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29 December 1966

Deuterium Oxide: Direct Action on Sympathetic Ganglia Isolated in Culture

Abstract. *Immature ganglia from chicks and rodents were maintained as organized, developing cultures for 2 months or more, during which time they were continuously exposed to deuterium oxide in their medium. Observations of the living cell communities with the light microscope indicated that deuteration within viable limits (up to 25 percent) accelerates and increases the growth of sympathetic neurons and favors their repeated subdivision as a very large size is attained, thus inducing them to recapitulate cyclically the early stages of neurogenesis. Living deuterated cells appear more opaque and heteromerous than control neurons; furthermore, electron micrographs reveal an unusual abundance of granular and fibrillar elements in the nuclei of both neurons and supporting cells. Sheaves of complexly organized fibrillar components appear in the neuronal perikaryon; and ribosomes, Golgi elements, and microtubules are conspicuously numerous. Both fine structure and function of these ganglia therefore appear to have been modified directly by action of the deuterium isotope.*

Soon after the deuterium isotope of hydrogen (mass 2) was discovered, and its biological significance began to be explored, Barbour (*1*) reported a sympathicomimetic effect in mice whose normal body water had been progressively replaced by D_2O to the sublethal extent of 20 percent as they received the compound with their drinking water. In this and subsequent studies summarized by Thomson (*2*), it was observed that with 15 to 20 percent replacement by D_2O the animals exhibited piloerection and exophthalmos and became hyperexcitable and hard to handle. Above this range, combativeness was very pronounced, and convulsions frequently followed stimulation. At 30 percent replacement the mice became comatose and refused to eat; death ensued as the ratio of heavy to normal water increased further. Anatomical and histological studies of lethally exposed animals have shown a variety of organs and systems to be modified, including the cortex but not the medulla of the adrenal (*3*). Bachner *et al.* (*4*) confirmed in mice the earlier reports of sympathetic erethism which was followed by lethargy, convulsions, and death as replacement of body water by D_2O advanced to one-third or more; the nervous system, which appeared grossly normal in these mice at autopsy, was not examined microscopically, nor were the parotid glands. The submandibular salivary gland, however, in histological section showed destructive changes, especially in its ductular epithelium.

Notwithstanding these highly suggestive incidental findings, very little study has been directed in vivo toward the deuterated nervous system per se, and none previously, as far as we know, toward deuterated nervous tissue isolated in culture (*5*). We therefore undertook to expose long-term organized cultures of sympathetic chain and ganglia to deuterium oxide, administered directly by incorporation into the feeding medium, for periods of 2 months or longer. The normal development of sympathetic ganglia in vitro has been under study for several years in this laboratory (*6*), as has their response (*7*) to continued administration of the nerve growth factor (NGF) isolated by Cohen and Levi-Montalcini from the ductal portion of the mouse submandibular salivary gland (*8*, *9*).

Superior cervical, stellate, and chain ganglia were explanted from embryonic chicks (14 to 16 days) and newborn rats and mice onto collagen-coated coverslips and were carried in Maximow double-coverslip depression-slide assemblies up to 83 days without passage or transfer. In this series 440 cultures were used. The biological feeding medium (equal parts of human placental serum, bovine serum ultrafiltrate, and saline extract of chick embryos of 9 days) was supplemented with glucose to a total concentration of 600 mg per 100 ml of medium. The D_2O was incorporated in a fourth fraction so as to compose 5, 12½, 25, or 33 percent of the complete medium. In three types of controls this fraction was replaced by normal distilled water, isotonic balanced saline solution (BSS), or NGF (Abbott), 2 unit/ml. Cultures were incubated at 35.5°C; twice weekly they were washed briefly in BSS and supplied with fresh medium. They were observed daily and photographed repeatedly in the living state. These observations on specific cultures with the light microscope also provided background data for electron micrographs of the same cultures.

The developmental sequence for autonomic ganglia cultured in normal media (BSS and water controls), as observed serially with the light microscope in living material, is outlined as follows:

First, sporadic bursts of fine neurites form a smooth outgrowing network after 48 hours; their interneuritic spaces are then slowly invaded by cells of supporting-tissue type until by 9 to 10 days the explant has thinned sufficiently for the relatively immobile neurons to be discernible. These consist of groups of very small cells so closely packed inside capsules as to appear epithelial; at this time the round nucleus is surrounded by a thin rim of cytoplasm, and the whole cell may be 12 μ in

diameter. Next, these neuroblasts enlarge, with predominant increase in cytoplasmic volume, and the capsule becomes less confining. As growth and maturation proceed, the young neurons become multipolar, and a quota of satellite cells associate themselves rather loosely with them. With increase in size to ~ 20 μ in diameter, the neurons perforce shift somewhat in relative positions, but do not migrate far. This degree of maturity is reached by chick material at about 30 days. Cultures may subsequently remain in this state for 2 or 3 months with slight, gradual increase in neuron size and dendritic complexity. Neurons in general do not multiply, in vivo or in vitro, after having reached the early stages of normal maturation (*10*).

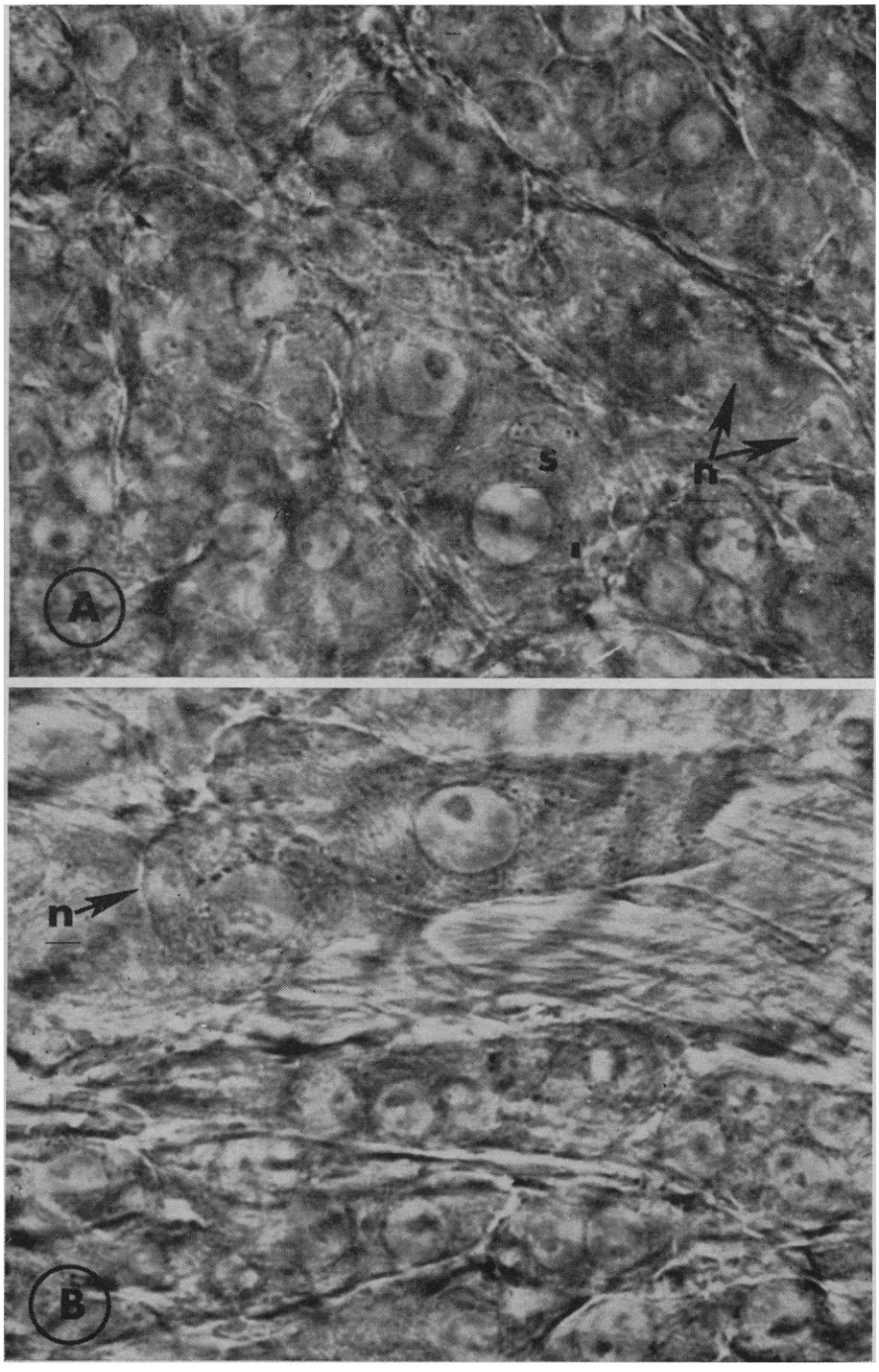

Fig. 1. Living deuterated cultures of sympathetic chain ganglia from chick embryo (14 days) 54 days in vitro (× 1000). Large, discrete neurons lie adjacent to smaller ones grouped in packages of a size roughly equivalent to the volume of the large, mature cells. (A) D_2O, 5 percent; (B) D_2O, 25 percent. *n*, Small neuronal nuclei in large cells which appear to be undergoing subdivision; *s*, satellite-cell nuclei.

The optimal dose of NGF for maintaining these cultures is 2 unit/ml. It is stimulating at the start, producing both the maximal neuritic halo described by Levi-Montalcini (*9*) and a precocious development of pyrenophore. Both nucleus and perikaryon attain mature size within 20 days. The nucleolus especially enlarges, concomitantly with accelerated production of cytoplasmic basophilic and fibrillar material. With increasing time in vitro, however, gross differences in size and maturity between NGF and other control cultures tend to level off.

In all concentrations used, D_2O produces an immediate acceleration of growth and development in both neurons and supporting cells, as compared to the water and BSS controls. Outgrowing neurites are very long and branching, but less numerous and bushy than in the NGF halo. Outgrowth of the deuterated supporting tissue is especially profuse and shows many mitotic figures. As soon as the nerve cells can be recognized in the explants of deuterated ganglia (by 8 days), their somas are already significantly enlarged 12 to 18 μ in diameter). Moreover, capsules containing small, undifferentiated, epithelial-type cells in oval or circular clusters are emerging along the edge of the chain—a phenomenon that is not observed in NGF or other control cultures at this stage. After approximately 30 days in vitro, the NGF-treated neurons are twice the size of the other control neurons, and the D_2O-treated neurons double the size of the former. At this time, deuterated cultures contain giant multipolar neurons (~ 70 μ in diameter) of a size never seen at all in control circumstances. The living cells are characterized by a peculiar opacity in bright-field observation, appearing densely packed with diversified subcellular elements. As the deuterium treatment continues, the neuron population increases markedly, so that after 60 days in culture it reaches two or more times that of control ganglia. Actual cell counts are rather inaccurate because of the thickness necessary to the ganglion explant (some six cell layers). However, the spreading of the neuronal area cannot be attributed simply to migration; there is an expansion of the central units which forces the whole mass outward. Large mature neurons have been seen from time to time in juxtaposition with several neuroblasts or a neuroepithelial cluster which were not observed there the day before (Fig. 1B). This suggested to us

the possibility that nuclear subdivision had occurred within the cytoplasmic mass of a large sister neuron. Fig. 1A supports this view; two other mitoses of mature neurons have been followed over a period of several hours to conclusion. Whatever their origin, the incidence of clusters of small undifferentiated nerve cells increases throughout the culture period in D_2O, their component units gradually developing in size and maturity and becoming interspersed with new clusters as the total cell population spreads peripherally in all directions. The inference is strong that these deuterated neurons are continuously going through cycles of growth and reproduction.

There is a perceptible concentration gradient in the stimulatory effects of D_2O up to the 25 percent level; this is more evident in the earlier stages of culture. However, 33 percent, although stimulating to early neuritic outgrowth, soon begins to exert toxic effects and is nearly always lethal within 2 weeks.

Studies by electron microscope (conducted by E. B. Masurovsky) confirm the greatly augmented growth and development of deuterium oxide–treated chick sympathetic neurons and reveal an unusual abundance of granular and fibrillar structures in their nucleus and, to a less marked degree, in their cytoplasm. Within the nucleus the quantity and type of such components is variable, possibly reflecting differences in nuclear synthetic or regulatory activity. Prominent bundles of ~ 60-Å fibrils (*11*), which sometimes extend entirely across the nucleus (Fig. 2C), are found in deuterated neurons, while relatively sparse fibrillar formations are seen in NGF-treated and other control neurons. These fibrils appear to have a beaded substructure and may branch into finer strands (*12*). Occasionally, other nuclear elements (granules) are observed within the meshwork of such fibrils. In the cytoplasm of deuterated chick neurons (especially at 25 percent D_2O) sheaves of intertwined, branched, and beaded fibrillar elements (90 to 120 Å), not seen in NGF-treated or other control neurons, are observed lying adjacent to elements of numerous, well-developed Golgi complexes and granular endoplasmic reticulum formations. Many microtubules traverse the perikaryon and the branched dendritic-neuritic processes which sprout from these cells. Unusually dense mitochondria are sometimes observed in both perikarya and processes. Structures which appear to be terminal boutons and contain aggregations of light- and dense-cored vesicles are found; typical synaptic profiles, displaying both types of vesicles on the presynaptic side, occur in both D_2O-

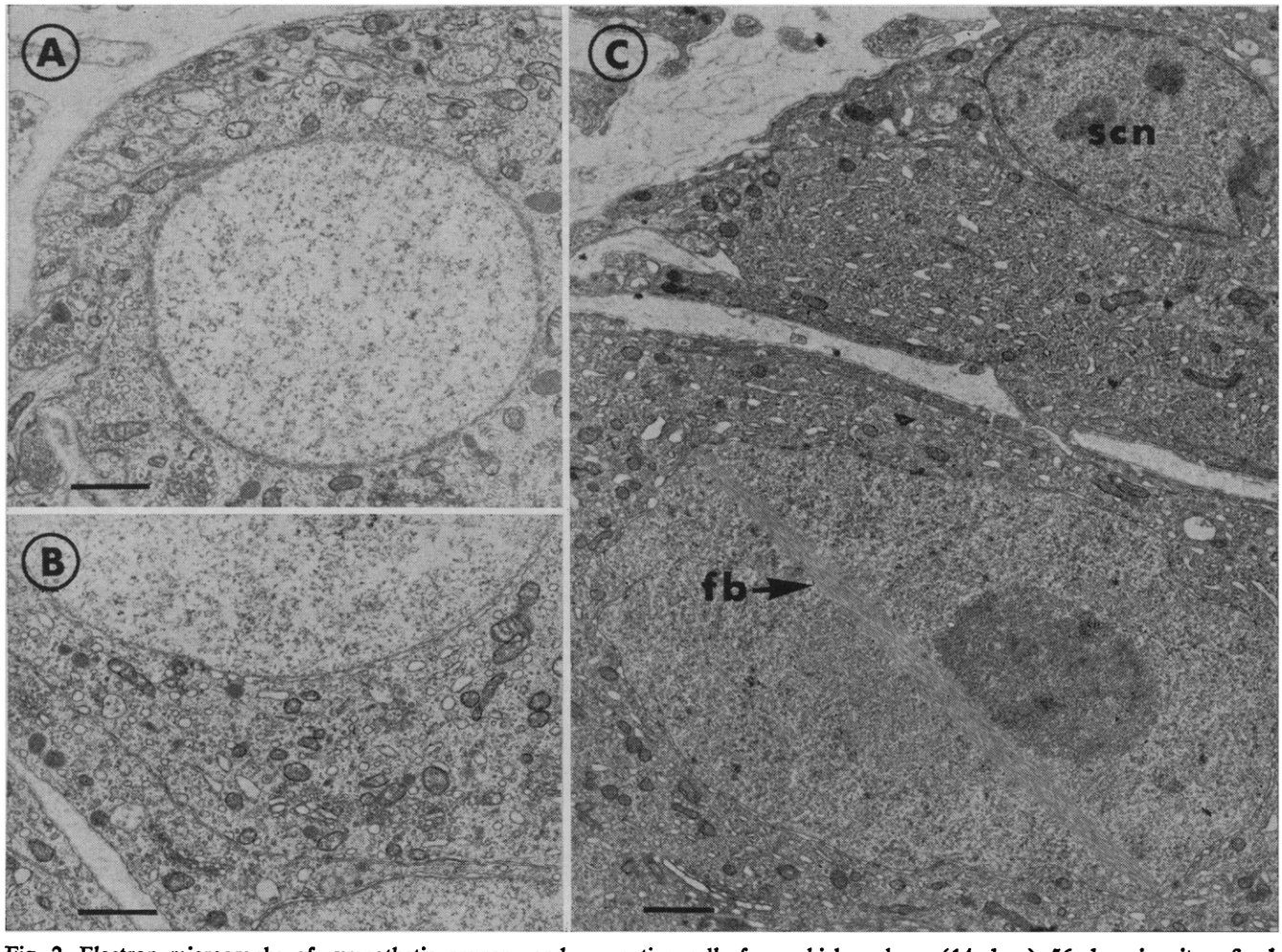

Fig. 2. Electron micrographs of sympathetic neurons and supporting cells from chick embryo (14 days) 56 days in vitro, fixed with 2 percent OsO_4 in veronal acetate buffer (*p*H 7.4), embedded in Epon, and stained with ethanolic uranyl acetate and lead citrate. Experimental and control cultures were regularly fixed at the same time and under exactly the same conditions. (A) Distilled H_2O control neuron with a cluster of neuritic processes along its upper margin. (B) NGF (2 unit/ml) control neuron, with a portion of satellite-cell cytoplasm and nucleus closely applied to its lower margin. (C) Neurons (25 percent D_2O-treated) and satellite cells with conspicuously abundant nuclear (and cytoplasmic) elements. Satellite-cell nucleus at *scn*; a prominent fibrillar bundle (*fb*) crosses the neuronal nucleus (scale bar, 1 μ).

treated and control cultures, especially those ≥ 60 days in vitro.

Like the neurons, satellite and Schwann cells of deuterated cultures (particularly at 25 percent D_2O) are notably rich in formed elements. Their nuclei contain numerous granules (~ 120 to 150 Å) and short filaments, some of which are clustered along the nuclear envelope; within the cytoplasm, ribosomes, microtubules, and fibrillogranular structures occur in abundance. Vacuoles and lipoid formations also appear sporadically in D_2O-treated nervous tissues. The proclivity of deuterated neurons and their supporting cells to display a greater quantity and variety of components (especially nuclear) than the controls is evidenced in cultures fixed in 3.5 percent glutaraldehyde or 2 percent osmium tetroxide, or in both, prepared either with normal water (H_2O) or with a portion of the H_2O replaced by an amount of D_2O equivalent to that in the culture medium. Previous work on microtubule-containing structures, such as the mitotic spindle, has shown that D_2O may influence their formation through solvent primary or secondary isotope effects, or both (*13, 14*). Deuterium isotope effects may induce significant conformational and functional changes in nucleic acids, proteins, and other cellular constituents (*3, 5, 14*). These actions of deuterium might be expected to lead to alterations in cytological and reproductive patterns of the tissues affected, such as those occurring characteristically in our isolated sympathetic ganglia exposed to D_2O.

Pilot studies on organized cultures of developing brain tissues from hypothalamus, cerebellum, and cerebral cortex (which differ from the sympathetic, in details of their early development, in the varying types of neurons involved and in the fact that they give rise to myelinated fibers in culture) indicate that these kinds of nervous tissues also are accelerated in growth and maturation by D_2O. Neurons are larger; both neurons and glia suffer fewer population losses than are normal in culture; myelin sheaths develop earlier and in greater quantity and extent. For central nervous tissue the optimal D_2O concentration appears to be less than 25 percent. Explanted murine submandibular gland dies in a medium containing 25 percent D_2O; 5 percent is unfavorable but not immediately lethal. Explanted parotid glands thrive at both exposure levels.

Although we can only surmise what was happening in the adult mice which exhibited symptomatic nervous disturbance during D_2O replacement in previous experiments (*1–4*), we conclude that some direct action was being exerted by deuterium oxide on their autonomic systems; our experiments do not support our original guess that these symptoms might have been induced by release of unphysiological amounts of nerve growth factor through structural deterioration which was occurring in the submandibular gland. In our hands, D_2O appears to afford a more potent stimulus to growth in sympathetic nervous tissue developing in isolation than nerve growth factor does. It also has an activating effect upon central nervous tissues. Nerve tissue is able to tolerate for periods of several months substantial amounts (5 to 25 percent) of D_2O in its ambient medium, while remaining within normal structural and functional limits as broadly defined. Specific metabolic pathways involved in this stimulative action of deuterium deserve investigation, especially in relation to the various unique aspects of neurochemistry.

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Photoinduced DNA-Protein Cross-Links and Bacterial Killing: A Correlation at Low Temperatures

Abstract. *The increased sensitivity of* Escherichia coli *to killing by ultraviolet irradiation when frozen and the variation in this sensitivity as a function of the temperature during irradiation have been correlated with changes in the amount of DNA that was cross-linked to protein by ultraviolet light. These variations in sensitivity to killing do not correlate with the production of thymine dimers.*

The sensitivity of *Escherichia coli* to killing (*1*) and to mutation (*2*) by ultraviolet light increases if the cells are irradiated while they are frozen, the relative sensitivity varying as a function of the temperature at which they are irradiated. It has been suggested (*1*) that a photochemical lesion, less amenable to repair than the thymine dimer, may be produced in *E. coli* irradiated at −79°C. Since the biological importance of the photochemical cross-linking of DNA with protein has been documented (*3*), we investigated the possibility that this lesion may be responsible for the enhanced killing of *E. coli* by irradiation while frozen. We therefore determined the sensitivity of cells of *E. coli* B/r,T⁻ to killing by ultraviolet light, the tendency of their DNA to become cross-linked to protein, and the production of thymine dimers at +21°, −79°, and −196°C.

Cells (*E. coli* B/r,T⁻ obtained from D. Freifelder) were grown to stationary phase (16 hours) in a salts-glucose medium (*4*) supplemented with thymine-2-C^{14} (2 μg/ml, 15.8 mc/mmole; Calbiochem). The cells were harvested and suspended in 0.1*M* phosphate buf-