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A simple and reliable method for the silver impregnation of nerves in paraffin sections of soft and mineralized tissues

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INTRODUCTION

Numerous silver impregnation methods for the demonstration of nerves in paraffin sections have been described (Davenport, 1930; Bodian, 1936; Holmes, 1943; Romanes, 1950; Ungewitter, 1951; Samuel, 1953*a*; Fearnhead & Linder, 1956; Rowles & Brain, 1959). However, many are technically difficult to perform and, tested by the criteria that all nerves should be impregnated, that they should be clearly differentiated from other structures and that their morphology should be clearly shown, most methods fall short. It was therefore decided to investigate the factors which may be responsible for specificity and quality of the silver impregnation.

Guided by previous investigations of the basic mechanisms underlying the reaction (Samuel, 1953*b*; Peters, 1955*a*, *b*; Wolman, 1955; Fearnhead & Linder, 1956; Rowles, 1960) these factors seem likely to include: methods of fixation and tissue preparation; the conditions under which the silver impregnation is carried out, in particular the concentration and type of silver compound used, pH and buffer type; the temperature and incubation time; the characteristics of the developing solution used to produce the final visible end result. Each of these factors has been examined, and a simple method for the demonstration of nerves has been evolved. The effects of variation, and the technique are described.

Recommended technique

Fixation and processing

Specimens are fixed where possible by perfusion, otherwise small thin pieces of soft tissue are immersed in the fixative. For study of the pulps of fully developed teeth the root apices must be cut off immediately after extraction, or else the tooth must be split longitudinally. The fixatives used are 4% formaldehyde saline, Baker's formaldehyde calcium chloride, Lillie's formaldehyde calcium acetate and Bouin's fluid (Lillie, 1965). Calcified tissues are fixed for about 1 week and then transferred to a 10% (w/v) solution of sodium EDTA at pH 7·0, or to a buffered solution of formic acid (Kristensen, 1948). The end-point of decalcification is determined by radiography, and the decalcifying agent carefully removed by washing the tissue in running tap water for several hours. The specimen is then dehydrated in ethyl alcohol, transferred to benzene or chloroform, and infiltrated in paraffin wax (melting point 56 °C). Sections ranging from 5 to 10 μm in thickness are cut and mounted on slides using a hot plate at 45 °C to flatten and dry them.

Solution required

(*a*) *Buffer stock solution.* A 0·1 M solution of 2,4,6-collidine (Hopkin & Williams, material for chromatography) is prepared by dissolving 6·5 ml of collidine in approximately 450 ml distilled water. The solution is then filtered to remove all traces of solid and a sufficient volume of 10% (w/v) solution of nitric acid (Analar) added to bring the pH to 7·2. The volume is then made up to 500 ml with distilled water. This solution can be stored indefinitely at +4 °C.

(*b*) *Diluted buffer.* To obtain a working solution 8 ml of stock solution is added to 92 ml distilled water, which is then heated to the temperature at which the impregnation is carried out, namely 40 or 60 °C.

(*c*) *The silver impregnating solution.* Eighty four ml distilled water is heated to about 60 °C, then, in the following order, are added to it: 4 ml 1% (w/v) silver nitrate, 4 ml 0·38% (w/v) sodium cyanate and finally 8 ml collidine buffer. (Stock solution.)

(*d*) *Developer stock solution.* Twenty g sodium sulphite ($Na_2SO_3 . 7H_2O$, Analar) and 4·75 g borax (Analar) are dissolved in about 400 ml distilled water by heating to about 50 °C. Ten g of sheet gelatine (Belgian Bronze Label) is then added to this solution while still hot and stirred until dissolved. The volume is then made up to 500 ml with distilled water.

(*e*) *Developer working solution.* To obtain a working solution 5 ml of 2% (w/v) quinol is added to 95 ml developer stock solution and thoroughly mixed. To activate the developer 2 ml 1% (w/v) silver nitrate (Analar) is added, with constant stirring. The temperature of the developer should be approximately 25 °C; if the temperature falls below 20 °C the gelatine solidifies, resulting in uneven deposition of silver; if on the other hand the temperature is above 30 °C the speed of development increases to such an extent that its control is difficult. The final solution can be kept until turbidity develops and non-specific precipitation of silver takes place. All the stock solutions, with the exception of the 2% quinol, are completely stable. The quinol can be used over several weeks, particularly if stored in a stoppered bottle and refrigerated; the slight brown discoloration which develops with time does not interfere with its activity.

The silver impregnation

Sections are de-waxed with xylene, transferred to absolute alcohol, and then covered with a thin film of celloidin by dipping the slide into a 0·2% (w/v) solution of celloidin in absolute alcohol–ether (50:50). The slides are then carefully drained and, after partial drying, the celloidin film is hardened by immersion in 70% alcohol. Sections are then washed, first in running tap water for 30 minutes, and then in distilled water (three changes) to remove all traces of fixative and/or acid. The sections are next placed in diluted buffer where they are left either overnight (about 16 hours) at about 40 °C or for 10–30 minutes at 60 °C. The longer incubation at the lower temperature is more suitable for decalcified tissue, but sections of soft tissue give good results under both conditions.

Sections are then transferred into the silver impregnating solution at the same temperature as that chosen for the previous buffer stage and incubated for 1½–2 hours at 40 °C or 10–30 minutes at 60 °C; they are washed in several changes of distilled water for 3 minutes and then transferred to developer at approximately 25 °C. The

development of the primary silver impregnation in the sections, which up till now have remained colourless, starts slowly, but as soon as the sections have taken on a pale golden tone, speed of development increases. Progress is monitored easily with the naked eye, or, if desired, by rinsing in distilled water and examining with the microscope. When results are judged to be optimal, i.e. maximum contrast between nervous tissue and background, the sections are washed in distilled water, dehydrated with ethanol, cleared in xylene and mounted in Canada balsam.

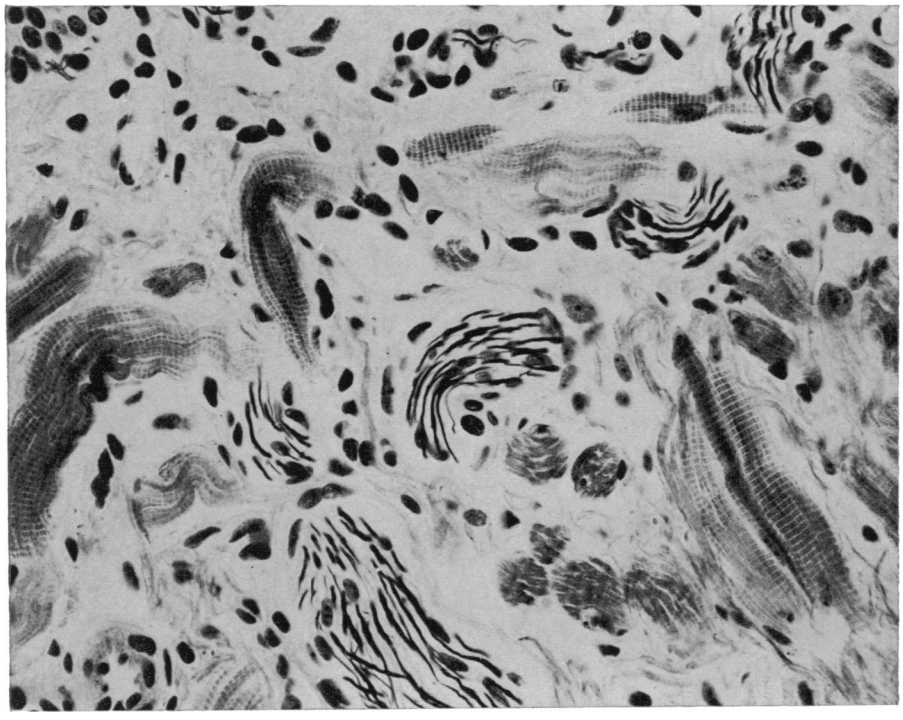

Fig. 1. Rabbit tongue (6 μm sector). Nerve fibres are well defined, strongly impregnated and clearly differentiated from other structures. $\times$ 400.

RESULTS

A typical result using this method is illustrated in Figure 1. Nerve fibres, both myelinated and non-myelinated, are strongly impregnated, well-defined, and clearly differentiated from other structures. Connective tissue fibres usually stain a diffuse pale gold colour; nuclei and, in particular nucleoli, are very discretely impregnated; striated muscle fibres and their cross-striations are usually very clearly impregnated; melanin, if present, has a strong affinity for silver, and is heavily impregnated. All other structures are stained in various shades of gold–brown.

DISCUSSION

Evolution of the technique

Tissue processing

(*a*) *Fixation.* All the formaldehyde-containing fixatives gave good results. The results obtained with Bouin's fluid were adequate, but the very finest nerve fibres did

not impregnate so clearly. It is important for tissues to be fixed thoroughly and promptly. Impregnation of sections prepared from poorly fixed tissue showed striking changes: large myelinated nerve fibres appeared vacuolated and showed reduced affinity for silver, possibly due to loss of lipid, and the finer nerve fibres and nerve endings were often unstained.

(*b*) *Decalcification.* Both Kristensen's formic acid solution and 10 % EDTA proved suitable. Whenever possible, however, especially for tissues which require only slight

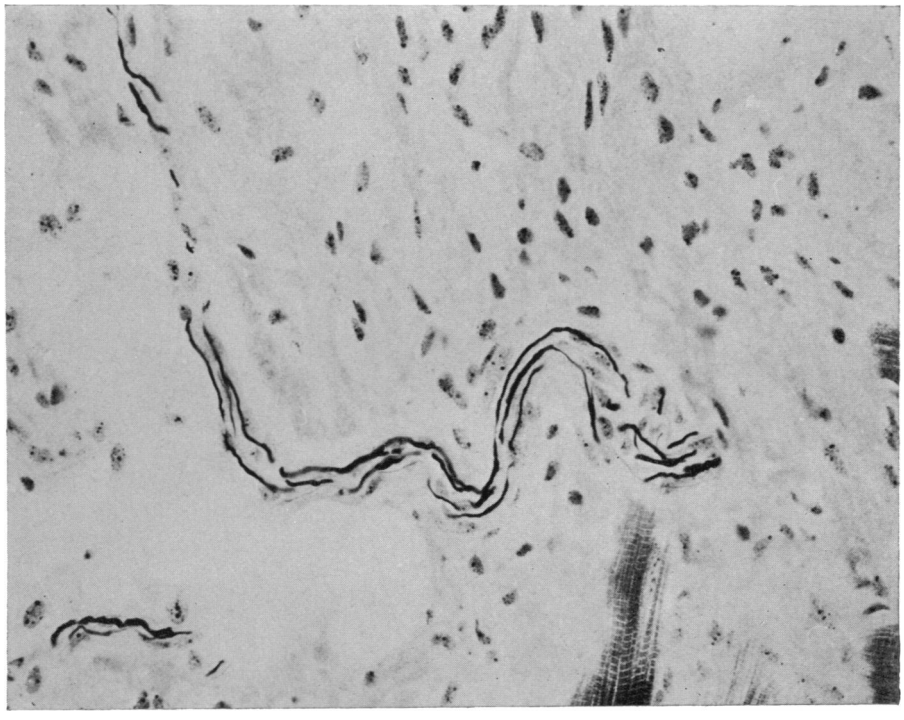

Fig. 2. Similar section of rabbit tongue to Fig. 1, but incubated at room temperature in Kristensen's formic acid mixture for 5 hours and then impregnated with silver. This has resulted in a weaker impregnation of nuclei, connective tissues and other structures, but nerve fibres have retained their affinity virtually undiminished. × 400.

decalcification, such as fetal tissues, the chelating agent is preferred. If speed is important, acid decalcification is adequate, although prolonged use of acid will decrease the amount of silver taken up during impregnation.

To test the effect of acid, paraffin sections of rabbit tongue were incubated at room temperature in Kristensen's formic acid solution for 5 hours, washed in tap water and distilled water, and then impregnated with silver at 40 °C. A control section not treated with acid was included. Impregnation of nuclei, connective tissue and other structures was much weaker in the acid-treated section, although nerve fibres retained their affinity for the silver (Fig. 2).

Silver impregnation

(*a*) *The buffer.* As the silver impregnation has to be carried out in a pH controlled environment, buffers are essential. The choice of buffer is important as it may react not only with the silver compound contained in the impregnating solution, but

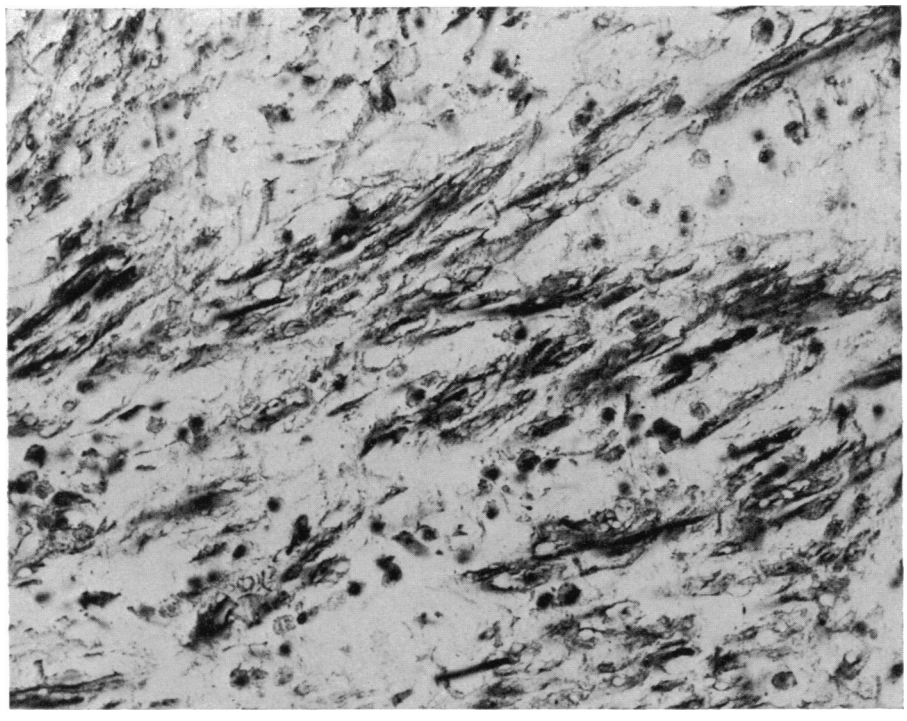

Fig. 3. Tooth pulp (6 μm). The section has been impregnated with a silver cyanate–Palitzsch buffer mixture. The resulting reaction is completely non-specific, with impregnation of nuclei and connective tissue in addition to nerves. $\times 400$.

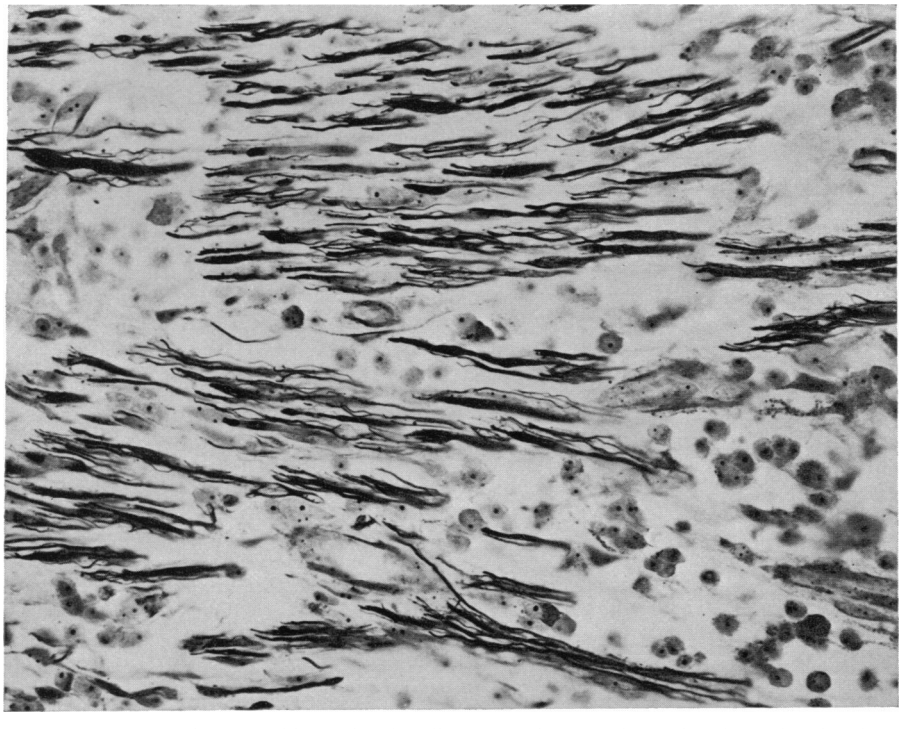

Fig. 4. Tooth pulp (6 μm). This section is adjacent to the one shown in Fig. 3, but has been impregnated with the standard silver cyanate–collidine buffer mixture. The resulting reaction is almost exclusively confined to nerves. $\times 400$.

also with the tissue, altering affinities of tissue components for the silver. Two types of buffer have been tested: Palitzsch's borax–boric acid buffer (Fearnhead & Linder, 1956) and the collidine–nitric acid buffer described above (Rowles & Brain, 1959). The latter is more useful as it is compatible with both silver compounds used (silver nitrate and silver cyanate), whereas Palitzsch's buffer can only be used with silver nitrate. When used with silver cyanate, borax reacts to form a completely non-specific impregnation (Figs. 3, 4). Collidine is closely related to pyridine, which was used by Bielschowsky (Brontë Gatenby & Beams, 1950) in his method for frozen sections to

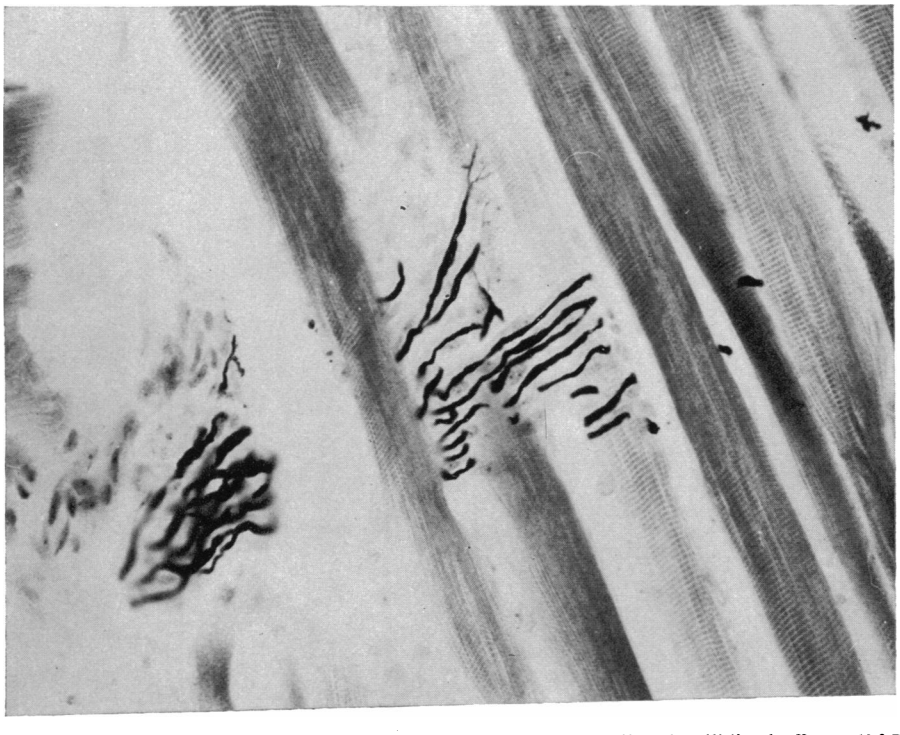

Fig. 5. Rabbit tongue (6 μm). This section has been incubated in diluted collidine buffer at 60 °C for 80 minutes and then impregnated with silver. Impregnation of nuclei, connective tissue and other tissue elements is virtually absent, but nerve fibres have retained their affinity for silver. In the centre of the photograph, innervation of muscle is seen with fine terminal fibrils indicating the position of a motor end plate. × 400.

reduce the argyrophilia of connective tissue and nuclei, achieving thus a more precise differentiation of axis cylinders. To test if collidine produced a similar effect, sections of soft and of decalcified tissue were incubated in diluted collidine buffer at 40 °C and at 60 °C for a controlled time sequence and then impregnated with silver. The results show that, with increasing exposure to the collidine buffer, the intensity of the impregnation of nuclei, connective tissue and other structures decreases, while nerve fibres fully retain their affinity for silver (Fig. 5).

(*b*) *Type of silver compound used.* Both silver cyanate (Rowles, 1960) and silver nitrate are suitable. Silver cyanate crystallises in colourless needles and is only slightly soluble in cold water. It is not commercially available but can be prepared by mixing equivalent amounts of solutions of silver nitrate and sodium cyanate. Solutions of silver cyanate are only weakly dissociated and will impregnate the nerve

fibres in sections of peripheral tissue more specifically than can be achieved with silver nitrate, which will frequently stain collagen and reticulin fibres very heavily. In the CNS, however, where there are few connective tissue fibres, silver nitrate is preferable, as the nerve fibres become more intensely impregnated. The impregnating solution can be adapted quite easily by omitting the sodium cyanate, leaving the remainder of the solution unaltered.

(*c*) *pH, time and temperature of incubation.* The specificity of silver impregnation depends largely upon an interrelationship between pH, time, temperature and silver concentration of the impregnating solution (Samuel, 1953*a*,*b*; Peters, 1955*a*,*b*; Fearnhead & Linder, 1956). Fearnhead & Linder used a silver impregnating solution buffered to pH 7·0 which gave consistently well differentiated staining of nerves in teeth; pH 7·0 was therefore selected for the present method. To adjust the pH of the impregnating solution to this level, the buffer stock should be at pH 7·2 at room temperature so that when the buffer is suitably diluted and incorporated in the warm impregnating solution the final pH will be about 7·0.

To evaluate the effect of time and temperature, sections were incubated at 40–45 °C and at 60 °C for 10, 20, 40, 80 and 120 minutes. Differentiation between nervous and non-nervous tissue was optimal after about 90 minutes at the lower temperature, but a corresponding degree of specificity was obtained after 10–15 minutes at the higher temperature. Specificity and completeness of impregnation is best in decalcified sections after impregnation at 40 °C while soft tissue will give equally good results at 40 °C or 60 °C. If sections are impregnated for a much longer time, not only will the overall intensity of silver impregnation increase, but other fibrillar structures, such as collagen and reticulin, may stain heavily.

(*d*) *Development.* A physical method of development is used to produce the final result by depositing silver contained in the developer on to silver nuclei formed in the tissue during impregnation. As specificity of development depends on the balance between quinol, sodium sulphite, the amount of available silver nitrate, and the type of gelatine used, only chemicals of high purity should be employed. The types of gelatine found suitable were Belgian Bronze and Gold Label sheet gelatine. The developer can be used until it has become turbid, after which it may deposit silver non-specifically. Progress of development can be followed by a change of colour taking place in the section. It will assume a pale golden colour after 1–2 minutes, which will gradually darken and become brown after about 5–6 minutes. If the progress of development is monitored with the microscope the first structures to stain can be seen to be large myelinated nerve bundles, voluntary muscle fibres and cell nuclei. Usually, after about 5–6 minutes' development when the section has become dark gold to light brown, there will be optimal contrast between nervous and non-nervous tissue. If development is carried on beyond this stage the contrast between nerves and other tissues will diminish as the intensity of the background increases. Collagen and reticulin fibres also begin to take up silver, simulating the appearance of nerve fibres, and specificity thus suffers. Development can be stopped at any time by removing the section from the developer and washing with distilled water. The final impregnation, although generally stable and permanent, may occasionally fade after the section has been mounted for some months in balsam. This may be due to the solvent action of clearing agents, such as excessive amounts of terpineol left on the sections prior to mounting. Faded preparations can be salvaged by redevelopment. Gold toning of the section, by means of which the reduced silver is replaced by gold, will also avoid this fading (Bielschowsky, 1927).

APPLICATION

Earlier silver impregnation techniques often appeared to be more successful with some species; for example, a method working extremely well with mammalian tissue is sometimes unsuccessful with fish, amphibians and reptiles. Fearnhead & Linder found that their technique worked very well on decalcified material, but was less predictable on soft tissues which had not been subjected to decalcification.

With the present technique, equally good results have been achieved repeatedly with mammalian and fish material, either decalcified or non-decalcified, in peripheral or central nervous system, and in either adult or embryo.

As the impregnation can quite easily be restricted to nervous tissue, the method has wide applications, for example, to investigations involving graphic reconstruction of the nerve supply of various tissues and organs.

SUMMARY

To improve existing methods for the identification of nerves in routine sections the factors most likely to be responsible for specificity, reliability and reproducibility of the silver impregnation have been investigated. These included methods of fixation and tissue preparation, concentration and type of silver compound used, pH and buffer type, temperature and incubation time, and the characteristics of the developing solution. Guided by the results of this investigation a simple, easily repeatable technique for the silver impregnation of nerves in histological sections has been evolved.

Tissue is fixed by immersion, or preferably by perfusion, with formaldehyde-containing fixatives. Hard tissue is decalcified either in 10% (w/v) Na-EDTA at pH 7·0 or in Kristensen's formic acid mixture, then embedded in paraffin and cut at 6–10 μm. Mounted sections are de-waxed, washed in tap water followed by distilled water, and transferred to an 8 mM (pH 7·2) collidine–nitric acid mixture, where they are left either overnight at 40 °C or for 10–30 minutes at 60 °C.

The sections are then transferred into a 2·3 mM silver cyanate solution buffered at pH 7·2 at the same temperature as the previous buffer stage, and incubated for 1½–2 hours at 40 °C or 10–30 minutes at 60 °C. They are then washed in distilled water, transferred to a physical developer containing 158·6 mM sodium sulphite ($Na_2SO_3 . 7H_2O$), 24·9 mM borax, 2% (w/v) sheet gelatine, 1·12 mM silver nitrate and 9 mM quinol. Development of the latent image of the silver impregnated tissue elements is monitored by washing in distilled water and checking with the microscope. Sections are then dehydrated, cleared and mounted in balsam. Myelinated as well as non-myelinated nerves, intradentinal fibres, and various types of nerve end organs such as motor end plates, as well as intraepithelial nerves, are impregnated with a high degree of specificity and contrast.

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