IMPREGNATION AND DEVELOPMENT IN SILVER STAINING

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In spite of the multiplicity of silver solutions and developing agents and the varied manner of their employment, the many different silver staining techniques for nervous tissues are capricious. This appears to be due in part to an inadequate understanding of the processes involved in impregnation and development. It was the purpose of this research to elucidate with greater exactitude the properties of the impregnating solution and the reactions occurring during the development phase of silver staining.

The investigations of Foot (1929) established the importance of controlling the silver concentration and pH of the silver bath for successful staining, and Holmes (1943), recognizing that the significant variables in the silver solution used in Bodian's method (1936) were the silver and hydrogen-ion concentrations, stained nerve fibres in paraffin sections with dilute silver nitrate solutions adjusted to a particular pH. Pearson & O'Neill (1946) and Romanes (1950) also elaborated methods, using acid or weak alkaline solutions of silver salts. These techniques, using very different impregnating solutions, were designed for tissues fixed in a similar manner. Consequently, there appeared to be some relationship between the pH, the silver concentration, the time, and the temperature of the incubation, for when suitably varied one with another they produced similar results. A relationship has been postulated, without direct experimental proof, by Kubie & Davidson (1928) and Kubie (1929) who stated that the differences in the activity of the silver salts (they used ammoniacal silver salts) must be compensated for by altering the concentration, duration, and temperature of the silver bath and the strength of the developer. But there was no evidence as to why, or how, this compensation took place, nor why certain hydrogen- and silver-ion concentrations were necessary in different methods, which, if departed from, resulted in failure of the staining process. Information was equally deficient regarding the action of the developer. Silver (1942) believed the pH of the developer to be all-important in the staining process, but Davenport, McArthur & Bruesch (1939) found that the pH of an amidol developer had little effect on the staining of paraffin sections impregnated in a protargol solution, except when it approached the isoelectric point of protargol. Holmes (1943) concurred with this view, but disagreed with Kubie's (1929) opinion that diffusion of silver from paraffin sections affected the specificity of the stain, and believed that differences in staining produced by different developers was due only to the more complete development of the silver in the section when the more active developers were used.

EXPERIMENTAL PROCEDURE

Before the present investigation could be undertaken it was essential to develop a reliable silver staining method which permitted complete control of the chemical and physical factors involved. This proved singularly difficult, but the following method was ultimately evolved from the Holmes (1943) technique. Dewaxed sections were:

(1) Washed in running water for 2 hr.

(2) Rinsed in three changes of distilled water.

(3) Placed in a slide bath containing borax-boric acid buffer solution (10 c.c. of buffer–90 c.c. distilled water)* and incubated. A flask containing 99 c.c. of a similar solution was also placed in the incubator. When the solutions in the flask and bath were stabilized at a temperature of 53·5° C., 1 c.c. of 1 % silver nitrate solution was added to the buffer within the flask and thoroughly mixed. The buffer was decanted rapidly from the slides and the silver buffer solution added to them. The impregnation was continued for a predetermined time at the same temperature.

(4) The sections were then developed.

(5) In some instances, the sections were toned in 0·2 % gold chloride for 10 min., washed with distilled water for 1 min., reduced in 2 % oxalic acid for 5 min., washed for 15 min., and finally dehydrated and mounted.

The term 'impregnation' is reserved for the reactions taking place between the section and the buffered silver nitrate solution. The term 'staining' refers to the final appearance of the section when cleared and mounted.

The impregnation method described possesses certain advantages. The time taken for solutions in the incubator to reach a stable level varies in different types of slide baths and in different parts of the incubator, and if the sections and solutions are allowed to reach a stable temperature before the silver is added this ensures:

(*a*) Precise control of the duration and therefore of the amount of reaction between the section and the silver nitrate at a constant temperature.

(*b*) That the sections are always in equilibrium with their chemical environment before reacting with the silver. This is important, for temperature variation causes variation in pH and if the pH of the solution affects the silver-reacting qualities of the section, it is essential that this should be constant for any particular pH.

The following experiments were carried out on 10 μ paraffin sections of sympathetic ganglia and spinal cord of rabbits fixed in the solution of Davenport & Kline (1938) consisting of formic acid 5 c.c., trichloracetic acid 5 g., *n*-propyl alcohol 25 c.c., *n*-butyl alcohol 65 c.c., dehydrated in ethyl and butyl alcohol and imbedded in paraffin.

* The buffer solutions consisted of an M/20 borax solution containing 19·1 g. $Na_2B_4O_7 . 10H_2O$ in 1 l., and an M/5 boric acid solution containing 12·4 g. H_3BO_3 in 1 l. For a particular pH the solutions were mixed in the proportions given in the Palitzsch buffer table. The dilution of the buffer and the omission of the sodium chloride from the boric acid solution caused insignificant differences in the value of the pH as given in the table and these have been ignored.

THE PROPERTIES OF THE IMPREGNATING SOLUTION

Hydrogen-ion concentration and the effects of varying the time of impregnation

Series of sections were impregnated in solutions containing 1 c.c. of 1 % silver nitrate buffered at pH 6·8, 7·4, 7·8, 8·2, 8·4, 8·7 and 9·1 for periods of 7 min., 2½ hr., and 24 hr. They were then developed in 1 % hydroquinone in a 10 % sodium sulphite solution and toned.

Seven min. impregnation. At pH 6·8, the section was covered with a grey granular precipitate and very faint neuronal staining was observed (Pl. 1, fig. 1). As the pH was raised to 7·8 the precipitate decreased markedly and some axonal staining was apparent, together with a general improvement in the definition of the neurons. With further increases in pH this improvement continued, until at pH 8·7 (Pl. 1, fig. 2) the cells and nucleoli were sharply stained in contrast to the clear background in which well-stained axons were visible; at pH 9·1 the staining became so heavy and diffuse that much of the contrast between the different constituents of the section was lost (Pl. 1, fig. 3).

Two and a half hours' impregnation. The most marked changes occurred at the extremes of the pH range. In the sections impregnated at pH 6·8 (Pl. 1, fig. 4) the axons, the neurons, and their nuclei, were now heavily stained, but at pH 9·1 the section was uniformly and evenly stained reddish brown in colour and had an undifferentiated flat appearance. The axons were no longer black as in the 7 min. impregnation, but were the same colour as the rest of the tissues and were scarcely distinguishable.

Twenty-four hours' impregnation. On removal from the silver bath the sections were a brown colour, which became intensified as the pH of the buffered silver solution increased. Microscopic examination of these undeveloped sections impregnated at high pH levels showed deep capsular staining and the cell cytoplasm was pale brown. Nuclear detail was evident, particularly at the periphery of the section, where pale brown axons could also be seen. When the sections impregnated at pH 6·8–7·8 were developed and toned, the axons were delicately stained in a feebly stained background; the neurons and nuclei too were sharply differentiated. Above pH 7·8, however, the sections had a flat undifferentiated appearance in which all structures were uniformly stained a reddish brown colour. Thus, whereas in the shorter impregnations of 7 min. and 2½ hr., the undifferentiated staining appeared only at the upper limit of the pH range, when the impregnation was prolonged for 24 hr., sections impregnated at much lower pH levels became evenly stained.

Progressive staining

Since the increase in incubation time to 2½ hr. resulted in the staining of the axons at pH 6·8, sections treated in this way were removed from the silver bath at 15 min. intervals, developed, and toned in order to study the progress of axonal staining.

After 15 min. the sections were almost completely unstained. With further impregnation, axonal staining commenced, and after 45 min. (Pl. 1, fig. 5) a coarse granular precipitate was distributed along the axons. This gradually increased in amount, and half an hour later the axons were clearly visible but still exhibited

granular staining which had also appeared in the cell cytoplasm and nucleus (Pl. 1, fig. 6). With a further increase in incubation time to 1 hr. 45 min., the axons became heavily but unevenly stained; they had begun to lose their granular appearance and neuronal staining was more complete (Pl. 1, fig. 7). After 2½ hr. impregnation, the axons were evenly and regularly stained in sharp contrast to the faintly stained background tissue. The neurones were completely stained and considerable nuclear detail could be seen (Pl. 1, fig. 4).

Silver concentration

To evaluate the role of the silver-ion concentration, buffered silver nitrate solutions at pH 6·8 containing increasing amounts, 0·5–10 c.c. of 1 % silver nitrate, were added to sections. These were then impregnated for 15 min., and then developed and toned as before. The amount of distilled water added to the 10 c.c. of buffer solution was adjusted so that the final volume was always 100 c.c.

When 0·5–1 c.c. of silver nitrate was added no staining occurred. As larger amounts were added the staining of the axons gradually increased, closely followed by increased staining in the neurons. After the addition of 10 c.c. of silver nitrate the cells and axons were clearly stained (Pl. 1, fig. 8). The improvement in staining generally followed a course very similar to that seen when sections were impregnated at pH 6·8 for increasing lengths of time.

THE ROLE OF THE DEVELOPER

The effects of different developers

Slides were immersed in buffered silver solutions at pH 6·8, 7·4, 7·8, 8·0, 8·7, 9·1, at 53·5° C. for periods of 15 min., 1 and 2½ hr., and then rinsed and developed in one of the following solutions at 20° C. for 5 or 10 min.

(*a*)	Hydroquinone	1 g.	(*b*)	Hydroquinone	1 g.
	Sodium sulphite	10 g.		Distilled water	100 c.c.
	Distilled water	100 c.c.			
(*c*)	Amidol	0·5 g.	(*d*)	Amidol	1 g.
	Sodium sulphite	10 g.		Sodium sulphite	10 g.
	Sodium bisulphite	2·5 g.		Distilled water	100 c.c.
	Distilled water	100 c.c.			

It is unnecessary to give a detailed description of the staining produced by each developer after each impregnation, and the discussion will be confined to the most significant aspects of the results.

As the pH was raised and/or the time of impregnation was prolonged, it was found that under similar conditions staining first appeared with the amidol sulphite developer, secondly with the hydroquinone solution, thirdly with the amidol bisulphite mixture, and lastly with the hydroquinone sulphite solution. For every equivalent pH and time of impregnation the staining intensity of the section as a whole also increased in that order. But every developer had its own staining spectrum, for apart from the difference in staining intensity the same structures were stained to varying extents in each developer. This may be exemplified by sections of sympathetic ganglia impregnated at pH 7·4 for 1 hr. and developed in

1 % amidol sulphite, and in the 1 % hydroquinone sulphite developer—i.e. the most and least active developer used. In the former the cytoplasm of the neurons was deeply and evenly stained, the nuclear detail was almost complete and the axons were barely visible in the diffusely stained background tissue (Pl. 2, fig. 9). In the section developed in the hydroquinone sulphite solution the neuronal cytoplasm was irregularly stained with little nuclear detail and the finely stained blue-black axons were in marked contrast to the clear background (Pl. 2, fig. 10). These differences were apparent over the entire pH range. The hydroquinone developed sections resembled the sections developed with the amidol sulphite solution, but were paler and more granular; the amidol bisulphite caused staining which resembled that produced by the hydroquinone sulphite, but with more intense staining of cells and background and the axons had a finer appearance.

Each developer produced different effects, and the more active and faster the developer the more diffuse and even was the staining, the slower developer giving a more precisely differentiated axonal stain. It appeared that the speed of development played an important role. But it was not only a question of the rate of development, because prolonged immersion in the slow hydroquinone sulphite did not produce the staining comparable with the rapid amidol sulphite developer. Why did similarly impregnated sections stain so differently with different developers? It did not appear, as Holmes (1943) believed, that the hydroquinone sulphite developed only a proportion of the silver in the section as compared with the amidol sulphite solution. For, when stained, the chromidial bodies in the neurons, and the axons in the spinal cord and ganglia, were always more heavily stained and clearly differentiated after hydroquinone sulphite, whereas they should have been less heavily stained if development was incomplete. These considerations suggested further experiments.

Amidol sulphite and hydroquinone sulphite development

To determine whether the differences in the appearance of the sections were due to complete development in the amidol sulphite solution and incomplete development in the hydroquinone sulphite solution, sections were impregnated at pH 7·4, and 7·8, at 53·5° C. for 1 and 2½ hr., as before. From each bath, sections were placed directly, without rinsing to avoid all possible interference with the sections, in a variety of solutions:

(1) 1 % hydroquinone in 10 % sodium sulphite at 20° C. for 5 min.;
or (2) 1 % amidol in 10 % sodium sulphite at 20° C. for 5 min.;
or (3) 1 % hydroquinone in 10 % sodium sulphite at 20° C. for 5 min., and then rinsed in a similar solution before being transferred to a 1 % amidol in 10 % sodium sulphite at 20% C. for 5 min.;
or (4) distilled water at 20° C. for 5 min. followed by immersion in 1 % amidol in 10 % sodium sulphite at 20° C. for 5 min.

If no silver was removed in the hydroquinone sulphite developer, a section placed in the hydroquinone sulphite and then in the amidol sulphite solution should resemble a section placed directly in amidol sulphite. Sections impregnated at pH 7·4 for 1 hr. and subjected to the double development procedure did not resemble sections placed directly in the amidol sulphite solution (Pl. 2, fig. 9). Their appearances were

those of sections developed in the hydroquinone sulphite developer above (Pl. 2, fig. 10). The control sections placed in distilled water, though paler, were stained like those placed directly in the amidol developer (Pl. 2, fig. 9).

It was clear that the differences between sections developed in the hydroquinone and amidol sulphite developers were not due to diffusion of silver from the section in the solvent of the developer; but a definite loss of silver from the section had taken place, for after double development, no, or little, further development ensued in the amidol developer. Davenport & Kline (1938) observed that increasing the concentration of sodium sulphite in the developer increased differential staining, i.e. it produced an increased 'hydroquinone sulphite' effect. It was considered possible that the sulphite constituent could be responsible for the differences in the staining of the sections reduced in the hydroquinone and amidol sulphite developers.

The action of the sulphite in the developer

Sections impregnated at pH 7·8 and 8·5 for 1 and 2½ hr. were treated with sulphite solutions whose concentrations ranged from 0·25 to 10 %, for times varying between 15 sec. and 10 min., and then developed either in a 1 % amidol in 10 % sodium sulphite solution or a 1 % hydroquinone in 10 % sodium sulphite solution.

The sections of spinal cord impregnated at pH 7·8 for 2½ hr. and developed in 1 % amidol in 10 % sodium sulphite solution evidenced less staining after treatment with 1 % sodium sulphite for 1 min. than when an 0·25 % sodium sulphite solution was used. In sections of ganglia impregnated at pH 8·5 for 1 hr. and developed in 1 % hydroquinone in 10 % sodium sulphite the axons and cells were completely stained (Pl. 2, fig. 11). After 15 sec. in 2·5 % sodium sulphite cellular staining had diminished, nuclear detail was almost absent, but the axons were clearly visible in the colourless background (Pl. 2, fig. 12). After 2 min. in the sodium sulphite the sections were completely unstained (Pl. 2, fig. 13).

The evident correlation between staining intensity and the time in, and concentration of, the sulphite before development suggested that the difference in the appearance of amidol sulphite and hydroquinone sulphite developed sections could be partially attributed to the sulphite constituent of the developer. The complete absence of staining following immersion in 2·5 % sodium after 2 min. showed that the sulphite removed the silver which would have been reduced and deposited in the section by the developer, i.e. it removed the reducible silver on which the staining of the tissues depended. In the sulphite developer solution, therefore, the sulphite competed with the developing agent and tended to remove the reducible silver from the section. Amidol, a powerful and rapid developer, outpaced the sulphite and developed the silver *in situ*. Hydroquinone, a slower developer, was partially outpaced by the sulphite which removed some of the reducible silver in the section.

Further experiments confirmed this conception of developer sulphite competition for the available silver, and established that a low pH, apart from depressing the activity of the developing agent, also facilitated the removal of the silver by the sulphite, and vice versa.

DISCUSSION

It is evident from the sections stained for increasing lengths of time at pH 6·8 that nerve staining is not an end-point phenomenon. It takes place after a certain amount of reaction between the section and silver solution. To render the axons visible a certain quantity of silver must be deposited in them, and this amount can be deposited under various conditions of impregnation, providing they possess a certain degree of affinity for silver. If it is assumed that the pH controls their silver affinity, the results of the experiments fall into an intelligible pattern. As the pH increases up to pH 8·2, 8·4, the silver affinity of the axons increases and thus a shorter time of impregnation is needed for an adequate amount of reaction and silver deposition. At lower pH levels the axons have less affinity for silver and a longer impregnation time is required. However, increasing the silver nitrate concentration tenfold shortens the impregnation at pH 6·8 from 2½ hr. (Pl. 1, fig. 4) to 15 min. (Pl. 1, fig. 8). Though a section impregnated at a low pH and high silver concentration (Pl. 1, fig. 8) resembles a section impregnated at high pH and low silver concentration (Pl. 1, fig. 2), there are important differences between the effects produced by increasing the silver concentration and by increasing the pH of the solution, for apart from the increased speed of staining as the pH rises, the pattern of staining alters. As the staining of the section is followed through the pH range after 7 min. impregnation, axonal staining reaches a maximum about pH 8·2–8·4, beyond which further addition of alkali produces no visible effect on the axon. But with increasing pH other structures absorb more silver until the axons become obscured by heavy cellular and background staining. When the impregnation is prolonged for 2½ hr., further changes take place at high pH levels—the whole character of the staining alters, and all the constituents of the section become stained an even reddish brown colour. This change also occurs at intermediate pH levels after 24 hr., but only to a slight extent at the lower end of the pH range. But the changes in staining effects associated with increase in the pH does not occur when the silver concentration alone is increased at pH 6·8. It would appear, therefore, that the effect of increasing concentrations of silver is limited by the pH of the solution and only decreases the time required for the full staining range of a particular pH to be revealed.

When an impregnated section with an adherent film of impregnating solution is placed in a developer, very complex reactions occur. As the developer penetrates the section and mixes with the adherent buffer, the pH fluctuates. These fluctuations affect: (*a*) the tenacity with which the silver is held by the section and thereby indirectly influences the amount of silver removed by the sulphite; and (*b*) the activity of the developing agent, particularly hydroquinone, which is more sensitive to the hydrogen-ion concentration than amidol which is affected only to a slight degree. The lower the pH of the development in hydroquinone sulphite the slower the development, the more silver is removed by the sulphite and less staining occurs, and vice versa. The resultant staining is, therefore, considerably affected by the outcome of the competition between the developer and sulphite, and the complex conditions in which it occurs. This is clearly evidenced by the variations in staining which follow absence of sulphite, or variations in its concentration in the developing

solution. It would be incorrect to conclude that the staining depends entirely on the speed of the developing agent and the silver removing properties of the sulphite and the *resultant amount* of silver deposited in the section. For a section treated with sulphite to remove a proportion of the reducible silver before hydroquinone development still stains diffusely, and does not resemble a section developed in a hydroquinone sulphite mixture. Therefore, differential staining appears only as a result of the integrated action of the sulphite and developing agent on the section. But although the *differences* in staining effected by different developers on similar sections can be explained in terms of their different rates and extent of reaction, and on the silver removing properties of the sulphite, it does not explain why a particular developer produces the effect that it does. Why, for example, are amidol sulphite sections diffusely stained, when the same sections developed in hydroquinone sulphite have a differentiated appearance? Apparently whilst the competition between the sulphite and developer is proceeding, some other factor is operating which is partially responsible for the final staining. This aspect of the problem has necessitated a more detailed consideration of the mechanism of silver staining which will be the subject of a future communication.

Holmes (1943) maintained that different developers did not affect the specificity of axonal staining, but that more active developers reducing the silver in the section more completely cause a greater degree of intense staining of all the tissues in the section. As other structures are stained by silver, the term 'specific axon staining' must be qualified, for the phenomenon to which this term really applies is one in which the axons are more intensely stained in contrast to the other tissues, in spite of the silver affinities of the latter. Defined in this way, it is clear from the present experiments that developers do affect the specificity of the stain.

In the last analysis the properties of the fixed tissue determine the amount and distribution of the silver affinities at a particular pH, and the many techniques which have been elaborated were attempts to adjust the silver concentration, pH, temperature and time in the silver bath to the properties of the tissue. But the present experiments indicate that it is incorrect to consider a particular pH and silver concentration as being 'specific' for axonal staining, as the pH, silver concentration, time, and temperature are so inter-related that *within limits* increase or decrease in one has the same effect as decrease or increase in any other. Furthermore, the adjustments of the conditions of impregnation must also ensure that after the certain *time* of reaction between the section and the silver solution demanded by the particular technique, the amount and distribution of the silver in the section is suited to the qualities of the developer employed. So that on development, there results the *same* adequate deposition of silver in the axons which stains them in *contrast to the surrounding tissues.*

SUMMARY

1. Paraffin sections were immersed in buffered silver nitrate solutions of varying pH and silver concentrations for different lengths of time, and developed with various developing agents.

2. The experiments showed an evident inter-relationship between the pH and silver nitrate concentration and the time of impregnation; within limits, increase in one produced effects similar to increase in any of the others.

3. There were, however, important differences between increasing the time, temperature, and silver concentration and increasing the pH.

4. At high pH the sections rapidly assumed an undifferentiated appearance, a change which occurred more slowly as the pH was lowered.

5. To render the axons visible a certain amount of silver must be deposited in them and this amount can be deposited under various conditions of impregnation.

6. Different developing solutions caused profound alteration in the specificity of axonal staining dependent in part on the outcome of the competition between the silver-removing properties of the sulphite and the reduction potential of the developing agent.

7. The effect that a developer could produce was limited by the impregnation.

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EXPLANATION OF PLATES

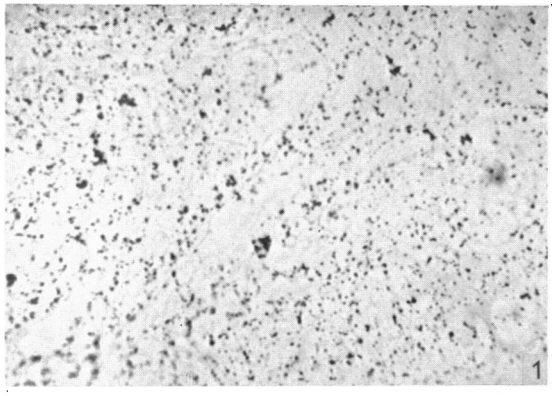
All the photomicrographs have been taken under standard conditions of light intensity, exposure time, development and printing. All the sections are of the sympathetic ganglion of the rabbit fixed in the solution of Davenport & Kline (1938).

PLATE 1

Fig. 1. The section impregnated at pH 6·8 for 7 min. is unstained. × 520.

Fig. 2. The section impregnated at pH 8·7 shows clearly differentiated axons and cells. × 520.

Fig. 3. In the section impregnated at pH 9·1 for 7 min., heavy diffuse staining obscures axonal detail. × 520.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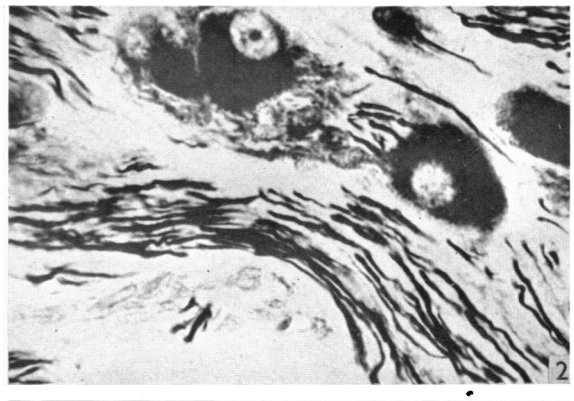
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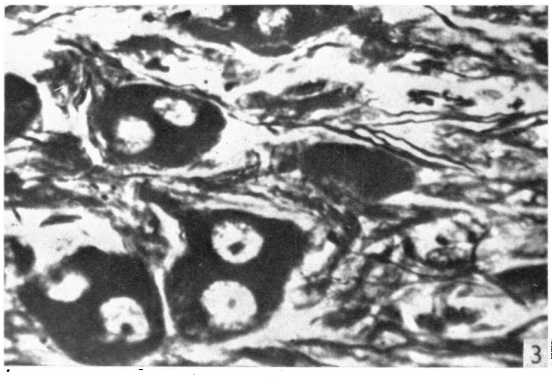
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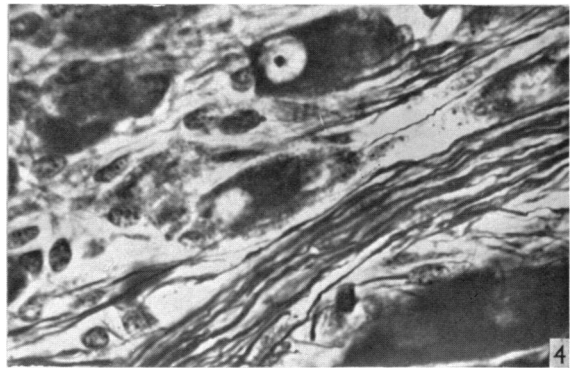
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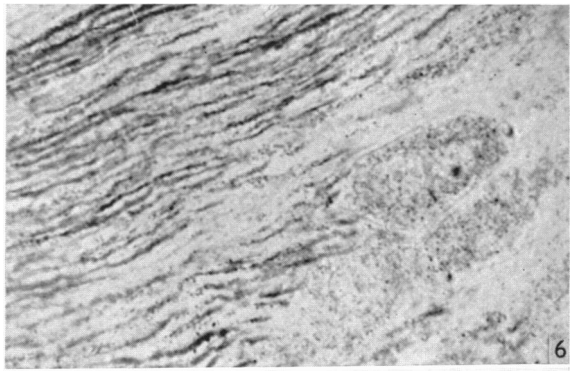
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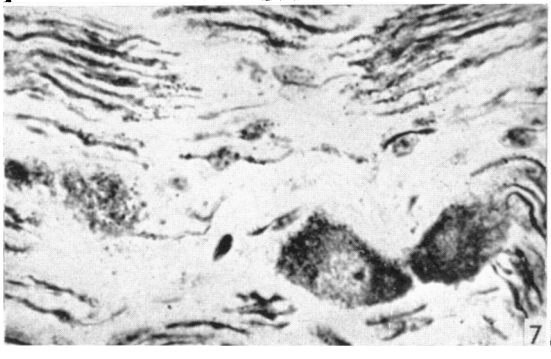
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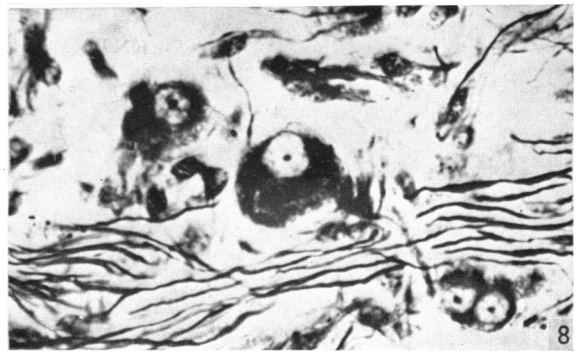
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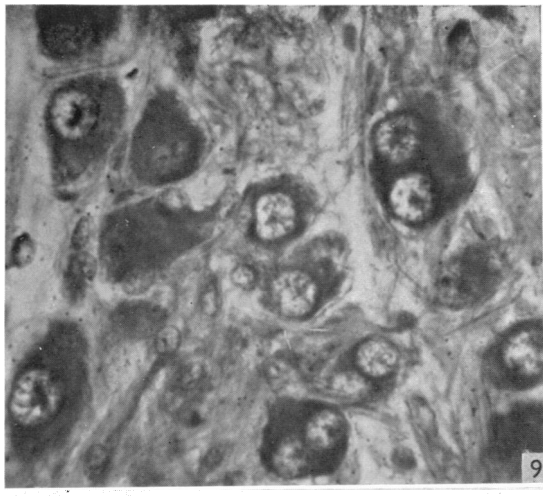
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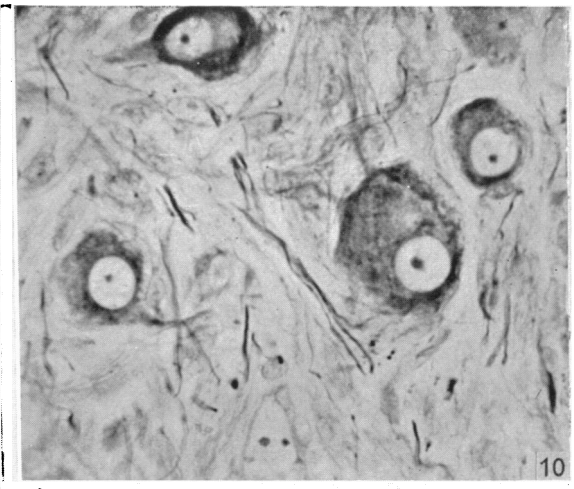
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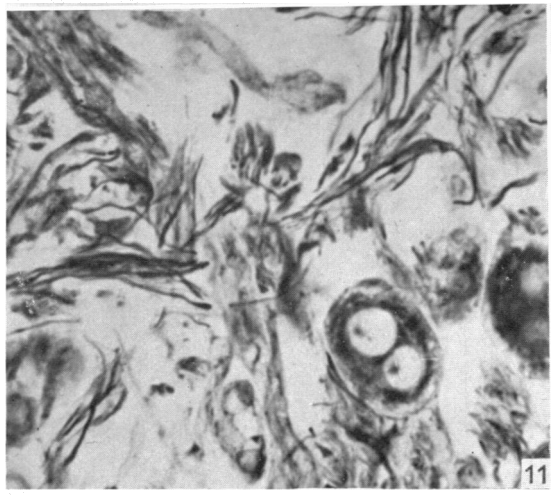
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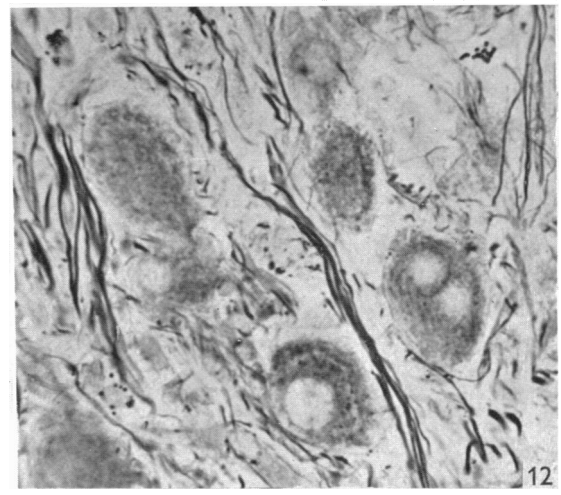
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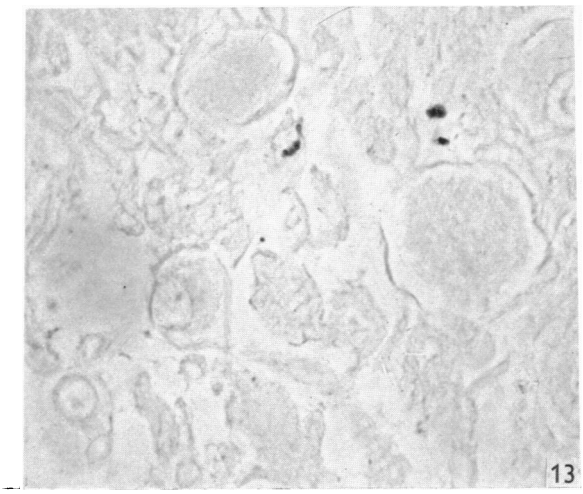
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Fig. 4. A section impregnated at pH 6·8 for 2½ hr. showing the effect of prolonged incubation at this pH, compare with fig. 1. × 520.

Fig. 5. A section impregnated at pH 6·8 for 45 min. × 520.

Fig. 6. A section impregnated at pH 6·8 for 1 hr. 15 min. × 520.

Fig. 7. A section impregnated at pH 6·8 for 1 hr. 45 min. Figs. 5, 6, 7 and 4 illustrate how axonal staining is a gradual process and is not an end-point phenomenon. × 520.

Fig. 8. A section impregnated at pH 6·8 for 15 min. with 10 c.c. of silver nitrate per 100 c.c. of buffer solution. Compare with fig. 1 which resembles a section impregnated for 15 min. when 1 c.c. of 1% silver nitrate was added to the buffer. × 520.

PLATE 2

Fig. 9. Section impregnated at pH 7·4 for 1 hr., developed in 1% amidol in 10% sodium sulphite. The axons are barely visible in the diffusely stained background. Note the intense staining of the cells. × 520.

Fig. 10. A similar section to that shown in fig. 9 but developed in a 1% hydroquinone in 10% sodium sulphite. The delicately stained axons are readily seen in the clear background. Note the uneven stain of the cell cytoplasm and lack of nuclear detail as compared with fig. 9. × 520.

Fig. 11. A section of sympathetic ganglion of rabbit impregnated at pH 8·5 for 1 hr. and developed in a 1% hydroquinone in 10% sodium sulphite. × 520.

Fig. 12. A section similar to that shown in fig. 11 but treated for 15 sec. in a 2·5% solution of sodium sulphite before development. There is much less staining in the cells and background but the axons are hardly affected. × 520.

Fig. 13. When the time in the 2·5% sodium sulphite is prolonged for 2 min. there is a complete absence of staining. × 520.