A SILVER - STARCH - GELATINE METHOD FOR THE DEMONSTRATION OF SPIROCHETES IN SINGLE TISSUE SECTIONS*

By ALDRED SCOTT WARTHIN, Ph.D., M.D., LL.D., Professor of Pathology in the University of Michigan, Ann Arbor.

In 1920 and 1921, Warthin and Starry published their two silver-agar methods for the demonstrations of Spirocheta pallida in single tissue sections. Since 1921 the second of these methods has been in constant use in this laboratory, where it has been considered a great improvement over all other existing methods in its great saving of time over the Levaditi and allied tissue-block methods, in the correlation of histologic lesion and spirochete localisation afforded by it, and in that in our hands we have been able to obtain by its use a much higher percentage of positive findings of spirochetes than by any other method. Nevertheless, we have not remained satisfied with this method, because of the fact that the results obtained by it are not always uniform, although the working conditions may have appeared to be identical. In certain material showing characteristic syphilitic lesions repeated staining would bring only negative results, or after repeated failures a given section might turn up filled with beautifully stained spirochetes. A rough calculation of our results might be given as 50 per cent. positive spirochete demonstration in histologic lesions of syphilis on first attempt. On repeated attempts and with slight variations of the method the positive correlation of spirochete and histologic lesion would be raised to 65–70 per cent. of cases. This is too far from being a perfect method, particularly when it is considered that our positive results run higher than those obtained by the same method in less expert hands. The apparently capricious results have been studied, and an attempt

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made to ascertain the reasons for them. In this we have been partly successful, and in the modification of our method herein presented we feel that we have a method that will give a much larger percentage of positive findings than any other one yet devised. At least, such has been our experience with it, but we do not claim for it a 100 per cent. result.

We have found that one of the most important factors in the reduction of silver on the spirochete is the hydrogen-ion concentration of the tissue. The silver impregnation for *Spirocheta pallida* is by no means a specific reaction. There is apparently no chemical affinity between the spirochete and the silver nitrate. The precipitation of the reduced silver on the organism depends on the properties of the tissue, of which the most important is the hydrogen-ion concentration. The spirochete plays the part of a foreign body, differing materially from the tissue of its host, so that it is possible under favourable conditions to bring about a silver precipitation more rapidly on the parasite than on its surroundings, and thus make possible its demonstration within the tissue. In the
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Warthin-Starry method it was demonstrated that the use of the double cover-glasses permitted only a minimum of contact between the silver nitrate and the section, and that in this way an entirely different effect was produced than when the tissue is given a greater degree of exposure to the silver nitrate solution. The obvious cause for this is that the tissue is changed in proportion to the amount of fluid with which it comes into contact, and if the reaction in the solution is less favourable than that of the tissue, the results will be less favourable. Therefore the

Fig. 2.—Congenital Syphilis of the Liver. Silver-starch-gelatine method. × 2500.

use of the double cover-slip method gives a very great advantage.

The size of the colloidal particles of the reduced silver nitrate varies with changes in the hydrogen-ion concentration of the tissue and the silver nitrate solution. Most methods demand the use of neutral formaldehyde in the fixation of the tissues, as it has been demonstrated by poor results that increased or decreased acidity produces a different reaction in the tissue to the silver nitrate which is unfavourable. One week's failure of staining was found to be due to an acidity of the distilled water used in the laboratory. Not only the hydrogen-ion concentration causes variations in the precipitate of silver

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upon the organism, but also the temperature, light exposure, impurities that may be present, and the length of the interval between the death of the tissue and the beginning of its fixation. All of these factors are of importance, and must be taken into account if uniformly good results are to prevail. In this laboratory the attempt is made to bring all the tissues to the same reactive stage, irrespective of their previous histories, by making the technique as simple as possible, and then

![Image](https://via.placeholder.com/150)

**Fig. 3.—Congenital Syphilis of the Liver. Silver-starch-gelatine method. \( \times 2500 \).**

using dilute nitric acid at the last moment before placing the tissue in the silver nitrate.

The most important effect of the nitric acid is to increase the acidity of the section, so that the precipitation of the silver will not take place so rapidly, and thus allow for a greater degree of differentiation between the organism and the surrounding tissue. The spirochete is evidently less affected by the acid than is the tissue, as the silver precipitates on it while the tissue remains almost colourless. The spirochete plays the *rôle* of a relatively basic object in an acid field, and as hydroquinone acts more rapidly to reduce silver nitrate when the reaction is basic, the result is a rapid precipitation around the spirochetes and a slow one in the tissues. By
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removing the tissue from the solution at the proper time a maximum differentiation can be obtained. Another useful action of the acid is the dissolving out of any inorganic precipitates that may be present which would otherwise cause the silver to precipitate and prevent the staining of the organism. The action of the acid must be prolonged for some time to produce this result, although in single sections it takes place rapidly. Further, the nitric acid acts as a weak oxidising agent to freshen the tissue, this being very important in over-fixed tissues or those which have been kept in alcohol in the laboratory for a very long time. The stronger solutions of the acid recommended, acting from half an hour to an hour, are best suited to obtain this effect. If the action of the acid be unduly prolonged, or if the spirochetes were on the point of breaking up when the tissue was fixed, the spirochetes will be broken up by the acid. While the use of nitric acid favours the precipitation of silver on the spirochetes, the latter will be broken up when over-exposed to the acid reaction. The action of the acid on the tissue itself is less important as far as damaging the tissue is concerned. Over-exposure to the acid will produce a fuzzy appearance, and if the tissue is too acid in reaction the silver will precipitate in larger aggregates, with a marked contrast between the tissue elements entirely different.

Fig. 4.—Syphilis of the Tonsil. Silver-starch-gelatine method. × 1500.
from the homogeneous yellow of the section with the proper acidity. Not all tissues containing spirochetes respond to the same treatment, but the use of nitric acid, as outlined below, permits the one method, the Warthin-Starry, to obtain a higher percentage of positive results than would otherwise be possible, and to do this more readily.

A. For material well fixed shortly after removal from body.
   (1) Remove paraffin from cover-glass preparation, pass through alcohol and water, and place in the oven in about 10 c.c. of 1 per cent. nitric acid for thirty minutes.
   (2) Wash in distilled water for ten to fifteen seconds, and then continue with the modified Warthin-Starry method.

B. For material poorly fixed or showing post-mortem changes.
   (1) Remove paraffin from cover-glass preparation, pass through alcohol and water, and place in 1 per cent. nitric acid for one minute.
   (2) Wash in distilled water for ten to fifteen seconds, and then continue with the modified Warthin-Starry method.

In the case of old tissues much over-fixed in alcohol the following routine may be used:
   (1) Remove the paraffin from the cover-glass preparation, pass through alcohol and water, and place in nitric acid of 2 to 10 per cent. strength for from fifteen to sixty minutes. The greater the acid concentration, the shorter the period of immersion in the acid necessary.
   (2) Wash in distilled water from fifteen seconds to three minutes. The stronger the acid used the longer should be the period of washing.
   (3) Dip in 2 per cent. silver nitrate and proceed with the starch-gelatine method. Longer development may be necessary with the increased acidity of the section, but there results a lighter background, with heavily stained organisms and a cover-glass almost free from precipitates.

This method of "freshening" the tissue has been used by us with great success in stock control tissue that has been kept in the laboratory for over twenty years, in which it was becoming increasingly difficult to obtain well-stained spirochetes. Hydrogen peroxide had been used with varying degrees of success, but at the best was uncertain. By the use of nitric acid as above directed
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beautiful preparations are now easily obtained with this old material. The spirochetes appear intensely black on an almost colourless background. Other material which had been preserved under similar conditions, but not for so long a period, gave even better results. The sections, dried on the cover-glass without the use of albumin fixative, become detached when the stronger acid solutions are used for an hour or longer at a temperature of 30° to 40° C. On the other hand, in dilutions too great the tissues are not sufficiently acted upon by the acid to produce the proper result. In fresh cases fixed for not more than several days and then imbedded in paraffin the acid treatment should not be prolonged in case the fixation was poor or the spirochete on the point of breaking up when fixed, since the action of the acid tends to break up the spirals and give the beaded effect of degenerating spirochetes. In such tissues the sections may be dipped in dilute acid for one minute, and a favourable tissue reaction thus established.

THE STARCH-GELATINE MODIFICATION OF THE WARTHIN-STARRY METHOD

The Warthin-Starry method depended on exposing the section to a minimum amount of silver nitrate and then slowing down the development by the colloidal silver-agar mixture. In order to produce uniform results it is necessary for the technician to practise the method for a long time. The mixture, which is rather complicated, should be made the same every day. The agar must have the gel formation broken up just at the right time when it is cooling, or the consistency will vary. The five different substances used must be kept separate and mixed each time a section is to be developed. Obviously the results will vary considerably with different workers, and with the same worker if inexperienced. In spite of these handicaps, the method when properly employed is very successful, and is widely employed.

In order to overcome these difficulties and in the direction of the simplification of the method various changes in the reducing mixture have been worked out. The agar-agar is replaced by starch, the glycerine by acetone, while the gelatine is still used. Five substances are still used in the developing mixture, but four of these are made
up at once in a homogeneous mixture, and only the fifth has to be added when ready for use. Also this mixture can be kept indefinitely simply by cooling when not in use and warming when ready for action. In addition to the simpler mechanism in technique, the starch provides a gel more perfectly suited to precipitating the silver on the spirochetes. Consequently darker organisms can be obtained against a lighter background, and the search for them is thus made much easier when they are present in small numbers. We have found the starch-gelatine developer to obtain good results in cases in which the spirochetes have either not been stained at all or were very poorly stained by the silver-agar developer, yet when the starch-gelatine mixture was used the organisms stood out beautifully. Further, a very important feature of this modification is that it simplifies an important staining procedure, thus extending its field of usefulness.

**Directions for using the Starch-gelatine Modification of the Warthin-Starry Method of Staining Spirochaeta Pallida in Single Tissue Sections on the Cover-glass, with the Preliminary Nitric Acid Treatment**

1. The tissues should be well fixed in formol, larger pieces requiring more time than smaller ones.
2. Transfer blocks of convenient size to 96 per cent. alcohol for one hour or longer. Follow by three changes of absolute ethyl alcohol for one hour each at a temperature of 50° to 55° C.
3. Run through two changes of xylol for half an hour and an hour respectively at room temperature.
4. Press the xylol out of the tissues on filter paper and pass through two changes of paraffin for half an hour and eight to twelve hours each at 50° to 55° C.
5. Block in paraffin.
6. Cut sections 6 to 10 microns thick and transfer on to water just warm enough to flatten the section without melting the paraffin at about 35° to 40° C. Distilled water free from bacteria must be used, or there will be a precipitate of silver between the tissue and the cover-glass.
7. The perfectly clean cover-slip is then immersed in the water perpendicularly at the edge of the floating section and then lifted out with the section on it. If the glass
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is wholly free from oily contaminations the tissue will adhere to it. No albumin fixative is used. Number 1 cover-slips of suitable dimensions are employed, and with a little practice the sections can be readily centred.

(8) The cover-glass preparations are then allowed to dry for two hours at 55° C., or overnight at 35° C.

(9) The paraffin is removed from the section by flaming slightly and putting through two changes of xylol, two changes of absolute alcohol, 96 per cent. alcohol and water.

(10) The cover-glass preparation may now be treated

with nitric acid solution as described above, or immersed directly in 2 per cent. silver nitrate, and placed face down upon another perfectly clean cover-slip (the tissue then being between the two cover-slips), and the two cover-glasses adherent by capillary attraction are put upright at the edge of a clean bottle containing sufficient silver nitrate solution to cover the lower half of the two cover-slips only. When nitric acid is used the section must be washed as directed in distilled water before it is put into the silver.

(11) The incubation in the silver nitrate solution should be carried out in a dark oven at 50° to 55° C. for from thirty minutes to two hours, depending on the type of

Fig. 5.—Syphilis of Aorta. Silver-starch-gelatine method.  x 2500.
tissue involved. A dense fibrous and elastic section, such as aorta, requires longer impregnation than does one of liver or lymph node. Fœtal tissues and those treated with nitric acid require less exposure to silver nitrate than do those of adults, especially if overfixed.

(12) The opposed cover-slips with the section between them are taken from the silver solution and the cover-glass forceps slipped between them, prying them apart. It is unwise to slide the glasses apart, as the tissue may be injured or partially loosened from the cover to which it is adherent. The cover-glass with the tissue is placed section side uppermost in a watch-glass or other flat container, and the developing mixture, consisting of 1 part of 2 per cent. silver nitrate added to the starch-gelatine-acetone-quinol 5 parts (see below), is poured over it, so that a layer at least 3 mm. thick is over the tissue. The reduction is allowed to proceed until the section is of a medium yellow or pale brown appearance.

(13) The section is then removed from the developer and washed for a few seconds in warm water to ensure removal of the starch and gelatine.
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(14) It is then passed through a 5 per cent. sodium-hyposulphite solution to remove any remaining silver nitrate.

(15) Wash in distilled water, 96 per cent. alcohol, two changes of absolute alcohol, two changes of xylol, and mount in balsam.

DEVELOPING SOLUTION

The developing solution is made up as follows:—

(1) Ten grams of gelatine are dissolved in 100 c.c. of distilled water, using a double boiler to prevent burning.

Strain while hot through a clean cloth into the stock bottle.

(2) Ten grams of starch (Argo or Kingsford's) are mixed with a few cubic centimetres of cold distilled water to make a thick paste, and then 100 c.c. of boiling distilled water are added. This is stirred and then poured into the hot gelatine in the stock bottle without straining.

(3) Five c.c. of acetone are dissolved with 7 c.c. of a fresh 5 per cent. solution of quinol and are added to the starch and gelatine in the stock bottle, which is immediately tightly stoppered and vigorously shaken.

(4) This mixture is allowed to cool, when it will
become solid. Whenever it is to be used it is placed in the oven and warmed until liquid.

(5) Just before the developer is poured over the section a 2 per cent. silver nitrate solution is added in the proportion of 1 to 5, and well mixed by pouring from one beaker into another several times.

The fewer the sections developed at any one time the better the results, but any number may be done, as long as there is sufficient of the developer to cover all to the uniform depth of 3 to 5 mm. In this laboratory never more than eight sections are developed at once.

(6) If the stock developer is allowed to stand in the melted condition for very long at a time it will settle to the bottom, in which case a vigorous shake will restore it to its original condition. It is unnecessary to add any preservative to prevent bacterial decomposition.

SUMMARY

(1) The uncertainty of silver precipitation methods is due largely to unrecognised variations in the hydrogen-ion concentration.

(2) Dilute nitric acid may be used on single sections or small blocks of tissue to produce a common reaction or a reaction best suited to the precipitation of the silver on the spirochetes in the given sections irrespective of previous changes in the acidity of the tissue.

(3) For the demonstration of spirochetes in all tissues except that of the central nervous system we believe that the starch-gelatine modification of the Warthin-Starry silver-agar method is much more convenient and specific, and yields more uniform results with a higher percentage of positive demonstration of the organism in association with the characteristic histological lesions of syphilis.

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