A reduced silver staining method applicable to dense neuropiles, neuroendocrine organs, and other structures in insects

Structural study of the insect brain has been hampered by a lack of adequate reduced silver impregnation methods. Several reduced silver methods have been offered in recent years for use with insects[2,3,5,7,9,11] and these or other reduced silver procedures have been employed in numerous recent studies[1,6,8,12,13], but it is evident from published photographs that successful impregnation, when present at all, is usually limited to coarse neuropiles. Methods for use on paraffin sections have not shown any general capability for impregnating fine fibers of dense neuropiles of particular interest, such as the calyces of the corpora pedunculata or the medulla externa of the optic lobes.

A reduced silver method is described here which has been successfully used in studies of the brain of the American cockroach, *Periplaneta americana* (L.), and the red-legged grasshopper, *Melanoplus femurrubrum f.* (de Geer)[14,15] (Weiss, in preparation), and which has also succeeded in other insects and a crustacean. The method provides excellent impregnations of fine fibers in dense neuropiles throughout the brain and optic lobes. It can also provide excellent fiber impregnations within (among other structures) the neuroendocrine organs of insects: this ability is of special interest in view of the fact that, so far as I am aware, nerve fibers have not previously been demonstrated within these much-studied organs through use of metallic impregnation techniques.

The method is an improved version of the Holmes–Blest collidine technique[3], which is modified primarily in the use of different (and in part more precisely specified) procedures for fixation, embedding, and toning. The fixative employed in the present technique, Bodian's fixative No. 2[4], produces excessive brittleness in the brain of many insect species, a fact which necessitates careful attention to further processing of the tissue if series of smooth sections are to be reliably obtained. The method, in its most recent form, is applied to the insect brain as follows.

(1) Anesthetize in CO_2, in which animal remains for 0.5 min after ceasing motion.

(2) Pin onto wax plate; carefully and quickly expose frontal and dorsal surfaces of brain, taking care to avoid subjecting the tissue to pressure, stretch, or drying.

(3) Plunge severed head into Bodian's fixative No. 2 (5 ml formalin, 5 ml glacial acetic acid, 90 ml 80% ethanol); fix 18 h (a fixation period of 3 h or even less, tested in *P. americana*, yields equally successful if not better impregnations in this species, and seems to decrease tissue brittleness).

(4) Wash in 3 baths of 70% ethanol over 1–2 days. Store in 70% ethanol. Dissect out brain before proceeding.

(5) Bring through this sequence, with occasional agitation in all steps (except as noted) prior to impregnation with pure molten wax: (a) 82% ethanol, 2 min; (b) 95% ethanol, 3 min; (c) 95% ethanol–terpineol, 3 : 1 (v/v), 4 min; (d) 95% ethanol–terpineol, 1 : 1, 5 min; (e) 95% ethanol–terpineol, 1 : 3, 6 min; (f) terpineol, 2 baths totaling 1.5 h; (g) benzene, 2 baths, 30 sec each, with *constant* agitation; (h) benzene –

paraffin, 1 : 1 (v/v), fully liquefied, 2 min; (i) paraffin in oven, 2 baths, 5 min each; (j) embed in fresh paraffin. 'Bioloid Paraffin Embedding Compound' (Scientific Products, Division of American Hospital Supply Corp.) of either 56–58° C mp (*M. femurrubrum*, *P. americana*) or, more recently, 50–52°C mp (*P. americana*, for which it seems preferable) has been used, with the oven maintained as close as possible to 60.0°C or 54.0°C, respectively.

(6) Section serially at 10 μm as slowly as practicable, using *sharp* blade. The exceptionally sharp razor blades currently sold under the name 'Gem Blue Star Super Single Edge' (American Safety Razor Co., Division of Philip Morris, Inc.) have been found useful for this purpose. For reliable ribboning under these conditions, coat trimmed block heavily on 2 sides with 'Parowax' household paraffin or a similarly sticky wax. The sections may smell faintly of terpineol, but its presence seems in practice to be harmless. Mount on slides generously coated with Mayer's albumin fixative. For sharpest results, it is probably safest to stain within 2–4 days.

(7) From this point on follow Blest's *collidine* modification of Holmes' method, with the following changes: (a) Tone in *1.0%* gold chloride for *7* min. (b) Following toning, limit the distilled water rinse to *1 brief dip*. (c) Reduce in oxalic acid for *10* min.

The slides are brought up through an ethanol series into xylene and mounted using 'Permount' (Fisher Scientific Co.).

Additional comments. Vertical Coplin jars are used in processing the slides. For convenience, 27.5 ml of one-fifth *molal* boric acid (*i.e.*, 1.24 g boric acid per 100 ml distilled water) and 22.5 ml of one-twentieth *molal* borax (*i.e.*, 1.91 g borax per 100 ml distilled water) have been substituted for the like amounts of *molar* solutions stipulated in Blest's formula. Both the impregnation solution *and* the jars containing it are prewarmed to $\sim$ 37°C before receiving the slides, and the jars are subsequently wrapped in aluminum foil to insure total darkness while in the oven. The slides should also be maintained in total darkness while in 20% silver nitrate.

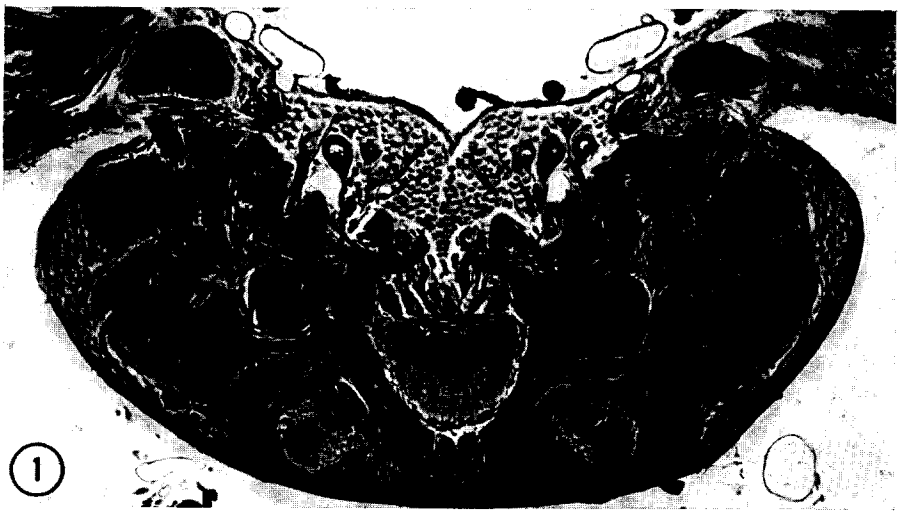

Fig. 1. *M. femurrubrum* ♂. Brain and portion of optic lobes. This and all other photographs are of 10-μm sections of adult material. × 86.

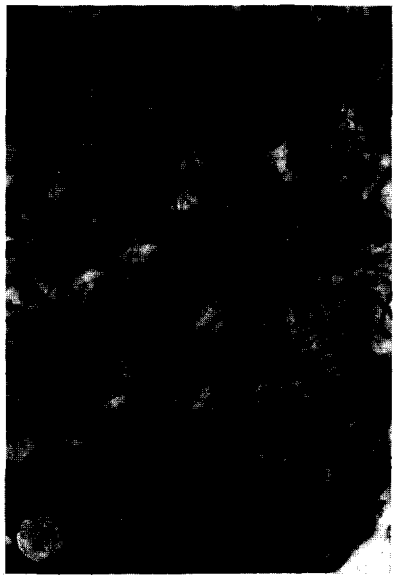

Fig. 2. *M. femurrubrum* ♂. Accessory calyx of corpus pedunculatum. The arrow points to a fiber of ~ 0.2 μm diameter. × 900.
Fig. 3. *M. femurrubrum* ♀. Medulla externa of optic lobe. The arrow points to a fiber of ~ 0.3 μm diameter. × 900. Impregnated at room temperature (~ 25°C) (comparable results follow impregnation at 37°C).

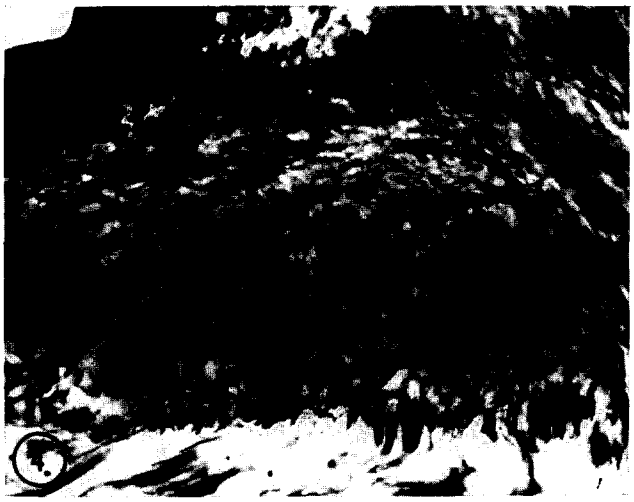

Fig. 4. *P. americana* ♂. Lower wall of lateral calyx of the corpus pedunculatum. × 460.

Comparison of Figs. 1–5, illustrating successful preparations of *M. femurrubrum* and *P. americana*, with photographs of both the original[3] and more recent[10,12] Holmes–Blest preparations from various insects indicates the improvement furnished by the present technique. In addition to a generally clearer and finer impregnation,

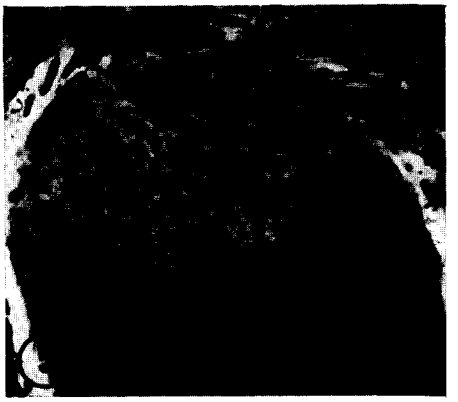

Fig. 5. *P. americana* ♂. Distal end of α lobe of corpus pedunculatum. × 360.

gross preservation is equal and perhaps superior to that furnished by the original Holmes–Blest method. Fibers measuring as little as 0.2–0.3 μm in diameter in the fixed condition are clearly visible. Useful chromatic differentiation, though not apparent in the accompanying illustrations, occurs within sections.

Comparable results have been obtained with the brain or optic lobes of other species: the acridid grasshoppers *Dissosteira carolina*, *Arphia pseudonietana*, and *Syrbula admirabilis*; the tettigoniid grasshopper *Orchelimum nigripes* and katydid *Amblycorypha oblongifolia*; and the bumblebee *Bombus bimaculatus* (Fig. 6). The success of a similar method (involving the same course of fixation and staining but

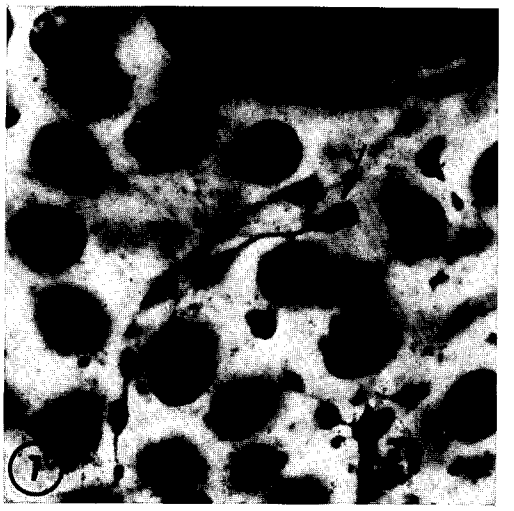

Fig. 6. *B. bimaculatus* queen. Medulla externa of optic lobe, showing branching fiber, which measures ~ 0.2 μm at the arrow. × 1290.

Fig. 7. *P. americana* ♂. Corpus cardiacum, showing short length of a narrow varicose fiber (arrow). A long series of varicosities of an out-of-focus, adjacent fiber can also be seen arching toward the lower left. × 1140.

Fig. 8. *M. femurrubrum* ♂. Corpus allatum. The arrows point to two groups of nerve fibers. × 490.

differing in pre-fixation, embedding, and sectioning procedures) in producing excellent impregnations of the brain of the spiny lobster *Palinurus vulgaris* indicates that the present technique, suitably modified when necessary to take into account such factors as organ size, may prove applicable over a range of arthropod groups.

Structures outside the central nervous system also may yield excellent preparations. Of significance for insect neuroendocrinology is the demonstration of fine, varicose fibers within the corpora cardiaca of *P. americana* (Fig. 7) and the demonstration of nerve fibers within both the corpora allata (Fig. 8) and corpora cardiaca of *M. femurrubrum*. In addition, successful impregnations have been observed in the ocelli of *D. carolina*, *A. pseudonietana*, *M. femurrubrum*, and *B. bimaculatus*, and in the hypocerebral ganglion of *M. femurrubrum*.

The consistency with which the method succeeds varies among different species. In adult *M. femurrubrum*, approximately one-half of the preparations of the brain and optic lobes resemble in quality those illustrated. A comparable, if not greater, proportion of successful impregnations occurs with the brain of *P. vulgaris*. In adult *P. americana*, on the other hand, the proportion is approximately one-fifth, and even then only perhaps one-half of the tissue may show this 'ideal' impregnation (nevertheless, many more of the impregnations are sufficiently demonstrative to provide useful information). As with other silver methods, therefore, the success rate of the present method when applied to a new organism cannot be predicted in advance. In species for which the method is suited, however, the preparations obtainable show a wealth of detail adequate for the most critical neuroanatomical study.

I am indebted to Drs. Richard D. Alexander and John S. Edwards for encouragement and advice.

This work was partly supported through Graduate Fellowships from the N.S.F. and Postdoctoral Research Fellowships from the National Institute of Neurological Diseases and Stroke (Nos. 1 F02 NS40187-01 and 2 F02 NS40187-02). A portion is taken from a dissertation submitted in partial fulfilment of the requirements for the Ph.D. degree at the University of Michigan.

Department of Zoology,
University of Michigan,
Ann Arbor, Mich. (U.S.A.)

MITCHELL J. WEISS*

1 ÁBRAHÁM, A., Die Struktur der Gehirnzentren des Gelbrandkäfers (*Dytiscus marginalis* L.), *Z. mikr.-anat. Forsch.*, 76 (1967) 435–465.
2 BACCETTI, B., L'impregnazione del sistema nervoso degli insetti esequita secondo una nuova modifica al metodo Samuel, *Boll. Soc. ital. Biol. sper.*, 34 (1958) 844–847.
3 BLEST, A. D., Some modifications of Holmes's silver method for insect central nervous systems, *Quart. J. micr. Sci.*, 102 (1961) 413–417.
4 BODIAN, D., The staining of paraffin sections of nervous tissues with activated protargol. The role of fixatives, *Anat. Rec.*, 69 (1937) 153–162.
5 CHEN, J. S., AND CHEN, M. G. M., Modifications of the Bodian technique applied to insect nerves, *Stain Technol.*, 44 (1969) 50–51.
6 DRESCHER, W., Regenerationsversuche am Gehirne von *Periplaneta americana* unter Berücksichtigung von Verhaltensänderung und Neurosekretion, *Z. Morph. Ökol. Tiere*, 48 (1960) 576–649.
7 GREGORY, G. E., Silver staining of insect central nervous systems by the Bodian protargol method, *Acta Zool.*, 51 (1970) 169–178.
8 HECKER, H., Das Zentralnervensystem des Kopfes und seine postembryonale Entwicklung bei *Bellicositermes bellicosus* (Smeath.) (Isoptera), *Acta Trop. (Basel)*, 23 (1966) 297–352.
9 LARSEN, J. R., The use of Holmes' silver stain on insect nerve tissue, *Stain Technol.*, 35 (1960) 223–224.
10 PEARSON, L., The corpora pedunculata of *Sphinx ligustri* L. and other Lepidoptera: an anatomical study, *Phil. Trans. roy. Soc. B*, 259 (1971) 477–516.
11 ROWELL, C. H. F., A general method for silvering invertebrate central nervous systems, *Quart. J. micr. Sci.*, 104 (1963) 81–87.
12 STRAUSFELD, N. J., AND BLEST, A. D., Golgi studies on insects. Part I. The optic lobes of Lepidoptera, *Phil. Trans. roy. Soc. B*, 258 (1970) 81–134.
13 VOWLES, D. M., The structure and connexions of the corpora pedunculata in bees and ants, *Quart. J. micr. Sci.*, 96 (1955) 239–255.
14 WEISS, M. J., Structure and fiber connections of the calyces in the brain of the American cockroach, *Periplaneta americana* (L.), with comparative observations on an acridid grasshopper, *Melanoplus femur-rubrum f.-r.* (DeGeer), Ph. D. Dissertation, University of Michigan, 1970, pp. vi + 182. (No. 71-4768, University Microfilms, Inc., Ann Arbor, Mich., U.S.A.)
15 WEISS, M. J., From soma to synapse: the tracing of fiber inputs of a complex brain center in the American cockroach, *Periplaneta americana* (L.), *Amer. Zool.*, 11 (1971) 696–697.

(Accepted January 18th, 1972)

* Present address: Department of Zoology, University of Washington, Seattle, Wash. 98195, U.S.A.