THE MORPHOLOGY AND STAINING CHARACTERISTICS OF THE *TREPONEMA PALLIDUM*. REVIEW OF THE LITERATURE AND DESCRIPTION OF A NEW TECHNIQUE FOR STAINING THE ORGANISM IN TISSUES*

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In 1905, sixty-eight years after Donne[31] (cf. Kolmer,[66] Hoffmann,[58] Ingraham[59]) described a regularly spiraled micro-organism in the exudates of primary syphilitic lesions of the genitalia, Schaudinn and Hoffmann[140, 141] recognized a pale, similarly spiraled organism in primary lesions and inguinal lymph nodes of syphilitic patients. Donne, who published his findings in 1837, believed that he had discovered the infective agent of syphilis. His observations were confirmed by Vanoye[156] in 1841 in a report that has apparently escaped the attention of syphilographers. Vanoye found the identical organism described by Donne in lesions of male and female syphilitic patients, and he believed that the identification of these organisms in genital lesions could be utilized as a method for the diagnosis of syphilis. Little credence was initially given to the earlier work of Donne and of Vanoye, but following the rediscovery of the organism by Schaudinn and Hoffmann, investigators in widely scattered centers soon confirmed the presence of spirochetes in early active lesions of syphilis (Russia: Zeleneff[165, 166]; Scotland: Taylor[152]; France: Brunet[18], Bodin[15], Levaditi[75]; England: Dudgeon[32]; Germany: Davidsohn[27], Giemsa[47]; Sweden: Almkvist and Jundell[2]; Argentina: deElizalde and Wernicke[26]).

Demonstration techniques

Numerous methods for demonstrating the organism were rapidly investigated after the spirochete of syphilis was identified by Schaudinn and Hoffmann. These techniques may be divided into two distinct groups. In the first, the spirochete is impregnated with a dye or metallic ion such as silver and rendered visible against a pale background. With the second technique, the background is stained black, or the illumination is altered so the background is darkened, and the unstained spirochete appears pale and sharply outlined against the dark background. The darkfield illumination method is based on this principle.

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The early staining techniques utilized the dyes commonly known as aniline dyes or coal tar derivatives. Generally in the case of the spirochete of syphilis some form of mordant is required to fix the dye, for in the absence of a mordant the spirochete is stained only slightly or not at all. Staining practices were first limited to smears, but subsequently techniques were devised for staining the organisms in histologic sections. Schaudinn and Hoffmann[140, 141] utilized an azure-eosin mixture to demonstrate the spirochete, first in primary lesions, and finally in lymph nodes of syphilitic patients. In the same year Giemsa,[47] using a similar mixture, likewise succeeded in staining spirochetes. Later he suggested minor improvements on his technique.[48] Subsequently Bruckner,[17] Davidsohn,[27] Dudgeon,[32] Ballenger,[8] Tilden,[153] Mühlpfordt,[119, 120] and more recently Olsen and Weller[130] successfully employed a variety of aniline dyes together with mordants.

Various forms of polychrome methylene blue have been utilized by several investigators including Bruckner, and Olsen and Weller. Other aniline dyes studied were the following: azure-eosinates (Schaudinn and Hoffmann, Giemsa, Bruckner), gentian violet (Tilden), basic fuchsin (Tilden, and Olsen and Weller), Victoria blue (Mühlpfordt), and cresyl echt violet (Davidsohn). At one time or another practically all the dyes utilized by the histologist have been employed in efforts to stain the spirochete. In all cases simple aniline dyes alone have not succeeded in staining the organism sharply, and only when a suitable mordant was employed was the stain at all reliable. The mordants were for the most part known protein precipitants such as phenol, tannic acid, acetic acid, phosphotungstic acid, and phosphomolybdic acid. With the resulting chemical alteration of the organism, the dye finally becomes at least partially fixed, revealing a sharply defined, generally more brilliantly stained spirochete against a pale but often stained background. Staining methods using aniline dye derivatives were never as successful as the later forms of metallic impregnation, since they were frequently inconsistent and usually showed considerable fading (Coles[25]). One method described by Olsen and Weller[130] utilized the mordant action of phosphomolybdic acid together with Unna's alkaline methylene blue, and carbol fuchsin or carbol iodine green. This staining procedure is probably the most satisfactory of the techniques utilizing the coal tar dye derivatives, although it also may give inconstant results.

Because of the general lack of consistency of staining techniques employing aniline or coal tar derivative dyes, a search for better staining methods was made. This soon revealed that impregnation with silver salts resulted in specific staining of the spirochete. In 1905, shortly after the discovery by Schaudinn and Hoffmann, Bertarelli, Volpino, and Bovaro,[13, 14] utilizing methods of silver impregnation of nervous tissue, demonstrated the Spirocheta pallida. In the same year Levaditi[75] and Petresco[121] devised similar techniques and likewise demonstrated the organisms. It was found that silver salts were reduced to metallic silver, resulting in sharpness of outline and marked contrast of the black or dark brown spirochete against the yellow hue of the background. The silver impregnation techniques were far from perfect, and the results varied widely. Artifacts, silvering of the sections, and inconsistency due to technical carelessness or impurity of chemical reagents occurred.

Special techniques for staining smears were devised by Fontana[39] and subsequently improved by Fontana,[40] Tribondeau,[154] and Hage.[50] Newer and preferred smear techniques have more recently been developed by Warthin and Starry,[169] Steiner,[148] and Krajian.[67] Silver impregnation techniques when applied to smears have for the most part resulted in atypical forms with marked changes in the regularity and shape of the

spirals, probably due to the drying and flattening of the organisms because they are unsupported by the stromal framework in tissue sections, or by the liquid vehicle in darkfield preparations.

Methods for staining spirochetes in histologic sections were devised for both single sections and block impregnation. Single section techniques have been utilized by many investigators because of the shorter processing time required, but block impregnation techniques have been found to be more reliable and show fewer artifacts (Turner[155]). In single sections as in smears following impregnation with silver, a considerable amount of reduced silver is deposited in and upon the sections, resulting in numerous black artifacts. In some single section methods silver nitrate has been used alone or in loose combination with proteins such as albumin. Bergel[11] believed the staining qualities of the spirochete to be related to changes in the lipoid nature of its cell wall as a result of the action of the strong alcohol frequently used in the processing. Other methods obviously alter the structure of the spirochete during staining, but there does appear to be some relationship between lipoid structure and the staining qualities of the organism. Of the single section techniques utilizing silver impregnation methods, those of Dieterle,[30] Nieto,[121-123] and Jahnel,[61-63] have been found to be the most consistent and satisfactory. The Warthin-Starry[159-161] techniques have been popular although they have shown inconsistency in results in some laboratories. Other methods have been developed by a number of investigators including Steiner,[149] Gyenes and Sternberg,[49] Armuzzi and Strempel,[8, 150] and Krajian.[68, 69] The use of metals other than silver for spirochetal impregnation was investigated by Ghoreyeb,[45, 46] who tried salts of lead and osmium. These methods are apparently not as satisfactory as the ordinary silver techniques.

Silver impregnation of entire blocks of tissue, while time consuming as compared with the twenty-minute staining processes of Krajian, offers greater freedom from artifacts and greater reliability. The methods of impregnation were for the most part devised, utilized, and improved by the French school under Levaditi.[76] Modifications in technique were made by Levaditi and Manouélian,[83] Manouélian,[92] Nyka,[127] and Haythorn.[52]

Staining or blackening the background to allow the unstained spirochete to stand out palely in contrast with the surrounding areas was studied by Burri.[19] Burri's method utilized India ink and has been investigated at one time or another by many students of syphilis. Cohn[23] and Barach[9] were among the early investigators. In addition to India ink, several dyes including Congo red (Benians[10]) and nigrosine (Dienst and Sanderson[29]) were similarly used. A method utilizing collargol was devised by Harrison.[51]

The most common and most widely employed method of demonstration of the Spirocheta pallida involves the alteration in the angle of lighting by special substage condensers. This method, known as the darkfield illumination technique, shows the spirochete in the motile, viable state, while all others reveal the organisms after they have been killed by fixation and manipulation. The darkfield techniques were studied carefully by many investigators, including Antoni,[1] Cares,[21] Coles,[24] and Hoffmann.[53-56]

A more recent but cumbersome method of demonstration of the Spirocheta pallida utilizes the electron microscope (Zworykin *et al.*[167]) which gives magnifications of between 4,000 and 40,000. With this technique many structures not seen by routine microscopy are visualized. Structures noted by early investigators with their staining techniques and long regarded as artifacts, such as flagella and intraspirochetal refractile

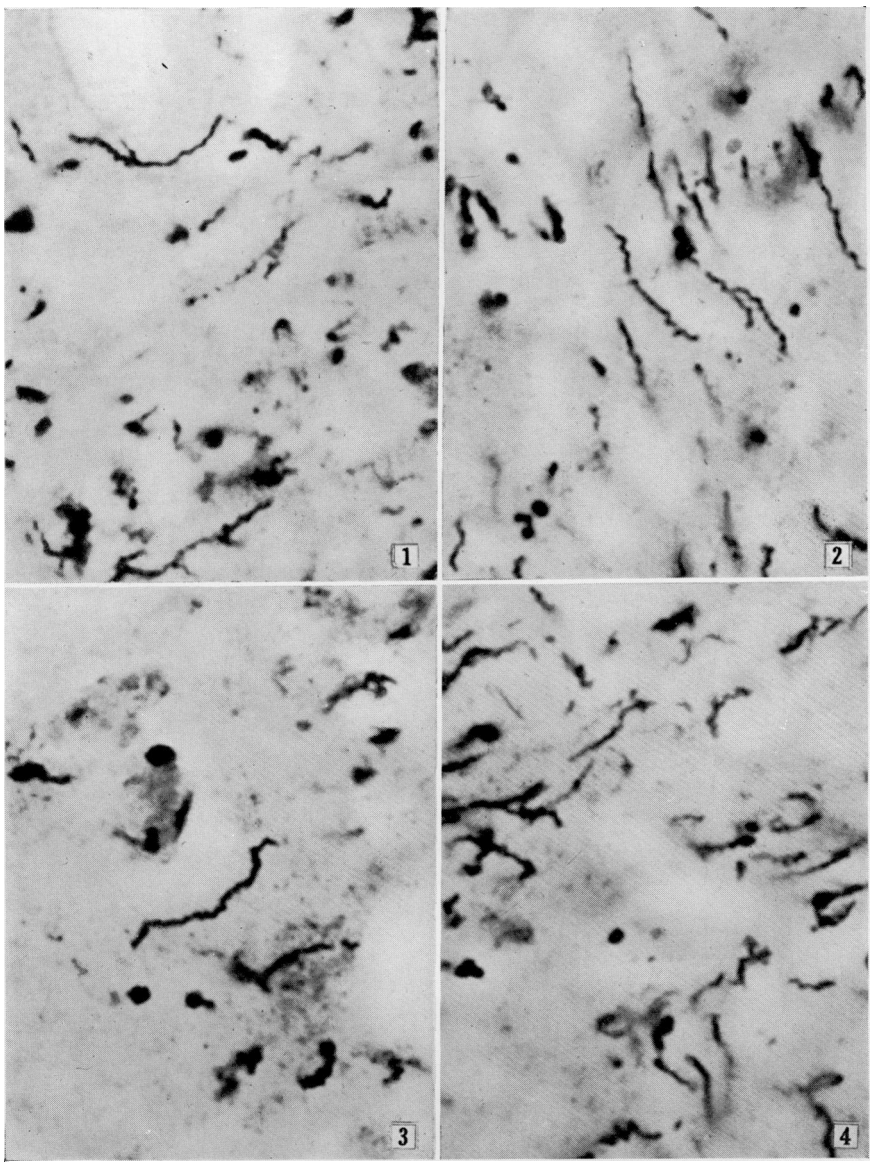
bodies, have been demonstrated through electron microscopy by Wile,[163, 164] Mudd, Polevitzky and Anderson,[117, 118] and Morton and Anderson.[115, 116] Phase contrast microscopy has also been recently employed.[28, 33]

Morphology of the Treponema pallidum

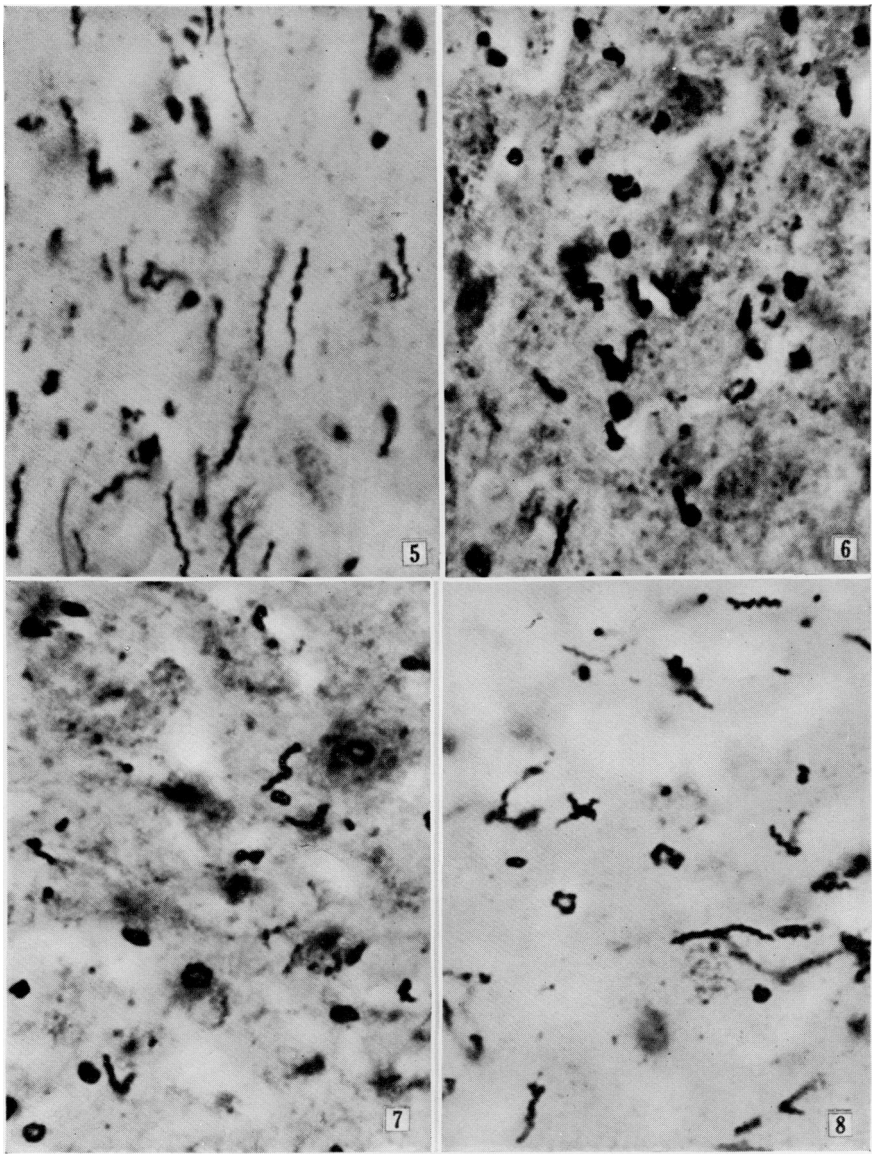
Soon after Schaudinn and Hoffmann described the spirochete, which measured between 6 and 14 microns in length, a number of investigators began to note shorter and longer forms and finally varieties of shapes. Within two years most of the forms ascribed to the evolution or involution of the spirochete of syphilis had been described by Krzystalowicz and Siedlicki,[70, 71] Bosc,[16] Berger,[12] Jacquet and Sézary,[80] Ciuffo,[22] Ewing,[35] Provazek,[132] and Sakurane.[136, 137] Of the forms considered to be part of the cycle of evolution are the so-called buds which are described as small oval argentophile structures attached either terminally or laterally. These buds are occasionally separated from the spirochete by a delicate filament or stalk, and sometimes they are free within the tissues. The investigators who considered them to be developmental forms or buds were Buschke and Fischer,[20] Meirowsky,[105–113] Kermorgant,[64, 65] Leishman,[72] Noguchi,[124, 125] and Geistfeld.[44] Others who demonstrated the forms included Nyka,[127–129] Manouélian,[93–97] Levaditi,[77] and Warthin and Olsen.[157, 158]

A second variant has been known as the granular form. The intracytoplasmic variety has been described in lymphocytes, histiocytes, large and small giant cells, and in fibroblasts. The granules are generally from 0.3 to 0.4 microns in diameter, and are best demonstrated by special modifications of the silver stains including those of Nyka, and Warthin and Olsen. These forms have been described by McDonagh,[98–104] Nyka,[127–129] Warthin and Olsen,[157, 158] Saleeby and Greenbaum,[138] Levaditi, Sanchis-Bayarri, and Schoen,[89] and Ehrmann.[34] The extracellular granular form is generally larger and varies more in size. The granule is described as round or oval measuring from 0.3 to 1.0 microns in diameter and is likewise argentophile. This form has also been described by many investigators including Balfour,[4, 5, 6, 7] Fantham,[36–38] Seguin,[142–145] Hoffmann,[57] Levaditi,[89] Warthin and Olsen,[157, 158] Manouélian,[93–97] McDonagh,[98–104] Noguchi,[124–126] Nyka,[127–129] and Gastinel and Mollinedo.[43] This type is seen routinely in smears and sections stained with any silver impregnation technique. Several early investigators described delicate small filamentous spirochetes arising from the granular forms, i.e., Balfour,[4, 5, 6, 7] Leishman,[72] Fantham,[36–38] and Sergent and Foley.[146] This occurrence has neither been confirmed nor recorded in recent literature.

A third group of forms commonly found in sections includes a variety of ring types which may be small or large, intracellular or extracellular,



FIGS. 1 TO 8: Spirochetal forms observed in an active syphiloma of the rabbit. All photographs taken at 1500 x magnification by Mr. Howard J. Reynolds. A variety of spirochetal forms are shown including the following: filamentous forms, short forms, irregular forms, thick long forms, circular forms, forms with terminal ovoid body, free ovoid bodies, incomplete serrated circular forms, comma forms, intracellular circular smooth and serrated forms, extracellular granular circular forms, and granular forms.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regular or irregular, stellate, rectangular, oval, elongate, serrated, or smooth. These have been described by Fouquet,[41, 42] Seguin,[142–144] Warthin and Olsen,[157, 158] Levaditi, Sanchis-Bayarri and Schoen,[89] Nyka,[127–129] and Sezary.[147]

A fourth group includes thin filamentous and beaded filamentous forms which stain only very palely, if at all, by the usual silver impregnation technique. These occur both intra- and extra-cellularly. They have been best demonstrated by Nyka,[127–129] and have also been noted by Manouélian,[93–97] and Levaditi, Sanchis-Bayarri, and Schoen.[89]

A final group of atypical forms has been hypothecated by Lepine,[73, 74] Levaditi, Sanchis-Bayarri, and Schoen,[89] Warthin and Olsen,[157, 158] and others to explain infectivity in certain conditions in which no visible form of spirochete can be demonstrated. This has been known as the ultramicroscopic or invisible type.

Life cycle theories

There has been much speculation on the significance of the various types of argentophile structures in the tissues of syphilitic patients in association with spirochetes and seemingly having a stream of relationship with them. From the beginning these forms were generally considered to be atypical and involutional. A few investigators, however, believed that the reverse was true, and from their interpretations arose schools of the evolutive cycle, i.e., those of Meirowsky, McDonagh, and of Levaditi. The investigations of these workers and their associates have attempted to fit the various forms into some kind of developmental cycle, but none of the theories has satisfactorily explained the structures or the conditions as they exist.

The simplest hypothesis of the evolutive cycle was proposed by the bacteriologist, Meirowsky.[108, 109] His original theory was based on the presence of a so-called bud form which had been frequently demonstrated in both darkfield and silver stain preparations. The "buds" were small oval argentophile globules attached to the mother adult form either terminally or laterally. Occasionally the "bud" was found attached to the spirochete by a thin filament or stalk, but sometimes it was free in the tissues or examined material. According to this theory, separation of the globule from the spirochete results in a "bud" from which typical adult forms arise. The newly derived spirochetes exhibit the usual delicate regular spirals of eight to twelve undulations, and then proceed by further budding to increase in number. The theory of evolution by budding forms was subsequently restudied and further elaborated on by Meirowsky.[111–113] These buds were also described by other investigators including Antoni,[1] McDonagh,[98–104]

Levaditi, Sanchis-Bayarri, and Schoen,[89] Levaditi,[77, 78, 81, 82, 84–87] Saphier,[130] Szilvási,[151] Warthin and Olsen,[157, 158] and Noguchi,[124–126] but there was no agreement as to their significance. Meirowsky considered the spirochete of syphilis to be one of the higher fungi.

A second more complicated theory of the evolutive cycle was expounded by McDonagh.[98–104] He classed the spirochete with the Protozoa, and his theory of the evolution of the spirochete parallels the development of the malaria parasite. According to McDonagh the life cycle begins with small granular forms or bodies demonstrated within the endothelial cells. These he designates as "sporozoites." The sporozoites or intracellular granules are considered to be the infective agent of syphilis. Development continues with the nuclear budding of the intra-endothelial sporozoites giving rise to a merozoite form. Further development of the merozoite form follows, with the formation of immature male and female forms. The endothelial cell walls subsequently burst, freeing these forms which further mature and by a process of fertilization begin the evolutive cycle once again. McDonagh claimed that he had been able to follow the complete cycle many times although no other investigator has substantiated his claims. Many workers have observed the small intracellular granules, not only in endothelial cells, but also in lymphocytes, fibroblasts, giant cells, and in other macrophages (Ross,[133–135] Moolgavkar,[114] Lundie and Goss,[91] Levaditi *et al.*,[77–86] Warthin and Olsen[157, 158] and Nyka[127–129]), but no investigator has been able to demonstrate the complete cycle.

A third evolutive cycle was proposed by Levaditi[88, 89] in 1927, and subsequently elaborated upon in 1928. This cycle was based entirely upon the morphological variations of the spirochete as demonstrated by many investigators. From the apparent changes in size, shape, and argentophile nature, a coherent scheme was evolved, with recognizable forms, each apparently completing a phase in the cycle. Subsequently, Levaditi turned away from his evolutive theories, but he left intact a sequence which may be ascribed without change to involution. Levaditi divided the spirochete into two phases: The first was composed of typical, regularly spiraled organisms, and the second of all the atypical forms. The atypical forms were considered developmental stages in the evolutive cycle. They were for the most part located intracellularly in the tissues, being found in giant cells, fibroblasts, and macrophages. The groups which he described are as follows: (1) Filamentous flattened forms with slightly bulging extremities; (2) Shortened club or dumb-bell forms; (3) Incomplete loop or ring forms; (4) Complete loop and ring forms; (5) Compact ball forms;

(6) Comma or question mark forms, with or without attached delicate filaments; (7) Marked argentophilic granules, ranging in diameter up to 3-4 microns; (8) Ultramicroscopic granular forms. Most of these variants have been observed by many of the aforementioned investigators.

Lepine[73, 74] hypothecated the existence of a virulent virus or ultramicroscopic organism as the actual cause of syphilis. According to this theory the spirochete is an avirulent organism. However, ultra-filtration experiments made by Lisi[90] and Levaditi[89] utilizing various filters, i.e., Chamberland Types L-1, L-2, and L-3, collodion membranes, and others have been unsuccessful in demonstrating a filterable virulent form.

Experimental

In an attempt to identify and photograph the various atypical forms of spirochetes found in syphilomas of the rabbit, a number of special techniques for silver impregnation and reduction were restudied. Staining single sections by the methods of Warthin and Starry, Nieto, Dieterle, and others, resulted in dark brown to black organisms outlined against a yellow to yellow-brown background of tissue structure. Photomicrographs of these preparations were lacking in sharpness, and besides, the sections often were spotted by a heavy precipitation of metallic silver. Only extreme uninterrupted care with each individual section throughout the techniques produced satisfactory results. The silver precipitation noted with the single-section technique occurred over the surface of the block and did not penetrate to the deeper levels. It therefore could be eliminated by cutting the sections well below the level of precipitation, but photomicrographs of the smaller atypical forms were difficult to obtain because of the colored background, and also because many of the forms were not revealed by these techniques.

The block techniques of Levaditi were generally more successful and required considerably less care during processing. One drawback was the relatively prolonged time required for impregnation and reduction of silver in the tissue blocks. An attempt to reduce the time through the use of alcoholic solutions was made with great success, but with alcoholic silver impregnation there occurred a precipitation of the metal in deeper levels of the tissue. Moreover, it was noted that exposure of the blocks to light during silver impregnation and reduction increased the rate of reduction to metallic silver, with resultant increased numbers of black silver artifacts. The adoption of light-free techniques and the use of amber bottles for the processing solutions effectively diminished the number and distribution of silver artifacts.

The next study was directed at efforts to reduce the intensity of the diffuse staining of the tissue framework and thus increase the sharpness of outline of the organisms. Much of the diffuse tissue staining appeared to result from the action of the reducing agent itself, rather than of the silver salt. Pyrogallic acid which Levaditi employed stains tissues yellow on oxidation, and hydroquinone, another reducing agent, also stains tissues yellow but to a lesser degree. A less soluble and less active reducing agent than these seemed indicated, and therefore p-hydroxyphenyl-glycine (photoglycine) was chosen for study. Alcoholic and aqueous solutions of this reducing agent were employed. It was found that although the solubility of photoglycine was much less in alcohol than in water, more rapid penetration was obtained with the alcoholic solutions. The results were even more satisfactory when sodium sulfite was added to the developer in order to stabilize it.

These changes in the technique of impregnation and reduction resulted in a gray-green diffuse tissue background instead of the usual yellow color of the classical methods. The spirochetes, however, were stained a deep blue-black, and many of the atypical forms were easily identified, indicating that a considerable degree of silver impregnation had been obtained. The gray-green tissue background was a distinct disadvantage. Attempts to clear the background without too great diminution of the spirochetal stain were then made. For this purpose several reducing agents frequently used in photography were tested. Both potassium ferricyanide and potassium permanganate gave good results, but potassium ferricyanide was found to be the best of the chemical reducers tested. Clearing with potassium ferricyanide was therefore adopted as an integral part of the processing technique. The entire section could be cleared of the stain, the spirochetes and atypical forms appearing as densely stained blue-black bodies against a colorless background. With the clearing of the background most of the atypical forms could be photographed with comparative ease.

From these experiments the following procedure was finally adopted:

1. Fix tissue blocks 2-5 mm. in thickness for 12-24 hours in buffered 10% formalin.
2. Transfer and wash tissue blocks in three changes of distilled water for 30 minutes.
3. Transfer to 95% ethyl alcohol for 12-24 hours.
4. Transfer to and incubate at 37° C. in a 3% silver nitrate solution in 50% ethyl alcohol in distilled water for 12-24 hours (amber bottle).
5. Transfer to and wash tissue in three changes of distilled water (amber bottle).

6. Transfer tissue to developer solution containing stabilizer for 12-24 hours at room temperature (0.5 gms.% photoglycine and 0.5 gms.% sodium sulfite dissolved in 50% ethyl alcohol in distilled water). Prepare fresh developing solution for each group of tissue blocks and filter before use.

7. Transfer tissues to and wash for one hour in three changes of distilled water.

8. Transfer tissue blocks to 80% alcohol (2 hours), 95% alcohol (2 hours), 100% alcohol (2 hours), aniline oil (one hour or until tissue blocks become transparent and sink), xylol (three changes, one-half hour, 1 hour, 1 hour), paraffin (two changes, 1 hour, 1 hour), and embed. Retain tissues in amber containers to prevent reduction of silver by light.

9. Block and cut sections at five microns. Mount on albuminized slides, fix by heat in paraffin oven.

10. Remove paraffin in xylols (3 changes) and pass through alcohols (100%, 95%, and 80% ethyl alcohol) to water.

11. Cover sections with freshly prepared solution of 1.5% of reagent grade potassium ferricyanide. The section changes from gray-green to colorless or cloudy white in from one to two minutes. Slight prolongation of time does not change the apparent silver content of the spirochetal forms.

12. Wash sections in three changes of distilled water (1 to 2 hours).

13. Transfer through and dehydrate in ascending alcohol concentrations, clear in xylol, and mount in Clarite or permount.

Spirochetes and spirochetal forms appear blue-black against a colorless background.

Innumerable tissues have been processed by the above technique. From this experience we have observed that several variables can be introduced without altering the end results. Tissue may be fixed for 24 hours or longer in neutral 10% formalin. It may be stained for from 24 to 72 hours in the silver solution, and allowed to remain in the developing solution for from 24 to 72 hours. The background may be decolorized either after the tissue has been cut and dried on slides, or immediately at the time of cutting by floating the ribbon on the warm decolorizing agent before mounting on slides. This latter method is especially desirable when time is an important factor for it saves eight steps in the handling of the slides. Fixation in acetone, alcohol, acid formalin, or alkaline formalin all proved unsatisfactory. Alkaline silver was also unsatisfactory.

The following schedule is used with excellent results: Fresh tissue is placed in the refrigerator at 5° C. for several hours. This prevents shrinkage. The tissue is then fixed in buffered 10% formalin for 24 hours. The blocks are trimmed and washed in distilled water for 30 minutes and then placed in 95% alcohol for 16-20 hours. A fresh solution of 3% silver

nitrate in 50% alcohol is made in a clean amber bottle, and the blocks of tissue are dropped into this where they remain at 37° for 24-48 hours. The solution is poured off and without removing the blocks from the bottle, they are washed in 6-8 changes of distilled water for 1 hour. This is done in the dark. The developer solution is made by dissolving 0.5 gm. of anhydrous sodium sulfite in 50 cc. distilled water. When this is dissolved, 0.5 gm. of photoglycine (p-hydroxyphenyl-glycine) is added. The bottle is shaken for several minutes until all of the photoglycine which will dissolve has gone into solution. Then 50 cc. of 95% alcohol are added, a few cc. at a time and with constant shaking. The solution is then filtered directly over the blocks of tissue which have been well drained of water. The bottle is kept at room temperature, in a dark closet for 24-48 hours. The solution is then poured off and the blocks are washed in three changes of distilled water for one hour. The tissue is then dehydrated, cleared in xylol, embedded in paraffin, and cut at 5 microns.

For decolorizing before mounting on slides, the ribbons of tissue are separated into suitable lengths for mounting, and transferred to 1.5% aqueous solution of potassium ferricyanide which has been warmed to about 54° C. When the sections appear opaque, they are transferred to two changes of distilled water to remove the decolorizing agent. They are then mounted from warm water on albuminized slides. The slides are allowed to dry in the paraffin oven, the paraffin is removed with xylol, and coverglasses are applied over Clarite. For decolorizing after mounting on slides and drying in the usual manner, the paraffin is removed and the slides carried through alcohol into distilled water before being placed for one or two minutes in the potassium ferricyanide solution. When the background is clear or opaque, the slides are washed in several changes of distilled water, dehydrated, cleared, and mounted. There is no appreciable difference in the end results with either method of decolorization.

If desired, counterstaining with hematoxylin and eosin can be performed as a final step. This does not decrease the intensity of the spirochetal stain and permits histologic study of the lesion on the same tissue block used to determine the presence of organisms.

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