THE NISSL SUBSTANCE OF LIVING AND FIXED SPINAL GANGLION CELLS

II. An Ultraviolet Absorption Study

By ARLINE D. DEITCH,* Ph.D., and MONTROSE J. MOSES,‡ Ph.D.

(From the Department of Surgery, College of Physicians and Surgeons, Columbia University, and The Rockefeller Institute for Medical Research)

Plates 139 and 140

(Received for publication, December 7, 1956)

INTRODUCTION

Ever since Nissl bodies were originally described in fixed nerve cells, there has persisted a controversy over their real or artifactual character. In a recent issue of this Journal, a phase contrast study of developing neurons grown *in vitro* was reported which bore directly on this question. In that study comparisons were made among images of the same neurons[1] in the living condition, after fixation, and subsequently after staining. In the cytoplasm of the unstained neurons, homogeneous areas were found which were clearly distinguishable from the surrounding cytoplasm with phase contrast. These corresponded in size, shape, and location to the Nissl bodies of the same cells seen after staining with basic dyes. Such evidence offers strong support to the concept that particulate Nissl bodies preexist in the living state and are not substantially changed by good cytological fixation.

There exists one recent report, however, that appears to contradict these observations. Koenig (4) has stated that discrete Nissl bodies are not resolved in ultraviolet images of living chick embryo neurons grown *in vitro*. Fixation by formaldehyde or ethanol resulted in the formation of aggregates which absorbed more ultraviolet light than the surrounding cytoplasm. Koenig concluded from this evidence that the Nissl pattern of the neuron is an artifact resulting from the fixation process.

The discrepancies between our observations using phase contrast and Koenig's findings with ultraviolet light have led us to reexamine this problem

* Research carried out while this author held a postdoctoral fellowship from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, United States Public Health Service.

‡ This author's contribution to the study was supported by a special purpose grant (INSP-85a) from the American Cancer Society, Inc.

[1] Cultured from chick embryo spinal ganglia.

of the nature and appearance of the ultraviolet-absorbing substances in the neuronal cytoplasm.

Materials and Methods

Treatment of Cultures.—Spinal ganglia explanted from the lumbar region of 9-day chick embryos were grown *in vitro* on quartz coverglasses in a new culture vessel (2) according to the procedures described by Deitch and Murray (3). The coverglass bearing the culture to be studied was sealed by vaseline to a quartz slide. The time allowed for microscopic examination of a culture never exceeded 2 hours. Following ultraviolet photomicrography of living cells, cultures were fixed with neutral 10 per cent buffered formalin (4 per cent formaldehyde) and the same cells were again photographed in the ultraviolet. The cultures were then stained with 5 mg./cc. azure A at pH 3.5 followed by differentiation overnight in tertiary butyl alcohol, or with cresylecht violet as described by Powers and Clark (8), and were rephotographed with visible light. Ribonucleic acid was removed enzymatically by 0.1 mg./cc. ribonuclease (Worthington Biochemical Company) at pH 6.2 at 37°C.; the cultures were again photographed in the ultraviolet and subsequently restained by the cresylecht violet or azure A procedures. Following this last treatment the basic dye was removed with acid alcohol and the culture restained with 0.1 mg./cc. acid fuchsin in 1 per cent acetic acid.

Ultraviolet Photomicrography.—A critical problem in ultraviolet studies of living cells is the possibility of radiation-induced change. The number of ultraviolet photographs that can be taken of a healthy living cell is limited by the radiation dosage that the cell will tolerate. However, there is no rigid criterion of radiation damage, and in these experiments where a particulate Nissl structure in the living cell was being examined, absence of visible changes in morphology or absorption patterns of the cell under study was taken as an index of "lack of injury." Undoubtedly, changes may have been induced by irradiation, but these were at a level which did not lead to the alteration of structure in the images studied. As noted by Walker and Davies (10) among others, the obvious changes in cultured cells become evident only with time. Thus, over a short period of time it is possible to collect a quantity of absorption information from "unchanged" cells, the amount depending on film speed, wave length, and speed of operation.

The color-translating ultraviolet microscope (UV-91)[2] incorporates a number of automatic facilities permitting unusually rapid and controlled photographic operation. It was used in this study primarily as a fast ultraviolet photomicrographic apparatus; the color-translating faculty was not utilized. The resolution permitted by the instrument (measured to be *ca.* 0.25 μ at 265 mμ) was limited by instrument vibration that could not be entirely eliminated, by inherent characteristics of reflecting optics, and by the coarse photographic grain resulting from rapid film processing at high temperature. This resolution was, however, sufficient to resolve the structures under study. Sets of three photographs were made at (*a*) 265 mμ near the absorption maximum of the purine and pyrimidine bases of nucleic acids and related compounds, (*b*) 287 mμ, near the peak absorption of tyrosine and tryptophane and of proteins containing these heterocyclic amino acids (there is also appreciable absorption by purines and pyrimidines at this wave length), and (*c*) 310 mμ where image formation results chiefly from non-specific light losses. The monochromator was set to deliver energy at a calculated band width (half-height) of 5.3 mμ from a high-pressure Hg arc (AH-6). No cell accumulated more than 45 seconds exposure at 265 mμ, or a total ultraviolet exposure of 49 sec. Exposures

[2] The UV-91 was manufactured by the Scientific Specialties Corporation, Boston, Massachusetts, and purchased by the American Cancer Society, Inc. for The Rockefeller Institute. It was patterned after the instrument developed by the Polaroid Corporation, Boston, Massachusetts (5, 9).

were made on Eastman Kodak spectrum analysis #1 film, developed in D-8 at 90° C. for 7 seconds. Total processing time was 25 sec. The total elapsed time from initiation of the first exposure to viewing of the finished negative was at most 40 seconds. Because of this speed, it was possible to first make a through-focus series of photographs at 265 mμ to find precise focus. A rhodium step wedge was used to check film characteristics, and exposure and processing were controlled to utilize the linear portion of the H. and D. curve. Thus, film densities reflect concentration of absorbing material. The optics used were a Bausch and Lomb 53 X, N.A. 0.7 reflecting objective and condenser, (Gray design) and either a 3.5 X or 6 X quartz ocular; magnification at the film was either 186 X or 318 X.

OBSERVATIONS

The Nissl Pattern of the Neurons.—The spinal ganglion cultures employed in this study were grown 19 to 25 days *in vitro*. When these neurons are examined after formalin fixation and basic staining, the Nissl pattern indicates that they are at an intermediate stage of differentiation, corresponding to stages 2 and 3 of Peterson and Murray (7). In all the neurons studied the most prominent cytoplasmic basophilia consists of a broad dense band lying near the cell periphery (Fig. 10, *NS*). This peripheral basophilic ring is somewhat granular and shows local variations in density. Additional smaller masses of Nissl substance are usually to be found lying in the more central cytoplasm. Frequently, small rounded basophilic masses are found arrayed against the nuclear membrane forming a nuclear cap (Fig. 10, arrows). In some cells, however, no evidence of these smaller Nissl bodies can be found and the only cytoplasmic basophil material present consists of the broad peripheral ring (Fig. 12, *NS*). In such cells, the nucleus has a somewhat eccentric location.

Ultraviolet Absorption of Living Neurons.—It is apparent from the ultraviolet photographs that there is abundant structure in the cytoplasm of all the living neurons studied. In living neurons photographed at 265 mμ (Figs. 1, 7, and 8) the most prominent ultraviolet-absorbing area is a dense peripheral band in the cytoplasm. In addition smaller absorbing masses (Figs. 1 and 8, arrows) can frequently be distinguished in the central cytoplasm. The nucleolus also absorbs heavily at this wave length and appears as one of the densest structures in the cell (Fig. 1 corresponding to *nl* of Fig. 2; Figs. 7 and 8). Several nuclei of satellite cells can usually be seen surrounding the neuronal cytoplasm or soma (Fig. 1 corresponding to *Sn* of Fig. 2). Because of its relatively weak absorption the satellite cytoplasm is obscure and the cell margin is difficult to distinguish.

The morphology of the living neuronal cell seen at 287 mμ (Fig. 3) is precisely that at 265 mμ, while, as is to be expected, the relative density of the absorbing regions at 287 mμ is less. In contrast, the photographs of living neurons taken at 310 mμ are almost structureless (Fig. 5), revealing only a small amount of non-specific light loss or "scatter" at the extreme periphery of the somata.

In subjecting living cells to a series of up to 12 exposures at 265 mμ, no

differences between the first and last photographs could be detected that were attributable to cell damage. (However, no attempts were made to determine how much irradiation and time were necessary before injury-induced changes could be seen). Thus, in successive photographs at constant focal level and wave length, the first exposure at 265 mμ produces an image showing the characteristic arrangement and relative densities of absorbing materials discussed above (Fig. 7), and this remains unchanged in subsequent pictures.

The Effect of Fixation.—After fixation of the cells in neutral 10 per cent formalin, the absorption pattern is essentially unaltered from the living state. Photographs of the fixed neurons taken at 265 mμ (Figs. 2 and 9) and at 287 mμ (Fig. 4) are almost indistinguishable from those of the same cells in the living state (compare with Figs. 1, 8, and 3). No increase, and possibly even a decrease, in non-specific light loss following fixation is indicated in photographs taken at 310 mμ (Compare Fig. 6 with Fig. 5).

Identification of Absorbing Components.—Comparison of the ultraviolet photographs with the images of the same neurons stained with basic dyes reveals excellent correspondence between the ultraviolet images and the Nissl pattern of these cells. The prominent cytoplasmic ultraviolet-absorbing areas (Figs. 8, 9, and 11, *NS* and arrows) can be readily identified as being the areas of maximum cytoplasmic basophilia (Figs. 10 and 12, *NS* and arrows). It should be emphasized that this holds true as well for the living cell (compare Fig. 8, living, UV, with Fig. 10, fixed, basic stain) as it does for the fixed (compare Figs. 9 and 11, fixed, UV, with 10 and 12, fixed, basic stain). That ribonucleic acid is a major component of the ultraviolet-absorbing regions is evident from the result of extraction with ribonuclease. Cytoplasmic basophilia is abolished by the digestion (Fig. 14) and absorption at 265 mμ is reduced to a level that can be accounted for by protein and light scatter (Fig. 13). The residual basophilia of neuronal and satellite cell nuclei is attributable to their deoxyribose nucleic acid content.

After ribonuclease treatment the absorption image at 265 mμ is comparable to, but slightly less dense than that at 285 mμ. That much residual absorption reflects protein content was indicated by staining digested cells with acid fuchsin. The nucleolus, which remains heavily ultraviolet-absorbing after digestion, is the most intensely acidophilic structure in the cell, another indication of its high protein content. The nucleus also stains densely. In the less heavily staining cytoplasm, the Nissl areas can be distinguished as somewhat more intense acidophilic structures.

DISCUSSION

The chemical characterization of Nissl bodies in nerve cells has usually been carried out on fixed, and hence chemically altered, specimens. Ribonucleic acid and proteins have been identified in such structures by ultraviolet ab-

sorption curve analysis (6) and ribonuclease extractable basophilia (1). Consistent with this are the ultraviolet absorption pictures of isolated living nerve cells (11, 12) and the absorption curves for living cultured neuron cytoplasm (4). Our ultraviolet photographs of living and fixed cells correlated with patterns of acidophilia and ribonuclease digestible basophilia are entirely in support of the ribonucleoprotein nature of the Nissl substance.

The point under examination here is whether the Nissl substance occurs in the living cell in the form of discrete nucleoprotein aggregates (Nissl bodies) as it does in the fixed cell or whether the aggregates are artifacts resulting from fixation. According to our observations the ultraviolet absorption pattern of a living spinal ganglion neuron is essentially unchanged by fixation. This means that with fixation of the cell there has been no gross alteration in concentration of absorber or in its distribution (Figs. 1 to 6, 8, and 9). The results of the basic staining and ribonuclease experiments clearly equate the ultraviolet-absorbing cytoplasmic masses of our living and fixed cells with the basophilic structures classically identified as Nissl bodies (Figs. 8 to 10, 11 to 14).

We have considered the possibility that some phase of our experimental treatment of the living cell may have led to the production of artifacts. The criteria for optimal culturing conditions and for considering the cells examined to be healthy have already been discussed (3). There remains only the possibility that changes may have been produced by ultraviolet irradiation. We consider this highly unlikely. By virtue of having utilized the automatic facilities of the UV-91,[3] we were able to work rapidly with the living cells and to expose them to a minimum of ultraviolet energy. The failure to detect alterations in cells from the beginning to the end of a series of exposures argues against artifact change. Fig. 7 is a photograph at 265 mμ of a previously unirradiated cell. Its morphology is essentially like other cells that have received ultraviolet irradiation (Figs. 1, 8). Moreover, the appearance of unirradiated living cells in phase contrast is no different from that of irradiated cells. Our observations thus support the contention that the ultraviolet-absorbing Nissl substance exists preformed in the living neuron just as it is seen after fixation.

While this conclusion confirms earlier ideas of the preexistence of Nissl bodies, it is inconsistent with the findings of Koenig (4). He examined living and fixed cultured chick embryo spinal ganglia and concluded that in living neurons the Nissl substance is homogeneously distributed with only local variations in density. From the photographs presented by Koenig, however, it

[3] It should be mentioned here that this work illustrates a unique application of the color-translating ultraviolet microscope. Quite aside from any use it may have as a color translator, the instrument is set apart from other photomicrographic systems by virtue of the great speed with which ultraviolet absorption information may be collected. It thus becomes of considerable value in studying living cells.

appears that although the cells he investigated are of different *in vitro* ages, they are all in a moderate to severe state of chromatolysis. In all, the nucleus lies in an eccentric position, in some cases bulging from the cytoplasm, and the Nissl substance is either almost completely lacking (reference 4, Plate I, Figs. 1 to 6), or restricted to a peripheral band (reference 4, Plate II, Fig. 3), with occasionally an additional juxtanuclear cap in evidence (reference 4, Plate II, Figs. 1 and 2). Deitch and Murray (3) have presented phase contrast evidence showing that the Nissl substance of neurons which are in a moderate state of chromatolysis is indeed quite homogeneous (reference 3, Fig. 24). The fixation and staining procedures used were found to result in some shrinkages. However, in contrast to Koenig's findings, no marked increases in inhomogeneity of cytoplasmic structure of these cells were noted after fixation, and it would seem that such changes in his material might represent procedural artifacts. In both this and the aforementioned study of Deitch and Murray (3) emphasis was placed on obtaining cells in healthy condition; cells in severe chromatolysis, such as employed by Koenig, were rejected from consideration as not being pertinent to a study of the distribution of Nissl material in healthy living neurons. It would seem that Koenig's results might best be explained if one assumes that his cultural conditions were suboptimal and that the neurons in his material apparently were unable to differentiate to a state in which discrete Nissl bodies occur. In view of this possibility and the present evidence, therefore, his conclusion that "the Nissl pattern appears to be an artifact" seems unwarranted.

The previous phase contrast study (3) demonstrated that structures corresponding to basophilic Nissl bodies exist in living cultured chick spinal ganglion cells. This together with the present evidence leads to the conclusion that these structures are present in the living neuronal cytoplasm as discrete masses containing high concentrations of nucleoprotein just as they are in the fixed cell.

SUMMARY

Living chick spinal ganglion neurons grown for 19 to 25 days *in vitro* were photographed with a color-translating ultraviolet microscope (UV-91) at 265, 287, and 310 mμ. This instrument was unique in permitting rapid accumulation of ultraviolet information with minimal damage to the cell.

In the photographs taken at 265 mμ of the living neurons, discrete ultraviolet-absorbing cytoplasmic masses were observed which were found to be virtually unchanged in appearance after formalin fixation. These were identical with the Nissl bodies of the same cells seen after staining with basic dyes. The correlation of ultraviolet absorption, ribonuclease extraction, and staining experiments with acid and basic dyes confirmed the ribonucleoprotein nature of these Nissl bodies in the living and fixed cells.

No change in distribution or concentration of ultraviolet-absorbing substance was observed in the first 12 ultraviolet photographs of a neuron, and it is concluded that the cells had not been subjected to significant ultraviolet damage during the period of photography.

On the basis of these observations, as well as previous findings with phase contrast microscopy, it is concluded that Nissl bodies preexist in the living neuron as discrete aggregates containing high concentrations of nucleoprotein.

BIBLIOGRAPHY

1. Brachet, J., *Compt. rend. Soc. biol.*, 1940, **133,** 88.
2. Deitch, A. D., *Lab. Inv.*, 1956, **5,** 513.
3. Deitch, A. D., and Murray, M. R., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 433.
4. Koenig, H., *J. Histochem. and Cytochem.*, 1954, **2,** 334.
5. Land, E. H., Grey, D. S., Flower, M. S., Husek, H., Jones, R. C., Matz, C. H., and Merrill, D. P., *Science*, 1949, **109,** 371.
6. Landström, H., Caspersson, T., and Wohlfahrt, G., *Z. mikr.-anat. Forsch.*, 1941, **49,** 534.
7. Peterson, E. R., and Murray, M. R., *Am. J. Anat.*, 1955, **96,** 319.
8. Powers, M. M., and Clark, G., *Stain Technol.*, 1955, **30,** 83.
9. Shurcliff, W. A., *Lab. Inv.*, 1952, **1,** 123.
10. Walker, P. M. B., and Davies, H. G., *Discussions Faraday Soc.*, 1950, **9,** 461.
11. Wiemann, W., *Z. ges. Neurol. u. Psychiat.*, 1925, **98,** 347.
12. Yamada, E., *Kyushu Mem. Med. Sc.*, 1953, **4,** 25.

EXPLANATION OF PLATES

PLATE 139

Ultraviolet photomicrographs of a single chick spinal ganglion neuron cultured 19 days *in vitro*. Figs. 1, 3, and 5 are of the living cell and Figs. 2, 4, and 6 of the same cell after fixation in 10 per cent neutral formalin for 1½ hours. Total previous ultraviolet exposure to the living cell, 14 seconds. Total ultraviolet exposure prior to fixation, 46 seconds. Photographed with a color-translating ultraviolet microscope (UV-91). × 1050.

nu, nucleus.
nl, nucleolus.
NS, Nissl substance.
Sn, satellite cell nuclei.

FIG. 1. Living cell, 265 mμ. Image formation is due primarily to absorption of purines and pyrimidines of nucleic acid.

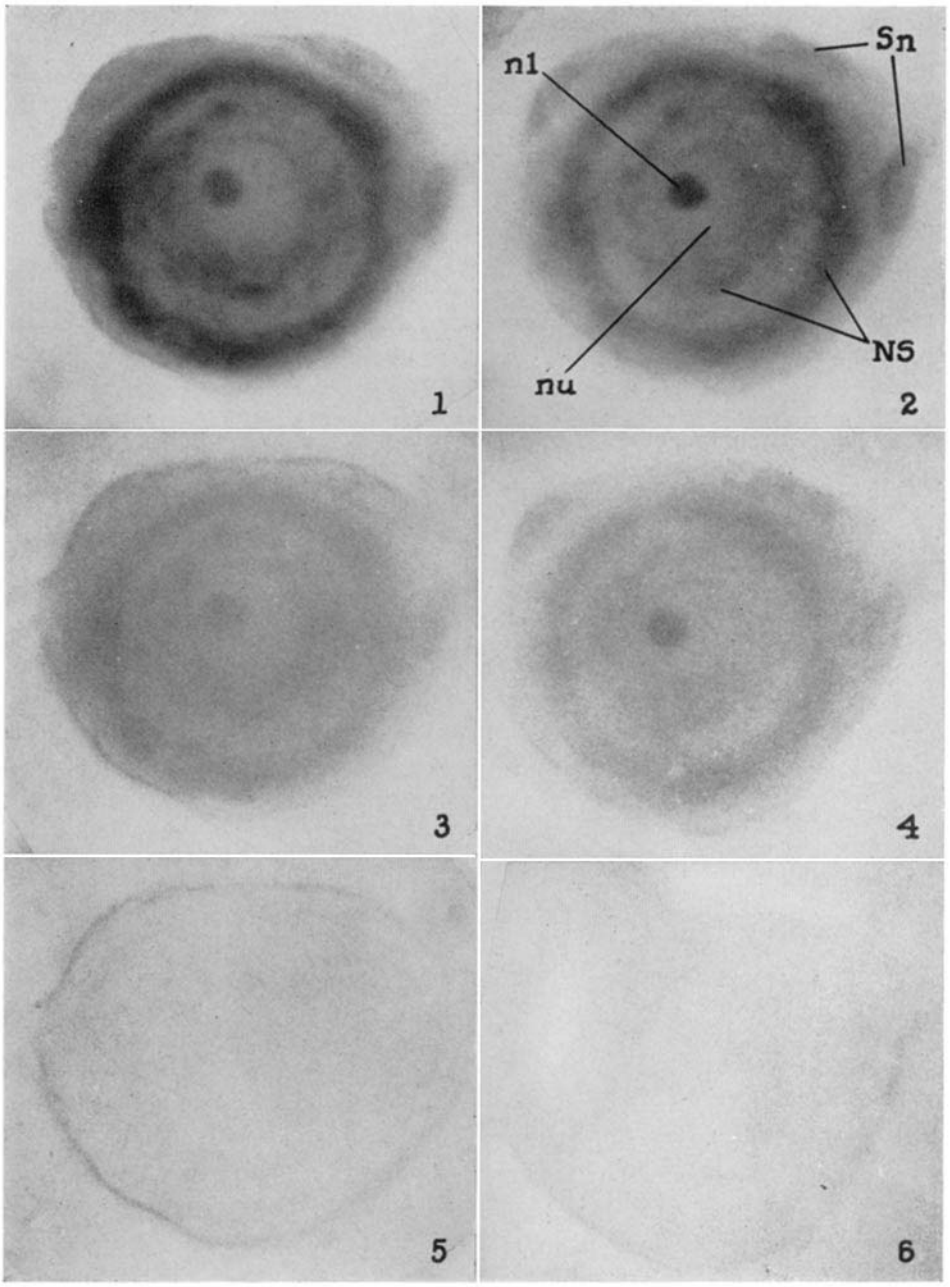
FIG. 2. Fixed cell, 265 mμ.

FIG. 3. Living cell, 287 mμ. Image formation is due to absorption both by nucleic acid and by protein (tyrosine and tryptophane).

FIG. 4. Fixed cell, 287 mμ.

FIG. 5. Living cell, 310 mμ. Image formation is due to scattered, rather than absorbed light.

FIG. 6. Fixed cell, 310 mμ.



(Deitch and Moses: Nissl substance of spinal ganglion cells. II)

nu, nucleus.
nl, nucleolus.
NS, Nissl substance.
cy, cytoplasm.

FIG. 7. Living neuron, 19 days *in vitro*, photographed at 265 mμ. This cell had received no prior exposure to ultraviolet energy. × 1010.

(FIGS. 8, 9, and 10. The Nissl material is arranged in a narrow peripheral band (*NS*) and as rounded masses forming a nuclear cap (arrows).)

FIG. 8. Living neuron, 19 days *in vitro*, photographed at 265 mμ. This cell had received 13½ seconds prior exposure to ultraviolet energy. × 1245.

FIG. 9. The same cell as in Fig. 8 after fixation in 10 per cent neutral formalin for 1½ hours, photographed at 265 mμ. The cell had received a total of 46 seconds of ultraviolet irradiation prior to fixation. × 1245.

FIG. 10. The same cell as in Figs. 8 and 9, stained with cresylecht violet and photographed through a red (*ca.* 620 mμ) filter. × 1245.

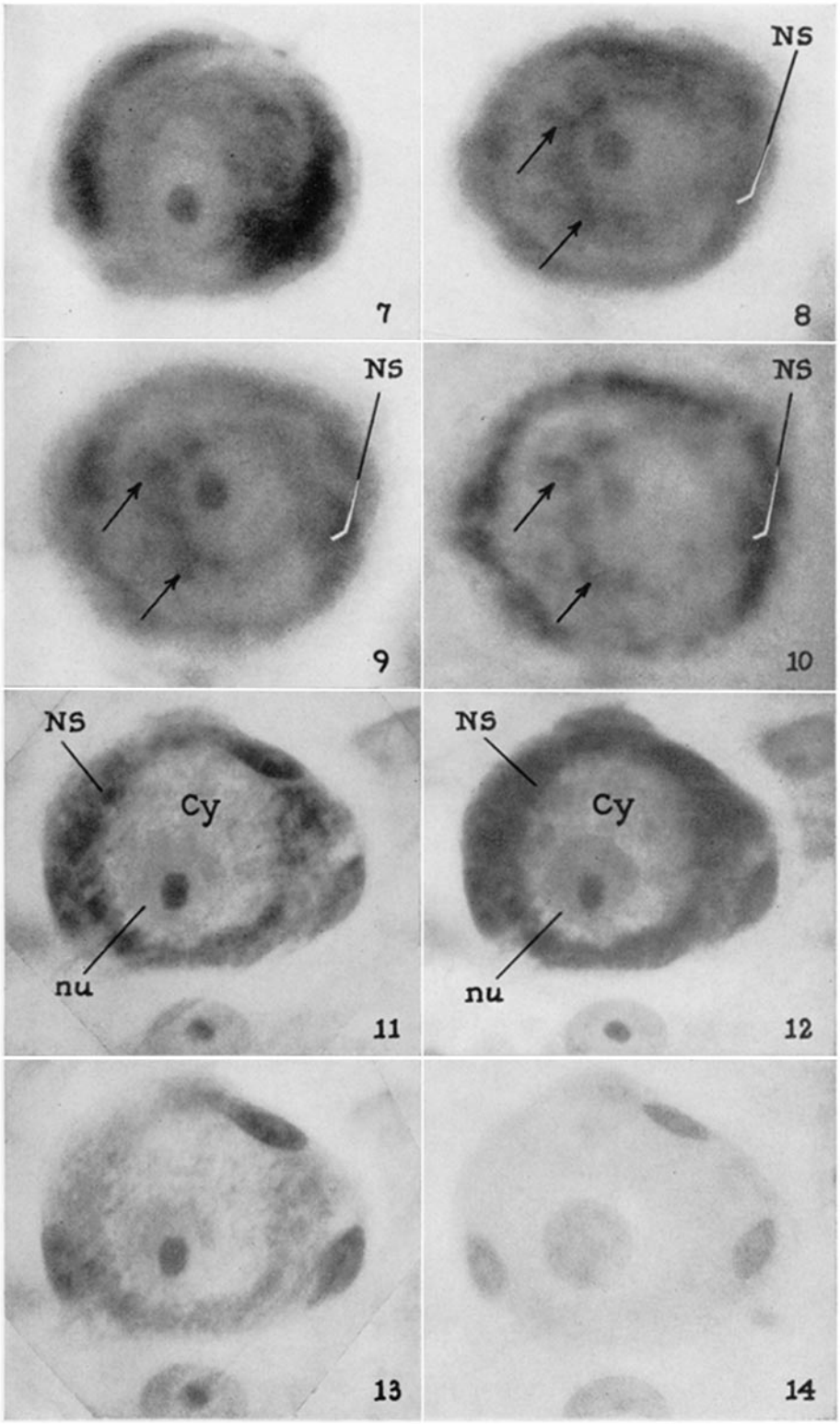
(FIGS. 11 and 12. The Nissl material is arranged as a broad peripheral band (*NS*). The central cytoplasm (*cy*) is devoid of Nissl substance.)

FIG. 11. Chick spinal ganglion neuron, 25 days *in vitro*, fixed in 10 per cent neutral formalin, and photographed at 262 mμ. This cell had received no exposure to ultraviolet energy prior to fixation. × 1190.

FIG. 12. The same cell as in Fig. 11 stained with azure A at pH 3.5 and photographed through a red (*ca.* 620 mμ) filter. × 1190.

FIG. 13. The same cell as in Figs. 11 and 12, after removal of the azure A stain and digestion with ribonuclease, photographed at 262 mμ. × 1190.

FIG. 14. The same cell as in Fig. 13, restained with azure A and photographed through a red (*ca.* 620 mμ) filter. × 1190.



(Deitch and Moses: Nissl substance of spinal ganglion cells. II)