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COMPARATIVE STUDY OF RECOGNIZED (CLASSIC) AND MODIFIED IMPREGNATION METHODS ON THE GANGLIONS OF *ANODONTA CYGNEA* L.

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For the examination of the nervous system of higher animals a number of useful silvering methods were developed. A serious drawback of the majority of recognized methods is that they cannot be applied to lower animals. This contributed to the fact that morphological research work on the nervous system of invertebrates that set on late in the past century came to a deadlock. Recently, the study of the nervous system of the lower animals was thrust again in the foreground since as a consequence of its comparatively simpler structure it proved a suitable test objective for neurophysiologists. This involves a supervision of cytological data demonstrated with primitive methods and described incompletely in the majority of cases. It is not due to mere chance that in these last years more and more nerve impregnation methods were described which can be used in molluscs (PALMGREN 1948, BETCHAKU 1960, MILDRED 1962, ROWELL 1963). However, for instance the impregnation of the nervous system of *Aplysia californica* with the method of PALMGREN (1948) and BETCHAKU (1960) can not be termed as successful (MILDRED 1962). ÁBRAHÁM and MINKER (1957, 1959) dealt with the impregnation of the adductor muscle of Lamellibranchiata but in the sources available no reference to the silver staining of the ganglions of the mussels was found.

The microstructure of the nervous system of Lamellibranchiata is insufficiently known and also for the ganglions of the fresh water mussel only few data are available which point to other directions (HANSTRÖM 1928, NAGY 1962, BARANYI—SALÁNKI 1963).

As the first step of our work we considered the development of the silvering procedure to be applied on the nervous system of the mussels the results of which are reported in the present communication.

Material and methods

The subject of the present study were the ganglions of the fresh water mussel *Anodonta cygnea*. The mussels were collected in the river Hortobágy and elaborated partly immediately, partly after a storage for considerable time in running Balaton water.

The more rapid freezing procedures, the silvering methods of the paraffin sections and the total (block) impregnation methods suitable for serial section were tested.

From the silvering methods of frozen sections the shortened method of JABONERO and the methods of JABONERO—BIELSCHOWSKY, CAJAL, BIELSCHOWSKY, BIELSCHOWSKY—ÁBRAHÁM and CAUNA were dealt with.

From the impregnation methods of paraffin sections that of ROWELL (1963) and from the total silvering processes the CAJAL I., CAJAL IV., WEBER, GOLGI's rapid and CAJAL—FAWORSKY's were given a trial.

The above methods were realized according to their original description. Since even after repeated trial none of the methods gave a satisfactory result we applied modifications to those which already according to the basic process offered some hope.

The modifications did not essentially differ from the specified methods (except for the method CAJAL I), they were related only to the $AgNO_3$ concentration, the period of incubation and to the reduction. For the paraffin section silvering method of ROWELL the pH-conditions were changed, while in the CAJAL I block impregnation method a pre-treatment with various metallic salts was applied. The detailed realization of the modifications is treated for easier survey together with the results.

Results

I. RESULTS OF ORIGINAL METHODS

A) *Impregnation of frozen sections*

Fixation, sectioning, washing, silvering, reduction etc. was performed according to ROMEIS (1948).

The BIELSCHOWSKY, JABONERO—BIELSCHOWSKY and JABONERO methods proved to be unsuccessful, so we did not try to modify them.

Some results were obtained with the BIELSCHOWSKY—ÁBRAHÁM, CAJAL and CAUNA methods. It should be noted that these methods even after repeated trials gave poor results. Even though specifications were most accurately adhered to, the microscopical picture of the sections did not improve. Therefore in contrast to the description we applied modifications to several processes (see later).

B) *Impregnation of paraffin material*

The application according to specifications of ROWELL's method which worked well with insects and can be also used for *Octopus* gave poor results in the case of *Anodonta* ganglions. It is assumed that the result obtained with mussels is poorer than that obtained by ROWELL with other groups of animals although it was not possible to compare our sections with those of this author who did not publish microphotograms in his article.

C) *Block impregnation methods*

Their advantage as against the section-silvering processes is that they are suited for series sectioning. We dealt in some detail with the modification of the CAJAL I., CAJAL—FAWORSKY, GOLGI rapid and WEBER methods.

II. THE RESULTS OF MODIFIED METHODS

A) *The modification of frozen section methods*

In impregnation according to CAJAL in contrast to the specifications the pyridine silvering was carried out, after neutral formaline fixation for 2 weeks, sectioning and washing, in the following $AgNO_3$-solutions: 1%, 2%, 4%, 8% and 10% silvernitrate 4 to 5 days in the dark. To the silver nitrate solutions we added amounts of pyridine and alcohol in conformity with specifications. In some cases instead of pyridine, dimethyl pyridine (lutidine) was used. The incubation temperature was 18 to 20 and 35 to 36 °C respectively. At higher temperatures according to our previous experience the nerve cells showed strong deformation. At lower temperatures mediocre staining of the ganglions was obtained in silvered sections *(Fig. 1)*.

Contrary to the description of the BIELSCHOWSKY—ÁBRAHÁM method (ÁBRAHÁM 1951) several processes were carried out with modifications. Prior to fixation the ganglions were placed in AFA for 1 hour* and subsequently in 10% neutral formol for 30 days. Formol was changed every 5 days. More essential changes were applied in impregnation. The substance fixed accordingly to the method was incubated in silver nitrate of different concentration at different temperatures.

A wide range of changing the $AgNO_3$ and incubation period was applied without success. The neurons at the periphery of the ganglions and even the fibres became hardly visible. The microscopic picture of the sections was not improved even if reduction was carried out in a more concentrated formol solution contrary to specifications.

Impregnation according to CAUNA (1956) is similar to the BIELSCHOWSKY—ÁBRAHÁM method, with differences in the silver nitrate concentration, in the period of impregnation, in the formol treatment and in the absence of glacial acetic acid rinsing. In general it is a simpler and quicker method. We have dealt for the most time with this method and obtained our test preparations of fibres with CAUNA's method *(Fig. 2)*. The impregnation according to CAUNA as it can be seen on the picture, is eminently suited to make visible the nerve fibres in the ganglions. The most successful preparations were obtained by the following prescription:

Preliminary fixation in AFA 1 hour.
Fixation in neutral formol 1 day at 20 °C in the dark.
Washing 1 day in distilled water.
Sectioning. — Washing for 1 hour, in distilled water.
Silvering in 10% $AgNO_3$ for 15 minutes in the dark.
Washing in distilled water for 10 minutes.
Formol treatment in a bath changed four times:

1. in 1% neutral formol 4 minutes
2. in 3% ,, ,, 4 ,,
3. in 3% ,, ,, 4 ,,
4. in 5% ,, ,, 3 ,,

* The composition of AFA: identical amounts of saturated arsenic acid, neutral formol, 96% alcohol.

Washing in distilled water 5 minutes.
Ammonium silvering 8 to 10 minutes.
Formol treatment in a bath changed several times:

1. 50 cu.cm distilled water + 2 drops neutral formol 1 min.
2. 0.5% neutral formol 3 minutes
3. 3 % ,, ,, 3 ,,

Washing in distilled water 2 min., in well water 25 to 30, in distilled water 2 min.

Preservation in the usual way.

B) *Impregnation of paraffin section*

With the ROWELL (1963) method — as referred to above — we did not succeed, when applying the specifications, to make visible the nerve elements in a satisfactory way *(Fig. 3)*. The impregnation of these sections was poor. The nerve elements, mainly the cell body is hardly visible and therefore unsuited for morphological examination.

The fixation of the ganglions was carried out in the usual way in CARNOY, FLEMMING, SUSA, BOUIN formol fixing solution in the usual way. The further treatment of the test material revealed that the CARNOY and formol fixations are best of all.

The sections were placed in 20 per cent $AgNO_3$ for 1 hour and subsequently in dimethylpyridine bath for 17 hours. The solution was prepared according to specifications (1% $AgNO_3$ 20 ml, dimethylpyridine 10 ml, distilled water 250 ml). ROWELL has buffered this solution for *Octopus* with a mixture of M/5 boric acid and M/20 borax to 7.6 pH. For the ganglions of *Anodonta cygnea* the silver nitrate with pyridine was established at 7, 7.6, 8 and 9 pH The repeated tests evidenced that the solution of dimethylpyridine and $AgNO_3$ established at 7 pH was the best.

The further treatment was performed according to the description of ROWELL. With after-gilding we succeeded in improving the quality of the sections *(Fig. 4)*. The comparison of the *microphotograms 3* and *4* makes it clear that in the examination of mussel-ganglions our modification of ROWELL's method supplies a better result.

C) *Block-impregnation methods*

With the CAJAL IV. method described on p. 428 of the book of ROMEIS (1949) impregnation of the mussel ganglions was very poor and therefore we have changed the fixation and silvering period of the substance. Formol fixation was performed for 3, 6, 10, 12 and 16 days. Continuing the treatment of the substances fixed for different periods according to specifications, the results were invariably unsatisfactory. Then we tried to change the period and temperature of impregnation. The material was kept in the 1.5 per cent $AgNO_3$ solution for 3—6—8 days at room temperature in the dark. The further treatment, paraffin embedding, sectioning, preservation was carried out in the usual way. The 6 day silvering at room temperature proved comparatively suitable for making visible the nerve fibres *(Fig. 5)*.

The Cajal I. method

From the CAJAL block impregnation methods also method No. I (ROMEIS 1789.) is unsuited for the purpose of making visible the ganglion cells and nerve fibres of the fresh water mussel. As in the literature data are found according to which preliminary treatment with some metallic salts improves the results of impregnation (MILDRED 1962) we tested the CAJAL I method after preliminary treatment for 24 hours in 2 to 4% $CuCl_2$, 2 to 4% $FeSO_4$, 1% $Pb(NO_3)_2$, 10% $FeCl_3$, 10% $FeNH_4(SO_4)_2$, $12H_2O$ solutions. From these solutions we obtained satisfactory results with $FeSO_4$. The march of the process is the following:

1. "Fixation" in 2 to 4 per cent $FeSO_4$ solution to 20 ml of which 1 drop glacial acetic acid was added.
2. Washing in distilled water 3 · 10′.
3. In 5 per cent $AgNO_3$ for 3 to 5 days at 37 °C or at room temperature in the dark.
4. 1 minute washing in distilled water.
5. Reduction in the mixture of 1 g hydrochinon, 5 ml formol, 100 ml distilled water for 24 hours in dark, at 37 °C or at room temperature.
6. Washing in 5′ distilled water.
7. Dehydration in alcohol series 2 · 10′ per grade.
8. Paraffin-embedding.
9. Sectioning, deparaffination.
10. In 0.2 per cent $AuCl_2$ 15′.
11. 30′ well water washing.
12. Dehydration, covering with balsam. The section attains its final colour about a day after covering. The result after this proceeding is satisfactory *(Fig. 6)*. The nerve cells, nuclei and the nucleolus are well visible, also the nerve fibres in the neuropile, the glia cells are to be recognized. The concentration of $FeSO_4$ can not be raised because *e.g.* at 10 per cent already strong shrikings arise evidently because of the hypertonic character of the solution. This CAJAL I. method as modified by us is thus well suited for the cytological examination of the ganglions of *Anodonta cygnea*.

Evaluation of results

According to our objective by the modification of the recognized procedures we succeeded in finding impregnation methods that can be applied to the ganglions of *Anodonta cygnea*.

Among the frozen sections the modification of *Cauna*'s method proved to be most suitable.

For the impregnation of the paraffin section the modified form of ROWELL's silvering method supplies satisfactory results.

From the block-impregnation methods the modification of CAJAL I is suitable for the impregnation both of the nerve cells and the appendices. The modification of the CAJAL IV process is well suited for the impregnation of the fibres within the ganglions. A number of literary data point to the fact that in the lower animals generally the application of $AgNO_3$ of higher concentration is successful. In our experiments in the case of several methods in several cases we made the experience that better results are obtained with silver nitrate of lower concentration.

Summary

Authors have silvered the ganglions of *Anodonta cygnea* L. with several generally known impregnation methods. The more rapid freezing procedures, the silvering processes of paraffin sections and the total impregnation methods suited for series sectioning were tested.

From the silvering methods of the frozen sections the shortened JABONERO the JABONERO—BIELSCHOWSKY, CAJAL, BIELSCHOWSKY, BIELSCHOWSKY—ÁBRAHÁM and CAUNA methods, from the impregnation methodics of paraffin sections the CAJAL I., CAJAL IV., WEBER, GOLGI quick, CAJAL—FAWORSKY methods were tried out.

The above methods were repeatedly tested according to specifications but none of them gave satisfactory results. Therefore several impregnation methods were modified by the authors.

In the individual methods the $AgNO_3$ concentration, the incubation period and the reduction process was changed. In the ROWELL method the pH of the demethylpyridine silver nitrate was modified while with the CAJAL I method $FeSO_4$ preliminary treatment was applied.

The impregnation of the frozen sections with the modification of CAJAL's process is moderately suited *(Fig. 1)* to make the fibres visible while the modified CAUNA method is excellently suited *(Fig. 2)*. The ROWELL method in its original form silvers the nerve cells of the mussel ganglions not at all, while the fibres very poorly *(Fig. 3)*; but the nerve fibres of ganglions silvered according to the authors modification resulted in beautiful preparations *(Fig. 4)*.

From the block impregnation methods CAJAL IV in its modified form is suited for the fibres to be made visible *(Fig. 5)* while CAJAL I. after $FeSO_4$ pretreatment proved to be excellent both for the impregnation of ganglion cells and appendices.

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KLASSZIKUS ÉS MÓDOSITOTT IMPREGNÁCIÓS MÓDSZEREK ÖSSZEHASONLITÓ VIZSGÁLATA *ANODONTA CYGNEA* L. GANGLIONJÁN

Gubicza András és Zs.-Nagy Imre

Összefoglalás

A szerzők az *Anodonta cygnea* L. ganglionjait több általánosan ismert impregnálási módszerrel ezüstözték. Kipróbálták a gyorsabb fagyasztott eljárásokat, a paraffinos metszetek ezüstözési eljárásait és a sorozatmetszésre alkalmas totál impregnálási módszereket.

A fagyasztott metszetek ezüstözési eljárásai közül a JABONERO rövidített, JABONERO—BIELSCHOWSKY, CAJAL, BIELSCHOWSKY, BIELSCHOWSKI—ÁBRAHÁM és a CAUNA-féle módszereket, a paraffinos metszetek impregnálási metodikák közül a CAJAL I., CAJAL IV., WEBER, GOLGI gyors, CAJAL—FAWORSKY módszereket próbálták ki.

A fenti módszereket a leírások szerint többször megismételték, de kielégítő eredményt egyik sem adott. Ezért több impregnálási eljárást módosítottak.

Az egyes módszereknél az $AgNO_3$ koncentrációját, az inkubációs időt és a redukálási folyamatot változtatták meg. A ROWELL-féle módszernél a dimethyl-piridines ezüstnitrát pH-ját módosították, a CAJAL I. módszernél pedig $FeSO_4$-os előkezelést alkalmaztak.

A fagyasztott metszeteki mpregnálása a CAJAL-féle eljárás módosításával a rostok feltüntetésére közepesen *(1. ábra)*, a CAUNA módosított módszer pedig kiválóan alkalmas *(2. ábra)*. A ROWELL-féle módszer eredeti formában a kagylók ducainak idegsejtjeit nem, a rostokat pedig rosszul ezüstözi *(3. ábra)*, míg a szerzők módosítása szerint ezüstözött ganglionok idegrostjai szép preparátumokat eredményeztek *(4. ábra)*.

A blokk-impregnációs módszerek közül a CAJAL IV. módosított formában a rostok feltüntetésére alkalmas *(5. ábra)* a CAJAL I. pedig Fe SO_4 előkezelés után a ducsejtek és nyulványok impregnálására egyaránt kitűnőnek bizonyult.

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ МЕТОДОВ ИМПРЕГНАЦИИ НА ГАНГЛИЯХ БЕЗЗУБКИ *(Anodonta cygnea L.)*

А. Губица и И. Ж.-Надь

Авторы окрашивали серебром ганглии беззубки по нескольким общеизвестным методам. Были применены метод быстрого замороженния, окрашивание серебром парафиновых срезов и методы общей импрегнации, пригодные для серийных срезов.

Для окрашивания серебром замороженных срезов были применены методы Jabonero, Jabonero—Bielschowsky, Cajal, Bielschowsky, Bielschovsky—Ábrahám, Cauna, для импрегнации парафиновых срезов употребляли методы Cajal I., Cajal IV., Weber, Cajal—Faworsky и быстрый метод Golgi.

Описанные выше методы были повторены многократно, но не давали удовлетворительного результата. Поэтому пришлось видоизменять несколько методов для импрегнации.

Видоизменение отдельных методов состояло в изменении концентрации $AgNO_3$, времени инкубации и процесса восстановления. В методе Rowell изменяли pH диметил-пиридиновый раствор ANO_3, а в методе Cajal I. применяли предварительную обратку $FeSO_4$.

При импрегнации замороженных срезов для выявления волокон видоизмененный метод Caina-a дает средние результаты (рис. 2). Исходный метод Rowell-a не окрашивает совсем нервные клетки ганглиев беззубки и волокна тоже окрашиваются очень слабо. (рис. 3), а видоизмененный авторами метод дает хорошо окрашенные срезы (рис. 4).

Из методов блок-импрегнации метод Cajal IV., в видоизмененной форме пригоден для выявления волокон (рис. 5), а метод Cajal I., после предварительного применения $FeSO_4$ успешно используется для импрегнации и клеток и их отростков в ганглиях.

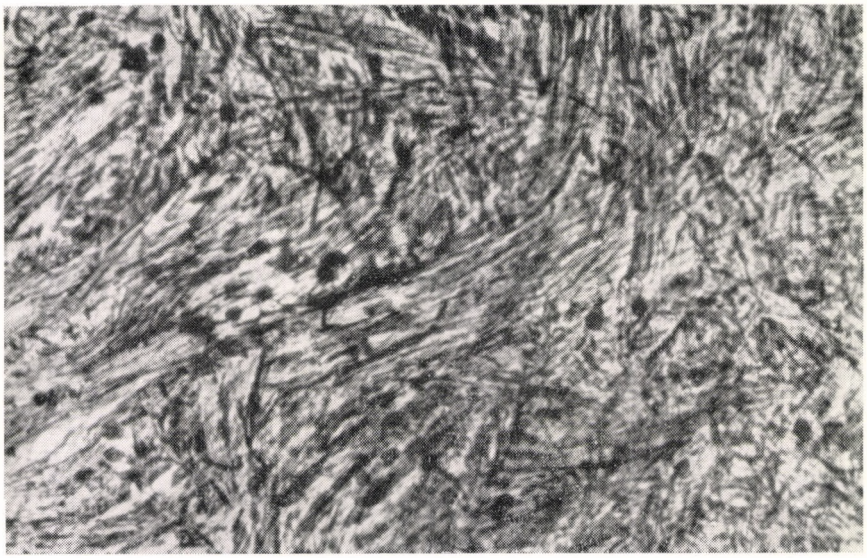

Fig. 1. The result of CAJAL's modified frozen section method in the neuropile of ganglion pedale. Incubation at 20° C with 4 per cent $AgNO_3$ concentration. 378× enlargement.

1. ábra. Módosított CAJAL-féle fagyasztott metszet eljárás eredménye ganglion pedale neuropiljében, 20° C-on inkubálva 4%-os $AgNO_3$ koncentráció mellett. 378× nagyítás

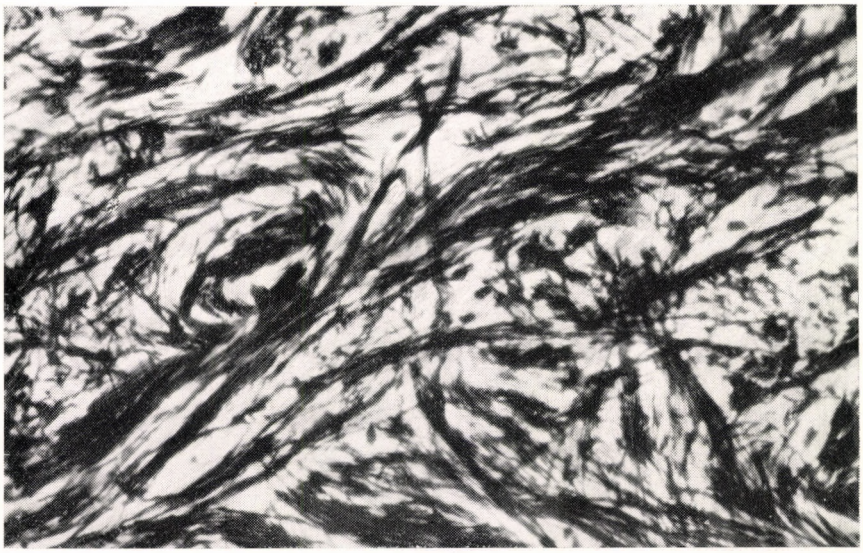

Fig. 2. The neuropile of ganglion pedale silver stained with the modified CAUNA method. 378× enlargement

2. ábra. Ganglion pedale neuropilje módosított CAUNA-eljárással, ezüstözve. 378× nagyítás

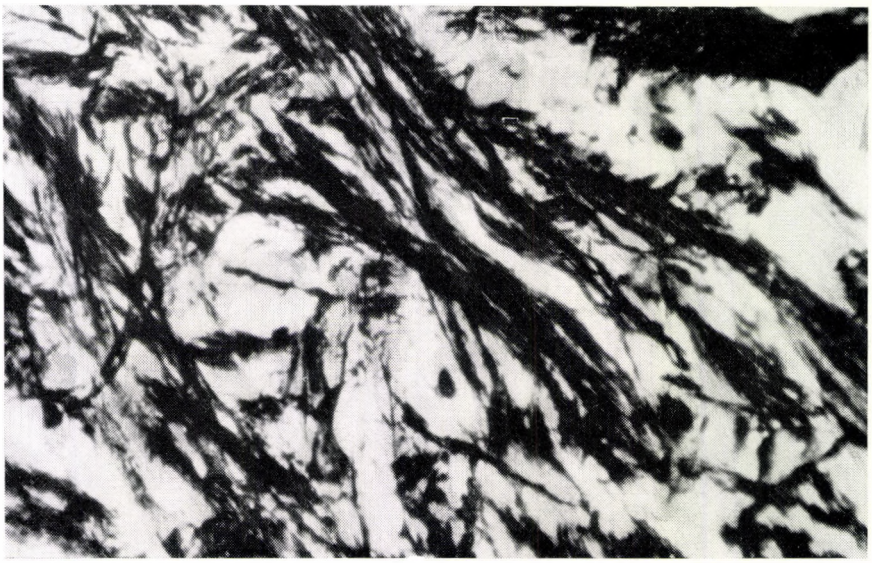

Fig. 3. Neuropile of ganglion cerebrale impregnated according to ROWELL's original method. The result is not satisfactory. 378× enlargement

3. ábra. ROWELL eredeti módszere szerint impregnált ganglion cerebrale neuropilje. Az eredmény nem kielégítő. 378× nagyítás

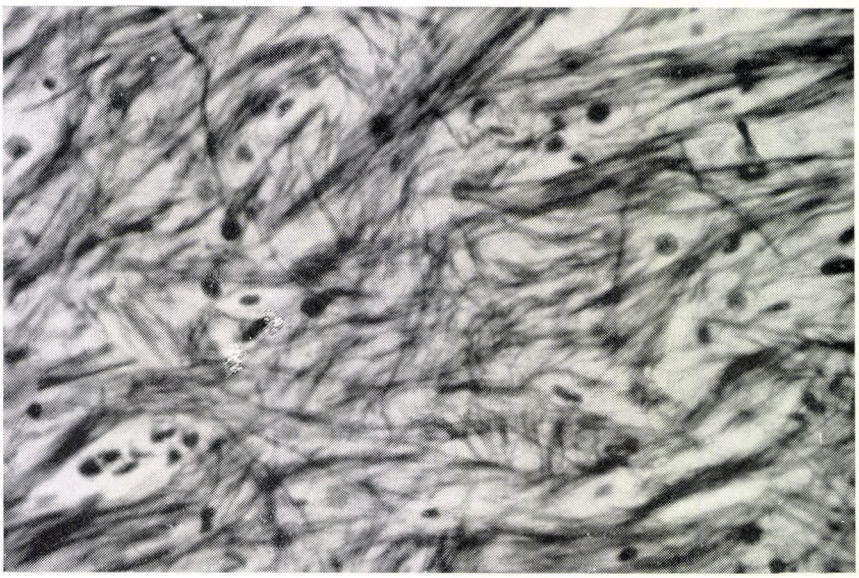

Fig. 4. The result of the modified ROWELL method in the neuropile of ganglion viscerale. Impregnation was performed at pH 7.0, also after-gilding was applied. 378× enlargement

4. ábra. Módosított ROWELL módszer eredménye ganglion viscerale neuropiljában. Az impregnálás pH 7,0 mellett történt, utóaranyozást is alkalmaztunk. 378× nagyítás

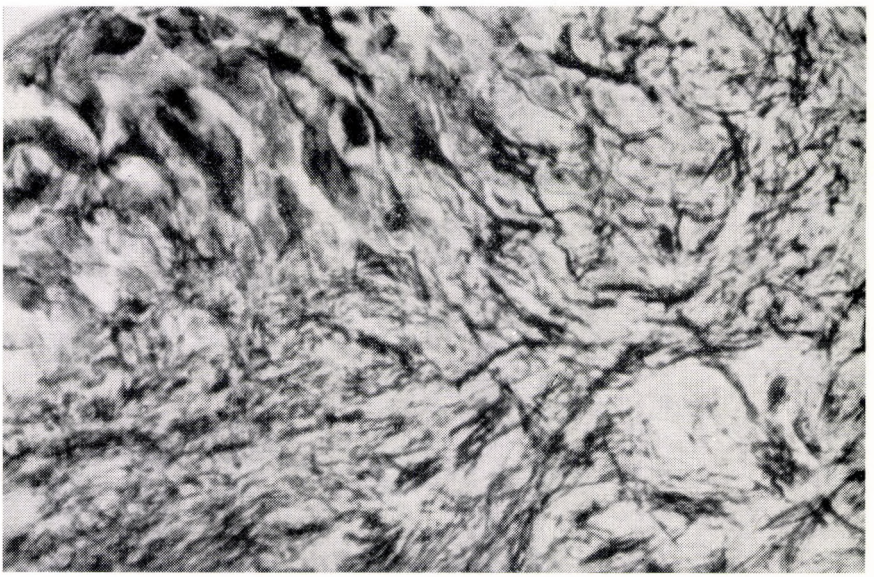

Fig. 5. Edge part of ganglion cerebrale silverstained according to the CAJAL IV. method. Incubation in 1.5 per cent $AgNO_3$ at room temperature for 6 days. The result is medium for fibres and definitely poor for cells. 378× enlargement

5. ábra. Ganglion cerebrale széli része módosított CAJAL IV. módszerrel ezüstözve. Inkubálás 1,5%-os $AgNO_3$-ban szobahőn 6 napig. Az eredmény rostokra nézve közepes, sejtekre vonatkozóan rossz. 378× nagyítás

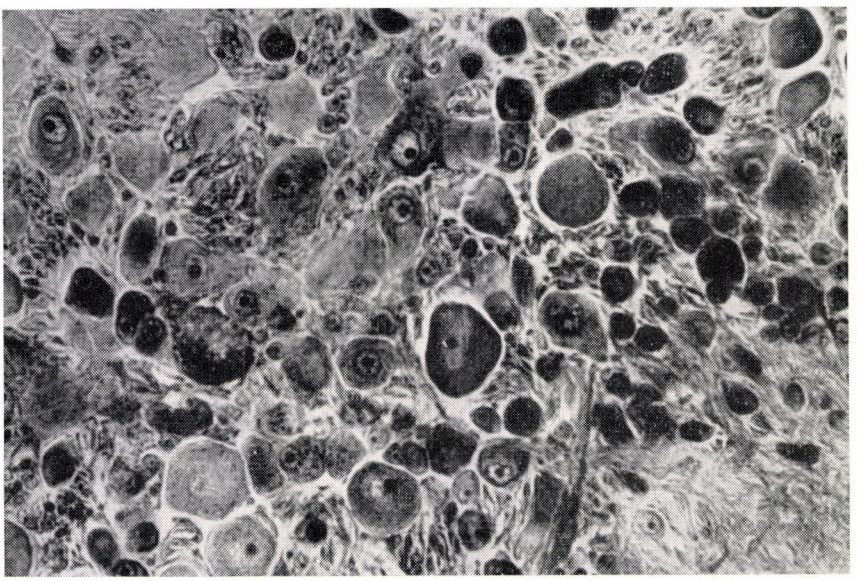

Fig. 6. The result of the CAJAL I. method modified by ferrosulphate pretreatment and after gilding at the edge part of ganglion viscerale. The result makes this method apt for cytological examinations. 378× enlargement

6. ábra. Ferroszulfátos előkezeléssel és utóaranyozással módosított CAJAL I. módszer eredménye ganglion viscerale széli részén. Az eredmény alkalmassá teszi ezt a módszert citológiai vizsgálatokra. 378× nagyítás