THE MECHANISM OF SILVER STAINING

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Though much research has been devoted to the elaboration of silver stains for nervous tissue, there have been few experimental investigations of the mechanism of the staining process. Liesegang (1911) claimed that during the impregnation in the silver bath, ultramicroscopic particles of metallic silver were deposited in the sections as silver 'nuclei', and it was on these 'nuclei' that further silver deposition occurred during development. Thus the final staining of the tissue was determined by the initial distribution of the silver 'nuclei'. This theory was generally accepted until Owens & Bensley (1929) and Nageotte & Guyon (1930) suggested that the staining was more dependent on the physical properties of the tissue. Zon (1936) concluded that the differences in stainability of the tissue elements were due solely to the differences in the protective properties of their colloids, which permitted varying degrees of precipitation of silver within them. Silver (1942) thought that during the development phase of silver staining negatively charged silver micelles were formed and these were precipitated by the positive charges carried by the tissues. The pH of the silver solution was important only in so far as it affected the charge on the micelle, rendering it more or less liable to flocculation, but the pH of the developing solution was of extreme importance in so far as it affected the charge on the tissues and hence the distribution of the silver.

As none of the previous opinions seemed to be based on incontestable evidence, it was considered that a further investigation into the mechanism of silver staining might throw further light on the problem.

MATERIALS AND METHODS

The experiments to be described below were carried out on 10 μ paraffin sections of the sympathetic ganglia and spinal cords of rabbits fixed in a modified Hofker's solution (Davenport & Kline, 1938). The sections were impregnated in a silver borate solution (for details see previous communication) and subjected to various experimental procedures.

EXPERIMENTS

Silver's (1942) *hypothesis*

I have already shown, in experiments described previously, that different developers produce different staining effects, and that the impregnation process exercises a controlling influence on the staining caused by a particular developer. But it was necessary to discover whether, as Silver (1942) believed, the staining was entirely dependent on the physical state of tissue colloids during the development phase, because, if this was the case, variously impregnated sections, after washing and drying, should be in a uniform physical state; the staining on development should

then depend solely on the state of the section in the developer *as determined by the properties of the developing solution*, and the staining of the sections should always be the same, irrespective of the conditions of the impregnation.

However, no significant difference was found between the sections impregnated at the same pH, whether they had been dried before development or not. Thus sections impregnated at pH 7·4 and 8·8 and washed and dried (Pl. 1, figs. 1, 3) resembled sections impregnated at pH 7·4 and 8·8 which had not been dried (Pl. 1, figs. 2, 4). There were, however, marked differences between the dried sections —the staining was much less complete at pH 7·4 (Pl. 1, fig. 1) than at pH 8·8 (Pl. 1, fig. 3).

Liesegang's (1911) *hypothesis*

Was it possible, as Liesegang (1911) postulated, that reducible silver was thrown down by the developer on silver 'nuclei' of reduced metallic silver, deposited in the section during the impregnation? Certainly sections were capable of reducing silver, for after prolonged incubation, particularly at high pH levels, silver was deposited in the section and the tissue elements were visible without development or toning. It was found that this silver was not removed by sodium sulphite.* If this type of silver was deposited in sections in short impregnations it was likely that this also would not be removed by sodium sulphite. But sections treated by sulphite after short impregnations were quite unstained and silver 'nuclei', if present, were invisible and could not be revealed by gold toning. Attempts were made to render them visible by a process of physical development, a technique used in miniature photography, in which the latent image is developed by depositing additional silver on it from a silver containing developer solution. The developer finally adopted was that used in the Pearson & O'Neill (1946) silver method (40 c.c. 3 % gelatin, 20 c.c. 2 % silver nitrate and 10 c.c. 1 % hydroquinone), a solution differing little from that of Liesegang (1911) who used gum arabic instead of gelatin. This provided a source of silver particles to deposit on any silver 'nuclei' present, without gross precipitation on the slide.

The routine adopted was:

(1) Impregnate in buffered silver nitrate solution.
(2) Rinse in three changes of distilled water.
(3) Remove the reducible silver by treatment with 2·5 % sulphite for 5 min.
(4) Wash in running tap water for 10 min. and rinse in distilled water.
(5) Place in Pearson's developer for 3 min. at 28° C. in a Coplin jar in a thermostatically controlled water-bath.

No toning was employed.

This method of processing will hereafter be referred to as physical development. It differed basically from that of Liesegang (1911), because he failed to remove the reducible silver from the sections prior to their treatment in his gum arabic-silver hydroquinone mixture.

The present method demonstrated that the impregnation affected the section, as staining occurred when the section was placed in the physical developer. Control

* Earlier experiments had shown that sections treated with sodium sulphite before development did not stain, i.e. the sulphite removed the reducible silver from the section.

sections subjected to identical treatment, but without the addition of silver to the second buffer solution, did not stain in any way. It was concluded that a *silver-section* reaction occurring during the impregnation resulted in the deposition of silver in the section. This determined the distribution and amount of further silver derived from the physical developer. The precise form of the silver deposited in the section during impregnation could only be surmised, but (*a*) it was ultramicroscopic, (*b*) it caused autocatalysis in a hydroquinone, gelatin, silver nitrate solution (see below), and (*c*) since reducing groups exist in the tissues (Liang, 1947) it was surmised that the silver was in the form of ultramicroscopic particles of reduced silver, the so-called silver 'nuclei'. By removing the reducible silver, physical development could be carried out completely independently of the conditions of impregnation, and because the conditions of physical development were constant any variation in the staining could be attributed solely to altered impregnation. It proved possible by this method to study the disposition and amount of the silver 'nuclei' under different conditions.

To demonstrate the reducible silver in the section under varying conditions, the sections were quickly rinsed in distilled water and then placed in a rapid developer, 1 % hydroquinone solution; the rinse served to remove any adherent buffer silver solution carried over mechanically from the impregnating bath which might affect the staining.

As a 1 % hydroquinone in a 10 % sodium sulphite solution had been found to produce well-differentiated staining, sections were also developed in this solution for comparison with the physically developed sections.

Sections were impregnated in a solution containing 1 c.c. of 1 % silver nitrate buffered over a pH range 6·8–9·1 for periods of 15 min., 2½ and 24 hr. They were then:

(1) treated with 2·5 % sodium sulphite and physically developed;
or (2) rinsed in distilled water and developed in a 1 % hydroquinone solution;
or (3) rinsed in distilled water and developed in a 1 % hydroquinone in a 10 % sodium sulphite solution.

The sections were not toned in these experiments.

Physical development, silver 'nuclei'

Fifteen min. impregnation. At pH 6·8 the axons and cells at the periphery of the section were stained varying shades of brown. Over the intermediate pH range 8–8·4 the staining became more even, but thereafter the staining decreased until at pH 9·1 (Pl. 1, fig. 5) only faint axonal staining could be seen—the cells and their nuclei stained at the periphery, with more nuclei staining towards the centre.

Two and a half hours' impregnation. At all pH levels the whole section was stained. In the sections impregnated at pH 6·8 (Pl. 1, fig. 6) the cells were dark grey green in colour, their nuclei were deep brown, and the axons were clearly stained brown and black in a clear background. As the pH rose the staining diminished, until at pH 9·1 (Pl. 1, fig. 7) the neuronal cytoplasm was feebly stained, the nuclei were brown, and the axons were almost invisible in the stained background tissue, but reticulin staining was clearly evident in arterial walls and in the capsule of the ganglion.

Twenty-four hours' impregnation. After prolonged incubation, the sections became less heavily stained on physical development and showed uniform brown staining, particularly in high pH impregnations.

Development in 1 % hydroquinone. Reducible silver

Fifteen min. impregnation. At pH 6·8 only a faint precipitate covered the section. With rising pH the amount of reducible silver greatly increased and in sections impregnated at pH 9·1 (Pl. 1, fig. 8) considerable staining of the cells, nuclei and background occurred.

Two and a half hours' impregnation. At each pH the reducible silver greatly increased as the impregnation was prolonged. Apart from the great overall increase in reducible silver, there was a difference too in its distribution. As the pH rose from 6·8 (Pl. 1, fig. 9), the staining of the background increased in intensity and the neurons changed from pale ye low through deepening shades of brown until they were almost black at pH 9·1 (Pl. 2, fig. 10). The axons, however, increased in staining from pale yellow at pH 6·8 to brown up to pH 8·2–8·4, but then the staining diminished and became scarcely visible at the high pH levels, partly because of the heavy granular staining of the background. At all pH levels, the sections developed in 1 % hydroquinone had a coarse granular appearance.

Twenty-four hours' impregnation. After prolonged incubation, there was little difference in the staining at the lower end of the pH range. As the pH increased the sections became increasingly brown before development, and consequently it was impossible to determine how much of the staining could be attributed to the reducible silver. But two changes were observed in the developed sections: (*a*) they were more evenly stained than after 2½ hr., and (*b*) they were less granular in appearance.

Hydroquinone sulphite development

Fifteen min. impregnation. Sections impregnated at pH 6·8 were unstained; staining did not appear until the pH rose above a value of 8. Thereafter the axons and cells and cell nuclei gradually increased in density until at pH 9·1 they were heavily stained (Pl. 2, fig. 11).

Two and a half hours' impregnation. After 2½ hr. the cells and axons were faintly stained at pH 6·8 (Pl. 2, fig. 12) and the sections impregnated at the intermediate pH levels also showed some increase in staining. At the highest pH levels there was *less* staining than before, and the distribution of the stain had altered. At pH 9·1, the sections were stained a diffuse even brown in colour and the axons were scarcely visible in background tissue (Pl. 2, fig. 13), cf. pH 9·1 at 15 min. (Pl. 2, fig. 11).

Twenty-four hours' impregnation. Prolonged incubation caused little change at low pH, but the sections became increasingly and uniformly brown in colour after development, as the pH rose.

DISCUSSION

Before considering the significance of the above experiments, it is necessary to relate briefly certain relevant information.

James (1939*a*, *b*; 1940) has shown that the reduction of silver nitrate in a gelatin solution by hydroquinone is:

(1) Catalysed by the addition of colloidal silver, and the colloidal silver initiates

an autocatalytic process in which the silver is adsorbed on to the metallic silver particles and reduced *in situ.*

(2) The rate of reduction is increased by increasing concentrations of silver ions up to a point beyond which further increase has no effect.

(3) The oxidation products of hydroquinone accelerate its action.

(4) In the gelatin silver nitrate hydroquinone, new particle formation does not occur for 16 min.

Though the conditions in my experiments are not identical with those described by James, it is reasonable to suppose that the staining which occurs when a section is placed in the physical developer is due to the catalytic action of the silver deposited in the section during the impregnation, particularly as control sections do not stain on physical development. The amount and distribution of the silver passing into the section in physical development is determined by: (*a*) the amount and distribution of the silver 'nuclei', and (*b*) the amount of physical development.

Sheppard (1944) mentioned several facts which are relevant to the present discussion:

(1) The deposition and development of silver ions on metallic silver 'nuclei' probably assumes a filamentous form, which becomes disrupted because of the thermal energy of the system.

(2) Silver ions react with sulphite

$$Ag^{+} + SO_3 = AgSO_3$$

and form perhaps even higher complexes. The silver ions in this way are more protected from the developer and the silver catalytic reaction is retarded.

(3) The presence of sulphite decreases the tendency for clumping of metallic silver particles.

In addition, it has been shown in the present research that: (*a*) the sulphite also removes reducible silver from the section, and (*b*) during impregnation, two essential fractions of silver are deposited in the section: (*a*) a fraction which is reduced by the developer, 'reducible silver'; (*b*) a fraction of silver which is not affected by sulphite and which is probably in the form of minute silver particles or 'nuclei'.

The colour of silver sols. varies from yellow to brown to red as the particle size increases. If, however, a section impregnated at pH 8·5 is subjected to physical development for increasing lengths of time up to 8–10 min., the colour changes in the section vary from yellow to deep brown to black, indicating that the progressive deposition of silver which occurs does not result in a uniform growth of particle size. This is probably due to the nature of the medium in which it occurs and the mechanism of growth of the initial 'nuclei'.

Bearing the above facts in mind and correlating them with the experimental findings, it is possible to postulate the following explanations of the staining effects produced on physical development, and on development in the hydroquinone and hydroquinone sulphite solutions.

Physical development

A section removed from the impregnating solution and rinsed and treated with sodium sulphite solution contains a number of ultramicroscopic metallic silver

'nuclei'. At pH 6·8, the axons and cells contain a large number of 'nuclei' after 2½ hr. impregnation; thus, when placed in the physical developer, vigorous catalysis of silver occurs, the silver ion coming in contact with the silver 'nuclei' and being reduced *in situ*. This freshly deposited metallic silver in turn becomes a silver ion receiving surface, and by this process of autocatalysis, rapid deposition of silver occurs in the axons and cells, which in 3 min. become black and grey greenish brown in colour respectively (Pl. 1, fig. 6). As the pH rises the amount of silver 'nuclei' per unit volume of tissue decreases, and less staining occurs under the same conditions of physical development, until at pH 9·5 with 15 min. impregnation only faint axonal staining (Pl. 1, fig. 5) is seen, the neurons having a pale cytoplasm and black nuclei; at 2 hr. 30 min. axonal staining has all but disappeared, though the section is now more evenly stained, showing pale yellow cells, brown nuclei, and extensive reticular staining (Pl. 1, fig. 7).

Development in the hydroquinone and hydroquinone sulphite solutions

As the pH rises the amount of reducible silver in the section rises but its final distribution, and hence the staining, varies with the developer used.

In the hydroquinone sulphite developer, development takes place more slowly than in hydroquinone, as the oxidation products combine with the sulphite, and as the sulphite forms more stable complexes with the silver it removes from the section. The formation of silver sulphite complexes simulates the condition in the physical developer, i.e. there is formed a solution of reducible silver in the developing agent. Development again commences on the silver 'nuclei' in the cells and axons, and proceeds at increasing speed as the silver available for catalysis increases in amount. Meanwhile, the sulphite removes silver from the section where development proceeds least rapidly. This silver partially diffuses away from the section, but is partially reduced and deposited on the increasing surface of metallic silver. In effect, therefore, a re-distribution of the reducible silver takes place in the section. Thus, in the hydroquinone sulphite, as the reducible silver increases with rising pH, its deposition is still maximal where the development commences; and where the silver 'nuclei' are present in greatest concentration, increasing amounts of silver are deposited in the cells and axons. Therefore, although at pH 9·1, with 15 min. impregnation, there are very few 'nuclei' in the axons and cells (Pl. 1, fig. 5), development commences on them. It then progresses at increasing speed with the deposition of the large amounts of silver removed from the adjoining regions providing additional catalytic silver, and intense staining results (Pl. 2, fig. 11). (This cannot occur in the rapid hydroquinone developer which does not contain sulphite and which reduces the silver *in situ*, cf. Pl. 1, fig. 8; Pl. 2, figs. 10, 11). But when the impregnation is prolonged for 2½ hr. at pH 9·1, and the section is developed in hydroquinone sulphite solution, the cells become deep brown in colour and the axons are barely visible in the brown background tissue—this is seen in Pl. 2, fig. 13. As the same developer is used, the change must be due to alteration in the properties of the section as a result of the increased incubation. Reducible silver is still present in large amounts (Pl. 2, fig. 10), and it is concluded that the change probably affects the silver 'nuclei'.

That change can occur in the 'nuclei' is evidenced by the changes in the response

to physical development of sections impregnated at pH 8·5 for 1 hr. and 24 hr. In the former (Pl. 2, fig. 14) all the structures are jet black; in the latter much less silver is deposited in the section (Pl. 2, fig. 15) indicating a diminution in the active catalytic silver. This changing response to physical development is slightly evident at lower pH values, but is more obvious, and occurs with increasing rapidity, as the pH of the impregnating solutions rises, until at pH 9·1 it appears within 2½ hr. Possibly in the course of the impregnation the larger 'nuclei' grow at the expense of the smaller ones, so reducing the catalytic surface of the silver 'nuclei'. Some slight evidence in favour of the conception of growth of particle size is that sections impregnated at pH 9·1 for 15 min. and physically developed, when viewed by dark ground illumination, show marked light diffraction in their cell nuclei (Pl. 2, fig. 16). Two hours fifteen minutes later this diffraction has almost disappeared (Pl. 2, fig. 17). This may perhaps indicate that in 15 min. a multifocal deposition of silver occurs on physical development, giving a greater irregularity of surface than when less silver deposition occurs more slowly in relationship to the fewer larger silver 'nuclei' present after 2½ hr. impregnation.

Thus in the sections impregnated at pH 9·1 for 2½ hr. this decrease in the rate of development in the hydroquinone sulphite developer allows greater diffusion of the sulphite-removed silver from the section, and results in less silver being deposited in the cells and axons (Pl. 2, fig. 13), but more in the background tissues due to increased amounts of silver 'nuclei' there. The decrease in catalytic surface has less effect on the more rapid hydroquinone developer and heavy deposition of silver still occurs (Pl. 2, fig. 10). With further prolongation of the development the hydroquinone sections are also stained a paler brown colour.

Do the charges on the colloids of the section play any part in the staining reaction? Silver (1942) believed that the reducible silver, under the influence of the developer, produced negatively charged silver micelles which are distributed and deposited according to the electrical charges of the tissues. The charges of the tissues are determined by their chemical environment. If this is true, sections impregnated at different pH levels and rinsed and dried in an incubator before development should exhibit staining determined only by the properties and pH of the developer. This is not so. Sections treated in this manner stain exactly the same as sections placed directly in the developer from the impregnating bath, i.e. the impregnation plays a decisive role. As the staining process takes place in the colloidal media of the tissue elements the properties of the colloids must play an important part in staining. The chemical and physical properties of the colloid of the section, however, have complex interrelationships and it is as yet impossible to say exactly how these interrelated properties, which change as the reaction proceeds, affect the silver staining.

The experiments indicate that when responding to developer and reducible silver, silver 'nuclei' exert a determining influence. Liesegang (1911) claimed that the silver 'nuclei' serve as centres on which the reducible silver is mechanically deposited on development. In his experiments he omitted to remove the reducible silver in the section, which would therefore be reduced by the hydroquinone in his developer solution. Thus his results do not demonstrate the true sites of deposition of the 'nuclei' as determined by the impregnation. Furthermore, his conception does not

explain why after 15 min. impregnation at pH 9·1 (Pl. 2, fig. 11) intense axonal staining occurs when very few 'nuclei' are present (Pl. 1, fig. 5). One would expect at least a degree of 'nuclear' deposition comparable with that produced at pH 6·8 after 2½ hr. impregnation (Pl. 1, fig. 6), if there is any quantitative correlation between staining and the 'nuclei'. In fact, in no case does the appearance of the untoned sections after hydroquinone development correspond with the distribution of silver 'nuclei' as seen by physical development, and there is no basis to explain why different developers, contrary to the opinion of Holmes (1943), produce such different effects. Nor does Liesegang's hypothesis explain why the staining should alter so profoundly at pH 9·1 between 15 min. (Pl. 2, fig. 11) and 2½ hr. (Pl. 2, fig. 13) though the identical developer is used. Zon (1936) recognized the presence of reduced silver in the tissue, but considered that it is so protected by the colloid in which it is embedded that it exerts no significant effect on staining. This view is not supported by the changing effects occurring on physical development when the conditions of impregnation with buffered silver nitrate are altered.

To summarize, it may be said that the changing distribution of silver 'nuclei' as seen by physical development, after varying impregnation conditions, can be related to the differences in staining produced by different developers. The present research seems to indicate that the silver 'nuclei' play a dynamic part in a reaction in which the properties of the developer, and the amount and distribution of reducible silver, also have their own decisive influence.

SUMMARY

1. When immersed in a buffered silver solution two types of silver are deposited in the section: (*a*) a fraction of silver reduced by the section probably as minute metallic silver particles or 'nuclei'; (*b*) a fraction of silver which is reduced by the developer, reducible silver.

2. The amount and distribution of the silver 'nuclei' and reducible silver depends upon: (*a*) the properties of the section; (*b*) the pH of the solution; (*c*) the silver concentration; and (*d*) the time and temperature of incubation.

3. The silver 'nuclei' play a dynamic role as catalytic centres for silver reduction.

4. There is a complex interrelationship between these centres, the reducible silver, the developer, and the tissues. All play fundamental roles in the final staining of the section.

I should like to express my appreciation of the keen interest shown by Prof. G. A. G. Mitchell in this work, and I am indebted to Dr L. P. Kendal, Lecturer in Physiological Chemistry, and Dr D. C. Henry, Reader in Colloid Physics, for the valuable discussions I have had with them on the various physico-chemical aspects of this problem. My thanks are also due to Mr P. Howarth for the careful preparation of the photomicrographs.

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EXPLANATION OF PLATES

All the photomicrographs have been prepared under standard conditions of light intensity, exposure time, development and printing.

PLATE 1

Fig. 1. Transverse section spinal cord of rabbit. After impregnation at pH 7·4 the section was washed and dried before development. Note the similarity to fig. 2 and the difference from fig. 3. × 105.

Fig. 2. Section treated as in fig. 1 but not dried before development. × 105.

Fig. 3. Transverse section spinal cord of rabbit. After impregnation at pH 8·8 the section was washed and dried before development. Note the similarity to fig. 4. × 105.

Fig. 4. Section treated as in fig. 3 but not dried before development. × 105.

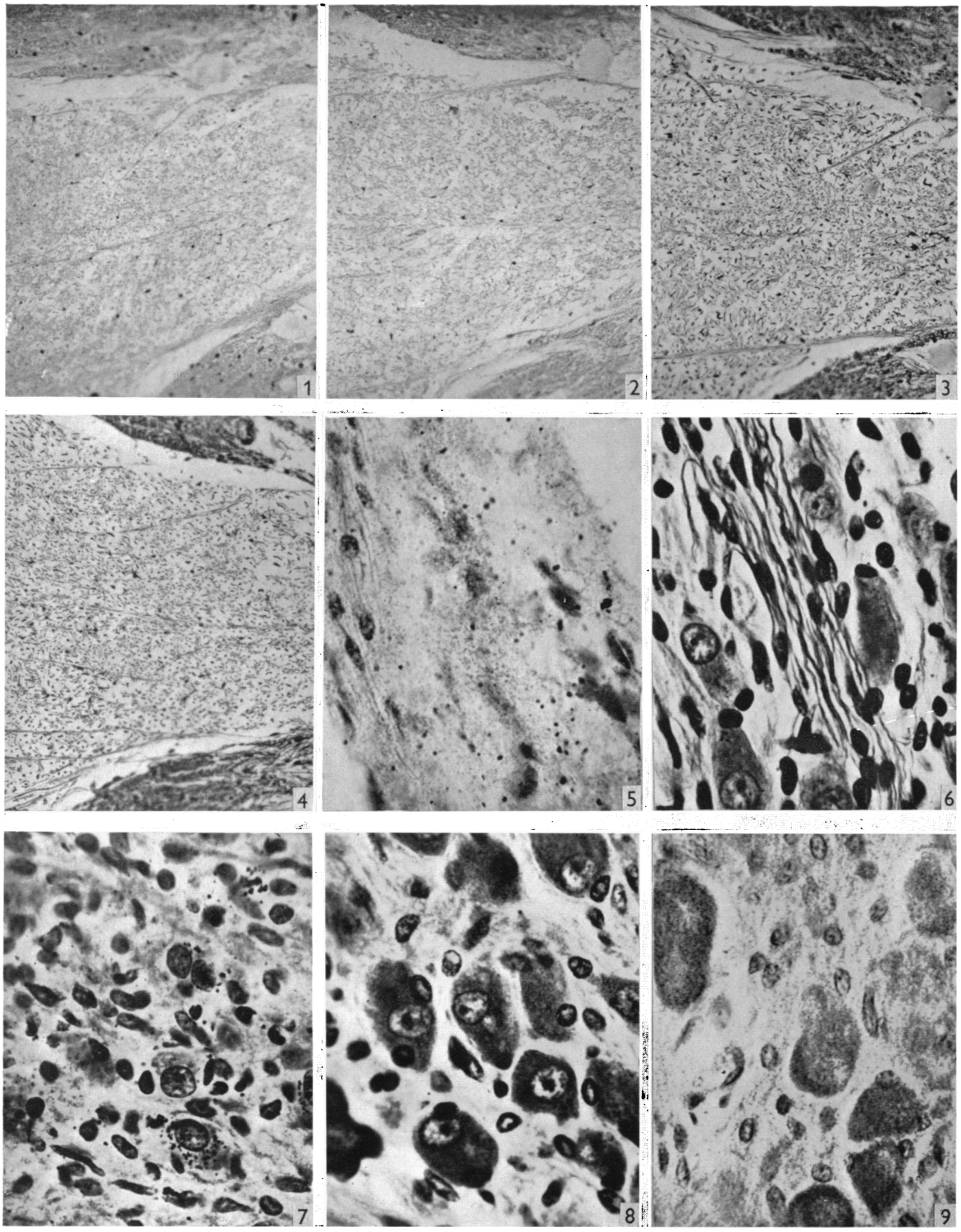
Fig. 5. Section of sympathetic ganglion of rabbit. Physical development after impregnation at pH 9·1 for 15 min. indicates that few silver 'nuclei' are present in the tissues. × 520.

Fig. 6. Section of sympathetic ganglion of rabbit. Physical development after impregnation at pH 6·8 for 2½ hr. The axons, the cells and their nuclei are well stained, compare with fig. 7. × 520.

Fig. 7. A section treated as in fig. 6 but impregnated at pH 9·1. The section is feebly stained, and no axonal staining can be seen. × 520.

Fig. 8. Section of sympathetic ganglion of rabbit impregnated at pH 9·1 for 15 min. and developed in 1% hydroquinone. After 15 min. there is a considerable amount of reducible silver in the section, compare with fig. 9. × 520.

Fig. 9. Section of sympathetic ganglion of rabbit impregnated at pH 6·8 for 2½ hr. and developed in 1% hydroquinone. Only a small amount of reducible silver is present in the section. × 520.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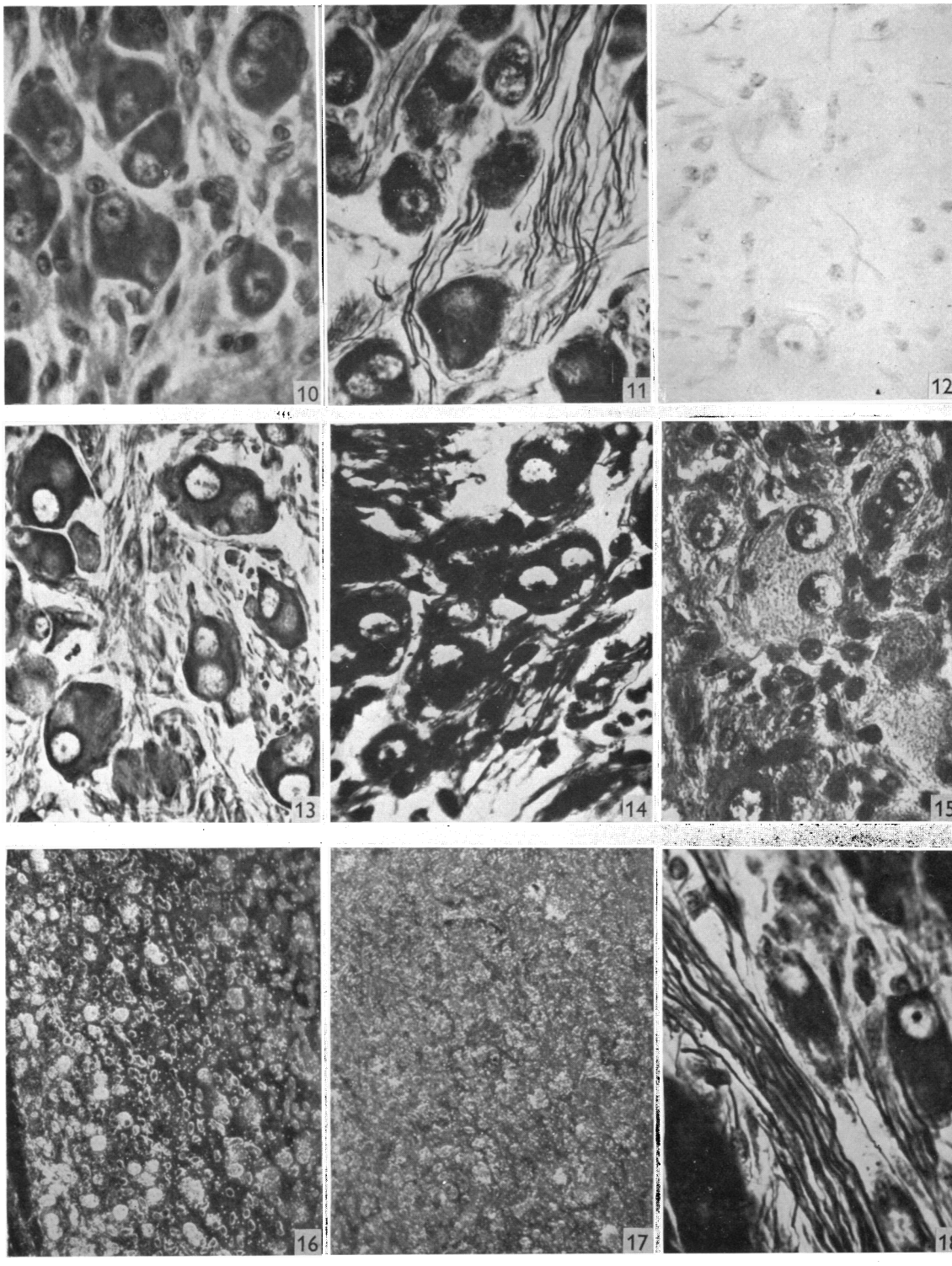
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PLATE 2

Fig. 10. A section treated as in fig. 9 but impregnated at pH 9·1. The heavy diffuse staining indicates how the reducible silver increases as the pH rises, compare with fig. 9, and as the time of impregnation is prolonged, compare with fig. 8. × 520.

Fig. 11. Section of sympathetic ganglion of rabbit impregnated at pH 9·1 for 15 min. and developed in 1% hydroquinone in 10% sodium sulphite. Deeply stained axons and neurons may be seen. This section has *not* been toned. × 520.

Fig. 12. Section of sympathetic ganglion of rabbit impregnated at pH 6·8 for 2½ hr. and developed in 1% hydroquinone in 10% sodium sulphite. Untoned. The section is faintly stained but similar sections when toned, see fig. 18, resemble fig. 6. × 520.

Fig. 13. A section treated as in fig. 12 but impregnated at pH 9·1. The diffuse staining should be compared with fig. 11 which was impregnated for 15 min. only. × 520.

Fig. 14. A section of sympathetic ganglion of rabbit impregnated at pH 8·5 for 1 hr. and physically developed for 10 min. at 53·5° C. The whole section is jet black in colour, compare with fig. 15. × 520.

Fig. 15. A section treated as in fig. 14 but impregnated for 24 hr. After the prolonged incubation there is much less staining on physical development. × 520.

Fig. 16. A section of sympathetic ganglion of rabbit impregnated at pH 9·1 and physically developed for 15 min. photographed with dark ground illumination. Note the highly refractile cell nuclei. × 220.

Fig. 17. A section treated as in fig. 16 but impregnated for 2½ hr. The cell nuclei are no longer refractile. × 220.

Fig. 18. A section of sympathetic ganglion of rabbit impregnated at pH 6·8 for 2½ hr., developed in 1% hydroquinone in 10% sodium sulphite and toned. When toning is used the close relationship between the distribution of the silver 'nuclei' and the staining effect produced by the hydroquinone sulphite becomes clearly apparent, cf. figs. 6, 12 and 18.