A RELIABLE AND SENSITIVE METHOD TO LOCALIZE TERMINAL DEGENERATION AND LYSOSOMES IN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT. After reconsidering the physicochemical mechanisms involved in the so-called degeneration methods for the demonstration of axons and nerve terminals, the method of Eager was fundamentally modified in order to stabilize the staining process. This resulted in a simple and reliable method which stains degenerating terminals and lysosomes with a high degree of selectivity and sensitivity. Frozen sections 30 to 50 μm thick are prepared from material fixed with formaldehyde by cardiac perfusion. The staining procedure consists of 5 steps: 1) alkaline pretreatment (pH 13), 2) silver impregnation, 3) washing, 4) development at pH 5.0–5.5 monitored by an indicator, and 5) washing in acetic acid. Possible faults can be easily detected by their specific effects on the staining results. Primary submicroscopic silver precipitates are localized selectively in the osmiophilic parts of lysosomes and those degenerating presynaptic elements that are surrounded by glial processes. In degenerating axons, precipitates originating from mitochondria can usually be distinguished from terminal degeneration by their different size, shape, or characteristic arrangement. Non-specific staining is restricted to glial fibrils, erythrocytes, and single cell nuclei. Dark field illumination can be applied routinely and television image analysis can be used for quantitative evaluation because of low background staining.

Recently, the formation of metallic silver and the binding of silver ions by tissue components has been investigated experimentally (Gallyas 1979a–c). Based on these results, it has been possible to study the physicochemical mechanisms involved in the silver staining of neuronal degeneration products (Gallyas et al. 1980). The so-called degeneration methods are rather capricious. The staining of degenerating axon terminals is especially inconsistent and has given rise to a number of modifications (see de Olmos et al. 1980). According to our findings, the technique of Eager (1970) was considered to be a suitable basis for developing a method by which terminal degeneration could be demonstrated with a high degree of reliability. This paper presents several basic modifications of Eager’s staining procedure which allow good control over the factors influencing it.

MATERIALS AND METHODS

Preparation of the tissue. Since the present method contains erythrocytes dark brown to black, it is preferable to prefix the tissue by vascular perfusion. The fixative may consist of 4 to 10 percent unbuffered formal. By using 4 to 5 percent paraformaldehyde buffered in 0.1 M sodium cacodylate (pH 7.2) the co-staining of glial fibrils can be suppressed to some extent, but not completely. Glutaraldehyde is an unfavorable fixative because it produces general staining of mitochondria. The optimum duration of fixation varies between 3 days and about a month. Sections

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299
can be stored for about one week in fixative, provided they are extensively rinsed in water before staining. Frozen sections of adult material should not be thicker than 60 μm.

**Stock solutions.** A. 9% Sodium hydroxide (make with analytically pure reagent which has been stored in an air tight bottle). B. 30 mg Orange G indicator (Code No. 21700, Gurr, Searle Diagnostics, High Wycombe, Bucks, England) dissolved in 1000 ml of 16% ammonium nitrate. C. 50% Silver nitrate. D. 1% Ammonium nitrate. E. Mix 300 ml of 96% ethyl alcohol with 600 ml of distilled water, dissolve 5 g sodium carbonate (anhydrous) in the mixture and make up to 1000 ml with distilled water. F. Dissolve 15 ml of 40% formol, 0.5 g of citric acid, 100 ml of 96% ethyl alcohol and 5-10 ml of Merck Universal Indikator (No. 9175, Merck, Darmstadt, FRG) in 700 ml of distilled water. Titrate the solution with 9% sodium hydroxide (2 to 3 ml) until its color changes from red to yellow (pH 5 to 5.5), then make up to 1000 ml with distilled water. G. 0.5% Acetic acid.

Solutions A to G remain stable for several weeks in closed flasks at room temperature.

**Working solutions. Pretreating solution:** Mix equal volumes of stock solutions A and D, pH 13; this solution is stable for 1 to 2 hours.

**Impregnating solution:** Titrate 100 ml of stock solution B with stock solution A until the mixture changes its color from yellow to pink (approximately 90 ml). Pipette into the mixture an additional volume of solution A equal to 10% of that required for the titration.

The optimum silver content of the impregnating bath should be determined as follows: Treat sections of each specimen according to the instructions below using impregnating solutions prepared by pipetting 0.05, 0.10, 0.15, 0.20 or 0.25 ml of stock solution C into 10 ml of the above mixture. Develop and examine the sections under the microscope. The sections vary in intensity of staining from transparent-colorless through yellow to dark brown. The silver concentration affects only the background staining, and the specificity of staining is not influenced. A pale background is usually preferable, because it produces higher contrast with the specific staining in both bright and dark field illumination. The bulk of the sections cut from the same material can now be treated in an impregnating bath containing the optimum concentration of silver nitrate.

**Washing solution:** Mix 100 parts of stock solution E with one part of D.

**Developing solution:** Mix 100 parts of stock solution F with one part of D.

**Staining procedure.** 1. Immerse 30–50 μm frozen sections of formol-fixed material into the pretreating solution for 2 × 5 min. At each stage of the procedure except step 4 the sections should be agitated for 1 min in the new solution before being unfolded. 2. Transfer them without washing into the impregnating bath for 10 min. 3. Treat for 5 min in three changes of washing solution. 4. Place into the developing solution for about 1 min. The time is not critical. 5. Wash in stock solution G three times for 10 minutes each.

**Technical notes.** The shrinkage of sections in the last step can be avoided by mounting them while they are still in the developer. They can then be dried and washed.

It is advisable to use a fine-meshed nylon sieve stretched on a plastic frame to transport the sections from one solution to another (Fig. 1a). Use of such a device is recommended particularly for the transfer into the developer, i.e. between steps 3 and 4. In this way even large sections can be treated simultaneously and stained uniformly. The sections can be agitated in each solution, but should not float on the surface of the solution. In the case of the developer step (4), the nylon net should be turned and immersed in the solution with the sections downward and should not be agitated.

The working solutions can be used several times; when exhausted the developer turns greenish yellow, indicating that the pH has risen above 6.0. About 20 sections of rat brain can be developed in 50 ml of the solution. All the vessels have to be covered. Otherwise the ammonia content of the pretreating, impregnating and washing solutions decreases and that of the developing solution may increase. Both changes are detrimental to the staining (see Table 1).

The present method works reliably, i.e., the sections take on the same microscopic appearance when the method is repeated with the same solutions and with sections of the same brains. However, there are a few cases in which the degenerating terminals fail to stain in spite of a correct staining procedure. According to our experience occasional failures to stain may originate either from poor fixation, from unfavorable conditions during the storage of the brains, or from some technical fault of the staining procedure. In such cases several sections of a material previously found to stain correctly should be treated together with the sections of the new material. When degenerated terminals stain only in the control sections, then the new material is unsuitable for further processing. When both sets of sections are unsatisfactorily stained, the failure must be due to some technical fault. In this case the microscopic appearance of the sections usually reveals the type of mistake that was made; for the criteria see Table 1.

**Results and Discussion**

The described method produces highly reproducible and selective staining of certain granular structures. In the normal brain, the stained particles show a characteristic distribution in certain parts of the central nervous system. Electron microscopic observations were undertaken on impregnated frozen sections which were osmicated, embedded in Epon, cut and analyzed with or without uranyl acetate and/or lead citrate treatment. These preparations demonstrated that in the normal brain small granular precipitates of silver 0.02 to 0.01 μm in diameter are almost exclusively located in the osmophilic parts ("black bodies") of lysosomes, residual bodies and lipofuscin granules. These may occur in all types of cells: neurons, glial cells, or cells of the blood vessel walls and meninges. A large proportion of the stained particles are, however, located in the somata and dendrites of specific neurons of the normal central nervous system (CNS).

After axotomy of afferent nerves and after brain lesions the number of stainable granules temporarily increases. This increase, as observed in all our experiments, was restricted to those parts of the CNS in which, according to other techniques (electron microscopy, staining of terminal degeneration by other silver techniques,
antegrade transport of tracer molecules), the injured axons terminate. The distribution of fine granules or dust-like precipitates resembles very much that seen in optimal preparations by the methods of Eager (1970) or Pind and Heinmüller (1967) or its modifications (Fig. 1, 1 to 4). One of the advantages of the present method is that unlike in the previously applied methods, optimum results can be obtained in every experiment. The high reproducibility allows densitometric comparisons to be made between serial sections as well as between different brains. Hence, the temporospatial course of the degeneration process can be easily followed (see Wolff et al. 1980). Another advantage is the high sensitivity of the described method. It depends on several factors: (1) the contrast of the staining is very high because of the low density of the background, (2) the high contrast allows dark field illumination to be used even at very low magnifications (Fig. 1, 1 and 2; see Holzgrafe and Wolff 1977), and (3) in regions containing terminal degeneration, the numerical density of stained structures increased. The latter increase is not only caused by degenerating axon terminals, but also by argyrophilic dense bodies and secondary lysosomes stained in the vicinity of degenerating terminals. Many of these lysosomes may not even originate from terminal degeneration, but presumably
represent phagocytosed residues of degenerated unmyelinated axons (Fig. 2, a and c) or cytolytic products in postsynaptic elements. The sharp borders of columnar and laminar subunits of the distribution pattern of cortico-cortical connections as demonstrated by the present method (Wolff and Záborszky 1978) suggest, however, that the vast majority of stained fine granular structures is directly or indirectly related to the degeneration of axon terminals or synapses (Fig. 2b,d,e). Many fiber degeneration products can be discriminated from terminal degeneration by their larger size and shape ("coarse granules," Teuchert et al., unpublished results) and by their characteristic arrangement in rows (Fig. 1d). Thus, the method is a highly selective indicator of terminal degeneration, although not all stained structures represent degenerating axon terminals.

The described method is a sensitive indicator not only of the spatial distribution, but also of the time course of terminal degeneration. For example, in the neocortex of rats small amounts of degeneration products exceeding the low nonspecific background level can be detected as soon as 12 to 16 hours after the injury (Wolff et al. 1980). Degenerating myelinated axons begin to show discrete aggregates of silver precipitates after survival times of 3 days or longer. At the earliest stages, the silver precipitates are limited to the mitochondria (granula mitochondriae) of degenerating axons and axon terminals (Fig. 2f).

Nonspecific deposits of silver sometimes occur in cell nuclei and in the cytoplasm of neurons and glial cells. These submicroscopic precipitates 10–100 nm in diameter do not show a consistent relation to any submicroscopic structure. In the light microscope they cause a diffuse staining which appears yellow to brown or green in bright or dark field illumination, respectively. Its intensity can be controlled or almost entirely suppressed by variations in the pH of the impregnation and developing solution (see technical notes). This controllable variability turned out to be a useful tool for demonstrating the spatial relation between terminal degeneration and cytoarchitecture in some sections, while in other sections of the same series this staining can be almost completely suppressed allowing an analysis of the specific staining in dark field illumination even at low magnifications (Holzgreve and Wolff 1977). Another nonspecific staining concerns glial fibrils. This can be suppressed to some extent by fixing the tissue with neutral formal (pH 7.2), although it cannot be completely avoided. However, as the staining of glial fibers produces a characteristic appearance, they usually do not hinder degeneration analysis. The tendency to stain normal axons is very low and staining of erythrocytes can be avoided by vascular perfusion.

The described method has been tested in several laboratories on various species. It turns out to be a very sensitive tool for localizing degenerating terminals of myelinated and unmyelinated axons. It works consistently in the CNS of rodents and birds, but good results have also been obtained in some invertebrate brains. The main advantages over other degeneration methods are the following: (1) the background staining is controllable and can be kept consistently low, (2) occasional technical faults can be rapidly diagnosed, (3) the high contrast of the specific staining increases the sensitivity for small amounts or small variations of terminal degeneration, and (4) the good reproducibility of the results allows quantitative analysis.

Fig. 2. Electron microscopy of silvered degeneration products in frozen sections of the rat neocortex 2 days after callosotomy; bar = 1 μm. a) Profile of a neuronal process containing two mitochondria. The cytoplasm seems to be condensed and there is some silver precipitate in one mitochondrion. b) Dense degeneration of a presynaptic element connected to a postsynaptic thickening (arrow). The silver precipitate consists of a grain lying in part over a mitochondrion and of irregular silver deposits extending to the outer limits of the degenerating terminal. c) Dense degeneration of a neuronal profile, probably an unmyelinated axon, containing a small silver deposit in its mitochondrion. d and e) Dense degeneration of neuronal profiles.
evaluation. Additionally, the spatial resolution is higher than in autoradiograms following anterograde axon transport of labeled amino acids.

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