Some Modifications of Holmes's Silver Method for Insect Central Nervous Systems

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With two plates (figs. 1 and 2)

Summary

Two main modifications of Holmes's method are described. In both the amount of silver nitrate in the impregnating bath is increased, and larger quantities of higher pyridine derivatives are substituted for pyridine. The preferred method for general purposes employs 2·4% 2:6 lutidine at pH 8·4, and gives a bluish picture with improved selectivity of fibre staining. The use of 1·6% 2:4:6 collidine gives a reddish picture, with highly selective fibre staining, and sharp resolution of neurosecretory cells. Minor modifications of pre-treatment and reduction procedures are also described.

Introduction

HOLMES'S method for the silver impregnation of paraffin sections was originally devised for the vertebrate peripheral nervous system (Holmes, 1947). It has since been used on invertebrate material with varying degrees of success (Vowles, 1955; Larsen, 1960). Certain materials, e.g. the brains of lepidopterous larvae (Van der Kloot and Williams, 1954) have proved difficult to stain.

Unsatisfactory impregnation of the brains of adult tussore silk moths (*Antherea mylitta*) and of *Automeris* spp. has led to an exploration of some modifications of Holmes's method other than mere manipulation of the pH of the impregnating solution.

Materials and Methods

Brains of the desert locust (*Schistocerca*), the honey bee (*Apis*), *Antherea*, and *Automeris* spp. were fixed for 24 h in alcoholic Bouin's solution, washed in 50% alcohol, and embedded in 56° C wax after dehydration through alcohols, cedarwood oil, and benzene. Sections were cut at 10 μ. After dewaxing, the sections were brought to distilled water as rapidly as possible. They were then processed as follows:

1. 20% silver nitrate.	2 or 3 h
2. Rinse in distilled water.	5 min

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3. Incubate in the following solution at 37° C:

M/5 boric acid	27·5 ml	
M/20 borax	22·5 ml	
1% silver nitrate solution	5 to 10 ml	
pyridine or derivative	2 to 6 ml	
distilled water	250 ml	16 to 20 h
(pH 8·4 at 20° C, by B.D.H. narrow-range indicator strips)		

4. Place without washing in the following solution:

hydroquinone	3 g	
sodium sulphite	30 g	
distilled water	300 ml	3 min

The initial temperature of this reducing bath was 60° C. It fell to 55° C at the end of the treatment.

5. Wash in running tap-water. 3 min
6. Rinse in distilled water. 3 min
7. Tone in 0·2% sodium gold chloride solution. variable (1 to 3 min)
8. Rinse in distilled water. 1 min
9. Reduce in 2% oxalic acid. 5 min
10. Wash in distilled water. 3 min
11. Fix in 5% sodium thiosulphate. 1 min
12. Wash thoroughly in distilled water, dehydrate, and mount.

The following substances have been used in the impregnating bath:

pyridine.	6 ml	with 10 ml silver nitrate in 250 ml of buffer
α-picoline (1-methylpyridine)	6 ml	
2:4 / 2:5 lutidine (dimethylpyridine)	6 ml	
2:6 lutidine (dimethylpyridine)	6 ml	
2:4:6 collidine (trimethylpyridine)	4 ml	
2-methyl-5-ethyl-pyridine	2 ml	with 5 ml silver nitrate in 250 ml of buffer

Solutions containing 2:4 / 2:5 lutidine were filtered before use. In all cases thorough shaking was necessary during the addition of the usually poorly miscible higher derivatives. The amounts of collidine and of 2-methyl-5-ethyl-pyridine are about the maximum which is miscible without separating out during incubation.

A few tests were made with cat and rat brains perfused with formalin solution and cut at 15 μ. For these materials the amount of silver in the impregnating baths was reduced to 3 ml of 1% silver nitrate.

FIG. 1 (plate). A, α-lobe of corpus pedunculatum of *Apis*, cut transversely towards the distal end. 2:6 lutidine method.

B, α-lobe of *Apis*, cut at the same level as A. Pyridine method.

C, transverse section of the medial part of the protocerebrum of *Automeris aurantiaca*. 2:6 lutidine method.

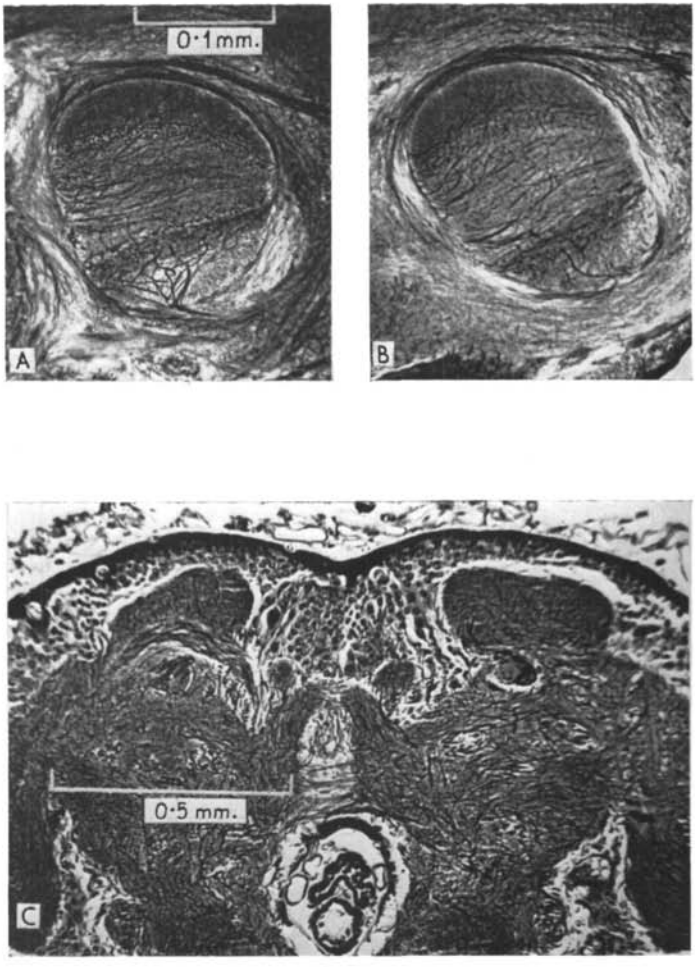

FIG. 1

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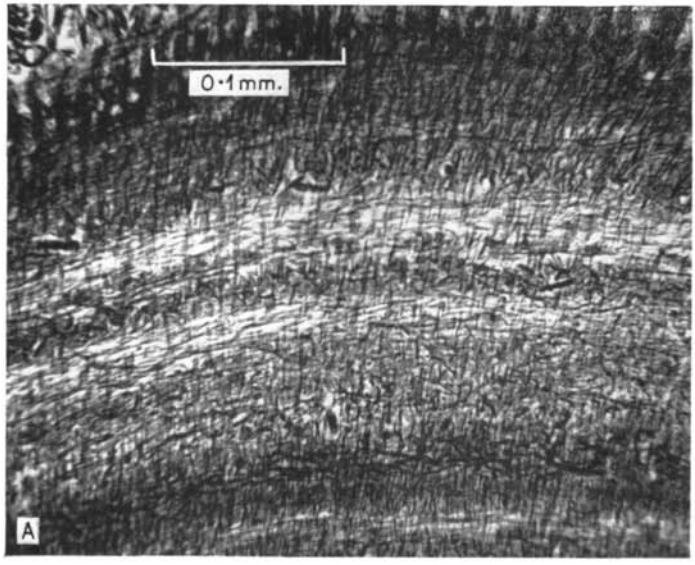

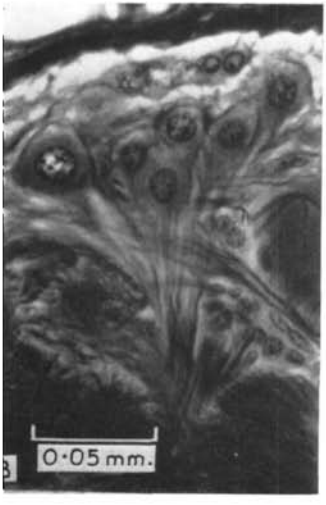

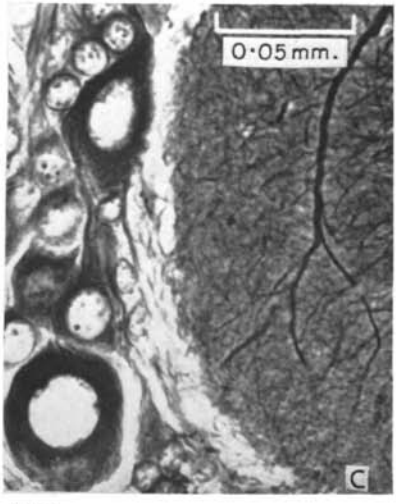

FIG. 2

Critical comparisons were confirmed with sections derived from single brains.

Results

Some of the results obtained are shown in figs. 1 and 2.

Differences between materials. Good impregnation of the locust and bee brains was obtained with all the derivatives tested. Results with *Antherea* and *Automeris* were always adequate and sometimes excellent.

Differences between the pyridine derivatives. Despite considerable variation between batches, the following differences were clear-cut and replicable. Increasing the number of alkyl groups on the pyridine molecule leads to an increase in the specificity of fibre staining, which does not appear to be the result of increased silver uptake, but due to a reduction of the degree of background staining of the neuropile. There is a consistent impression that the proportion of small fibres separately stained is greatest in the lutidine preparations. There is also an increase in the differential staining of the fibre tracts. Pyridine preparations show, for example, some differences in the staining reactions of the large tracts linking the optic centres, and the fine fibres of the calyces of the mushroom bodies. These differences are exaggerated in the lutidine and collidine preparations and also in some *Automeris* brains processed with 2-methyl-5-ethyl-pyridine. Further, there is a decrease in the staining of the normal nerve-cell bodies supplying the neuropile. In lutidine preparations the nuclei are well stained, while the cytoplasm is clear. In collidine preparations the nuclei, too, are more weakly stained. Beyond this, there is an increase in the differential staining of the neurosecretory cells both in the medial and medio-lateral groups of the protocerebrum, and in the suboesophageal region. This increase does not extend to the neurosecretory cell nuclei, and neurosecretory cells do not invariably show denser staining than that of the neurones surrounding them; the latter variable may perhaps prove to relate to secretory phase.

In addition there are differences in the general colour of sections stained in the presence of the various derivatives. The usual colours assumed by fibres of medium thickness of the protocerebral neuropiles of both *Schistocerca* and *Antherea* are:

pyridine—reddish purple
α-picoline—reddish-bluish
lutidines—very bluish
collidine—reddish
2-methyl-5-ethyl pyridine—reddish-purple (*Automeris* only)

The few results obtained with vertebrate tissues showed similar trends.

Fig. 2 (plate). A, second optic glomerulus of *Schistocerca*. 2:6 lutidine method.

B, group of 6 medio-lateral protocerebral neurosecretory cells from *Antherea*. 2:6 lutidine method.

C, portion of a calyx of corpus pedunculatum of *Schistocerca* with a large branching afferent fibre (on right), and adjacent secretory and non-secretory cell-bodies (on left). 2:4:6 collidine method.

Discussion

The mechanisms of silver impregnation are still imperfectly understood (Peters, 1955; Wolman, 1955) and these modifications to Holmes's method must be regarded as empirical. The results obtained with the various pyridine derivatives are sufficiently striking to suggest that they deserve to be tried as possible alternatives to pyridine in all those silver-staining methods which involve treatment or pre-treatment with solutions containing pyridine.

The following general conclusions may be reached. α-Picoline shows no advantages over pyridine that do not appear in more marked form in lutidine preparations. The blueness of the lutidine preparations is a definite advantage, and recommends its routine use. No distinction could be made between 2:6 lutidine and the 2:4 / 2:5 lutidine mixture; both yielded equally good preparations, which were invariably superior to pyridine preparations from the same batch; but since none of the samples of 2:4 / 2:5 lutidine used was completely miscible with water, the use of wholly miscible 2:6 lutidine is to be preferred. This modification is now used as a standard procedure for the examination of *Antherea* and *Automeris* brains after surgical interference in early pupal development, and it has yielded uniformly satisfactory results.

The results obtained with solutions containing collidine suggest that this substance may well be of value where a sharp discrimination of neurosecretory cells is required, and for fine detail. Sections treated with 2-methyl-5-ethyl pyridine, however, have tended to show a poor definition of fine fibres in the neuropile, and a coarsely granular impregnation.

The general trends observed with insect material were also noted in the few preparations which were made of vertebrate material. Here the decrease in the staining and resolution of background structure which follows the use of higher pyridine derivatives is a disadvantage. While it has proved that lutidine and collidine can be usefully employed for special purposes, e.g. in modifications of the Nauta-Gygax technique for degenerating fibres (Guillery, Shirra, and Webster, 1961), most probably the benefits which it confers over pyridine for vertebrate brains are no more than marginal.

Although the methods described have invariably given satisfactory results with brains of *Schistocerca*, *Apis*, *Antherea*, and *Automeris*, a few attempts to stain sphingid brains were not successful. As with the pyridine-silver technique, particular materials will doubtless demand appropriate alterations to the pH and silver concentration of the impregnating bath, and the selection of an optimal fixative solution.

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REFERENCES

GUILLERY, R. W., SHIRRA, B., and WEBSTER, K. E., 1961. Stain Tech., **36**, 9.
HOLMES, W., 1947. In *Recent advances in clinical pathology*, ed. by S. C. Dyke, p. 402. London (Churchill).
LARSEN, J. R., 1960. Stain Tech., **35**, 223.
PETERS, A., 1955. Quart. J. micr. Sci., **96**, 301.
VAN DER KLOOT, W. G., and WILLIAMS, C. M., 1954. Behaviour, **6**, 233.
VOWLES, D. M., 1955. Quart J. micr. Sci., **96**, 239.
WOLMAN, M., 1955. Ibid., **96**, 337.