SILVER STAINING OF THE SYNAPSE

IN THE

HUMAN CEREBRUM

by

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ABSTRACT.

There has been recent tendency on the part of certain investigators, because of their failure to demonstrate boutons terminaux in the cerebral cortex by the silver impregnation methods, to suggest that the pericellular fibers in the cerebral cortex end freely on the cells and that such free terminals are the normal form of synapse in this part of the central nervous system. In the present work a histological study was made of certain areas in the frontal cerebral and the visual cortex to show the presence of normal boutons terminaux. It was demonstrated that boutons occur in these areas in sufficient numbers to indicate that these are the normal means of synapse. The direction which further research should take is suggested.

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INTRODUCTION

The purpose of the present work has been to establish and standardize the simplest reliable and consistent methods for the demonstration of nerve fibers and interneuronal connections in the cerebral cortex of man in order to apply these methods to the brains of mental patients coming to autopsy following prefrontal lobotomy. The methods to be studied are reduced silver and double impregnation silver procedures such as those used by Cajal, Bielschowsky and Del Rio Hortega. In addition to the main search for dependable methods of staining for degenerating nerve fibers, there has been an interest in the " contact vs. continuity " dispute which has so long pointed up the mystery of the interneuronal connection. Some re-examination of the literature pertaining to this controversy, therefore, has been thought advisable, confined largely to a discussion of the histological evidences. A number of methods have been recorded and discussed in detail and many others mentioned. Some evidence of the nature of the ' boutons terminaux ' in the cerebral cortex of man is recorded.

(a) The Neurone Theory - Contact vs. Continuity.

Knowledge of the structure of the interneuronal connections
can only be as accurate as the means used for their demonstration
are dependable. Since Golgi in 1885 developed a technique for staining
chrome-hardened nervous tissue with a silver solution many mysteries of
the working of the brain have unfolded under the microscope. From
observation of spinal cord stained by his new method Golgi became con-
vinced that " in the grey substance of the spinal cord there exists
an exceedingly extensive and delicate network " , which he considered
was due to anastomosing of the collateral branches and not as Gerlach,
the originator of the reticular hypothesis before supposed, to anasto-
mosing of the dendrites. Retzius, von Kolliker and others (Retzius,
1908) failed to reproduce Golgi's results satisfactorily at that
time.

Retzius, using Ehrlich's methylene blue method , could find
no such network as described by Golgi but in the ganglia of crawfish
he was able to see " not a network of reticulum, but a twistwork ".

His and Forel (1883) could not imagine that any protoplasmic
connection existed between one nerve cell and the next and proposed
the theory that the processes of the nerve cells end freely in the
grey matter and are not intertwined in elaborate network. This theory
was not, however, supported by objective evidence and had never been
widely accepted.

At about this time a young Spanish neurologist and professor
of Histology, Santiago Ramon y Cajal, published a series of works on

the minute structure of the brain. Wilhelm His of Leipzig had formed

the conviction long before (1883) that nerve cells developed as organs

independent of one another. This view was now accepted by Cajal on

the basis of his own researches using the Golgi method. Cajal (1891)

was unable to see the reticulum described by Golgi and could not see

in any place the processes of two different nerve cells anastomosing.

" Les fibres s'intrelacaient d'une maniere fort compliquée, engendrent

un plexux enchevêtre et serré, mais jamais un réseau ". From his ob-

servations on the cerebellum of the bird " on dirait que chaque élément

est un canton physiologique absolutment autonome ".

Delivering the Croonian Lecture to the Royal Society in 1894

he said, " Les connexions établies entre les fibres et les cellules

nerveuses ont lieu au moyen de contact, c'est-à-dire à laide d'une

veritable articulation entre les arborizations variqueuses des cylindres-

axes d'une côté, le corps et les prolongments protoplasmiques de l'autre.

Aussi est on amené à se représente l'axe encéphalo-spinal comme un édi-

fice compose d'unités nerveuses superposées, de neurones , suivant

l'expression de Waldeyer." Waleyer, a German anatomist, had written

a resumé of Cajal's ideas and popularized the neurone doctrine.

In 1891 Held reported his discovery of the " calices " or syn-

apses of the nucleus of the trapezoid body using the methods of Golgi

and Nissl. In 1897 he published a report of his " Endefusse " which

Auerbach confirmed in 1899 and called " boutons terminaux ". The con-

ception of the continuity between the Endefusse and the reticulum of

the neurone was published by Held (1904 - 1905) and finally accepted

by Auerbach. Held believed the Endefusse to be imbedded in the inter-

stices of the " pericellular nerve net " and saw continuity with the inner reticulum as well. Auerbach, however, thought that a transparent cuticle intervened.

There existed, then, in Europe two opposing theories on the intimate structure of the nervous system. Golgi, Gerlach and Held strongly advocated the concept of the Gargantuan spider-web - the hypothesis of continuity. His, von Kolliker, von Lenhossek, van Gehuchten and Retzius all supported the neurone doctrine of Cajal and Waldeyer - the hypothesis of contact and the autonomy of the neurone.

In 1897 an Hungarian zoologist, Stephen Apathy, published a work " Das leitende Element des Nerven-systems und seine topographischen Beziehungen zu den Zellen " in which he built up a theory regarding the minute structure of the nervous system. In this he theorized that the neurofibrils form the specific constituent of the nervous system and that they were the independent structural parts, the conducting elements. In the nerve fibres they preserved their individuality, forming reticula only in specific places. He maintained that they formed large continuous reticula in the organs of the body and above all in the central grey substance. The evidence for these conclusions was obtained with the help of a new gold staining method.

Apathy was later vigorously supported by a German histologist, Stephen Bethe, (   ) who became one of the chief opponents of the neurone theory.

The Golgi technique on which these opposing theories are largly bases was crude compared to the more delicate present day silver methods.

Fresh tissues were placed in solutions of potassium dichromate beginning with a low concentration, 2 1/2 %. The solution was changed frequently each time increasing the concentration to 3%, 4% or 5%. The exact degree of hardening of the tissue necessary for optimum silver impregnation had to be attained. The time required varied between summer and winter from fifteen days to four months. After hardening the tissues were placed in a large volume of 0.75% silver nitrate solution. The silver bath also was changed several times. The time required for the impregnation was 24 to 48 hours. Excess of the silver nitrate was washed out with 80% to 90% alcohol. Thick sections were made freehand, cleared and mounted in thick xylol-damar, without a cover slip. By this technique the nerve cells and delicate fibers were opaquely stained so that the intracacies of the nerve cell processes could be demonstrated but no intracellular detail could be seen.

In 1906 Cajal and Bielschowsky simultaneously published a new reduced silver nitrate method for staining nervous tissue. By this new technique Cajal was able to demonstrate for the first time the intracellular structure and to prove that in the nerve cell processes the neurofibrils always remain within their substance and are not, as Apathy asserted, capable of emerging from it. Retzius (1908) summarized the investigations on the subject stating that " Neurone fibrils are to be found in the nerve cells and their processes, that they form in the cells abundant reticula, which are plainly to be seen even in some of the peripheral terminal organs, and that they do not anastomose outside the particular domains of the cell unit or neurons, ie. that they

do not outside these form reticula but plexus. The several neurones are connected one with another _per contiguitatem,_ not _per continuitatem._ Finally, there does not exist any certain proof that the fibrils constitute the sole conducting element ".

After Cajal's severe rebuttal (1908) of Apathy's objections to the neurone theory only weak opposition has been raised to the neurone doctrine up to the present. A controversy arose between Bartelmez (1915) and Marui (1918) over the vestibular club-endings on the Mauthner's cells of Ameirus. " The contact theory is a histological impossibility " states Marui. Bartelmez (1930), with non-metallic stains, settled the question in favour of the contact theory and the neurone doctrine.

Tiegs, using the reduced silver methods of Cajal and Bielschowsky, revived the controversy by describing the pericellular net which had also been described by Cajal, insisting that it was the neurofibrillar system which conducted and that there was " no visible gap in the neurofibrillar system at the neurone junction ". In 1926 he concluded that a " true neurone continuity occurs at the junction of the neurones in the spinal cord ---------- in which collaterals from various neurones penetrate the various dendrites, and , passing into the substance of the nerve cells anastomose, the anastomosis with collaterals from other neurones thus permitting evidently of integration and the whole of the integration occurring within the body of the nerve cell ".

As late as 1931 Tiegs maintained that " whether there are end-bulbs or not there is protoplasmic continuity between the axone terminals and the intracellular fibrils of the cell or dendrite ". His histological preparations demonstrated to Sherrington at that time were not convincing.

Ballantyne (1925) explains bouton terminaux and pericellular

networks as optical illusions due to the use of monocular microscopes !

Windle and Clark (1928) reviewed preparations made by Ranson and found evidence to support the disconuity or contact theory . In answer to Tiegs, they said, " failure to observe free ends of nerve fibres in the young material is not sufficient evidence on which to assume a nervous sycytium ". They assume that fibres could end on dendrites without end-bulbs - as Cajal assumed with the climbing fibres on the Purkinje cells in the cerebellum. This view has lately been express- ed by Glees (1946).

In 1933 Bartelmez and Hoerr gleaned valuable information on returning to a study of the bullhead, Ameirus, in which the club-shaped endings of certain root fibres of the VIII nerve on the lateral dendrite of Mauthner's cells offer excellent subjects for preparation. They critically examined the fixation and staining techniques used in studies of the synapse. They suggest that the differences in opinion concerning the nature of the synapse are due to differences in technique. After shrinkage of the tissue in fixation most synapses are so minute as to be on the verge of the resolving power of the optical microscope. They also suggest that the personal factor plays an important role. It is not to be wondered at that Cajal and Held came away from their celebrated conference each certain that his own view was correct, although each had studied the other's most convincing preparations. Even scholars like Bielschowsky and Cajal have not discussed the reliability of their reduced silver techniques. The need to study living tissue as a control was obvious.

Bosler (1927) has been able to see the synapse in the living tissue of the medusa Rhistoma. The nervous elements were stained with a methylene blue leucobase. It was previously accepted that the coe-

lenterate nervous system was sycytial in character. Bosler's preparations showed only contact at the synapse and no evidence of continuity. He also observed the neurofibrillae in the living cells. Their presence in the vertebrate systems had not yet been established. Woollard ( ) confirmed Bosler's work.

At the junction of the club endings and dendrite of the Mauthner's cell in the Ameirus, Bartelmez and Hoerr could resolve only a single line with no indication of any pericellular network between the two. They could distinguish clearly that the neurofibrillae were sharply differentiated but in no instance could they be seen to pass from the end-feet across the synapse and into the Mauthner cell's dendrite.

Bartelmez and Hoerr emphasized the unreliability of all reduced silver techniques from the cytological point of view and suggested that they be checked by other methods. From their experiments with the Bielschowsky technique they concluded that the block method of impregnation is unreliable and is inferior to the individual section method. In staining with silver the time allowed for adsorption of the silver solutions is the most important variable. Ammoniacal alcohol used before mordanting in silver prepares the tissue for an overlay of silver which may give untrue results. Bartelmez maintains that formol is only to be used as a fixative and must be followed by a mordant to avoid gross shrinkage.

Bouton degeneration studies have contributed further convincing evidence in favour of the neurone theory. The 'bouton method ' also serves as an invaluable means of tracing fibre tracts to their destination.

As early as 1893 Nikolajew investigated the innervation of the

frog's heart by cutting the vagus nerve to to the heart and studying microscopically the degeneration of the pericellular endings on the cardiac ganglion cells. By comparing what he saw with the description of normal endings given by Smirnow (1890) he could determine which of the endings was affected by the lesion. Since then several investigators have studied neurofibrillar degeneration. Marinesco (1904-1906) studied the degenerating effects of various pathological conditions such as myelitis and hemiplegia on the boutons terminaux. Lache (1906) studied post mortem changes in neurofibrils sixteen hours after death in humans. Golgi (1908), Achucarro (1909) and Mott (1912) contributed to the information.

More recently Lawrentjew (1925) has applied the bouton method to the sympathetic nervous system. He showed that maximal degeneration of the boutons terminaux in the superior cervical ganglion was present five to six days after section of the preganglion fibres. He confirmed the work of Nikolajew (1893) and showed that degeneration of the end-feet did not necessarily affect the cells themselves.

The work of Hoff and Gibson finally proved the nervous nature of the bouton terminaux and indicated a new method for studying fiber tract degeneration. Hoff (1932) published a modification of Cajal's reduced silver method which he used in his studies of bouton degeneration. Experimental animals were fixed, by perfusion, with 10% chloral hydrate. The central nervous system was then removed to a 10% solution of chloral hydrate for 24 hours and stored in 96% alcohol and ammonia. To impregnate, the tissue was placed in 1.5% silver nitrate for six days at 37° C. After impregnation the tissue was washed, reduced in 2% hydroquinone,

dehydrated, imbedded, sectioned at 15 microns and mounted.

Studying the nature of the normal synapses in the cat, Hoff found no bouton terminaux at birth. The first boutons to appear, 21 days after birth were boutons de passage. In the adultt cat he showed boutons in the spinal cord, medulla and on the bodies of nerve cells in the granular layer of the cerebellar cortex. These normal synapses stained as round or elliptical loops of 2 to 4 microns in diameter. Around the dendrites Hoff found a meshwork of tortuous fibers some of which ended in bouton terminaux while others passed out of the field. At no time was he able to demonstrate continuity between boutons and the intracellular neurofibrils.

Hoff published several papers (1933-1934) on his bouton degeneration studies in the cat, monkey and human. A series of experiments was performed to determine the effect upon boutons of cutting the roots of the afferent fibers to the lumbar and cervical enlargements of the cord. Animals were sacrificed from twenty-four hours to two weeks after the operation and studies made of sections of the cord prepared with the silver technique (1932). After twenty-four hours the boutons were swollen, enlarged and elongated. The swelling had increased after forty-eight hours and the boutons no longer showed the loop-like appearance but were completely solid. The synapses may reach diameters of four to seven microns in degeneration. The seventy-two hour sections showed some boutons almost completely obliterated. Few abnormal boutons could be found after four days, and not at all after six days. There was still no evidence to show protoplasmic continuity between the boutons and the intracellular structures. The experiments showed that separation of a nerve fiber from its cell body is followed by the degeneration of its termination in the grey matter.

Foerster, Gagel and Sheehan (1933) confirmed Hoff's work. Sereni and Young (1932) observed a similar course of degeneration in the synapses of cephalopods. Here the degeneration took place earlier and regeneration of the fibres took place at seven to eighteen microns per hour. Measurement of ' degeneration time ' showed that it varies inversely as the temperature of the water in which cephalopods are kept.

Lawrentjaw (1934) considers the boutons to be constant structures in the synapses of the autonomic nervous system.

Gibson (1937) published the results of a carefully correlated series of degeneration experiments as a basis for further work on degeneration,in which it is important to differentiate between boutons of longer and those of shorter periods of degeneration.

THE SYNAPSE

The reticular hypothesis was finally rejected after experiments on bouton degeneration showed that disintegration of the end-feet had no effect on the effector cell. Boeke (1932) and Stohr (1935) demonstrated minute threads passing from the terminal nerve fibers to the nerve cell. Ninidez, however, smothered this final gasp of the defunct theory by proving that these fibers do not degenerate when the axons leading to the terminals are severed. The way was then open for intensive study of the morphology and the physiology of the synapse in health and disease.

The Morphology of the Synapse

The structure of the normal bouton terminaux or endefusse had been variously represented very early by Held, Cajal and Golgi in an effort to settle the contact vs. continuity controversy. (see earlier review) The contribution of Nikolajew, Smirnow, Marinesco, Lache and Lawrentjew as well as the work of Hoff and Gibson have already been discussed. The contributions of Sereni and Young were also mentioned, and of Foerster et. al.

After the introduction of the bouton method in the tracing of fiber pathways, researchers began to develop measuring sticks for bouton degeneration studies. The first problem was to set down accurate data as to the size, shape and distribution of the normal end-bulbs in each area to be investigated. Bodian (1937) stained the axon endings on the Mauthner's cell in the goldfish using a protargol method. He described them as blackened loops varying in diameter from 0.5 microns to 7 microns. Phalen and Davenport (1937) demonstrated boutons in the spinal cords of several vertebrates using a modified Cajal's stain. They found a marked variation

in the size and form of the endings. They also noted that in Mammals, with the exception of the monkey, the end-bulbs tended to vary in size with the species. Because of the variation in size of the boutons, Phalen and Davenport did not consider degeneration methods of fibre tracing to be satisfactory. Barr (1930) showed that axon termination on cells of the lateral groups of the ventral horn are smaller than those on cells of the medial group in the cat's spinal cord. In his preparations he found that the boutons tended to become smaller on cells undergoing retrograde degeneration.

Barnard found no significant difference between normal boutons and those at the end of axones that had been cut, thus emphasizing the unreliability of the bouton method of study.

An invaluable contribution to the study of normal terminals was made by Jeff Minckler (1940,41,42). Under his name a series of publications appeared defining five types of terminals in the human cord ; small loops, large loops, filamented loops, fibrillated loops and opaque or granular loops. He found that each of these might appear as regular, thickened or granular. These endings were usually found in contact with the cell or robust process. They ranged in size from 0.5 x 1 micron to 3.5 x 5 microns. Varying as a fundtion of the cell size, the number of boutons per cell ranged from 144 on small sensory cell bodies to 1832 on the large cell bodies of the posterolateral column. Minckler showed (1941) that the morphological types of endings in a given area of the human cord remain fairly constant from one individual to the next, regardless of the age, if the staining technique is carefully controlled. It was also shown that the different types of terminal occur in about the same numbers on different parts of the nerve cell. However, granular forms occurred more frequently in older persons. It was realized

that the appearance of the thickened forms may be due to technique
such as prolonged fixation in formaldehyde but Minckler stated that
autolytic processes operating up to 24 hours seemed to have little
effect on the bouton morphology demonstrable with the Cajal technique
used. (See also Hoff 1931).

Reports of the demonstration of normal bouton endings in the
cerebral cortex are remarkable for their scarcity. Hoff, using block
silver methods, stained cortical end-feet in the cerebral cortex of
the cat, but with great difficulty. Cajal (1934) reported boutons in
the cortex.

Poorly rewarded efforts to stain boutons in the cortex have led
some investigators to believe that these are not the normal types of
synaptic points in this part of the nervous system. Meyer and Meyer (1945)
concluded that " while bouton-like structures around cortical nerve cells
can be demonstrated by our present methods under favourable circumstances,
it is obvious that they constitute merely a portion of the terminals ".
Bielschowsky, (1935) and Cajal (1934) described plexuses formed by the
terminal fibres around cortical striatal cells and suggest that these
may make connection with the dendrites. Degenerating boutons were dem-
onstrated by Greenfield (1939) in patients with cerebral oedema. King
(1942) described briefly pericellular argyrophylic structures in relation
to pyriform cells in the pyramidal layers which reduced ammoniacal
silver solution without further chemical treatment. In 1946 Glees
stated that " It seems that the synapse within the cortex is mainly
represented by free terminals of the pericellular plexus ". However he
showed that the pericellular fibres in the caudate nucleus degenerate
after cortical ablation (Glees, 1944) and suggests that these can be

used in degeneration experiments. The fallacy of this view will be reviewed in the discussion.

In spite of its apparent potentialities, the ' bouton ' method has not enjoyed the popularity which one might expect. However, in the hands of a number of investigators it has yeÉlded many valuable contributions to the knowledge of the nervous system. Hoff (1935) used the bouton method to investigate the terminal distribution of the cortico-spinal fibers arising in the premotor area of the monkey. The structure of the lateral geniculate body and the projection of the retina in the lateral geniculate body were studied, using the bouton method, by Le Gros Clark and Penman (1934). A series of experiments was carried out (Le Gros Clark, 1941; Glees and ᴸe Gros Clark, 1941; Glees 1941 and 1942) to study the termination of the optic nerve tract fibres in the lateral geniculate body of the monkey, cat and rabbit. O'Leary (1940) contributed to the knowledge of the lateral geniculate body. The optic centres in the rat were investigated by Nauta and Van Straaten (1947). Furhher studies have been carried out by Glees (1944, 1946 and 1947) and by Glees and Meyer (1946) on the cortico-striate connections and the frontal cortex. The reference, by Glees, to the so-called free terminals in the cortex has already been mentioned. Brodal (1949) used the Glees method in an experimental study of the spinal afferents to the lateral reticular nucleus of the medulla oblongata in the cat. Recently (1951) Wall, Glees and Fulton were able to demonstrate direct projections from the orbital surface of the frontal lobe to the ventromedial and paraventricular nuclei of the hypothalamus and the causate nucleus.

A report on the use of intravenous methylene blue for studying

fiber degeneration in the central nervous system has recently been published by W. H. Feindel and A. C. Allison (1948). A critical discussion of the methods used to study fiber degeneration and suggestions for the use of methylene blue were published by Feindel, Allison and Weddell the same year. By their new method they were able to stain enlarged terminal boutons in the lateral geniculate body in rabbits.

The need for reliable methods of studying fiber degeneration in the cerebral cortex became more pressing with the introduction of the surgical treatment of certain mental disorders by frontal lobotomy. Most of the knowledge of the projection to and from the frontal cortex has been derived from experimental observations in monkeys and apes. In a report on the effects of prefrontal leucotomy, Meyer, Beck and McLardy (1947) state that " There is no need to emphasize the importance of a detailed neuro-anatomical investigation of the brains of patients dying at various intervals after prefrontal leucotomy. " The methods which can be used for such an investigation are few, and to date the most promising staining procedures are reduced silver methods.

The Mechanism of Synaptic Transmission

A brief discussion of the earlier literature on the mechanism of the synaptic transmission is thought to be important as giving context to the problem of synaptic structure.

The physiology of nerve activity has received much attention during the past half century and one can say that great strides have been made toward the solutions of many of the problems involved. However, the precise nature of the synaptic transmission has not yet been completely solved. Early in the century T. R. Elliot (1905) thought that some

chemical was responsible for the transmission of the nerve impulse across the synapse. In 1921 Otto Loewi showed that acetylcholine was the substance released by the vagus nerve to the heart and acted directly on the heart muscle. Dale (1933) tried to extend the ' neurohumoral ' theory to the neuromuscular junction and the ganglionic synapse. The acetylcholine hypothesis (Dale 1937, Clark 1936) simply stated that a presynaptic impulse liberated, at the synapse, a minute amount of acetylcholine, which excited the post-synaptic cell, thus setting up a synaptic potential. They held that it was quickly removed by the locally concentrated cholinesterase. (Brown 1937, Dale 1937, Nachmansohn 1940).

This met with two strong points of opposition. The neuromuscular transmission occurs much too quickly (milliseconds) to be explained by a chemical reaction. Secondly, the electrical signs of nervous action do not support the assumption that transmission of the nerve impulse along the axon differs fundamentally from that across the synapse since the excitable properties of the axon and of the cell are basically the same. Thus the neurohumoral theory seemed to be unsatisfactory.

The electrical theory of nervous transmission, in its early form, was also unsatisfactory. (Eccles 1936, Erlanger 1939, Lorente de No 1939, Monnier 1934) In general it was merely stated that the electrical currents of the presynaptic impulses set up impulses in the post-synaptic cell. The significance of the pre- and post-synaptic responses and of the rheobase of the post-synaptic cell necessitated so much modification of this vague formulation that it had to be abandoned.

Nachmansohn (1945) postulated that the release and removal of acetylcholine was an intracellular process occurring along the neuronal surface and directly connected with the nerve action potential. Thus the transmitting agent was considered to be the electric current, the

action potential, but the current was generated by changes in the membrane
in which a release of acetylcholine is an essential event. Nachmansohn
was able to show that the formation and removal of acetylcholine could
take place at a rate consistent with the speed of the nerve impulse.
However, Lorente de No (1944) had shown previously that the transmission
of the nerve impulse across the synapse is unaffected by high concentra-
tions of acetylcholine.

A later hypothesis on the synaptic transmission is the culmina-
tion of several years of intensive research by J. C. Eccles, (1947).
Three assumptions are made: first, that the geometrical situation at the
synapse is schematically represented by the pre-synaptic fiber ending as
a cylindrical membrane with a closed and direct apposition to the large
plane surface membrane of the post-synaptic cell. Thus he assumes that
a traverse membrane at right angles to the axon exists at the synapse
as described by Cajal (1934) and other histologists. There is, also,
electrical evidence for a highly resistant transverse membrane. Second,
he assumes that, in general the surface membranes have the electrical
properties demonstrated for peripheral nerve and muscle membranes, such
as resistance, electromotive force, capacity and rectification. Third,
that the membrane of the immediate post-synaptic region is specialized,
so that large and graduated local responses are set up by polarizing
currents.

With these basic assumptions the following sequence of events in
synaptic transmission was described. First, the impulse in the pre-synap-
tic nerve fiber generates a current which gives a diphasic effect at the
synaptic region of the post-synaptic cell with a total duration of not
more than 1 millisecond in mammalian muscle and the spinal cord; an
initial anodal focus with cathodal surround; a more intense cathodal

focus with anodal surround. Second, this cathodal focus sets up a brief and intense local response at the synaptic region. Third, from this local response, a catelectrotonus spreads decrementally over the post-synaptic cell membrane. Finally a propagated impulse is set up in the post-synaptic cells, if this catelectrotonus is above a critical value.

This summary of two of the more recent theories on the synaptic transmission gives some indication of the complexity of this problem. Any attempt to discuss the investigations of the past five years would be far beyond the scope of this review. An excellent monograph on the subject has been published by Rosenbleuth (1950). Reviews by Bullock (1951) and Rushton, W. A. H. (1951) indicate the vast and rapidly expanding literature. It can be said that both electrical and humoral transmission are involved in relay of a nerve impulse but the extent to which each agent is involved must yet be determined. Rushton concludes his summary " It is natural to try to explain the two kinds of conduction upon a common basis, but what has been proved is that along the nerve fiber the transmission is electrical; from the endings of peripheral nerves it is pharmacological. "

EXPERIMENTAL PROCEDURE

The experimental work has been concerned mainly with the staining of nerve fibers and the terminal synapses in the cerebral cortex. Several modifications of the reduced silver technique have been tried. The stepwise procedure for each of these has been included for reference purposes in an appendix. Certain procedures have been discarded for reasons which will be discussed under the heading of experimental results. The Cajal's modification for frozen sections and the Gros-Bielschowsky method have been used to stain the spinal cord and cortex in the dog. The double impregnation method for frozen sections and the Urea-Silver Nitrate method of Ungewitter were used to stain the cerebral cortex in humans. All tissues were fixed in 10% neutral formol regardless of the special fixatives mentioned in each method. In all cases frozen sections were cut at 15 microns.

Cajal's Modification for Frozen Sections. (See Appendix A for detailed
                                                      Instructions)

It was found advantageous to receive sections from the freezing microtome in water containing a few drops of concentrated ammonia. In step 2, three to five drops of pyridine per 15 c.c. of $AgNO_3$ solution proved sufficient. Adding greater amounts than this resulted in a dust-like precipitation of silver. An impregnation time of 6 hours gave the best results although longer in the silver bath seemed to do no harm.

Slides of spinal cord, cerebral cortex and cerebellum of dog were stained by this method. Alternate sections were toned in gold.

The <u>Gros-Bielschiwsky Method</u> (See Appendix B for details)

The block of tissue was soaked in water containing a few drops of ammonia. Frozen sections were cut at 15 microns and received in a dish containing a few drops of neutral formalin. The remainder of the procedure was followed as outlined in McLung's Handbook. (Ferdov's Modif.)

Sections of the cerebral cortex of dog were stained by this method. Alternate sections were toned in gold.

<u>Double Impregnation Method</u> (See Appendix C)

The procedure outlined in the Appendix is Gibson's modification of Rio Hortega's Silver Carbonate Stain for neurofibrils. Frozen sections cut at 15 microns were received in 15 c.c. of water plus 10 drops of ammonia. Petri dishes of 96 per cent alcohol and ammonia, 2 per cent silver nitrate plus 5 drops of pyridine, and the silver carbonate solution were maintained at a constant temperature of 45°C. in a shallow water bath. Five minutes in the gold chloride were found to be ample when the impregnation was reinforced by heating one minute over a spirit lamp.

Sections of human and dog cerebral cortex were stained by this method.

Because of the prominence to which the frontal lobes have been raised due to the treatment of certain mental disturbances by frontal lobotomy, it was decided to determine whether bouton terminaux could be satisfactorily demonstrated in certain areas of the frontal cortex. For this purpose frozen sections were taken from certain areas of the brain of a mental patient* who died twenty-nine hours after frontal lobotomy,

* Brain supplied by Dr. Paul Yakovlev, of the Connecticut Lobotomy Project.

and stained by the double impregnation technique. A transverse slice was removed from the brain immediately anterior to the bilateral lesion. (Fig. 1). Portions were cut from this slice as indicated in the diagram (Fig. 2), and frozen sections taken from each portion for silver staining. A few sections were also stained which had been taken from the visual cortex, area 18 of Brodmann.

EXPERIMENTAL RESULTS

The silver impregnation of Glees (1946) and the protargol
method of Stetler were found to be unsatisfactory in this laboratory
for our purposes. The method of Glees involved an unnecessarily long
procedure which is a modification of the shorter Gros-Bielschowsky tech-
nique. The neurofibrils stain quite black with some tendency for excess
precipitation of the silver. This method was tested on the human cortex
and no bouton terminaux could be found. The protargol procedure gave
rather spectacular ' low power ' results from a histological point of
view, producing a brilliant section in tones of gold and brown. However,
in our hands the procedure did not show neurofibrils, and moreover, no
terminal end-bulbs could be found. Another disadvantage to this method
is the use of protargol which is difficult to obtain.

Cajal's modification for frozen sections proved to be a rather
delicate and exacting method. The amount of pyridine used was found to
be very important, an excess usually resulted in the undue precipitation
of silver giving ' muddy ' preparations. However this is a good proced-
ure for demonstration of the larger neurofibrils and boutons in the cord.
The fibers and fibrils in the cortex were well shown, but no boutons
could be demonstrated. The background of the untoned sections also
stained fairly heavily so that tracing of the fibrils is more difficult.
The nuclei of the neuroglia pick up the stain fairly heavily. Much of
this unnecessary background is subdued by toning in gold. The fibers and
fibrils then appear more pronounced and are more easily traced. In the
cerebral cortex these conditions also hold true.

The Gros-Bielschowsky technique produced quite good results in the dog cortex. (Figs. 6 and 7) The nerve fibers take up the stain much more heavily with this stain so that even some of the larger dendrites appear black. Of several silver nitrate methods used successfully, this, in my hands, was the most capricious. Using identical times and conditions on consecutive sections and renewing the formalin, silver and ammoniacal silver each time, it was still impossible to predict the degree of impregantion. The very high concentration of silver nitrate used might have something to do with this.

The Double Impregnation technique was found to be the most dependable and satisfactroy. By carefully following the specified conditions and times, excellent preparations could be made almost without fail. In cell preparations of the human cerebral cortex it was possible to identify distinct, ring-like bouton terminaux. The fibers appear to be well impreganted but not to excess. In a few cases it was possible to distinguish the neurofibrils leading to the end-feet. (Figs. 9, 13 and 14).

The Lobotomized Brain. The results here are recorded in the numerical order of the portions as they are numbered on the diagram of the brain slice used. (Fig. 1 - 2).

Portion A. This is from the cingulate gyrus on the medial side of the cerebral hemisphere. Elongated torpedo-like swellings along many of the medium fibers. Distinct ring-like bouton terminaux, ranging in size from 1 micron to 3 microns in diameter, could be found in every field. On the average between three and five boutons could be found in every field. Many of these were adjacent to or apparently in contact with cells or dendrites. (Figs. 9 and 10).

Portion B. This is from the superior frontal gyrus. Torpedo-like swellings were again found in large numbers in the area. Many of

the fibers seem to have taken up the silver to a greater extent making them appear heavy and irregular. Boutons could be found in almost every field and often in contact with cell or dendrite. (Figs. 11 and 12).

Portions C and D. These are in the areas 8 and 9 of Brodmann anterior to the premotor area 6. The pattern of the previous section is carried out in these. The boutons could be found in all the layers below layer 1. The fibers show elongated swellings, and an increased take-up of silver. Many of the fibers also appear somewhat tortuous. (Fig. 13).

Portion E. Boutons on dendrites and cell bodies. Fibers show elongated swellings. (Fig. 14).

Portion F. Many large pyramidal cells. Boutons in almost every field. Fibers swollen at intervals and sometimes tortuous. (Figs. 15, 16 and 17).

Visual Area. Typical visual area showing line of Gennari. Boutons in this region appeared to be somewhat larger than general, ranging from 1 to 4 microns, in diameter. (Fig. 18).

DISCUSSION

The multitude of modifications of the reduced silver techniques of Cajal and Bielschowsky which have been published are as an hundred variations on a theme. In spite of the extensive literature on the silver impregnation of nerve fibers and tissues, nearly all silver methods can be classified into three basic techniques. All involve the adsorption and subsequent reduction of silver. In the early procedures of Cajal and of Bielschowsky, tissues were immersed in solutions of pure silver nitrate in varying concentrations. Del Rio Hortega immersed the already impregnated tissue in a carefully prepared solution of dilute silver carbonate before reduction. Finally Bodian used, as a source of the silver ion, a solution of silver albuminose produced by the Winthrop Chemical Company, called ' Protargol '. Each of these techniques in the laboratories of competent investigators, has produced excellent results.

The modification of the basic techniques have usually been introduced into one or more of three important stages in the staining of a piece of tissue. First, much emphasis has been attached to the tissue fixative used. It would be ridiculous to attempt to list the fixing solutions which have been suggested to prepare nervous tissues for impregnation with silver. The most widely used fixative, and that used for all tissues in this work, is neutral formalin. Formalin has the advantages of universal availability, cheapness, rapid penetration and minimal colour change. Most hospitals and mental institutions use neutral formalin for the preservation of autospy and biopsy material. It is therefore an additional advantage that silver techniques can be successfully applied to formol-fixed tissues. It is interesting that Spiegel-Adolf, Henny and Ashkenaz (1944) have pointed out that even X-ray diffraction studies re-

veal hardly any changes in the X-ray diffration pattern of formalin-fixed muscles, in marked contrast to results following the use of other fixatives.

The second stage at which modifications may be introduced is in the concentration of the silver solution. Concentrations recommended vary between 1% and 20% silver nitrate. Most authors suggest a 2% solution. This seems to be a sufficient concentration to give a rapid impregnation without resulting in too great a deposition of silver. Silver solutions of 10% and above have a tendency to encrust the nerve fibers, and, in addition , may cause much confusing precipitate.

Finally, the reducing solutions may be modified. However, once the silver has been deposited, then any standard reducing solution should be satisfactory provided the correct degree of impregnation has been attained.

There are few neurological methods which can be used to show nerve fibers and to trace nerve tracts. The Marchi method for myelin degeneration has been widely used and a great part of our anatomical knowledge of the pathways in the central nervous system has been provided by the Marchi technique. The method depends upon the observation that the products of myelin degeneration can be stained black with osmic acid, while staining of the normal myelin can be prevented by preliminary treatment with a chromic salt. It's failure to demonstrate degenerating non-myelinated fibers and degenerating finely myelinated fibers however, is a serious limitation of this technique. The tracing of such important pathways as the spinothalmic tract, many pathways projecting to and from the hypothalamus and the projection from the cortical suppressor areas to the basal ganglia are beyond the scope of the Marchi method. Many of the projections to and from the frontal areas are thought

to be unmyelinated fibers and therffore not demonstrable by the osmium

reaction. The myelin sheaths come to an end some distance from the

precise destination of the degenerated tract and cannot be demonstrated.

Thus, ins pite of its many uses and advantages, the Marchi method

alone cannot satisfy the needs of neuroanatomical investigations in

the frontal areas.

Recent investigations (Fiendel and Allison, 1948, Fiendel

Allison and Weddell, 1948) have shown that intravital methylene blue

can give a striking picture of nerve fiber degeneration following ex-

perimental lesion of the brain. Feindel, Allison and Weddell have

stained bouton ending in the lateral geniculate body. However, satis-

factory results have not yet been obtained using previously fixed ex-

perimental material. Therefore the method is not yet applicable to

the investigation of human brains obtained at autopsy. It should be

mentioned here that preliminary experiments in this laboratory using

methylene blue and other non-metallic stains for synapses have so far

met with little success.

It seems therefore that we must return to the silver impreg-

nation methods for the satisfactory demonstration of normal and de-

generating nerve fibers and endings.

This brings us to a discussion of the stainabilty of the bouton

endings in the cerebral cortex. They can be shown with varying success

in most other areas of the central and peripheral nervous system, as

has been mentioned previously. Only with great difficulty have these

fine endings been seen in the cortex and then they have been few and

far between. The failure of the end-feet to show themselves in this

area has led some investigators to believe that they donot represent

the normal method of nerve fiber termination. The statement of Meyer and Meyer (1945) that " While boutons-like structures around cortical nerve cells can be demonstrated by our present methods --------- it is obvious that they constitute merely a portion of the terminals " may well be reiterated here. But why should the character of the synapse be so different in the cerebral cortex ? Glees statement that " It seems that the synapse within the cortex is mainly represented by free terminals of the pericellular plexus " is too easy a rationalization. These pericellular fibers have been clearly demonstrated in this labor-atory. After careful study of many sections, however, no case of con-tact between these fibers and the cells could be seen except by means of bouton terminaux. The system of neurofibers and neurofibrils in the cerebral cortex is extremely complex. The processes of some nerve cells, such as those from the foot area of the cortex, have been shown to travel as far as three feet to reach their destination. The fact that a fiber, or groups of fibers, pass very close to a nerve cell en route to their destinations is no reason to believe that the fiber has any-thing whatsoever to do with that cell. Most theories on the nature of synaptic transmission would require the existance of some form of spec-ialized ending. Only with discrete endings such as boutons could one perform purposeful movements.

In this laboratory we have been able to see as many as five boutons incontact with one cortical cell. (Doctor Gibson tells me that he has seen as many as fifteen boutons in contact with one corti-cal cell.)

A solution to the problem of staining the endings in the cortex must be found. Why is it so difficult to stain boutons even though in many cases the nerve fibers right up to the synapse-stain easily ? It

was thought that acetylcholine might have something to do with the adsorption of silver on nerve fibers and especially in synapses. In this laboratory two dogs were fixed in an identical manner by perfusion with 10% formol. One of the dogs was perfused with a solution of eserine in physiological saline just before the fixation. Eserine is an anti-cholinesterase and its presence at the synapse would prevent the removal of acetylcholine by the cholinesterase. Thorough histological study of various areas in the spinal cord, cerebellum and cerebral cortex showed no difference in the stainability of the two nervous systems.

Perhaps the difficulty in staining boutons is a reflection of the rapid chemical change associated with death, which would be greater in the synapse because of the blood supply ? If this is so, would prevention of this chemical change by some means increase the staining ?

A positive approach to the problem must be made. Only after its solution can a thorough investigation of the frontal areas in the human cerebral cortex be made.

- SUMMARY -

A survey of several reduced silver techniques for the staining of nerve fibers and boutons terminaux in the cerebral cortex is reported. A double impregnation method which is a modification of Del Rio Hortega's silver nitrate - silver carbonate technique was found most satisfactory.

The method was used to study certain areas of the human frontal cerebral cortex and to demonstrate the boutons terminaux in these areas.

Evidence is sufficient to indicate that these boutons are the normal form of interneuronal synapse in the cerebral cortex of the human and further research on the staining problems in the nervous system is suggested.
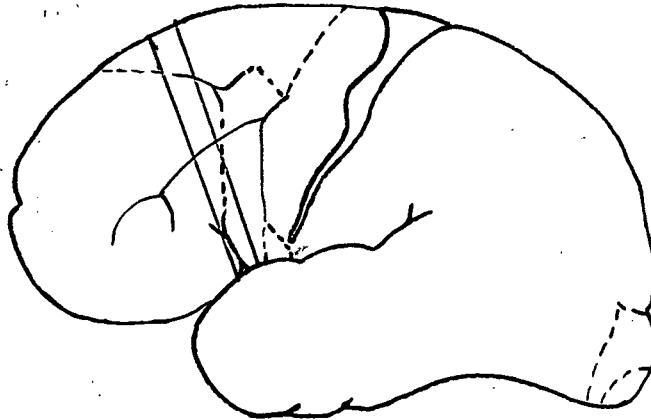
Fig. 1. Diagram of lateral view of brain to indicate the angle and region of lesion and the area from which the slice was taken.
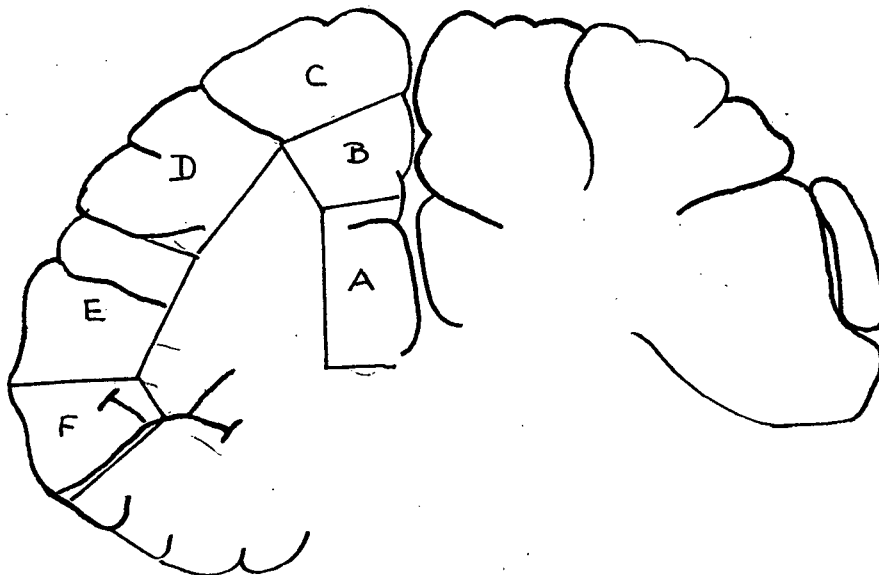
Fig. 2. Diagram of posterior face of slice indicating the portions mentioned in the text.

Figs. 3 and 4.

A few normal boutons terminaux on lateral

horn cell of the dog spinal cord. (arrows)

These two photographs were taken, at slightly

different focal levels, of the same cell to

indicate the difficulty in showing the number

of boutons to be found on a cell by means of

photographs. (mag. 1500 diameters)
Stained by Cajal's modification for frozen

sections and toned in gold.
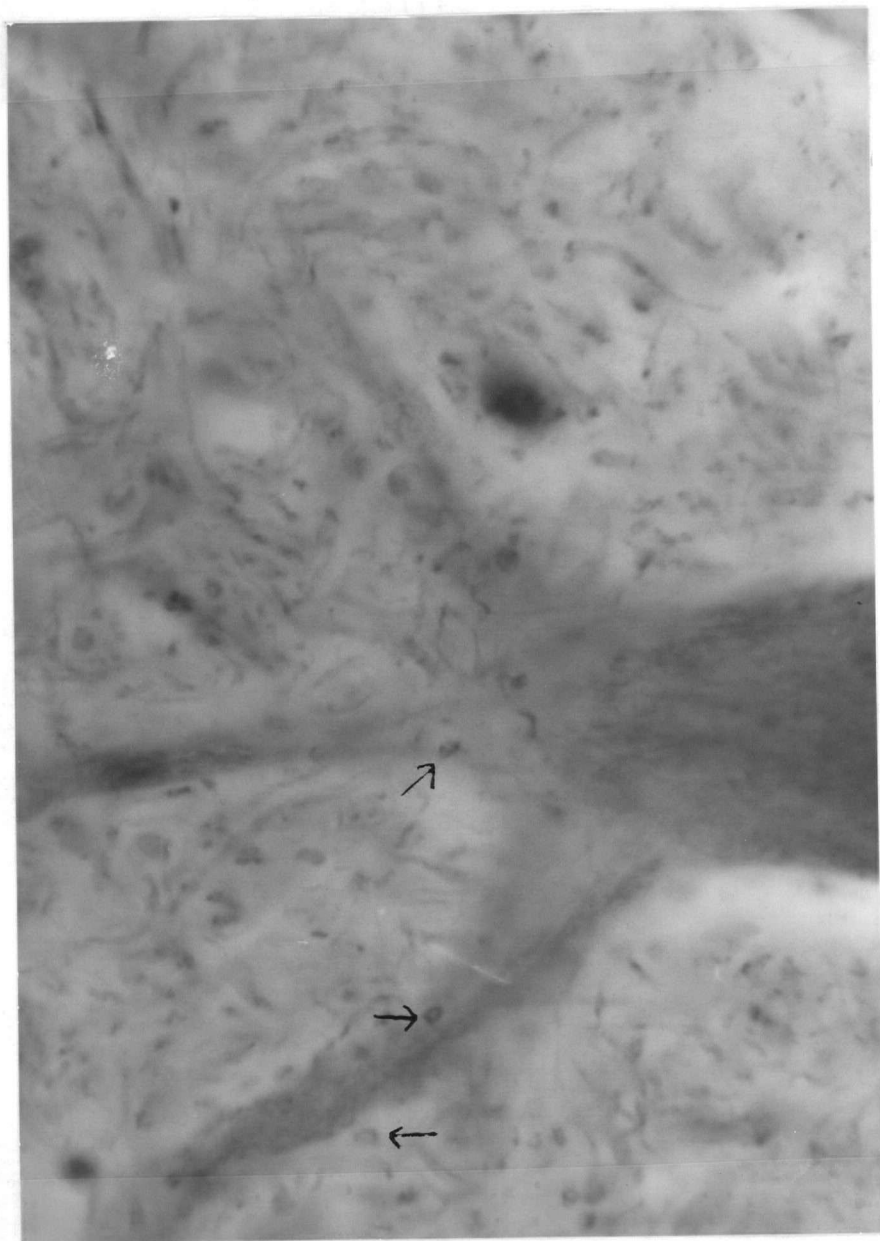
Figure 3
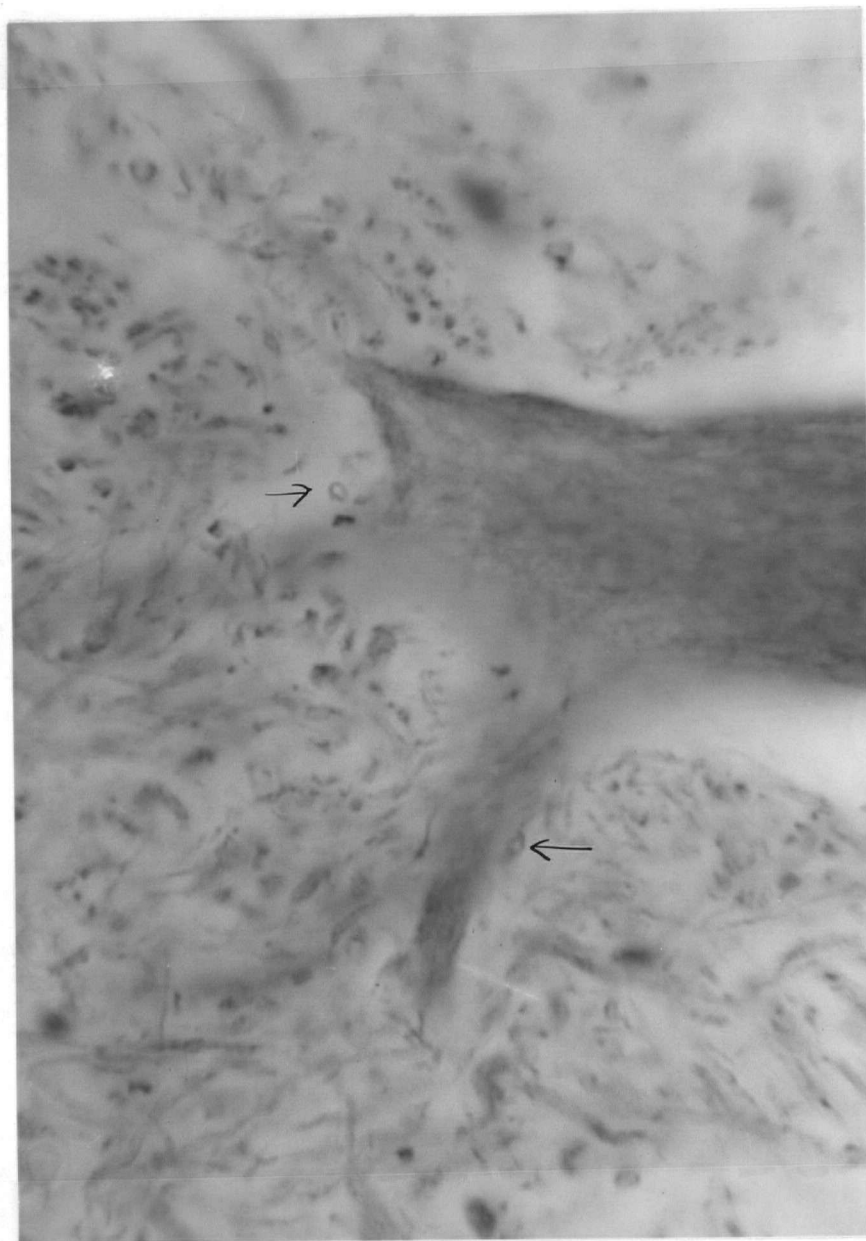
Figure 4

<u>Fig.</u> 5

Cell in the cerebral cortex of a dog.

Stained with Cajal's modification. Note

the differentiation of nuclei and fibers.
(mag. X 1500)
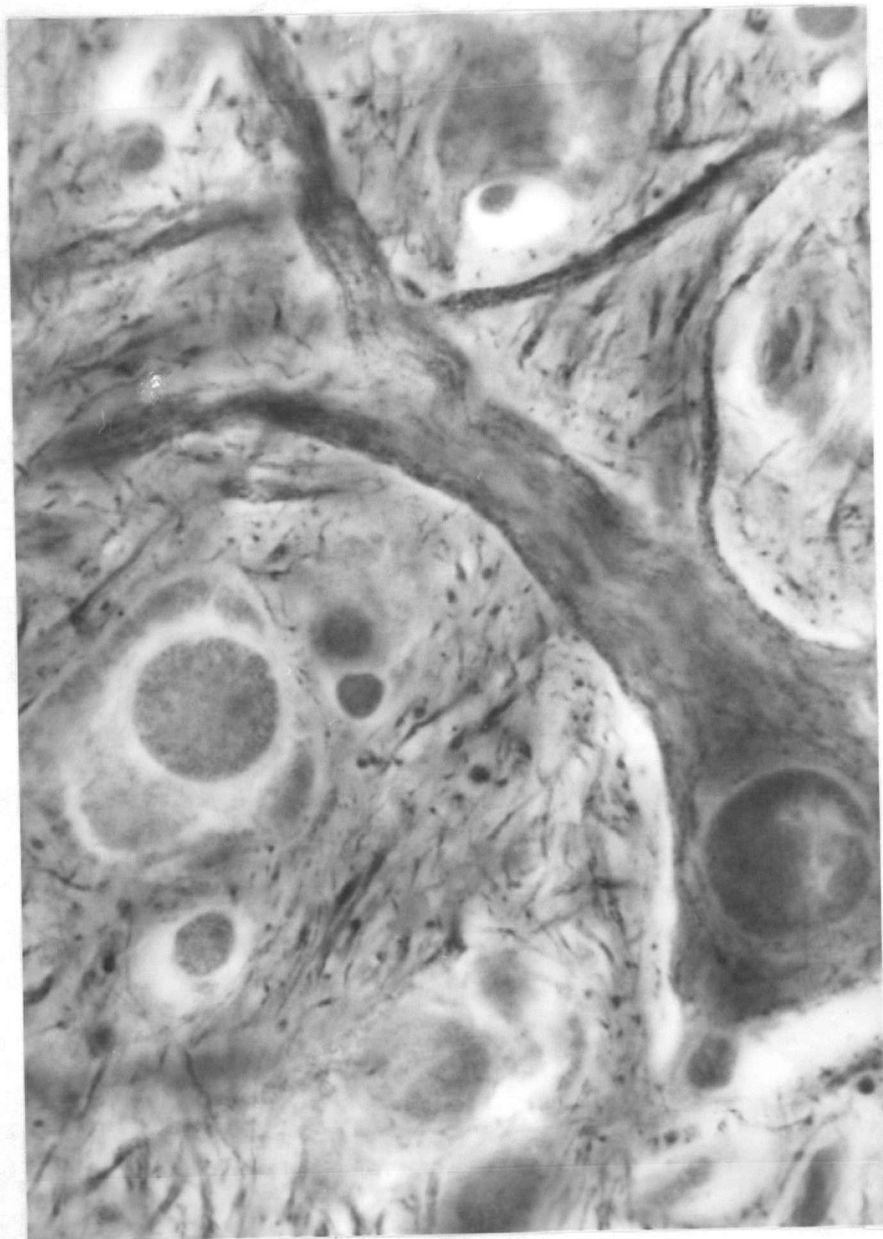
Figure 5

Figs. 6 and 7.

Cerebral cortex of dog stained by the Gros-
Bielschowsky technique. Note the apparently
degenerating bouton in fig. 7 and the large
darklyyimpregnated one in fig. 6. The fibers
in general are heavily impregnated with silver.
(mag. X 1500)

Figure 6
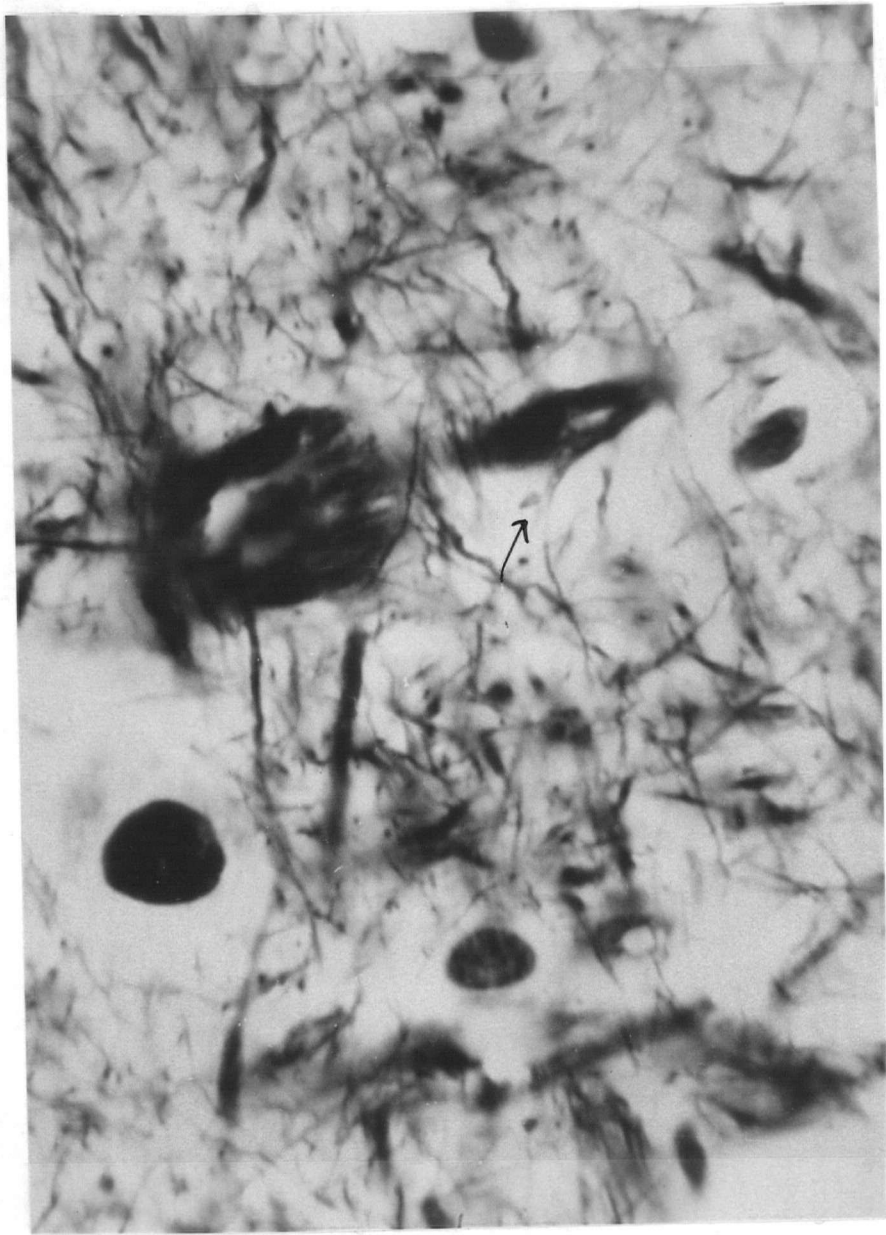
Figure 7

<u>Fig. 8.</u>

Motor area in cerebral cortex of a dog.

Stained by the double impregnation method,

and toned in gold. (mag. X 1500)
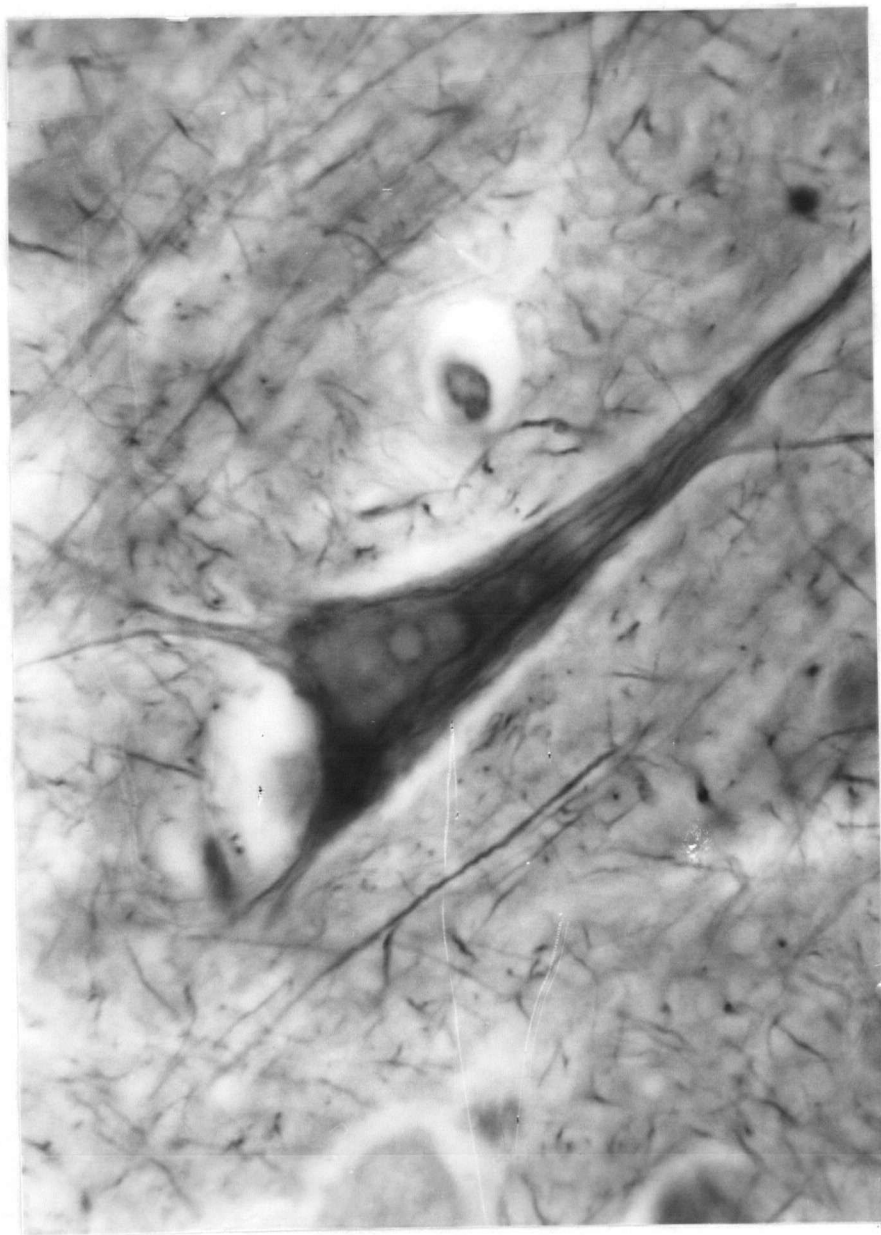
Figure 8

Fig. 9 and 10.

   Cortex on the cingulate gyrus. Stained by
   double impregnation method. Toned in gold.
   Boutons terminaux indicated by arrows.
   (mag. fig. 9 - X 3000, fig. 10 - X 1500)
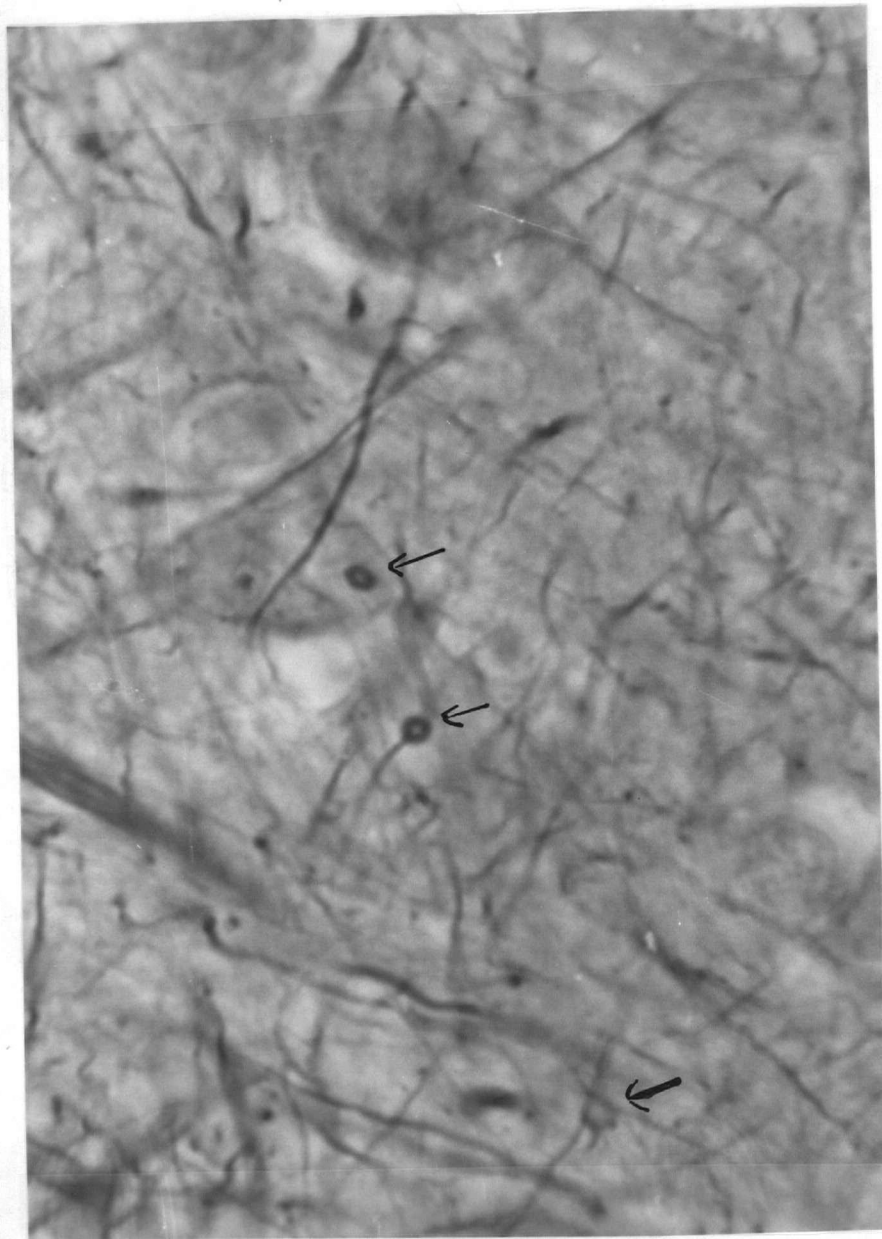
Figure 9
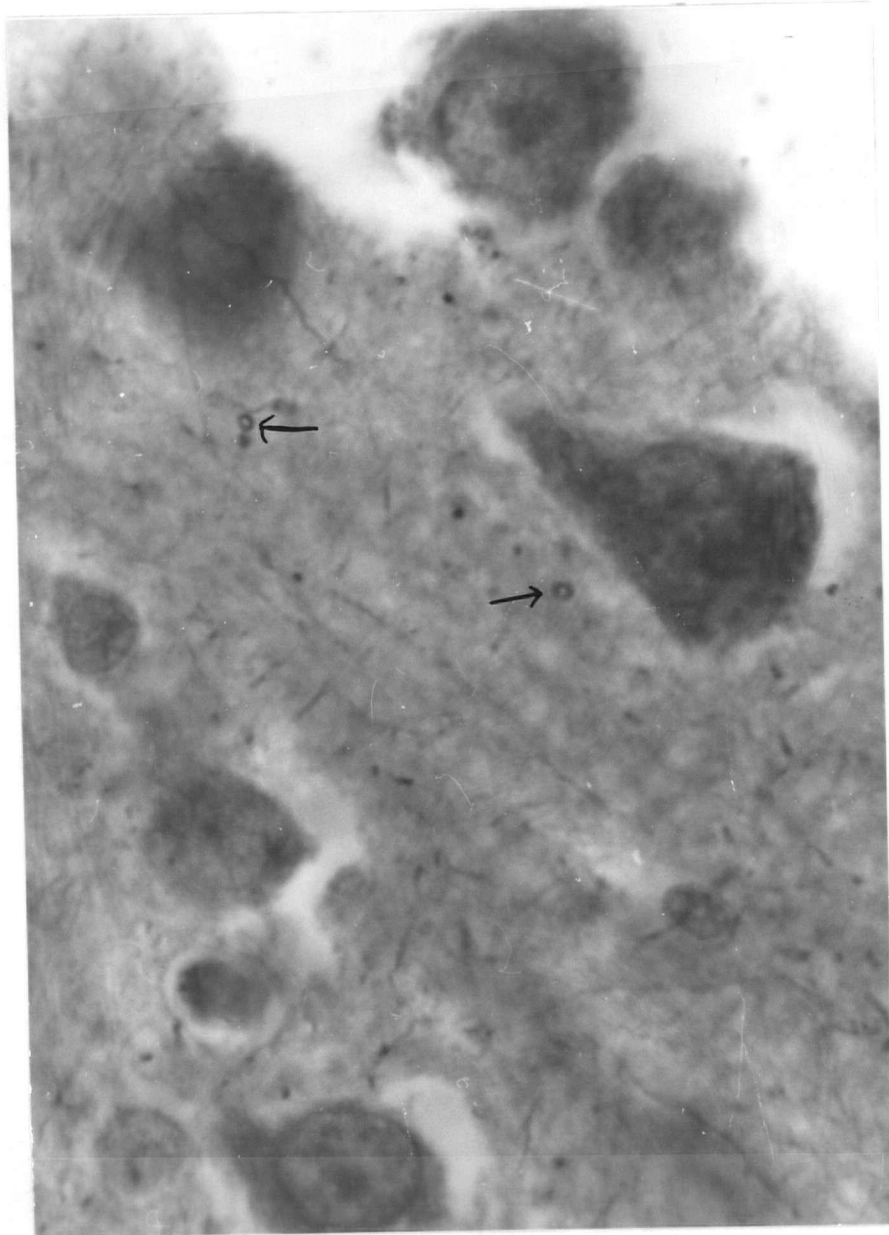
Figure 10

Fig. 11.

Human cortex from portion B (diagram, fig. 2)
Double impregnation. (mag. X 1500)

Fig. 12.

Human cortex from portion B. Dbl. Impr.
(mag. X 1500)

Fig. 13.

Human cortex from portion C
Dbl. Impr. (mag. X 1500)

Figure 11
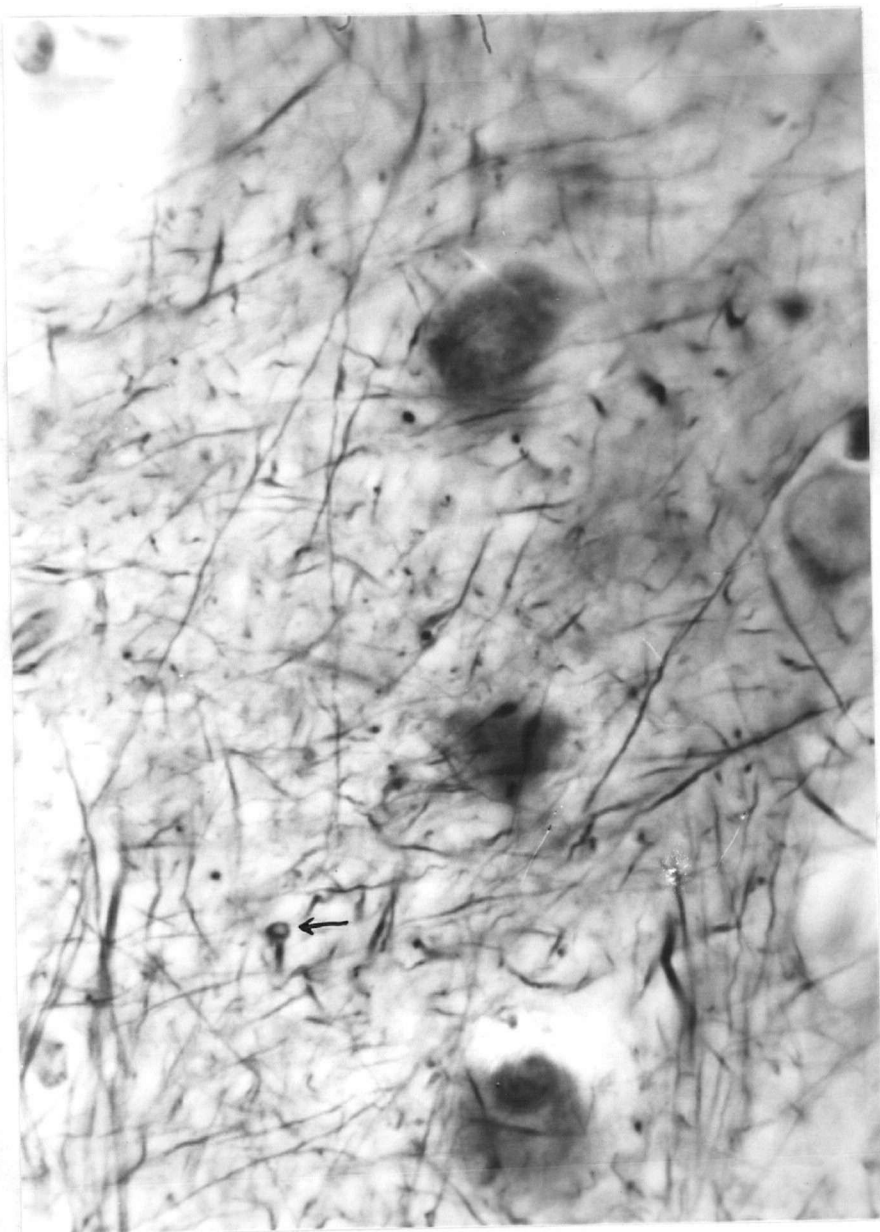
Figure 12
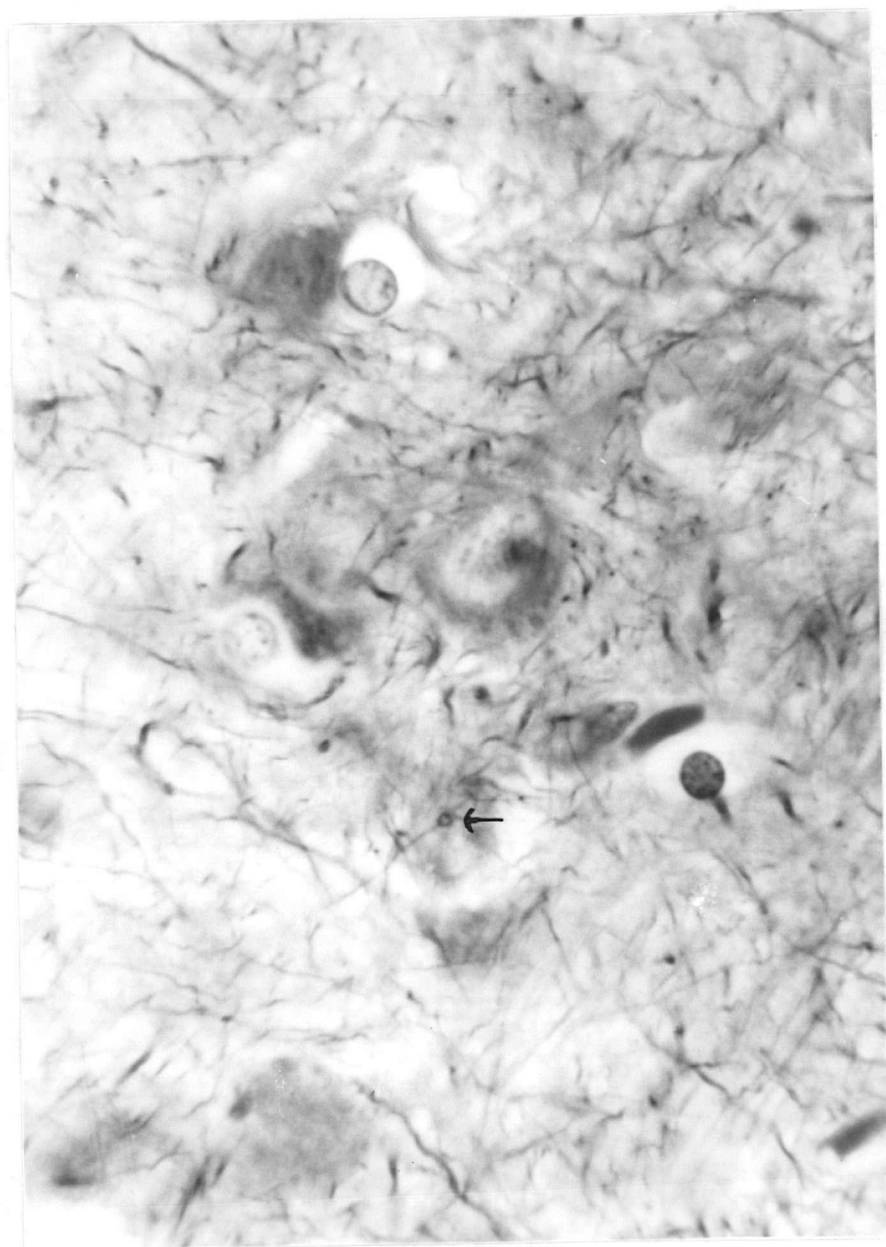
Figure 13

Fig. 14.

Human cortex from portion E. Dbl. Impr.

(mag. X 1500)
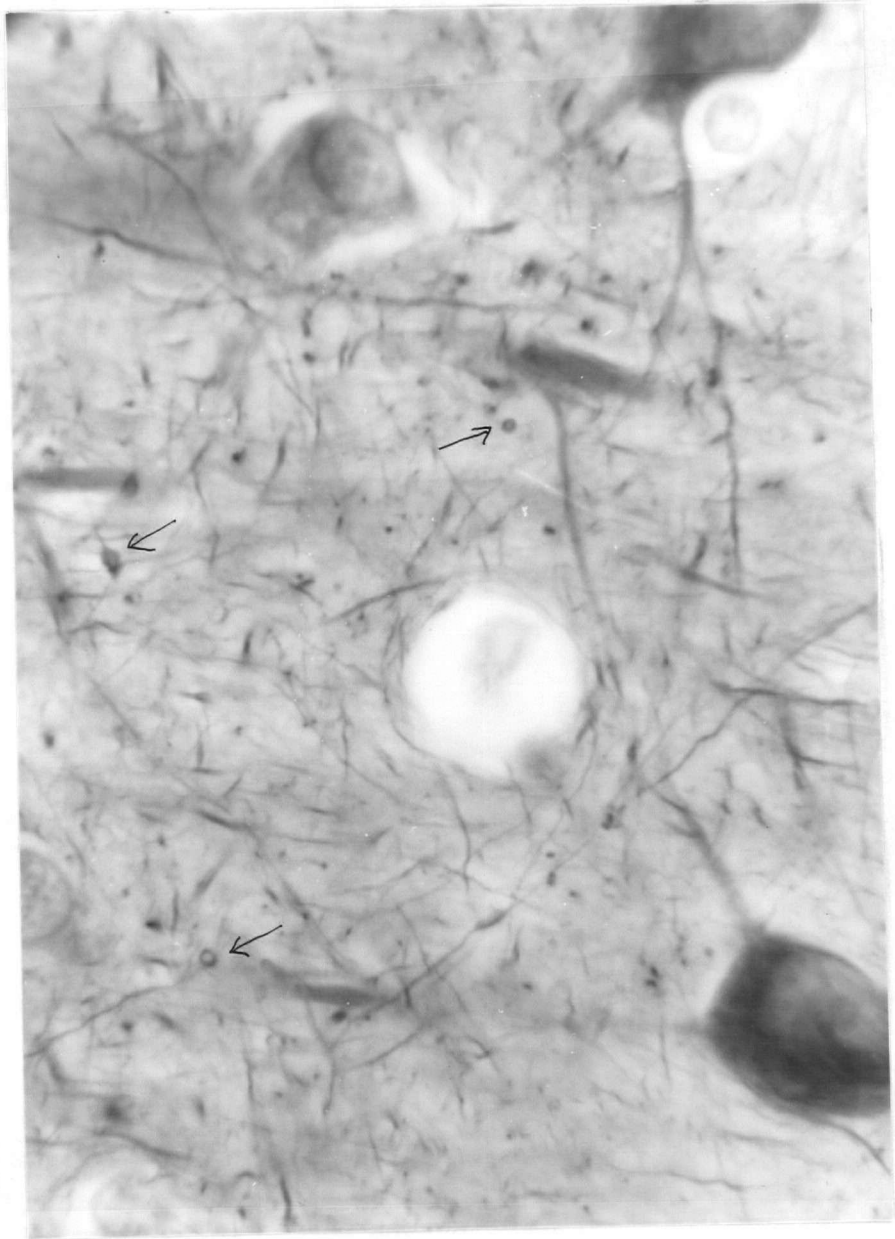
Figure 14

<u>Figs. 15, 16 and 17.</u>

Human cortex from portion F. Dbl Impr.

(mag. X 1500)
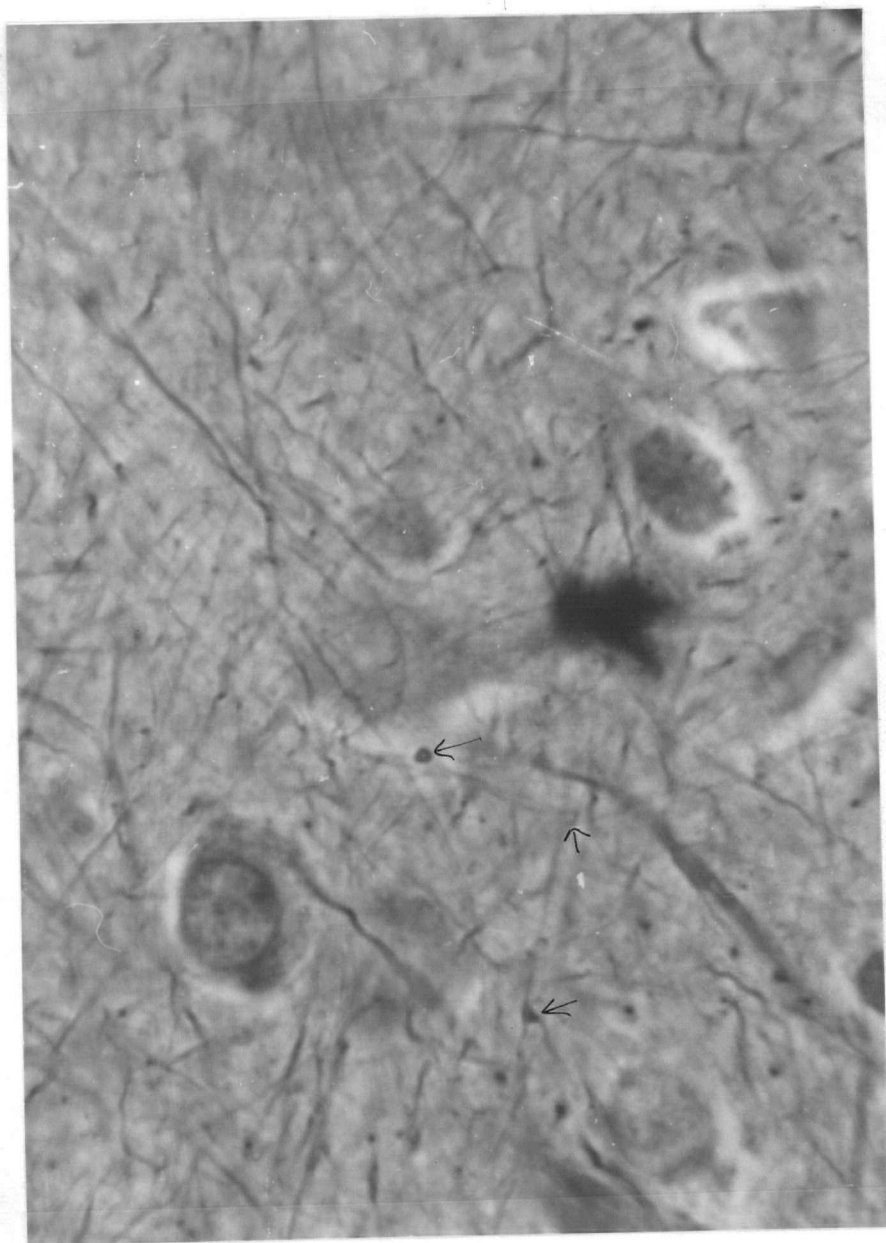
Figure 15
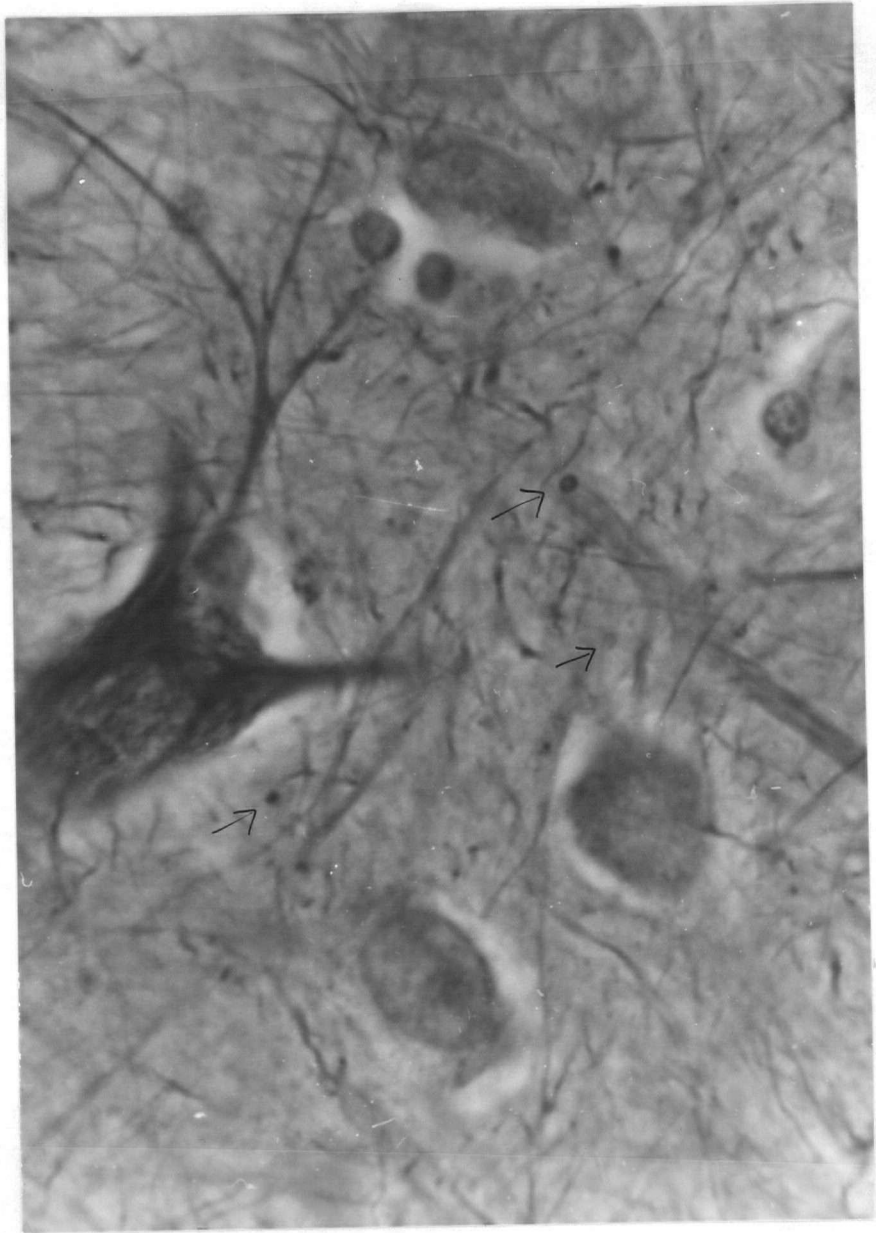
Figure 16
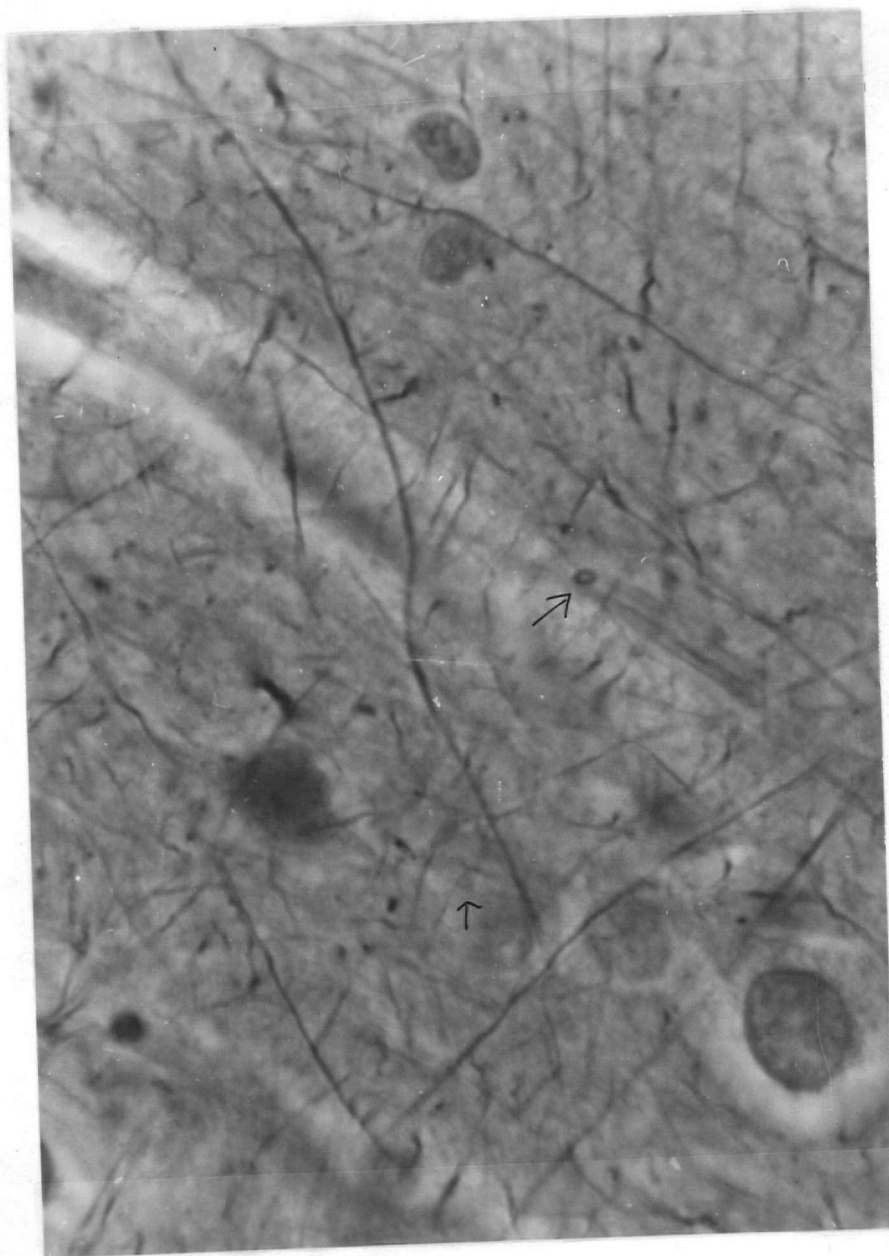
Figure 17

Fig. 18.

Human cortex from the visual cortex - area
18 of Brodmann. Dbl. Impr. (mag. X 1500)
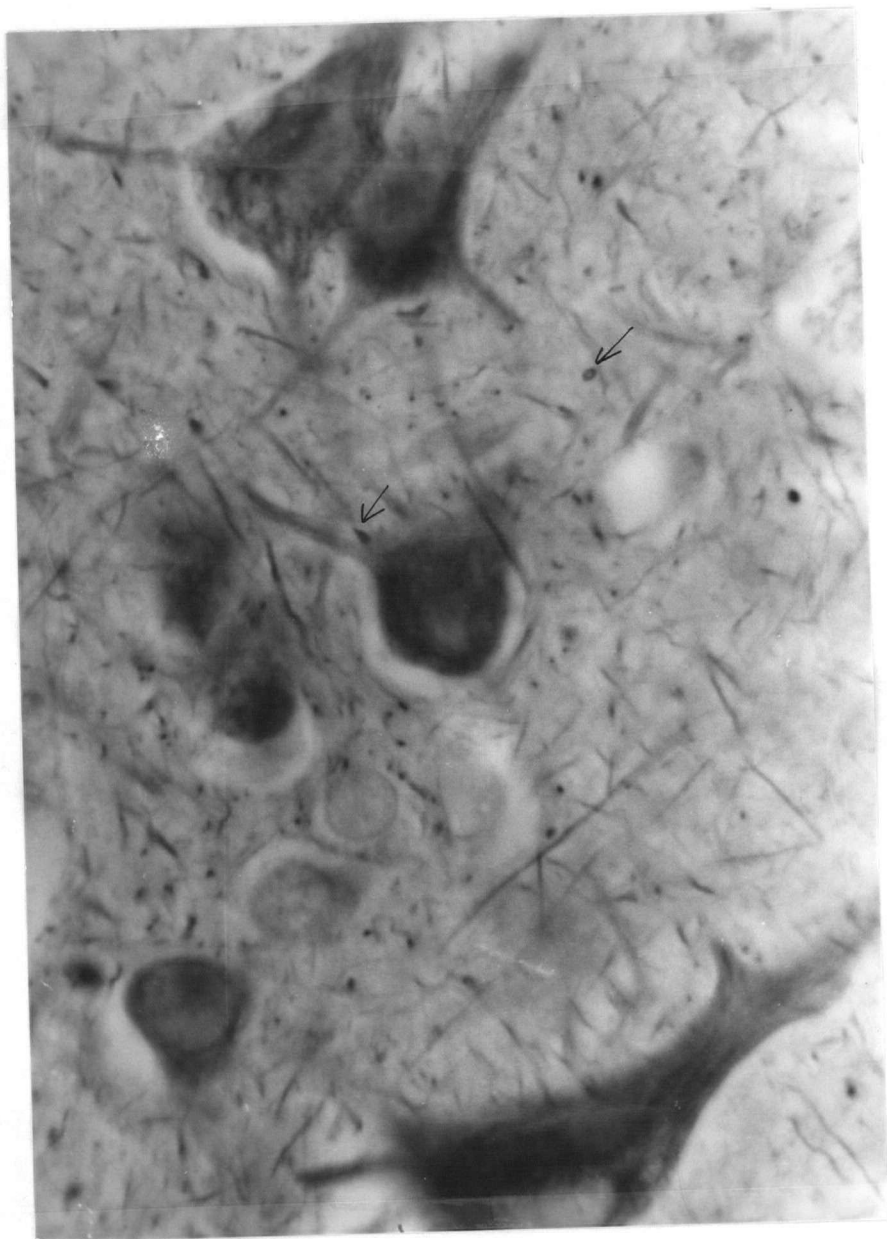
Figure 18

- APPENDIX -

A Cajal's Modification for Frozen Sections

(Gibson, 1950)

(1) Blocks of tissue, fixed for at least one week in 20 per cent formol, are cut on the freezing microtome at 12-15 microns.

(2) Wash the section well in water and place in a solution of 12 c.c. of 2 per cent silver nitrate, 6 c.c. of 96 per cent alcohol, and 5-10 drops of pure pyridine, for six to ten hours at 37° C., or twelve to twenty-four hours in the cold. The latter is desirable for the finest detail. Silver nitrate solution up to 5 per cent has been employed successfully in the cold.

(3) Transfer sections to a dish of 98 per cent alcohol where they may remain up to three minutes, depending on the depth of the silver impregnation.

(4) Reduce for three minutes in a solution of 0.3 gm. of hydroquinone, 70 c.c. of water, 20 c.c. of formol, and 15 c.c. pure acetone.

(5) Wash in water and tone in yellow gold chloride solution, 1 to 500, for five to ten minutes.

(6) Fix in 5 per cent hypo, wash, mount, dehydrate, balsam, etc.

B An Intensified Protargol Method for Paraffin Sections

(W.A. Stotler)

(1) Blocks of tissue, fixed in 15 per cent formalin, Bouin's solution or acetone, are imbedded in paraffin. Sections cut at 15 microns, paraffin removed, sections through alcohols to water.

(2) Impregnate 18 to 24 hours in 0.1 per cent aqueous solution of protargol. Place 5-10 gm. of copper (shot) in staining dish and dust 0.25 gm of protargol on 250 c.c. of water containing the sections. The addition of 4 drops of pyridine and 0.25 gm. of sodium glycerophosphate apparently improves the stain.

(3) Place sections without washing on a staining rack and flood with a few drops of the reducing solution. Follow the process of reduction and intensification under the microscope and when optimum, wash, dehydrate, and mount.

Prepare the unstable reducing solution immediately before use by combining the following solutions in order: Solution A, 6% AgNO3, 10 c.c.; Solution B, 20 gm. sodium sulphite in 330 c.c. water, 10 c.c. Solution C, 35 gm. sodium thiosulphate in 330 c.c. water, 10 c.c.; Solution D, 5 gm. sodium sulphite, 8 gm. Kodak Elon in 1000 c.c. water, 30 c.c.

- APPENDIX -

C Gros-Bielschowsky Method

(Gibson, 1950)

(1) Fix material in 12 per cent formalin neutralized with calcium carbonate, from one week to several years.

(2) Soak the block of tissue in water, and cut frozen sections from 20 to 50 microns into a dish of water containing a few drops of neutral formalin.

(3) Place sections in 20 per cent silver nitrate, one to five minutes.

(4) Arrange four capsules of 20 per cent neutral formalin in a row and lift one section only into the first. As soon as a white cloud forms at the edge of the section move to the next dish, and similarly into the third and fourth dishes. If clouds still appear use a fifth dish.

(5) Lift the section carefully with a glass needle, and touch it momentarily on a piece of clean filter paper to draw off the formalin. Place the section in a watch glass containing the ammoniacal silver solution (see below). From three to five drops of ammonia should now be added to the watch glass which is placed under an observation microscope to control the impregnation. A fairly rapid coloration is best, and when the nerve trunks in the section are seen to reach a dark, almost opaque brown-black color the section is transferred immediately to a solution containing 4 parts of ammonia to 5 of water, for five minutes.

(6) Wash until no odor of ammonia is detectable.

(7) Tone in a solution of 2 c.c. of 1 per cent gold chloride in 20 c.c. of water. This suffices for 40 sections if a drop of gold chloride is added periodically.

(8) Fix in 5 per cent hypo, dehydrate, and mount in balsam.

Ammoniacal Silver Solution - This must be prepared in small quantities as required by adding concentrated ammonia to 20 per cent silver nitrate solution until the precipitate just disappears, leaving a brown tinge throughout the solution. A glass rod should just be visible in this. A crystal clear is too ammoniacal.

Fresh formalin in the four reducing capsules, and fresh ammoniacal silver in the watch glass must be used for each section, regardless of its size. The watch glass is never washed. Its contents are renewed each time.

For boutons it is best to leave the section only a short time in the 20 per cent silvernitrate dish, and to use the minimum of ammonia in the watch glass stage.

- APPENDIX -

D Glees' Modification

(P. Glees, 1946)

(1) Fix material in 10 per cent formalin or 5 per cent for-
malin-saline for a period of at least 5 days up to 6 months.

(2) Cut frozen sections at a thickness of 15 to 20 microns.

(3) Place sections for 12 hours in a dish filled with 50 per
cent alcohol to which 6 drops of concentrated ammonia have been
added. (Use 6 drops of ammonia for every 50 c.c. alcohol and main-
tain at a constant temperature of 30° throughout.)

(4) Wash sections in distilled water.

(5) Place in 10 per cent silver nitrate solution for another
12 hours at room temperature.

(6) Wash sections three separate dishes filled with 10 per
cent formalin made up with tap water.

(7) Transfer sections into the following solution to be left
for 30 seconds - 3 parts 20 per cent silver nitrate, 2 parts 96 per
cent alcohol, then add concentrated ammonia intil the brown precip-
itate first formed has redissolved, and then add another 5 drops
of ammonia.

(8) Transfer into a 10 per cent formalin solution where sec-
tions are stained deep brown after 30-60 seconds.

(9) Wash briefly in distilled water.

(10) Place for 10 seconds in a 10 per cent hypo solution.

(11) Wash several times in distilled water and mount via
alcohol and creosote. The latter procedure makes the sections soft
and transparent. Any excess of creosote is removed by pressing the
section firmly under blotting paper.

- APPENDIX -

E   Double Impregnation Method for Neurofibrils

(Gibson, 1950)

        This technique was developed from a method first published by
Rio-Hortega in 1921. Is consists essentially of a preliminary imppeg-
nation with a solution of silver nitrate, followed by treatment with
silver carbonate.
        Fixation : Fix in small blocks or thin slices in 10 per cent
formol in distilled water. To this mat be added one drop of pyridine
or one drop of ammonia, per cubic centimeter of formol. The optimum
fixation period is 10 days at 37° C. or one month in the cold.

        (1)   Cut fixed material on the freezing microtome at 12-15
microns . Wash the sections well in a petri dish of water containing
10 drops of ammonia. Carry through two dishes of pure water.
        (2)   Place in a 12 c.c. pyrex glass cup containing a 2 per
cent solution of silver nitrate, to which 3-4 drops of pyridine have
been added.
        (3)   Heat gently for ten minutes at 45° C., whenthe gray matter
will become yellow.
        (4)   Wash very quickly and place in a similar pyrex dish con-
taining a 5 per cent solution of silver carbonate (sosa) with thrée
to four drops of pyridine.
        (5)   Heat gently for ten minutes at 45° C., after which the
tissue will take on a tobacco colour.
        (6)   Wash for 15 seconds.
        (7)   Reduce in 10 per cent formol.
        (8)   Tone in yellow gold chloride sãlution, 1 to 500, in the
cold for five minutes. Reinforce by heating the toning bath gently
for one minute. Wash quickly.
        (9)   Fix im a 5 per cent solution of hypo, dehydrate in 96
per cent alcohol, creosote, blot dry, balsam, etc.

        For material fixed in F.A.B., or for refractory material
fixed in formol for several months:
        (1)   Cut sections into water containing ten drops of ammonia.
        (2)   Heat in a pyres cup containing 10 c.c. of 96 per cent
alcohol and 10 drops of ammonia, for ten minutes at 45 °6.
        (3)   Wash well in three dishes of water.
        Employ the double impregnation technique as above, reducing
in 1 per cent formol without washing after the silver carbonate treat-
ment
        Five per cent silver carbonate (sosa)

Solution of 10 per cent silver nitrate   .... 67 c.c.
Solution of sodium carbonate 5 per cent ....267 c.c.
Water .... to1000 c.c.
        Add ammonia drop by drop while shaking the solution until the
precipitate just dissloves. Filter and store in a dark bottle.

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