

A Method of Silvering the 'Golgi Apparatus' (Nissl Network) in Paraffin Sections of the Central Nervous System of Vertebrates

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With two plates (figs. 1 and 2)

SUMMARY

1. It was accidentally found that methods of silvering synaptic end-feet sometimes blackened Golgi's 'internal reticular apparatus' in neurones of the central nervous system of the cat.

2. A method of achieving this consistently was worked out: (a) paraffin sections are coated with a collodion membrane; (b) the collodion membrane is soaked in silver nitrate; (c) the silver nitrate is reduced to metallic silver with a buffered formaldehyde solution; (d) steps (b) and (c) are repeated until the sections appear quite black; (e) the silver attached to structures other than the Golgi apparatus is removed with a ferricyanide/thiosulphate bleach; (f) the section is 'toned' with gold chloride, fixed in thiosulphate, and washed thoroughly; (g) the section is dehydrated, cleared, and finally mounted in Canada balsam, DPX, or similar media. *Results*: Golgi-apparatus, black; connective-tissue fibres, black; axons, grey to black; everything else is light grey or colourless.

3. A tentative hypothesis is advanced to explain the results obtained.

4. The following advantages are claimed for the new method: the cytoplasmic reticulum thus blackened resembles that seen in living neurones with the interference microscope; special methods of fixation are not required; the cytoplasmic reticulum of *given cells* can be studied before and after silvering; and serial sections of the same piece of tissue can be used for histochemical purposes.

INTRODUCTION

THE purpose of the work described here was to find a consistent method of silvering the 'Golgi apparatus' in paraffin sections of vertebrate nervous tissue. During a study of the synapse in the central nervous system of the cat (David, 1957; David, Brown, and Mallion, 1959), we found that our method of silvering synaptic end-feet sometimes blackened certain objects within the cytoplasm of neuronal perikarya (fig. 1). These objects resemble those seen by Golgi in the cytoplasm of the Purkinje neurones of the owl, and called by him 'internal reticular apparatus' (Golgi, 1898, especially his fig. 2). We then silvered a number of pieces of the spinal cord of a cat by one of the classical 'Golgi' techniques (Aoyama, 1929): similar objects were blackened in the cytoplasm of some of the neuronal perikarya. Therefore, it is convenient to call these objects 'Golgi apparatus'. Here the use of the term 'Golgi apparatus' does not imply a homology between the objects seen in the cytoplasm of vertebrate neurones (illustrated in Golgi's fig. 2 (1898) and our fig. 1) and the objects in other cells that have been referred to by the same name (see Cain, 1954).

Very recently, Malhotra (1959) discovered that certain objects, essentially similar in structure to the Golgi apparatus, could be seen with the interference microscope in living neurones of birds and rodents. This discovery inspired us to look for similar structures in living neurones isolated from the ventral horns of the cervical enlargement of the spinal cord of the cat. We chose to work with these cells because we were already studying them for other purposes and because they were suitable objects with regard to size, ease of isolation, and uniformity of function. When the neurones were mounted in a saline solution (Baker, 1944), and were carefully examined with the 2-mm double-focus lens of the Smith interference microscope, a richly organized cytoplasmic structure could be seen. It was not possible to see this structure with any other method of vital examination. It seemed to us (David, Mallion, and Brown, 1959) that this structure, that described by Malhotra, and that blackened by silver in our sections, were one.

Since the objects under consideration were not artifacts and could legitimately be called Golgi apparatus, we set out to silver deliberately what before had been blackened by accident. This work was simplified by the fact that the appearance of successful preparations was known beforehand from our work with living cells. Eventually, a reasonably reliable technique was worked out. This method works with paraffin sections of material fixed and after-treated by routine methods.

FIXATION AND AFTER-TREATMENT

Two requirements must be met: (i) the Golgi apparatus must be present in the sections; (ii) no appreciable concentrations of extraneous substances capable of interfering with the process of silvering must remain in the sections.

The preservation of the Golgi apparatus is best verified by examining with the interference microscope those sections one intends to silver (fig. 2). Glycerol (r.i. = 1.473) is a suitable immersion medium. It is convenient to prepare permanent mounts of some of the sections processed in a given way, but not actually silvered. Kaiser's (1880) glycerol jelly is a suitable mounting medium for the purpose. Whether a given method of processing interferes with the silvering must be discovered empirically. It is customary to fix material to be silvered by the classical Golgi methods in aqueous solutions of formaldehyde containing substances that are supposed to facilitate the silvering, such as arsenious acid (Golgi, 1908) and salts of uranium (Ramón y Cajál, 1914), cobalt (Da Fano, 1920), or cadmium (Aoyama, 1929). Ethanol (Golgi, 1908) or methanol (Ramón y Cajál, 1914) are sometimes added to these solutions. We found that the Golgi apparatus could frequently be seen with

FIG. 1 (plate). Optical sections through a neurone of the ventral horn of the cervical spinal cord of the cat, fixed in 'FAM', double-embedded, and cut at $10\ \mu$; silvered by a method for end-feet (David, Brown, and Mallion, 1959).

A, photographed with the Golgi apparatus of the large dendrite on the right of the perikaryon in focus.

B, photographed with the plasmosome and parts of the nuclear membrane in focus.

the interference microscope, and blackened with silver, in paraffin-sections of neurones of the spinal cord of the cat, that had been fixed in formaldehyde solutions containing only an isotonic concentration of sodium or calcium chloride. We tried the adjuvants given above and found that none appeared to affect the preservation or the silvering of the Golgi apparatus. Irrespective of the fixative used, objects similar to Golgi's (1898) fig. 2 could only be seen in some of the neurones. Since aqueous formaldehyde fixatives do not prevent severe shrinkage and distortion in the after-treatments required to produce paraffin sections, it is better to avoid them.

The most consistent results were obtained with material fixed in a reagent, 'FAM', introduced as a substitute for Carnoy's fluid (1887), when it is wished to fix nervous tissue for work on retrograde degeneration (David, 1955):

formaldehyde (undiluted commercial solution)	1 volume
glacial acetic acid	1 volume
absolute methanol	8 volumes

Methanol is used to coagulate proteins. It is preferable to ethanol, since it forms a finer coagulum; it diffuses more rapidly, but dehydrates more slowly, and therefore shrinks less. Acetic acid precipitates nucleoprotein and counteracts the shrinkage produced by the alcohol. Formaldehyde stabilizes proteins, lipoproteins, and proteolipids, and irreversibly inactivates ribonuclease. Formaldehyde, in 'FAM', does not fix tissues in the same way as it would in an aqueous solution. Since proteins of tissues fixed in 'FAM' do not become markedly basiphil and oxyphobe, it is unlikely that, in this instance, formaldehyde forms methylenic bridges between basic groups in peptide chains. Tissues fixed in 'FAM' fluoresce in ultra-violet light as formaldehyde polymers would. 'FAM' has a pH of 2.55, and will fix about 9 mm of cat-brain in 20 h (this corresponds to a *K*-value (Medawar, 1941) of 2.0). Small pieces of spinal cord are adequately fixed overnight at room temperature. After fixation in 'FAM', the pieces should be dehydrated in absolute methanol and double-embedded in collodion and paraffin. Pieces of tissue processed in this way are often of approximately the same volume as before fixation (David, 1955). It is a good plan to cut series of sections at different thicknesses. Details of the Golgi apparatus are best resolved in thin sections, of about 3 to 5 μ ; the ramifications and general appearance of the network are best seen in 7- or even 10- μ sections. Unfortunately, it is extremely difficult to obtain good photomicrographs of the thicker sections. Double-embedded sections are mounted exactly as if they were ordinary paraffin sections. It is essential that the slides be thoroughly dry before silvering is attempted.

FIG. 2 (plate). Neurone of the ventral horn of the cervical spinal cord of the cat, fixed in 'FAM', double-embedded, and cut at 5 μ .

A, photographed with the interference microscope before silvering; mounted in glycerol.

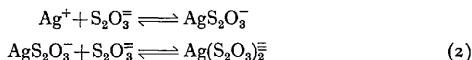
B, photographed with direct illumination after silvering by the method described in this paper.

SILVERING

The silvering of tissues is based upon the reaction



The whole problem is to ensure that metallic silver is attached only to those structures that one wishes to blacken. In the past hundred years, countless ways of doing this have been proposed. Techniques differing only in minor respects will blacken selectively neuronal perikarya, 'neurofibrils', synaptic end-feet, axons, different types of neuroglia, and connective-tissue fibres. Practically all methods of silvering are modifications of the original methods for axons, invented long ago by Golgi and Ramón y Cajál. It will be recalled that Golgi (1898) discovered his 'internal reticular apparatus' when he tried to blacken neuronal processes in pieces of nervous tissue that had been fixed too long. The mechanism of silvering axons has been studied in great detail by Liesegang (1911), Silver (1942), Holmes (1943), Nauta and Gyax (1951), Samuel (1953), Peters (1955), and Wolman (1955); the reader is referred to their papers for details. Methods of silvering axons comprise three basic steps: 'impregnation', 'reduction', and 'fixation'. Tissue is impregnated by soaking it in solutions containing silver ions (generally as silver nitrate, Ag^+NO_3^-). Some of the silver combines chemically with the tissue—probably with terminal amino-acid residues of peptide chains—and some is attached to the tissue as minute silver nuclei: in neither case is an image formed. In reduction, appreciable quantities of metallic silver are produced when the tissue is treated with a reagent capable of supplying the electron required for the reaction given in equation (1) to proceed. This reaction is catalysed by the silver nuclei in the tissue. Finally, the silver is 'fixed' with sodium thiosulphate, $\text{Na}_2\text{S}_2\text{O}_3$. In this step, any unreduced silver remaining in the tissue passes into solution as complex argyrosulphates (Mees, 1946, pp. 511-12):



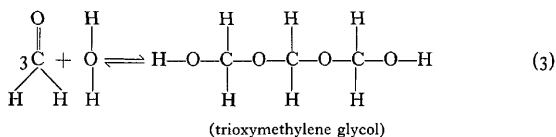
Unfortunately, the mechanism of silvering structures other than axons has not been studied in detail. It has tacitly been assumed that the same basic reactions were involved, but there has been no proof of this. So far, no theory has been advanced to explain why certain structures are selectively blackened in some methods of silvering, and completely different structures, often containing completely different substances, in others.

It occurred to us, when working on the synaptic end-feet, that differences in the argyrophilia of certain tissue-constituents, when silvered in certain ways, might be due to differences in the permeability of these tissue-constituents to silver that was reduced to particles of different sizes. We then developed a technique for silvering end-feet in which the tissue was not impregnated at all. Instead, ionic silver was reduced to particles of metallic silver of uniform size, which passed through a collodion membrane attached

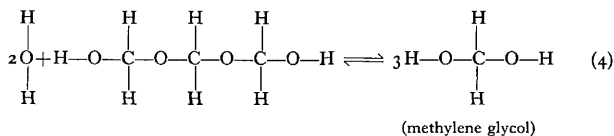
is not important—from about 0.2 to 0.5 M—but the pH should not be less acid than 3.8. The reason for this is that 'impregnation' (i.e. the formation of silver nuclei in the sections, and the chemical combination between the silver ions and the tissue) does not take place rapidly at acid levels (Peters, 1955c). When impregnation is allowed to proceed (for instance, when the membrane is silvered overnight), it is difficult to blacken the Golgi apparatus: the silvering then proceeds as in the methods for axons.

To silver the collodion membrane, place a coated slide on a staining rack, with the sections facing upwards. Flood the slide with the silvering solution (1 ml is ample), drain, and flood once more. One minute is long enough for the silvering, but no harm results from slightly longer periods.

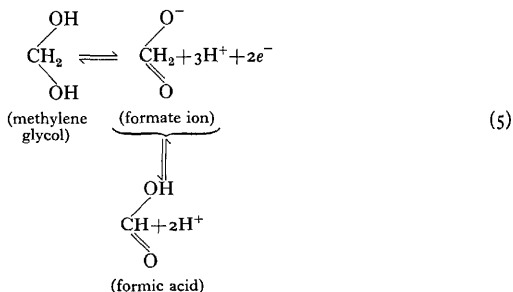
The next stage is reduction. This supplies the electron required for ionic silver to be reduced to metallic silver. Golgi (1908) reduced in a mixture of hydroquinone, formaldehyde, and sodium sulphite, and said that any 'common photographic developer' would do. We got more consistent results with formaldehyde alone. A simple aqueous solution of formaldehyde will not do. 'Formaldehyde solution' of analytical quality, as supplied to us, contains about 37% w/w formaldehyde, and has a pH of 3.1 (measured with the glass electrode). Its main impurity is formic acid, formed by the autocatalytic oxidation of formaldehyde (Walker, 1953). It is not a strong reducing agent. Formaldehyde gas in aqueous solution polymerizes reversibly, to form polyoxymethylene glycols; most of the formaldehyde in the commercial solution is found as trioxymethylene glycol:



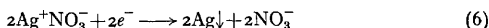
In dilute aqueous solution, trioxymethylene glycol depolymerizes to yield methylene glycol, the active monomer:



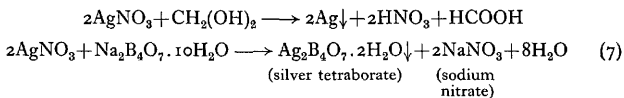
The point of equilibrium of the reaction given in equation (4) can be shifted to the right by rendering the formaldehyde solution alkaline. In alkaline solutions, methylene glycol is readily oxidized, with the liberation of the electron required to produce metallic silver:



The reduction of silver nitrate then proceeds as follows:



We found it convenient to buffer an aqueous solution, containing 16% w/v formaldehyde, with 5.1 g borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) per 500 ml; this has a pH of 8.5. Silver nitrate and borax react, to produce silver tetraborate and sodium nitrate. This side reaction slows down the precipitation of metallic silver, since a little silver is abstracted by the sodium tetraborate, and the pH of the solution drops rapidly. The metallic silver then comes down as particles of colloidal dimensions. If the reaction is carried out in a test tube, it can be seen that the silver thus formed is at first so fine that it only produces a slight, golden opalescence of the solution. If this is allowed to stand, progressively larger aggregates of silver become visible. The complete reactions between the silvering solution and the reducing fluid are as follows:



The insoluble silver tetraborate floats to the surface of the pool of liquid on the slide.

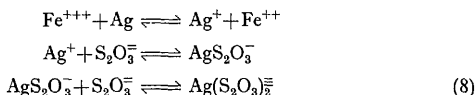
To start the reduction, drain the silvering solution from the slide, and replace it on the staining rack, *without washing*. Pour the formaldehyde reducer from a dropping bottle, using only enough of this to cover the sections (about 5 to 10 drops). Since the collodion membrane was soaked in an alcoholic solution of silver nitrate, turbulence can now be seen at the surface of the collodion membrane. This provides adequate agitation and ensures that the silver nitrate is reduced at the surface of the collodion membrane. Metallic silver, thus formed, reaches structures within the sections by a process of ultra-filtration (Ostwald, 1919). Obviously, only particles smaller than the pores of the collodion membrane can reach the section. The silver tetraborate formed in the secondary reaction given in equation (7) is carried to the surface

of the fluid on the slide. As soon as the turbulence on the surface of the slide slows down (this takes about 30 sec), the reaction must be stopped. If this is not done, the silver tetraborate will be precipitated at random on the collodion membrane, and the particles of metallic silver within the membrane will increase in size to a point where the membrane is ruptured. The reaction is stopped by washing the slide for a minute or two under a direct jet of running water (tap-water is suitable). It is important to wash vigorously; the sections are not damaged by the jet of water, since they are protected by the collodion membrane. During the washing, the sections become perceptibly darker; this is probably due to the catalytic reduction of the ionic silver remaining within the membrane (Mees, 1946).

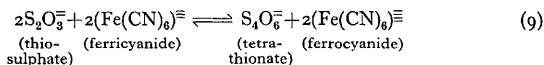
If the sections are now examined, preferably with a 3.6-mm water-immersion objective, the axons appear dark brown, the neuronal perikarya, golden; the Golgi apparatus is, at the most, golden reddish; the connective-tissue fibres are reddish brown; different types of neuroglia are light yellow to dark gold. We found that by repeating the silvering and reduction several times, the Golgi apparatus could eventually be blackened. Since metallic silver has a catalytic action upon the reduction of ionic silver, so that silver is deposited upon the silver already in the tissue, the blackening of those structures that remained colourless after the first silvering will only start when the structures that were silvered from the beginning become saturated with metallic silver. For this reason, the Golgi apparatus only begins to take up appreciable quantities of silver when the remaining structures in the section (with the exception of the cytoplasmic ground-substance) are almost black. If the permeability hypothesis to explain argyrophilia (given in p. 210) is correct, one would expect that structures not very permeable to silver—such as the Golgi apparatus—would resist de-silvering as much as they resisted silvering. Fortunately, this is so. For the Golgi apparatus to be successfully differentiated it is essential that it should be saturated with silver before bleaching is begun. To do so, it is necessary to repeat the silvering and reduction procedures from 3 to 5 times. It is convenient to examine the first slide of a batch after each silvering: if the conditions remain constant, the remaining slides will require the same number of treatments.

(iii) *Differentiation of the Golgi apparatus.* The purpose of this step is to oxidize the silver attached to structures other than the Golgi apparatus. When this is done, the Golgi apparatus stands out clearly because the background is very nearly transparent. At first we bleached the sections in iodine, exactly as recommended by Adamstone (1952). This was not satisfactory since the sections bleached irregularly and it was difficult subsequently to remove all of the silver iodide formed in this reaction. Better results were obtained with a reagent introduced by Farmer (1883, 1884) for bleaching over-exposed photographic negatives. Unfortunately, Farmer called this a 'convenient reducing agent': the only thing that is reduced is the intensity of the photographic image; the silver is oxidized. Farmer's reagent is a 0.3% solution of potassium ferricyanide ($K_3Fe(CN)_6$) in 5% sodium thiosulphate. Silver is

oxidized to silver ferrocyanide ($\text{Ag}_4\text{Fe}(\text{CN})_6 \cdot \text{H}_2\text{O}$), and passes into solution as an argyrosulphate complex. The ionic reactions are:



Photographs and sections oxidized in ordinary Farmer's reagent tend to be yellowish. This is due to the deposition of a small quantity of silver sulphide (Ag_2S) from the unstable higher argyrosulphate complexes (Lumière and others, 1912; Lüppo-Cramer, 1912). The deposition of sulphide can be prevented by treating the sections for a minute or two in a solution of 1% potassium iodide (KI) in 5% sodium thiosulphate after silvering, and before bleaching (Reindorp, 1935). Farmer's reagent is unstable. When freshly prepared, it is pale yellow-green; after a few hours, it becomes light blue. Ferricyanide is reduced to ferrocyanide by the thiosulphate ion, thus:



For this reason, the working solution of Farmer's reagent should be prepared immediately before use. Stock solutions of potassium ferricyanide and of sodium thiosulphate, however, are quite stable.

To oxidize unwanted silver, put a slide in a jar of Farmer's reagent, after treating it with iodide/thiosulphate. Watch carefully. The silver attached to the collodion is removed first. That attached to tissue structures is only dissolved after the collodion appears quite free of silver. Once the silver within the sections begins to dissolve, it is advisable to watch the rest of the differentiation under the microscope. The slide should be dipped in a jar of Farmer's reagent from time to time to replace the exhausted ferricyanide and to remove the reaction-products. When the contrast between the Golgi apparatus and the remainder of the tissue is adequate, stop the differentiation by washing under running water (the direct jet of tap-water is adequate) for several minutes.

(iv) *Finishing procedures.* If it is not intended to keep the slide permanently, or if this is later to be bleached and stained, the slide should be mounted now. To do this, rinse in distilled water, and dehydrate in ascending concentrations of ethanol. One can mount directly from absolute ethanol in Gilson's (1906) euparal. This has a conveniently low refractive index, and will, in time, dissolve the collodion membrane. Alternatively, clear in xylene and mount in a synthetic resin, such as DPX (Kirkpatrick and Lendrum, 1939) or the 'synthetic neutral mountant' sold by Flatters and Garnett (309 Oxford Road, Manchester, 13). Canada balsam is not suitable. Even when mounted in the synthetic media, the preparations are not permanent: in time, the silver goes into colloidal solution, especially if subjected to bright illumination.

If it is intended to keep the slide permanently, it is advisable to replace the silver with gold ('gold toning'). Gold toning was introduced more than a century ago as a means of rendering daguerreotypes permanent. Gold, being a 'nobler' metal than silver, will replace silver, exactly as silver would replace copper, or copper would replace zinc. Gold, being very unreactive, produces a permanent image. Proceed as follows: rinse the slide in distilled water and place it in a jar of 0.2% yellow gold chloride ($\text{AuCl}_3 \cdot \text{NaCl} \cdot 2\text{H}_2\text{O}$). After 5 to 10 min, rinse in distilled water and leave for a minute or two in 5% sodium thiosulphate to remove any silver remaining in the sections. Wash under running tap-water; dehydrate and mount as before. However, Canada balsam can now be used as a mounting medium. Thick sections can be made to appear thinner (see fig. 1) by mounting them in one of the media of high refractive index intended for mounting diatoms (for instance, the medium of r.i. 1.666 sold by G. T. Gurr as 'Clearax').

SOME CAUSES OF FAILURE

All methods of silvering the Golgi apparatus are somewhat capricious. By this it is meant that sometimes, for no apparent reason, the Golgi apparatus is not adequately blackened or does not stand out in sufficient contrast to be profitably studied. The method of silvering recommended here is no exception. But, as the sections can be examined before, during, and after silvering, and as the silvering can be repeated on different sections of the same piece of tissue, it is sometimes possible to ascertain the cause of failure.

Not all neuronal perikarya, after fixation in any of the reagents that we have tried, enclose cytoplasmic objects that resemble those described by Golgi (1898). Some enclose only a dense mass of much smaller objects, similar to those described by Flemming (1882). At present it is impossible to say whether these cells never had a Golgi apparatus, or whether the Golgi apparatus was destroyed by the fixation and the after-treatments. Where there is no Golgi apparatus before silvering, no Golgi apparatus can be blackened. In cases of failure, this possibility should be investigated; for this purpose, the interference microscope is invaluable.

Sometimes the Golgi apparatus is blackened to a greater extent in some parts of a section than in others. This is usually due to variations in the thickness of the collodion membrane. Considerable practice is required before the membrane is successfully produced on every slide. Practice is also required before one develops the knack of stopping the differentiation of the Golgi apparatus at the right moment. Once the differentiation of the tissue constituents begins, things happen rather quickly: one should have all the reagents required close to hand, and one must act without hesitation.

An occasional complication is the presence of a heavy granular deposit on the sections, usually as concentric rings, up to about 2 mm in diameter. This may be due to uneven flooding with the silvering solution, to an insufficient quantity of the reducing fluid, or to delays in washing off the reaction products of the reduction. The remedies suggest themselves. Finally, as in all methods

of silvering, extreme cleanliness is required: grains of dust are just as argyrophil as tissue constituents.

When neurones were photographed to scale before and after silvering (as was done in fig. 2), it became obvious that silvering produced considerable shrinkage, and that all parts of a neurone did not shrink at the same rate. The cytoplasm shrank more than the nucleus. It is strange that firmly mounted sections could be made to shrink. Most of the shrinkage occurs during oxidation in Farmer's reagent.

DISCUSSION

At first sight it is strange that the Golgi apparatus should have resisted fixation in 'FAM' and the after-treatments required to produce double-embedded sections. Acetic acid is generally supposed to destroy the Golgi apparatus, and ever since Nussbaum's investigations (1913), it has been assumed by many that lipids must be preserved. Baker (1957*a*) has recently demonstrated that acetic acid does not necessarily destroy cytoplasmic inclusions whether these are mitochondria or the Golgi apparatus. Concerning lipids, the situation is less clear. Organic solvents do not readily extract lipids from pieces of tissue that were fixed in formaldehyde (Baker, 1944). Treatment of formaldehyde-fixed material with organic solvents has the further effect of 'unmasking' certain lipids (i.e. splitting from any protein conjugated with them) (Ciaccio, 1926; Clayton, 1958), so that these now become more reactive, even if present in smaller quantities. 'FAM' preserves a surprising amount of lipid, though this fixative should not be used for histochemical studies of the distribution of lipids. If the permeability-hypothesis (p. 210) is correct, the preservation of different substances in the tissue, when it is wished to silver the Golgi apparatus, is important only in that enough of the substances contained in this structure must be preserved for the structure to retain its characteristic shape. Silvering, particularly as done here, cannot be considered a histochemical test for the demonstration of any particular substance.

Do the classical Golgi methods, in which whole pieces of tissue are silvered before embedding, also depend upon the relative permeability of different tissue constituents, rather than upon a chemical reaction between the Golgi apparatus and the silver? There is no proof of this, but the available evidence indicates that this may be so. For instance, in Aoyama's method (1929), small pieces of tissue are fixed in formaldehyde / cadmium chloride for 4 to 12 h; after a brief rinse in distilled water, they are 'impregnated' in silver nitrate for 13 to 17 h. Some reduction takes place during this period, since the tissue contains, at all times, a mixture of formaldehyde and silver nitrate in the presence of cadmium and chloride ions. The silver is reduced very slowly and then only to a colloidal state, presumably because the tissue acts as a protective colloid and because the pH of the mixture in the tissue is not conducive to rapid reduction. Obviously, a certain amount of 'impregnation' occurs at the same time, but it is difficult to say how much. Then, after another rinse

in distilled water, the tissue is put in a hydroquinone / formaldehyde / sodium sulphite reducer for about 5 h. It seems probable that this leads to the further reduction of silver upon those sites in the tissue that already contain some colloidal silver after the treatment with silver nitrate. This step would correspond to the repeated silvering and reduction in the method described here. Since in Aoyama's and similar methods some impregnation is unavoidable, and since it is not easy to control each step, inexplicable failures are not uncommon. There remains to be explained the so-called 'adjuvants', such as the salts of cadmium, cobalt, uranium, &c. We have shown that they do not directly influence the preservation of the Golgi apparatus or the ease with which this can be silvered in paraffin sections. Yet the classical methods definitely do not work without 'adjuvants'. Two explanations seem reasonable. Colloidal silver can be precipitated or 'salted out' by the addition of small quantities of salts of certain metals (e.g. lithium, sodium, potassium). However, salts of heavy metals, such as cobalt, cadmium, and uranium, behave abnormally in this respect: they protect the colloid rather than precipitate it (see Ostwald, 1919). This might help to produce particles of silver of the correct size for entering structures that have the permeability of the Golgi apparatus. The second possibility is this: that the meshes of the fixed tissue perform the same function as the collodion membrane in our method, that is to say, form a protective colloid round the particles of silver. The size of the particles that do penetrate the tissue would depend upon the size of the meshes. Since salts such as cadmium chloride, *when used with the fixative*, are powerful coagulants of protein (Baker, 1944, 1950), they would affect the size of the meshes, and therefore of the particles of silver.

Concerning the applications of the method described here, certain limitations should be taken into account. If a certain type of cell encloses a cytoplasmic network, this can almost certainly be blackened by our method. The structure of the deposit of silver then resembles very closely the cytoplasmic network of the living cell. The use of the term 'Golgi apparatus' to denote this type of network is unexceptionable (Cain, 1954). However, objects of various shapes are also blackened by our method (and other Golgi methods) in cells that do not contain similar objects when in the living state. For instance, in the cytoplasm of invertebrate neurones, objects known as dictyosomes are readily blackened: these are the distorted remnants of what, in the living neurones, are separate spherical globules of hydrated phospholipid (Chou, 1957a, b; Chou and Meek, 1958). The use of the term Golgi apparatus to denote separate lipid globules is clearly unjustifiable. We do not believe that all the objects blackened by our technique should be homologized, unless evidence can be found that they all have approximately the same structure in the living cells, contain the same or approximately the same substances, develop in approximately the same way, and perform the same type of function (see Baker, 1957b).

Malhotra (1959) has recently brought new evidence in favour of Legendre's hypothesis (Legendre, 1910) that the objects coloured by basic dyes in the

cytoplasm of vertebrate neurones (Nissl, 1885, 1888, 1894), and the Golgi apparatus, were one. When Malhotra silvered the Golgi apparatus of vertebrate neurones, and, after bleaching it, stained the same cells with a basic dye, the structures that had been silvered now took up the dye. He proposed that these structures be called the 'Golgi-Nissl complex'. One possible objection to this theory is that the silver used to blacken the Golgi apparatus and the iodine used to bleach it might have affected the tissue in such a way that the basic dye would become attached selectively to those structures that, before, had contained silver. With the new technique, Malhotra's evidence can be re-examined by performing the experiment the other way round. We have performed this and allied experiments, and hope to describe them in full in a subsequent paper (David, Mallion, and Brown, 1960). Some of the work described here (especially fig. 2) supports Malhotra's thesis. When fixed sections are examined with the interference microscope before silvering the Golgi apparatus, and with direct illumination after silvering (see fig. 2), it becomes obvious that no structure of sufficient size or of the right shape to be the objects described by Nissl, remains unsilvered. As our unsilvered sections take up basic dyes in the usual way, it follows that only one type of structure is involved in the silvering and the dyeing. Therefore, Malhotra's hypothesis can be accepted as a basis for further experimental work.

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APPENDIX

The reagents used in techniques of silvering should be carefully prepared and stored. All chemicals should, if possible, be of analytical quality. Below are given the special solutions required. Unless otherwise indicated, they should be weight/volume solutions, i.e. they should contain x g of solute per y ml of finished solution.

Collodion solution

Absolute ethanol	250 ml
Diethyl ether	250 ml; add
'Low-viscosity nitrocellulose' (G. T. Gurr)	5 g

Keep in a well-stoppered bottle.

Silvering fluid

Stock solution: 17% aqueous silver nitrate (about 1 M AgNO_3).

Immediately before use dilute 25 ml of this with 25 ml absolute methanol.

Reducer

Sodium tetraborate, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$	5.1 g
Undiluted commercial formaldehyde solution	200 ml
Distilled water	to make 500 ml

Farmer's reagent (unstable)

10% aqueous potassium ferricyanide $\text{K}_3\text{Fe}(\text{CN})_6$	1.5 ml
5% aqueous sodium thiosulphate, $\text{Na}_2\text{S}_2\text{O}_3$	50 ml

Sulphide inhibitor

2% aqueous potassium iodide, KI	25 ml
5% aqueous sodium thiosulphate, $\text{Na}_2\text{S}_2\text{O}_3$	25 ml

Gold chloride

0.2% aqueous yellow gold chloride, $\text{AuCl}_3 \cdot \text{NaCl} \cdot 2\text{H}_2\text{O}$

Sodium thiosulphate

5% aqueous sodium thiosulphate, $\text{Na}_2\text{S}_2\text{O}_3$