the marrow was shielded there was neither immune responsiveness nor rapid return of competence. Return of competence at 1 month in this group may be related to regeneration of thymus or gut, or both, implicated in item 2. (iv) The possibility that spleen may play a role in the development of competent cells cannot be evaluated since in the S group the picture was dominated by competent cells already present. (v) Tolerance will develop in one-third to one-half of adult recipient mice given allogenic skin grafts from donors with a non-H2 genetic disparity when few competent cells are present. (vi) From the morphological studies there is again evidence that after x-irradiation the thymus in the adult mouse is responsible for reconstitution of a population of mainly small lymphocytes, and that germinal center cells and plasma cells stem from other sources. Even in the absence of these other cellular elements in the peripheral lymphoid organs, if small lymphocytes were present in abundance, as at 7 and 14 days after x-irradiation in T and GBM, transplantation immunity was quite vigorous. Plasma cells may be derived from cells produced in the gut. Germinal center cells, however, only returned rapidly in animals with intact spleens. It is possible that some lymphoid tissue associated with gut, tissue that is analogous to the bursa of Fabricius in avain species, may be found as a source for both of these cell types as postulated by Good (*8*).

JOSHUA MILLER
Yale University School of Medicine, New Haven, Connecticut

References and Notes

1. J. F. A. P. Miller, *Lancet* **1961-II**, 748 (1961); O. Archer and J. C. Pierce, *Fed. Proc.* **20**, 26 (1961).
2. H. N. Claman and D. W. Talmadge, *Science* **141**, 1193 (1963); J. F. A. P. Miller, S. M. Doak, A. M. Cress, *Proc. Soc. Exp. Biol. Med.* **112**, 785 (1963).
3. F. M. Burnet, in *The Thymus in Immunobiology*, R. A. Good and A. E. Gabrielson, Eds. (Harper and Row, New York, 1964), p. 2732.
4. A. H. E. Marshall and R. G. White, *Brit. J. Exp. Pathol.* **42**, 379 (1961); J. N. Blau and B. H. Waksman, *Immunology* **8**, 332 (1964).
5. K. Isakovic, S. B. Smith, B. H. Waksman, *Science* **148**, 1333 (1965).
6. G. D. Snell and H. P. Bunker, *Transplantation* **3**, 235 (1965).
7. L. O. Jacobson, E. K. Marks, M. J. Robson, E. Gaston, R. E. Zirkle, *J. Lab. Clin. Med.* **34**, 1538 (1949).
8. R. A. Good, *Fed. Proc.* **24**, 160 (1965).
9. I thank Dr. W. W. L. Glenn for help and suggestions and Dr. B. H. Waksman for critical review of the manuscript. Supported by Victoria Fund, American Cancer Society, and a gift from Charles W. Ohse. I thank USPHS for a postdoctoral research fellowship in surgery.

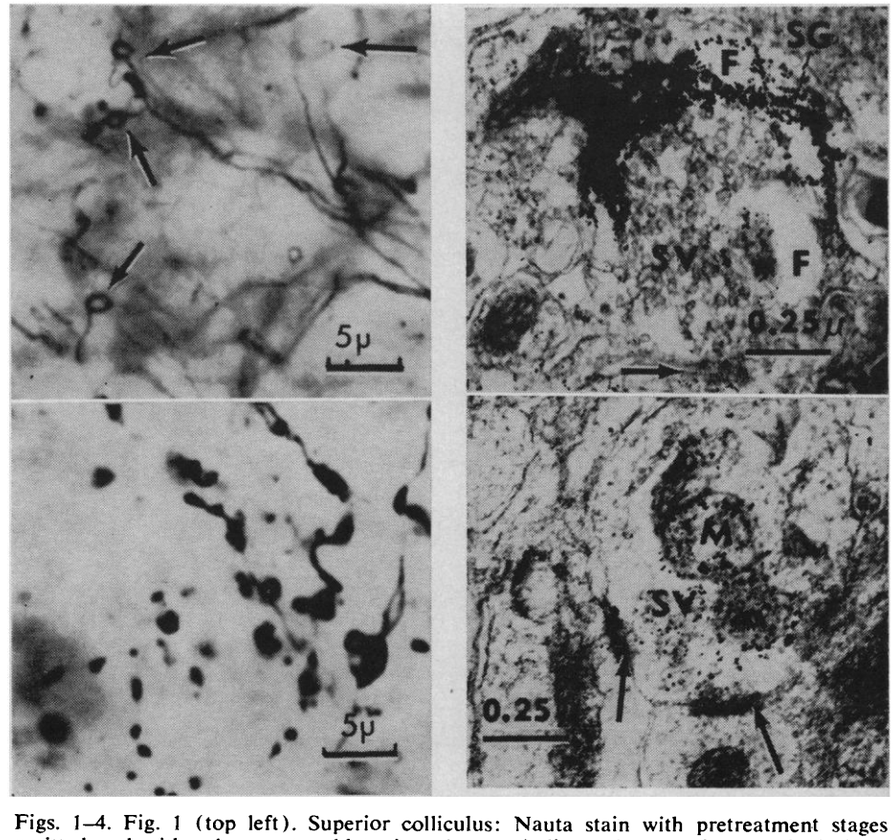
7 February 1966

Neurofibrils and the Nauta Method

Abstract. *When the pretreatment stages are omitted, the Nauta method for degenerating axons stains neurofibrils. Under the electron microscope the stain is closely related to neurofilaments. When one uses the complete Nauta technique, the stain is specific for membrane structures.*

The differential value and merits of the Nauta (*1*) and Glees (*2*) methods have been discussed in detail by many authors (*3*). It has been suggested from chemical studies that the Nauta method stains a product of membrane breakdown (*4*). An electron-microscopic study of Nauta degeneration in the spinal cord (*5*) indicated that, under the particular experimental conditions, silver granules occurred in some terminals; but it was not clear in which component of the cytoplasm they were found or whether this was a special case. Reduced-silver techniques, comparable to the Glees method, stain neurofilaments or a component closely related to them (*6*). The filaments proliferate in degenerating nerves, giving characteristic rings or other formations in terminals. At present, the correlation between filaments and the component stained by the Glees (or other neurofibrillar) methods is largely implied, although direct correlation is strongly suggested by Gray and Guillery (*6*).

We now report on part of a larger study investigating the ultrastructural basis of silver staining of the nervous system; we wish here to emphasize similarities between the Glees and Nauta methods in an attempt to understand the mechanism of both more fully.



Figs. 1–4. Fig. 1 (top left). Superior colliculus: Nauta stain with pretreatment stages omitted and with subsequent gold toning. Arrows indicate degenerating terminal rings. Fig. 2 (bottom left). Superior colliculus: Nauta stain with subsequent gold toning. No degeneration rings; degeneration appears as swellings and granules. Fig. 3 (top right). Superior colliculus: stained as for Fig. 1 and showing stain granules (*SG*) forming part of a terminal ring in a bouton. The synaptic vesicles (*SV*) are not stained with granules. Arrows indicate the synaptic cleft. The damage caused by freezing (*F*) does not appreciably alter the distribution of stain granules. Fig. 4 (bottom right). Superior colliculus (stained as for Fig. 2): a bouton showing degeneration stain granules in mitochondria (*M*) and synaptic vesicles (*SV*). Synaptic clefts are indicated by arrows.

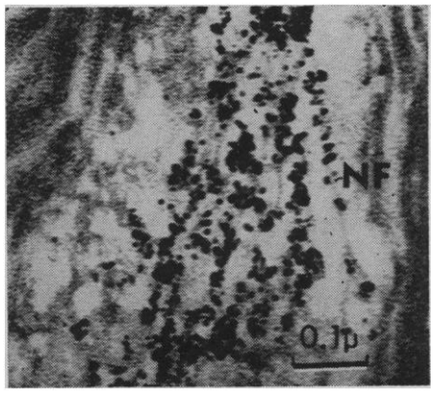


Fig. 5. Superior colliculus: detail showing the close relation of stain granules to neurofilaments (*NF*) in a large axon.

The principal areas studied have been the strata opticum and griseum superficiale of the superior colliculus of the albino rat three or more days after the contralateral optic nerve was cut in the orbit; work has also been done on the prepyriform cortex after olfactory tract lesions.

The most satisfactory fixation for this material has been found to be the formalin-perfusion technique of Pease (*7*), which has been detailed elsewhere (*8*). After biopsy, the brains were left in perfusate for 24 to 48 hours, cut on a freezing microtome, and stained by the methods of Glees (*2*) and Nauta (*1*) and by a modification of the latter. In the last, the pretreatment stages of the normal Nauta technique were completely omitted, sections being put, immediately after washing with distilled water, into the 1.5-percent silver nitrate bath. Some sections stained by each method were subsequently gold toned; this stage conferred the advantage that sections could be osmicated and membranes could be stained for electron microscopy without removal of the stained degeneration. Without gold toning, all silver granules are removed from the tissue within 1 minute in osmic acid. Moreover, stain-granule size was smaller after gold toning, and it was easier to see how the stain granules were related to ultrastructural features. After osmication, sections were treated according to the double-staining procedure of Westrum (*9*).

Glees-stained degeneration was well demonstrated with the light microscope in the superior colliculus 3 to 7 days after section of the optic nerve. Demonstration was, however, less satisfactory under the electron microscope because the "defatting" procedure in the staining technique rendered membranes unstainable, and it became difficult to recognize boutons; but stain granules could be recognized in close relation to a filamentous component of the cytoplasm, particularly within myelinated axons.

Use of the Nauta method with the pretreatment stages omitted gave light-microscopic results similar to results with the Glees-stained material (Fig. 1). Staining of ring formations was facilitated by the gold-toning procedure, and no characteristic Nauta degeneration was present. The electron microscope showed granules on the surfaces of filamentous structures in axons and in terminals (Fig. 3, *5*). Such filaments showed all the characteristics of neurofilaments in this and other (control) tissue and are not considered to be an artifactual precipitate induced during the staining procedure. A dispersed background granulation was situated mainly on membrane structures. The characteristic electron-dense degenerating axons and terminals seen under the electron microscope rarely contained more than a few random granules within them.

Material stained by the normal Nauta-Gygax method, with the same survival times, gave a completely different picture from that described above: in the light-microscope preparations there were no rings and the axons showed swellings along their courses; dispersed granules also were stained. This picture was not altered by gold toning (Fig. 2). Under the electron microscope, neurofilaments were not stained either in axons or terminals; the electron-dense degenerating axons and terminal fibers were packed with stain granules, and granules commonly occurred in other degenerating terminals—mainly in relation to vesicles and mitochondria (Fig. 4). Considerable concentrations of stain granules were also associated with myelin lamellae of some of the degenerating axons.

The general feature in the distribution of all the "Nauta" granules was that they were always associated with membranes of axoplasmic organelles as well as of myelin. There was no evidence to suggest that the stain was related in any way to neurotubules. In control samples of undegenerate material there were a few silver granules, often closely related to myelin lamellae or mitochondria but never as frequent or as large as those in degenerating axons. There was no indication that any of the characteristic features of the staining seen under the light microscope might have been produced by stain granules being specially oriented around the areas of tissue damage inevitably produced by the freeze-cutting.

Our results indicate that the Nauta method, with the pretreatment stages omitted, can be used routinely to stain neurofilaments and their light-microscopic correlate, neurofibrils. This practice has obvious practical applications in routine neurohistology, since our results confirm earlier suggestions that neither the Nauta nor the Glees (or associated neurofibrillar) method singly stains all the degeneration produced by a lesion (*3*, *5*). The degeneration seen under the electron microscope as dense areas of cytoplasm clearly represents part of the Nauta-positive component but little of the Glees-positive component; the latter is much less obvious.

Our results also offer an interesting basis for analysis of the differences in staining reaction between the Nauta and other reduced-silver methods. The failure to demonstrate neurofibrils by the Nauta-Gygax method (*1*) clearly derives from the pretreatment stages, which "facilitate" the Nauta-positive component in addition to "suppressing" the staining of filaments. In light-microscopic studies, for which phosphomolybdic acid or potassium permanganate was used alone in the preteatment stages, the former proved particularly important in suppressing the staining of neurofibrils. Since the closely related phosphotungstic acid is used routinely to stain neurofilaments for electron microscopy, phosphomolybdic acid, in its Nauta-pretreatment stage, may act by combining with sites on the filaments that may otherwise have been available for silver staining. This blocking of staining, as well as the oxidative action of potassium permanganate, the chemistry of which has been described in detail (*4*), redirects the staining reaction to the membrane component of the cytoplasmic organelles and myelin. If the chemical affinity of the Nauta method is as specific as chemical analysis suggests (*4*), with further refinement our technique may be a useful addition to the range of histochemical techniques now available for electron microscopy.

R. D. LUND
L. E. WESTRUM
Department of Anatomy, University College, London, W.C.1, England

References and Notes

1. W. J. H. Nauta and P. A. Gygax, *Stain Technol.* **29**, 91 (1954).
2. P. Glees, *J. Neuropath. Exp. Neurol.* **5**, 54 (1946).
3. ——— and W. J. H. Nauta, *Monatsschr. Psychiat. Neurol.* **129**, 74 (1955); D. H. L. Evans and L. H. Hamlyn, *J. Anat. London* **90**, 193 (1956); D. Bowsher, A. Brodal, F. Walberg, *Brain* **83**, 150 (1960).
4. R. P. Eager and R. J. Barrnett, *Anat. Record* **148**, 368 (1964); R. A. Giolli, *J. Histochem. Cytochem.* **13**, 206 (1965).
5. R. W. Guillery and H. J. Ralston, *Science* **143**, 1331 (1964).
6. E. G. Gray and R. W. Guillery, *J. Physiol. London* **157**, 381 (1961).
7. D. C. Pease, *Anat. Record* **142**, 342 (1962).
8. L. E. Westrum and R. D. Lund, *J. Cell Science*, in press.
9. L. E. Westrum, *J. Microscopie* **4**, 275 (1965).

10. We thank D. H. L. Evans for advice, B. G. Cragg and E. G. Gray for reading the manuscript, and E. Mansell for technical assistance. One of us (L.E.W.) is a postdoctoral fellow of NIH.

27 December 1965

Deep Scattering Layer Migration and Composition: Observations from a Diving Saucer

Abstract. *The distribution of a myctophid fish and physonect siphonophores observed during dives in the Soucoupe off Baja California closely correlates with scattering layers recorded simultaneously with a 12-kcy/sec echo sounder. These organisms were observed while they were migrating vertically, and at their night and daytime levels. They are capable of rapid, extensive changes in depth.*

Many meso-pelagic animals undergo diurnal vertical migrations of several hundred meters. Keyed to ambient light (*1, 2*), they move upward from mid-depths at dusk, remain near the surface throughout the night, then descend at dawn to their daytime levels. Parallel movements of ubiquitous, stratified zones of sonic reverberation in the oceans discovered during World War II (*3*) led Johnson (*4*) to the conclusion that similar organisms were the cause of these deep scattering layers. In the intervening 20 years diverse methods have been used in an effort to specifically identify the responsible animals (*5*). The preponderance of evidence implicated meso-pelagic fishes, particularly the Myctophidae (*5, 6*). Of acoustic importance, many of these diminutive lantern fishes have gas-filled swim bladders of such a size as to be resonant for the sound pulses used (12 to 24 kcy/sec) (*7*), and most layers studied by appropriate methods are sharply peaked in narrow frequency bands. This strongly implies resonant scattering from bubble-containing organisms (*8*). Recently, direct observations from the bathyscaphe *Trieste* showed that another type of organism at the opposite end of the phylogenetic scale, siphonophores of the suborder Physonectae (*9*), must be considered as the cause of some layers (*10*). These polymorphic coelenterates consist of various types of individuals arranged along a contractile stem. Because of its gelatinous nature, the colony should have a sound impedance similar to that of water. A terminally born, buoyant individual (pneumatophore), however, generates and retains carbon monoxide bubbles which approximate the resonant size for echo sounder frequencies (*11*). Thus, much of the acoustical evidence implicating fishes with swim bladders (*5, 8, 12*) is also applicable to physonects.

The causative animals, in addition to being efficient sound scatterers, must form widely distributed populations spatially related to the layers. Obviously, they must also undergo vertical migrations of the extent and rate of the recorded layers. Both fishes and physonects are inadequately sampled by standard net tows, and only scant data on their migratory movements are available from this source. Earlier observations from submersible vehicles also leave this question in doubt. Because of operational limitations, the *Trieste* observations had been made on vertical penetrations of the layers at their daytime depths, and apparently the French bathyscaphe dives have also been confined to daylight hours (*13*). With the Cousteau Soucoupe Sous Marine "diving saucer" (*14*), four dives were made on 3 and 4 February 1965, in about 1300 m of water, approximately 10 miles (16 km) southeast of Cape San Lucas, Baja California. Observations were made while the layers were at the surface; at intermediate depths while the layers were migrating upward and downward; and in the upper regions of the main layer while it was at its daytime level. The saucer is a small, two-man vehicle which is limited to a depth of 300 m. This is too shallow to penetrate through most scattering layers at their daytime levels, but maneuverability, ease of launching and recovery, and hovering ability make it ideally suited for such an operation. Thus, more than 14 hours of a 36-hour period were spent in underwater observations, and two complete cycles of scattering layer migrations were studied in detail. This probably constitutes the first *in situ* observation of migrating myctophids and physonects, and permits correlation of these organisms with components of a complex scattering layer.

High-resolution echo sounder records show that multiple and double component layers are common (*2, 5*). The deep scattering layer off Cape San Lucas was no exception. On 31 January, and during the dives, scattering layers were surveyed from the Scripps Research Vessel T-441 with a hull-

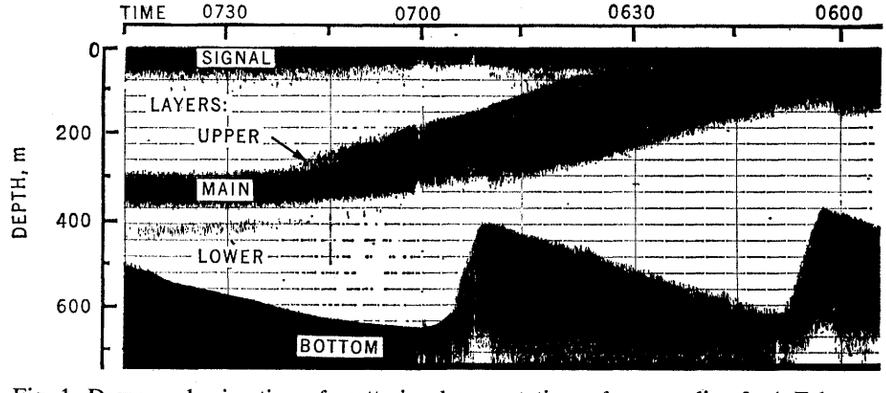


Fig. 1. Downward migration of scattering layers at time of saucer dive 3, 4 February 1965. Resolution between the two components of the "main" layer is vague in the photograph, but clearer in the actual recording. During most of the time, vessel T-441 was drifting. For the brief periods, from about 0601 to 0610 and again from 0650 to 0710, the ship was under way, returning to station. At these times screw and water noise affected the resolution and intensity of the recording, and details of the separation of the "lower" layer from the "main" layer were lost. Time is recorded from right to left, and the 20-fathom (about 40-m) depth lines are broken at 3-minute intervals. Bottom is being recorded on the second cycle, and 732 m should be added for its true depth.