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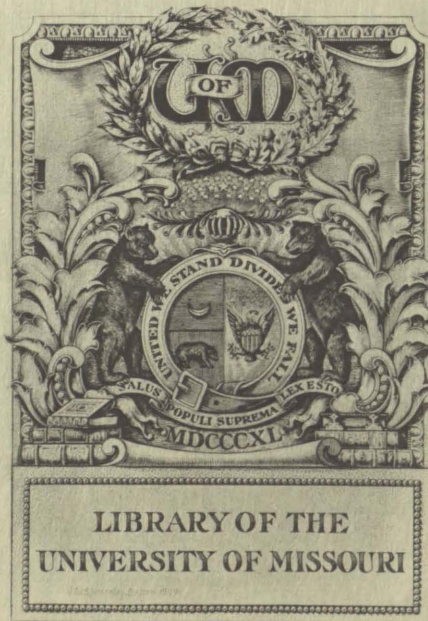
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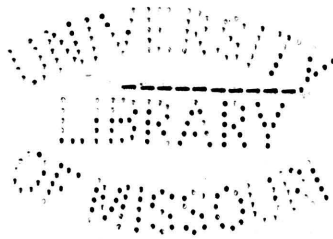
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THE PRESENCE OF NISSL'S BODIES AND NEUROFIBRILLAE  
IN THE FRESHLY-FIXED SPINAL NERVE CELL

by

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" "



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The Presence of Nissl's Bodies and Neurofibrillae  
in the Freshly-Fixed Spinal Nerve Cell.

Synopsis :-

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Review of Recent Literature.

Material and Technique.

Source of material and method of killing.

Fixation and staining of smear preparations, taken and treated under varying conditions.

Observations.

Presence and nature of Nissl's bodies and neurofibrillae in spinal nerve cells taken less than a minute after death.

Alterations in Nissl's bodies and neurofibrillae under varying conditions of technique.

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### Introduction.

Since the discovery of Nissl's bodies and neurofibrillae much work has been done on these structures. Nevertheless, some have even questioned their presence in the living nerve cell and have considered them as artefacts. Möllgaard (10), especially, in a recent article strongly doubts the existence of both in the "vitaly-fixed" nerve cell. Thus it has seemed desirable to do some further work in this direction.

This investigation was carried out in the Anatomical Laboratory, University of Missouri, and under the supervision of Dr. C. M. Jackson.

### Review of Recent Literature.

Möllgaard (10) emphasizes the fact that to exclude the greatest number of artefacts a tissue should, before its study, undergo as little preparation as possible. He remarks, that the very simplest of methods is demanded, and, that with simpler technique comes ease in making controls. If one would succeed, he says, in studying the finest structural relationships of the living cell, one must, firstly, be prepared to study a definite physiological condition of the cell. Secondly, since a physiological function is progressive, one must, in order to study this condition, be prepared to interrupt that function at any given point. He would interrupt the functional state and immediately preserve the tissue in that vital condition until such time as would be convenient to continue the technique. The physiological processes of the central nervous system proceed with great rapidity and post-mortem changes enter in like manner, so for a physiological-histological investigation he regards our general neurocytological technique as unsuitable.

Möllgaard's paramount aim is therefore the elimination of post-mortem changes. To attain this end he takes the tissue from the living animal and quickly drops it into a freezing mixture in which it is at once frozen. Since at the low temperature (ca.  $-40^{\circ}\text{C}$ ) of the freezing mixture all chemical reactions are interrupted, Möllgaard maintains that the tissue is preserved in the same vital condition in which it was when committed to the fluid. The next step rests upon the fact that the central nervous system has, at a temperature of about  $-20^{\circ}\text{C}$ , a consistency like that of paraffin and may be cut into sections of 5 to 10  $\mu$  in thickness without previous fixation or imbedding. The sections as fast as made are consigned to a fixative of similar temperature which at once fixes them and so permanently preserves the sections in a "vitaly-fixed" condition.



More in detail, Möllgaard's technique is briefly as follows :- From the living animal (rats, dogs, etc.) upon which, with the aid of an anesthetic, he has previously performed a craniotomy or a laminectomy, he excises a small piece of brain or cord tissue, and immediately drops the same into a freezing mixture, consisting of carbon tetrachloride, xylol, and absolute alcohol. The temperature of this mixture has been reduced to about  $-40^{\circ}\text{C}$  by the addition of carbon dioxide snow. The animal is then killed, generally by cutting its throat, and pieces of the brain or spinal cord taken and placed in the freezing mixture at various intervals after death for the purpose of later comparison. After a sojourn of varying length in the freezing mixture, the pieces of tissue are removed to a fixing fluid, generally 96% alcohol, of practically the same temperature (ca.  $-40^{\circ}\text{C}$ ) where they are then allowed to acquire a temperature of about  $-35^{\circ}$  to  $-15^{\circ}\text{C}$ , according to the desired thickness of the subsequent sections. At the appropriate temperature sections are cut with a specially constructed microtome into the cold fixative. The sections are thus fixed at once and at a low temperature. The fixative with the contained sections is now allowed gradually to acquire room temperature, after which the sections are studied in the unstained condition, or stained with toluidin-blue or nileblue-base.

After a study of the sections, obtained and prepared according to the foregoing technique, Möllgaard concludes :-

Firstly, concerning the Nissl's bodies :

1. "Nissl-Körner existieren nicht in vital fixierten Nervenzellen..
2. "Nissl-Körner sind Kunstprodukte, hervorgerufen während der Alkoholfixation..

He notes however that it is not the alcohol alone that produces the Nissl's bodies, for by his method of "vital fixation" he excludes a factor which is present in the usual Nissl's method, namely, post-mortem change. Since he has observed that the first result of post-mortem change is the production of a meshwork\* in the unstained protoplasm, and a later result that of an aggregated or conglomerated meshwork ("zusammengeballtes Maschenwerk.") in the stained protoplasm, he holds that the Nissl's bodies are due to the simultaneous action of post-mortem change and alcohol

\* This, however, seems to be practically identical with the "glia-network" (to be described later) which Möllgaard finds in his "vitaly-fixed" nerve cell, and which he considers normal. Regarding this and some other points his various statements seem vague and inconsistent.



fixation. He regards the post-mortem changes as acid in character, stating that : "Die Nissl-Körner sind darum als Kunstprodukte anzusehen, und zwar als solche, die durch eine Verbindung der säuren postmortalen Spaltung und der langsamen Alkoholfixation entstanden sind."

Although he finds no Nissl's bodies, Möllgaard observes in the "vitaly-fixed" and stained nerve cell a network, which he interprets as a glia-network, and whose richness depends on the time after death the preparation is made. In the cells of a piece of tissue taken from the living animal and "vitaly-fixed" and stained he finds a network consisting at most of three to four coarse meshes, while in cells of preparations made 10 to 12 minutes after death the network reaches its greatest development. In "vitaly-fixed" tissue the cell protoplasm lying between the meshes of this network remains wholly unstained with toluidin-blue for some time after the tissue is taken, and only becomes stainable  $1\frac{1}{2}$  to 4 hours after death, during which time the reaction of the brain substance has become increasingly acid. As the time after death increases the cellular protoplasm within the meshes of the networks of the cells becomes more and more stainable. But at about 15 to 20 hours post-mortem the network itself, on the other hand, begins to dis<sup>in</sup>tegrate and forms peculiar aggregated masses. The significance of these masses will be mentioned later.

The technique may be varied as follows : A small piece of nerve tissue is taken 7 minutes after death, is frozen, and placed in 96% alcohol at room temperature and left for  $1\frac{1}{2}$  to 2 hours. Then it is cut and fixed in alcohol at  $-20^{\circ}\text{C}$ , and finally stained with toluidin-blue. With this treatment Möllgaard says that the network begins to shrink and dis<sup>in</sup>tegrate. If the tissue is left in the alcohol at room temperature for about 12 hours before being cut and stained he finds an additional structure present, namely, granulations. These granulations, in his own words : "gleichem ... vollständig den Nissl-Körnern." The networks too, of the cells in which the granulations are present, he finds, on close examination, to be composed of a series of closely placed granules. Hence Möllgaard believes that in tissue taken at various intervals after death the networks of the "vitaly-fixed" nerve cells gradually degenerate into the peculiar masses and granulations mentioned above, which are apparently Nissl's bodies. To quote again, he says : "Wir haben gezeigt oder jedenfalls im höchsten Grade wahrscheinlich gemacht, dass die Nissl-Körner von diesen Netzen gebildet werden." The origin and nature of the network discussed now becomes Möllgaard's principal question. For the purposes of the present paper however it is not necessary to follow him farther in his attempts to explain its presence. But it might be added that his final conclusion



regarding its nature is that it is a glia-network, which is both inter-cellular and intracellular.

Secondly, regarding the neurofibrillae, Möllgaard's conclusion is evident from the following quotations : "Ich habe nun gleichzeitig an vital alkoholfixierten Schnitten die allgemeinen Fibrillmethoden versucht, doch überall mit negativem Ergebnis. ... Auf Grund dieser Versuche darf ich nicht wagen, die Existenz der Neurofibrillen zu verneinen. Dazu scheint mir, dass ich die Verhältnisse vorläufig zu wenig untersucht habe. Doch kommt mir unleugbar vor, dass die ebenerwähnten negativen Ergebnisse recht stark gegen die Existenz der Neurofibrillen sprechen. ... Falls dagegen Neurofibrillen Kunstprodukte gleichwie Nissl-Körner sind, so würden diese vermutlich ganz dasselbe Schicksal wie die Nissl-Körner erleiden, nämlich dass sie erscheinen, wenn man langsam fixiert und die postmortalen Prozesse verlaufen lässt, während sie nicht vorkommen, wenn die postmortalen Prozesse ausgeschlossen sind, d. h., wenn man das Gewebe vital fixiert.. It may be further noted that he remarks : "Die Zellen lassen sich überhaupt sehr schwierig imprägnieren." He is of course speaking of cells "vitaly-fixed". In speaking of the neurofibrillae he also says that one cannot deny that the appearances which the "vitaly-fixed" nerve cells present lie nearest to the reality; and so one can maintain with considerable certainty that what is not present in the "vitaly-fixed" nerve cell is likewise not present in the living cell.

Retzius (12) strongly criticizes Möllgaard for adopting a method which in all its essentials is like that which he and Axel Key discarded 37 years ago. He says that Möllgaard without doubt started out with the right object in view, namely, to secure a simple method for the study of the central nervous system, realizing that our present methods demand too much preparation before the tissue is ready for study. But of all the methods for the study of the central nervous system, Retzius holds the freezing method to be one of the poorest. Of this fact he says he convinced himself in the year 1874, when he carried out a large number of experiments in this connection, some of which may be briefly mentioned. Under the microscope he followed the very formation of the network which Möllgaard describes at such great length. He likewise examined the freezing process in all its phases for a large series of tissues and fluids (blood, glue, egg-albumen, etc.) and arrived at the certain conviction that this "vital" method is very treacherous for scientific purposes and should be strongly condemned. From his studies Retzius concludes that the network observed in the frozen preparations is due to a system of clefts filled with crystals of ice.





He says that if to the frozen preparations a fixing fluid (alcohol, osmic acid, etc.) is applied, the system of clefts is preserved in all detail; but, if, on the contrary, the preparations are permitted to thaw out without previous fixation, the water reappears anew in the entire mass, and only here and there a small cleft is left behind. From a consideration of the entire phenomenon it is evident to him that the water contained in the tissue or the fluid mass at the moment of the freezing passes out of the parenchyma and collects itself in the passages and lacunae which then appear in the frozen condition filled with ice. The water apparently collects where it meets with the least resistance. The foregoing results and others arrived at by Retzius together with Key were published by them in the Swedish language in 1874 and in the German language in 1882, so they should have been quite accessible to Möllgaard. Retzius in brief affirms that Möllgaard's glia-network is an artefact due to the method of "vital fixation"; but further than this he does not criticize Möllgaard's ideas and results.

Möllgaard (11) in replying to Retzius' criticism, says that he very much regrets that the articles by Retzius and Key so long ago escaped his notice, for on duplicating some of their experiments he is forced to acknowledge that his glia-network is wholly an artefact. He however entertains the hope that the method may still be utilized in studies dealing with physiological and pathological changes in nerve tissue.

From the foregoing it will be observed that it may still be considered questionable :

- (1), whether the Nissl's bodies and neurofibrillae are present in the freshly-fixed nerve cell ;
- (2), whether freezing the fresh nerve tissue at a low temperature so changes the cytological structure that neither Nissl's bodies nor neurofibrillae can be demonstrated ;
- (3), whether the Nissl's bodies and neurofibrillae are due to post-mortem changes ; and,
- (4), the relation of Möllgaard's reticulum to the Nissl's substance.

The observations made in the present study will afford at least a partial answer to these questions.



## Material and Technique.

With one exception, the tissue studied was taken from the spinal cord of the dog. The exception was that of a horse, <sup>killed</sup> by the Department of Veterinary Medicine of the University. The number of dogs used was eight. They were obtained as a rule the day before they were to be killed, from the city pound or from individual owners. Such public source of course means that the dogs were of varied breed and description. Care however was taken that only healthy, well-nourished, active dogs were used. Likewise much care was exercised in avoiding exciting or in any way injuring the animal before it was killed. It was sought in general to use dogs of medium size but this was not always possible. The weight of the animals used varied between 10 to 19.7 kilos.

Regarding the method of killing, the technique should produce the least possible change in the nerve cell and at the same time permit of taking the tissue in a minimal length of time. This excludes the use of anaesthetics, narcotics, and even hypnotics, as well as death by shooting, hanging, or stabbing. The method finally decided upon was one of decapitation with a double-guillotine, thus removing a segment of the neck including the spinal cord. The apparatus used was composed of two large butcher's cleavers, heavily weighted, and so bolted together that they could be adjusted according to the length of segment of the cord desired. The necessary accessories to the method consist of: a block sufficiently firm to receive the impact of the blow of the decapitating apparatus without jolting, and thus permitting of obtaining the segment desired at one blow; and a strongly made animal-board that can be closely and carefully adjusted to the foregoing block. The animal-board is provided with straps permitting of fast<sup>en</sup>ing the dog upon it quickly and conveniently. The dog's neck, by means of a strap and collar, is stretched out upon the block. For recording the time of decapitation and the subsequent taking of the tissue a stop-watch was used.

In taking the tissue it is of course essential that the assistants be fully instructed as to the particular place they fill, and that everything is in absolute readiness. For the decapitation is practically instantaneous and thereafter the tissue is immediately at the disposal of the investigator. The segment of the animal's neck obtained by the decapitation contains a portion of the spinal cord varying from the first to the fifth cervical segments according to the adjustment of the apparatus and as to the particular place on the



neck where the blow happens to fall. This neck segment is quickly deprived of the greater part of its muscle, etc., by a large heavy knife. This then leaves the vertebra, or vertebrae, enclosing the cord, which is now removed by a long slender knife passed around it between its meninges and the wall of the canal. The grey substance of the isolated cord segment is now hastily laid bare by paring away the white substance. From the exposed grey substance (anterior horns) smears are made on glass slides which are dropped into Coplin jars containing the fixing fluids. When the foregoing was carefully and successfully carried out it was found that the smears could be placed in the fixing fluids 42 seconds after the moment of execution.

Fixation was carried out both with and without freezing; i. e., in the one case the jar containing the fixing fluid was surrounded by a freezing mixture while in the other case it was not. In general, 96% alcohol was used as a fixative for Nissl's bodies, while the fluid (24 parts absolute alcohol, and 1 part ammonia) recommended by London (8) was used as a fixative for the neurofibrillae. For comparison and checking of results on the Nissl's bodies, Ohlmacher's fluid and formol-corrosive were used in a few cases. For a like purpose 12% formol was used as an additional fixative for the neurofibrillae in a few instances. The length of time the smear preparations were left in the fixatives varied in the case of the Nissl's bodies from  $1\frac{1}{2}$  hours to 3 days, and for the neurofibrillae from 5 hours to 4 days.

Fixation with freezing, to be more explicit, was carried out as follows: A medium-sized vessel (capacity approximately 1 gallon) containing 95% alcohol was placed within a larger container. In the smaller vessel with the alcohol were placed small Coplin jars containing 96% alcohol, London's fluid, and 10 or 12 glass slides respectively. The larger container was now filled with ice and salt which was carefully packed about the smaller vessel. When the temperature of the alcohol and the fixatives had been reduced to  $-3^{\circ}$  or  $-10^{\circ}$  C, carbon-dioxide snow was added to the 95% alcohol in the smaller of the containers until the temperature was further reduced to  $-20^{\circ}$  or  $-25^{\circ}$  C. At this time the dog was immediately killed and smears made on the cold slides upon which they froze instantly. Then they were quickly dropped into the cold fixatives. These smears were left in the fixatives for a time varying as above noted. When the time was prolonged the whole of course acquired room temperature in the meantime.



The staining methods used are comparatively simple. For the staining of the Nissl's bodies the method outlined by Dolley (3) was used. The method is in the main as follows: The smears, fixed in 96% alcohol, are brought through a series of alcohols of decreasing strength to distilled water, then stained with warm (ca. 40°C) erythrosin for three minutes, well washed in water. They are then brought into a 1% aqueous solution of toluidin-blue for five to eight minutes, again well washed in water, after which they are dipped in 95% alcohol and differentiated, until the Nissl's bodies and nuclear structures are clearly defined, in a mixture of 96% alcohol 9 parts, anilin oil 1 part. The differentiation is stopped by bringing the slide into absolute alcohol from which it is brought into xylol and mounted in Canada-balsam.

To stain the neurofibrillae, London's method was followed. The method is essentially the following: After fixation, the smears are brought into a 1½% aqueous solution of silver nitrate for 3 to 7 days, after which time they are treated with a solution of pyrogallol<sup>acid</sup>, 2 grams, formal 5 cc. and distilled water 100 cc., for 24 hours. The smears are then placed in a 1% aqueous solution of gold chloride for 5 to 10 minutes, brought into a 5% aqueous solution of sodium hyposulfite for 10 minutes, carried through distilled water and a series of alcohols of increasing strength to xylol and mounted in Canada-balsam.

The foregoing methods were used almost exclusively. Variations were made only occasionally and in fact so rarely that they need not be mentioned, excepting that some of the smears were stained for Nissl's bodies without any previous fixation, and that in staining for neurofibrillae a few control preparations were made according to Legendre's (7) modification of Bielschowsky's method.

It is thought unnecessary to describe the various magnifications employed in the study of the smears. An ordinary Leitz oil immersion lens was in all cases quite sufficient to determine the points in question.





### Observations.

The observations on Nissl's bodies may be noted under two headings: First, as to their presence in the unfrozen nerve cell, and second, in the frozen.

In smears not subjected to freezing, but fixed in alcohol at ordinary room temperature, the Nissl's bodies are present, clearly and definitely defined, in cells 42 seconds after decapitation (Fig. I). They were also found present at all intervals thereafter up to 24 hours after death, after which time no further observations were made. Similarly Nissl's bodies were found present in the cells of smears of spinal grey matter of the horse, fixed in alcohol three minutes after the instant of the shooting of the animal. And again, the Nissl's bodies are present, distinctly defined, in the nerve cells of smears placed, without fixation, in the toluidin-blue stain  $1\frac{3}{4}$  minutes after decapitation. Not only are the Nissl's bodies distinctly present in the foregoing preparations, but also their <sup>form,</sup> and their distribution in the cell-body and the dendrites is clearly evident. In some of the best smears, and with good magnification, it is even possible to see that the Nissl's bodies are composed of granules which lie imbedded in a matrix, the "gerinnselartige Masse," of Held (5). In Nissl's bodies in the periphery of a well differentiated cell this matrix appears of slightly purplish hue while the Nissl's granules are a deep blue. These last observations on the matrix are in agreement with those of Held (5 & 3) and Becker (1).

For the frozen cell however the picture is quite different. In smears frozen and fixed in alcohol 53 seconds after decapitation the cell-body on staining presents a striking network which stains deeply with toluidin-blue (Figs. II, III, & IV). It shows a considerable variety of forms. Furthermore, these various forms seem to be transitions between a distinct network on the one hand, and more or less well-defined Nissl's bodies on the other. Then too, the transition forms, and in fact the entire system of networks, seem very much dependent upon the conditions of the technique, such as, different degrees of cold, thickness of the smear, and rapidity of fixation. And although the network has no very constant characteristics it seems more or less dependent too on the size and type of the cell. The networks were found in cells frozen at various intervals after death up to 20 minutes. After this time no further preparations were



made, for it seemed quite evident that the networks were in some way related to the process of freezing rather than to the length of time after death. To determine whether the networks could be produced in cells that had first been fixed, some smears were made approximately 7 minutes after death, fixed in 96% alcohol at room temperature for one hour, and then subjected to a temperature of -15 C. At this temperature (which was possibly not low enough) no networks could however be observed in the cells. The Nissl's bodies had remained unchanged. The significance of the network described above, and its relation to Möllgaard's reticulum or "glia-network" will be discussed later.

The observations on the neurofibrillae, like those on the Nissl's bodies, may be considered under two headings; namely, their presence in the unfrozen and in the frozen cells. The neurofibrillae are unquestionably present in the unfrozen cells of smears fixed 42 seconds after decapitation, and likewise in cells taken at various intervals up to 24 hours after death, after which time no further observations were made. It may also be noted that they were found to be present in the cells of spinal grey matter smears of the horse, fixed 3 minutes after the animal was shot. Although the perinuclear network and other finer details of the endocellular neurofibrillar structure of the nerve cell (which may be seen in thin sections) are not visible as such in smear preparations, the neurofibrillae are distinctly seen at the origin of the cell processes and an endocellular neurofibrillar network of varying richness is more or less faintly discernible in the cell-body (Fig. V).

In the frozen cell-body it is somewhat questionable whether the neurofibrillae are actually demonstrable. It would seem that in the processes and where these leave the cell that they can, though faint, be made out in cells fixed 55 seconds after decapitation. They were also found at various intervals afterwards up to 20 minutes, after which time the observations were discontinued. In the frozen cells stained for neurofibrillae a network may be observed, but it does not resemble the typical neurofibrillae, but rather, seems to be a product of the freezing. It is possible that the freezing may impair the capacity of the neurofibrillae for impregnation with silver nitrate or otherwise cause their failure to stain. On the other hand, it may be noted that even in control (unfrozen) preparations the stain is somewhat capricious, and further observations are necessary to decide this point definitely.



### Discussion.

It was one of Möllgaard's primary objects to produce a method which would be simpler than our present neurocytological methods. His procedure however is not so strikingly simple as he would have us believe. In the first place, the production of a temperature as low as  $-40^{\circ}\text{C}$  and the necessity for maintaining it for a definite length of time requires a difficult and complicated technique. Again, sectioning the tissue at a temperature of  $-20^{\circ}$  to  $-15^{\circ}\text{C}$  at best, and that with a specially constructed microtome is not easy. Finally, his method requires the preparation of the animal, its anaesthetization, the performance of a craniotomy or a laminectomy, and the waiting for the effects of the anaesthetic and the shock of the operation to disappear. In connection with the latter features, it may be well to mention that Dolley (4) has conclusively shown that the effects of the anaesthetic and the shock of the operation do not disappear in so short a time (7 to 3 hours) as Möllgaard assumes. So that for various reasons, in addition to that urged by Retzius, Möllgaard's technique is by no means ideal.

It was in order to avoid the foregoing objections that the technique previously described was decided upon for the present investigation. The decapitation avoids all preliminary preparation of the animal, and likewise excludes the effects of an anaesthetic and the prolonged shock of the operation. The apparatus for decapitation permits one to obtain practically instantaneously a segment of the animal's neck, after which the isolation of the cord, with a little experience, requires but a few seconds. By resorting to smear preparations, the freezing process, which the sectioning of an unbedded tissue requires, is avoided. The smear preparations have the further advantage of being fixed the very instant they are consigned to the fixative. Thus it is evident that the entire procedure, namely, the taking of the tissue, putting the tissue in a form suitable for study, and the fixation, all occur in less than a minute of time. It should be noted that the cumbersome freezing method of Möllgaard is designed merely to preserve the tissue structure in the "vital condition" until thin sections can be made upon which the fixative can act. The smear method accomplishes this far more easily and quickly, and eliminates the production of artefacts by the freezing. It is of course freely granted that the smear preparations do not show the minute details of structure as



readily as do thin sections. Nevertheless, for the points in question the smear preparations are amply sufficient. Regarding the endeavor, made by the writer, to duplicate, in its essentials at least, Möllgaard's freezing procedure, for purposes of comparison, it may be well to note that, since the technique used was less elaborate and complete, the lowest temperature employed was  $-25^{\circ}\text{C}$ . There may be some question as to whether a temperature of  $-40^{\circ}\text{C}$  would not produce changes in the nerve cell which are not present at  $-25^{\circ}\text{C}$ . In all essential respects however, the results obtained at  $-25^{\circ}\text{C}$  are very similar to those described by Möllgaard at  $-40^{\circ}\text{C}$ .

In smear preparations not subjected to the freezing process the Nissl's bodies and the neurofibrillae are unquestionably present 42 seconds after decapitation. It has<sup>been</sup> stated that the smear preparations do not permit of the detailed study that sections do. It is therefore unnecessary to discuss the various details of observation noted under this heading. It is particularly the time element with which we are here concerned. Möllgaard found no Nissl's bodies or neurofibrillae in freshly-frozen and fixed nerve cells. He considers at least seven minutes post-mortem change, followed by several hours of slow alcohol fixation, necessary to produce the Nissl's bodies even imperfectly. But the results submitted in the present paper prove that in unfrozen smear preparations (which have the advantage of excluding the production of artefacts by freezing and allow immediate fixation) both the Nissl's bodies and the neurofibrillae are found in tissue fixed less than one minute after decapitation, while the cells are still practically in a living condition. Hence Möllgaard's contention that Nissl's bodies are produced by post-mortem change and slow fixation is totally wrong. The freezing is responsible for his misleading results.

Whether the sojourn in the fixative be comparatively long or short seems to have no noticeable influence on the Nissl's bodies. In smears taken at various intervals after death the Nissl's bodies show no noticeable change till about 12 to 13 hours post-mortem, when they gradually begin to disintegrate. To determine whether the alcohol fixation could in any way be responsible for the presence of the Nissl's bodies in the freshly-fixed cell, some smears, instead of being dropped into the fixative, were immediately consigned to the toluidin-blue stain, after which they were carefully washed, mounted in water, and studied. Such smears show the Nissl's bodies





to be undeniably present. They show distinctly, but it seems that they are somewhat more granular and possibly a little more diffuse than those of cells fixed in alcohol. In this connection it may be mentioned that Dogiel (2) in 1896 stained the Nissl's bodies in unfixed nerve cells, with dilute solution of methylene-blue in warm physiological salt solution 5 to 10 minutes after death.

Regarding the alterations which freezing produces in the smear preparations, it may be stated that the network observed after staining, in the nerve cells subjected to a temperature of  $-20$  to  $-25^{\circ}\text{C}$ , seems to be definitely related to the Nissl's bodies. This relation is oftentimes quite apparent from the number of transition forms which occur in the cells of a single smear. These transition forms seem to ~~to~~ depend upon, and vary according to, the size of the nerve cell, the thickness of the smear at the point where the cell occurs, on the intensity of the freezing, and the rapidity of fixation. In some cells frozen at  $-10^{\circ}$  to  $-15^{\circ}\text{C}$ , and stained, the Nissl's bodies show no noticeable change while <sup>in</sup> other cells, though still preserving their shape more or less, they seem to be peculiarly vacuolated as if they were gradually being broken up by a system of spaces of some kind. In still other cells, generally those subjected to a temperature of  $-20^{\circ}$  to  $-25^{\circ}\text{C}$ , and afterwards stained, the Nissl's bodies no longer show any of the preceding forms but some few cells show a system of minute networks, as it were, more or less loosely joined together (Fig. II). Other cells show what would seem to be the most extreme form, namely, a network, whose meshes are of various sizes, which extends quite uniformly throughout the entire cell (Figs. III & IV). These transition forms do not correspond to those which Möllgaard regards as being dependent upon the time after death that the cells undergo the "vital fixation". In the present investigation no such transition forms as Möllgaard figures and describes were observed. From less than a minute to 20 minutes after death all the cells of smears subjected to a temperature of  $-15^{\circ}$  to  $-25^{\circ}\text{C}$ , and afterwards stained, showed networks that answered in some way or another the description already given, but none, for example, showed the 3 or 4 coarsely meshed network to which Möllgaard calls attention.

From the observations made upon these networks it seems evident that they are counterparts of, and in fact are derived from, the substance constituting the Nissl's bodies: and, that they are identical with Möllgaard's reticulum or "glia-network" there can be no question.

Just how the Nissl's bodies are transformed into these networks,



and just what changes they undergo during the transformation is beyond the scope of the present paper. But whatever the solution of these questions may be, it is plainly evident that the formation of these networks is brought about by the freezing.

Regarding the difficulty, or the failure, of demonstrating the neurofibrillae in the frozen nerve cell, it may further be remarked, as Legendre (7), Marinesco (9), and many others, have noted, that the reliability of our present neurofibrillar methods is not sufficient to admit of constant results. So it is entirely possible that in case of the present investigation, in which the observations upon this particular point are comparatively few, that the failure is due to the inconstant results of the method used. And in close relation to this inconstancy lies the possibility that the proper conditions necessary for the success of the method in dealing with frozen tissues may not have been secured. Likewise the smears may require a modification of the technique ordinarily used for sections.

#### Conclusions.

From the observations presented and the discussion made the following conclusions may be drawn :-

1. With a simplified smear method, both the Nissl's bodies and the neurofibrillae are found present in the spinal nerve cells of the dog fixed 42 seconds after decapitation. There is no evidence that they are artefacts due to post-mortem changes.
2. In spinal nerve cells frozen and fixed 53 seconds (or later) after decapitation, a peculiar, variable network is found, which stains deeply with toluidin-blue.
3. This network is identical with the reticulum or "glia-network" described by Möllgaard. Although Möllgaard originally held that the Nissl's bodies are artefacts due to post-mortem changes in this network, the converse is nearer the truth ; for the network is an artefact produced from the Nissl's substance by the process of freezing.



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### Explanation of Figures.\*

All the cells drawn are anterior horn cells of the spinal cord of the dog. With the exception of Figure V, all are from smears fixed in 96% alcohol and stained with erythrosin and toluidin-blue. The individual differences will be noted in connection with the separate legend for each figure.

#### Plate I

Figure I. Magnification 460 diameters. Fixed  $42\frac{3}{5}$  seconds after decapitation. The Nissl's bodies are distinctly present, even showing the "gerinnselartige Masse," of Held in which their individual granules lie imbedded.

Figure II. Magnification 1000 diameters. Frozen and fixed at about  $-25^{\circ}\text{C}$ , 74 seconds after decapitation. The figure represents one of the early transition forms, the Nissl's bodies appearing as minute networks loosely joined together.

Figure III. Magnification 600 diameters. Frozen and fixed at about  $-25^{\circ}\text{C}$ , 1 minute and 43 seconds after decapitation. This is a more advanced stage than figure II, but there are still evident traces of the Nissl's bodies from which the network was derived. The nucleus is faintly discernible.

#### Plate II

Figure IV. Magnification 875 diameters. Frozen and fixed at about  $-25^{\circ}\text{C}$ , 1 minute and 43 seconds after decapitation. From the same smear as figure III. This represents one of the extreme forms in which practically all evidence of the former Nissl's bodies is lost. The nucleus is represented by the area having but few meshes. The nucleolus is much distorted.

Figure V. Magnification 1000 diameters. Fixed in London's fluid  $53\frac{3}{5}$  seconds after decapitation. Stained for neurofibrillae (which show distinctly) according to London's method.

\* For figures II and III, I am indebted to Mr. George T. Kline, Biological artist of the University.



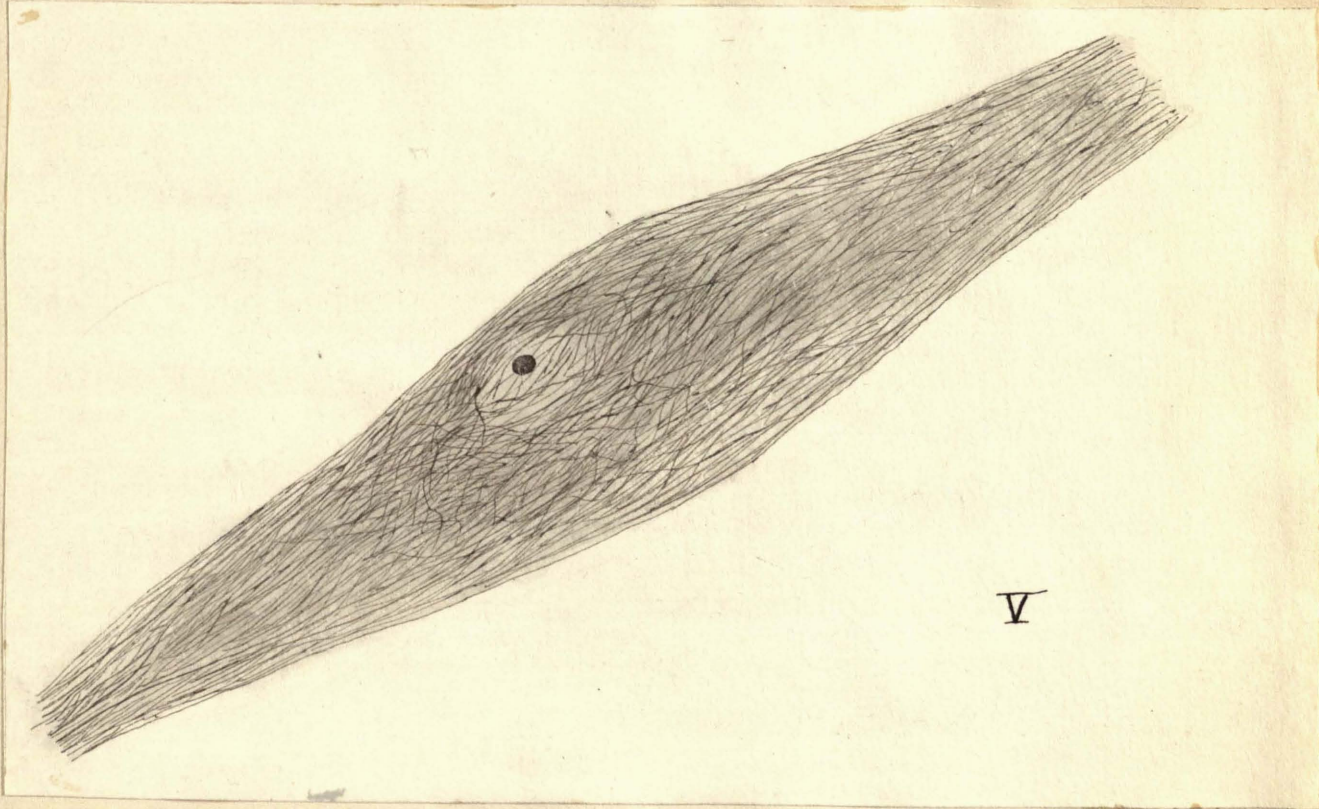
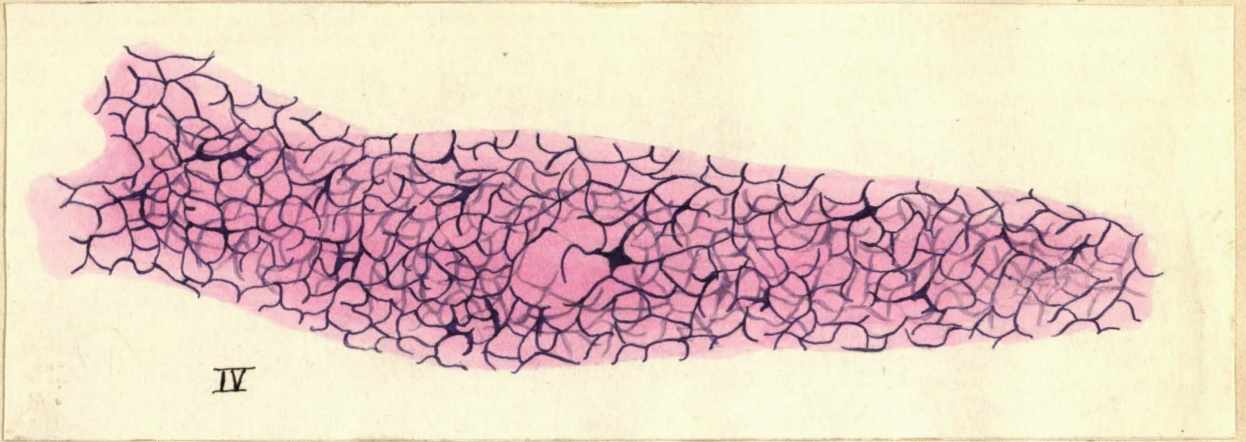


Plate I





Plate II





# Möllgaard's Reticulum

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## MÖLLGAARD'S RETICULUM

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SIX FIGURES

Since the appearance of Möllgaard's article: "Die Vitale Fixation des Zentralnervensystems," in the Anatomische Hefte (Bd. 43, July, 1911) much comment has been made upon his method and his conclusions. Considerable difference of opinion still seems to exist however regarding his results, so it has been thought profitable to do some further work in this direction. Prominent among Möllgaard's conclusions is the fact that he strongly doubts the existence of Nissl's bodies and neurofibrillae in the 'vitaly-fixed' nerve cell. Thus, one of the objects of the present paper will be to determine whether or not Nissl's bodies do exist in tissue so fixed, and a few observations incidentally made on neurofibrillae will be included. An endeavor has also been made to ascertain how and why Möllgaard's 'glia-network' in neural tissue is formed during the process of freezing.

This investigation was undertaken on suggestion of Dr. C. M. Jackson by whose criticism and advice the author has greatly profited and for which aid he here expresses his indebtedness.

### REVIEW OF RECENT LITERATURE

Möllgaard ('11 a) emphasizes the fact that to exclude the greatest number of artefacts a tissue should, before its study, undergo as little preparation as possible. He remarks, that the very simplest of methods is demanded; and, that with simpler technique comes ease in making controls. If one would succeed, he says, in studying the finest structural relationships of the living cell, one must, firstly, be prepared to study a definite physio-

logical function of the cell. Secondly, since a physiological function is progressive, one must, in order to study this condition, be prepared to interrupt that function at any given point. He would interrupt the functional state and immediately preserve the tissue in that vital condition until such time as would be convenient to continue the technique. The physiological processes of the central nervous system proceed with great rapidity and post-mortem changes enter in like manner, so for a physiological-histological investigation he regards our general neuro-cytological technique as unsuitable.

Möllgaard's paramount aim is, therefore, the elimination of post-mortem changes. To attain this end he takes the tissue from the living animal and quickly drops it into a freezing mixture in which it is at once frozen. Since at the low temperature (ca.  $-40^{\circ}\text{C}.$ ) of the freezing mixture practically all chemical reactions are almost completely interrupted, Möllgaard maintains that the tissue is preserved in the same vital condition in which it was when committed to the fluid. The next step rests upon the fact that the central nervous system has, at a temperature of about  $-20^{\circ}\text{C}.$ , a consistency like that of paraffin and may be cut into sections of 5 to  $10\mu$  in thickness without previous fixation or embedding. The sections as fast as made are consigned to a fixative of similar temperature which at once fixes them and so permanently preserves the sections in a 'vitality-fixed' condition.

More in detail, Möllgaard's technique is briefly as follows: From the living animal (rats, dogs, etc.) upon which with the aid of an anaesthetic, he has previously performed a craniotomy or a laminectomy, he excises a small piece of brain or cord tissue, and immediately drops the same into a freezing mixture, consisting of carbon tetrachloride, xylol, and absolute alcohol. The temperature of this mixture has been reduced to about  $-40^{\circ}\text{C}.$  by the addition of carbon dioxide snow. The animal is then killed, generally by cutting its throat, and pieces of the brain or spinal cord taken and placed in the freezing mixture at various intervals after death for the purpose of later comparison. After a sojourn of varying length in the freezing mixture, the



pieces of tissue are fastened to the object carrier of the microtome. The 'inner vessel of the calorimeter' containing the fixing fluid, generally 96 per cent alcohol, which by the addition of carbon dioxide snow has been reduced to a temperature of  $-15^{\circ}$  to  $-35^{\circ}\text{C}$ ., according to the desired thickness of the subsequent sections, is now brought up about the tissue block. At the appropriate temperature sections are cut with the specially constructed microtome into the cold fixative. The sections are thus fixed at once and at a low temperature. The fixative with the contained sections is now allowed gradually to acquire room temperature, after which the sections are studied in the unstained condition, or stained with toluidin-blue or Nile-blue base.

After a study of the sections, obtained and prepared according to the foregoing technique, Möllgaard concludes:

*Firstly*, concerning the Nissl's bodies:

1. "Nissl-Körner existieren nicht in vital fixierten Nervenzellen."
2. "Nissl-Körner sind Kunstprodukte, hervorgerufen während der Alkoholfixation."

He notes, however, that it is not the alcohol alone that produces the Nissl's bodies, for by his method of 'vital fixation' he excludes a factor which is present in the usual Nissl's method, namely, post-mortem change. Since he has observed that the first result of post-mortem change is the production of a meshwork<sup>1</sup> in the unstained protoplasm, and a later result that of an aggregated or conglomerated meshwork ('zusammengeballtes Maschenwerk') in the stained protoplasm, he holds that the Nissl's bodies are due to the simultaneous action of post-mortem change and alcohol fixation. He regards the post-mortem changes as acid in character, stating that: "Die Nissl-Körner sind darum als Kunstprodukte anzusehen, und zwar als solche, die durch eine Verbindung der sauren postmortalen Spaltung und der langsamen Alkoholfixation entstanden sind."

<sup>1</sup> This meshwork, however, seems to be practically identical with the 'glianetwork' (to be described later) which Möllgaard finds in his 'vitaly fixed' nerve cell, and which he considers normal. Regarding this and some other points his various statements seem vague and inconsistent.

Although he finds no Nissl's bodies, Möllgaard's observes in the 'vitaly-fixed' and stained nerve cell a network, which he interprets as a 'glia-network,' and whose richness depends on the time after death the preparation is made. In the cells of a piece of tissue taken from the living animal and 'vitaly-fixed' and stained he finds a network consisting at most of three to four coarse meshes, while in cells of preparations made ten to twelve minutes after death the network reaches its greatest development. In 'vitaly-fixed' tissue the cell protoplasm lying between the meshes of this network remains wholly unstained with toluidin-blue for some time after the tissue is taken, and only becomes stainable one and one-half to four hours after death, during which time the reaction of the brain substance has become increasingly acid. As the time after death increases the cellular protoplasm within the meshes of the networks of the cells becomes more and more stainable. But at about fifteen to twenty hours post-mortem the network itself, on the other hand, begins to disintegrate and forms peculiar aggregated masses. The significance of these masses will be mentioned later.

The technique may be varied as follows: A small piece of nerve tissue is taken seven minutes after death, is frozen, and placed in 96 per cent alcohol at room temperature and left for one and one-half to two hours. Then it is cut and fixed in alcohol at  $-20^{\circ}\text{C}.$ , and finally stained with toluidin-blue. With this treatment Möllgaard says that the network begins to shrink and disintegrate. If the tissue is left in the alcohol at room temperature for about twelve hours before being cut and stained he finds an additional structure present, namely, granulations. These granulations, in his own words: "gleichen . . . vollständig den Nissl-Körnern." The networks of the cells in which the granulations are present, he finds, on close examination, to be composed of a series of closely placed granules. Hence Möllgaard believes that in tissue taken at various intervals after death the networks of the 'vitaly-fixed' nerve cells gradually degenerate into the peculiar masses and granulations mentioned above, which are apparently Nissl's bodies. To quote again, he says: "Wir haben gezeigt oder jedenfalls in höchsten Grade

wahrscheinlich gemacht, dass die Nissl-Körner von diesen Netzen gebildet werden." The origin and nature of the network discussed now becomes Möllgaard's principal question. For the purposes of the present paper, however, it is not necessary to follow him through all the details of his attempts to explain its presence. But it might be added that his final conclusion regarding its nature is that it is a 'glia-network,' which is both intercellular and intracellular.

*Secondly*, regarding the neurofibrillae, Möllgaard's conclusion is evident from the following quotations:

Ich habe nun gleichzeitig an vital alkoholfixierten Schnitten die allgemeinen Fibrillmethoden versucht, doch überall mit negativem Ergebnis. . . . Auf Grund dieser Versuche darf ich nicht wagen, die Existenz der Neurofibrillen zu verneinen. Dazu scheint mir, dass ich die Verhältnisse vorläufig zu wenig untersucht habe. Doch kommt mir unleugbar vor, dass die ebenerwähnten negativen Ergebnisse recht stark gegen die Existenz der Neurofibrillen sprechen. . . . Falls dagegen Neurofibrillen Kunstprodukte gleichwie Nissl-Körner sind, so würden diese vermutlich ganz dasselbe Schicksal wie die Nissl-Körner erleiden, nämlich dass sie erscheinen, wenn man langsam fixiert und die postmortalen Prozesse ausgeschlossen sind, d. h. wenn man das Gewebe vital fixiert.

It may be further noted that he remarks: "Die Zellen lassen sich überhaupt sehr schwierig impragnieren." He is of course speaking of cells 'vitaly-fixed.' In speaking of the neurofibrillae he also says that one cannot deny that the appearances which the 'vitaly-fixed' nerve cells present lie nearest to the reality; and so one can maintain with considerable certainty that what is not present in the 'vitaly-fixed' nerve cell is likewise not present in the living cell.

Retzius ('11) strongly criticises Möllgaard for adopting a method which in all its essentials is like that which he and Axel Key discarded thirty-seven years ago. He says that Möllgaard without doubt started out with the right object in view, namely, to secure a simple method for the study of the central nervous system, realizing that our present methods demand too much preparation before the tissue is ready for study. But of the methods for the study of the central nervous system, Retzius

holds the freezing method to be one of the poorest. Of this fact he says he convinced himself in the year 1874, when he carried out a large number of experiments in this connection, some of which may be briefly mentioned. Under the microscope he followed the very formation of the network which Möllgaard describes at such great length. He likewise examined the freezing process in all its phases for a large series of tissues and fluids (blood, glue, egg-albumen, starch-pasté, etc.) and arrived at the certain conviction that this 'vital' method is very treacherous for scientific purposes and should be strongly condemned. From his studies Retzius concludes that the network observed in the frozen preparations is due to distortions and lacerations brought about by a system of spaces, passages, lacunae, clefts, and tubules filled with crystals of ice. He says that if to the frozen preparations a fixing fluid (alcohol, osmic acid, etc.) is applied, the system of clefts and lacunae, with the distortions and lacerations incurred, is preserved in all detail; but, if on the contrary, the preparations are permitted to thaw out without previous fixation, the water reappears anew in the entire mass, and only here and there a small cleft is left behind. From a consideration of the entire phenomenon it is evident to him that the water contained in the tissue or the fluid mass at the moment of the freezing passes out of the parenchyma and collects itself in the passages and lacunae which then appear in the frozen condition filled with crystals of ice. The water apparently collects where it meets with the least resistance. The foregoing results and others arrived at by Retzius together with Key were published by them in the Swedish language in 1874 and in the German language in 1882, so they should have been quite accessible to Möllgaard. Retzius in brief affirms that Möllgaard's 'glia-network' is an artefact due to the technique employed, but farther than this he does not criticise Möllgaard's ideas and results.

Möllgaard ('11 b) in replying to Retzius' criticism, says that he very much regrets that the articles by Retzius and Key so long ago escaped his notice, for on duplicating some of their experiments he is forced to acknowledge that his 'glia-network'

is wholly an artefact. He, however, entertains the hope that the method may still be utilized in studies dealing with physiological and pathological changes in nerve tissue.

From the foregoing it will be observed that it may still be considered questionable:

1. Whether the Nissl's bodies and neurofibrillae are present in the freshly fixed nerve cell.

2. Whether freezing the fresh neural tissue at a low temperature so changes the cytological structure that neither Nissl's bodies nor neurofibrillae can be demonstrated.

3. Whether the Nissl's bodies and neurofibrillae are due to post-mortem changes.

4. What the relation is of Möllgaard's reticulum to the Nissl's substance.

5. How and why Möllgaard's reticulum, or 'glia-network,' is formed during the freezing.

The observations made in the present study will afford at least a partial answer to these questions.

#### MATERIALS AND TECHNIQUE

With one exception, the tissue studied was taken from the spinal cord of the dog. The exception was that of a horse killed by the Department of Veterinary Medicine of the University of Missouri. In addition to neural tissue, several other tissues and different fluid masses were used, but as these were employed expressly for control observations they will be referred to only as occasion demands. The number of dogs used was twenty-four. They were obtained, as a rule, the day before they were to be killed, from the city pound or from individual owners. Such public source of course means that the dogs were of varied breed and description. Care however was taken that only healthy, well-nourished, active dogs were used. Likewise much care was exercised in avoiding exciting or in any way injuring the animal before it was killed. It was sought in general to use adult dogs of medium size and of an age that did not lie below one year

nor above five years. The age of all the animals used lay well within the limits noted but their weight varied between 10 to 19.7 kgm.

Regarding the method of killing, the technique should produce the least possible change in the nerve cell and at the same time permit of taking the tissue in a minimal length of time. This excludes the use of anesthetics, narcotics, and even hypnotics, as well as death by shooting, hanging, stabbing, or asphyxiating. The method finally decided upon was one of decapitation with a double-guillotine, thus removing a segment of the neck including the spinal cord. The apparatus used is composed of two large butcher's cleavers, heavily weighted, and so bolted together that they can be adjusted according to the length of the segment of cord desired. The necessary accessories to the method consist of a block sufficiently firm to receive the impact of the blow of the decapitating apparatus without jolting, and thus permitting of obtaining the segment desired at one blow; and a strongly made animal-board that can be closely and carefully adjusted to the foregoing block. The animal-board is provided with straps permitting of fastening the dog upon it quickly and conveniently. The dog's neck, by means of a strap and collar, is stretched out upon the block. For recording the time of decapitation and the subsequent taking of the tissue a stop-watch was used. The various details of construction and other minor aspects of the method, so briefly and simply stated above, as well as the several little difficulties confronted in its use are obvious and readily remedied and so need no lengthy description.

In taking the tissue it is of course essential that the assistants<sup>2</sup> be fully instructed as to the particular place they fill, and that everything is in absolute readiness. For the decapitation is practically instantaneous and thereafter the tissue is immediately at the disposal of the investigator. The segment of the animal's

<sup>2</sup> It is with a feeling of deep obligation that the author expresses his indebtedness to Messrs. M. D. Ott, H. L. Kearney, M. M. Miller, J. S. Homan, S. H. Snider, W. H. Taylor, T. K. Kruse, and Geo. Klinkerfuss, for the assistance so willingly and kindly granted.

neck obtained by the decapitation contains a portion of the spinal cord varying from the first to the fifth cervical segments according to the adjustment of the apparatus and as to the particular place on the neck where the blow happens to fall. This neck segment should of course be obtained as soon as possible after the blow is struck. Then, with or without depriving the segment of its adherent musculature, a long slender knife is passed around the spinal cord just within its dural sheath. Simultaneously, one of the assistants carefully grasps an end of the cord and drawing it from the canal quickly hands it to the investigator. The isolated cord segment is now cleft longitudinally in the region of the anterior horns. From the exposed gray substance (anterior horns) smears are made on glass slides which are dropped into Coplin jars containing the fixing fluids. When the foregoing was carefully and successfully carried out it was found that the smears could be placed in the fixing fluids twenty-five seconds after the moment of decapitation.

Fixation was carried out both with and without freezing; that is, in the one case the jar containing the fixing fluid was surrounded by a freezing mixture while in the other case it was not. In general, 96 per cent alcohol was used as a fixative for Nissl's bodies; and, when the smears were frozen, for the neurofibrillae also. For fixing the neurofibrillae of unfrozen smears the fluid (24 parts absolute alcohol, and 1 part ammonia) recommended by London ('05) was more frequently used. For comparison and checking of results on the Nissl's bodies, Ohlmacher's fluid and formol-corrosive were used in a few cases. For a like purpose 12 per cent formol was used as an additional fixative for the neurofibrillae in a few instances. The length of time the smear preparations were left in the fixatives varied in the case of the Nissl's bodies from one-half hour to three days, and for the neurofibrillae from five hours to four days.

Fixation with freezing, to be more explicit, was carried out as follows: A medium sized vessel (capacity approximately one gallon) containing 95 per cent alcohol was placed within a larger container. In the smaller vessel with the alcohol were placed small Coplin jars containing 96 per cent alcohol and ten or twelve

glass slides respectively. The larger container was now filled with ice and salt which was carefully packed about the smaller vessel. When the temperature of the alcohol and the fixative had been reduced to  $-8^{\circ}\text{C}$ . or lower, carbon dioxide snow was added to the 95 per cent alcohol in the smaller of the containers until the temperature was further reduced to  $-20^{\circ}$  to  $-50^{\circ}\text{C}$ . At this time the dog was immediately killed and smears made on the cold slides upon which they froze instantly. Then they were quickly dropped into the cold fixative. The time consumed in making the preparations was judged from the instant of decapitation to the exact moment the fixative received the smear. The smears were left in the fixative for a time varying as above noted. When the time was prolonged the whole of course acquired room temperature in the meantime.

In those cases where preparations were made at various intervals after death the cord segment was in all cases kept under the ordinary conditions of the laboratory and at ordinary room temperature. The majority of the long post-mortem interval preparations, however, were made during the winter months so it may be said that the temperature of the laboratory ranged from  $5^{\circ}$  to  $23^{\circ}\text{C}$ .

The staining methods used are comparatively simple. For the staining of the Nissl's bodies the method outlined by Dolley ('11) was used. The method is in the main as follows: The smears fixed in 96 per cent alcohol are brought through a series of alcohols of decreasing strength to distilled water, then stained with warm (ca.  $40^{\circ}\text{C}$ .) erythrosin for three minutes, and well washed in water. They are then brought into a 1 per cent aqueous solution of toluidin-blue for five to eight minutes, again well washed in water, after which they are dipped in 95 per cent alcohol and differentiated, until the Nissl's bodies and nuclear structures are clearly defined, in a mixture of 96 per cent alcohol 9 parts, anilin oil 1 part. The differentiation is stopped by bringing the slide into absolute alcohol from which it is brought into xylol and mounted in Canada-balsam or damar.

To stain the neurofibrillae, London's method was followed. The method is essentially the following: After fixation, the smears



are brought into a 1.5 per cent aqueous solution of silver nitrate, kept at a temperature of about 37°C. for three to seven days, if unfrozen, and for one to three weeks if frozen, after which time they are treated with a solution of pyrogallie acid, 2 grams, formal 5 cc., and distilled water 100 cc., for twenty-four hours. The smears are then placed in a 1 per cent aqueous solution of gold chloride for five to ten minutes, brought into a 5 per cent aqueous solution of sodium hyposulphite for ten minutes, carried through distilled water and a series of alcohols of increasing strength to xylol and mounted in damar or Canada-balsam.

In the case of the neural tissue, the foregoing methods were used almost exclusively. Variations were made only occasionally and in fact so rarely that they need not be mentioned, excepting that some of the smears were stained for Nissl's bodies without any previous fixation, and that in staining for neurofibrillae a few control preparations were made according to Legendre's ('06) modification of Bielschowsky's method.

For control purposes too, smear preparations of hepatic and pancreatic tissues were subjected to the above technique, and the freezing of distilled water and egg-albumen was carefully studied under various experimental conditions.

It is thought unnecessary to describe the various magnifications employed in the study of the smears. An ordinary Leitz oil immersion lens was in all cases quite sufficient to determine the points in question.

#### OBSERVATIONS

The observations made may be conveniently assembled under the following headings:

##### Nissl's bodies

1. In unfrozen smears
2. In frozen smears
  - a. Fresh, frozen, unfixed, and unstained smears
  - b. Frozen, fixed, and stained smears considered as a whole
  - c. Nerve cells of smears frozen at  $-5^{\circ}$  to  $-10^{\circ}$ C.
  - d. Nerve cells of smears frozen at  $-10^{\circ}$ C. and lower

##### Additional observations

## NISSL'S BODIES

*1. In unfrozen smears*

The observations on Nissl's bodies in nerve cells of unfrozen tissue may be very briefly considered.

In smears not subjected to freezing, but fixed in alcohol at ordinary room temperature the Nissl's bodies are present, clearly and definitely defined in cells fixed twenty-five seconds after decapitation (fig. 2). They were also present at all intervals thereafter until they were finally lost in the complete post-mortem disintegration of the tissue. Similarly Nissl's bodies are present, distinctly defined, in the nerve cells of smears placed, without the usual fixation, in the toluidin-blue stain one and three-fourths minutes after death. Not only are the Nissl's bodies distinctly present in the foregoing preparations, but also their form and their distribution in the cell body and the dendrites are clearly evident. In some of the best smears, and with good magnification, it is even possible to see that the Nissl's bodies are composed of granules which lie embedded in a matrix, the 'gerinnselartige Masse' of Held ('95). In Nissl's bodies in the periphery of a well differentiated cell this matrix appears of slightly purplish hue while the Nissl's granules are a deep blue. These observations on the matrix are in agreement with those of Held ('95 and '97) and Becker ('06).

*2. In frozen smears*

In the case of the frozen tissue the picture is quite different from that of tissue that has not been frozen, and as an aid to its interpretation a few preliminary notes on the effect of freezing on some fluids and fluid-masses may first be mentioned.

Since such a great percentage of all tissues and fluid masses is water and because so much separates out during the process of freezing, it is of interest to note the appearance of a frozen drop of water or of the ice as it fills the interstices of the tissue. The appearance is one of large and small prismatic and spherical foam-cells (Quincke) filled with clear pure or nearly pure congealed water with here and there small air bubbles between their

adjacent walls. This appearance is observed of course only in freshly frozen preparations, not after their fixation, and is mentioned only to insure a more correct association between the appearances of the aqueous and less aqueous portions of the preparations.

The appearance of ice in animal tissues seems to have been quite neglected, but its appearance in plant tissues has been variously noted by Müller-Thurgau ('80 and '86), Fischer ('11), Wiegand ('11), and others. The details of ice formation however need not be entered into here; for such details, with physico-chemical explanation, are given at length by Quincke ('05).

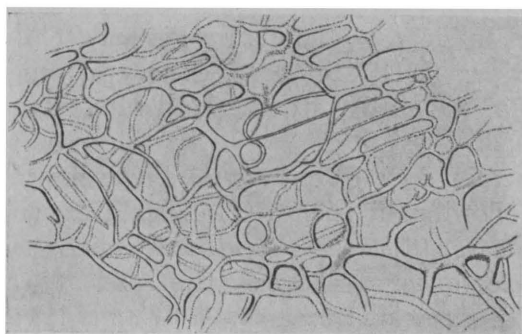


Fig. 1 This figure is a reproduction of Molisch's ('97) figure 6, with a reduction of one-sixth. It represents the reticulum observed in a thin film of starch-paste, first frozen and then permitted to thaw out.

The appearance of frozen egg-albumen is also instructive. Depending on the conditions of the freezing, many variations occur, but in general the appearance is one of a variable network or reticulum the meshes of which are filled with ice. Disregarding the variations, the network is not unlike that figured by Molisch ('97) for frozen starch-paste with subsequent thawing (fig. 1). Small air-bubbles are usually present, and intermingled with the reticulum, if the temperature employed be low enough, many small clefts occur.

It should be remarked that Molisch ('97 and '11) has made extensive observations on the action of freezing on many substances, solutions, and emulsions, colloidal and otherwise. From

his studies Molisch concludes: Freezing causes a separation of water; this water at numerous points forms ice crystals which by molecular force continue to extract water from the substance in question and by their consequent enlargement push aside the substance, and in this way give rise to the network or reticulum observed. Several of his figures, one of which is reproduced here in figure 1, strikingly illustrate this point.

The observations on various gums and other colloids all agree, in their essentials, with those of Molisch: Ambronn ('91); on a number of inorganic and organic colloids, at temperature reductions of  $-10^{\circ}$ ,  $-70^{\circ}$ , and  $-180^{\circ}\text{C}$ . by Bobertag, Feist, and Fischer ('08); on starch-paste, various gums, and hemoglobin, at temperatures as low as  $-180^{\circ}\text{C}$ ., by Fischer ('11); and, on gelatin by Liesegang ('11). Likewise, except for some details, the observations of the author are in accord with those of the investigators named.

The observations on frozen neural tissue may be considered under the following divisions: (a) fresh, frozen, unfixed, and unstained smears; (b) frozen, fixed, and stained smears considered as a whole; (c) nerve cells of smears frozen at  $-5^{\circ}$  to  $-10^{\circ}\text{C}$ .; (d) nerve cells of smears frozen at  $-10^{\circ}\text{C}$ . and lower.

*a. Fresh, frozen, unfixed, and unstained smears.* A smear of gray matter from the spinal cord of dog, frozen over the gas-escape of a carbon dioxide tank or by making the smear on a very cold slide, becomes of whitish opacity and at very low temperatures develops clefts not unlike those observed in ice and frozen egg-white, though in general it takes a much lower temperature to develop such clefts in neural tissue. The appearance presented under the microscope varies considerably according to the thickness of the smear, the rapidity of the freezing, the degree of temperature reduction, and undoubtedly too on the surface tension and other intrinsic forces of the individual neural elements. In general, however, it may be said that small foam-cells resembling those of aqueous ice, variously arranged, are as a rule to be observed around the margin of the smear; the smear itself appearing as a coarse network or reticulum with large and small, irregular or multiangular meshes. Within the meshes is

the more liquid frozen matrix or ice, while the network itself consists of the more solid portions of the tissue. In many places the largest of the nerve fibers found in gray matter appear to form initial strands upon which the network is formed, in other places however they extend into or through the lacuna-like spaces quite unaccompanied by other neural elements. Regarding the various kinds of cells (nerve cells, blood cells, and glia cells) nothing can be made out with certainty in these frozen and unstained preparations.

Molisch ('97) has shown that the reticulum produced by freezing in such colloids as gelatin and starch-paste is more or less permanent after thawing out, others such as gum tragacanth, gum arabic, and egg-albumen the reticulum disappears quite completely. The author's observations on the last named colloid confirm this statement and so too for the non-fixed neural tissue described above no noticeable trace of the effects of the previous freezing can be noted after thawing.

*b. Frozen, fixed, and stained smears considered as a whole.* Smears frozen, fixed and stained according to the directions given under the heading 'Material and technique' show, when frozen at a temperature of  $-5^{\circ}$  to  $-10^{\circ}\text{C.}$ , the first effects of freezing. Like the fresh, frozen, unfixed, and unstained smear, the preparation, when looked upon as a whole, appears to consist of a more or less coarse network or reticulum the interstices of which are of variable size and form. With greater temperature reductions ( $-10^{\circ}$  to  $-40^{\circ}\text{C.}$ ) this network becomes more definite and distinct, and presents a number of special features which will be discussed under other headings. The network observed is stained in part blue (by the toluidin-blue) and in part pink (by the erythrosin). Coarse and fine nerve fibers and blood capillaries lie, apparently unaffected by the freezing (at  $-5^{\circ}$  to  $25^{\circ}\text{C.}$ ), surrounded by the network. The pink stained portion of the reticulum appears to be composed of the most delicate nerve fibers and condensations, as it were, of the tissue fluids; in fact, it may be regarded as being composed of all the acidophilic substances of the tissue. The blue stained portion, on the other hand, may be looked upon as composed of the basophilic sub-

stances of the tissue, especially the chromatin. The relation of the pink and blue portions to each other, and to the different conditions of the technique, will be considered more in detail under subsequent divisions.

In these stained preparations the various kinds of cells may be clearly distinguished. Red and white blood cells and glia cells appear, at temperatures above  $-25^{\circ}\text{C}$ . quite unaffected by the freezing, excepting of course those that have been crushed or otherwise distorted in making the smear. Such crushed forms produce areas of coarser network. At lower temperatures ( $-25^{\circ}$  to  $-40^{\circ}\text{C}$ .) these cells, and the nerve cells as well, show a considerable variety of changes which will be discussed under the headings following.

*c. Nerve cells of smears frozen at  $-5^{\circ}$  to  $-10^{\circ}\text{C}$ .* The multipolar nerve cells of smears frozen at the temperature stated present appearances that vary somewhat depending, in addition to the reduction in temperature, on the thickness of the smear; the rapidity of the freezing, and no doubt also on the surface tension and other intrinsic forces of the different cellular elements. The size of the cell does not appear to play so great a part in these variations as one might expect. The change which is the most marked and characteristic of this temperature interval, and, in fact, the first change to become noticeable in the cells, is in the Nissl's bodies which become minutely reticular or vacuolated, thus forming many small blue-staining networks, each network corresponding to a single Nissl's body. These networks are loosely connected together by finer trabeculae of the same blue-staining substance (fig. 3). The trabeculae appear to be direct extensions of the Nissl's substance which has probably a lower freezing point. By virtue of this property these delicate processes have been squeezed out into the surrounding cytoplasm under the force of the displacement brought about by the enlarging and expanding ice masses. None of the networks extends beyond the walls of the cells. Aside from the Nissl's bodies, the cellular cytoplasm, the nucleolus, and the other nuclear structures show as yet no change, unless the nuclear chromatin, exclusive of the nucleolus, may be said to be beginning to suggest

reticular arrangement. Though this description applies particularly to smears frozen at  $-5^{\circ}$  to  $-10^{\circ}\text{C.}$ , it also applies in part to smears frozen at temperatures as low as  $-25^{\circ}\text{C.}$

*d. Nerve cells of smears frozen at  $-10^{\circ}\text{C.}$  and lower.* The changes next in occurrence to those described under the heading just preceding are to be observed in cells of smears frozen at  $-10^{\circ}$  to  $-25^{\circ}\text{C.}$  In such preparations some of the cells show many of the Nissl's bodies still appearing as individual networks but with larger meshes and more closely interconnected, all however being still confined within the cell wall (fig. 4). In the cellular cytoplasm no changes are yet to be noticed. The nuclear chromatin (and also the achromatic substance of the nucleus which stains pink with erythrosin) has become distinctly reticular, apparently forming one continuous network limited to the area within the nuclear membrane; or, extending beyond the nuclear membrane and becoming continuous with the blue-staining network derived from the Nissl's bodies. The nucleolus may be unaffected, or may take part in the formation of the nuclear chromatin reticulum, forming as it were its starting point by sending out a varying number of processes. Some nerve cells show these changes much intensified with the cellular cytoplasm, exclusive of Nissl's bodies, also becoming reticular. This cytoplasmic reticulum, although continuous with the blue-staining network, is readily distinguished, when once fully formed, by the fact that it stains pink with the counterstain employed. In general, too, this pink-staining cytoplasmic reticulum has smaller meshes than the blue-staining network, and lies within the interstices of the latter. In order to avoid confusion, only the blue-staining portion of the reticulum is shown in the figures. Still other cells at this temperature ( $-25^{\circ}\text{C.}$ ) and at lower temperatures ( $-25^{\circ}$  to  $-50^{\circ}\text{C.}$ ) show a reticulum with much larger meshes. The elements composing this reticulum are none other than those already mentioned, namely, the blue-staining network derived from the Nissl's bodies, nuclear chromatin, and nucleolus; and, the pink-staining network derived from the cellular cytoplasm (exclusive of Nissl's bodies) and the achromatic substance of the nucleus. The continuity of these two networks is one of

intimate blending and so a single double-staining reticulum results. This single reticulum extends throughout the entire cell and even beyond (fig. 5), and thus becomes directly continuous with the pink-staining extracellular network previously described under the observations on the stained smear as a whole. In these cells there is of course a complete obliteration of the Nissl's bodies as such. Thus, to repeat, it appears quite evident that the intracellular networks are directly continuous with each other, and again, are similarly continuous with the network formed in the cellular interstices. These last changes described may be called the final form the networks assume, for the appearances just described are quite constant and do not change to any noticeable extent in a temperature range of 20° to 30° (-40° to -60°C.). It cannot be said that any of the appearances described have an absolutely definite temperature at which they appear. For instance, the last form described may even be found to a certain extent in smears frozen at -15°C., none of the earliest forms, however, occurs as a rule at temperatures below -25°C.

The significance of the networks described above and their relation to Möllgaard's reticulum or 'glia-network' will be discussed later.

#### ADDITIONAL OBSERVATIONS

The perivascular network described by Möllgaard may also be satisfactorily studied in the foregoing preparations. Its origin is plainly from the chromatin of the nuclei of the endothelial cells of the blood and lymph capillary and precapillary vessels (fig. 6). The variations and various relations of the network merit no description. The chromatin of the nuclei of the glia cells also form networks but their relation to both the networks of the nerve cells and the perivascular networks seems to be merely one of accident. The rather constant appearance of what Möllgaard believes to be a glia cell at one pole of the nerve cell nucleus the author did not observe and it is probably explained as being a modification of a chromatin nuclear cap.

All the observations on frozen neural tissue apply to smears frozen and fixed as soon as thirty-two seconds after decapitation



and at various intervals thereafter up to twenty-five hours post-mortem. After this time no further preparations were made, for it seemed quite evident that the networks were in some way related to the process of freezing rather than to the length of time after death. In this regard the author was entirely unable to confirm Möllgaard's statement that the earliest form of the reticulum (in nerve cells of tissue excised from the living animal) consists of a network of 3 to 4 meshes, and that the reticulum becomes finer and reaches its greatest development ten to twelve minutes post-mortem. The reticula of nerve cells in smears frozen and fixed thirty-two seconds after decapitation, and this time probably does not correspond very unfavorably with the time consumed by Möllgaard in excising and transferring the tissue from the living animal, were found as fine and as extensive as the reticula of cells in smears frozen and fixed at various later intervals up to twenty-five hours after death. It seems apparent, therefore, that very little, if any, connection exists between the fineness and extensiveness of the reticulum on the one hand and the time after death, up to at least twenty to twenty-four hours, on the other.

Neither was the author able to note any disintegration of the reticulum in nerve cells of frozen smears left in 96 per cent alcohol for a time varying from half-an-hour to three days. Even when Möllgaard's modification of his technique, as previously explained under 'Material and technique,' was carefully followed out, as nearly as the technique and conditions of the present investigation would permit, no such changes as noted by Möllgaard were observed in the nerve cells of the smear preparation.

Alterations in the staining properties of the protoplasm, as observed by Möllgaard in frozen preparations made at various intervals after death, were given but little attention because smear preparations do not lend themselves readily to a reliable study of such details. Furthermore, very much depends on the thickness of the smear and its degree of differentiation after staining. It may be noted however that in smears frozen twenty-four to twenty-five hours after death there is a more diffuse staining with the basic stain in consequence of which the blue and

pink staining networks become less distinct and in places ill-formed.

That post-mortem changes have a marked influence on the cytological appearance of the tissue there can be no question. But it is questionable if the post-mortem changes produce alterations sufficiently great to be detected by our present technique until a considerable time after death, at least half-an-hour. The relationship between the acid reaction of the neural tissue and the post-mortem changes produced are not so strikingly noticeable as Möllgaard's emphasis on this point would lead one to believe. Möllgaard states that the cerebrum of dog shows a marked acid reaction to moist neutral litmus paper ten minutes after death. It was however fully twenty minutes before the author could be sure of such acid reaction. Nevertheless, it is quite possible that with a more delicate indicator an acid reaction might be observed somewhat earlier.

To determine whether the network could be produced in cells that had first been fixed, some smears were made approximately seven minutes after death, fixed in 96 per cent alcohol at room temperature for one hour, and then subjected to a temperature of  $-15^{\circ}\text{C}$ . At this temperature (which was possibly not low enough) no networks could be observed in the cells. The Nissl's bodies had remained unchanged. This question of the possibility of structural change through freezing after fixation, however, was insufficiently investigated to warrant any definite conclusions; yet, it is a matter of common knowledge that in the use of various neurological methods where the tissue is frozen after fixation no *marked* changes at least have been reported.

Smears of fresh hepatic and pancreatic tissues, prepared after the manner of the neural tissue preparations, show networks which are markedly similar to those of the neural tissue, the chromatin of the nuclei giving rise to a blue-staining network while the non-chromatic elements give a pink-staining network. Thus it may be said that Möllgaard's blue-staining reticulum is not a characteristic of neural tissue alone but may be formed from the chromatin of the nuclei of hepatic and pancreatic cells as well.

The networks in the liver and pancreatic tissues, as well as those of frozen smears of egg-albumen, may also be stained with silver nitrate.

In corroboration of the foregoing observations on animal tissues it may be mentioned that Molisch has observed very similar appearances in the freezing of amoebae, fungi, yeasts, and various algae. Molisch's observations however apply principally to cytoplasmic changes. Equally instructive and showing chiefly the nuclear changes due to freezing are the numerous figures of Matruchot and Molliard ('02) in their work on the influence of freezing on plant cells.

The observations on the neurofibrillae, like those on the Nissl's bodies, may be considered under two headings; namely, their presence in the unfrozen and in the frozen cells. The neurofibrillae are unquestionably present in the unfrozen cells of smears fixed twenty-five seconds after decapitation and thereafter until lost in the total disintegration of the tissue. Although the perinuclear network and other finer details of the endocellular neurofibrillar structure of the nerve cell (such as may be seen in thin sections) are not visible as such in smear preparations, the neurofibrillae are distinctly seen at the origin of the cell processes and an endocellular neurofibrillar network of varying richness is more or less discernible in the cell-body.

In regard to neurofibrillae in frozen preparations it may be noted that smears frozen at  $-5^{\circ}$  to  $-20^{\circ}\text{C}$ . fixed in 96 per cent alcohol, and impregnated with 1.5 per cent silver nitrate for from nine to twenty-one days, show networks corresponding more or less closely to the networks stained with toluidin-blue and erythrosin. Large numbers of darkly stained nerve fibers render the appearance somewhat difficult of recognition at first sight, especially where the smears are thick. In the larger meshes of these networks lie the nerve cells whose individual reticular structure may or may not be continuous with the walls of the meshes about them. In these preparations, when not too darkly stained, the neurofibrillae are unmistakably present in the nerve cells and their processes as well as in the intercellular nerve fibers. They are well stained and quite distinct. The neuro-

fibrillae however are best observed in preparations frozen quite rapidly and at moderately low temperatures ( $-5^{\circ}$  to  $-15^{\circ}\text{C.}$ ), for when thus treated the intracellular neurofibrillae do not seem to take part in the formation of the cellular reticulum corresponding to that stained with toluidin-blue and erythrosin. At temperatures below the foregoing, especially if frozen slowly, the intracellular neurofibrillae cannot be made out with any degree of certainty though they are still quite distinct in many of the cell processes and in the fine nerve fibers of the intercellular spaces. The above observations were made on smears frozen and fixed forty-two seconds after decapitation and at different times afterwards up to twenty-five hours after death. These observations on the neurofibrillae in frozen tissue are quite in agreement with Liesegang's ('11) conception; but, on the other hand, quite opposed to that of Auerbach ('11).

In the above study of neurofibrillae both in the unfrozen and the frozen preparations many variations were met with. In this regard, as Legendre ('06), Marinesco ('09), and many others have noted, it may be mentioned that the methods for the demonstration of neurofibrillae are not sufficiently adequate or reliable to permit of constant results. Only by extensive observation and many controls can definite conclusions be reached. So it may be questioned whether the above observations on the neurofibrillae in frozen nerve cells, in this one investigation, are sufficient to determine the point at issue; yet, since this point is merely the presence of the neurofibrillae in such frozen cells, without reference to the details of their distribution, and so forth, the author believes the results may be regarded as quite reliable.

It may be of interest to note that both the Nissl's bodies and the neurofibrillae were unmistakably present in nerve cells from the cervical portion of the spinal cord of horse (cf. 'Material and technique'), fixed three minutes after the instant of the shooting of the animal.

In imbedded tissue, that is, tissue fixed, dehydrated, imbedded in paraffin (or celloidin), cut, stained, and mounted; both the Nissl's bodies and the neurofibrillae were clearly and distinctly

present in cells fixed *twenty-two* seconds after decapitation. Small pieces of the cervical portion of the spinal cord of dog were quickly dropped in the fixative at the time stated. Then in the cutting, staining, and mounting of the sections care was taken to use only those sections cut from the most superficial parts of the tissue block. Thus one may be reasonably sure that the fixation of the superficial cells was practically simultaneous with the committal of the tissue to the fixative.

No observations were made on imbedded specimens of frozen tissue.

#### DISCUSSION

It was one of Möllgaard's primary objects to produce a method which would be simpler than our present neurocytological methods. His procedure however is not so strikingly simple as he would have us believe. In the first place, the production of a temperature as low as  $-40^{\circ}\text{C}$ . and the necessity for maintaining it for a definite length of time requires a painstaking and rather cumbersome technique. Sectioning the tissue at a temperature of  $-20^{\circ}$  to  $-15^{\circ}\text{C}$ . at best, and that with a specially constructed microtome, is not easy. Even in the fixation, if the author has correctly interpreted Möllgaard's following statement, complicating factors enter: "Die Flüssigkeit, in der man zu schneiden wünscht, wird in den innersten Kasten des Kalorimeters gegossen und durch Zusatz von fester  $\text{CO}_2$  auf eine Temperatur von  $-20^{\circ}$  bis  $-25^{\circ}$  heruntergebracht." Judging from the description of his apparatus this seems an unnecessary step, yet he so directs us. Bohr (Annalen der Physik, IV F, Bd 1, S. 244, 1900) states that at  $-20^{\circ}$  and  $-40^{\circ}\text{C}$ . (760 mm.?) 98.7 per cent (by weight,  $15^{\circ}$ , 760 mm.) ethyl alcohol will absorb 7.16 cc. and 13.89 cc., respectively, of  $\text{CO}_2$  ( $0^{\circ}$ , 760 mm.). In 96 per cent (the percentage used by Möllgaard) alcohol the absorption is of course less. However, Möllgaard thus leaves without control the possible changes the tissue may suffer from the effects of the  $\text{CO}_2$ . At the temperature stated it may be questioned if there is any effect on the tissue, still it cannot be nil. The presence, and its liberation as the temperature rises, of the  $\text{CO}_2$  in the fixative does not simplify the technique to say the least.

Finally, his method requires the preparation of the animal, its anesthetization, the performance of a craniotomy or a laminectomy, and the waiting for the effects of the anesthetic and the shock of the operation to disappear. In connection with the latter features, it may be well to mention that Dolley ('09 and '10) has conclusively shown that the effects of the anesthetic and the shock of the operation do not disappear in so short a time (seven to eight hours) as Möllgaard assumes. So that for various reasons, in addition to that urged by Retzius, Möllgaard's technique is by no means ideal.

It was in order to avoid the foregoing objections that the technique previously described was decided upon for the present investigation. The decapitation avoids all preliminary preparation of the animal, and likewise excludes the effects of an anesthetic and the prolonged shock of the operation. The apparatus for the decapitation permits one to obtain practically instantaneously a segment of the animal's neck, after which the isolation of the cord, with a little experience, requires but a few seconds. By resorting to smear preparations, the freezing process, which the sectioning of an unimbedded tissue demands, is avoided. The smear preparations have the further advantage of being fixed the very instant they are consigned to the fixative. Thus it is evident that the entire procedure, namely, the taking of the tissue, putting the tissue in a form suitable for study, and the fixation, all occur in less than a half a minute of time, or at most a minute. It should be noted that the cumbersome freezing method of Möllgaard is designed merely to preserve the tissue structure in the 'vital condition' until thin sections can be made upon which the fixative can act. The smear method accomplishes this far more easily and quickly, and eliminates the production of artefacts by freezing. It is of course freely granted that the smear preparations do not show the minute details of structure as readily as do thin sections. Nevertheless, for the points in question the smear preparations are amply sufficient.

In smear preparations not subjected to the freezing process the Nissl's bodies and the neurofibrillae are unquestionably pres-

ent twenty-five seconds after decapitation. It has been previously mentioned that in nerve cells of imbedded tissue the Nissl's bodies and neurofibrillae were found in tissue fixed twenty-two seconds after death. It has been stated too that the smear preparations do not permit of the detailed study that sections do. It is therefore unnecessary to discuss the various details of observation noted under this heading. It is particularly the time element with which we are here concerned. Möllgaard found no Nissl's bodies or neurofibrillae in freshly-frozen and fixed nerve cells. He considers at least seven minutes post-mortem change, followed by several hours of slow alcohol fixation, necessary to produce the Nissl's bodies even imperfectly. But the results submitted in the present paper prove that in unfrozen smear preparations (which have the advantage of excluding the production of artefacts by freezing and allow immediate fixation) both the Nissl's bodies and the neurofibrillae are found in tissue fixed less than half a minute after decapitation, while the cells are still practically in a living condition. Hence Möllgaard's contention that Nissl's bodies are produced by post-mortem change and slow alcohol fixation is totally wrong. The freezing is responsible for his misleading results.

Whether the sojourn in the fixative be comparatively long or short seems to have no noticeable influence on the Nissl's bodies. In smears taken at various intervals after death the Nissl's bodies show no appreciable change till about twelve to eighteen hours post-mortem, when they gradually begin to disintegrate. To determine whether the alcohol fixation could in any way be responsible for the presence of the Nissl's bodies in the freshly-fixed cell, some smears, instead of being dropped into the fixative, were immediately consigned to the toluidin-blue stain, after which they were carefully washed, mounted in water, and studied. Such smears show the Nissl's bodies to be undeniably present. They show distinctly, but it seems that they are somewhat more granular and possibly a little more diffuse than those of cells fixed in alcohol. In this connection it may be mentioned that Dogiel ('96) stained the Nissl's bodies in unfixed nerve cells, with dilute solution of methylene-blue in warm physio-

logical salt solution five to ten minutes after death. It may be possible however, as Held ('95) has pointed out, that the toluidin-blue or the methylene-blue (Dogiel) may fix, or partially fix, the tissue put into it.

Regarding the alterations which freezing produces in the smear preparations, very much depends upon the conditions under which the freezing occurs. The factors previously referred to may be repeated, namely, the composition of the substance or tissue in question, the thickness of the smear, the degree of temperature reduction, the rapidity of the freezing, the surface tension and other intrinsic forces of the individual elements of the substance or tissue investigated (e.g., the forces of molecular attraction, imbibition, osmosis, etc.), to say nothing of thawing and fixation. These factors have been wholly or partially recognized by all investigators of the phenomenon of freezing.

The freezing of water has been sufficiently discussed. The physico-chemical explanation of ice-formation, though of fundamental importance in the following considerations, are given in detail by Quincke and so will be referred to here only as occasion demands.

The appearances observed in the freezing of egg-albumen are concisely expressed in Molisch's following statement in which he describes his microscopical findings for the freezing of a 2 per cent aqueous solution of gelatin:

An zahlreichen Punkten tauchen unter Abscheidung von Luftblasen rundliche Eismassen auf, die, der benachbarten Gelatinegallerte das Wasser entziehend, sich rasch vergrössern und dabei die immer wasserärmer werdende Gelatine ringsum zur Seite schieben, so dass diese, wenn die Eisbildung ihr Ende erreicht hat, als ein höchst complicirtes Maschenwerk zwischen den Eisklumpchen ausgespannt erscheint. Die ursprünglich homogene Gelatine ist nun in eine Art Schwamm umgewandelt, in welchem das höchst complicirte Gerüstwerke aus Gelatine, die Hohlräume aber aus Eis bestehen.

Molisch's observations on frozen egg-white are similar to his above observations on gelatin, excepting that in the case of the egg-albumen the network disappears on thawing, while that produced in gelatin is quite permanent for some time. It is of interest here to note that Ambronn ('91) in his study of frozen gelatin and agar-agar states that the appearance of the fine network



due to the freezing of dilute solutions of these substances is exactly similar to the appearance of a section through a parenchymatous plant tissue. Molisch casually remarks that his studies confirm this statement. Furthermore, Ambronn states that in an optical respect the walls of the meshwork in the frozen colloids named are entirely similar to the walls of plant cells, showing a strong double refraction and the same orientation of the optical elasticity ellipsoid as do the cell-walls.

From the observations and references presented, it must be clear then that on freezing there is a separation of water from the substance in question. This fact has long been known to many observers (Müller-Thurgau, Molisch, Fischer, Wiegand, and others). The water separating out under the reduced temperature forms ice, which, omitting for the present the details, by displacement of the substance produces the network or sponge-like structure described. The great part played by this water separating out during the freezing of the substances and tissues under consideration, may be further realized by a study of smears of egg-albumen, fresh neural, liver, and pancreatic tissues thoroughly evaporated or desiccated in an oven. Such preparations show networks somewhat suggestive of those produced by freezing. So similar are the processes of freezing and desiccation, in producing a loss of water, says Fischer, that the curve for the loss of water by freezing may be calculated, for some colloids at least, from the curve for the loss of water by desiccation. Matruchot and Molliard have compared the separation of water during freezing to plasmolysis; and, wilting, or slow and rapid desiccation, as well.

Noting the comparative uniformity of the results arrived at by many observers regarding the fundamental principles underlying the freezing of numerous simple substances, colloids, and plant and animal tissues we may consider, as does Molisch, and with considerable support, that the cells of a plant or animal tissue may be regarded, as far as their behavior in freezing is concerned, as aggregate masses of solutions, emulsions, and colloids. Thus, for explanatory purposes, we may consider that neural tissue with all its various elements consists of a complex aggregate of solutions, colloids, and emulsions, one within the

other, most delicately interrelated and adjusted, specialized and differentiated, if such terms are permissible in this connection, to a high degree. There is however the one common element, water, whose proportional presence depends on the various conditions to which the tissue may be heir. If now this complex aggregate be subjected to a freezing temperature, there is, as is already evident from previous statements, a separation of water. This separation of water is strikingly dependent on the factors before noted. If the freezing is slow the water as a rule collects in the interstices of the tissue and thus in the subsequent ice formation there are comparatively few centers of crystallization. If however, the freezing is rapid the rapidity of the process does not permit the water to collect in a few, apparently the least resistant, interstices but forces it to crystallize in numerous places. It would seem that in the employment of low temperatures, the more rapid the reduction the more numerous the centers of crystallization. Liesegang states that if the reduction in temperature be great enough the centers of crystallization become so numerous as to warrant the designation colloidal ice, the existence of which has been proven by Ostwald and Weimarn (cited by Liesegang).

Since it is in the more slowly frozen tissue that the more typical reticular structure occurs, we must return to that condition. Just how and why the water of the various tissue elements collects in the interstices of the tissue during the process of freezing is explained in considerable detail, in the case of plant tissue, by Wiegand ('06 b). Just what, at a freezing temperature, determines the formation and location of a center of ice crystallization Wiegand does not state. Many factors no doubt are involved, the more important ones however are probably the following: the minimal amount of solute present in the water, and the relative molecular capillarity with which this water is held, together with the molecular distribution and arrangement, at that particular instant and position. With the formation of the ice crystals of course comes the molecular force of crystallization which continues to abstract water from the particular tissue element in question with a simultaneous increase

in the size of the ice crystal, or crystals. This process of abstraction of water and enlargement of ice crystals continues until the force of crystallization is equal to the force of imbibition, or molecular capillarity, of the tissue element or cell, that is, until an equilibrium is reached. With a new reduction in temperature the process is again set up until the equilibrium is once more restored.

Whatever the exact details of the process may be, it is evident that with the first formation of the center of ice crystallization the tissue elements, and cellular elements especially, are subjected to a displacement which is increased in extent both by the expansive force of the water changing to ice and the actual increase in size of the ice crystals under the force of crystallization. With a moderate rate of freezing (such as making smears on slides cooled to  $-20^{\circ}$  to  $-40^{\circ}\text{C.}$ , with fixation at the same temperature) and a like temperature reduction this displacement gives rise to the various networks or sponge-like reticula described for the egg-albumen, nerve cell, and so forth. These reticula are therefore the resultant of aqueous abstraction, and displacement of the subsequent tissue or cellular residue, as it were, by the formation and growth of ice crystals or ice masses within the tissue or cell. The expansion and contraction of the ice, as the maximum and minimum temperatures for these phenomena are reached and surpassed, augment the displacement, while the force of imbibition is probably the main retarding force. With very rapid freezing at very low temperatures ( $-50^{\circ}\text{C.}$  and below) the formation of multiple centers of crystallization subjects the individual cellular elements to the contraction of the frozen mass of course much more than at higher temperatures. That the networks discussed arise in consequence of the displacement by the enlarging ice masses is particularly emphasized by Müller-Thurgau, Molisch and Wiegand, as before stated.

The blue and pink staining properties of the reticulum are explained as being due to the fact that some of the substances composing it are basophilic while others are acidophilic, that is, some of the composing substance stains with toluidin-blue while other portions of it stain with erythrosin. If now, we regard

the Nissl's bodies as composed of substances of the nature of chromatin, as is generally done, then we may say that the blue-staining (with toluidin-blue) networks or reticula are derived from the Nissl's bodies and nuclear chromatin of the nerve cells as well as the nuclear chromatin of all other cells present in the preparation. The pink-staining (with erythrosin) networks, on the hand, may be regarded as arising from all the achromatic substances of cellular cytoplasm and tissue (blood, lymph, etc.) alike. That such is indeed the origin of the networks or reticula is confirmed by this investigation. Möllgaard's reticulum therefore arises from the Nissl's bodies and nuclear chromatin. The reticulum is a product of the Nissl's bodies rather than the converse as believed by Möllgaard. The close relationship between the reticulum and the Nissl's bodies is further supported by the many transition forms that may be noted in nerve cells of smears frozen at moderately low temperatures ( $-5^{\circ}$  to  $-25^{\circ}\text{C}.$ ) (figs. 3 and 4).

Retzius in his study of the freezing of various tissues and fluid masses lays much stress on the formation of a system of clefts and lacunae due he says to the collection of the water at the moment of freezing at the points of lowest resistance. The formation of ice in this system produces distortion and laceration. With fixation the entire picture is preserved and is known to us as the artefacts due to freezing. Excepting for the undue stress laid on the formation of a system of clefts and lacunae and the production of real laceration, it is evident that Retzius' results are in the main quite similar to those arrived at in this investigation. The cleft and lacunar system of Retzius, however, must not be confused with the clefts spoken of here as due to the contraction of the ice present.

#### CONCLUSIONS

From the observations presented and the discussion made the following conclusions may be summarized:

1. With a simplified smear method, both the Nissl's bodies and the neurofibrillae are found present in the spinal nerve cells of the dog, fixed *twenty-five* seconds after decapitation.

There is no evidence that they are artefacts due to post-mortem changes as described by Möllgaard.

2. Nissl's bodies and neurofibrillae may also be demonstrated, in a more or less modified condition, in frozen neural tissue. The freezing causes the Nissl's bodies and nuclear chromatin to assume the form of a reticulum. This reticulum is identical with Möllgaard's reticulum, or 'glia-network.'

3. Möllgaard's reticulum is produced during the process of freezing, and is due to the displacement incurred by the enlarging and expanding ice-masses which form in the cell or tissue at the reduced temperature.

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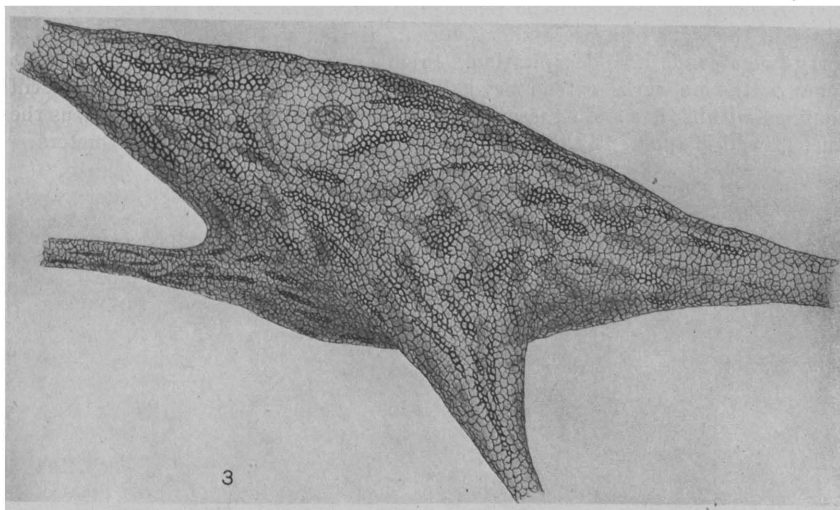
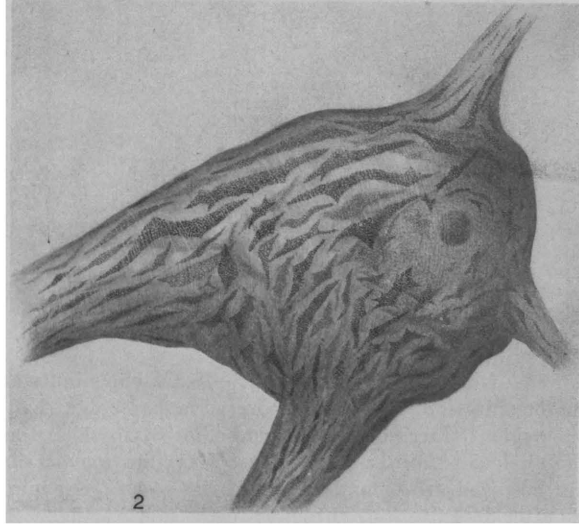
## PLATE 1

## EXPLANATION OF FIGURES

Figures 2 to 6 were all drawn with the aid of a camera lucida. Figures 2 to 5 are anterior horn cells of spinal cord of the dog, from smears fixed in 96 per cent alcohol and stained with erythrosin and toluidin-blue. In the case of the frozen preparations only the blue-staining reticulum is represented, with this exception, that in figure 5 a small portion of the pink-staining extracellular reticulum is represented to show its continuity with the blue network. The individual differences will be noted in connection with the separate legend for each figure.

2 Anterior horn cell of an unfrozen smear, fixed twenty-five seconds after decapitation. The Nissl's bodies are distinctly present. Magnification 750 diameters.

3 Anterior horn cell, frozen and fixed at about  $-20^{\circ}\text{C}$ ., seventy-four seconds after decapitation. The figure represents one of the early transition forms, the Nissl's bodies appearing as minute networks loosely joined together. Magnification 750 diameters.



## PLATE 2

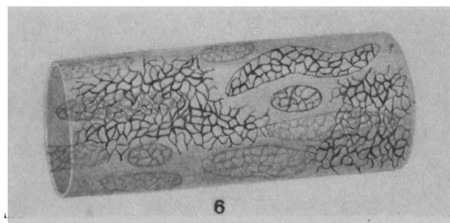
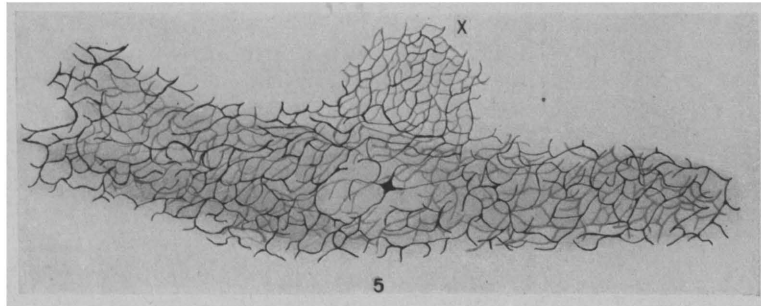
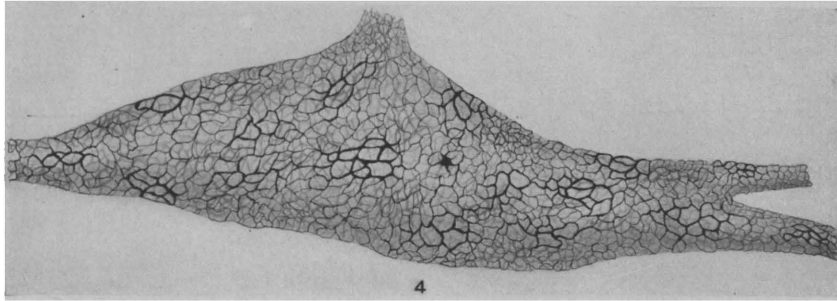
### EXPLANATION OF FIGURES

4 Anterior cell, frozen and fixed at about  $-25^{\circ}\text{C}$ ., one minute and forty-three seconds after decapitation. This is a more advanced stage than figure 3, but there are still evident traces of the Nissl's bodies from which the network was derived. The nucleus is faintly discernible. Magnification 400 diameters.

5 Anterior horn cell, frozen and fixed at about  $-25^{\circ}\text{C}$ ., one minute and forty-three seconds after decapitation. From the same smear as figure 4. This represents one of the extreme forms in which practically all evidence of the former Nissl's bodies is lost. In the area marked X there is observed a small portion of the extracellular pink-staining network which is continuous with the intracellular blue-staining reticulum. This area in this figure is the only place where any of the pink-staining network is shown. The nucleus is represented by the area having but few meshes. The nucleolus is much distorted. Magnification 600 diameters.

6 A somewhat diagrammatic representation of Möllgaard's 'perivascular network' in the walls of a precapillary vessel. Frozen and fixed at about  $-40^{\circ}\text{C}$ ., forty-two seconds after decapitation. From a smear of neural tissue. Note that some of the networks extend beyond the nuclear walls while others are still confined within them. In those extending beyond the limits of the nucleus the nuclear wall is apparently lost in the network. Magnification 1000 diameters









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