METABOLIC REQUIREMENTS FOR INTERACTIONS BETWEEN NUCLEAR AND CYTOPLASMIC MEMBRANES IN THE REPAIR OF DAMAGED AMOEBA NUCLEI

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SUMMARY
Amoeba nuclear envelopes were damaged using microsurgery, and metabolic requirements for the steps in their repair were studied, by placing the cells in a solution containing one of several metabolic inhibitors. The first step in repair, the association of pieces of endoplasmic reticulum with holes in the nuclear membranes, appears to be a passive process since it was not affected by inhibitors of energy production, RNA synthesis, or protein synthesis. In contrast, fusion of pieces of endoplasmic reticulum with the nuclear membranes at the margins of the holes was blocked by KCN and dinitrophenol, indicating that membrane fusion requires energy derived from respiration, but RNA and protein synthesis inhibitors did not prevent fusion of pieces of endoplasmic reticulum with the nuclear membranes. The subsequent completion of repair and restoration of intact nuclear membranes was almost completely blocked by inhibitors of respiration, and it was reduced in the presence of actinomycin and emetine, suggesting that in addition to a requirement for energy, some later steps in the repair of the nuclear membranes require RNA and protein synthesis.

INTRODUCTION
Using microsurgery it is possible to test interactions between parts of cells by altering their normal relationships. For example, nuclear transplantation from one cell to another has been used to study functional interactions between nucleus and cytoplasm (Jeon & Danielli, 1971; Goldstein, 1973), and microsurgical methods have been utilized to investigate the formation of the Golgi apparatus in amoebae (Flickinger, 1969, 1973).

Similarly, a means for study of interactions between different types of intracellular membranes is provided by the observation that the endoplasmic reticulum is involved in the repair of amoeba nuclear membranes damaged microsurgically (Flickinger, 1974). Both in the fraction of nuclei that sustain injury during nuclear transplantation (Flickinger, 1970) and in nuclei that are intentionally damaged microsurgically (Flickinger, 1974), pieces of endoplasmic reticulum intrude into gaps in the nuclear membranes and then fuse with the nuclear membranes at the margins of the holes, resulting in restoration of continuous nuclear membranes (Flickinger, 1974). This process thus appears to represent an example of the transfer of pieces of membrane
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from one location to another and subsequent incorporation of endoplasmic reticulum membranes into the nuclear envelope.

In the present investigation, possible requirements for RNA synthesis, protein synthesis, and energy obtained from oxidative phosphorylation and glycolysis for nuclear envelope repair were studied. This was accomplished by damaging amoeba nuclei and exposing the cells to inhibitors of these processes. The effect of an inhibitor on each of the following steps in nuclear envelope repair was analysed: (1) association of endoplasmic reticulum with holes in the nuclear envelope; (2) fusion of endoplasmic reticulum with the nuclear membranes; and (3) the ultimate restoration of continuous nuclear membranes.

**MATERIALS AND METHODS**

Cultures of *Amoeba proteus* were maintained in Prescott’s amoeba medium (Prescott & Carrier, 1964) with daily feedings of washed *Tetrahymena*.

Amoeba nuclear envelopes were damaged microsurgically according to a method used previously (Flickinger, 1974). Amoebae were placed individually in small drops of medium on the surface of an agar-coated slide. The excess medium was withdrawn, flattening the amoebae on the agar (Jean, 1970). Under a compound microscope at a magnification of 180 x, the nucleus was pushed from each amoeba with a glass probe controlled with a de Fonbrune micromanipulator. The nucleus was placed on the agar alongside the cell and damaged by striking it 8–10 times with a vertical motion of the probe. The nucleus was then re-inserted into the amoeba and the cells were transferred for recovery to a Syracuse watch glass containing medium.

After manipulation, groups of amoebae were placed in a solution of one of the following inhibitors in amoeba medium: (1) 7.5 x 10^-3 M KCN; (2) 5 x 10^-4 M dinitrophenol; (3) 7.5 x 10^-3 M NaF; (4) 1 x 10^-4 M or 1 x 10^-3 M emetine hydrochloride (Eli Lilly & Co., Indianapolis, Indiana); and (5) actinomycin D, 1 mg/ml. These concentrations of KCN and NaF were used to inhibit oxidative phosphorylation and glycolysis respectively because they are the highest concentrations that permit survival of 90% of normal amoebae for at least 6 h (Flickinger, 1972). This concentration of KCN is capable of inhibiting Golgi apparatus formation in *Amoeba proteus* (Flickinger, 1972), and 1 x 10^-3 M KCN (less than that used here) inhibits 67% oxygen consumption in the giant amoeba *Pelomyxa carolinensis* (Pace & Belda, 1944). The concentration of dinitrophenol used was the highest that could be readily dissolved in amoeba medium. Emetine, an antiparasitic drug, was used because it inhibits protein synthesis.

Figs. 1–7. Electron micrographs of portions of the nuclear envelopes of control and treated amoebae fixed 30 min after nuclear damage.

Fig. 1. Electron micrograph of a portion of a control amoeba nucleus from a cell placed in normal medium for 30 min after damage. Several holes (h) in the nuclear membranes (m) are visible, and pieces of endoplasmic reticulum (er) are associated with some of the holes. c, cytoplasm; f, fibrous lamina or honeycomb layer of the nuclear envelope; n, nucleus. x 33,000.

Fig. 2. Part of a control amoeba nuclear envelope 30 min after damage. Fusion of a piece of endoplasmic reticulum (er) with the nuclear membranes (m) at the margin of a hole is indicated by connection between the two (arrow). The piece of endoplasmic reticulum is readily identified by the presence of ribosomes (r) on the surface facing the fibrous lamina and the interior of the nucleus. c, cytoplasm; n, nucleus. x 37,000.

Fig. 3. Part of the damaged nucleus of an amoeba exposed to KCN for 30 min. The inhibitor did not prevent the association of pieces of endoplasmic reticulum (er) with holes (h) in the nuclear membranes (m). n, interior of the nucleus. x 33,000.
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synthesis in amoebae (Maruta & Goldstein, 1975). One of the more commonly used substances such as cycloheximide was not employed because if the concentration is elevated to a point sufficient to inhibit protein synthesis the amoebae are killed (D. M. Prescott, personal communication; Flickinger, 1971). Two different concentrations of emetine were tested. A solution of $1 \times 10^{-3}$ M was tried because this has been reported to inhibit more than 97% protein synthesis in amoebae (Maruta & Goldstein, 1975). Observations were made on experimentally manipulated amoebae treated for 30 min with this amount of emetine, but cells treated for 5 h could not be studied because most of the amoebae died. Therefore, the effects of emetine were also studied at a concentration of $1 \times 10^{-4}$ M, which has previously been used to inhibit formation of the Golgi apparatus in amoebae and permits survival of the majority of normal amoebae for more than a day (Flickinger, 1971). The concentration of actinomycin utilized is greater than that used in mammalian cells, but has been used to inhibit RNA synthesis in amoebae because amoebae are relatively insensitive to this antibiotic (Stevens, 1967; Rao & Prescott, 1970; Flickinger, 1971). The concentration used inhibits all detectable RNA synthesis within 18 min (Rao & Prescott, 1970).

Samples of amoebae were prepared for electron microscopy at intervals of 30 min and 5 h after damaging the nuclear envelope. These intervals were selected because previous work (Flickinger, 1974) indicated that the main steps in the repair process could be observed at these two intervals. Amoebae were fixed for 1 h in Karnovsky's fixative (Karnovsky, 1975) and rinsed in water. The cells were embedded in a small cube of agar to prevent their subsequent dispersion (Flickinger, 1969). The samples were postfixed in 1% OsO$_4$ in 0.1 M cacodylate buffer at pH 7.3, dehydrated in a graded series of ethanols followed by propylene oxide, and embedded in Araldite. Silver to pale gold sections were cut with a diamond knife on a Porter-Blum MT-i microtome, mounted on copper grids, and stained with lead citrate (Reynolds, 1963). The preparations were examined in a Philips EM-300 electron microscope.

Because of the use of microsurgery, the size of the samples was limited to between 50 and 120 amoebae. In each sample, sectioning at several levels in the block permitted the study of 15-20 nuclei. Most experiments were performed 2 or 3 times.

The grids were scanned systematically and each nucleus encountered was studied in the following way. The entire perimeter of the nucleus was studied at a magnification of 5000-10000 × on the fluorescent screen and an additional optical magnification of 10 ×. All areas exhibiting damage were photographed in their entirety. Representative micrographs were obtained of undamaged profiles.

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Fig. 4. Damaged amoeba nucleus exposed to dinitrophenol for 30 min. A cisterna of endoplasmic reticulum (er) is aligned on the surface of the fibrous lamina (f) in the position normally occupied by the nuclear membranes, but it is not fused to the membranes (m) at the margin of the hole. × 36000.

Fig. 5. Part of a damaged amoeba nucleus following exposure to NaF for 30 min. A piece of endoplasmic reticulum (er) is fused to the nuclear membranes (m) at the margin of a hole (h). n, nucleus. × 47000.

Fig. 6. Nuclear envelope of an amoeba exposed to emetine ($1 \times 10^{-3}$ M) for 30 min after damage. A piece of endoplasmic reticulum (er), identified by the presence of ribosomes on the surface facing the interior of the nucleus, is fused to the nuclear membranes (m) in 2 locations (arrows). It may be noted that, in this cell, fusion of endoplasmic reticulum with nuclear membranes took place in the presence of a concentration of emetine that inhibits 97% protein synthesis in Amoeba proteus. × 32000.

Fig. 7. Portion of the nucleus from an amoeba placed in actinomycin D for 30 min after manipulation. A piece of endoplasmic reticulum (er) is associated with a hole (h) in the nuclear membranes. c, cytoplasm; n, nucleus. × 42000.
RESULTS

Thirty minutes after damage

The nuclear envelopes of control amoebae placed in normal amoeba medium following damage conformed to those described previously (Flickinger, 1974). In cells fixed 30 min after manipulation (Figs. 1, 2), there were numerous holes in the nuclear membranes, distributed among nearly all the nuclear profiles observed. Pieces of endoplasmic reticulum intruded into the holes (Fig. 1), and in the case of larger stretches lacking nuclear membranes, cisternae of endoplasmic reticulum were disposed on the surface of the fibrous lamina or honeycomb layer of the nuclear envelope in the position usually occupied by nuclear membranes. Examples of fusion of pieces of endoplasmic reticulum with the nuclear membranes at the margins of the holes were observed in many control nuclei (Fig. 2). Pieces of endoplasmic reticulum fused to the nuclear envelopes were identified as such by the following criteria: the presence of ribosomes on the surface facing the interior of the nucleus as well as that facing the cytoplasm; the absence of nuclear pores; extension into the cytoplasm and connexion with other portions of the endoplasmic reticulum; and the general configuration of the element which, like the rest of the endoplasmic reticulum, was more irregular in shape than the normal nuclear envelope. Further details of the various steps in the repair process are described in a previous publication (Flickinger, 1974), and the normal fine structure of the amoeba nuclear envelope is well documented (Roth, Obetz & Daniels, 1960; Flickinger, 1970, 1973; Daniels, 1973).

None of the inhibitors prevented the association of pieces of endoplasmic reticulum with holes in the nuclear envelope of cells fixed 30 min after damage (Figs. 2–7). Pieces of endoplasmic reticulum were found lying within gaps in the nuclear membranes and along the cytoplasmic surface of the fibrous lamina of amoebae placed in KCN, dinitrophenol, NaF, emetine, or actinomycin.

Instances of apparent fusion of endoplasmic reticulum with nuclear membranes were observed in many cells exposed to NaF, emetine or actinomycin (Figs. 5–7). In contrast, examples of fusion of endoplasmic reticulum and nuclear membranes were rare in the presence of KCN or dinitrophenol (Figs. 3, 4). No more than 1 or 2 examples of connexions were detected in each sample of amoebae exposed to KCN or DNP, compared with approximately 10–15 instances observed in each of the other samples, including the control.

Five hours after damage

By 5 h after the operation, the majority of profiles of control amoebae had intact nuclear membranes (Fig. 8), and in only a few did some small holes persist. As noted previously (Flickinger, 1974), this indicates that repair of the amoeba nuclear membranes occurred after damage, since all the profiles showed numerous holes 30 min after operation, while most of the cells survived and their nuclei had intact nuclear membranes 5 h after manipulation.

Repair of nuclear membranes was greatly reduced in amoebae placed in KCN or dinitrophenol for 5 h. In these amoebae, large holes in the nuclear membranes were
Figs. 8–13. Parts of nuclear envelopes from cells prepared 5 h after damage.

Fig. 8. Part of the nuclear envelope of a control amoeba placed in normal medium for 5 h after damaging the nucleus. The nuclear membranes (m) of control amoebae are almost completely intact, indicating that repair occurred between 30 min and 5 h after manipulation. x 17000.

Figs. 9, 10. Nuclear envelope of an amoeba placed in KCN for 5 h after damage. Numerous holes (h) in the nuclear membranes persist. Some pieces of endoplasmic reticulum (er) remain in association with holes in the nuclear membranes, but they are not connected to the nuclear membranes (m). c, cytoplasm; n, nucleus. Fig. 9, x 31000; Fig. 10, x 37000.
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encountered so frequently that almost none of the nuclear profiles displayed intact nuclear membranes (Fig. 9). In addition, the interior of the nucleus was less dense than normal. Some pieces of endoplasmic reticulum continued to be associated with gaps in the nuclear membranes, but the endoplasmic reticulum had a distended appearance and was not connected to the nuclear membranes at the margins of the holes (Fig. 10).

The nuclei of amoebae exposed to NaF, actinomycin, or emetine for 5 h after damage lay between the 2 extremes represented by the nearly complete repair in control amoebae and the virtual absence of repair in cells exposed to an inhibitor of respiration. Although this fact can be appreciated adequately only through the study of numerous electron micrographs, examples of nuclei of amoebae treated with these inhibitors are illustrated in Figs. 11–13. In some nuclear profiles large stretches of nuclear membranes were intact, suggesting that repair had proceeded in these regions. In other locations, however, holes persisted. Thus in amoebae exposed for 5 h to actinomycin, emetine or fluoride, some repair of the nuclear membranes occurred, but the extent of the repair process was less than in the absence of the inhibitor.

DISCUSSION

The results indicate that the steps in repair of amoeba nuclear membranes differ in their metabolic requirements. The first step, the association of endoplasmic reticulum with the nuclear envelope, represented by the intrusion of pieces of endoplasmic reticulum into holes in the nuclear membranes, seems to be a passive process requiring neither energy, protein synthesis, or RNA synthesis, since it was not affected by any of the inhibitors. It is difficult to speculate on the mechanism of this association, but perhaps during the normal movement of organelles within the cell, portions of the endoplasmic reticulum contact the nuclear envelope. If this occurs at damaged regions the endoplasmic reticulum might then adhere to the edges of the nuclear membranes or to the exposed fibrous lamina.

In contrast, the next step in repair, the fusion of pieces of endoplasmic reticulum with nuclear membranes at the margins of the holes, appears to be an energy-requiring process. The results suggest that the necessary high-energy compounds are obtained through oxidative phosphorylation, because connexions between endoplasmic reticulum and nuclear membranes were greatly reduced in the presence of KCN or dinitrophenol. This inhibition of fusion between pieces of endoplasmic reticulum and nuclear membranes may account for the virtual absence of nuclei with intact nuclear

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Fig. 11. Nuclear envelope of an amoeba exposed to NaF for 5 h after damage. The nuclear membranes (m) are intact in a part of the field, but some holes (h) persist also. n, interior of nucleus. × 22000.

Fig. 12. Portion of a nucleus from a cell exposed to actinomycin D for 5 h after manipulation. The nuclear membranes (m) are intact in most of the field, but a hole (h) with a piece of endoplasmic reticulum (er) in it is also present. n, nucleus. × 23000.

Fig. 13. Part of the damaged nucleus of an amoeba placed in emetine (1 x 10^{-4} M) for 5 h. The nuclear membranes (m) in one region are intact, while a hole (h) is visible in a corner of the micrograph. n, nucleus. × 24000.
membranes in amoebae fixed after 5 h in one of the inhibitors of respiration. The results suggest that glycolysis is less important than respiration for nuclear envelope repair, since fusion of endoplasmic reticulum with nuclear membranes was not inhibited by fluoride in samples fixed 30 min after damage. However, since the nuclei of amoebae exposed to NaF for 5 h after damage did not achieve as high a level of repair as control amoebae, glycolysis may make a contribution to energy requirements for repair over a longer time span, or perhaps fluoride has some other effect.

RNA and protein synthesis were not required for the initial interactions between endoplasmic reticulum and nuclear membranes, since neither association of endoplasmic reticulum with the nuclear envelope nor fusion of endoplasmic reticulum with nuclear membranes was prevented by actinomycin or emetine. However, some RNA and protein synthesis appeared to be required ultimately for repair of the nuclear envelope, because in cells exposed to one of these inhibitors for 5 h the extent of restoration of intact nuclear membranes was less than in control amoebae. It is uncertain which steps in repair were inhibited, but since fusion of endoplasmic reticulum with the nuclear membranes was not blocked, subsequent stages in reconstruction of the nuclear envelope were apparently involved. These might include pore formation, removal of ribosomes from the membrane surface facing the interior of the nucleus, and flattening of the endoplasmic reticulum cisternae to the configuration of the perinuclear cisterna.

The finding that energy is required for fusion of pieces of endoplasmic reticulum with the nuclear membranes is in accord with previous observations and suggestions that membrane fission-fusion processes in general are energy-requiring steps (Jamieson & Palade, 1968, 1971). For example, the transport of secretory protein from the endoplasmic reticulum to the Golgi apparatus in the exocrine pancreas is an energy-requiring process that also relies on oxidative phosphorylation, and it has been suggested that the energy-requiring step is the production of small vesicles by budding from the endoplasmic reticulum (Jamieson & Palade, 1968). Similarly, discharge of zymogen granules requires respiration and it is thought that this too may be related to the processes of membrane fusion and fission (Jamieson & Palade, 1971). In these instances also concomitant protein synthesis was not found to be necessary for membrane fusion and fission, although protein synthesis is, of course, necessary for continuation of secretion in the long term.

Maruta & Goldstein (1975) have presented evidence that labelled nuclear envelope lipids (and possibly proteins as well) are dispersed to the cytoplasm during mitosis in amoebae but subsequently return in their entirety to the daughter nuclear envelopes formed in telophase. These observations imply that nuclear envelope components are distinctive and that the nuclear envelope is not simply formed from the general endoplasmic reticulum after mitosis. However, as these authors point out, their observations on conservation of nuclear envelope components do not exclude the possibility that endoplasmic reticulum membranes contribute to the formation of the nuclear envelope either during formation of new nuclear envelopes after mitosis (Porter & Machado, 1960; Porter, 1961; Feldherr, 1972) or in experimentally induced repair. Instead, the observations on the nuclear envelope described in the present study
and a previous one (Flickinger, 1974) suggest that, despite the special characteristics of the nuclear envelope components, pieces of membrane derived from the endoplasmic reticulum can be transferred from the cytoplasm to the margin of the nucleus and become incorporated into the nuclear membranes.

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REFERENCES


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