Experiments on the Mechanism of Silver Staining

II. Development

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With one plate (fig. 1)

SUMMARY

The effect of a series of photographic developers on the final silver-staining picture has been investigated. Ten common developers were used, but of these only hydroquinone, chloroquinol, pyrogallol, and \( p \)-aminophenol, were found to be of general use. The other developers were either so weak in their action that the final staining was light and incomplete, or so powerful that a differentiated nerve staining was not produced.

For silver staining to be effected nuclei of reduced silver should be present in the section. These nuclei act as centres for the deposition of additional silver reduced by the developer; the additional silver may either be derived from that combined with the sections during impregnation or from the developing solution itself. Whether or not the additional silver is deposited in such a way as to produce differentiated nerve staining depends on the properties of the developer and on the composition of the developing solution. The redox- and 'bromide'-potentials, the sulphite and hydrogen ion concentrations in the developing solution, and the protective action of the tissue components of the section all play a part in determining the final staining picture.

A new glycine-containing physical developer and a gold thiocyanate physical developer are described.

CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Introduction</td>
<td>103</td>
</tr>
<tr>
<td>II</td>
<td>Development</td>
<td>104</td>
</tr>
<tr>
<td>(a)</td>
<td>Physical development</td>
<td>105</td>
</tr>
<tr>
<td>(b)</td>
<td>Chemical development</td>
<td>106</td>
</tr>
<tr>
<td>(c)</td>
<td>Discussion</td>
<td>109</td>
</tr>
<tr>
<td>III</td>
<td>Gold thiocyanate development</td>
<td>111</td>
</tr>
<tr>
<td>IV</td>
<td>General discussion</td>
<td>112</td>
</tr>
<tr>
<td>References</td>
<td></td>
<td>115</td>
</tr>
</tbody>
</table>

I. INTRODUCTION

So far as I am aware no systematic investigation has been carried out into the use of different developing agents for the reduction of silver taken up during impregnation. The present investigation was undertaken to determine the effect of different developing agents on the final picture obtained in silver staining.

In a previous paper (Peters, 1955) it was shown that during impregnation two essentially different reactions take place between the silver ions in the impregnating bath and the sections of fixed tissue. A substantial part of the
silver is combined in the unreduced form with the histidine and other amino-
acids of the tissue, and a smaller part is reduced to form silver nuclei. The
combination of the unreduced silver is a rapid process which is complete
within about 15 minutes at 37° C. and pH 9, but the formation of the silver
nuclei is a slower process and accounts for the long period of impregnation
that is necessary to obtain good staining. During development the silver
nuclei act as centres on which the developing agent can deposit additional
silver derived from the combined (reducible) silver fraction.

Liesegang (1911) considered that the characteristics and distribution of the
silver nuclei determined the final specificity of the stain. To some extent this
is true because they act as the centres for the reduction and deposition of the
reducible silver. However, the characteristics of the developing solution also
play a part in the final distribution of developed silver. Therefore the final
staining picture depends both on the distribution of the silver nuclei and on
the action of the developing agent in depositing additional reduced silver in
relation to these nuclei.

II. DEVELOPMENT

In photography developers are classified into two main types, namely,
‘physical’ and ‘chemical’ developers (Mees, 1944). In physical development
the silver which is deposited onto the latent image centres of the exposed
emulsion is derived from the developing solution, so that the emulsion need
contain no silver other than that of the nuclei which constitute the latent
image. Such a developing solution contains a reducing agent, free silver ions,
and a protecting or complexing agent to retard the action of the developer on
the free silver. In contrast, in chemical development, the silver reduced to
form the visible image is that of the silver halide crystals in the emulsion.
Therefore a chemical developing solution contains no free silver ions initially.
In general the developing agents used in chemical development are stronger
than the ones used in physical development. The developing agents most
widely used in silver staining are hydroquinone and formol.

In the case of an impregnated section, development is always physical in
nature since free silver ions are involved; there is no mechanism like the
chemical development of an emulsion, which involves crystals of silver halide
containing nuclei of reduced silver. There are, however, two possible sources
of silver ions which can be reduced by the developer. The silver ions may be
derived either from the reducible silver combined with the tissue during the
impregnating stage (see Peters, 1955), or from the developing solution. If the
reducible silver is not removed from the section before it is immersed in a
developing solution, itself containing free ions, then the developed silver will
be derived from both the section and the solution.

For the sake of clarity, in the present discussion, although there is no parallel
with chemical reduction in the photographic sense, developing solutions con-
taining free silver ions initially will be referred to as ‘physical developing
solutions', and the developing solutions containing no free silver ions initially as 'chemical developing solutions'.

(a) Physical developing solutions

Pearson and O'Neill (1946), suggested the use of a physical developer containing hydroquinone with gelatine as a protecting agent. The developer that they used had the following formula:

\[
\begin{align*}
1 \text{ per cent. hydroquinone} & : 2 \text{ ml}.
2 \text{ per cent. } \text{AgNO}_3 & : 5 \text{ ml. at } 60^\circ \text{C.}
3 \text{ per cent. gelatine} & : 20 \text{ ml.}
\end{align*}
\]

The pH value of the solution was adjusted to 4.4 by citric acid. This developing solution was tested but was found to produce rather a granular development which was only specific when the sections had been impregnated at pH 7 or 8. Hydroquinone is probably too strong a reducing agent to be used in this type of development, and consequently other developing agents were tried. The best results were obtained with a developer having the composition:

Stock solution: glycine . . . 1.25 gm.

\[
\begin{align*}
\text{Na}_2\text{SO}_3 \text{(anhyd)} & : 2.5 \text{ gm.}
5 \text{ per cent. gelatine} & : 25 \text{ ml.}
distilled water & : 225 \text{ ml.}
\end{align*}
\]

\[
0.1 \text{ M citric acid-sodium citrate buffer at pH 6.3} : 20 \text{ ml.}
\]

1 per cent. silver nitrate solution . . . 1 ml.

The mixing of the stock solution with the silver nitrate and buffer is carried out just before the solution is to be used because the mixture is unstable: silver begins to plate out from the solution within 10-15 minutes of mixing. The usual time for development is of the order of 5 minutes or even less. Slides should be rinsed in distilled water before immersion in the solution (fig. 1, H).

In general the optimum pH value for development was pH 6.3, but in some cases a more selective staining was obtained at pH 6.0. However, the isoelectric point of the gelatine is important in such a solution, and for the sample used here it was at pH 5.3. To obtain the best results tests should be carried out over the range pH 5.5-6.5 since on either side of the optimum the deposition of silver is granular. This is especially noticeable on the acid side. The citric acid-sodium citrate buffer is used to control the pH value at the points of reaction, since a change of pH at the deposition sites during development may influence the further deposition of silver.

Two other means of improving the results may be used:

(i) The development is carried out at low temperatures, which retards the deposition, so that finer grains of silver are produced, or

(ii) The volume of silver nitrate solution in the formula is reduced to 0.5 ml. This has virtually the same effect as (i).
Other developing agents were added to the above type of solution. With hydroquinone, metol, and chloroquinol the staining was unspecific and the silver deposition was granular.

(b) Chemical developing solutions

Ten common photographic developers were used; hydroquinone, chloroquinol, metol, glycine, pyrogallol, pyrocatechin, amidol (2:4 diaminophenol), phenylendiamine, p-aminophenol (base), and oxalic acid. Most of the tests were carried out on alcohol-fixed sections of frog spinal cord, but many experiments were repeated on alcohol-fixed rat cerebellum and formol-fixed human cerebrum and cerebellum. In many cases the sections were afterwards toned with gold. The developing solutions also contained sodium sulphite, and in a few tests citric acid was added. The only developers which were found to be of general use were hydroquinone, chloroquinol, p-aminophenol, and pyrogallol; others gave unspecific or faint results, so that the details of the nervous system were not clear.

The amidol solution used had the composition:

\[
\text{amidol} \quad 0.5 \text{ gm.} \\
\text{Na}_2\text{SO}_3 \text{ (anhydrous)} \quad 5 \text{ gm.} \\
\text{distilled water} \quad 100 \text{ ml.}
\]

The pH value of the solution was varied by the addition of 10 per cent. caustic soda or 25 per cent. acetic acid, and determined just before use by a pH meter. This solution gave the best results at pH 6.5, which agrees with the value obtained by Davenport, Bruesch, and McArthur (1939), who used a similar solution after impregnation in protargol. (The developer used by them was similar in composition to the one used here, but with the addition of 5 gm. of crystalline sodium sulphite.) At other pH values the silver deposit is granular. The developer is poor for normal routine staining, since there is a tendency for the connective tissues and myelin sheaths to stain. This lack of specificity was also found by Samuel (1953a), who used a 1 per cent. amidol-
sulphite developer. It is, nevertheless, a useful developer for fine connexions and cell bodies.

Metol, at a concentration of 0.5 per cent. or 0.25 per cent. with the addition of sulphite, gave a very unspecific staining picture (fig. 1, A). The addition of citric acid to the solution produced very little improvement. In the presence of citric acid alone the development was weak.

Para-phenylenediamine, as in the case of metol, gave an unspecific and granular deposition of silver, which resulted in an extensive staining of connective tissue.

Formol, used as a 10 per cent. solution (4 per cent. formaldehyde), was very active and produced a deep, but unspecific staining. This is not surprising because formol is commonly used for development in silver methods employing frozen sections.

Glycine, either in the presence of sulphite or with the addition of citric acid, produced very light development in which few details were visible (fig. 1, B). This is also true of oxalic acid and pyrocatechol.

Good results were obtained with the remaining four developing agents. The p-aminophenol was used in the form of the base, which is not soluble in water, and consequently the developer was initially dissolved in absolute alcohol. The composition of the solution employed was:

\[
\begin{align*}
p\text{-aminophenol (dissolved in 40 ml. abs. alcohol)} & \quad 0.5 \text{ gm.} \\
\text{Na}_2\text{SO}_3 \text{ (anhyd)} & \quad 5.0 \text{ gm.} \\
distilled \text{ water} & \quad 60 \text{ ml.}
\end{align*}
\]

The solution gave good staining, with a small amount of connective tissue staining, which did not obscure the details of the fibres appreciably (fig. 1, C). If the concentration of the sulphite was reduced to 2 per cent. or the p-aminophenol was used in a completely aqueous solution (when caustic soda had to be added to dissolve the developer), the results were not so good.

The developing solution containing pyrogallol had the composition:

\[
\begin{align*}
\text{pyrogallol} & \quad 2 \text{ gm.} \\
\text{Na}_2\text{SO}_3 \text{ (anhyd)} & \quad 2 \text{ gm.} \\
distilled \text{ water} & \quad 100 \text{ ml.}
\end{align*}
\]

This solution produced deep development, with quite extensive nuclear and fibre staining (fig. 1, D). The addition of extra sulphite to the solution, resulted in a less intense staining.

Chloroquinol produced results very like those obtained after development in warm solution of hydroquinone. This might be expected, since the two compounds have a similar structure. The formula of the developing solution used was:

\[
\begin{align*}
\text{chloroquinol} & \quad 1 \text{ gm.} \\
\text{Na}_2\text{SO}_3 \text{ (anhyd)} & \quad 4 \text{ gm.} \\
distilled \text{ water} & \quad 100 \text{ ml.}
\end{align*}
\]

To make up this solution, the whole was warmed to dissolve the chloroquinol, cooled, and then filtered. Development took 4 minutes at room
temperature. The results were more complete than those produced by hydroquinone at room temperature, but very similar to those obtained when the hydroquinone developer was warmed to 20° C. (fig. 1, f).

The hydroquinone solution had a similar composition to that employed by Holmes (1943) and Romanes (1950), namely:

- hydroquinone . 1 gm.
- Na₂SO₃ (anhyd) 10 gm.
- distilled water . 100 ml.

This contains twice the amount of sulphite used by Holmes and Romanes, but the addition of extra sulphite resulted in a more differentiated staining (fig. 1, e). The effect of sulphite in increasing the differentiation of the stain was described by Davenport and Kline (1938). If the concentration of the sulphite was less than 5 per cent. then the staining was granular and less specific. It can be shown that the depth of staining on development decreases with the addition of sulphite to the solution and, conversely, increases with the addition of more hydroquinone. Temperature is also an important factor, and Romanes (1950) put the optimum temperature at 15° C. Experiments showed that the intensity and extent of staining increased with temperature. At 0° C. the staining was very light and few cell nuclei were stained, but as the temperature was raised, the numbers of nuclei stained, together with the details of nerve fibres, increased. It is therefore suggested that the developer should be warmed to a temperature of about 20° C. before use. Samuel (1953a), showed that at low pH values the activity of hydroquinone developers is reduced.

Reinders (1934) produced a series of ferrous citrate-ferric citrate developing solutions. These solutions consisted of 20 ml. of a solution containing 0.125 M ferrous ammonium sulphate and 0.125 M ferric ammonium sulphate, to which was added different volumes of 1 M sodium citrate to vary the redox potential. A redox potential of +0.06 volts was obtained by the addition of 10 ml. of citrate, while the potential of the solution without the citrate was +0.645 volts. Hence, by varying the amount of citrate added the redox potential of the solution could be varied and adjusted to intermediate values.

To find the effect of redox potential on the development of the silver-imregnated sections, frog central nerve cord sections were developed in solutions of different potentials. There was only a slight development above +0.45 volts, but immediately below this value a rapid rise in the intensity of staining occurred, followed by a gradual rise in intensity as the potential fell from +0.45 volts to 0.06 volts. The staining produced by this developer was too light to be of general use, but this experiment showed the importance of the redox potential of the developer in staining.

The varying types of staining produced by different developers suggested that there was some movement of the reducible silver during development. This was shown by the following experiment. Three slides were impregnated in the same solution. The first was developed in hydroquinone-sulphite, th
second in amidol-sulphite, and the third in hydroquinone-sulphite for 10 seconds followed by amidol-sulphite. Specific staining was obtained in the first and last slides, both having approximately the same intensity of staining. In the slide developed in amidol alone, the staining was more intense and less specific than in the other two. One must therefore conclude that in the third slide, during the short immersion in hydroquinone and before it was transferred to the amidol, silver was lost from the section. Otherwise the amidol would have produced some connective tissue staining and the staining would have been as deep as that obtained in the second slide where the amidol alone was used. A similar experiment was carried out by Samuel (1953a), but he immersed his sections in hydroquinone for 5 minutes before transferring them to the amidol solution. His experiment does not show the rapidity of the loss of reducible silver from the section when it is immersed in the developing solution.

(c) Discussion

From the above experiments on development it is seen that of the chemical developers considered only four are useful. Once the silver nuclei have been formed in the section the staining depends to a great extent on the developer although the tissue of the sections also plays an important part. Thus, even among the four good developing agents, pyrogallol produced poor staining in the rat cerebellum sections although it produced good results in the other tissues which were tested.

The effect of the potential of the developer on the reduction of the silver has been shown to be important; to some extent the development is governed by the equation:

\[ E = E_{Ag} - E_{\text{redox}} \]

where \( E_{Ag} \) is the silver potential, \( E_{\text{redox}} \) the potential of the developer, and \( E \), the resulting potential.

For the silver ions to be reduced, it is necessary that \( E \) should reach a minimum value for any given system (Reinders, 1934). By variation of \( E_{\text{redox}} \), the minimum value for this factor can be determined, and for the ferrous-ferric citrate system the value has been shown to be \( +0.45 \) volts. In practice, however, the value of the redox potential is of little help in determining the type or rate of development that will be produced by a series of different developing solutions, since it is merely an index of the ratio of reduced to oxidized form of the developing agent. The actual rate of development depends upon the activity of the developer at the site of action; this is determined by such factors as the rate of diffusion of the developer.

Bromide retards the action of a developer, and the amount of bromide that it is necessary to add to a series of developers to reduce their activity to a given level provides a characteristic index for each developer. This is called the 'bromide potential' of the developer (Mees, 1944, p. 352). The bromide potentials
of some of the developing agents used in the experiments are shown in table 1.

In general the developing agents which produce specific development are those with low bromide potentials, while the ones that produce unspecific development have higher bromide potentials. One exception to this is $p$-phenylenediamine, which has a low bromide potential and yet produced unspecific staining.

The various types of development brought about by the different developing solutions brings out another important point. If the silver nuclei are confined to the nervous elements then the staining should be specific irrespective of the developing agent used. The fact that strong developing agents, such as metol, produce unspecific staining, suggests that all elements in the section contain silver nuclei. With a weak developing agent the nerve fibres stain, but when stronger developing agents are used the cell-nuclei, cell-body, and connective tissue stain. Which silver nuclei in the section act as centres for development depends on a series of factors, of which the most important are the size of the silver nuclei, the numbers of the silver nuclei, the activity of the developing solution, the distribution of reducible silver, and the protective action of the protein at the site.

The protective action of protoplasm in staining has been discussed at length by Zon (1936). The protective action of a system may be regarded as the influence which that system exerts in retarding a chemical reaction, in this case the reduction of silver by the developing agent. With any given developing solution the elements which stain most readily are the ones with the lowest protective action and the largest silver nuclei. When strong developing agents are used the protective action of the elements containing the nuclei is overcome, so that elements in the section stain in the sequence outlined above. Specific staining is therefore the result of the deposition of developed silver on the nuclei contained only in certain tissue elements.

Variations in development may be brought about by the action of sulphite in the solution. Sulphite can change the potential of the developing solution

### Table 1

<table>
<thead>
<tr>
<th>Developing agent</th>
<th>Bromide potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous oxalate</td>
<td>0.3</td>
</tr>
<tr>
<td>$p$-phenylenediamine</td>
<td>0.3</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>1.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.6</td>
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<tr>
<td>$p$-aminophenol</td>
<td>6.0</td>
</tr>
<tr>
<td>Chloroquinol</td>
<td>6.0–7.0</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>16.0</td>
</tr>
<tr>
<td>Metol</td>
<td>20.0</td>
</tr>
<tr>
<td>Amidol</td>
<td>30–40</td>
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</table>
II. Development

quite appreciably (Evans and Hanson, 1937). The action of sulphite is complicated, but it is known to form complexes with silver and to increase the rate of development by rapidly removing the oxidized developer in the form of sulphonate from the site of development (Evans and Hanson, 1937). Samuel (1953a) has shown that sulphite competes with the developer for the reducible silver held by the section. He found that immersion of an impregnated section in a 2.5 per cent. solution of sodium sulphite for 2 minutes before development removed the silver which would have been reduced and deposited by the developer. Therefore no visible staining occurs at the development stage if the developing solution does not contain free ions of silver. Samuel concluded that hydroquinone, which is a relatively weak developing agent, was partially outpaced by the sulphite, which removed a greater portion of the reducible silver from the section than when amidol was used. Thus amidol, a more powerful developer than hydroquinone, outpaced the sulphite and reduced the silver in situ. Samuel also established that low pH values facilitated the removal of silver by the sulphite. This is to be expected, because lowering the pH value reverses the ionization of the basic groups of the proteins and so releases the silver ions from combination.

Holmes (1943) stated that the pH of the developing solution is not critical in determining the specificity of staining. To some extent this is true in chemical development since, within limits, the pH value of some solutions can be changed by the addition of citric acid without producing any great effect, but the experiments with amidol show that the pH can affect the rate and type of development. In physical development, on the other hand, pH is a much more critical factor, and is only variable within small limits.

Thus the final staining picture is dependent on the activity of the developing agent, the concentration of sulphite, and hydrogen ions in the solution, and the protective properties of the elements of the section at the site of development. Whether the silver reduced by the developer is derived from the section or the developing solution, no staining can be obtained unless the section has been previously impregnated, so that silver nuclei have been formed in it.

III. Gold Thiocyanate Development

James, Vanselow, and Quirk (1948) showed that treatment of exposed photographic emulsions with an aurous thiocyanate solution increased the rate of subsequent development. The silver of the latent image appeared to be either replaced, or plated over, by gold. However, the action of gold did not end with a replacement because if the exposed emulsion was allowed to remain in the aurous thiocyanate solution, then a visible image appeared, thus showing that physical development of the latent image, by the gold, was taking place.

James (1948) later showed that prolonged treatment of exposed emulsions by aurous thiocyanate, produced gold particles which were visible under the electron microscope. James considered that the deposition was autocatalytic,
and that the effect of the gold was to build up the latent image nuclei far above their normal size.

To obtain further evidence of the formation of silver nuclei during the impregnation of nervous tissue and to investigate aurous thiocyanate as a possible developing agent, sections pretreated with $1/20,000$ silver nitrate, at pH 9 and $37^\circ$ C. for 20 hours, were immersed in an aurous thiocyanate solution. The basic solution of aurous thiocyanate was made as follows: 40 ml. of a 0.1 per cent. solution of KAuCl$_4$ was heated to boiling, when 0.6 gm. of potassium bromide and 0.5 gm. of potassium thiocyanate were added. When the solution of the latter was complete, the whole was cooled and diluted to 160 ml.

After impregnation in the silver nitrate solution the sections were washed in several changes of distilled water for 30 minutes to 1 hour to remove uncombined silver ions, and then immersed in the following solution at $37^\circ$ C.:

- 25 ml of solution of AuCNS complex
- 5 ml of 0.5 per cent. gelatine
- 20 ml of distilled water.

The period of immersion in the gold complex was generally of the order of 24 hours at $37^\circ$ C., but the sections can be removed when a sufficient colour depth is obtained. The aurous thiocyanate is rather unstable and tends to reduce to gold easily. As in the case of the physical developer containing silver, the gelatine acts as a protective colloid to retard the deposition of gold from the solution.

The results were generally good and the staining specific in almost every case (fig. 1, G); the staining was rather like that obtained after gold toning of silver stained sections.

Experiments were carried out with the thiocyanate solution in an attempt to obtain staining without pretreatment with silver nitrate. In these experiments the pH, concentration, temperature, and absence of potassium bromide were tested, but the results were much inferior to those obtained after pretreatment with silver nitrate or after normal silver staining.

The production of specific development by the gold thiocyanate after pretreatment with silver nitrate is further evidence for the formation of silver nuclei during the period of impregnation in the silver solution.

The outstanding features of the staining produced by this method of development were the deep staining of the nerve-cell nuclei and the extensive staining of the cytoplasm of the cell-bodies (fig. 1, G).

**IV. General Discussion**

In photography the 'latent image' determines the sites of development, and it is believed to consist of sensitivity specks of reduced silver which, on development, allow the silver bromide crystals containing them to be preferentially reduced by the developer. This being the case there is a parallel between the sensitivity specks of the emulsion and the silver nuclei in the sections.
The mechanism of development in photography is far from understood (James, 1950). The fundamentals are known, however, and in simple form a corresponding sequence taking place during chemical development of the impregnated section may be outlined as follows. On immersion of the section in the developing solution an unstable system results such that the reducible silver combined during the impregnation stage tends to diffuse away from the section and to form complexes with the developing agent or any other complexing agents such as sulphite, which may be present in the solution. The rapidity of the reaction is seen from the experiment in which a section was transferred to amidol after development for 30 seconds in hydroquinone. Initially, therefore, there will be a high concentration of complexed silver in the interstices of the section. When a strong developing agent such as metol is used there will be little diffusion of silver away from the section, and it will be reduced in situ, so that an unspecific staining results (fig. 1, A). With other developing agents, such as hydroquinone and chloroquinol, the silver will be less readily reduced and will be deposited at specific points so that differential staining occurs in relation to the position and number of silver nuclei present (fig. 1, E and F). Taking this even further, weak developing agents which reduce even less rapidly deposit only a small portion of the reducible silver, the rest being lost to the solution, so that the staining is only light (fig. 1, B). The deposition of silver on to the nuclei is autocatalytic. It is probable that during differential development, only certain silver nuclei act as centres for the deposition of silver. On the other hand, in development with stronger developing agents, the silver may be deposited on a more extensive range of nuclei, so that an unspecific staining results. This of course implies a differential deposition of the developed silver in relation to different silver nuclei.

In physical development it is only necessary for the silver nuclei to be present in the section for development to be possible. Thus the silver, which is complexed with the tissue elements and reduced during chemical development, may be removed from the section either by washing or by the action of sodium sulphite (Samuel, 1953b). If the reducible silver is not removed from the section before physical development then it will be available to the developer.

During development it is believed that an activated complex is formed between the silver and the developer (James, 1950). In the case of hydroquinone it is thought that the complex has the form, hydroquinone:Ag⁺:Ag metal, which splits up into oxidized developer and reduced silver. This type of reaction is probably common to a large number of developers, although in those like p-phenylenediamine the absorption of the developer by the silver nuclei is important. Whether the silver ions are derived from the section or from the developing solution as in physical development, the reaction is virtually the same.

A further important factor is the ‘protective action’ of the tissue of the section. This has been discussed at length by Zon (1936) who found that when silk fibres were immersed in a solution of silver nitrate and potassium dichromate
in gelatine, the fibres took on the distinct red colour of silver dichromate. A precipitate was not formed in the gelatine solution for several hours, showing that the protective action of the silk fibres was less than that of the gelatine. Thus the protective action of the gelatine may perhaps be likened to that of the connective tissue, and that of the silk fibres to the nerve fibres. Consequently, the different protective properties of the tissue elements may play an important part in the deposition of silver, both during impregnation and development. In development using strong developing agents, the protective action of the tissues is not so important, so that an unspecific staining results, but with weaker developing agents it is more important and produces a more specific deposition of developed silver in relation to the silver nuclei.

Before any development can take place it is necessary that silver nuclei should be formed in the section. These are formed during the impregnating stage (Peters, 1955), and the reactions which take place between the sections and the silver ions in the impregnating bath can exert a considerable influence on the final staining picture. Thus Silver (1942) has shown that the pH value at which impregnation is carried out determines which elements in the section stain on development.

Silver (1942), in his paper on the colloidal factors controlling silver staining, states that the absorption of negatively charged micelles of silver by the regions of the tissue bearing positive charges is the factor controlling the specific deposition of silver during staining. The negatively charged micelles are assumed to be formed by the action of the developer on the reducible silver, so that specific deposition does not take place until the sections are immersed in the developer. Samuel (1953b) has shown that Silver's hypothesis is incorrect. Although the developing agent plays some part in the specificity of staining, as pointed out by Holmes (1943), Silver makes no mention of the presence of silver nuclei, and assumes that specific deposition is determined solely by the charges on proteins. While the charge on the proteins may determine the sites of formation of the nuclei, it is principally the nuclei that determine the specific deposition sites.

Palmgren (1948) has given a theoretical treatment of the mechanism of silver staining, and the present theory is largely in agreement with the theory put forward by him. He stresses the importance of the formation of silver nuclei, and states that the nuclei have a negative charge, so that they absorb the positively charged silver ions. However, the initial charge on the nuclei is unknown, but it is probable that on immersion in the developing solution they assume a negative charge. Palmgren also points out that a weak developing agent is necessary to ensure specificity of staining, so that the silver, as yet unreduced, has time to move out of the tissue between the nervous elements. To slow down the rate of development by pyrogallol he added alcohol to the developing solution.

The action of the developer is therefore seen to be complex. Development can be affected, and to some extent controlled, by a number of factors, so that the deposition of developed silver is varied in relation to the silver nuclei.
Thus it is clear that in a series of sections impregnated under identical conditions the type of final staining picture obtained depends on the developing solution which is used to reduce the developable silver.

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