# SPIRILLUM VIRGINIANUM NOV. SPEC.[1]

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## INTRODUCTION

Spiral microörganisms living in stagnant water and in infusions made from decaying hay, leaves, mud, etc., have been described by the early investigators on "infusion animalcula." It was not until the latter half of the seventeenth century that the Jesuit, Kircher, in 1659, and the Dutch linen-draper, van Leeuwenhoek, in 1675, actually saw and described living things too small to be seen with the naked eye. During the century following the work of these pioneers, the efforts of investigators were chiefly devoted to the more exact morphological description of some of the forms of unicellular life, already known. From that time to the present many species of spiral microörganisms, pathogenic and saprophytic have been described.

The term spirilla was originally used to describe spiral, cork screw organisms regardless of whether they were vibrios, spirilla or spirochaetes. The definition of spirilla as accepted at present is as follows: The microörganisms belonging to the genus Spirillum are free-living or parasitic, non-flexuous, spirals of various thicknesses, length and pitch of spiral, curved in three planes, forming either long spirals or a portion of a turn, non-motile or motile by means of flagella at one or both poles. Reproduction is by transverse division. The difference between spirillum and spirochaete is that the former possesses a rigid, non-flexuous cell body, while the latter has a flexuous cell body and no true flagella.

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The difference between spirillum and vibrio is that the former curves in three planes and the latter curves in only two.

This investigation was devised to study the spirilla according to the standard methods for studying pure cultures of bacteria as follows:

1. Experiments on the cultivation of spirilla.
2. The separation of the spirilla from the other bacteria associated with them.
3. The morphological study of pure cultures of spirilla.
4. The physiological study of pure cultures of spirilla.
5. Their identification and classification.

## REVIEW OF LITERATURE

The term "Spirillum" originated with Ehrenberg (1838) when he described *Spirillum undula*, *Spirillum volutans*, and *Spirillum tenue*. He defined spirillum as follows: "animal belonging to the family Vibrios, multiplication takes place by spontaneous imperfect (oblique?) division, forming tortuous spiral, rigid and cylindrical chains (filaments)." Ehrenberg's *Spirillum undula* was probably the same organism which Moeller (1786) had designated *Vibrio undula*.

No reports of researches on environmental spirilla seem to have been made from 1838 until Sorokin (1886) discovered a branching spiral organism, *Spirillum endoparagicum* in the white exudate from an old hollow black poplar tree. The spirillum seemed to have existed in pure culture but no attempt to cultivate it was reported.

In the following year Esmarch (1887) discovered *Spirillum rubrum* in pure culture in the pale red, dry, crumbling remains from a putrified mouse that had fallen in a basin of rain water.

During April Kutscher (1895) reported his observation on the vibrios and spirilla found in the juice obtained from decaying manure and succeeded in isolating *Spirillum tenue*, *Spirillum undula*, and *Spirillum volutans*. During October of the same year Kutscher reported his further observations on what he called *Spirillum undula*. In order to check up his conclusion Kutscher sent a culture of his spirillum to Professor Zettnow who observed

that this organism was smaller than what was considered at that time *Spirillum undula* (Cohn) and accordingly suggested the name *Spirillum undula-minus* to distinguish it from *Spirillum undula* (Cohn) later called *Spirillum undula-majus.*

Among other forms Swellengrebel (1909) described *Spirillum volutans* and *Spirillum parvum* as possessing "finely honeycombed protoplasm with chromatin substance distributed through the cytoplasm in transverse and zigzag bands."

Fuhrmann (1909) described in great detail the origin of the flagellum in *Spirillum volutans* as "arising from the external membrane and meeting the cytoplasm in immediate union."

Hölling (1911) distinguished between "Spirochaeta" and "Spirillum" by finding rigid, non-flexuous cell bodies in the spirilla, in contradistinction to the flexuous cell bodies of the spirochaetes.

## EXPERIMENTAL

### *a. Methods of cultivation*

The initial culture studied in the present investigation was obtained from the mud that had adhered to the outside of an oyster shell. This mud was emulsified with tap water and incubated in the ice box for twenty-four hours when a hanging drop preparation revealed rapidly motile spiral microörganisms in great numbers swarming in the emulsion. Many unsuccessful attempts were made to cultivate this spirillum on the various laboratory media and in combinations of Ringer's solution and sea water. A combination of 75 per cent sea water and 25 per cent egg cube medium[1] proved a good medium for growing the first cultures of this spirillum, although it supported life only a few days. Many other kinds of media and combinations of various media were tried and eventually one other gave good results. It was prepared as follows:

200 cc. physiological salt solution
50 cc. egg cube medium
30 cc. liquid 2 per cent agar in distilled water

[1] Egg cube medium is prepared by putting a small cube of hard boiled egg white in 10 cc. beef extract broth and sterilizing it in the autoclave.

In this medium the spirillum grew well but it appeared smaller than the spirillum originally seen.

Later a 0.7 per cent semisolid medium was obtained on which these spirilla grew luxuriantly. They grew very scantily on the surface, but in a stab made in the medium they produced an abundant, filiform growth all along the line of puncture. This medium was prepared as follows:

40 cc. liquid 2 per cent agar in distilled water
68 cc. distilled water
20 cc. sterile egg cube medium

After adaptation to laboratory conditions these spirilla were studied in pure cultures obtained by plating on beef extract agar to which egg cube medium was added in the following proportions:

75 cc. liquid beef extract agar
25 cc. egg cube medium

On this medium the spirilla produced small, dew-drop, convex, glistening, slightly opaque colonies. Discrete colonies were fished out and transferred by the stab method to 0.7 per cent semisolid beef extract agar. The procedures of plating, fishing out the colonies and transferring them into semisolid medium were repeated until pure cultures of spirilla were obtained. In the course of the experiment a 0.7 per cent beef extract agar (without the egg cube medium) was used and on it the spirilla grew luxuriantly.

### *b. Methods of staining*

The morphological study of these spirilla was carried on with pure cultures grown on the semisolid medium mentioned above for forty-eight hours at 18°C. During the preliminary experiments many staining methods were tried. Neither Heidenhain's nor Wright's iron haematoxylin yielded satisfactory results. Giemsa's solution was not satisfactory in studying the spirilla because of the amount of time it required for staining the preparations and because the details of morphology were not accen-

tuated. Noguchi's (1921) method for staining cristispiras gave fair results, but the spirilla appeared shrunken. Loeffler's methylene blue stained them lightly. By Gram's method they stained red. Carbol fuchsin stained them fairly well, while Ziehl-Neelsen's method for staining acid-fast bacteria rendered the spirilla distorted, swollen, and hardly recognizable. With Benigetti's method for staining flagella as given by Besson the spirilla appeared very large and swollen. Loeffler's flagella stain did not stain the flagella of these spirilla. Fontana's method for staining spirochaetes yielded very poor results. It was found that if heat was applied during the process of staining, the spirilla were altered to such an extent that the study of their morphological characteristics was impossible. A combination of Fontana's solution no. 1, with Sterling's anilin gentian violet-carbol fuchsin proved the most satisfactory stain. Its application was carried out as follows:

1. The air-dried film was covered with Fontana's solution no. 1 (acetic acid, 1 part; formalin, 2 parts; distilled water, 100 parts) for two minutes and washed in water
2. The film was then immersed from two to five minutes in a stain prepared as follows:
   *a.* 2 cc. Sterling's anilin gentian violet
   *b.* 2 cc. of 10 per cent basic carbol fuchsin
   *c.* 60 cc. distilled water

This method was effective in staining the flagella. In routine work the Fontana's solution was omitted.

For the study of internal structure moist films were fixed in freshly mixed Zenker's fluid and stained by the gentian violet-carbol fuchsin method mentioned above. Vital staining with malachite green, methyl green, crystal violet, neutral red, and safranin was used to advantage in the study of internal morphology.

Dark-field illumination proved especially useful in studying the refractive granules and the position of the flagella.

Air-dried films were prepared and stained as already described. They show the spirilla stretched out rather than spiral (plate 5).

Figure 10 suggests, in a small degree, the normal, living spirillum. Plate 5 also shows the type and position of the flagella. The spirilla were stained solidly when they were kept in the dye longer than two minutes, but exhibited a barred appearance when they were stained for one minute (fig. 7, plate 2; figs. 5 and 6, plate 4). The appearance of the barred staining was perhaps due to the contraction of the cytoplasm in transverse bars during the drying and fixing processes.

The internal structure of the spirilla was studied in moist films prepared as follows: Albumin fixative was spread very thinly over a perfectly clean glass slide, in order to hold the spirilla and to prevent them from being washed off during staining and rinsing. A loopful of the culture was spread over the albumin fixative and exposed to osmic acid vapor for one minute. The glass slide with the film on it was then immersed in freshly mixed Zenker's solution for fifteen minutes, gently rinsed with water, stained with very dilute (0.1 per cent) gentian violet-carbol fuchsin for one minute, rinsed in water, dried, and examined under the oil immersion objective. It is essential to have the dye very weak if a delicate, distinct staining of the chromatin material is desired. Most of the spirilla were washed off during this process, but enough remained to permit study.

This method of staining apparently did not cause the cytoplasm to segregate in the form of transverse bars, although some element in the cytoplasm, chromatin material probably, was stained heavily as is shown by figures 4, 8, 9, 10, plate 2. The deeply staining material was distributed as small dots along the cell wall, as is shown in figure 4, plate 2, although in figure 9 most of the deeply staining material appears at the poles. The spiral threads running longitudinally through the cytoplasm were the most noticeable elements in the internal structure, although their accurate tracing was impossible (figs. 8 and 10, plate 2). In specimens that were almost straightened out the spiral threads could be followed definitely (fig. 9, plate 2). It should be noted that only one spiral thread is represented in figure 8, while two are represented in figure 9, plate 2. The spirillum represented by figure 10 was still motile when the drawing was made.

### *c. Vital Staining*

Further study of the internal morphology was made by means of staining according to the method recommended by A. B. Lee. Methyl green, malachite green, and crystal violet were used to stain the chromatin material, while neutral red and safranin were employed to stain the plasma.

A 1 per cent aqueous solution was prepared from each of the five stains and filtered. The preparations were made by mixing one loopful of the culture and one loopful of the dye directly on the slide; a cover slip was placed over the film which was examined immediately under the high power, then under the oil immersion objective. Unfortunately, the spirilla died as soon as they came in contact with the dye, so the process of infiltration of the stain into the living organism was not observed.

Methyl green showed a marked affinity for the chromatin material, as is shown by the deeply stained portions in the cytoplasm (figs. 1 to 7, plate 4). The chromatin material was not distributed alike in all the organisms. Figure 2, plate 4, shows a large chromatin granule that appeared like a nucleus, but no similar observations were made on other specimens. Figures 4 and 7, plate 4, show the spiral thread running longitudinally through the cytoplasm, while the chromatin was distributed along the cell membrane at the points of contact between the cell wall and the spiral threads.

It was found that a 1 per cent aqueous solution of malachite green was too strong for vital staining so a 0.25 per cent solution was prepared which was found satisfactory. This dye showed a marked affinity for the chromatin in the spirilla, as is shown in figures 8, 10, 11, 12, plate 4

The spiral threads and the chromatin granules were plain in each specimen except that shown in figure 10, in which the chromatin granules appeared surrounded by thread-like septa. A lateral view of the spirillum was best suited for the study of the internal structure.

Crystal violet also proved effective in the staining of the chromatin material, as is shown by figure 9, plate 4. The dye seemed to stain the plasma as well as the chromatin.

Neutral red stained the chromatin fairly well, although it was used primarily to stain the plasma. Figures 13 and 14 show the spiral threads, but they are more pronounced in figure 13 than they are in figure 14.

Safranin showed the least affinity for the chromatin, while its affinity for the cytoplasm was strong, although the spiral threads were also seen (fig. 15, plate 4).

*d. Morphology*

This microörganism has a spiral, non-flexuous cell body consisting of ½ to 3 complete turns in young cultures, while in old cultures individuals are found which possess 7 turns. It is 3 to 11 micra long and 0.6 to 0.9 micron wide. The spiral amplitude varies from 4 to 5.6 micra and the width of the spiral is 1.2

TEXT-FIG. 1. SHOWING THE RELATIVE POSITION OF THE AXIAL FILAMENT AND THE FLAGELLUM DURING MOTILITY

micron. The contours of the cell body are parallel and the ends are rounded. It possesses a single, spiral flagellum at each pole, of the same spiral amplitude and width of spiral as the cell body. In fully grown individuals the flagellum is shorter than the cell body, but in young individuals the flagellum is longer. The spirilla appear barred when they are stained lightly, while they appear solid when they are overstained (plate 5). The morphology of the individual spirillum varies from a short comma to individuals possessing several turns. This variation is especially common in young vigorous cultures. In older, less vigorous cultures, longer spirilla are more numerous.

*Observations upon the living Spirilla as seen under the dark field illumination.* The motility of these spirilla is so rapid that no accurate observations are possible except as their movements become retarded. Motility is brought about by a typical corkscrew movement and a boring through the medium as the spirillum rotates on its long axis. The organisms possess no "anterior-posterior" polarity, but move in either direction with equal

rapidity and facility. They move in one direction and then suddenly dart in the opposite direction. The effect of the flagella on the motility of the spirillum is clearly observed in slowly moving organisms. Both flagella are stretched out, as shown in text-figure 1. The forward movement of the spirillum is caused by the spiral rotation and circular switchings of the "posterior" flagellum, which appears to impart the rotating movement to the cell. In some instances the "anterior" flagellum was observed folded along the cell body during motility (text-fig. 5).

Further observations showed that the average spirillum possesses a single refractive flagellum at each pole, while the young (short) spirillum possesses a single flagellum at one pole. The flagella in the average spirillum are slightly longer than the cell body, whereas the flagellum in the young (short) spirillum is

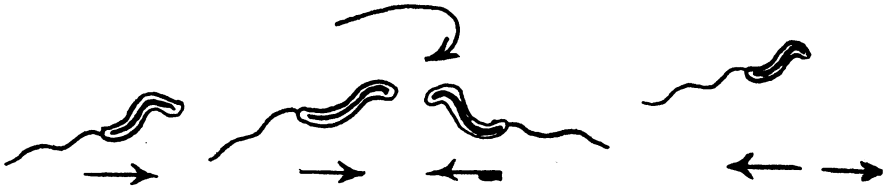

FIG. 2 FIG. 3 FIG. 4

The arrow indicates the direction of the movement

twice as long. The origin of the flagella appeared to be in the cell membrane, since no refractive material was observed in the cytoplasm opposite the "base" of the flagellum. The cell membrane and the flagellum refract the light with the same degree of intensity as is indicated by the dark areas represented by these organs. Figures 3, 5, 7, 9, 10, 14, and 15, plate 3, show that the flagella arise from the cell wall and not from the cytoplasm. The cell membrane (white area) appeared to be devoid of cytoplasm, since it did not take the stain (figs. 3, 13, 14, 15, plate 3).

The short spirillum, possessing one flagellum, during forward movement has the flagellum extended "posteriorly" (text-fig. 2), but in reversing its direction the spirillum makes a quick "about face" and moves in the opposite direction (text-fig. 3). In other instances the direction of movement is reversed after the spirillum

comes to a dead stop; it then darts in the opposite direction without turning about (text-fig. 4).

Observations under dark field illumination showed the spirilla to possess a refractive axial filament, running longitudinally through the cytoplasm, but not extending to the tip of the poles (text-figs. 1 and 5).

The area between the axial filament and the cell wall is translucent and at times very delicate, thread-like processes are observed extending from the axis to the cell membrane. Great care was exercised to determine whether or not these thread-like processes are the same as the spiral threads that were observed in plates 2 and 4. Their identity is not established because the thread-like processes are very indistinct and it is impossible to trace them accurately under the dark field illumination.

TEXT-FIG. 5. SHOWING THE RELATIVE POSITION OF THE FLAGELLA DURING MOTILITY

The arrow indicates the direction of the motion

*Multiplication.* A loopful of a vigorous culture of spirilla grown on semisolid medium for forty-eight hours at 18°C. was mixed with a loopful of liquefied gelatin medium. The preparation was made directly on the glass slide, a cover slip was laid over the film, sealed with vaseline, and the preparation examined under the high power objective. The rapid motility of the spirilla was retarded by the solidifying gelatin, and extensive observations on the living specimens were made possible. Long and short spirilla were observed, but the organisms of average length appeared to divide most frequently in the following manner:

The spirillum that is approaching the state of cell division becomes very sluggish. At this time a faint transverse line appears in the middle, in some organisms, while in others it is a little away from the middle (fig. 2, plate 1). As this transverse

line becomes more pronounced the cytoplasm gradually separates and contracts away from it, toward the poles, leaving a transparent area on each side of this transverse line (figs. 3 and 4, plate 1). At this time a vibrating movement of the middle portion of the dividing spirillum is seen, as is indicated by the dotted lines in figure 3, plate 1. After a few moments the vibration ceases, but is followed by oscillating movements of the ends which swing like a pendulum, with the fixed point at the line of division, as is indicated by the dotted lines in figure 4, plate 1. During the oscillating movements the cell membrane begins to constrict at the transverse line, and gradually the constriction is completed (figs. 5 and 6, plate 1).

The new daughter cells remain attached to each other for some thirty minutes, at the same time lashing violently in a hinge-like fashion, each struggling to detach itself from the other. During motility the "posterior" cell causes the forward movement, while the other hangs on quiescent. When the direction of motion is reversed the quiescent spirillum becomes motile, while the other becomes immotile. Finally the organisms separate.

*Involution forms.* The figures shown in plate 3 represent the morphological types that were found in cultures one month old, stained as described on page 24. The characteristic contraction of the cytoplasm toward the poles is represented in figures 2, 3, 5, 9, 11, 13, and 15, plate 3. "Coccoid bodies" were also frequently observed (fig. 4, plate 3). Specimens as shown in figure 4, plate 2, figure 4, plate 3, figure 12, plate 5, are typical of "coccoid bodies" as they are found in old cultures of these spirilla. Some "coccoid bodies" possess flagella while others do not. Segregation of the cytoplasm in definite, heavily stained areas, suggests an analogy to spore formation in bacteria.

What have been called "budding" spirilla were found frequently in old cultures (figs. 1, 2 and 3, plate 2). Figure 2 was stained for flagella, and it is clear that each pole (bud) possesses a flagellum. On one occasion a budding spirillum was observed in a hanging drop preparation, but its motility was very sluggish.

The exact meaning of these involution forms is not clear. Further observations may be helpful in clearing up the life-cycle of the spirilla and of the spirochaetes.

*e. Physiology*

It was found that these spirilla were able to grow on the usual laboratory media after the organisms had been grown on the special semisolid medium for 40 generations and had become accustomed to artificial cultivation. This adaptation to laboratory conditions made it possible to study the cultural reactions of 26 pure cultures of spirilla on the following media: beef extract broth, lead acetate, egg cube medium, agar slant, nitrate solution, gelatin, lactose broth, mannitol infusion broth, glycerol broth, dulcitol broth, sucrose broth, dextrine broth, glucose broth, Uschinsky's protein-free medium. The media were adjusted to the optimum pH 8.2 to 8.4 before sterilization.

The inoculation of the media was made by introducing three drops into each tube from a four-day culture grown either on beef extract broth or on egg cube medium. A heavy inoculum was necessary for obtaining good growth. All the cultures were incubated in the ice box and daily reading of the cultural reactions were made for seven days. The cultures were kept for one month and during this time frequent observations were made.

The cultural characteristics of these spirilla can best be studied in a tabular form. Analysis of table 1 which gives the properties of *Spirillum volutans*, *Spirillum undula*, and of this new species shows that the points of similarity among these spirilla are found in their inability to produce indol and their inability to reduce the nitrates. The colonies produced on gelatin plates by *Spirillum volutans* and by the species described in this investigation are similar, while their growths in gelatin stab differ. The former produces porcelain-white, crumpled surface growth, with slight growth in the stab and slow liquefaction. *Spirillum virginianum* grows very scantily on the surface, but produces luxuriant, filiform, ivory-white growth in the stab without liquefaction. This species produces cloudiness (no flocculation) in broth, while the others produced turbidity. This species is Gram-negative, while the others are Gram-positive. The habitat of *Spirillum volutans* is stagnant water, and that of *Spirillum undula* is putrid and stagnant water, while this new species inhabits the muddy

bottom of brackish water. The optimum temperature for *Spirillum volutans* is 20°C., for *Spirillum undula* 25°C., and for *Spirillum*

TABLE 1

*Cultural characteristics of Spirillum volutans, Spirillum undula, and Spirillum virginianum*

| MEDIUM | SPIRILLUM VOLUTANS (BERGY*) | SPIRILLUM UNDULA (BERGY*) | SPIRILLUM VIRGINIANUM |
|---|---|---|---|
| Gelatin colonies. | Gray, smooth, entire glistening | Circular, granular, greenish-yellow | Entire, convex, circular, moist, colorless |
| Gelatin stab..... | Porcelain-white, crumpled, surface growth, slight growth in stab, slow liquefaction | White, rugous, surface growth, no liquefaction | Growth along entire stab, no liquefaction |
| Agar colonies.... | | | Dew-drop, convex, entire moist, colorless |
| Agar slant..... | | | Dew-drop, isolated |
| Broth..... | Turbid | Turbid | Cloudy, seldom turbid |
| Litmus milk..... | Unchanged to slight alkalinity | | No growth |
| Potato..... | Dry brown streak | | No growth |
| Indol..... | Negative | Negative | Negative |
| Nitrates..... | Not reduced | Not reduced | Not reduced |
| Gas in sugars.... | | | None |
| Acid in sugars... | | | None |
| Uschinsky's protein-free medium..... | | | Abundant growth |
| Loeffler's coagulated serum.. | | | Convex, dew-drop isolated colonies, no liquefaction |
| Lead acetate.... | | | Negative for $H_2S$ |
| Voges-Proskauer..... | | | Negative |
| Methyl red..... | | | Negative |
| Gram..... | Positive | Positive | Negative |
| Habitat..... | Stagnant water | Putrid and stagnant water | Muddy botton of brackish water |
| Optimum temperature..... | 20°C. | 25°C. | 18°C. |

* According to Bergy's Manual.

*virginianum* 18°C. On the basis of these cultural differences this spirillum is described as a new species.

### *f. Discussion of morphology*

*Spirillum virginianum* has a spiral, non-flexuous cell body consisting of ½ to 3 complete turns in young cultures, while in old cultures individuals are found which possess 7 turns. It is from 3 to 11 micra long and 0.6 to 0.9 micron wide. The spiral amplitude varies from 4 to 5.6 micra and the width of the spiral is 1.2 micron. The contours of the cell body are parallel and the ends are rounded, possessing a single, spiral flagellum at each end with the same spiral amplitude and width of spiral as the cell body. The spirilla appear barred when they are stained lightly, but solid when they are overstained.

Ehrenberg describes *Spirillum volutans* as possessing "a single whip-like flagellum" at each pole. Sternberg describes it as being from 1.5 to 2 micra wide and from 25 to 30 micra long; wave length from 9 to 13 micra; a single whip-like flagellum at each end, with opaque granules in the cytoplasm. Migula describes *Spirillum volutans* as a long, regular, spiral organism 30 by 1.5 micra, with spiral amplitude 6.6 to 7 micra and width of spiral 13 to 14 micra, 2 to 3½ turns, with 10 to 15 flagella at one pole. Hoelling is satisfied that it possesesses but one flagellum at each pole. Kutscher observed a tuft of flagella at each pole. Cohn reports only a single flagellum at each pole. Bergey describes *Spirillum volutans* as possessing spirals from 2 to 3 micra thick by 30 to 50 micra long, with 3 to 8 flagella at each pole. It is obvious that no two descriptions of *Spirillum volutans* are alike.

In 1909 Swellengrebel described in *Spirillum volutans* and in *Spirillum parvum* "finely honey-combed protoplasm with chromatin substance, distributed through the cytoplasm in transverse and zig zag bands." The description of these chromatin spirals stimulated much research and some controversy regarding the inner structure of the spirilla. Zettnow repeated Swellengrebel's work and confirmed the existence of "Chromatinbänder" in the cytoplasm of *Spirillum volutans*. Hoelling denied their existence, although his drawings certainly suggest it. He de-

scribed the protoplasm as being alveolar. Swellengrebel's "finely honey-combed chromatin, distributed in zig zag bands," and Hoelling's "alveolar plasma" of *Spirillum volutans* appear to be the same structure, the apparent differences being due to dissimilar methods of staining.

In 1909 Fuhrmann described the flagellum in *Spirillum volutans* as "arising from the external membrane and meeting the cytoplasm in immediate union." In 1911–1912 Hoelling confirmed Fuhrmann's observations in every detail, but added "the flagellum penetrates the outer membrane by means of a hole." He further stated: "They must have their origin in the inner plasma and we differentiate them from the plasma. Therefore they consist of two elements, central elastic fiber, and an outer contractile plasma." Hoelling's final conclusion was that *Spirillum volutans* possesses only one flagellum at each pole.

A similar variation in the description of *Spirillum undula* is found in the literature. Sternberg describes it as a rigid organism having a spiral filament 8 to 12 micra long by 1.1 to 1.4 micron wide with spiral amplitude 4 to 5 micra, from ½ to 3 turns, and with a whip-like flagellum at each end. Migula describes it as a curved bacterium 4 to 5 micra long, ½ to 3 turns, and the number of flagella may reach 15. Bergey describes it as a stout, thread-like organism 1.2 to 1.5 micron wide by 8 micra long, spiral amplitude 4 to 5 micra, ½ to 3 turns. There are bundles of 3 to 9 flagella at each pole. It is obvious that these descriptions of *Spirillum undula* do not agree.

The morphological characteristics of these three types of spirilla are expressed in table 2, which shows two points of similarity between *Spirillum undula* and the spirillum described in this investigation. The first similarity is that they have the same wave length of the spirals, and the second is that they have the same number of turns. On the other hand, they differ in length and in the width of the cell bodies, in the number of flagella, and in the width of the spiral. Chromatin spirals are described in *Spirillum volutans* but not in *Spirillum undula.* The morphological characteristics presented in table 2 show that the organism in this paper is a new species. It is therefore named *Spirillum virginianum.*

*Classification of Spirilla and Vibrios.* It has been shown that the organism described in this investigation differs morphologically and physiologically from *Spirillum volutans* and *Spirillum undula.* On account of its rigid, spiral cell body, it can be classified under the genus *Spirillum.* On the other hand the presence of a single flagellum at each pole makes it possible to classify it under the genus *Vibrio.* Considering these two possibilities of classification this organism must be classified under the genus *Spirillum,* because it is curved in three planes, forms either long spirals or a portion of a turn, in contradistinction to the short, curved rods of the genus *Vibrio.* Furthermore, there is no valid reason why a

TABLE 2

*Comparative morphology of Spirillum volutans, Spirillum undula and Spirillum virginianum*

| | SPIRILLUM VOLUTANS (BERGY*) | SPIRILLUM UNDULA (BERGY*) | SPIRILLUM VIRGINIANUM |
|---|---|---|---|
| Length of cell | 30–50$\mu$ | 8.16$\mu$ | 3–11$\mu$ |
| Width of cell | 2–3$\mu$ | 1.2–1.5$\mu$ | 0.6–0.9$\mu$ |
| Spiral amplitude | 6.6–7$\mu$† | 4–5$\mu$ | 4–5.6$\mu$ |
| Width of spiral | 13–14$\mu$† | | 1–2$\mu$ |
| Number of turns | 2½–3½† | ½–3 | ½–3 |
| Number of flagella | 3–8 | 3–9 | 1 |
| Chromatin spirals | Present | | Present |

* Description according to Bergy's Manual.

† Description according to Migula's Manual.

spiral organism, possessing a single flagellum at each pole, should not be classified under the genus *Spirillum.* It is also clear from the original descriptions that the spirilla mentioned by Ehrenberg, Swellengrebel, Zettnow, Cohn, and Hoelling possessed a single flagellum at each pole, although the text books describe the spirilla as possessing tufts of flagella. Therefore this new organism is regarded as a spirillum.

### *g. Discussion of coccoid bodies*

The granules found in the cultures of spirilla are called "coccoid bodies" while similar structures found in cultures of spirochaetes are called granules. "Coccoid bodies" are common in old cul-

tures of *Spirillum virginianum*. In hanging drop preparations they appear like single cocci, although larger than staphylococci. During certain stages of their life-cycle the "coccoid bodies" are motile and in their movements resemble very young tadpoles swimming about, although no flagellum could be seen in hanging drop preparations. Immotile "coccoid bodies" were also observed in hanging drop preparations made from old cultures of spirilla. "Coccoid bodies" with and without flagella were observed in stained preparations made from the same cultures. It is apparent that the motile "coccoid bodies" possess flagella, while the immotile do not. Plate 3 shows "coccoid bodies" in various stages of formation. Similar granules are described by Sorokin in the cell body of *Spirillum endoparagicum*. He interpreted these granules as analagous to spores.

In 1914 Wolbach and Binger described "bud-like forms either free or attached to the cell body" of the free living *Spirochaeta elusa*. They think that these buds are part of the life-cycle of this spirochaete.

In 1914 Todd and Wolbach studied the filterability of two strains of *Spirochaeta duttoni* and made some observations on the granules they found in their cultures. On the basis of their experiments these investigators came to the following conclusions: "The minute granules and comma bodies found in epithelial cells, and probably those in other tissues, are not stages in the development of spirochaetes. Large granules of coiled and encysted forms derived from spirochaetes, occur in various connective tissue structures, and may possibly represent resting or multiplication stages."

Meirowsky described red staining wart-like granules in the middle of *Spirochaeta nodosa* and in *Spirochaeta pallida*. He also reports granules in the living organisms and concludes that "they are the forms by which the spirochaetes sporulate in the process of multiplication." He further mentions bud-like granules attached to the cell body of the spirochaete. Finally he states that "the granules are part of the evolution cycle of the spirochaete."

Balfour has studied the granules in *Spirochaeta granulosa-penetrans* in connection with spirochaetosis of Sudanese fowl.

He first noticed granules in the erythrocytes of the peripheral blood of fowls suffering with spirochaetosis. Under the dark field illumination he observed spirochaetes entering the red blood cell and breaking into granules. He also described the pouring out of these granules by the host erythrocyte. His observations on the granules found in ticks infected with *Spirochaeta granulosa-penetrans* lead him to conclude that these granules develop into young spirochaetes. The granules that he observed in the blood of diseased fowl are described thus: "The spirochaetes shed the granules during a state of violent contortions, shaking themselves to and fro, like a dog shaking water from its coat." He succeeded in producing spirochaetosis in fowl by injecting them with mascerated ticks which showed only granules, and spirochaetes were found in the blood of the fowl in the course of the disease.

Dutton and Todd working with African Tick Fever were the first to describe coiled and encysted spirochaetes, containing a large number of small granules. Later Leishman advanced the view that the granules into which spirochaetes disintegrate are capable of developing into new spirochaetes. He laid particular stress on the significance of granules in the epithelial cells of the Malphighian tubules and in the ova. He further believes that the granules could multiply and were therefore not merely resting forms of spirochaetes, but represented a definite stage of the life history. Nuttall observed that *Spironema gallinarum* penetrates into the Malphighian tubules of infected ticks. There the spirochaetes break up into a large number of small coccoid bodies which he says multiply by fission. Hindle found that if infected ticks were kept at 21°C. no spirochaetes were present, but if kept at 35°C. they were. He further states that the granules develop into spirochaetes with a favorable temperature. On the other hand Wittrock and Couvy-Machoux brought much evidence to show that the granules above described were not connected with the spirochaetes. In the infected ticks the spirochaetes become very delicate and are not demonstrable with Giemsa stain, but they are by gentian violet, and by the dark field illumination.

## CONCLUSIONS

1. The initial culture of these spirilla was obtained from the mud adhering to the outside of an oyster shell.

2. A medium consisting of 0.7 per cent beef extract agar containing egg cube medium was found satisfactory for growing them.

3. The spirilla were grown for many generations on 0.7 per cent beef extract agar before they were able to grow on the usual laboratory media.

4. The spirilla were isolated from the contaminating bacteria by plating them on 1.5 per cent beef extract agar containing egg cube medium and five days' incubation at 18°C. was necessary for producing colonies.

5. A combination of 2 cc. Sterling's anilin gentian violet, 2 cc. of 10 per cent basic carbol fuchsin in 60 cc. distilled water was a very satisfactory stain, while the flagella were stained well by first immersing the film in Fontana's solution no. 1.

6. The living spirilla possess a single, spiral flagellum at each pole; a refractive axial filament running longitudinally through the cytoplasm; and non-flexuous cell bodies.

7. Stained specimens show granular (barred) structure and chromatin spirals in the cytoplasm.

8. Motile and non-motile coccoid bodies were observed in hanging drop preparations from old cultures of spirilla.

9. Complete cycles in the process of transverse cell division were observed microscopically in hanging drop preparations.

10. The cultural characteristics were determined on the several laboratory media.

11. The name, *Spirillum virginianum*, is proposed as describing this new species.

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# PLATES

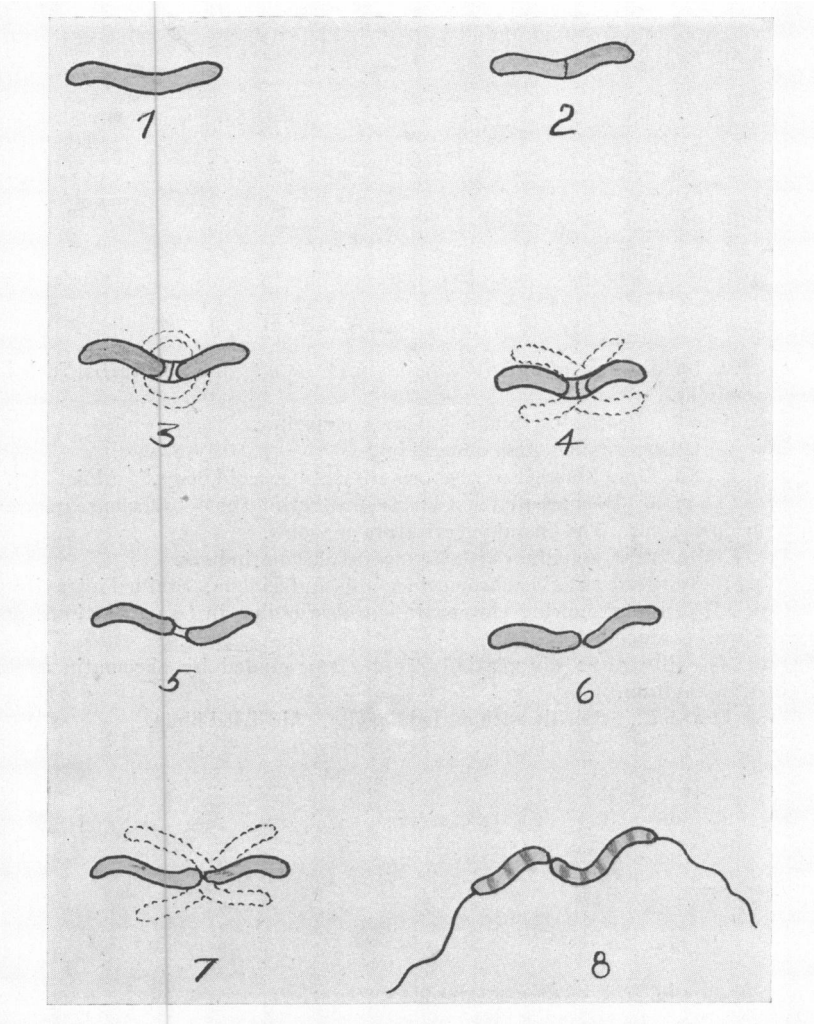
## PLATE 1

FIGS. 1 TO 7. Illustrate the process of transverse division in *Spirillim virginianum* as observed in hanging drop preparation.

FIG. 3. Dotted lines indicate the bendings of the dividing spirillum.

FIGS. 4 AND 7. Dotted lines indicate the positions of the lashing daughter-cells in the process of division.

FIG. 8. A dividing spirillum stained by carbol fuchsin-gentian violet.




(Dimitroff: *Spirillum virginianum* nov. spec.)

## PLATE 2

Camera lucida drawings of *Spirillum virginianum* stained by carbol fuchsin-gentian violet.

FIGS. 1 TO 3. Branching (budding) forms of spirilla.

FIG. 2. Illustrates flagellum on each bud.

FIG. 4. Spirillum illustrating the formation of "coccoid body." Moist film.

FIGS. 5 AND 6. Degenerated specimens illustrating the cytoplasm segregated into the dark areas. The unstained areas are vacuoles.

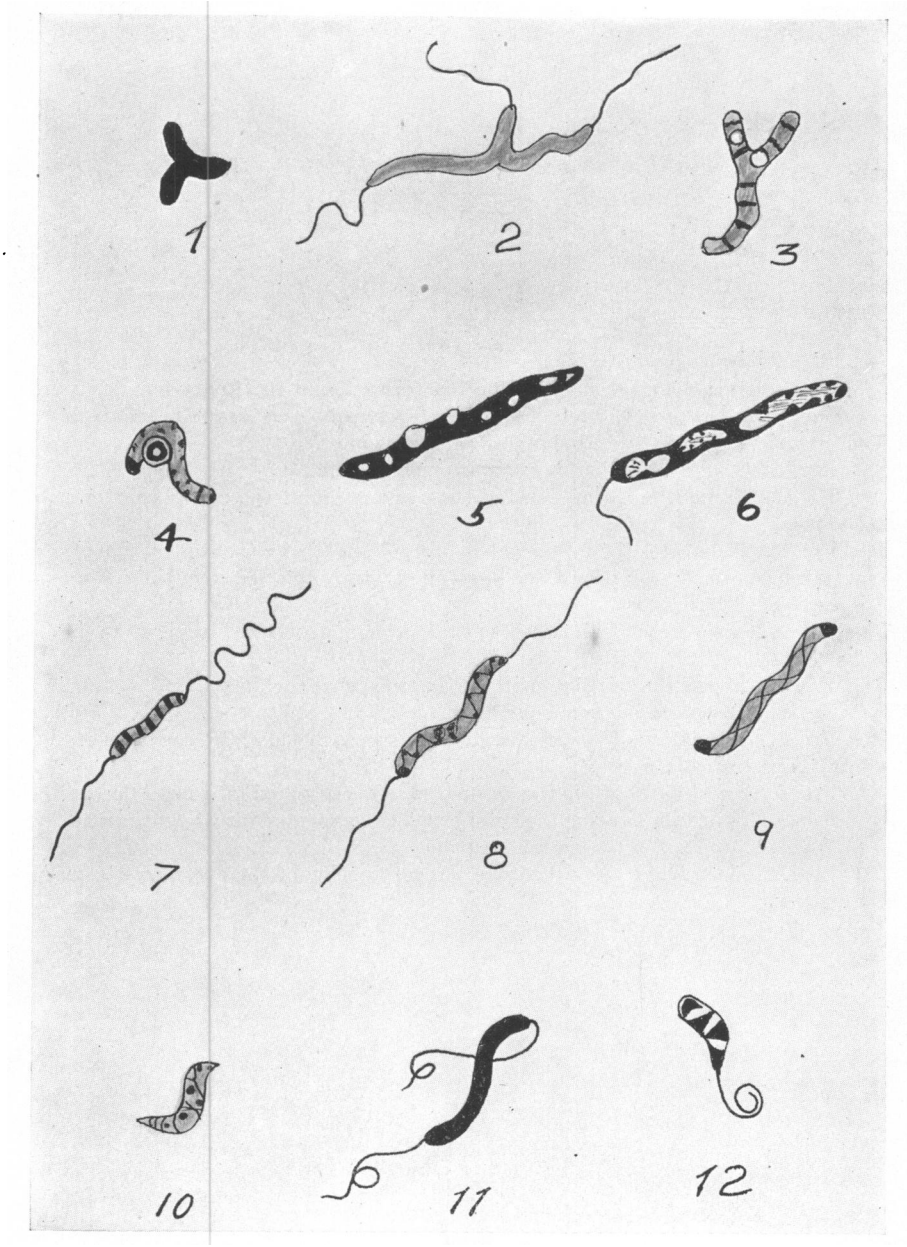
FIG. 7. Illustrates spirillum with transverse chromatin bars.

FIG. 8. Spirillum showing chromatin spirals and flagella. Stained intravitam.

FIG. 9. Spirillum showing chromatin spirals running in two directions, and polar kaps. Stained intravitam.

FIG. 10. Illustrates chromatin granules surrounded by chromatin septa. Stained intravitam.

FIGS. 11 AND 12. Spirilla with curled flagella. Air-dried films.




(Dimitroff: *Spirillum virginianum* nov. spec.)

## PLATE 3

Camera lucida drawings of involution forms found in twenty-five-day pure culture of *Spirillum virginianum*. All the specimens were drawn from air-dried preparations stained by carbol fuchsin-gentian violet.

FIG. 1. Young individual possessing one flagellum.

FIG. 2. Involution form showing the contraction of the cytoplasm towards one pole.

FIG. 3. Illustrates the formation of "coccoid body" by the contraction of the cytoplasm towards one pole. The flagellum appears to arise from the cell membrane.

FIG. 4. Two "coccoid bodies" one at each end of what appears to be a flagellum.

FIG. 5. Spore-like spirillum with the cytoplasm at each pole.

FIG. 6. Degenerated, dead spirillum.

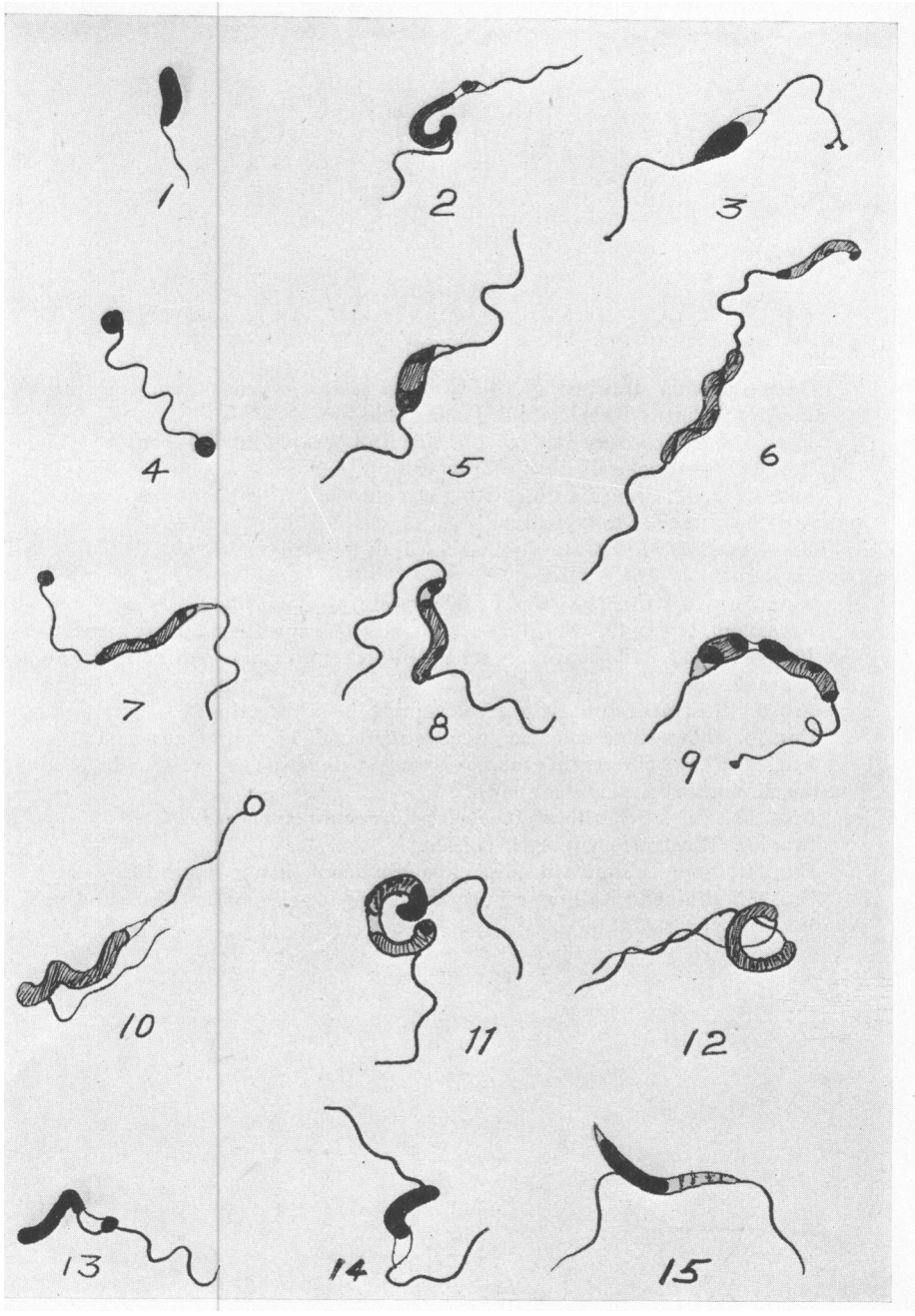
FIG. 7. Formation of "coccoid bodies" one at each end of the flagellum on the left as illustrated in figure 4.

FIG. 8. Spirillum showing the cytoplasm has segregated into small dots.

FIG. 9. Spirillum showing the cytoplasm has segregated into definite areas.

FIGS. 10 AND 12. Illustrate dead spirilla.

FIGS. 11, 13, 14 AND 15. Illustrate the contraction of the cytoplasm towards the poles.




(Dimitroff: *Spirillum virginianum* nov. spec.)

## PLATE 4

Camera lucida drawings of *Spirillum virginianum* made from preparations stained by the intravitam method of moist films.

FIGS. 1 TO 7. The spirilla were stained with 1 per cent methyl green.

FIG. 1. Spirillum showing no chromatin.

FIGS. 2 AND 3. Spirilla illustrating the chromatin distributed in dots along the cell membrane of the spirillum.

FIGS. 4 AND 7. Illustrate chromatin spirals in spirilla as demonstrated by the intravitam method of staining.

FIGS. 5 AND 6. Illustrate the barred appearance of spirilla.

FIGS. 8, 10, 11 AND 12. Spirilla stained with 0.25 per cent malachite green.

FIGS. 8 AND 12. Illustrate chromatin spirals running in two directions. Polar kaps are shown.

FIG. 9. Illustrates chromatin spiral running in one direction.

FIG. 10. Shows the chromatin granules surrounded by chromatin septa.

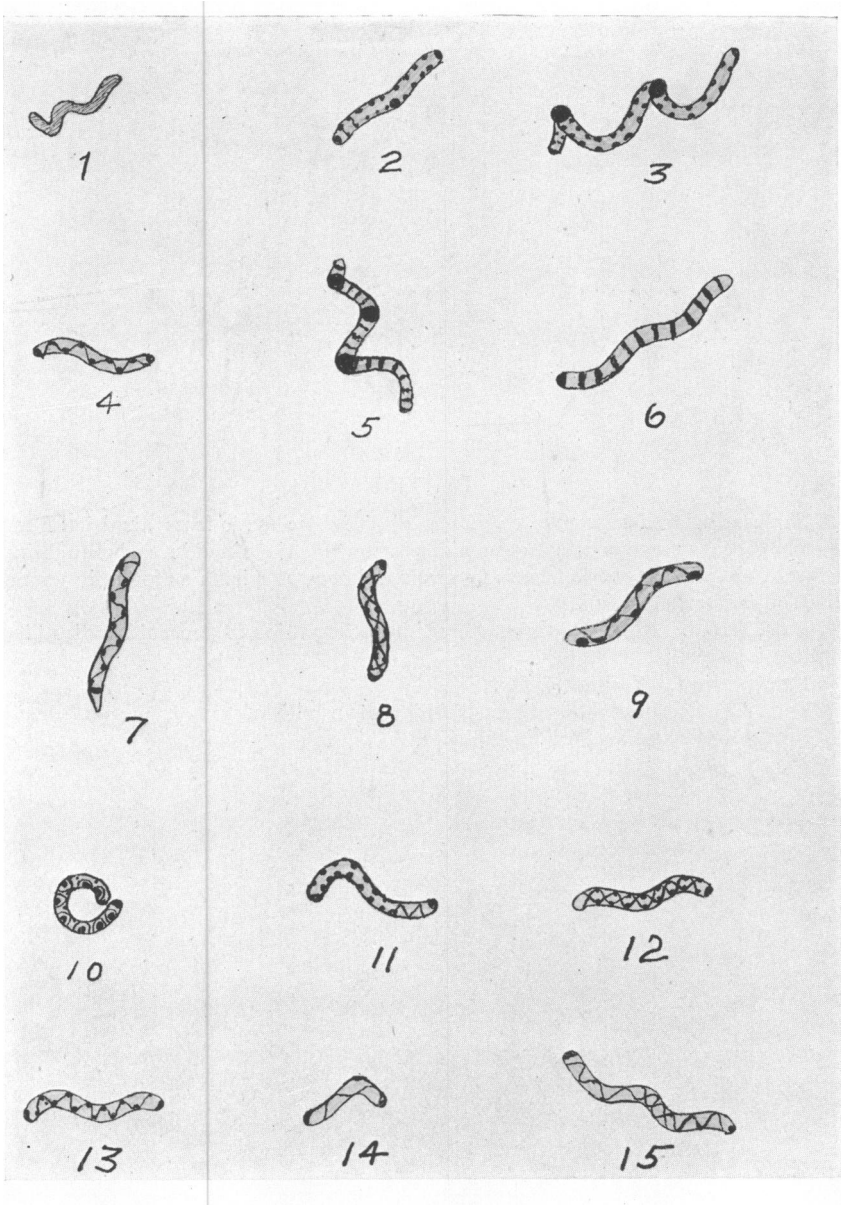
FIG. 11. Shows chromatin granules arranged along the cell wall, and a portion of the chromatin spiral is also shown.

FIGS. 13 AND 14. Spirilla stained with 1 per cent neutral red.

FIG. 13. Illustrates chromatin spirals.

FIG. 14. Shows chromatin spirals less prominent than in figure 13.

FIG. 15. Stained with 1 per cent safranin showing chromatin spirals and polar kaps.




(Dimitroff: *Spirillum virginianum* nov. spec.)

## PLATE 5

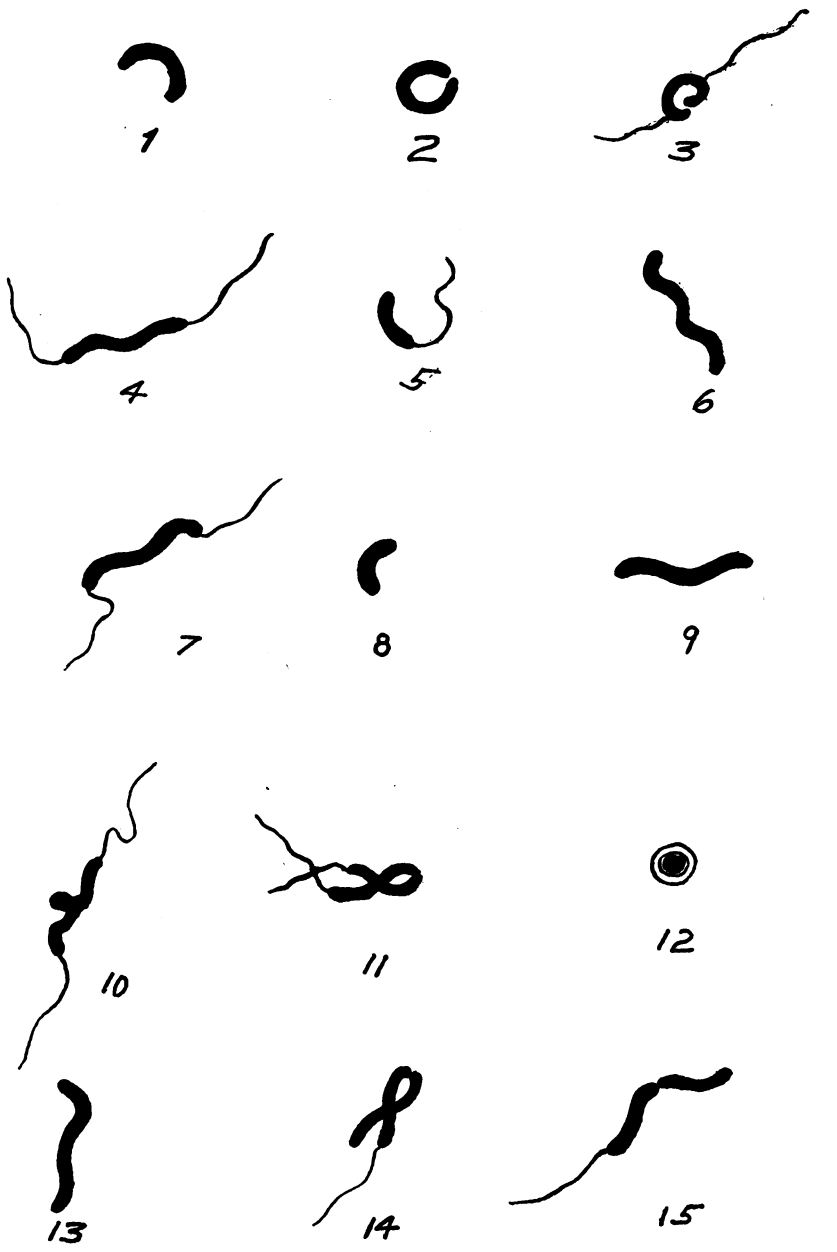
Camera lucida drawings of *Spirillum virginianum* made from air-dried films fixed by heat, immersed in Fontana's solution no. 1, stained in carbol fuchsin-gentian violet. This plate shows typical specimens of spirilla as found in young pure cultures of spirillum.

FIGS. 1 TO 3. Illustrate three stages in the formation of "coccoid bodies" by the coiling spirilla.

FIGS. 5 AND 8. Young spirilla.

FIGS. 4, 6, 7, 9, 10 AND 13. Normal, fully grown spirilla.

FIGS. 11, 14 AND 15. Dividing spirilla.




(Dimitroff: *Spirillum virginianum* nov. spec.)