MODIFICATION OF NAUTA'S METHOD

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Nauta's method of silver impregnation of degenerated axons was first described in 1952 [1]. Subsequently, several modifications have been developed (celloidin, paraffin).

The method attracted the attention of investigators because it allowed degenerated fibers to be selectively demonstrated along the whole of the path to the site of damage. However, it had certain shortcomings which limited its utilization and affected the results adversely. In the method described by the author the effects due to illumination and temperature of the room were not excluded, so that there might be a variation from day to day in the intensity of impregnation. In the author's recipe the stage of passing the material through ammoniacal silver is particularly capricious and causes variation of impregnation from one section to another in a series; test sections must be tried each day to determine the optimal time.

A shortcoming of Nauta's method for the study of serial section of the brain is the limitation on the number of sections which can be handled; according to their size, the number is limited to from 5 to 20 [2]. In a working day, even with highly qualified technicians, not more than 20-30 sections can be impregnated.

We here described the results of an attempt to improve the method and to increase the output.

Even the first tests showed that the rate at which the brain sections react in the ammonical silver solution varies greatly with time: it is relatively high at the moment the ammonical silver solution is prepared (immediately before use), but falls, at first rapidly and then more slowly. According to Nauta's recipe, the impregnation in ammoniacal silver is carried out at the time that the reaction is slowing down. Naturally, as the velocity of the reaction falls the time required for treatment of the sections is correspondingly increased, and depends upon the temperature of the solution. The result is that the time for which the sections must remain in the solution has to be determined experimentally.

The nature of the impregnation is influenced greatly according to whether or not the reaction of the sections is carried out at the time the ammoniacal silver solution is prepared, when it is comparatively insensitive to the influence of room temperature.

There is a difference of 6-8 times in the rate of the reaction on the steep or slow portions of the curve. Because on the shallow portion the reaction is very much less vigorous it does not greatly affect the degree of impregnation already effected on the steep part.

Many experiments with Nauta's impregnation method confirmed completely these ideas as affecting the quality of the sections. Because impregnation of the sections in ammoniacal silver was almost independent of the time and of a variation of room temperature, a large number could be treated simultaneously.

The proposed modification was confirmed on an extensive experimental material (cats and rabbits) and in all cases it gave good results.

The brain was first fixed intravitally by perfusion through the heart and aorta with 10% neutral formol in physiological saline. The vascular system had first been washed out with physiological saline. The brain was fixed in 10% neutral formol for not less than two weeks. On the day before staining the blocks of cerebral tissue were cut on a freezing microtome into sections 30 μ thick. The serial sections in 10% neutral formol and physiological saline were placed for the night in a refrigerator at -5° as recommended by Nauta. To pass the whole series of sections through

the solutions it is best to use 150 ml glass vessels. For the cerebral hemispheres of the cat 100 ml of each solution is sufficient. With this amount 70-80 sections can be treated simultaneously. The sections are most conveniently taken through the solutions by means of a small piece of gauze through which the excess solution is strained off, before the sections are transferred to the next vessel. In this way they are prevented from breakage, and there is a great economy of time because several series of sections can be passed through the solutions in parallel.

To prevent contamination several pieces of gauze should be used which are changed as the sections pass through the solutions. All the pieces of gauze are kept in glass vessels containing distilled water, and they may be used several times.

All the reactions, particularly with the silver solutions, are carried out in a clean vessel to avoid contaminants causing precipitation, and the used vessel may be employed without further washing out for further treatment of the sections with the same solutions; vessels containing ammoniacal silver are an exception and must be washed free from alkali in running water each time, and then in distilled water.

The passage through the solution is carried out as follows:

1. The sections are washed free from formalin three times and then treated successively with a 0.5% solution of phosphomolybdic acid for 45 min, with an 0.1:200 potassium permanganate solution for 5-10 min, and are then bleached in a 1% solution of hydroquinone and oxalic acid (previously mixed in equal volumes, as recommended by Nauta). In between treatments with each of these solutions it is recommended that the sections be washed in distilled water.

In summer the sections should remain in the 1.5% silver nitrate solution for 35 min, and in winter for 45 min. The silver nitrate solution should remain completely clear after the sections have been placed in it. It must be replaced if there is even the slightest turbidity.

2. The passage through ammoniacal silver is made in a chemically pure 300 ml vessel and in a small flask. A mixture consisting of 60 ml distilled water, 30 ml absolute alcohol, and 3.0 g of silver nitrate is placed in the vessel.

Sections from the silver nitrate solution are washed briefly in water and then transferred to the vessel. The following mixture is made up and placed in the flask: 5-8 ml of a 25% concentrated solution of ammonia and 4.5 ml of 2.5% caustic soda solution.

After lightly shaking the vessel containing the sections the contents of the flask are quickly emptied into it. The whole mixture then becomes turbid, takes on a muddy appearance, and then becomes clear. When the passage through the ammoniacal silver is made in this way impregnation of the sections is complete after 15-25 sec. It is recommended that the sections should remain in ammoniacal silver until the color of the solution changes from clear to a slightly pinky shade; subsequently the solution begins to darken, which indicates that too long a time has elapsed.

The amount of concentrated ammoniacal solution necessary for the optimal reaction of the sections in ammoniacal silver must be accurately determined by titration for each reagent as it is obtained from store. Failure to observe this condition may result in failure of impregnation, because of the degree of ionization of this reagent varying according to the solution, so that the optimal equilibrium of ions for the formation of ammoniacal silver may be disturbed when the solutions are mixed.

In the present investigation, when one part of the concentrated ammonia was used for optimal results, we required to measure out 7.5 ml of it into the flask; however, when the other part of the reagent was used 5.4 ml were required. When there is insufficient ammonium in the solution instead of the formation of ammoniacal silver, a dark precipitate of silver oxide is formed, and with an excess of ammonia the ammoniacal silver solution for long fails to turn pink and does not darken. In both cases there is practically no impregnation.

3. The sections are transferred rapidly into the following solutions recommended by Nauta: distilled water 400 ml, absolute alcohol 45 ml, 10% acid formol 13.5 ml, 1% citric acid 13.5 ml.

In this solution the sections acquire their final golden color, which is noticeably enhanced after subsequent treatment of the sections with a 1% thiosulfate solution; before immersion of the sections in this solution they should be briefly washed in water.

4. The sections are washed three times in water.

5. The sections are attached to glass with gelatine in the usual way, and after dehydration in alcohols and clearing in xylol they are placed in balsam under a cover slip.

If before sectioning the brain was embedded in gelatine, the stage between washing the sections in 1.5% silver nitrate and ammoniacal silver solution should be replaced by a treatment in a solution consisting of 1.5 ml concentrated ammonia and 100 ml water; if this is not done silver oxide may precipitate from the ammoniacal silver, and impregnation will be impaired.

The real advantage of our modification of Nauta's method is the consistency of the results, the high yield, and the considerable economy of silver. The sections of the cerebral hemispheres of a cat can be treated and mounted under cover slips in a single day.

SUMMARY

A modification of the Nauta method of impregnation of degenerated axons is described. With this method, the quality of axon impregnation depends upon a number of factors which can only be determined experimentally during staining. On this account impregnation may be unsuccessful. The Nauta method is not applicable to a study of serial brain sections because of the comparatively small number of sections which can be treated. The essential advantages of the described modification are the consistency of the results, the greater number of sections which can be handled, and the considerable economy of silver.

LITERATURE CITED

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2. W. Nauta. Selective silver impregnation of degenerating axons in the central nervous system, Stain Technology, 1954, Vol. 29, No. 2, p. 91.