CELL DEATH IN THE EMBRYONIC CHICK SPINAL CORD

THOMAS M. O'CONNOR and CHARLES R. WYTTENBACH

From the Department of Physiology and Cell Biology, University of Kansas, Lawrence, Kansas 66044. Dr. O'Connor's present address is the Department of Biology, Washburn University, Topeka, Kansas 66621.

ABSTRACT

The events which occur in the death of visceromotor neurons of the cervical region of the chick embryo's spinal cord have been analyzed by electron microscopy. These normal degenerative events are compared with those in the lumbosacral cord where nerve cell death was induced by removal of peripheral organs. The initial set of degenerative changes include a decrease in nuclear size, the clumping of chromatin beneath the nuclear envelope, an increase in electron opacity of the cells, the disappearance of Golgi bodies, and the disaggregation of polysomes. These events are followed by the loss of the nuclear envelope and most of the endoplasmic reticulum, the appearance of bundles of filaments, and the formation of many ribosome crystals. Ribosome crystals are seen only in the dying cells. Their abundance may indicate a drastic reduction in RNA synthesis as one of the initial events which lead to the death of these neurons. The neurons are finally subdivided and engulfed by cells of the normal glial population, and further breakdown of the cell fragments occurs in large phagocytic vesicles of the gliocytes.

Death of cells plays a very prominent part in the embryonic development of vertebrate organisms (1–3). It occurs during the differentiation and sculpturing of various organs, and in the elimination of structures that are useful only during a limited phase of the embryonic period. The mechanism of cell death in embryonic systems has not for the most part been identified. Many studies have indicated that it is under genetic control (4), but how this genetic control is mediated on a cellular level is completely unknown.

During normal development, massive cell death occurs in the cervical motor column of the chick spinal cord between the 4th and 5th days of incubation (5). As a result of this degenerative process, at least two-thirds of the cell population in this region is reduced to cellular debris and pycnotic cells within a 24 h period. Careful analysis of this area using the Cajal silver staining method revealed that the dying cells are preganglionic neurons and part of an abortive visceral system (5), a conclusion supported by experiments involving transplantation of the cervical cord to the thoracic region (6). Previous work has indicated that the dying cells are removed by macrophages which invade the motor column and ingest the degenerating cells (5, 7). After the 6th day of incubation, however, macrophages could not be identified; therefore, it was presumed that they had migrated from the cord.

In the chick embryo, massive cell death does not normally occur in any other regions of the spinal cord. However, most of the motor neurons in the lateral motor column of the lumbosacral cord will die after extirpation of the ipsilateral leg bud of a 2½ day old embryo (8). Nerve processes are not severed in the course of the extirpation, since leg nerves do not reach the base of the leg bud until the 4th day; therefore, this cell death does not result from nerve cell damage. Light

microscope analysis of this induced massive cell death (8) indicated a sequence of events similar to that already described for the abortive visceral system in the cervical region. These experiments support the idea that developing neurons will die if they fail to achieve adequate connections with peripheral organs.

This study extends these earlier observations by analyzing the sequence of ultrastructural changes which occurs in the cervical neurons, where cell death is a normal ontogenetic event, and in the lumbosacral neurons, where cell death is a consequence of removal of the peripheral field. In both regions, the same degenerative sequence emerges. This sequence begins with nuclear changes which include clumping of the chromatin material beneath the nuclear envelope. These nuclear changes are followed by a release of ribosomes from polysomes and the creation of a pool of "free" ribosomes within the perikaryon. As the degenerative events proceed, most of the ribosomes become assembled into crystals. These observations indicate that a significant reduction in RNA synthesis may be one of the initial events which lead to the death of these cells. After undergoing considerable degradation, the neurons are subdivided and engulfed by cells of the normal glial population.

A preliminary account of this work has appeared in abstract form (9).

MATERIALS AND METHODS

Fertile eggs were incubated at 39°C for 4–6 days. Windows were then cut in the egg shells, the embryos excised from the yolk, and the extraembryonic membranes removed. The embryos were then transferred to a watch glass containing cacodylate-buffered Karnovsky's fixative (10) and staged (11). Embryos of stages 23–29 were used. The dorsal one-third of the cervical region was removed and placed in a fresh solution of Karnovsky's fixative. Using glass needles, the spinal cord was dissected out of this segment and placed in a third change of the fixative. Fixation was continued at room temperature for 2 h. The tissue was then washed for 1 h in cold 0.1 M cacodylate buffer and postfixed for 2h in 1.3% osmium tetroxide buffered with *s*-collidine at pH 7.4. After a 30 min wash in three changes of 10% sucrose, the tissue was stained *en bloc* in 2% aqueous uranyl acetate for 1 h. The cord segments were then dehydrated in graded ethanol solutions, cleared with propylene oxide, and embedded in Epon. Thick sections were cut with glass knives, stained for light microscope observation, and used for the purposes of orientation and trimming. Thin sections (gray-to-silver) were placed on uncoated copper grids, stained successively with aqueous uranyl acetate and lead citrate, and examined with a Philips EM 300 at 60 KV.

The above procedures were followed in the leg bud extirpation experiments except for the following modifications: Fertile eggs were incubated for approximately 57 h. Windows were then cut in the egg shells and the embryos staged within the eggs. Only stage 16 and 17 embryos (11) were used. The right leg bud was then removed after the procedure outlined by Hamburger (12). The windows were sealed, and the eggs placed back in the incubator for 3½ days. The embryos were then harvested, staged, verified for complete absence of the right leg, and the lumbosacral cord segments obtained and prepared for electron microscopy as outlined above. The embryos at the time of cord removal ranged in age from stage 29 to state 31.

RESULTS

Structure of Nondying Motor Neurons

Nondying motor neurons (top, Figs. 1 and 2) are large cells which have numerous processes extending from their cell bodies. Each contains an oval nucleus in which is found highly dispersed chromatin and one or two centrally positioned nucleoli. Within the perikaryon are numerous Golgi complexes, many of which appear to be in the process of budding off small vesicles which have diameters of approximately 450 Å. The endoplasmic reticulum (ER) contains wide cisternal spaces and is generally studded with ribosomes. Almost all of the ribosomes which are not bound to the ER appear in small clusters. These small free polysomes are characteristic of nerve cells (13).

Normal Cell Death Within the Cervical Motor Column

Alterations in nuclear structure provide the first indication that a particular neuron has started into a sequence of events which will ultimately lead to its death. Initial nuclear changes in the dying neuron include a condensation of chromatin beneath the nuclear envelope (bottom, Fig. 1) and a decrease in nuclear size. The karyoplasm is more dense than that of the nondying neuron, and small electron-opaque masses are scattered throughout the nucleus. The nucleoli of the dying cells remain unchanged in appearance during these early degenerative changes.

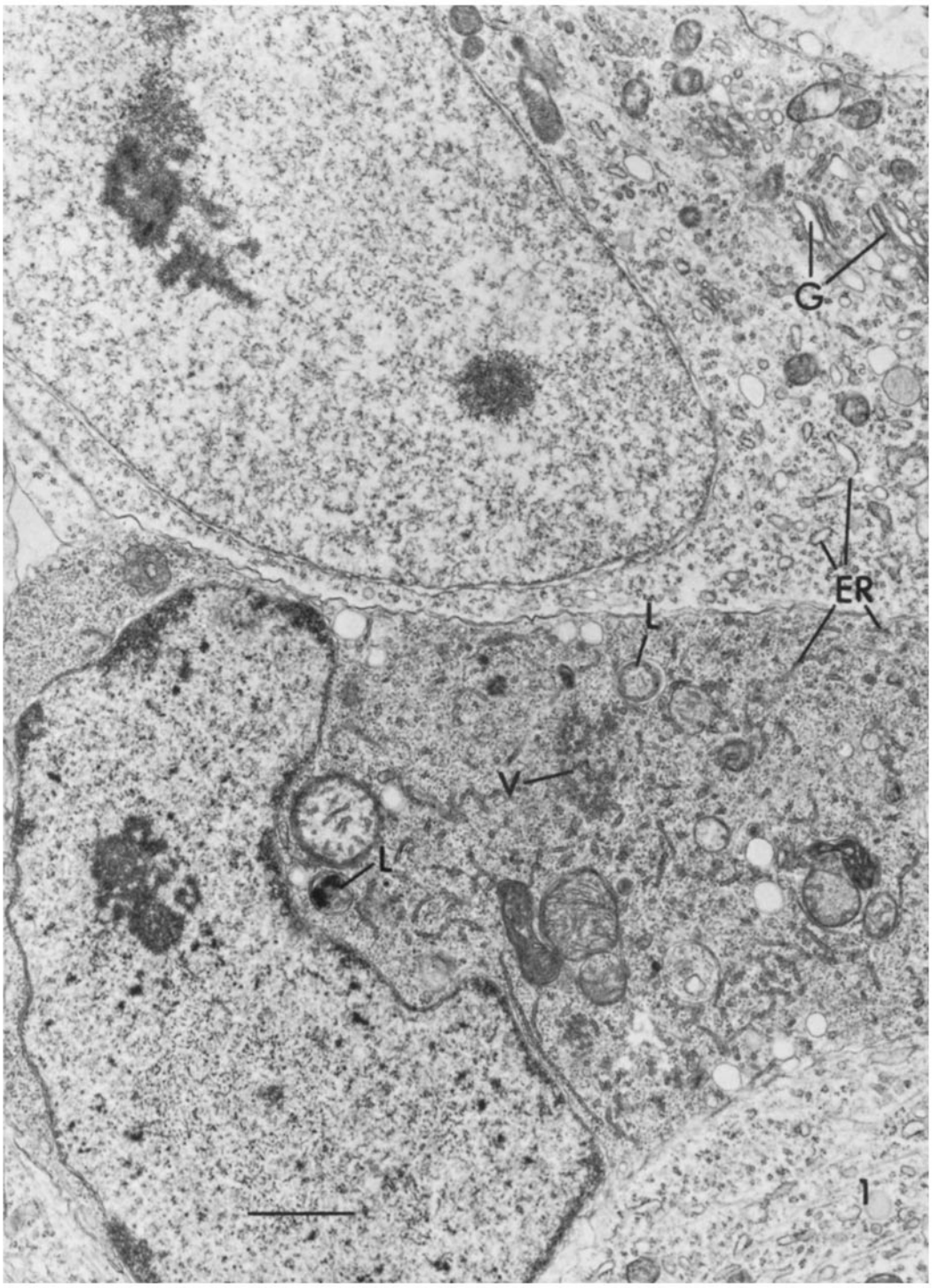


FIGURE 1 Section of the cervical motor column of a stage 24 chick embryo showing the cell body regions of a nondying neuron (top) and a neuron in which initial degenerative changes have occurred (bottom). *G*, Golgi complex; *ER*, endoplasmic reticulum; *V*, cluster of small vesicles; *L*, lysosomes. Scale marker, 1 μm. × 15,900.

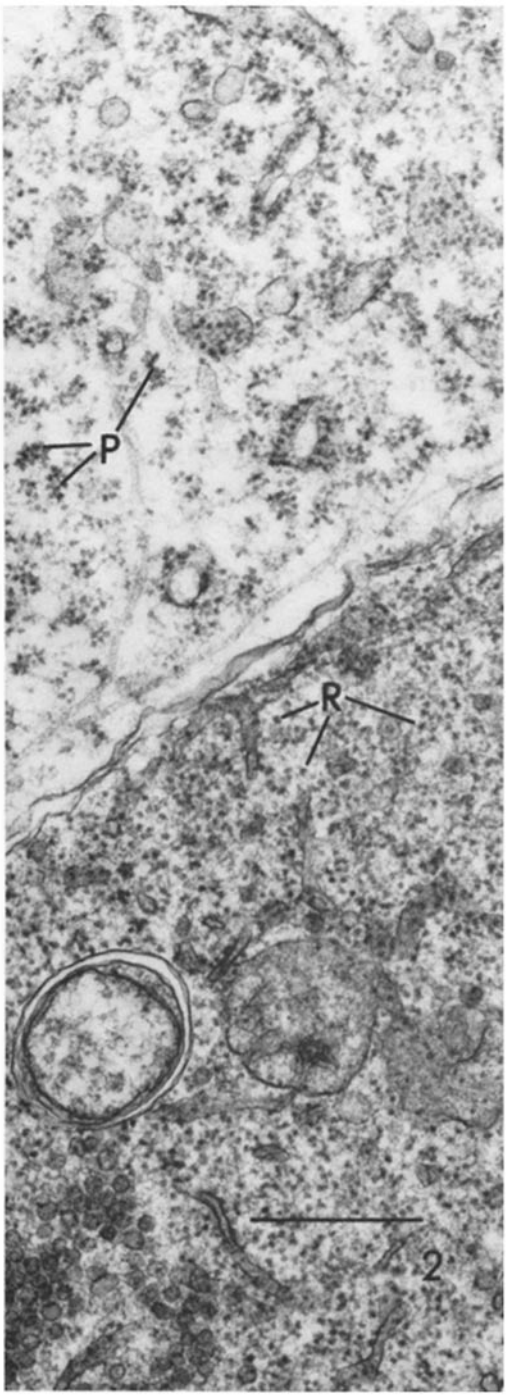


FIGURE 2 Enlargement of the neurons seen in Fig. 1 showing the polysomes (*P*) in the perikaryon of the non-dying neuron and the free ribosomes (*R*) in the dying neuron. Scale marker, 0.5 μm. × 46,000.

As the degenerative process continues, the nucleus becomes quite irregular in shape (Fig. 3). By this time a distinct nucleolus usually cannot be seen. In addition, the chromatin which has accumulated beneath the nuclear envelope is found compacted into large, dense, usually spherical or oval-shaped bodies which are located in pockets at the periphery of the nucleus.

The initial degenerative nuclear events are followed by characteristic cytoplasmic changes. The Golgi complexes disappear, but the perikaryon continues to possess for some time rather large clusters of 450-Å diameter vesicles (Figs. 1 and 3). The ER assumes a fragmented appearance with its cisternal spaces reduced in width to about one-third those of the cisternae of nondying cells, and most of the ribosomes now appear as free and separate elements instead of in polysome complexes or bound to ER (Figs. 1, 2, and 3).

The nuclear envelope then breaks down, and the karyoplasm, with its large dense chromatin bodies and its smaller dense masses, becomes contiguous with the perikaryon. In such cells, most of the ribosomes are seen in the form of ribosome crystals (Fig. 4). These crystals are identical with those found in chick embryos after hypothermic treatment (14, 15). Fig. 5 shows one of these crystals in surface view. The crystal is made up of basic units of four ribosomes in a square tetramer. These tetramers are bonded together into a net which is described crystallographically as a member of plane group p4 (14). Although single sheetlike crystals of this nature are numerous, frequently a number of these sheets are arranged into three-dimensional structures with a p422 configuration (15). Fig. 6 shows one of these three-dimensional crystals in edge view. This crystal is made up of a stack of six of the p4 sheets. Note that solid-line views alternate with dotted-line views—a pattern which reflects the alternation in polarity of the stacked sheets and their specific orientation relative to one another (15). Ribosome crystals were never seen in any cells other than the degenerating nerve cells.

Another characteristic feature of dying neurons is the appearance of bundles of parallel 70-Å diameter filaments within the perikaryon (Fig. 4). These bundles are first seen in cells where the the nuclear envelope is beginning to break down. Occasionally numerous bundles are seen within one cell, and in such cases they are arranged in different directions with no apparent orientation

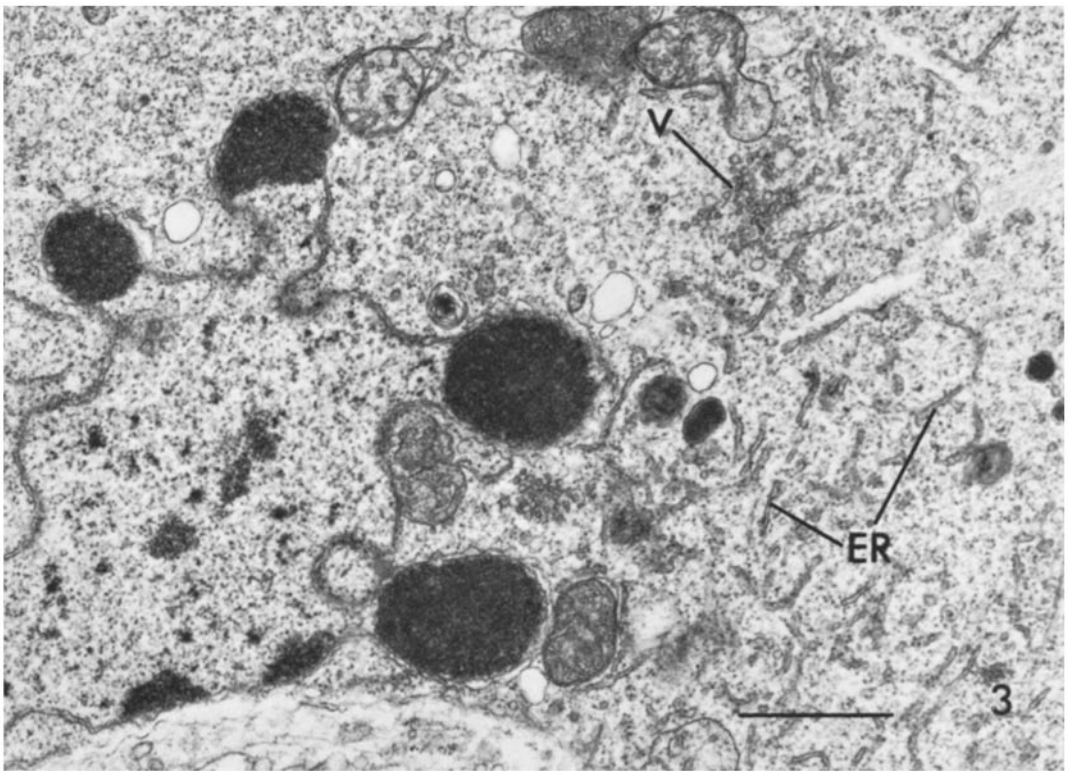


FIGURE 3 Section of a motor neuron in which degenerative changes have progressed further than in the dying neuron of Fig. 1. The nucleus is now quite irregular and contains large, oval masses of condensed chromatin. Most of the ribosomes appear as free and separate elements. *ER*, endoplasmic reticulum; *V*, cluster of small vesicles. Scale marker, 1 μm. × 20,400.

relative to each other. The origin and significance of these bundles remain unknown.

The mitochondria of the dying cells also show certain degenerative changes (Figs. 1, 4, and 7). These usually include a decrease in the volume of the matrix with an increase in its electron opacity. Mitochondria often appear swollen, and in more advanced stages of cellular necrosis they tend to aggregate (Fig. 7). Within such clusters two mitochondria are occasionally seen sharing a common membrane.

Fate of Degenerating Cervical Motor Neurons

Gliocytes are scattered throughout the cervical motor column during the period of massive cell death, stages 23-25. Most of these glial cells are cytologically similar to adult mammalian astrocytes (13). Occasionally cells that can be identified as oligodendrocytes are also seen, but these do not become numerous until later developmental stages.

The nuclei of the astrocytes are smaller than the nuclei of neurons, tend to be somewhat irregular in shape, and have some chromatin condensation on the inner side of their nuclear envelope (Fig. 9). Within the perikaryon are found randomly dispersed mitochondria and elements of the ER which are unevenly studded with ribosomes. Most of the ribosomes not found to the ER occur in polysome clusters. These aggregates are numerous and fill the perikaryon. Golgi complexes are generally seen within the cell body region as are membrane-bounded vesicles of various sizes.

The processes which extend from the astrocyte's cell body have a rather watery appearance (Figs. 4, 7, and 8). These processes generally lack ER, ribosomes, and microtubules. They usually contain mitochondria, electron-lucent vacuoles, and numerous filaments. The filaments have diameters of approximately 70 Å are and usually oriented parallel to the long axis of the process (Fig. 8).

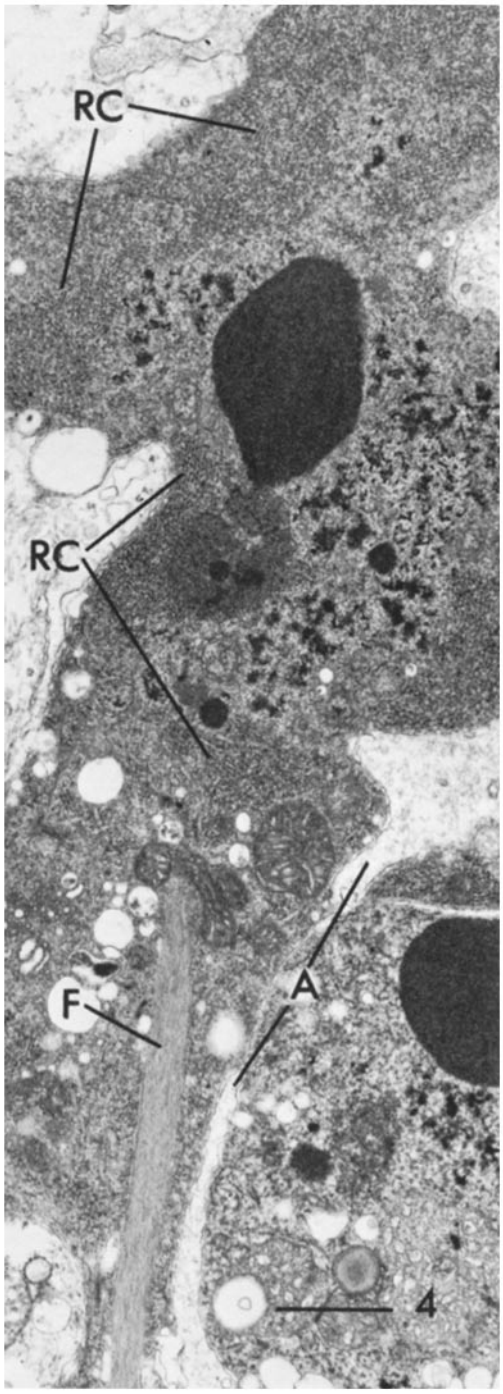


FIGURE 4 The nuclear envelope has broken down in both of these dying neurons, and the perikaryon of one of the cells is filled with ribosome crystals (*RC*). *F*, bundle of filaments; *A*, processes of astrocytes. Scale marker, 1 μm. × 15,600.

The degenerating cervical neurons are subdivided, ingested, and thus removed from the motor column by the normal astrocyte population. By the time the degradative events have progressed to the point of disappearance of the nuclear envelope, the immediate environment of the dying neuron's cell body usually contains a number of astrocytic processes (Fig. 4). These processes can often be seen penetrating deeply into the degenerating neurons (Fig. 7), and eventually segments of the dying cells become completely surrounded by these invasive astrocytic extensions.

Several isolated fragments of degenerating neurons are shown in Fig. 8. Each fragment is either partially or completely engulfed by the cytoplasm of an astrocyte. Extending diagonally across the figure is a gliocyte process containing two large phagocytic vesicles; the smaller one contains material in an advanced state of degradation, the larger contains a portion of a dying neuron which appears to have just recently been ingested. The obvious disparity in the extent to which degenerative changes have occurred in two fragments suggests that a given astrocyte may ingest at different times parts of more than one neuron. Support for this suggestion is provided by Fig. 9. Within the perikaryon of this astrocyte are numerous large phagocytic vesicles containing material showing different degrees of degradation. The large chromatin masses seen in several of these vesicles leads to the conclusion that some of the degenerating material is from different cells.

Induced Cell Death Within the Lumbosacral Cord

Dying neurons in the lateral motor column of the lumbosacral cord show the same sequence of degenerative changes as observed within the cervical region. Fig. 10 shows the cell body of a dying neuron of the lateral motor column on the side of the embryo from which the leg bud had been removed 3½ days earlier. The nuclear envelope is incomplete, and the karyoplasm contains the large chromatin bodies and the small electron-opaque masses as described in dying neurons of the cervical region. Only a few elements of the ER are seen, and no Golgi complexes are found. The most striking structures seen in the perikaryon are the numerous ribosome crystals. Both surface and edge views are seen,

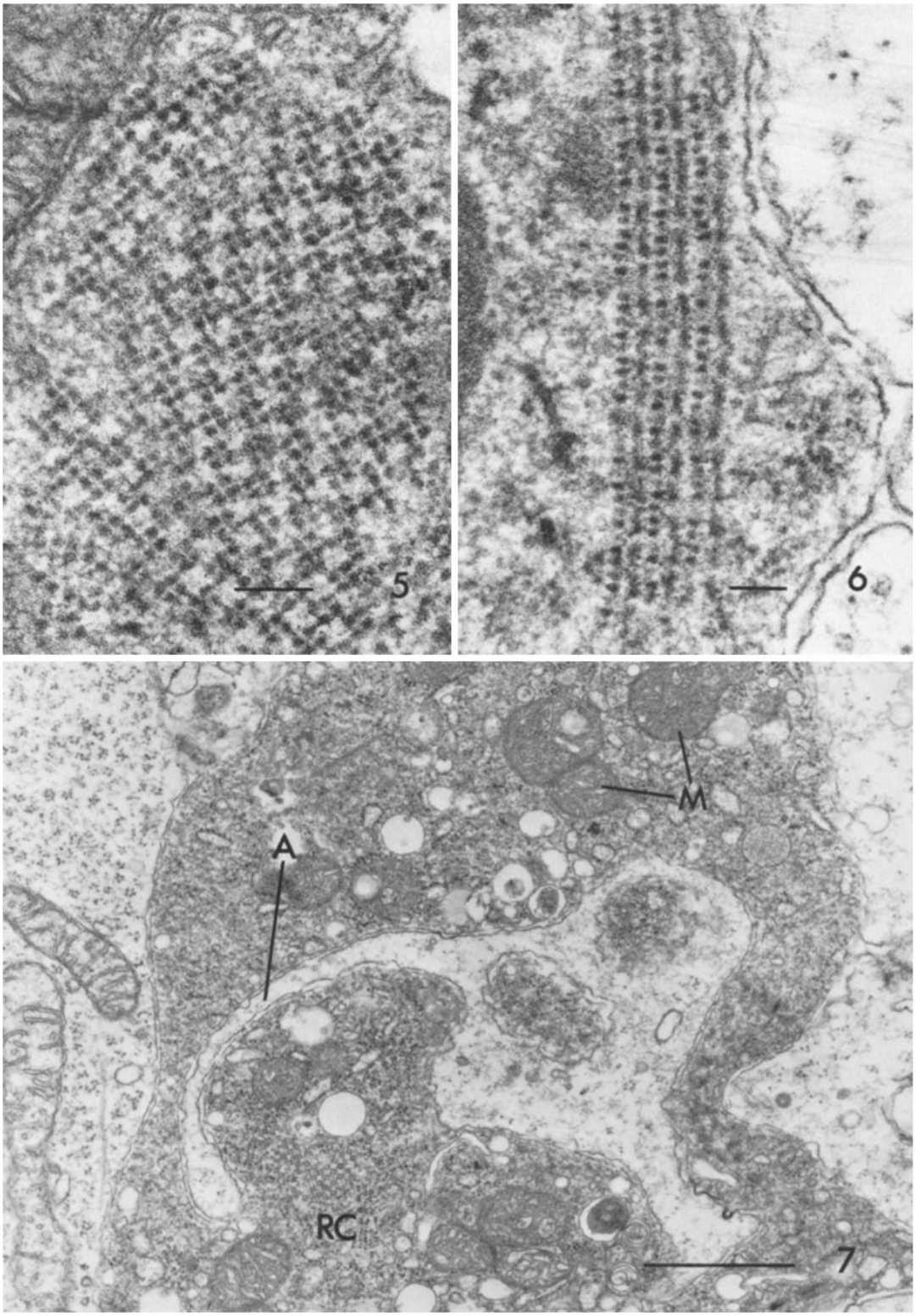
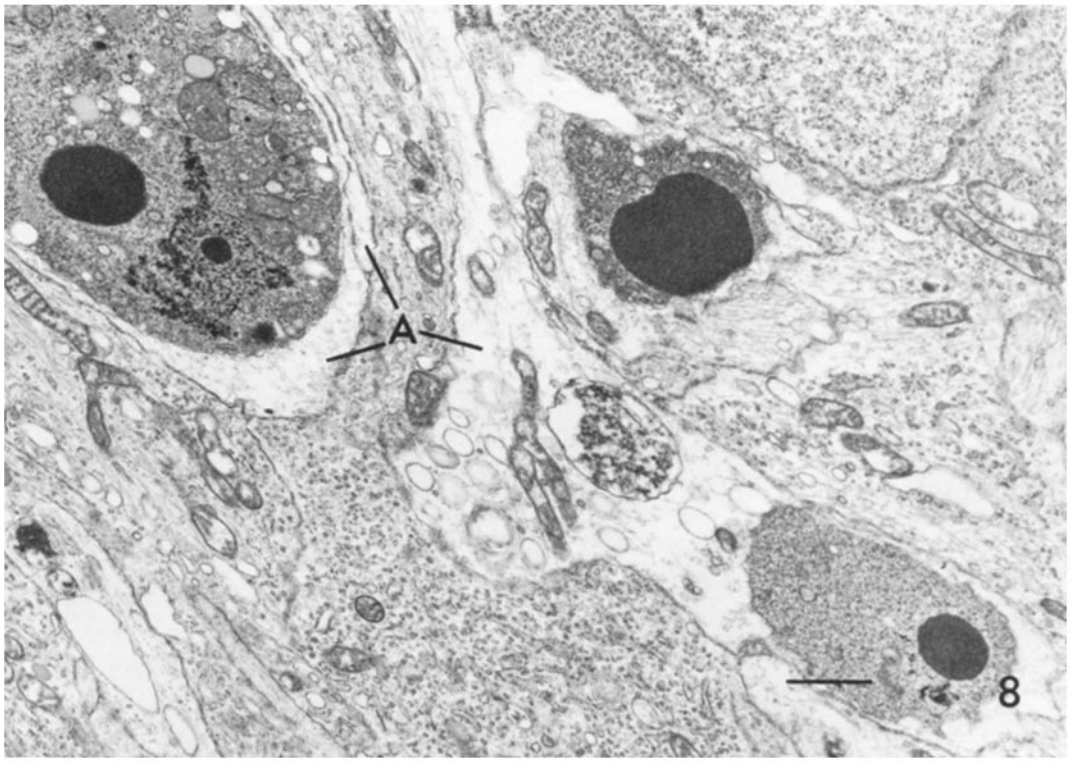


FIGURE 5 A ribosome crystal in surface view. Scale marker, 0.1 μm. × 124,700.

FIGURE 6 Edge view of a ribosome crystal composed of a stack of six crystalline sheets. Scale marker, 0.1 μm. × 91,400.

FIGURE 7 Section showing an astrocyte process (*A*) penetrating deeply into a degenerating neuron. Part of the perikaryon of a nondying neuron is seen to the left of the dying cell. *RC*, ribosome crystals; *M*, mitochondria. Scale marker, 1 μm. × 23,700.



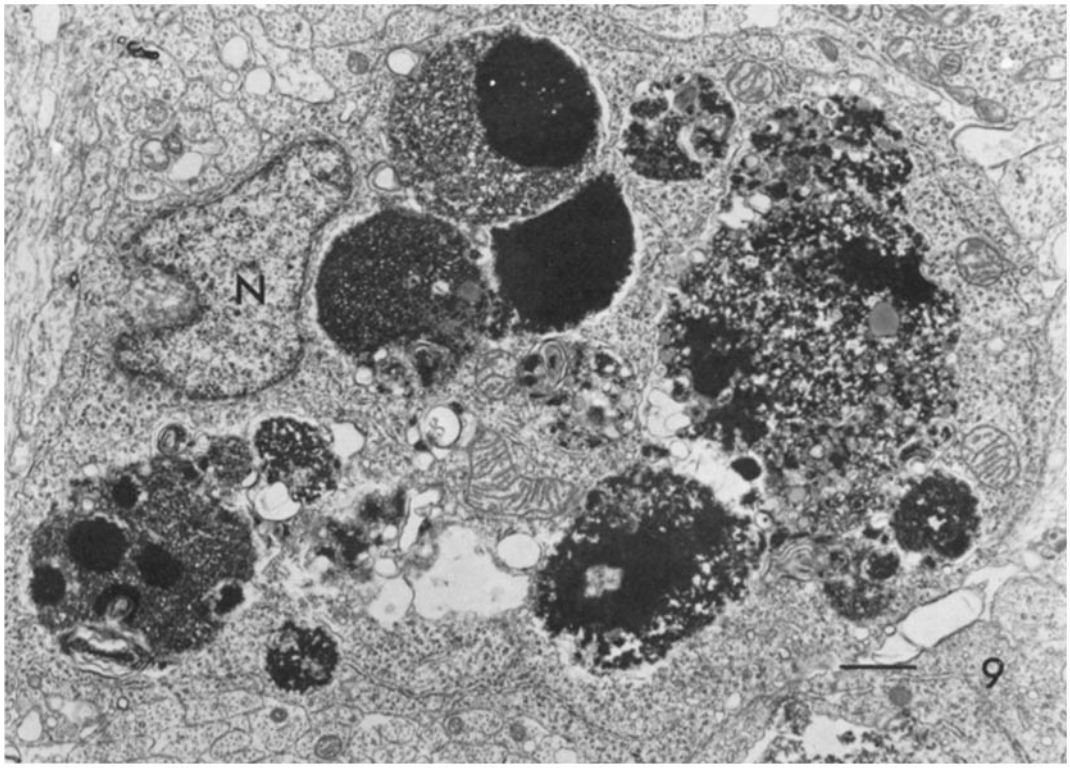


FIGURE 8 Section showing fragments of degenerating neurons which are either partially or completely engulfed by the processes of astrocytes (*A*). Scale marker, 1 μm. × 11,200.

FIGURE 9 Section passing through the nuclear region of an astrocyte positioned at the lateral edge of the cervical motor column. Numerous large phagocytic vesicles containing cellular debris in varying degrees of degradation are seen. *N*, nucleus. Scale marker, 1 μm. × 10,200.

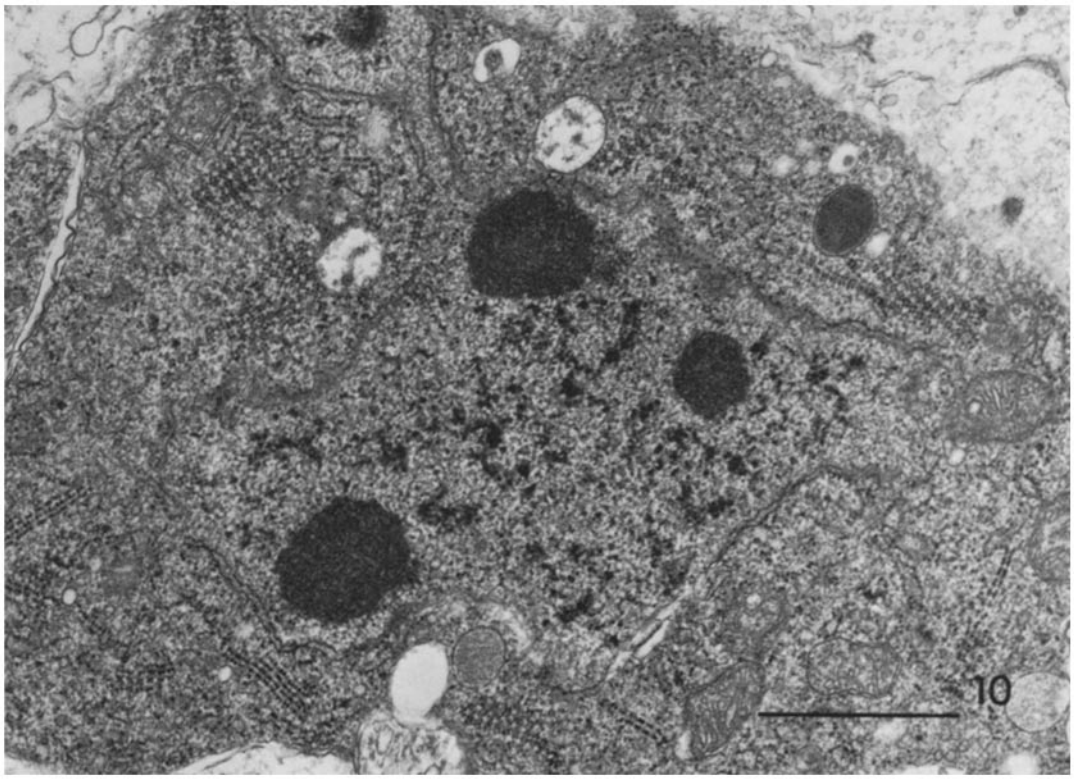


FIGURE 10 Cell body region of a dying neuron in the lateral motor column of the lumbosacral cord. Numerous ribosome crystals are seen within the perikaryon. Scale marker, 1 μm. × 26,800.

and the edge views show that the ribosome sheets are in stacks. These crystals of ribosomes, which in the lumbosacral cord are found only in the dying cells, are identical with those described in degenerating neurons of the cervical region.

Two distinct types of gliocytes are found, generally in equal numbers, within the lateral motor column. One is identical with the astrocyte already described in the cervical region. The other type is very electron opaque and has a small nucleus and prominent Golgi complexes. This cell conforms to the general description for oligodendrocytes presented by Peters et al. (13). This second gliocyte type is only rarely seen in the cervical region during the period of massive cell death, but is seen much more frequently at later developmental stages. It should be re-emphasized that massive degenerative events in the cervical region occur around 4½ days of incubation, whereas the induced cell death within the lumbosacral cord occurs in embryos which have been incubated for approximately 6½ days. Therefore the oligodendrocytes are not unique to the lumbosacral region, they are just not as numerous at 4½ days as they are at 6½ days. In the lumbosacral region, both gliocyte types are involved in the subdivision and ingestion of dying neurons.

DISCUSSION

Numerous experiments have shown that the life of spinal motor neurons appears to be unaffected by the peripheral field during the early stages of neuron differentiation (16). Proliferation of neuroepithelial cells and the initial differentiation of neurons occurs normally in the absence of the peripheral organs. However, contact between the axon and the peripheral tissues is essential for final maturation and maintenance of the neuron. Levi-Montalcini (5) suggested that cell death in the motor column of the cervical cord is a result of the failure of preganglionic neurons to establish synaptic contact with sympathetic ganglia, and this suggestion was later confirmed by transferring segments of the cervical cord to the thoracic region (6). In this new location not all cells in the trans-

planted segment which normally undergo degeneration were spared this fate, but the axons of those which did survive were traced to the adjacent paravertebral sympathetic ganglia.

If contact between the axon and the peripheral tissue is essential for the maintenance of a differentiating neuron, then experimental removal of peripheral organs should result in death of the nerve cells which would have innervated them at a later time. Hamburger (8) demonstrated that removal of a hindlimb bud results in extensive degeneration of motor neurons in the ipsilateral lumbosacral region of the embryonic chick's spinal cord. That study and others (5, 6, and 7) have indicated that in both the normal cell death of the cervical region and the induced cell death of the lumbosacral cord no cells are genetically programmed for death as has been proposed for other embryonic regions (4). Instead, cell death is contingent upon peripheral factors, and differentiating neurons that fail to meet specific functional requirements die.

In this study one of the unique features observed in both normal cervical cell death and induced lumbosacral cell death is the appearance of ribosome cyrstals within the perikaryon of the dying cells. Since these crystals are seen only in the dying neurons, their presence may indicate something about the causes of cell degeneration. Ribosome crystals were first identified and described in detail by Byers (14, 15) in chick embryo cells after hypothermic treatment of the embryo. Gradual cooling, but not rapid cooling, of embryos yielded crystals in interphase cells; therefore, he concluded that crystallization within interphase cells must be preceded by the release of ribosomes from polysomes during slow cooling. Free ribosomes occur naturally, however, in mitotic cells and form stacked sheets under rapid cooling. Morimoto et al. (17) analyzed the tetramer subunits which they isolated from cells of hypothermic chick embryos and found them to consist of mature 80S ribosomes which contain all species of ribosomal RNA and a complete set of ribosomal proteins. The tetramers were shown to lack nascent polypeptide chains and endogenous messenger RNA (mRNA) activity; thus, they are not a special type of polysome. Subsequently, Morimoto et al. (18) showed that slow cooling of chick embryos permits the elongation and termination of nascent polypeptides in polysomes but prevents the initiation of new protein chains. This leads to polysome disaggregation and to the formation of a pool of inactive ribosomes that are able to crystallize. In summary, their results show that tetramers are formed from ribosomes not "programmed" for protein synthesis, thus indicating that crystallization is one means of storing unprogrammed but functionally viable ribosomes in hypothermic eggs.

While most of the ribosomes of nondying neurons are seen in polysome clusters, most of the ribosomes within dying neurons appear as free and separate elements early in the degeneration process. Morimoto et al. (18) have indicated that tetramer formation requires the release of ribosomes from polysomes, and, as is shown here, this prerequisite is seemingly fulfilled by the dying nerve cells.

What could account for the apparent release of ribosomes from polysomes within the dying neurons? One possibility is a termination of some degree of the transcription within the nucleus. It has been demonstrated (19) that dense chromatin is not active in RNA synthesis. As one of their first degenerative signs, nerve cells show a condensation of chromatin beneath the nuclear envelope, which could reflect transcriptional termination.

After the degenerative stage in which the ribosomes appear as free and separate elements, most of them are aggregated into ribosome cystals. In these nerve cells exposed to a constant incubation temperature of 39°C, one would not expect such extensive crystallization to occur if the cytoplasm contained "normal" amounts of translatable RNA. Byers (14, 15) demonstrated that hypothermically induced ribosome crystallization is reversible; thus when treated cells are returned to normal incubation temperatures, temperatures to which the cells in our system were constantly exposed, the crystals disappear and the ribosome population resumes the translation of available RNA. Therefore, the nuclear changes and extensive ribosome crystallization seen in the degenerating neurons may indicate a drastic decrease in mRNA production.

Although numerous studies of cell death in embryonic systems have been made (1, 2), only a few have been conducted at the ultrastructural level. In some of these (3, 4, and 20) ribosome crystals were not seen, while in others they were (21–23). Bellairs (21) observed "bands of cytoplasmic granules" in dying cells of primitive streak stage embryos. Dawd and Hinchliffe (22) saw "banded granules" in degenerating cells of the opaque patch of the developing limb, and Mottet and Hammar (23) described ribosome crystals in the

dying cells of the posterior necrotic zone of the limb. In none of these three studies was an attempt made to correlate the ribosome crystals with a sequence of degenerative changes, and so the pathogenesis and significance of their presence remained unknown.

Within the dying neurons investigated in this study, the sequence of morphological changes, beginning with certain nuclear alterations and followed by polysome disaggregation and ribosome crystallization, leads us to the conclusion that cell death is caused by nuclear inactivation. But just what might trigger the nuclear changes remains unknown. Because contacts between the terminals of axons and peripheral organs are essential for neuron maturation and maintenance, Jacobson (16) suggests that materials might be transferred from the peripheral tissues to the tips of the axons, and a message then transmitted up the axon to the neuron cell body, resulting in growth and maturation of the neuron. It is also possible that as a neuron differentiates a critical period is reached; if functional activity is not achieved at that time, then a negative feedback of some cytoplasmic substance may cause nuclear inactivation. Other possibilities exist as well. However, the observations reported here do not allow us to choose among them.

Ever since their discovery and characterization by de Duve (24), lysosomes have been mentioned as organelles intimately involved in cellular degeneration. Although the role they play in the neuron degeneration described here has not yet been established, they do not appear to be the cause of death. Golgi bodies, the structures generally associated with primary lysosome production, are numerous and appear quite active in nondying neurons. Vesicles with diameters of approximately 450 Å can generally be seen either budding off from Golgi bodies or in the immediate environment of these organelles. The Golgi complexes are among the first cytoplasmic organelles to disappear as the neuron begins to degenerate, but the cytoplasm continues to possess for some time rather large clusters of the above-mentioned vesicles. These may be primary lysosomes. However, they have the same size and appearance as synaptic vesicles. If synaptic vesicles are derived from Golgi bodies and move to the axon terminals by axoplasmic flow, and if axoplasmic flow ceases as one of the early degenerative events, then these clusters may be synaptic vesicles stranded in the perikaryon of the dying neuron.

The gliocyte processes which subdivide and engulf the dying neurons generally lack structures which might be considered lysosomes. Therefore, it is proposed that the release of hydrolytic enzymes from the neuron's lysosomes mediate the initial degradation which occurs within the phagocytic vesicles of the gliocyte. Once formed, the digestive vacuoles are apparently moved into the cell body region of the gliocyte, possibly by withdrawal of cell processes, as digestion continues. Within the perikaryon of the gliocyte are found numerous Golgi complexes and lysosome-like structures. These may be involved in the final breakdown of the neuronal remnants.

In most studies concerned with the fate of degenerating cells in embryonic systems, it has been found that the cells are either engulfed by macrophages or ingested by mesenchymal cells which later become macrophages (2, 3, 5, 8, 22, and 25). Levi-Montalcini (5) stressed that macrophages are present in large numbers within the motor column of the cervical spinal cord during the period of neuronal degeneration. Their identification as macrophages was based on light microscope examination and on the uptake of trypan blue. She proposed that the macrophages engulf the remnants of the dead cells and then migrate from the area. Hamburger (8) also reported the presence of macrophages among the degenerating neurons of the lumbosacral cord after leg bud removal. Contrary to these earlier reports, in the present study no "macrophages" are seen in the cervical or lumbosacral regions of the developing spinal cord. Found among both nondying and dying neurons are cells which conform to the general descriptions for gliocytes (13), and some of these gliocytes contain ingested debris. Based on ultrastructural identification, the present study clearly indicates that it is the normal gliocyte population that is involved in the ingestion and removal of dead neurons in the motor column of these two regions. In the cervical region, glial cells identified as astrocytes subdivide and engulf fragments of necrotic neurons, while in the lumbosacral region both astrocytes and oligodendrocytes engulf degeneration products.

In summary, this study of neuron degeneration in an embryonic system has made it possible to propose a sequence of events which occurs during cell death and to account for the removal of cellular debris. By extending these studies, it should be possible to gain even more insight into the mechanisms of cell death in general.

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