REVERSIBLE ALTERATIONS IN THE GOLGI COMPLEX OF CULTURED NEURONS TREATED WITH AN INHIBITOR OF ACTIVE NA AND K TRANSPORT

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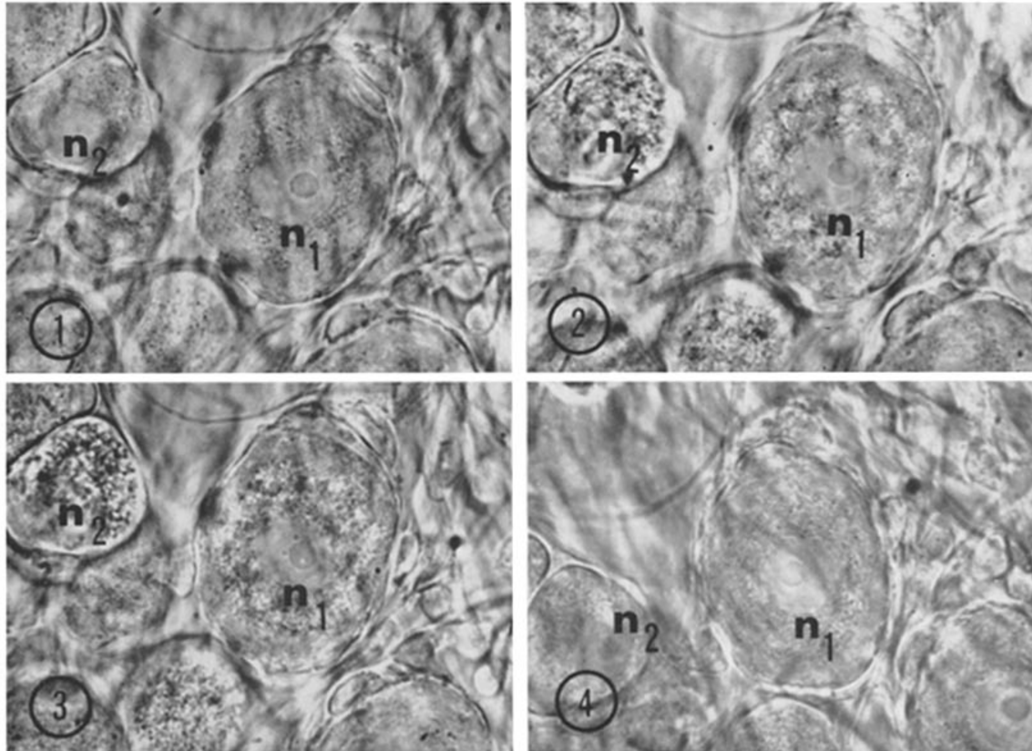
ABSTRACT

Highly differentiated cultures of rat and mouse sensory ganglia were treated for varying periods (up to 6 hr) with selected doses (1×10^{-3} M to 5×10^{-5} M) of the cardiac glycoside, ouabain (Strophanthin G). This inhibitor of active Na and K transport produced a selective and progressive swelling of elements of the Golgi complex in many, but not all, neurons. After 6 hr of treatment, virtually all Golgi complexes in affected neurons were either altered or absent; large cytoplasmic vacuoles limited by agranular membrane were prominent in these neurons. This response was not observed in satellite cells and Schwann cells. Within a few hours of ouabain withdrawal, the neuronal vacuoles disappeared and normal Golgi areas were again observed. These observations suggest that there is a site for active transport of Na and K on the Golgi membrane of these neurons. In discussing the possible significance of this observation, it is suggested that if this site were directed so that cation was actively pumped from Golgi cisternae into cytoplasm (and if there were differential water and ion permeabilities in various parts of the endoplasmic reticulum–Golgi system), then such a pumping mechanism could provide an explanation for the concentrating function of the Golgi apparatus.

INTRODUCTION

This study derives from an initial light microscopic observation that the application of the cardiac glycoside, ouabain, to cultures of rat dorsal root ganglia results in the formation of cytoplasmic vacuoles within neurons.[1] This neuronal response is the first and most obvious alteration after ouabain treatment. There is extensive documentation that ouabain inhibits active Na and K transport across cell membranes (1–3, among others). There is also evidence that this active transport involves an enzyme system located within cell membranes which hydrolyzes ATP, is activated by Na and K, and is specifically inhibited by ouabain (4, 5, among others). This plasmalemmal system for active Na and K pumping against concentration gradients is widely distributed among many cell types (6), and must be distinguished from the system of special transmembrane channels, characteristic of nerve fibers, which allow Na and K to

[1] This initial observation was made in collaboration with Miss Joanne Mire and Dr. Walter Hendelman.



FIGURES 1–4 Fig. 1 shows normal, healthy neurons in a mature culture of rat sensory ganglion. Fig. 2 shows the same neurons after 3 hr of exposure to ouabain; Fig. 3, after 6 hr of exposure to ouabain; and Fig. 4, 12 hr after removal of ouabain. In Fig. 4, the field is slightly rotated counterclockwise. Neuron (n_1) is shown to undergo a moderate transient increase in perinuclear granularity in response to ouabain treatment. The smaller neuron (n_2) shows a more marked response. In Fig. 3, the granular cytoplasm in both n_1 and n_2 appears to contain vacuolar components. Note that satellite cells and supporting cells do not show discernible change. A complete description of normal cytology in these cultures is given in reference 12. All figures, × 725.

move down their concentration gradients during the generation of an action potential (reviewed in 7).

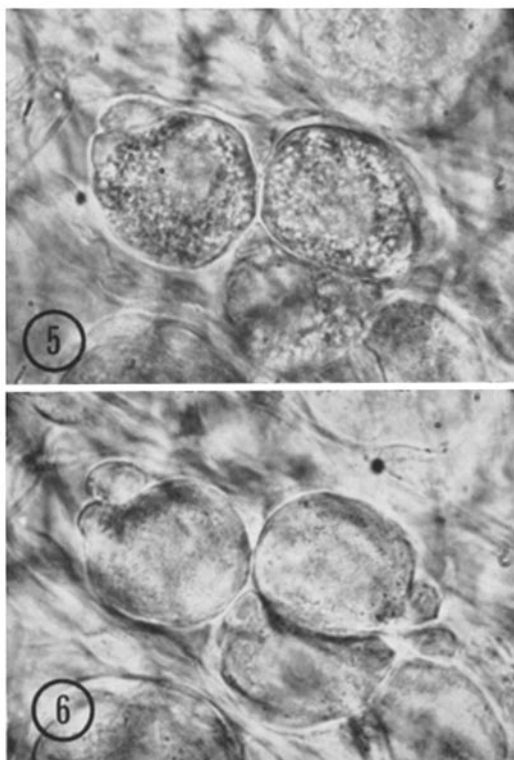
It has been suggested that there may also be microsomal sites of active ion transport (e.g., 8, 9) since an enzyme system which hydrolyzes ATP, is activated by Na and K, and is specifically inhibited by ouabain, has been located on the microsomal membrane fraction of neurons (10, 11, among others). In the present experiments, the vacuoles which form in neurons in the presence of ouabain were found to arise in the region of the Golgi complex, often by direct dilation of Golgi components; the vacuoles were observed to disappear and the Golgi complex to resume its normal configuration upon ouabain withdrawal. This discrete cytoplasmic response and the known specificity of ouabain's action suggest that an active ion-pumping mechanism is located on membranes of the Golgi complex. The possible significance of such a pump in the function of the Golgi apparatus is considered in detail in the Discussion.

METHODS

The experiments utilized cultured dorsal root ganglia prepared from rat or mouse fetuses and carried on collagen-coated coverslips in Maximow double-coverslip assemblies (for details, see 12). Cultures were incubated at 35°C. Rat cultures were fed twice weekly with one drop of a mixture containing 50% Eagle's Basal Medium, 25% human placental

serum, and 25% chick embryo extract, supplemented with 900-mg % glucose, the usual feeding medium for this tissue. Mouse cultures were fed twice weekly with a drop of a mixture of 25% Eagle's Basal Medium, 25% human placental serum, 25% chick embryo extract, and 25% Simms' Balanced Salt Solution supplemented with 600-mg % glucose, standard for this tissue. Mature cultures containing many large neurons (Fig. 1) and displaying substantial myelination were treated for up to 6 hr with solutions of ouabain (Strophanthin G, Sigma Chemical Company, St. Louis, Missouri) of the lowest concentration that produced an easily discernible effect in the living neuron. The final concentrations used were 1×10^{-3} M for the rat and 5×10^{-5} M for the mouse tissue, applied to the cultures as a component of the normal feeding medium.

Some cultures were fixed after exposure to ouabain for specific lengths of time; others, not fixed immediately, were washed to remove ouabain, refed normal medium, and carried for subsequent observation for periods up to 12 hr. Cellular responses were photographed in the living state with bright-field optics and after fixation and staining by various methods. Cultures for electron microscopic observation were fixed (in 2% OsO_4 buffered with veronal acetate) and embedded (in Epon) directly on the carrying coverslip following methods previously detailed (12).



FIGURES 5 and 6 A group of rat sensory neurons is shown in Fig. 5 at 6 hr after exposure to ouabain and in Fig. 6 at 12 hr after removal of ouabain. The level of focus is selected to show the condition of the cytoplasm; the nucleus is generally out of focus. The coarse cytoplasmic granularity (Fig. 5) disappears after ouabain withdrawal (Fig. 6). Both figures, × 750.

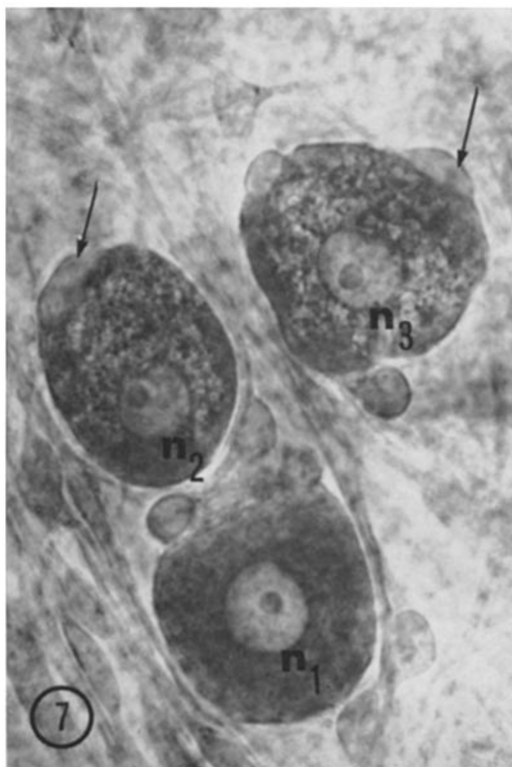
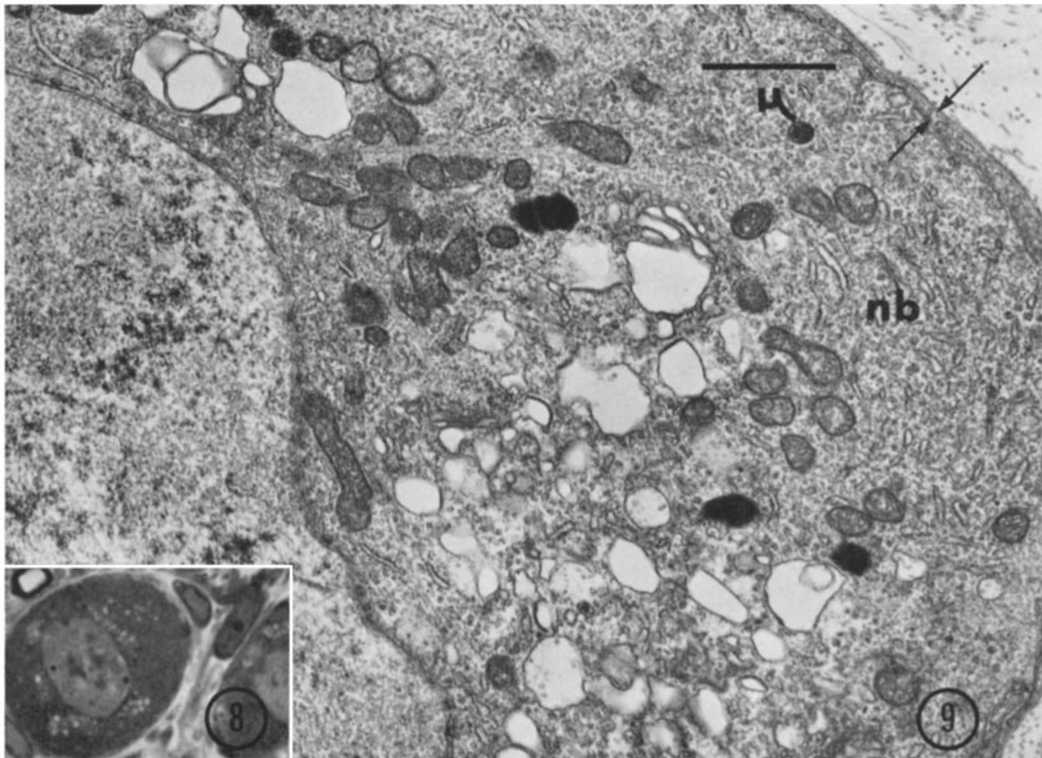


FIGURE 7 These three rat neurons are from a culture treated with ouabain for 6 hr. They are in an intact, unsectioned culture fixed in OsO_4, stained with Sudan Black, and mounted whole on a glass slide. One neuron, n_1, appears completely unaffected and two others, n_2 and n_3, show prominent cytoplasmic vacuolization. Note normal-appearing satellite cells (arrows) and supporting cells. × 950.



FIGURES 8 and 9 Rat neurons in a culture exposed to ouabain for 3 hr. Fig. 8 (× 700) shows discrete perinuclear aggregates of vacuoles within neuronal cytoplasm. Supporting elements appear normal. Fig. 9 (× 17,000) illustrates the occurrence of vacuoles in the region of altered Golgi complexes and the distortion of these complexes caused by dilation of their component parts. The vacuoles are bounded by agranular membrane and, at this early stage, are largely confined to the perinuclear region. Note the normal granular endoplasmic reticulum in a Nisslbody (*nb*) and the normal-appearing satellite cell sheath (arrows).

RESULTS

Observations on Rat Tissue

Observations on living cultures indicated that some neurons treated with ouabain (1×10^{-3} M) developed a slightly increased perinuclear granularity after 1–2 hr of incubation. At this stage, as in later stages, smaller neurons often showed a more marked response to ouabain treatment (Figs. 2, 3, 5, 6). Not all neurons were affected; approximately 15–30% of neurons in each culture showed no discernible response (Fig. 7). After 3 hr of ouabain treatment, the affected neurons showed unmistakably abnormal perinuclear granules; these granules were disposed in tiny clusters around the nucleus (Fig. 2). The nuclear membrane remained visible at this stage, but became progressively more difficult to discern as the perinuclear changes developed. In the peripheral cytoplasmic areas of some neurons, a diminution of the fine cytoplasmic granularity resulted in an abnormally clear zone around the cell periphery.

After 4 hr, the cytoplasmic changes in the affected neurons had increased, resulting in a punctate appearance of the perinuclear area. The nuclear membrane remained visible, but difficult to define, and the peripheral zones of cytoplasm were unchanged.

After 6 hr of ouabain, much of the cytoplasm of the affected neurons (including the peripheral zones of smaller neurons) appeared coarsely granular and some areas contained clearly discernible, though tiny, vacuoles (Fig. 3). In most neurons, the nucleus remained centrally located and visible, but the nuclear membrane was

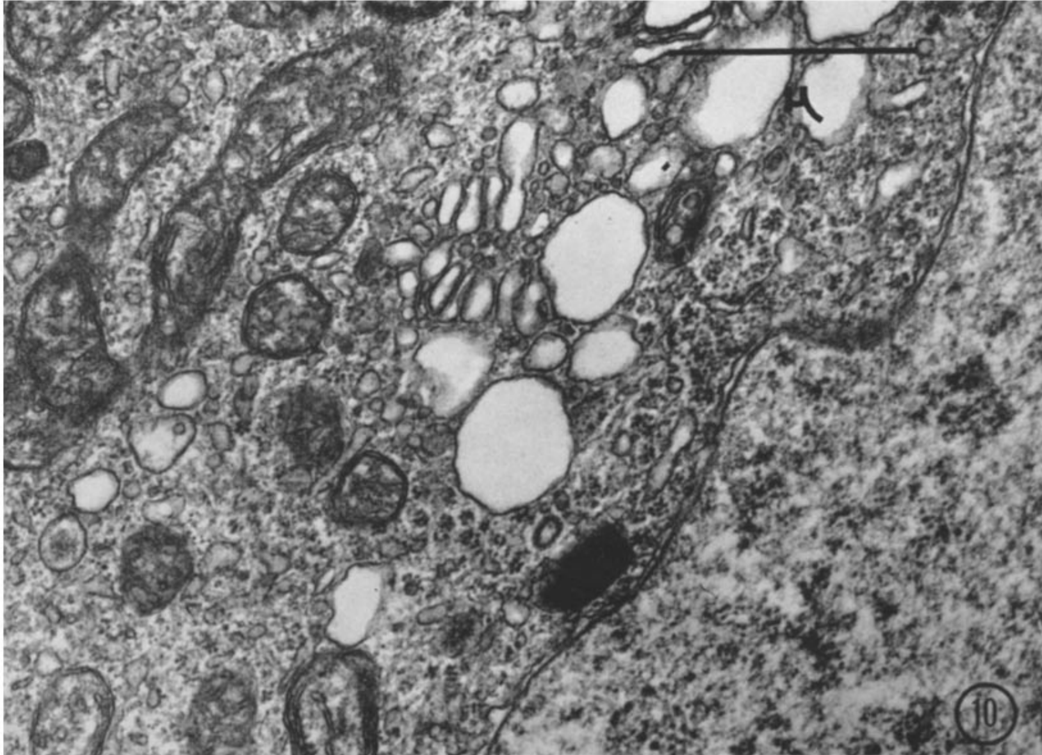


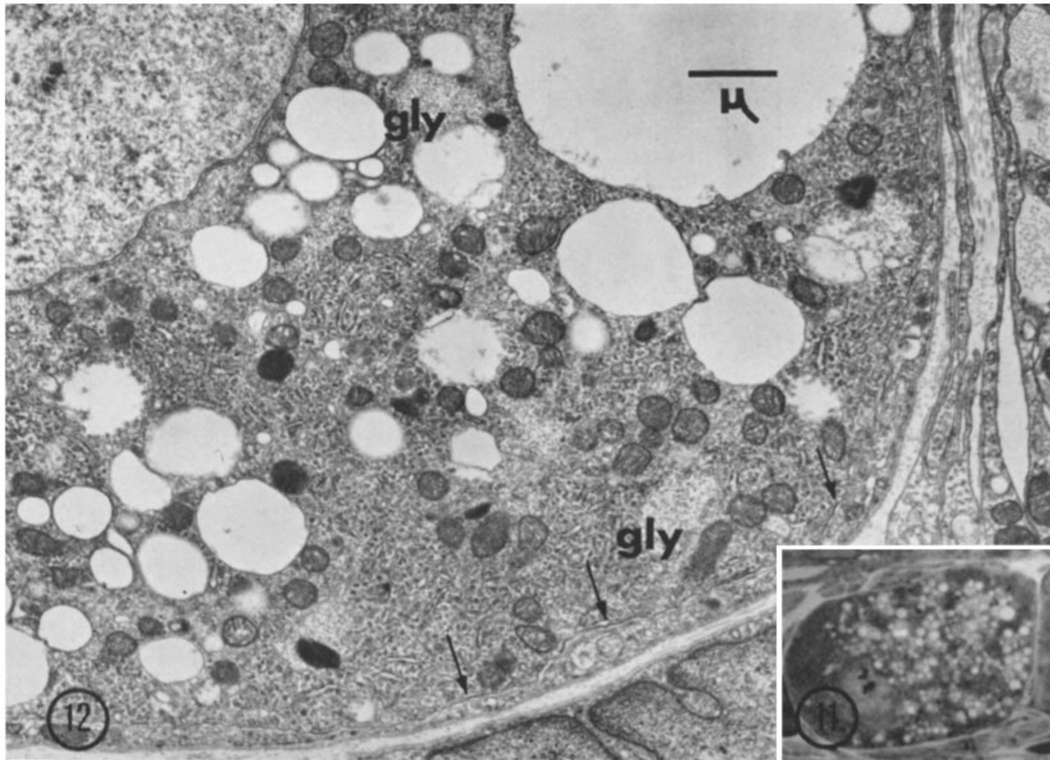
FIGURE 10 The perinuclear region of a rat neuron in a culture exposed to ouabain for 3 hr. The dilation of various components of an altered Golgi complex is shown. The mitochondria and nuclear envelope are considered normal. × 33,000.

obscured. Measurements on serial photographs indicated that no substantial change in neuron diameter had occurred at this time. No changes were observed in other nervous tissue elements in this complex culture system (12), i.e., satellite cells, Schwann cells, or axons.

Cultures that had been treated with ouabain for 6 hr showed a completely normal appearance within 6–12 hr after ouabain removal. Neurons of these cultures had centrally placed nuclei with crisp, clear nuclear membranes and a normal cytoplasmic configuration (Fig. 4).

Semithin sections of cultures fixed after 3 and 4 hr of ouabain treatment showed central nuclei of usual size and appearance. The cytoplasm of many of these neurons showed discrete clusters of rounded vacuoles in the perinuclear area (Fig. 8). The peripheral cytoplasmic zones often showed a smooth homogeneous appearance interrupted occasionally by vacuolated structures. *Electron microscopic* study of cultures fixed at this stage showed clusters of distinct cytoplasmic vacuoles varying from 0.1 to about 0.7 μ in diameter in the perinuclear zones of affected neurons (Figs. 9 and 10). These vacuoles, always seen arising in areas of Golgi complexes or as direct dilation of Golgi cisterns, were bounded by a single agranular membrane. At this and at all later stages, other neuronal organelles (including subsurface cisterns) did not appear altered.

Cultures fixed after 6 hr in ouabain showed many neurons with accumulations of rounded vacuoles throughout the cytoplasm (Fig. 11). Electron microscopic study showed accumulations of larger vacuoles, now widely dispersed through the cytoplasm (Fig. 12). The vacuoles often occupied the usual areas of the Golgi complex, but at this stage, normal-appearing Golgi regions were not noted in affected neurons. It appeared that in these neurons, virtually all membranes directly associated with the Golgi complex had been taken up in vacuole formation. At this late stage, the



FIGURES 11 and 12 Rat neurons in a culture exposed to ouabain for 6 hr. Fig. 11 (× 700) shows vacuoles of varying size now quite widely distributed throughout the neuronal cytoplasm. Fig. 12 (× 11,000) shows a portion of an affected neuron without a visible normal Golgi complex. The vacuoles are bounded by agranular membrane except in some cases in which the bounding membrane appears to be broken down or in the process of breaking down. Despite the extreme vacuolization, the granular endoplasmic reticulum appears normal, as do subsurface cisterns (arrows), nuclear envelope, and supporting cells. The deposits of glycogen-like material (*gly*) are sometimes seen in control neurons.

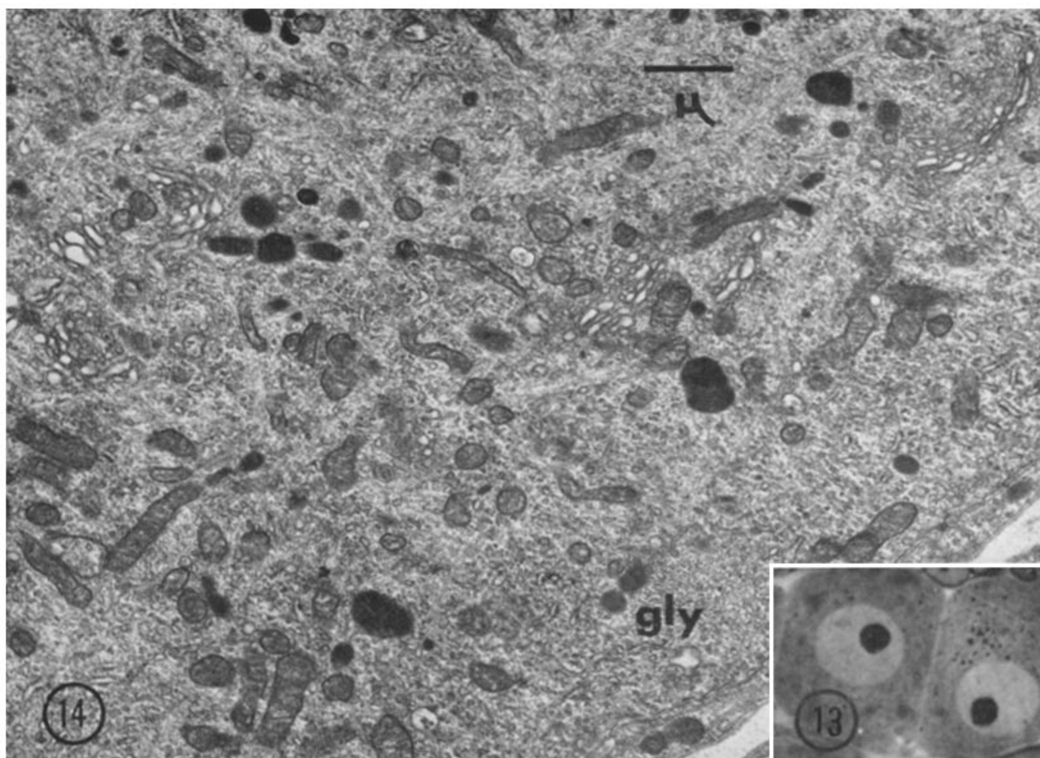
bounding membranes of some of these vacuoles appeared to be breaking down (Fig. 12). The vacuoles, bounded by single, agranular membranes, now varied in size from 0.1 to 2.5 μ or more in some instances.

In cultures that were reversed after 3 or 4 hr of ouabain treatment and fixed 12 hr later, neurons appeared normal by light microscopic examination, and electron microscopic study indicated completely normal cytoplasmic configuration, including numerous normal Golgi regions. Neurons in cultures reversed after 6 hr of ouabain treatment (Figs. 13 and 14) showed normal cytoplasmic configurations by 12 hr, though increased accumulations of glycogen-like material appeared in the cytoplasm of a few neurons.

Observations on Mouse Tissue

Observation of *living cultures* of mouse dorsal root ganglia treated with 5×10^{-5} M ouabain indicated that some neurons (approximately 50%) developed a slightly increased generalized cytoplasmic granularity after about 3 hr of incubation. After 6 hr of ouabain treatment, the cytoplasmic granularity in affected neurons became quite distinct, but these changes were never so dramatic as those seen in rat tissue treated for a similar length of time. Upon removal of ouabain from these cultures, the neurons began to revert to a normal appearance by 8–10 hr, but they did not appear completely normal until about 18–24 hr.

Semi-thin sections of cultures fixed after incubation in ouabain for 6 hr showed both normal-appearing



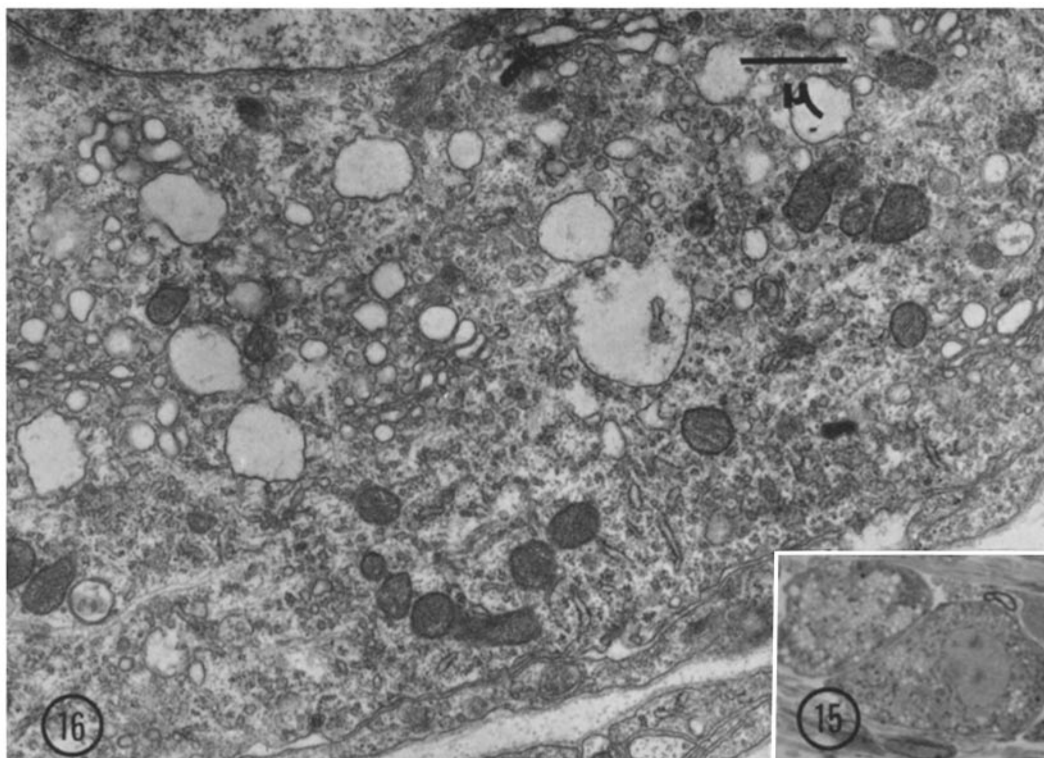
FIGURES 13 and 14 Rat neurons in a culture exposed to ouabain for 6 hr, washed to remove ouabain, refed normal feeding medium, and fixed 12 hr after ouabain withdrawal. Fig. 13 (× 700) shows neurons with normal nuclear and cytoplasmic configuration. Fig. 14 (× 12,000) illustrates cytoplasm which is typical of that of large neurons of rat dorsal root ganglion in culture. Occasional deposits of glycogen-like material (*gly*) are seen in these neurons. The satellite cell sheath appears normal.

neurons and neurons with easily discernible clusters of cytoplasmic vacuoles (Fig. 15). The vacuole clusters did not seem to be disposed in a perinuclear distribution as in the rat, but instead appeared throughout the cytoplasm. *Electron microscopy* of this tissue indicated that the vacuoles in the cytoplasm of affected neurons arose in the areas of the Golgi complex, apparently by distension of the Golgi cisterns and vesicles (Fig. 16). The widespread distribution of these vacuoles within the neuronal cytoplasm is explained by the fact that in mouse dorsal root ganglion neurons, the Golgi profiles are normally located not only in the perinuclear areas (as in the rat), but also more peripherally (Fig. 17).

Though smaller in size, the vacuoles seen in affected mouse neurons appeared quite similar to the vacuoles seen in affected rat neurons. As in the rat, other neuronal organelles appeared unaffected.

DISCUSSION

There is extensive documentation (as cited in the Introduction) that a membrane-associated ATPase is directly involved in transport of Na and K across cell membranes. The distinguishing properties of this Na-pump system have been recently summarized by Baker (7) as including: (*a*) the ability to move ions against an electrochemical gradient, (*b*) the inability to pump Li ions out of the cell efficiently, (*c*) linkage between Na and K movement, (*d*) inhibition by metabolic poisons, (*e*) dependence on energy-rich phosphate compounds, and (*f*) specific inhibition by ouabain (among other properties). An enzyme system with many of these properties has been found in a variety of cell types (13). Though this system is generally considered primarily plasmalemmal, the presence of a similar system in microsomes prepared from



FIGURES 15 and 16 Mouse neurons in culture exposed to ouabain for 6 hr. Fig. 15 (× 700) shows scattered aggregates of vacuoles in the neuronal cytoplasm; these aggregates are not specifically concentrated in the perinuclear area. Supporting tissue appears normal. Fig. 16 (× 13,500) shows the cytoplasm of a medium-sized neuron. Various components of the numerous altered Golgi complexes appear dilated. Some of the larger vacuoles appear to have bounding membranes which are breaking down. Granular endoplasmic reticulum, mitochondria, subsurface cisterns, and the satellite cell sheath appear normal.

nervous tissue has suggested to several workers (8–11) the possibility that active transport is associated with some part of the endoplasmic reticulum.

Although the characterization of this Na-K–dependent ATPase has come primarily from studies of cell fractions, the specific blocking action of ouabain has suggested that observations on whole tissues after ouabain application might also provide valuable information. This approach has been rewarding in studies of tissues from several species, including rabbit cornea (14), human erythrocytes, frog skin, toad bladder (15), and lens from cat, calf, and rabbit (16). Drug levels required to obtain an effect in whole tissues have been higher than those used with cell fractions; the level initially administered in such preparations is apparently greater than the amount of ouabain actually reaching the sites of action.[2]

Studies of direct effects of ouabain on intact nervous tissue have been few. In a study of the effects of the cardiac glycosides on sympathetic nervous tissue, Birks (17) observed that cat superior cervical ganglion perfused for 3 hr with plasma solutions containing 5×10^{-6} g/ml digoxin and fixed in 1% OsO_4 showed neuronal swelling accompanied by shrinkage of mitochondria and swelling of granular endoplasmic reticulum. He observed no alteration of smooth endoplasmic reticulum or Golgi apparatus, and the nonnervous tissue was unaffected. Cornog et al. (18) studied rat cerebral cortex after injection of ouabain solutions directly into the intact hemisphere in the living,

[2] G. I. Kaye, 1968. Personal communication.

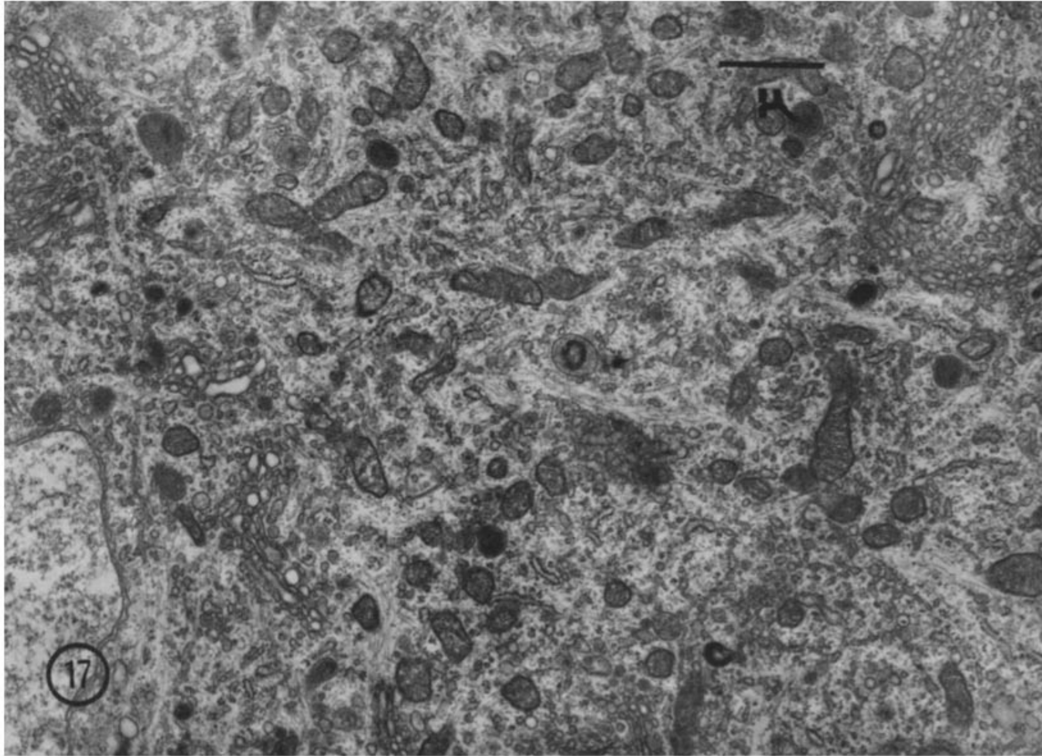


FIGURE 17 Normal cytoplasm of a large neuron of mouse dorsal root ganglion in culture. Golgi complexes are scattered throughout the cytoplasm. × 13,500.

anesthetized animal. After fixation in 5% glutaraldehyde, they observed swelling of astrocytes and presynaptic terminals, but neuronal perikarya, axons, dendrites, and oligodendrocytes showed little effect. Steffanelli et al. (19) incubated relatively undifferentiated chick spinal ganglion cultures in medium containing 1×10^{-4} M ouabain for periods up to 24 hr. Their light microscopic study showed vacuolization in fibroblast-like cells. They suggested that the vacuolated cells are of a morphologically undifferentiated "glial" type; they reported no observed effect on neurons.

In the present study, it was possible to make observations in living tissue, as cellular changes developed and reversed, as well as in fixed and sectioned material. Cytoplasmic vacuoles were observed to develop in cultured dorsal root ganglion neurons of rat and mouse upon incubation in feeding solutions containing ouabain. These vacuoles appeared to arise by progressive distension of Golgi vesicles and cisterns. Normal Golgi configurations gradually disappeared as the cytoplasmic vacuoles increased in size and number. Other cytoplasmic organelles appeared to be unaltered in the presence of ouabain. Upon removal of the ouabain, the usual Golgi profiles reappeared in the neuron cytoplasm and the cells returned to normal. Other nervous tissue elements in these cultures remained relatively unaffected. We have never observed this type of change in control cultures or in response to washing and handling procedures.

A Proposed Mechanism of Golgi Swelling in Ouabain-Treated Cultured Neurons

It seems reasonable to suggest that the primary target of ouabain in these cultured neurons is an especially active (or sensitive) Na-K transport system associated with the Golgi membrane. This must be considered in light of the evidence for transport of materials into the Golgi region from other parts of the intracellular cisternal system (discussed in 20). Varying amounts of cytoplasmic

water and ions may be admitted into these interrelated cisternal systems, especially along with secretory products in the region of the granular endoplasmic reticulum. These ions and water may then pass with (or aid in transporting) secretory material to the Golgi regions where a portion of the dominant cation (and the water which follows) might be transported back into the cell cytoplasm. The concentration function of the Golgi components could thus be achieved at the same time, preserving ions and water for reuse by the cell.

The present study gives no indication of which ion might be accumulating in the Golgi components. There is some evidence that Na is present within the cisternal system of neurons (21), and preliminary data (22) suggest that Na accumulates within dilated Golgi components in the presence of ouabain. If the ion actively pumped out of Golgi components and into cell cytoplasm were Na, then control of Golgi volume would occur in a manner in some ways analagous to the control of volume of the whole cell (23).

There is a striking similarity between the Golgi alterations we have observed in cultured neurons and the changes observed in an hereditary motor neuron disease of mice recently reported by Andrews and Maxwell (24). Studying "wobbler" mice at 1 month of age, these authors observed changes in motor neurons of the brain stem and spinal cord, consisting of the formation of large vacuoles arising from enlargements of Golgi cisterns. In early stages, these vacuoles were often seen in the perinuclear area; other organelles were normal in appearance. In cells with apparently more advanced pathology, vacuolization of cytoplasmic components was more widespread and involved other membrane systems of the cell including granular endoplasmic reticulum.

In concluding, it should be stated that hypotheses other than that given above might be considered to explain the effect of ouabain on cultured neurons. The observed response could be a change developing secondary to failure of plasmalemmal pumping mechanisms. It should also be noted that the amount and type of Golgi component containing ouabain-sensitive pumping mechanisms may vary greatly in different cell types. In the exocrine pancreatic cell, for instance, there is evidence that intensive concentrating action occurs primarily in condensing vacuoles, rather than in all components of the Golgi system (discussed in 20). The involvement of all components of the Golgi complex in ouabain-treated neurons may indicate a widely distributed pumping mechanism in the neuronal Golgi complex, or it could indicate progressive recruitment of components following failure in one region.

This study was supported by National Institutes of Health grants NB-04235, GM-00256, and NB-05242 and by National Multiple Sclerosis Society grant 428.

We gratefully acknowledge the assistance of Mrs. Julia Shen and Miss Joanne Mire in the preparation and handling of cultured material.

A preliminary report of this work was presented before the American Society for Cell Biology, Boston, 1968 (*J. Cell Biol.* 1968. **39** [No. 2]: 141a.).

Received for publication 7 February 1969, and in revised form 24 March 1969.

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