OBSERVATIONS ON THE SILVER IMPREGNATION OF NERVE FIBRES IN TEETH

By R. W. FEARNHEAD and J. E. LINDER

Departments of Anatomy and Dental Histology, The London Hospital Medical College

It is widely accepted that small beaded nerve fibrils form a marginal plexus in the predentine parallel to the pulpo-dentinal surface. Whether or not branches from the marginal plexus continue into the calcified dentine, following an intratubular course (Tojoda, 1934), or are situated in the dentine matrix, is uncertain. To examine this problem it was decided to make, in the first instance, a carefully controlled investigation into the method of impregnating nerves in teeth with silver, taking into account Samuel's (1953*a*, *b*) recent evaluation of the many factors which influence the mechanism of silver impregnation. In view of the observations made by Weddell, Pallie & Palmer (1954), use was also made of the *in vivo* and *in vitro* methylene blue methods (Schabadasch, 1935) as a control for the results of the impregnation with silver.

MATERIAL AND METHODS

Formalin-fixed $10\,\mu$ paraffin sections of decalcified human teeth, and of the pulps removed from human premolars and molars, were used for the staining tests. The method of staining was based on that described by Holmes (1943), and was chosen because each stage of the procedure could be adapted for experimental purposes and tested separately.

The deparaffinized sections were washed thoroughly in running tap water to remove traces of formalin or acid, followed by three changes of glass-distilled water, and buffered overnight (approx. 16 hr.) at 37° C. The sections were then transferred to fresh buffer solution containing 1 ml. of 1 % solution of silver nitrate to every 100 ml. of buffer. The sections were left in this solution in the dark for periods varying from 1 to 10 hr. After this initial impregnation, the sections were rinsed *quickly* in distilled water and transferred to 2·5 % sodium sulphite for periods varying from 1 to 10 min. After sulphite treatment the sections were washed in running tap water, followed by three changes of distilled water and then placed in the developing solution. Development was carried out for 3 min. at 29° C. in a mixture used by Pearson & O'Neill (1946), which consisted of 3 % gelatine, 2 % silver nitrate, 1 % hydroquinone in the proportions of 4 : 2 : 1. After development, sections were washed thoroughly in distilled water, toned with 0·2 % gold chloride for 10 min, and washed in distilled water. The gold was reduced with 2 % oxalic acid for 5 min. or with 1 : 10,000 resorcinol for 30 min. Sections were then washed and fixed in 5 % sodium thiosulphate, and finally washed, dehydrated and mounted in neutral canada balsam. The stages in what is at present employed as the standard technique for sections of teeth are shown in Text-fig. 1, which also indicates the various points which were found to have profound effects on the impregnation.

RESULTS

In evaluating the results it was borne in mind that any method should possess the following properties:

(1) The impregnation should demonstrate completely all the nervous elements present in the dental tissues.

(2) The impregnation should show clearly the morphology of the smallest axons.

(3) The impregnated nerves should be well differentiated from other tissue elements.

(4) The results should be repeatable with certainty on routinely prepared dental tissues.

Silver technique adopted after experimentation

Step		Note
1. Washing deparaffinized sections	←	2 hr. in running tap water for formalin fixed tissues 16–18 hr. in running tap water for other fixatives, Bouin, Rossman, etc.
↓		
2. Three changes of distilled water	←	1 hr. each change—the last change in double glass-distilled water
↓		
3. Overnight 12–14 hr. in concentrated Palitzsch boric acid borax buffer pH 7·0 maintained at 37° C.	←	Mix twice amount required, use half for initial buffering and remainder for silver impregnation
↓		
4. 2 hr. in 1 : 10,000 silver nitrate in Palitzsch buffer pH 7·0 maintained at 37° C.	←	When transferring to silver impregnating solution agitate sections to remove bubbles which may otherwise adhere to section and interfere with the impregnation.
↓		
5. Rinse quickly in double-distilled water		
↓		
6. Place in 2·5 % sodium sulphite for 5 min. at room temperature 20° C.		
↓	←	Mix the developer at this point and maintain at 29° C. in a water-bath for the next 14 min.
7. Wash in running tap water for 4 min.		
↓		
8. Distilled water for 10 min.	←	Three changes, double glass-distilled
↓		
9. Developer for 3 min. at 29° C.		
↓		
10. Wash in distilled water	←	Until all trace of developing solution is removed
↓		
11. Tone in 0·2 % gold chloride for 10 min.		
↓		
12. Wash 1 min. in distilled water		
↓		
13. Reduce gold with 1 : 10,000 resorcinol in distilled water for 30 min.	←	The time taken for the reduction of the gold may be decreased by using a stronger solution of resorcinol
↓		
14. Wash in distilled water		
↓		
15. 5 min. in 5 % sodium thiosulphate		
↓		
16. Wash, dehydrate, clear and mount		

Text-fig. 1

It will be seen from the following that the factors which have the greatest influence on the impregnation are simple and obvious ones, but inadequate control of any one of them may spoil the resulting impregnation. Our early results were inconsistent, some sections giving an excellent staining (Pl. 1, fig. 1 *a–c*), some showing a good impregnation of nerves spoiled by a very heavy staining of other tissues (Pl. 1, fig. 2), and in some an indiscriminate staining of non-nervous elements (Pl. 1,

fig. 3). These results, at first very puzzling, became more comprehensible as each factor was evaluated.

Washing

Preliminary washing in running tap water for 2 hr. was found to be the shortest time adequate for sections of formalin-fixed material. Tissues fixed in Bouin, Zenker or Rossman fluids required from 12 to 18 hr. washing in order to remove completely all traces of salts and metals which might either react with the silver or cause local fluctuation in the pH. In all, thirty-eight different fixatives have been tested, of which Bouin's fluid seemed to be the most promising. The results of the series of experiments described here, however, were obtained on formalin-fixed material.

Buffer

The Palitzsch buffer solutions were prepared with 19·0715 g. $Na_2B_4O_7.10H_2O$/litre of double glass-distilled water for solution A and 12·386 g. H_2BO_3/litre for solution B, weighing error being within $\pm 0{\cdot}1$ mg. It is common practice to use buffer solution diluted 1 in 10. At this dilution it was thought that at the extreme ends of the pH range covered by the solution the buffering capacity might be reduced. Solutions A and B were mixed and checked with electrodes on a direct reading pH meter to give a pH reading of 6·8, diluted 1 : 10 with double glass-distilled water (pH 7·0) and tested again at room temperature 20° C., at 37° and 53·5° C. At this dilution, at 20° C., the pH of the diluted buffer slowly rose from 6·8 to 7·2 over a period of 30 min. At 37° C. a similar rise in pH occurred but more rapidly, and at 53·5° C. even more rapidly. Concentrated buffer solution, mixed to give a pH 6·8, maintained this pH even when the temperature was raised. Concentrated buffer solutions with and without silver or sections were maintained at 37° C. over a period of 24 hr. without any change in the pH occurring. Maintaining sections at this temperature fits into routine laboratory work very well, and the use of concentrated Palitzsch buffer for this technique is recommended.

Because of the dense nature of the dentine matrix, a considerable time must be allowed for the complete penetration of the section by the buffer solution. For this reason sections were allowed to remain in buffer overnight in a thermostatically controlled oven at 37° C. In practice it was found convenient to prepare twice the amount of buffer required to cover the sections and to use half for overnight buffering and the remainder for the actual silver impregnation.

Using sections of sympathetic ganglia and spinal cord from rabbits fixed in a special fixing solution (Davenport & Kline, 1938), Samuel (1953*a*) has shown that within limits there is an inter-relationship between temperature, pH, silver nitrate concentration and time of the impregnation. Sections of tooth pulps were therefore impregnated for increasing periods from 1 to 10 hr. in 1/10,000 buffered silver nitrate solution maintained at 37° C. over a pH range of 6·2, 6·4, 6·8, 7·0, 7·4 and 7·9. In order to maintain the pH at 6·2 an acetic acid-sodium acetate buffer was used (Pearse, 1953), Palitzsch buffer being used for the remainder. The results supported the observations of Samuel (1953*a*). The optimal time of impregnation found to be necessary, however, was always longer than in his experiments which he carried out at 53·5° C., but this was probably due to the lower temperature during impregnation. It was found very difficult to assess within fine limits the optimal conditions of

impregnation, because at the time these experiments were carried out, control over development had not been obtained. Pl. 1, figs. 4–6, show sections impregnated at pH 6·8 for 6 hr., pH 7·0 and 7·4 for 2 hr. Impregnation at pH 7·0 and 7·4 appeared to give the best results. Even at pH 6·2 an impregnation of nervous elements was obtained, however, but only after prolonged immersion in the silver solution. In sections impregnated for short periods at this pH the quality of the impregnation was poorly defined and granular (Pl. 1, fig. 7). In all later experiments, therefore, the silver impregnation solution was buffered to pH 7·0, and the section impregnated for 2 hr. at 37° C.

Sulphite treatment

After the initial impregnation, sections were immersed in 2·5 % sodium sulphite solution for 5 min. to remove the reducible silver from the non-nervous tissues in the sections. It was found that if sections were left longer than this the silver was progressively removed, first from the finest nerve fibrils and later from nuclei and larger nerves. If sections were left for long periods in distilled water instead of the sulphite solution the silver was removed from the sections in a similar manner but more slowly. Removal of silver by distilled water was accelerated if the sections were first treated for a short time with sulphite. It was also found that sections could be left in running tap water for considerable periods without apparent loss of silver. Furthermore, removal of silver from the section did not occur if they were placed in distilled water after the tap-water treatment. This effect is probably due to the precipitation of the silver by impurities in the tap water, but the exact nature of these impurities has not been elucidated. It was found, however, that the loss of silver from sections could be prevented by placing them in a solution of 0·1 g. of sodium chloride per litre of glass-distilled water instead of tap water. Impurities in tap water are known to vary considerably from place to place, and may well play a more important role in successful silver impregnation than has hitherto been generally accepted. As a result of these observations, after immersion in the sodium sulphite bath for 5 min., sections were washed in running tap water for 4 min., and then transferred to distilled water for 10 min. prior to development.

Physical development

A greatly improved impregnation of the nerves was repeatedly obtained under the modified conditions already described, but differentiation was poor, and nerves and nuclei appeared densely black on a pale background of connective tissue which contained granular deposits of silver in the cytoplasm. It was thought that some improvement might result if greater control over the developing technique could be achieved.

It was noticed that the solution used for physical development exhibited striking changes during the first few minutes after mixing, when the opalescent straw colour of the freshly mixed solution changed rapidly to a deep amber and finally to an opaque 'muddy' grey-green fluid. In view of these observations it was necessary to decide whether to place impregnated sections in the freshly mixed physical developer, to wait until the clear amber stage was reached or to start development when the mixture had reached the 'muddy' stage. A series of impregnated sections were therefore immersed in the developing solution at intervals increasing by 3 min.

increments from the time of mixing the developer. Each section was allowed to remain in the developer for 3 min. The total time covered by the experiment was 27 min. In addition, in order to eliminate the possibility of the developing power of the solution being reduced by the successive development of sections, the experiment was repeated using a separate pot for each section. In both series the cleanest impregnation, with good nuclear detail and well-differentiated background, was obtained in sections developed 12 or 15 min. after mixing the developer.

Gold toning

It was believed that a slower rate of reduction of the gold chloride during toning might result in a deposit of gold of finer grain size in the tissues and thus enhance the colour differentiation between nervous and non-nervous elements. Among the many substances tried, reduction with 1 : 1100 aniline water for 30 min. or 1 : 10,000 resorcinol for 30 min. appeared to give results superior to those obtained with oxalic acid, a substance more commonly used in gold toning techniques. The longer time taken for the toning proved to be an advantage since the progress of differentiation could be watched and checked under the microscope (Pl. 1, figs. 8–10).

DISCUSSION

Weddell & Zander (1950) have made a critical evaluation of methods for the demonstration of peripheral sensory nerves. They compared the results given by silver, methylene blue and phase-contrast techniques with the appearance of living nerves in the cornea viewed by the slit lamp and corneal microscope. They came to the conclusion that methylene-blue techniques gave the most accurate representation of axis cyclinder in stained preparations. A series of specimens of rat skin, and the pulps of teeth from the cat, goat and man were therefore prepared using the methylene-blue method described by Schabadasch (1935) for comparison with the results of our silver experiments. The fine beaded terminal filaments could be seen clearly in both methylene blue and silver preparations, and the vesicular character of some of the larger fibres was clearly demonstrated by the silver sections (Pl. 1, figs. 11, 12).

Recently, Weddell & Pallie (1954) have shown that even in the most carefully fixed tissue distortion takes place sufficient to confuse the interpretation of sections prepared with both methylene blue and silver methods. They have found that a great deal of this distortion can be avoided and more rapid fixation obtained by subcutaneous injection of hyaluronidase a short time before introducing the dyes or fixatives. Use of hyaluronidase with this technique was not included in our experiments, but since the rate of penetration of dyes or fixatives is extremely slow in dentine, future investigations along these lines might prove very profitable.

On decalcified tissues the results of the silver technique described were consistent and gave a clear and well differentiated impregnation of the nerves. The nerve plexus described by Raschkow (1835) was well demonstrated, and very thin branches could be easily traced from it passing between the odontoblasts. Loops of fine nerves in the predentine as described by Bradlaw (1939) were clearly seen, and, in addition, beaded fibres almost at the limit of optical resolution could be traced forming a subdentinal or marginal pulp plexus. Numerous tiny branches could be traced

entering the dentinal tubules, many of them passing a considerable distance into the tubules well past the zone of predentine.

Some criticism of the use of physical development methods in the demonstration of nerve fibres has been made by Romanes (1950) on the basis that the nerve fibres are made to appear larger than they are in fact. Fernández-Morán (1952) has described the presence of nerve fibres of submicroscopic dimension in the central nervous system, and Robertis & Sotelo (1952) have described fine processes which are below the limits of optical resolution at the tips of neurites grown in tissue culture. It seems, therefore, not impossible that submicroscopic fibres could exist as fine prolongations of nerves within the dentinal tubules in teeth. If this should be so, to increase the diameter of such fibres so that they come within the resolving power of the light microscope would appear to be a justifiable technique.

SUMMARY

An attempt has been made to evaluate the factors which may influence the silver impregnation of nerve fibres in teeth. The method of impregnation was based on that described by Holmes (1943). It was found that insufficient control over any one of the factors summarized below could prevent the successful impregnation of nerve fibres in teeth.

1. Inadequate removal of fixatives during the preliminary washing of deparaffinized sections.
2. Instability of the pH of the impregnating solution (even more important than the pH level chosen), although neutral or alkaline pH gave better results than sections buffered on the acid side of neutrality.
3. Impurities in tap water have an important role in preventing the loss of too much silver from the sections after treatment with sodium sulphite solution.
4. The developing solution used in this technique rapidly undergoes striking changes after mixing; consequently there is a critical period during which development of impregnated sections should be commenced. This period was found to be 12–15 min. after the time of mixing when the temperature of the developing solution is 29° C.
5. More delicate differentiation between nerves and non-nervous elements was obtained after toning with gold chloride if the gold was reduced with aniline or resorcinol instead of oxalic acid.

The standardized technique was found to give consistently a reproducible impregnation of nerve fibrils in decalcified sections of teeth. By comparison with preparations of nerves stained by *in vivo* and *in vitro* methylene blue methods, the silver technique was regarded as giving a reliable histological representation of the nervous elements in teeth.

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REFERENCES

BRADLAW, R. (1939). The histology and histopathology of the dental innervation. *Proc. R. Soc. Med.* **32**, 1040–1053. (Sect. Odontol., pp. 68–81).

DAVENPORT, H. A. & KLINE, C. L. (1938). Part 2. Use of *n*-propyl and *n*-butyl alcohol in Hofker's fixative. *Stain Tech.* **13**, 147–160.

FERNÁNDEZ-MORÁN, H. (1952). The submicroscopic organization of vertebrate nerve fibres. *Exp. cell. Res.* **3**, 5–83.

HOLMES, W. (1943). Silver staining of nerve axons in paraffin sections. *Anat. Rec.* **86**, 157–187.

PEARSE, A. G. E. (1953). *Histochemistry, Theoretical and Applied.* London: J. and A. Churchill Ltd.

PEARSON, A. A. & O'NEILL, S. L. (1946). A silver-gelatin method for staining nerve fibres. *Anat. Rec.* **95**, 297–301.

RASCHKOW, J. (1835). *Die Gesundsheitslehre des Mundes.* Ludwigsburg.

ROBERTIS, E. & SOTELO, J. R. (1952). Electron microscope study of cultured nervous tissue. *Exp. cell Res.* **3**, 433–452.

ROMANES, G. J. (1950). The staining of nerve fibres in paraffin sections with silver. *J. Anat., Lond.*, **84**, 104–115.

SAMUEL. E. P. (1953*a*). Impregnation and development in silver staining. *J. Anat., Lond.*, **87**, 268–277.

SAMUEL, E. P. (1953*b*). The mechanism of silver staining. *J. Anat., Lond.*, **87**, 278–287.

SCHABADASCH, A. (1935). Theoretische und experimentelle Studien zur Methylenblaufärbung des Nervengewebes. *Acta morphologia*, H. 1. Staatsverlag, Gorkij. (Cited by Romeis, B. (1948). *Mikroskopische Technik*, Leibniz Verlag, München.)

TOJODA, M. (1934). Die Innervation des menschlichen Zahnbeins, *Dtsch, Zahnärztl, Wchschr.* **37**, 641–645, 670–673.

WEDDELL, G. & PALLIE, W. (1954). The value of 'spreading' factors in the demonstration of tissue neural elements. *Quart. J. micr Sci.* **95**, 389–397.

WEDDELL, G., PALLIE, W. & PALMER, E. (1954). The morphology of peripheral nerve terminations in the skin. *Quart. J. micr. Sci.* **95**, 483–501.

WEDDELL, G. & ZANDER, E. (1950). A critical evaluation of methods used to demonstrate tissue neural elements, illustrated by reference to the cornea. *J. Anat., Lond.*, **84**, 168–195.

EXPLANATION OF PLATE

Abbreviations: O = odontoblasts; P = predentine zone; d = dentine; n = nerve.

Fig. 1 (*a*). Small beaded nerve fibres passing between the odontoblast processes close to the pulpal surface of the predentine. Tangential section through the pulpo-dentinal junction of a human premolar. Formalin fixation, decalcified, silver impregnation, 2 hr. at pH 7·0, gold toned/resorcinol reduction. ×1000. (*b*) Small beaded nerve crossing the pulpo-predentinal junction, close to an odontoblast process and passing into a tubule in the predentine. Human premolar, formalin fixation, decalcified, silver impregnation, 2 hr. at pH 7·0, gold toned/resorcinol reduction. ×800. (*c*) The same field as (*b*). Difference in focus level approximately 0·5 μ.

Fig. 2. A small beaded nerve situated in the predentine, notice however that the preparation is spoiled by a very heavy deposit of silver in the dentinal tubules and the odontoblasts. Human premolar, formalin fixation, decalcified, silver impregnation, 2 hr. at pH 7·0, Palitzsch buffer diluted 10 ml. buffer : 100 ml. impregnating solution (washed 2 hr. running tap water, buffered for only 2 hr. before impregnation) untoned. ×800.

Fig. 3. Silver impregnated connective tissue fibres at the pulpo-predentinal junction. Human premolar, decalcified, silver impregnation, 2 hr. at pH 7·0, Palitzsch buffer diluted 10 ml. buffer : 100 ml. impregnating solution (washed 2 hr. in running tap water, placed in buffer solution only 2 hr. before impregnation) untoned. ×90.

Figs. 4–10 are photomicrographs of paraffin sections of the pulp removed from an undecalcified human premolar after formalin fixation. Sections illustrated in Figs. 4–7 are from series impregnated in 1 : 10,000 silver nitrate. The variable factors were pH and time of impregnation. They were then developed in freshly mixed 'physical developer' and the gold toning reduced with 2 % oxalic acid.

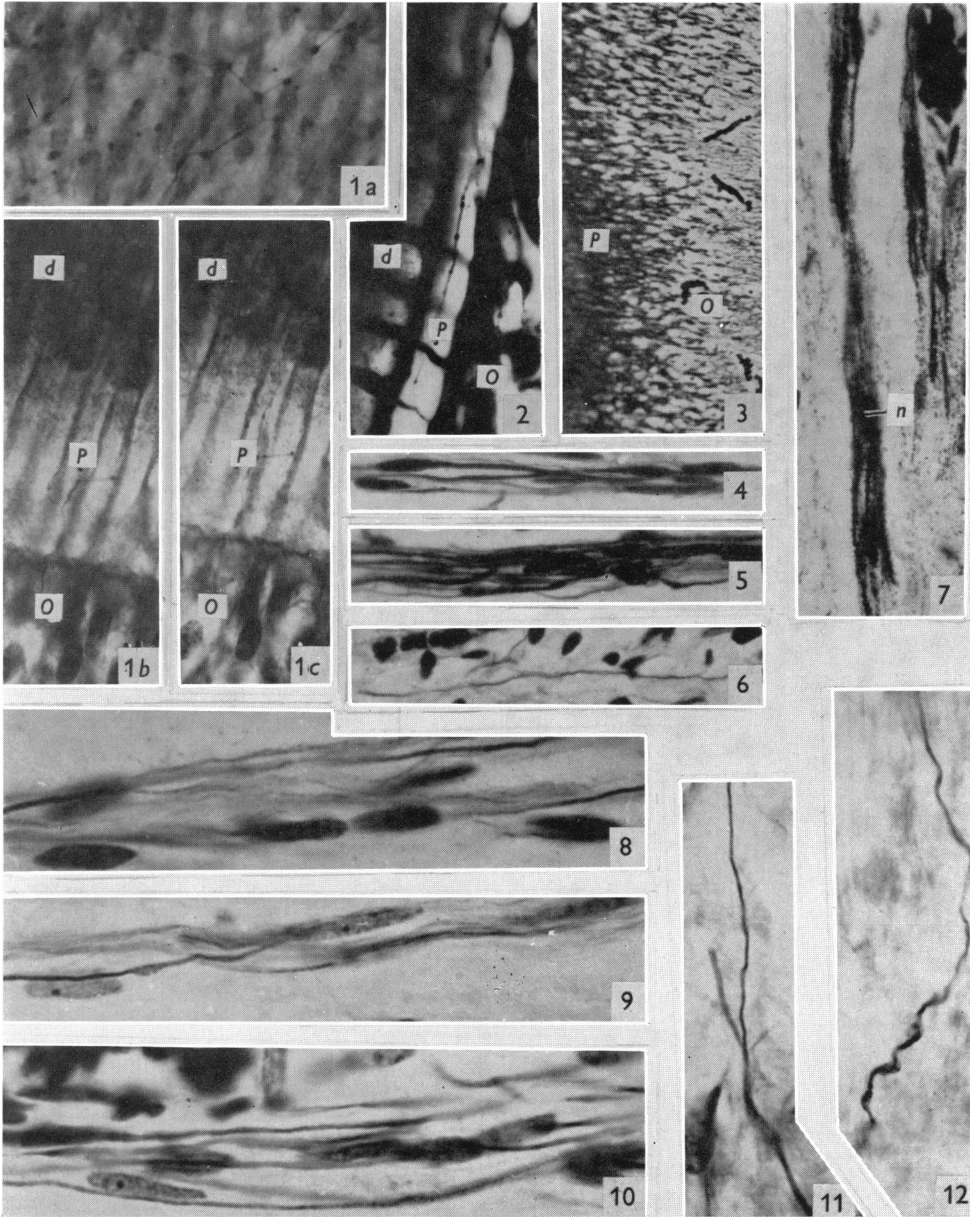

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Fig. 4. 6 hr. impregnation at 37° C. with dilute silver solution buffered to pH 6·8. Gold toned/oxalic acid reduction. × 390.

Fig. 5. 2 hr. impregnation at 37° C. with dilute silver nitrate solution buffered to pH 7·0. Gold toned/oxalic acid reduction. × 390.

Fig. 6. 2 hr. impregnation at 37° C. with dilute silver solution buffered to pH 7·4. Gold toned/oxalic acid reduction. × 390.

Fig. 7. 10 hr. impregnation at 37° C. with dilute silver solution buffered to pH 6·2. Gold toned/oxalic acid reduction. × 390.

Sections in figs. 8–10 were impregnated for 2 hr with 1 : 10,000 silver nitrate solution, buffered to pH 7·0 and development commenced 15 min. after mixing the developer, but the method of gold toning was varied.

Fig. 8. Gold toning reduced with 2 % oxalic acid for 5 min. × 1200.

Fig. 9. Gold toning reduced with 1 : 1000 aniline water for 30 min. × 1200.

Fig. 10. Gold toning reduced with 1 : 10,000 resorcinol for 30 min. × 1200.

Fig. 11. Nerves in pulp of a human molar formalin fixed, decalcified, silver impregnation, 2 hr. at pH 7·0. × 1200.

Fig. 12. Nerve in the pulp of human premolar. Methylene blue (Schabadasch) *in vitro* preparation. × 820.