Studies of the Impregnation of Nervous Tissue Elements

I. Impregnation of Axons and Myelin

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SUMMARY

All metallic impregnation methods depend on the presence of reducing groups in tissues. By testing the effect of various reduction indicators on sections of nervous tissue it was found that some reduction systems do not have a high enough oxidizing potential to serve for the visualization of either axons or myelin. Others can visualize myelin sheaths. The systems having the highest potential visualize also axons. Although myelin can be oxidized more easily than axons, axons are probably relatively richer in reducing groups.

Impregnation by AgNO₃ or Ag-diammine results in the formation of silver nuclei in both axons and myelin. Subsequent exposure of such sections to the action of reducing agents increases the visible impregnation of the axons much more than that of the myelin. This effect can be ascribed to the low dielectric constant of myelin.

Gold toning serves a dual purpose: (a) coating with gold the more heavily impregnated structures, and (b) clearing the background through the displacement of the silver from the less intensely impregnated areas.

The mechanism of metallic impregnations and the rationale of the different steps in Bielschowsky’s procedure for axons are discussed.

INTRODUCTION

ALTHOUGH metallic impregnation methods are of the utmost import-ance in the study of normal and pathological nervous tissue constituents, they are still universally considered to be most capricious. For no understandable reason they sometimes succeed and at other times fail. Furthermore, the chemical basis of these methods is not clearly understood, and their practical application represents more often an art than a science. The possible pitfalls in studies which are based on specific impregnations are numerous, as a slight change in technique, which may pass unnoticed, might result in the staining of structures other than those for which the method is considered to be selective.

The present series of studies aims at showing that all the metallic impregnation methods are based on a single general principle which can be deduced from the available information. According to this principle all metallic impregnations depend on the reducing capacity (redox potential) of the different structures, an idea expressed although not substantiated by Palmgren (1948). The various structures differ from each other and allow differential staining because of the following: (1) The reducing groups may be either free, or may...
be formed, or made available, by different preliminary treatments. (2) The redox potential of the structures may be different. (3) The rate of reaction of the chemical reducing groups with different reagents may be different. (4) The amount of reducing activity (number of reducing groups per unit of volume) may vary in the different structures. (5) Treatment subsequent to the reduction of the metal may effect differently various structures.

The experiments reported in this paper were performed in order to clarify the mechanism of the metallic impregnation of axons and myelin. The mechanism of impregnation of other nervous tissue elements will be dealt with in subsequent papers.

**Material and Methods**

Most experiments were performed on human autopsy material fixed within less than 8 hours after death. In some experiments dog and cat material was used for comparison. Pieces of cerebral cortex and of medulla or spinal cord were used, and their sections were usually processed simultaneously. Tissue sections were prepared as follows:

(a) *Frozen sections.* Preliminary experiments showed that the floating of sections, or parts of them, on the surface of the impregnating solution, and folding of the sections introduced disturbing effects and artifacts. These effects were probably due in the first case to the disturbing effect of atmospheric oxygen on oxidation of tissue elements by the metal ions, and, in the case of folding, to the different speed of penetration of reagents into the sections. Frozen sections cut at 15–20 μ were therefore affixed to albuminized slides according to the method of Iwanoff (cf. Roulet, 1948) in accordance with Feyrter's (1951) suggestion.

(b) *Carbowax-embedded sections.* Further experiments showed that carbowax-embedded material gave identical results with those obtained with frozen sections.

The following method of carbowax embedding and cutting was found empirically to be suitable. Pieces of fixed tissue, 2–4 mm. in thickness, were washed thoroughly in running water and embedded, after drying with filter paper, in a mixture of 4 parts carbowax 1540 and 1 part of carbowax 4000 for 3–5 hours in a paraffin oven at 58°. Sections 8 μ thick were picked up from water on albuminized slides and affixed to them directly or by Ivanoff's method.

In some experiments paraffin-embedded sections were used. The various methods of fixation and the treatments given to the sections are described with the experiments.

**Experiments Dealing with the Primary Impregnation (or Staining) of Axons and Myelin**

The following experiments were performed in order to find out which reduction indicators are suitable for the visualization of axons and myelin and
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the effect of some contributing factors on the visualization. The sections were not subjected to the action of secondary reducers.

(a) Treatment of sections by 2% AgNO₃ with subsequent 'fixation' in 3% sodium thiosulphate resulted in a weak impregnation of myelin and a much lighter, hardly visible one, of axons. It may be remarked that Bielschowsky (1904) called this part of his procedure 'Myelinfärbung'.

(b) Treatment of sections by Bielschowsky's silver-diammine solution ('ammoniacal silver nitrate') resulted in impregnation of myelin and a lighter impregnation of axons.

(c) The effect of pH on the staining by silver-diammine solutions was studied by the use of solutions of silver acidified (by HNO₃) or alkalinized (by ammonia). A double strength silver-diammine solution was prepared and aliquots of it were brought to the desired pH by the use of varying amounts of HNO₃ and NH₄OH; the solutions were then diluted to an equal final concentration of silver. After primary impregnation the sections were fixed in thiosulphate. In agreement with the results of Silver (1942) and Samuel (1953a), neither myelin nor axons were impregnated below pH 5. Myelin was well impregnated in pH ranges above 5. The impregnation of axons began at about pH 6-7, increased with the pH, and was optimal around pH 11-12 (at the upper limit of which lies the pH of Bielschowsky's solution). In the optimal zone the axons were impregnated as intensely as the myelin. Further alkalinization of the solution resulted in a rapid decrease of the impregnation of axons and a somewhat slower decrease in the impregnation of myelin.

(d) Impregnation was also attempted by silver methenamine solutions prepared according to Gomori (1953) and brought to pH levels between 7 and 8.5. At the lower pH levels only myelin was stained; at pH 8.5 the axons stained lightly.

(e) Treatment of sections: (1) by 1% osmium tetroxide for ½–1 hour, (2) by 0.2–1% gold chloride for 3–24 hours, (3) by neutral or alkaline 1% potassium permanganate solutions for between 10 minutes and 1 hour, or (4) for 2 hours in leucofuchsin (Schiff's solution prepared according to Lillie's prescription, 1954), resulted in staining of nerve cells and myelin, but not of axons.

(f) Treatment of sections by a 1% blue tetrazolium (BT) for 2 hours, and by Tetrazol-Purpur (Bayer) at various pH ranges, resulted in no staining in the neutral range and in a progressively increasing staining of myelin in the pH ranges 8–10. At pH 12 the myelin was stained quite intensely, while the axons were stained much more weakly. (These reagents were kindly supplied by Dr. A. M. Seligman and Messrs. Bayer respectively.)

(g) No visible impregnation occurred by treatment for 24 hours with potassium tellurite, HgCl₂, Bi(NO₃)₃, CuSO₄, CuCl₂, Pb acetate, and FeCl₃.

(h) The ferricyanide ferric-chloride reaction gave good staining of axons with a very light staining of myelin. It is interesting to note that this was one of the reactions recommended by Unna (1927) for the visualization of reduction-sites in the body. The technique used was that recommended by
Conclusions

The results obtained with the reduction indicators tested were of three types:

(a) No staining was obtained with: silver-diammine solutions below the pH of 5, potassium tellurite, HgCl₂, Bi(NO₃)₃, CuCl₂, Pb acetate, and FeCl₃.

(b) Staining of myelin only was obtained with: silver-diammine between pH 5-6 to 7, silver methenamine below pH 8, osmium tetroxide, gold chloride, KMnO₄, leucofuchsin and tetrazolium salts below pH 11.

(c) Staining of myelin and of axons was obtained with: AgNO₃ (axons very light), Ag diammine at pH levels above 6-7, tetrazolium salts at pH levels above 11, and Chèvremont’s reagent.

These results indicate that myelin can be oxidized more easily than axons under ordinary conditions.

It must be noted here that the lack of staining by some of the indicators does not necessarily mean that the redox potential of the indicators was not high enough for the structure concerned. In some instances the reduced forms of the compounds might have been soluble, in other cases proof will be given below to show that the reduced forms of the compounds, although precipitated, were invisible microscopically.

Experiments Dealing with the Effect of Reduction and Toning on the Metallic Impregnations

In order to study the effect of reducing agents and of gold toning on the primarily impregnated sections, the following experiments were carried out:

(a) Sections treated by AgNO₃ at different pH levels, or by Ag-urotropin, or by Ag-diammine, or by AgNO₃ for 24 hours followed by Ag-diammine (the procedure used in Bielschowsky’s method), were put into a reducing bath of either formalin, pyrogallol, or hydroquinone. The sections were removed from the reducing baths at different times and fixed in thiosulphate.

Whenever the reduction was not unduly prolonged, an existing (even slight) impregnation of axons was markedly increased. The intensity of the impregnation of myelin remained practically unchanged. A prolonged reduction resulted in a complete blackening of both axons and myelin in these cases. No length of reduction could render visible completely unimpregnated structures.

(b) Sections impregnated by various silver solutions were treated with 0.2-1% gold chloride for 4-16 hours; this had the folllowing dual effect. Those structures which were strongly impregnated already changed their colour and appeared more strongly stained; on the other hand, the background staining, i.e. the colour of the less strongly impregnated areas, became much paler.
Sections which were treated for 24 hours in HgCl₂, Bi(NO₃)₃, CuSO₄, CuCl₂, Pb acetate, and FeCl₃, were exposed to gold chloride as above. This treatment resulted in an intense impregnation of axons and a slight one of myelin in the case of HgCl₂-treated sections. In those treated with bismuth nitrate the gold chloride visualized myelin and nerve cells. No staining appeared in the other sections.

Conclusions
Reduction strengthened the impregnation of axons much more than that of myelin. Gold toning revealed that HgCl₂ and Bi(NO₃)₃ did impregnate nerve tissue elements, but the precipitates were invisible before the treatment by gold.

Experiments Dealing with the Effect of Fixation on the Impregnation

The effect of fixation on impregnation was studied by using many of the methods mentioned above. For the sake of simplicity only the results pertaining to impregnation by the method of Bielschowsky, in which gold toning was omitted, will be reported here, as the results with the other methods were of a corroborative nature.

Good staining of axons, neurofibrils, and nerve cells could be obtained in material fixed for many months in 10% formalin solution neutralized by Sörensen's phosphate buffer or in commercial (USP) formalin solutions, the pH of which ranged between 4.5 and 5.5.

Fixation in 10% formalin solutions buffered to pH values between 2 and 7 resulted in a progressive decrease of the impregnation with duration of fixation in the solutions of lowest pH. Although fixation in commercial unbuffered formalin is known to be only seldom detrimental to the impregnation of axons, pH values below 4 appear to weaken the impregnability. Thus, at pH 3 and below there was a marked decrease in the staining of axons already after three weeks. Fixation in formalin-ammonium bromide (FAB) had the same effect on the staining of axons as fixation in formalin acidified by HCl to the same pH.

A similar effect, i.e. weakening of the impregnation of axons and nerve cells, was also obtained in material fixed in neutralized formalin solutions by treatment of the sections with N/1 HCl at 58° for 15 minutes.

Treatment of tissue sections by N/5-N/100 NaOH for 20 hours at room temperature resulted in a weaker impregnation of the axons the higher the pH.

The impregnation of myelin depended much less on the pH of the fixing solution.

Conclusions
Fixation in strongly acid solutions and treatment by strong acids and bases is detrimental to the visualization of axons.
DISCUSSION

It is clear from the experiments reported above as well as from the data available in the literature (cf. Liesegang, 1911; Holmes, 1943; Samuel, 1953) that the most important step in the impregnation procedures is the primary nucleation.

This process is similar in principle to the formation of the latent image in photography, an image which is considered to consist of nuclei of metallic silver (cf. James and Higgins, 1948). During this step the metal is reduced at certain sites, which are most probably those sites where the reducing groups are concentrated in the tissue. Reduction in one site with subsequent migration and deposition at other sites by adsorptive forces (Zon, 1936) cannot be considered probable. No such phenomenon is known to occur in photographic emulsions, and besides, it seems highly improbable that reduced silver particles which have lost their charge would travel a distance great enough to be resolved by the light microscope.

The possibility (Liesegang, 1911; Seki, 1940; Voigt, 1952) that the impregnation depends on the activity of the tissue as a protecting colloid does not seem probable. Voigt compares the protecting activity of the tissue to the activity of the photographic gelatin layer, but migration of silver in the gelatin does not take place to a sufficient extent to diminish the sharpness of a photographic negative. And the proteins of fixed tissue are denatured and cannot be rightly considered to be in a colloidal solution.

It seems obvious, therefore, that impregnation of any structure depends on the adequate choice of an agent of which the oxidizing potential is sufficiently high for the oxidizable groups present in the structure. The fact that most reagents which stained axons stained also myelin, while of those which stained myelin only a few visualized axons indicates that axons are harder to oxidize than myelin. The behaviour of the tetrazolium salts supports this assumption, as their oxidizing potential is known to depend on their pH. Indeed, in the neutral and near-neutral zone, neither axons nor myelin were stained. At higher pH values the tetrazolium salts stained only myelin. The axons were stained slightly at pH 12.

The fact that potassium permanganate, a strong oxidizing agent, did not stain the axons seems puzzling at first. It should be remembered, however, that the reduction of KMnO$_4$ to MnO$_2$ is a step-by-step process, and that microscopical observation reveals only the result of the step in which a soluble intermediate is transformed into an insoluble product (MnO$_2$). Obviously the oxidizing potential of this step is lower than that of silver-diammine at pH 11–12.

On the assumption that axons are harder to oxidize than myelin, the results obtained with Chèvremont's method and with HgCl$_2$ followed by gold toning, where axons were stained more intensely than myelin, can best be explained by assuming that the axons, although hardest to oxidize, contain more numerous reducing groups than myelin.
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The effect of reduction by formalin, pyrogallol, and hydroquinone in increasing the impregnation of axons without affecting the myelin can be explained on the basis of the current opinions on the nature of photographic development. It is assumed (James and Higgins, 1948) that the primary nuclei of the exposed photographic films may act as tiny electrodes which accept electrons from the reducer and surrender them to the silver ions of the solution, thus reducing the ions to metallic silver. Under the conditions prevailing in the photographic plate the amount of secondary reduction is directly proportional to the amount of primary change (i.e., to the amount of light absorbed), as the film represents a homogeneous medium. In the impregnation procedures, the electrode effect responsible for the secondary impregnation cannot be as effective in the lipid medium of myelin (which has probably a low dielectric constant) as in the axons. Reduction, which is not unduly prolonged, results therefore in a stronger final impregnation of axons than of myelin. The fact mentioned by Voigt (1952) that primary nuclei differ in their composition and location in different primary impregnations, corroborates this conclusion, as any metal particle or reducing group may serve as a nucleus, provided its potential is suitable.

The difference in the dielectric constants also explains Liesegang's finding (1911) that the bleaching of sections by ferric chloride (which transforms black silver into white silver chloride) affects the grey matter before it affects the myelin sheaths. Thus the impregnation of axons may be increased or decreased by the various treatments, the axons reacting much more quickly than the myelin.

The intensification of impregnation by reduction depends obviously on the presence of non-reduced silver ions in the sections. These ions must be bound to the tissue as they are not washed away by rinsing with water. The situation is again similar to that prevailing in the photographic plate where unreduced silver is present, bound to gelatin, and serves as a reserve of silver ions which can be reduced during development. In the tissue sections the silver is probably bound to the tissue proteins forming colourless ionic silver proteinates.

As to the nature of gold toning, it is clear that the gold chloride alone, because of its low oxidizing capacity, can impregnate only the myelin sheaths. Gold toning is therefore not an independent reduction of auric ions by the tissue, but rather the coating by gold of reduced silver particles, i.e., reduction of gold ions by the reduced silver, as in the process of industrial gold plating (cf. Samuel, 1953c). The differentiating effect of the gold chloride solution is most probably due to its acidity, which causes dissolution of the smaller silver precipitates, or their transformation into white silver chloride.

Treatment by gold chloride of sections in which both axons and myelin were impregnated, also increased the impregnation of axons more than that of myelin. This effect may also be explained in terms of the lower dielectric constant of myelin.

In the impregnation experiments with HgCl₂ and Bi(NO₃)₃, it appears
obvious that there was a primary impregnation of tissue structures by invisible precipitates which could be visualized by the subsequent gold toning. The intensive primary impregnation of axons indicates that mercuric chloride has a higher oxidizing potential than bismuth nitrate, and probably also higher than any of the previously studied silver compounds.

As for the effect of acid fixatives on the impregnation of axons, the experimental findings corroborate the findings of Cajal (1910). It has been shown that treatment by acids is detrimental also to sections of fixed material.

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REFERENCES

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