised between these 2 extremes and elected to carry out the study by pushing the nucleus from the cell and administering 8 strokes with the probe before returning it to the cell. This procedure resulted in electron-microscopically visible damage to virtually all (more than 90%) nuclei, but
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still permitted reactivation of more than half of the amoebae and survival of a sufficient number of cells for electron microscopy for periods of several hours to a few days (Table 1). As described below, the ultrastructural studies indicate that when damaged in this way repair of the nuclear membranes took place. The injury was sufficiently severe, however, to prevent subsequent cell divisions.

Table 1. The survival of amoebae after injury to the nucleus

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<th>Days after injury</th>
<th>No. of amoebae</th>
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<tr>
<td>0</td>
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In retrospect, it seems that a lesser amount of injury might have been optimal in providing sufficient examples of lesions for electron-microscope study, coupled with a better survival of the manipulated cells. The large amount of work involved in performing the microsurgery and handling such small samples of cells for electron microscopy, however, precluded a survey of the varying effects of different degrees of injury and dictated that a particular protocol be selected and continued throughout the series of experiments.

**Normal amoeba nuclear envelope**

The amoeba nucleus is bounded by a nuclear envelope consisting of 2 nuclear membranes and a fibrous lamina or honeycomb layer (Fig. 1). The nuclear membranes are each ~7 nm thick. They are separated by a perinuclear cisterna and are fused at intervals to form pores. The fibrous lamina is composed of very fine filaments organized into a series of adjacent prisms, which are arranged roughly in a hexagonal pattern. The long axes of the prisms are directed perpendicular to the surface of the nucleus, in such a way that one end of each prism abuts the inner aspect of the inner nuclear membrane and is centred on a nuclear pore, while the opposite end is open to the interior of the nucleus. The centre-to-centre spacing of the prisms and pores is about 150 nm. The thickness of the fibrous lamina varies with the stage of the cell cycle (Feldherr, 1968), but throughout most of interphase it is 250-350 nm.

The interior of the nucleus contains fibrillar material identified as chromatin (Wise & Goldstein, 1971), helices, and groups of granules (Stevens, 1967). Multiple nucleoli are found at the periphery of the nucleus, immediately inside the nuclear envelope (Flickinger, 1968).
Damaged nuclei

Nuclei examined 10 min to 1 h after manipulation virtually all exhibited extensive damage (Figs. 2, 3). Most important in relation to later observations were gaps in the nuclear membranes. These ranged from small holes 200 nm in diameter (Fig. 3) to segments several μm wide, in which the nuclear membranes were missing and the nucleus was bounded only by the fibrous lamina. Frequently folds of the nuclear envelope, including the fibrous lamina and fragments of the nuclear membranes, were displaced into the interior of the nucleus. However, long finger-like projections of the nuclear envelope, such as were present in some transplanted nuclei (Flickinger, 1970), were not observed in nuclei damaged by the present method. In some cases membranous sheets, vesicles and cisternae were found in the interior of the nucleus. Except for some holes at which the nucleus apparently had been penetrated by the probe, the honeycomb layer remained largely intact, even when portions were dislocated into the interior of the nucleus or when segments were completely denuded of nuclear membranes. In some instances pieces of thick membrane resembling the plasma membrane surrounded the nucleus, but these did not persist in later samples. In some severely damaged nuclei, representing about 15% of the total observed 10–15 min after manipulation, the nuclear membranes were almost completely absent and the nuclear contents of chromatin and nucleoli were clumped together and greatly increased in density.

As early as 15 min following injury and continuing through 1 h samples, portions of rough endoplasmic reticulum were associated with the gaps in the nuclear membranes (Fig. 4). These pieces of endoplasmic reticulum intruded into the smaller holes as if to form a plug (Fig. 5). In the larger stretches of nuclear surface that were denuded of nuclear membranes, cisternae of rough endoplasmic reticulum often were aligned on the outer surface of the fibrous lamina in the position formerly occupied by the nuclear membranes (Fig. 6). In some instances, the surfaces of these elements of endoplasmic reticulum were studded with numerous ribosomes (Fig. 6), while in other cases ribosomes were sparse and most of the membrane was smooth (Fig. 5).

Beginning with the sample prepared 20 min after manipulation, images were encountered suggesting that the pieces of rough endoplasmic reticulum within gaps in the nuclear membranes were connected to the nuclear membranes at the margins of the holes (Fig. 7). It was difficult to be certain that true fusion of the membranes had occurred because of the possibility of overlap within the plane of section, but the impression gained was that pieces of endoplasmic reticulum had established a connexion with the nuclear membranes and that a continuous nuclear envelope had been restored at some locations. In some places, ribosomes were observed on the nuclear surface of the inner nuclear membrane facing the fibrous lamina (Fig. 8), apparently as the result of fusion of a piece of rough endoplasmic reticulum with the nuclear envelope.

By 5 h to 1 day after the injury, many cells had died, but those that survived displayed largely intact nuclear membranes, within the plane of the sections examined. In many cases remnants of membranous material were found within the nucleus.
Until this time, the fibrous lamina was resistant to changes, except for a few places at which it appeared to have been directly cleaved by the probe. At this stage, however, large stretches of the fibrous lamina lost the characteristic honeycomb-like organization. Instead, the region of the fibrous lamina was occupied by a thick feltwork of fine filaments (Fig. 9) in which dispersed, randomly oriented plates similar to those comprising the walls of the prisms were sometimes discernible.

In samples observed 4 days after the injury, the nuclear membranes also were intact. Regions of alteration in the fibrous lamina persisted. In contrast to the 1-day samples, however, the fibrous lamina was not so disorganized and plates and portions of prisms were more readily visible.

The contents of many nuclei were also altered. Often the number of nucleoli decreased, while their size increased. The appearance of the chromatin was variable. Some large clumps of condensed chromatin were observed, but in other cells at early intervals after manipulation the interior of the nucleus was unusually electron lucent, particularly when the nuclear membranes were absent over large areas.

**DISCUSSION**

The results indicate that some nuclear membranes were repaired, since there was moderate to severe damage to the nuclear membranes of almost all nuclei 10 min to 1 h after injury, but the nuclear membranes appeared intact and no longer contained holes in those cells that survived for more than 5 h. The sequence of images observed suggested that the repair of the nuclear membranes may take place in the following series of steps. Shortly after injury, pieces of rough endoplasmic reticulum became intimately associated with the gaps in the nuclear membranes. These pieces of rough endoplasmic reticulum intruded into the small holes. In the case of larger areas of disrupted nuclear membranes, a cisterna of endoplasmic reticulum became aligned on the external surface of the fibrous lamina. The endoplasmic reticulum then appeared to fuse with the nuclear membranes at the margin of the hole, re-establishing continuity of the 2 nuclear membranes. This process resulted in ribosomes being found temporarily on the inner surface of the nuclear membranes in some locations. These ribosomes must subsequently have been removed and the endoplasmic reticulum cisterna flattened to attain the normal configuration of the nuclear membranes. In addition, to reconstruct a normal nuclear envelope it would be necessary that pores form in the nuclear membranes, but the present study has not revealed the extent to which pore formation took place.

The changes observed in the present study following severe damage to the nuclear envelope closely resemble those detected in a small number of transplanted amoeba nuclei (Flickinger, 1970), and the present study supports the previous speculation that the rough endoplasmic reticulum can participate in the repair of damage to the nuclear membranes. It may be noted, however, that this proposed mechanism for repair of the nuclear envelope by intercalation of pieces of rough endoplasmic reticulum differs from mechanisms postulated for repair of injuries to the plasma membrane in amoebae and in various kinds of eggs. In the case of the plasma membrane, a role
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has been attributed to one or more of the following structures, which were not detected in the present experiments: (1) bundles of cortical filaments (Gingell, 1970; Luckenbill, 1971; Bluemink, 1972), which may cause contraction of the margin of holes in the membrane; (2) a 'new membrane' formed external to the plasma membrane (Szubinska, 1971); or (3) dense droplets of lipid, which adhere to the cytoplasmic surface of the membrane and are thought to insert into it (Szubinska, 1971; Bluemink, 1972).

The participation of the rough endoplasmic reticulum in repair of injury to the nuclear envelope is in accord with the view that these 2 structures are very closely related. The nuclear envelope and the rough endoplasmic reticulum are known to be in direct structural continuity in most cells and examples of connexions between the two are a common observation (Fawcett, 1965). In addition, the nuclear envelope is formed by fusion of pieces of endoplasmic reticulum around the chromosomes at the conclusion of mitosis (Porter, 1961; Feldherr, 1972). Ribosomes stud the surfaces and a similar content is frequently observed in both (Fawcett, 1965). On the other hand, differences between the two also exist. The chemical composition of the nuclear envelope differs in some respects from that of the rough endoplasmic reticulum, the nuclear envelope being richer in protein (Franke et al. 1970) and esterified cholesterol (Kleinig, 1970) and varying in the activities of certain enzymes (Franke et al. 1970). The presence of pores and the pore complex, the absence of ribosomes on the inner surface, and the presence of a fibrous lamina are additional morphological features of the nuclear envelope that differ from those of the endoplasmic reticulum.

The sequence of steps in repair of damaged nuclear membranes bears a marked resemblance to the stages in the reformation of the nuclear envelope after mitosis. Reconstruction of the nuclear envelope takes place by fusion of pieces of endoplasmic reticulum during telophase in most eukaryotic cells, with the exception of fungi and certain protozoa (Porter, 1961; Feldherr, 1972). In Amoeba proteus, the dissolution of the nuclear envelope during mitosis is not as complete as in most metazoan cells, and recognizable pieces of nuclear envelope, separated by large discontinuities, remain associated with the chromosomes during mitosis. With this qualification, however, the process is comparable to that of higher cells (Roth, Obetz & Daniels, 1960). In the present study, the rough endoplasmic reticulum became associated with damaged parts of the nuclear envelope within a few minutes after the injury. Thus it seems that under the appropriate stimulus, in this case that of mechanical injury, incorporation of pieces of rough endoplasmic reticulum into the nuclear envelope can occur rapidly during interphase as well as during telophase of mitosis. Whatever triggers this activity, therefore, is not exclusively a mitotic event, but can be activated during other parts of the cell cycle as well.

Some tears in the fibrous lamina were observed at early time intervals after manipulation of the nucleus, but in general the fibrous lamina was more resistant to direct early effects of the injury than the nuclear membranes. The main change in the fibrous lamina occurred later, developing hours to days after the injury, and consisted of a loss of the normal honeycomb-like organization and its replacement by a thick disorganized feltwork of filaments. Since this change occurred only after several hours
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and did not become highly developed until 1 day had elapsed it may not be a direct effect of the trauma. It might be considered a degenerative change that follows damage to the nuclear membranes, but the nuclear membranes usually were intact in the affected regions of nuclei that survived this long. An alternate possibility is that this disorganization was a preliminary step in the reorganization of the fibrous lamina to accommodate altered nuclear size or contour. This conjecture is supported by the observation that a progression in the organization of the affected regions to contain visible plates and prisms occurred between 1 and 4 days after manipulation.

The fibrous lamina has been considered to provide structural reinforcement to the nuclear membranes (Fawcett, 1966). An elaborate honeycomb-like organization of the fibrous lamina has been found only in amoebae, some gregarines (Beams, Tahasian, Devine & Anderson, 1957), and cells in the central nervous system of the leech (Coggleshall & Fawcett, 1964). Although the role of such a differentiation in nature is not clear, it has been argued that this extreme development of the fibrous lamina in amoebae is important in permitting the transplantation of nuclei by the method of pushing the nucleus from one cell to another (Flickinger, 1970; Jeon & Danielli, 1971). It seems likely that this unusual fibrous lamina was also important in the present study in permitting the amoeba nucleus to survive the extensive manipulations to which it was subjected.

Although some damaged nuclear envelopes showed evidence of repair, the nuclei did not return completely to normal, since no cells were observed to divide. The reason for this arrest is not known, although speculations might be entertained concerning the difficulty of nuclear pore formation, reconstruction of a normal fibrous lamina, or restoration of normal relationships between the nuclear envelope and intranuclear structures. Alternatively, perhaps some nuclear contents were irretrievably lost during the manipulation.

The extent to which patches of membranes or membrane components are transferred from one organelle to another remains an important problem in cell biology. For example, the possibility of transfer of portions of membranes and mixing of membrane components between endoplasmic reticulum, the Golgi apparatus, and plasma membrane during the normal secretory process continues to be debated (cf. Amsterdam et al. 1971; Meldolesi, Jamieson & Palade, 1971; Meldolesi & Cova, 1971, 1972; Siekevitz, 1972). The events described in the present study and the well known formation of the nuclear envelope from endoplasmic reticulum in telophase seem to present rather clear examples of exchange of large intact membrane fragments between 2 classes of cellular membranes, the rough endoplasmic reticulum and the nuclear envelope. Furthermore, in this instance cisternae of rough endoplasmic reticulum appear to be incorporated into the nuclear envelope and subsequently to form part of it. As discussed above, however, the endoplasmic reticulum and the nuclear envelope are similar, and it might be argued that this exchange actually represents the transfer of membranes from one part of the same membranous organelle to another.
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Fig. 1. Normal amoeba nucleus, which is bounded by 2 nuclear membranes (m). The fibrous lamina (f), or honeycomb layer, is apposed to the inner aspect of the nuclear membranes. The honeycomb layer is composed of a series of prisms that are oriented with their long axes perpendicular to the surface of the nucleus. The prisms are sectioned longitudinally on the left and obliquely on the right of the micrograph. c, cytoplasm; n, interior of the nucleus; nu, nucleolus. x 26000.

Fig. 2. Part of a damaged nucleus 15 min after injury shows numerous holes (h) in the nuclear membranes. The cytoplasm extends into a fold in the nuclear envelope (a). c, cytoplasm; f, fibrous lamina; n, nucleus. x 20000.
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1

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Fig. 3. A portion of a damaged nucleus 15 min after injury is shown at higher magnification. Three holes (h) appear as abrupt discontinuities in the nuclear membranes, but the fibrous lamina (f) is intact beneath the holes in the membranes. c, cytoplasm; n, nucleus. × 36000.

Fig. 4. Damaged nucleus 15 min after injury. Beginning as early as 15 min after injury and continuing for about 1 h, pieces of endoplasmic reticulum (er) become associated with holes in the nuclear membranes. Details of this association are shown in Figs. 5–7. c, cytoplasm; f, fibrous lamina; m, nuclear membranes; n, nucleus. × 32000.
Fig. 5. In the case of small holes in the nuclear membranes, a piece of endoplasmic reticulum (er) intrudes into the hole as if to form a plug. One element of endoplasmic reticulum may, as shown here, intrude into a hole (1), return to the cytoplasm, and be inserted subsequently into a second hole (2) nearby. Damaged nucleus 20 min after injury. c, cytoplasm; f, fibrous lamina; m, nuclear membranes; n, nucleus. × 68,000.

Fig. 6. The nuclear membranes are absent from large areas of some damaged nuclei. When the fibrous lamina (f) remains intact in these regions, cisternae of endoplasmic reticulum (er) frequently become aligned on the outer surface of the fibrous lamina, in the position normally occupied by the nuclear membranes. Nucleus 20 min after damage. c, cytoplasm; n, nucleus. × 28,000.
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Fig. 7. In damaged nuclei between 15 min and 1 h after manipulation, images are encountered suggesting that pieces of endoplasmic reticulum (ER) associated with holes in the nuclear envelope can establish connexions with the nuclear membranes (m) at the margins of the holes. Nucleus of an amoeba 15 min after injury. c, cytoplasm; n, nucleus. × 29000.

Fig. 8. In nuclear envelopes undergoing repair between 15 min and 1 h after injury, ribosomes (arrow) are occasionally found on the inner surface of the nuclear membranes (m) next to the fibrous lamina (f). This apparently occurs transiently as the result of incorporation of a cisterna of rough endoplasmic reticulum into the nuclear envelope at the margin of a hole. Damaged nucleus 1 h after injury. c, cytoplasm; f, fibrous lamina; n, nucleus. × 46000.

Fig. 9. The fibrous lamina is relatively resistant to changes at early intervals after injury, when the nuclear membranes are primarily affected. By 1 day following injury the nuclear membranes (m) have been repaired and are intact. At this time, however, large sections of the fibrous lamina (f) become disorganized, the normal honeycomb-like configuration being replaced by a feltwork of disoriented filaments. By 4 days after injury, however, these parts of the fibrous lamina are less disorganized than this and show a return toward a normal pattern, with parts of prisms being visible. Damaged nucleus 1 day after manipulation. c, cytoplasm; n, nucleus. × 36000.