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A simple method of rapid freezing adequately preserves brain tissue for immunocytochemistry, light and electron microscopic examination

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Abstract. A simple and reproducible method for cryopreservation of brain tissue from patients with Alzheimer's disease is described. Fresh brain slices (1 cm thick) obtained less than 6 h postmortem are placed in sealed plastic bags, sandwiched between 0.3-cm-thick aluminium sheets, and frozen by placing the entire "sandwich" between layers of dry ice pellets. The frozen brain slices are stored at −85 °C. Specific anatomic areas can be retrieved at any time for light and electron microscopic, immunocytochemical, autoradiographic and neurochemical studies.

Key words: Brain – Freezing – Immunocytochemistry – Microscopy – Ultrastructure

As part of the neuropathological evaluation of demented patients and nondemented elderly controls studied at the University of Washington's Alzheimer's Disease Research Center, "rapid autopsies" are performed within 6 h after death. To meet the present and future needs for adequately preserved brain tissue for morphologic and neurochemical studies, we devised a simple technique for cryopreservation of brain slices. Specific areas including the deeper structures can later be easily identified and retrieved, and neurochemical, immunocytochemical and microscopic analyses done in serial cryostat sections or after fixation. This technique is readily adaptable for other tissues and diseases and allows valuable tissue samples to be shared by many investigators.

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Material and methods

The brains are removed in the usual fashion [1] usually within 6 h after death. After the weight of the whole fresh brain is recorded, the cerebrum and brain stem with the cerebellum are divided in the mild-sagittal plane. The intact left cerebral hemisphere is fixed by immersion in 10 % phosphate-buffered formaldehyde solution for routine neuropathologic evaluations. The right cerebral hemisphere is sectioned serially in the coronal plane at 1-cm intervals, and the right half of the brain stem and cerebellum transversely, and the brain slices are rapidly frozen, then catalogued and stored at −85 °C for future use.

Freezing technique

To determine the time necessary to achieve a core temperature of −70 °C within individual slices a Type T # 36 Gauge thermocouple probe was used. The brain slices for freezing are put in plastic storage bags which are sealed and placed between two aluminum sheets (30 × 20 × 0.3 cm). This "sandwich" is then placed on top of a bed of dry ice pellets and covered by another layer of dry ice pellets. Subsequent slices are treated in the same manner creating alternating layers of dry ice pellets and the sandwiched brain slices.

To retrieve a structure of interest, the appropriate slice which contains that structure is placed on a stainless steel surface chilled with dry ice and the structure of interest can be broken off after scoring the margins with a scalpel.

Light and electron microscopic studies

Frozen slices from seven brain specimens that had been stored for a minimum of 3 weeks and maximum of 57 months (Table 1) were retrieved. Coronal sections of frontal or occipital pole from seven cases were thawed by immersion fixation in 10 % buffered formaldehyde at room temperature for 7 days or in one case for only 24 h. Entire thickness tissue blocks from these sections were dehydrated and embedded in paraffin in the usual fashion for sectioning [6]. The following stains were utilized: hematoxylin and eosin (H&E), luxol fast blue/periodic acid Schiff reagent-hematoxylin (LFB-PAS-H), luxol fast blue-Nissl (LFB-N) and Weil stains for myelin, modified Bielschowsky (MB) and Holmes' silver, Holzer and thioflavine-S (for UV fluorescence) [6].

Immunocytochemical staining was performed in four cases (Table 1) using polyclonal or monoclonal antibodies against β/A4

Table 1. Cases studied

Age	Sex	Diagnosis	Postmortem interval	Storage time (months)
64	M	A.D.	4 h 34 min	57[a,b]
65	F	Angina	4 h 30 min	7[a,b]
67	F	A.D., D.S.	5 h 25 min	6[a]
92	F	A.D.	3 h 55 min	2[b]
57	F	A.D., Ca	1 h 45 min	2
86	F	Depression	4 h 00 min	1
94	M	A.D.	3 h 10 min	3 weeks[b]

A.D., Alzheimer Disease; D.S., Down syndrome; Ca, Cancer
[a] For electron microscopic examination
[b] For Immunocytochemical studies

Alzheimer amyloid protein (generously provided by Drs. C. Master and K. Beyreuther), β-AP 1-28 (10D5) (Athena Neurosciences, S. San Francisco, Calif.), glial fibrillary acidic protein (GFAP; Dakopatts, Denmark) and a neuronal microtubule-associated protein Tau-2 (Sigma Immunochemicals, St. Louis, Mo.). Antibodies were diluted 1:400, 1:5000, 1:1000, and 1:2000, respectively, and a standard peroxidase-anti-peroxidase [9] or avidin-biotin complex (ABC) [4] techniques with hematoxylin counterstain were used. Formic acid pretreatment was used for the amyloid protein antibodies [5].

For electron microscopic (EM) studies, small fragments of frozen cerebral cortex and white matter from three cases (Table 1) were immersed in chilled Karnovsky's fixative for 4 h, post-fixed in buffered osmium tetroxide, dehydrated and embedded in epon following standard techniques [2]. Ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips 401 electron microscope at 60 kV.

Results

Of 87 cases 82 were autopsied within 6 h and 5 between 7 and 9 h of death, with a range 0.5–9 h (average 4.12 h). A core temperature of −70 °C was reached in individual brain slices within 10 min. Even after storage periods of up to 5 years duration, the various deep structures such as the striatum, thalamus and hypothalamus were easily identified in the individual brain slices.

Light microscopy

Histologic stains. Morphologic studies with all stains at the light microscopic level revealed excellent tissue preservation with little evidence of prior existence of ice crystals. The staining characteristics of the tissue elements were identical to those after primary fixation (Fig. 1A,B) and various argyrophilic deposits [16] were

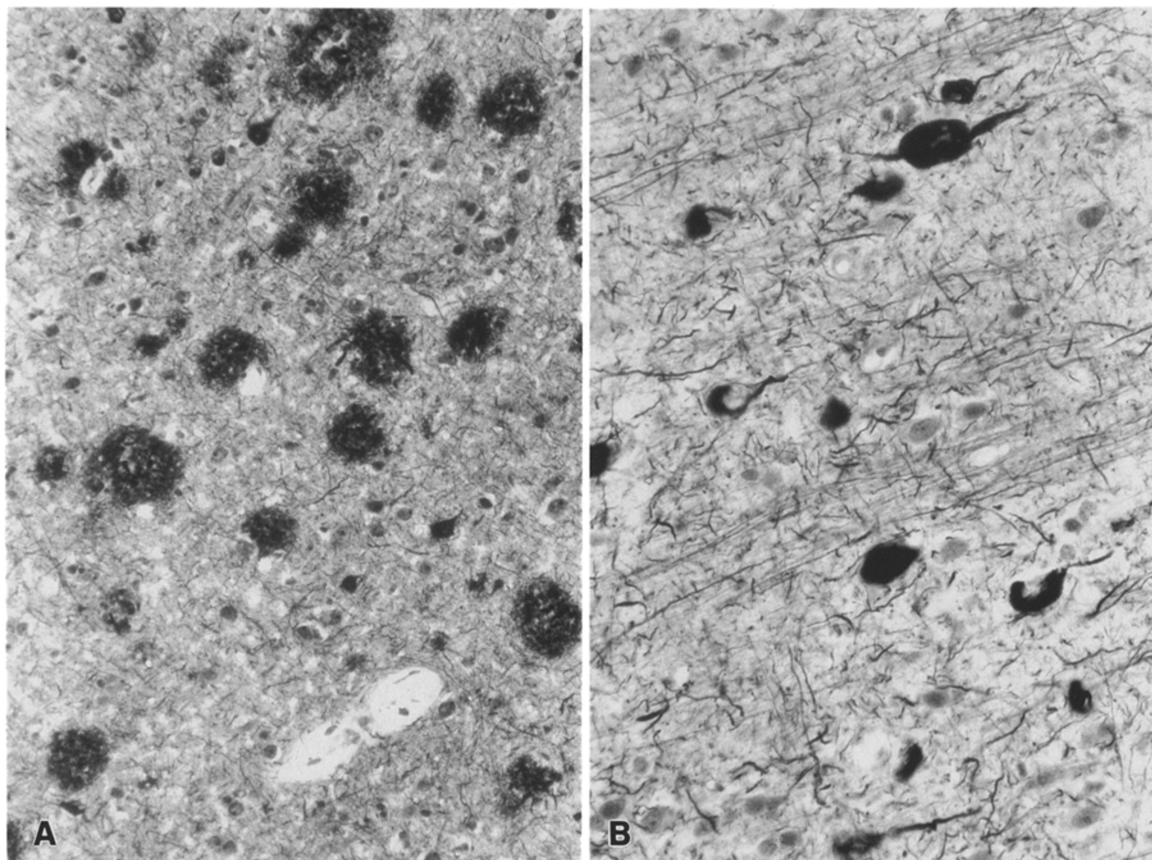

Fig. 1. A Light photomicrograph of cerebral cortex illustrating neuritic plaques with conglomerates of dystrophic neurites without amyloid cores. **B** Light photomicrograph of cerebral cortex illustrating neurofibrillary tangles. **A,B** Modified Bielschowsky silver method, × 1280

readily demonstrated. Small amounts of microscopic ice crystal artifact were present in one of the seven frozen-fixed cases. They consisted of empty vacuoles, a few micrometers in diameter, which occurred in rows running parallel or perpendicular to the pial surface. They were confined to short segments of the cortical subpial region or molecular layer and in some areas the ice crystals extended into the external granular layer but not into deeper cortical layers. They did not interfere with identification of cell types.

Immunocytochemical staining

The anti-β/A4 antibody and anti-β-AP 1–28 (Fig. 2A,D), anti-tau-2 (Fig. 2B), and anti-GFAP (Fig. 2C) antibodies also demonstrated amyloid plaques, neurofibrillary tangles and fibrillary astrocytes, respectively, and they did not differ in appearance from those seen after primary formalin fixation.

Electron Microscopy (Fig. 3)

Tissue preservation at the electron microscopic level was also good with, surprisingly, no evidence of ice crystal formation, and the various cell types (neurons, astrocytes, oligodendrocytes), and the neuronal and astrocytic cell processes were easily identified. The disruption of some cell membranes and variable swelling of mitochondria was similar to that often seen in non-frozen-fixed autopsy material. Paired helical filaments, amyloid fibrils and Hirano bodies were also well preserved.

Discussion

At the light microscopic level, the tissue morphology and staining characteristics of individual components (normal or pathologic) of our frozen-thawed-fixed brain tissue, were comparable to fresh autopsy tissue fixed in buffered formaldehyde. In most samples ice crystal formation was not evident even on electron microscopic examination. When it was present, it was confined to the cortical subpial region or to the molecular layer without spreading to deeper cortical layers, and was not confused with spongiform changes that may be found in some dementing diseases [7], including Alzheimer's disease [8]. At the ultrastructural level, tissue preservation was also good. Tissue degradation was similar to that seen in non-frozen-routinely fixed autopsy material and appeared not to be related to the freezing, but to the postmortem interval to freezing. Preservation of antigenic sites for immunocytochemistry was equally as good as in buffered formalin-fixed paraffin-embedded tissue for the β/A4, β-AP, 1–28, tau-2 and GFAP antibodies.

Rapid freezing of tissues provides adequate preservation of biologic specimens for gross morphologic studies and for studying the elemental composition of cells and tissues by more rigorous techniques, e.g., X-ray microanalysis [17] and electron microscopy [10, 13]. A variety of freezing methods for cryopreservation of tissue have been described: placing tissue slices on a block of dry ice [12], quenching them in cooled isopentane or liquid propane [12], bringing the tissue into contact with polished silver mirrors maintained at temperatures of about −207 °C [15] or by pressing tissue against a cold metal block [3, 11, 14]. All these methods suffer from the small size of tissue which can be adequately preserved or are limited by the depth to which the cold penetrates.

We made no attempt to compare our technique with any of the above-mentioned methods. Our purpose was to develop a simple, reliable and rapid method of freezing for the preservation of brain tissue that can be implemented in neuropathology laboratories, where the storage of tissues at low temperature is becoming a routine requirement to meet the needs for adequately preserved frozen tissue of easily identified anatomic sites, for later examination by immunocytochemical and molecular genetic techniques with adequate morphologic preservation to allow light and electron microscopic monitoring, when necessary, using adjacent tissue sections. This simple method for tissue freezing can be easily adapted to the needs of most laboratories.

The most commonly utilized freezing method in neuropathology laboratories is to place an entire hemisphere in a deep freezer. This technique has several obvious short comings, which include a slow rate of penetration of the cold into the depth of the hemisphere with questionable preservation of some enzyme systems of potential interest and formation of ice crystals with disruption of microscopic and ultrastructural tissue architecture. When it is necessary to retrieve deep nuclear structures for study, the entire hemisphere will have to be warmed to an appropriate temperature for brain sectioning, thus rendering the brain unusable for any further study. Another method used by some investigators consists of sectioning the frozen hemisphere in a custom-made cold saw (personal communication, Dr. Melvyn Ball). Because of the heat generated by friction during the sawing procedure, a variable but unknown thickness of the specimen is also rendered unusable. Another drawback is the high cost of acquiring or custom building the appropriate device. By freezing coronal brain slices we need to retrieve only the appropriate slice which contains the structure of inter-

Fig. 2. A Light photomicrograph of cerebral cortex illustrating positive staining of plaques with and without amyloid cores. Immunocytochemistry, anti-β/A4 Ab, peroxidase-anti-peroxidase method. **B** Light photomicrograph of cerebral cortex illustrating positive staining of dystrophic neurites of plaques and several neurofibrillary tangles. Anti-tau-2 Ab, peroxidase-anti-peroxidase method. **C** Light photomicrograph of cerebral cortex illustrating positive staining of stellate astrocytes. Anti-GFAP Ab. peroxidase-anti-peroxidase method. **D** Light photomicrograph of cerebral cortex illustrating positive staining of plaques. Immunocytochemistry, anti-β-AP 1–28, avidin-biotin complex method. **A,B,D** × 640; **C** × 1280

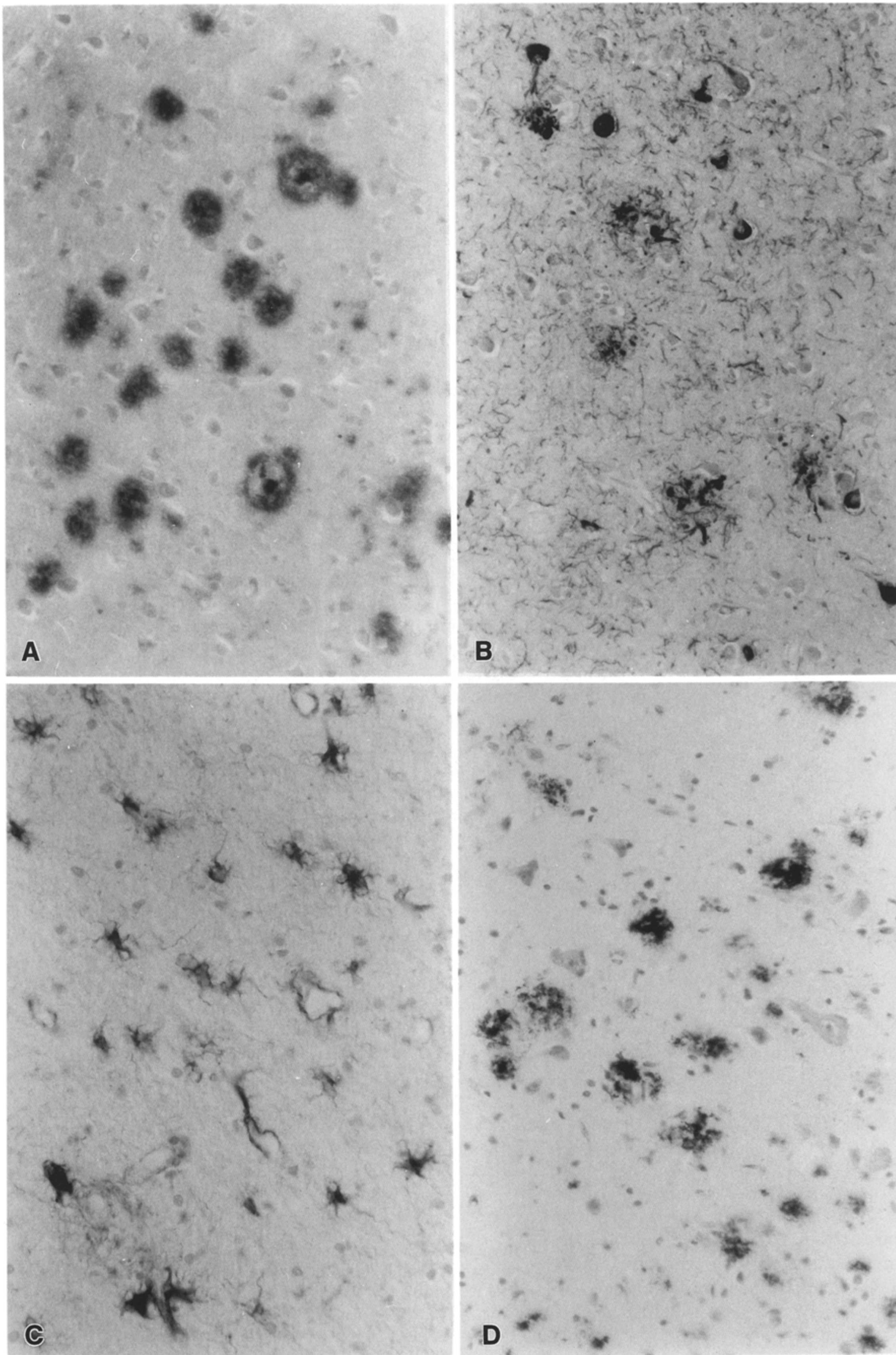

Fig. 2A–D.

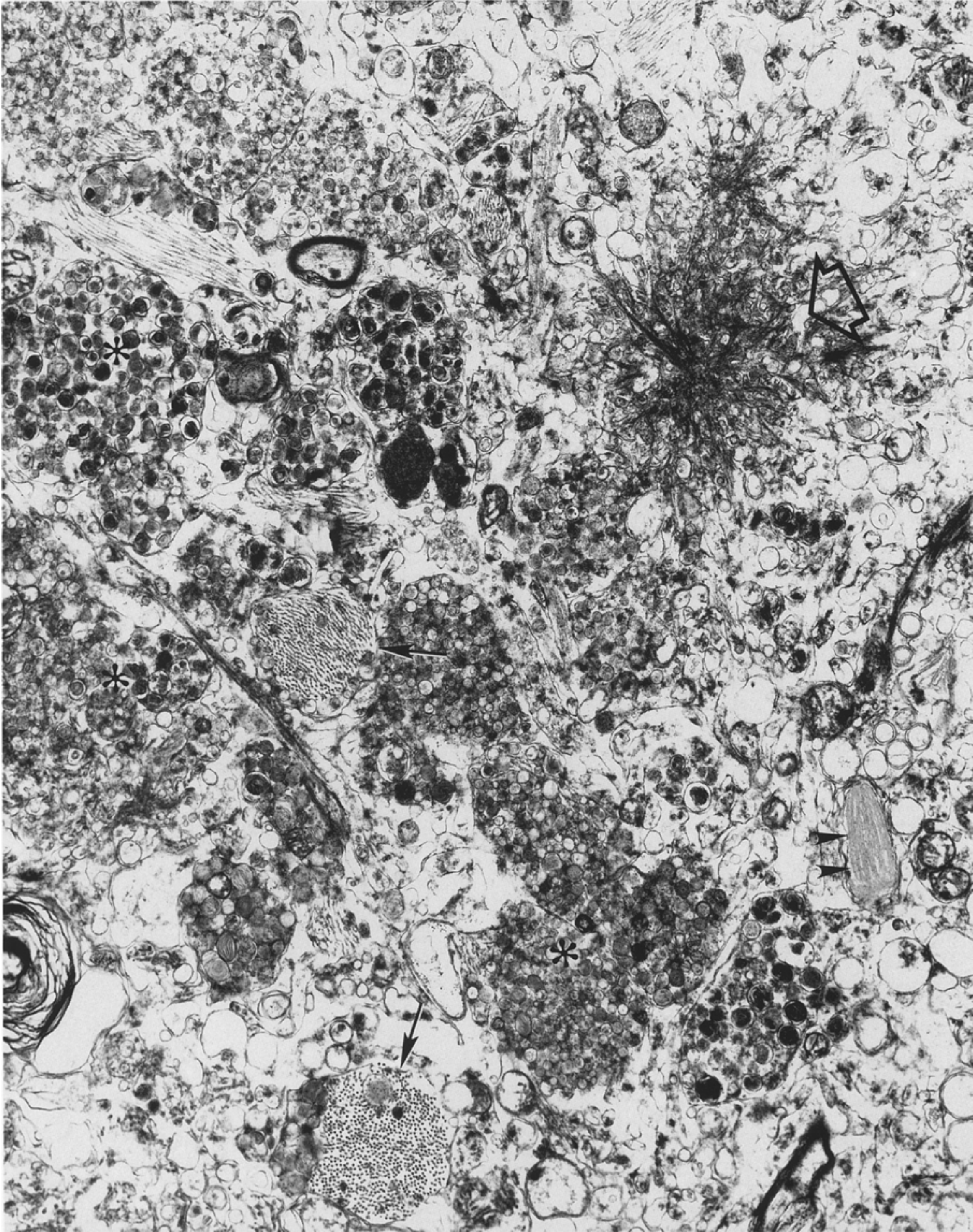

Fig. 3. Electron photomicrograph showing a neuritic plaque with an eccentrically placed amyloid core (*open arrow*), frequent dystrophic neurites some full of dense bodies (*asterisks*) and others with longitudinally, tangentially or transversely sectioned paired helical filaments (*arrows*). Also notice one Hirano body (*arrowhead*). Lead citrate and uranyl acetate, × 17640

est, and that structure can be carved out on a stainless steel surface chilled with dry ice, and the remaining brain slice returned to the freezer.

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