MONTANA STATE UNIVERSITY

A cytological and serological study of Spirillum serpens
by Curtis J Wilder

A THESIS Submitted to the Graduate Committee In partial fulfillment of the requirements for the degree of Master of Science In Bacteriology
Montana State University
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Abstract:
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Serological studies using the precipitin reaction were made of this same species using three portions of the cell material—the flagella, the soma, and the whole cells. From the results of this serological study it is believed by the writer that there is possibly a different system or basic morphological pattern between the soma and the flagella; that the flagella contain a much greater amount of precipitinogenic material than the soma; and that the soma contain a substance capable of reacting with the flagellar-precipitin in the immune serum.

A CYTOLOGICAL AND SEROLOGICAL STUDY OF SPIRILLUM SERPENS

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CURTIS J. WILDER

A THESIS

Submitted to the Graduate Committee

in

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ABSTRACT

A cytological and serological study was made of the bacterial organism Spirillum serpens (Muller) Winter. An effort was made to determine the way in which this species forms its flagella and where the flagella originate in the cell. A staining technic was developed by which certain polar granules could be demonstrated in the cell while it was in the process of flagella formation, and also granules were stained actually lying within the flagella. The flagella arise in close association with granular activity as new structures with serological properties differing from the soma. The flagella have been observed to be forming at different stages of cell division.

Serological studies using the precipitin reaction were made of this same species using three portions of the cell material--the flagella, the soma, and the whole cells. From the results of this serological study it is believed by the writer that there is possibly a different system or basic morphological pattern between the soma and the flagella; that the flagella contain a much greater amount of precipitinogenic material than the soma; and that the soma contain a substance capable of reacting with the flagellar-precipitin in the immune serum.

INTRODUCTION

Work has been done but very few conclusions have been reached concerning the formation of bacterial flagella and their connection with the cell. Some workers believe that the flagella are a continuation of the ectoplasm and others that the flagella spring from the endoplasm or cytoplasm and extend through the outer wall.

That there is an outer structure has been generally accepted, but its exact composition is still unknown. Mudd, Polevitsky and Anderson (1941) by the use of the electron microscope have demonstrated the existence of a solid cell membrane, differentiated from an inner fluid or potentially fluid protoplasm for several representative bacteria; streptococci, Bacillus subtilis Cohn emend. Prazmowski, "coliform" and typhoid bacilli. They state also that the inner protoplasm may shrink away from the cell membrane on drying or may escape in cytolisis, leaving the membrane as a "ghost". It has been suggested by Polevitsky (1941) of Mudd, Polevitsky and Anderson that the flagella of Eberthella typhosa (Zopf) Weldin and "coliform" bacteria may be tubular structures; and further that the rhythmic contraction of the protoplasm in the flagella causing pressure changes in the lumen of such tubules might well determine flagellar movement. This hypothesis suggests that the flagella are of both ectoplasm and endoplasm. It has been demonstrated quite conclusively, however, that the flagellar

material and the somatic material show a serological difference. This suggests three phases of the whole cell--endoplasm, ectoplasm and flagella.

Leifson (1930) pointed out that Migula (1900) in his "System der Bacterien" concluded that flagella were outgrowths of the bacterial ectoplasm which seemed to be the general conception at that time. Leifson worked with preparations of Eb. typhosa and Proteus vulgaris Houser and suggested that, from the appearance of flagella on these preparations, a different conclusion from that of Migula may be drawn. He went on to say that neither of these organisms, Eb. typhosa and Proteus vulgaris, stains uniformly with the flagella stain. The flagella, the ectoplasmic zone and a central column are stained red. The rest of the organism, unless a counterstain is used, is colorless, and when it is used the rest of the organism takes the color of the counterstain. In a number of cases the flagella were seen to originate in the central column and only rarely did a flagellum originate between the central column and the ectoplasmic zone. Although he stated that most frequently the flagella seem to originate on the surface, Leifson also pointed out from his data obtained with Eb. typhosa especially, it might be concluded that bacterial flagella are not of ectoplasmic origin but originate in the endoplasm, and perhaps in some definite structure within the endoplasm.

He found in the experiments on the effect of addition of strong hydrochloric acid to the flagella stain that it was noticed that when a concentration of 0.1 normal was reached the flagella would no longer stain evenly but as a series of evenly spaced round dots. But if the acid concentration was increased still further the flagella did not stain at all. This granular appearance of the flagella was also demonstrated by the addition of carbol fuchsin to a hanging drop of Vibrios (and other organisms as well) that some flagella can be seen --not stained solidly but as a series of dots. The probability would seem to be from their staining behavior that they are nuclear in nature. He concluded his experiments by stating that evidence is produced to show that flagella may not be of ectoplasmic origin, but may originate somewhere in the endoplasm and that there are some indications that flagella are not homogeneous but contain masses of nuclear material distributed at regular intervals.

Also in agreement with the theory that flagella originate in the endoplasm are Topley and Wilson (1937), page 24, who refer back to Trenkmann (1890), Ellis (1902-03), Fuhrmann (1910) and Meyer (1912), who believe that the central portion of the flagellar thread passes through the cell membrane and is in direct connection with the cytoplasm. Fuhrmann believes that the proximal extremity is connected with a granule of chromatin. This would then be analogous to the blepharoplast, first

described and named by Webber (1897), in his work on Zamia and the pollen tube apparatus of Ginkgo. He stated: "I am not aware that any distinguishing name has been applied to such an organ, and I would here suggest the name blepharoplast to distinguish them from other organs of the cell." Webber derived the word from two greek words meaning "eyelash" or "cilium"; and "formed". Topley and Wilson pointed out that the conclusion that the flagellar thread is in direct connection with the cytoplasm is adversely criticized by Zettnow (1918).

In his work with the fungi Blastocladia, Cotner (1930) found that the cilium is inserted on the plasma membrane in a highly refractive body, the blepharoplast, which is definitely connected to the tip of the nucleus by a single thread-like connection. He summarized this work in connection with the cilia and stated that the zoospores of the species studied all have a definite basal granule (blepharoplast) at the insertion of each cilium. This granule is connected with the nucleus by a definite strand (rhizoplast). The cilia develop as outgrowths from the region of a basal granule which has reached a position near the plasma membrane as a result of nuclear activity. The basal apparatus is always of nuclear origin and seems to be composed of several chromatic bodies, one of which forms the basal granule normally retained or drawn back into the nucleus and usually forms the tip or

beak-like part of the organ during zoospore activity. When the zoospores of either swimming stage come to rest, the basal granule or granules, as the case may be, migrate back to the nucleus and apparently become part of that organ.

In the writers investigation there has been stained by the use of a modified Casares-Gil's mordant and a high dilution of a mixture of borax and thionin, a definite basal granule or group of granules just inside the ectoplasmic membrane at the base of the flagella. In all indications these granules appear to be chromatin material.

In order to make a more clear differentiation between the flagella and the soma, a series of serological tests was made. The results of this study substantiate the cytological work done on the flagellated cells of Sp. serpens.

SEROLOGICAL STUDY

Many workers have noted the specificity of antigen material in the flagella of bacteria differing from that in the soma. These have been designated as the flagellar "H" and the somatic "O" antigens. The flagellar-somatic hypothesis was advanced by Smith and Reagh (1903) to explain the action of agglutinating sera prepared from a motile strain of the hog cholera bacillus and from its non-motile variant. The serum for the motile strain contained agglutinins which were demonstrated by absorption tests. Floccular and granular agglutination respectively were produced by the anti-serum of the motile strain while the anti-serum for the non-motile variant caused granular agglutination only. The non-motile strain was unable to absorb the flocculating agglutinins of the serum for the motile strain. It was thought by microscopical examination that the textural difference between floccular and granular clumping was a structural one dependent on the presence or absence of a foundation, presumably agglutinated flagella. Orcutt (1924) confirmed these results and concluded that the flagella possessed an individual antigenic property. She also demonstrated that the flagellar suspension, when heated to 70° C., was no longer agglutinable but retained its antigenic qualities. After shaking, the flagella were detached from the bodies of Eb. typhosa by Yokota (1925) and innoculated into rabbits to obtain an appropriate serum. He demonstrated

that the clumping which developed in flagellar suspensions when an appropriate serum was added was actually due to the aggregation of flagella. Yokota concluded that the flagella of Eb. typhosa contained a specific agglutinating and agglutinogenic property which distinguished them from the body of that organism. Arkwright (1926) confirmed these results by showing by means of hanging drop preparations that a pure somatic serum agglutinated the bodies of the bacilli without impairing their motility while a pure flagellar serum led primarily to an immobilization of the organisms. Hadley (1927) suggested that the serological differences between flagellated organisms and these same organisms deprived of or lacking their flagella might be explained in terms of soluble specific substances without any reference to morphological elements. The writer believes there can possibly be a morphological element entering into this difference which is explained later.

Craigie (1931) ascertained that the "H" antigen of Weil and Felix (1917) is associated with the flagella of an organism and summarized the results as follows:

H antigen	Flagella
Present only in flagellated organisms.	
Responsible for large-flaking agglutination.	Responsible for floccular agglutination.
Heat labile.	When heated to 75° C. are no longer agglutinable or demonstrable microscopically.
Labilotropic agglutinin is not impaired by degree of heating which destroys stabilotropic agglutinin.	Flagellar agglutinin is not impaired by degree of heating which destroys somatic agglutinin.

Craigie stated that this evidence does not necessarily mean that flagella consist entirely of "H" antigen.

Gibson (1932) agrees with the many workers in this field that the clear supernatant liquid of Vibria comma (Schroeter) may be employed as an "H" antigen in agglutination reactions. Various motile organisms were treated to obtain such flagellar suspensions. The results were quite definite, large, flocculent masses appearing rapidly in such tests. It was found that such appearances could be produced in all cases where a sample of serum reacted in even moderately high dilution with an untreated suspension of a motile organism. It is now generally accepted that the "H" antigen is found only in motile organisms and is associated with the flagella.

In most of the work carried out upon flagellar and somatic antigens, the agglutination reaction was used as a measure of the serological specificity of the antigen and its titer. In the author's work with the flagellated Sp. serpens, the precipitin reaction was used with excellent results.

Materials and Methods.--Sp. serpens was grown on dextrose agar slants and harvested twenty-four hours after innoculation. A suspension of the organisms was made in physiological salt solution and an aliquot part was set aside to be innoculated into the rabbit as whole cells. The remaining suspension was placed in a sterile bottle with sterile glass beads and shaken by hand approximately five to ten minutes. It was then centri-

fuged in small agglutination tubes, the clear liquid containing the suspended flagella was drawn off by a sterile pipette and placed in a separate tube. The remaining soma portion of the cells was re-suspended with physiological salt solution up to the original mark and used as a somatic vaccine. The rabbits were innoculated intraperitoneally at five day intervals for seven innoculations and then bled aseptically from the heart six days after the last innoculation. The serum was separated from the blood cells, placed in sterile tubes and stored in the refrigerator.

The details of the rabbit immunization are given in Table I, which shows the number of innoculations and the amount of innoculum.

The bacterial suspensions to be tested against the sera were prepared as above but the bacterial count was 925 million per cc. After separating the three antigens--whole cells, soma, and flagella--the suspensions of each were frozen quickly by placing the tubes containing the suspensions into a mixture of ice and NaCl and then thawed immediately. The procedure was repeated five times to enable the solid cellular and flagellar systems to be liquified or to liberate the protein systems from the solid into solution which was necessary for the precipitin reaction.

It would be interesting to explain at this time that after the freezing-thawing process just described, the flagellar

Table I

Suspension of organisms.		Number of Innoculations and Amount of Innoculum.
Whole cells	1.	0.3 cc. of 200 million per cc.
	2.	0.3 cc. " " "
	3.	0.6 cc. " " "
	4.	0.6 cc. of 725 million per cc.
	5.	1.0 cc. " " "
	6.	1.5 cc. of 800 million per cc.
	7.	1.4 cc. " " "
Soma minus flagella	1.	0.3 cc. of 200 million per cc.
	2.	0.3 cc. " " "
	3.	0.6 cc. " " "
	4.	1.2 cc. of 725 million per cc.
	5.	1.7 cc. " " "
	6.	2.4 cc. of 800 million per cc.
	7.	2.6 cc. " " "
Flagella	1.	1.0 cc. of 200 million per cc.
	2.	1.0 cc. " " "
	3.	1.9 cc. " " "
	4.	2.2 cc. of 725 million per cc.
	5.	2.5 cc. " " "
	6.	3.0 cc. of 800 million per cc.
	7.	3.0 cc. " " "

and whole cells suspension developed a dinstinct yellow cast, while the somatic suspension remained pure-white in color. This may be interpreted as an indication of a distinct difference between the soma and the flagella. This color difference might simply be due to pigmentation changes.

The results of the precipitin reactions are given in Table II. In the indicated dilutions the first dilution, 1/1000, contained 925,000 cells per cc. or in the case of the flagella the first dilution contained the flagella separated from 925,000 whole cells per cc. The cells and the flagella

have presumably been ruptured following the freezing-thawing action and can now be used in the precipitin reaction.

The reactions were read in small precipitin tubes. Approximately 0.10 cc. of serum was placed in the bottom of the tube by means of a capillary pipette and the same amount of diluted antigen was then layered above the serum. Care was taken to obtain a precise line of contact between the two fluids. The reactions were read at the end of the first and second hours after being set up.

In recording the reactions, a profuse precipitate showing considerable depth at the junction of the immune serum and the antigen extract was read as a four-plus reaction (++++), a ring with less depth was read as a three-plus reaction (+++), the lighter but definite rings as two-plus reactions (++), and a reaction in which no definite ring was formed but in which a slight precipitate was visible was read as a one-plus reaction (+).

By referring to Table II, which shows the reactions between the immune sera and antigens, it will be noted that the strong reactions take place in the high dilutions with the F (flagellar) immune serum. It is obvious that the serum from the rabbit immunized with the flagellar suspension has a very high titer--much higher than the serum from the rabbit immunized against the soma and the one immunized against the whole cells. There is a definite titer in the last two

Table II

Precipitin Reactions

Sera	Ant. ext.	Dilutions of Extract 1/ 1000	1/ 5000	1/ 10,000	1/ 20,000	1/ 40,000	1/ 60,000	1/ 80,000	1/ 100,000
F	F	+++	+++	++++	++++	+++	+++	++	+
		+++	++	++++	++++	+++	+++	+++	++
F	S	++	+++	+++	++	+++	++	+	++
		++	+++	++++	+++	+++	++	+	++
F	W	++	++	+++	+++	+++	++	++	++
		++	++	+++	+++	+++	++	+++	++
S	F	+	+	+	++	++	-	+	-
		-	-	++	++	++	-	+	-
S	S	+	+	++	++	+++	+	+	-
		-	-	++	+	++	+	-	-
S	W	+	+	+	-	+	+	+	-
		+	-	+	-	-	-	-	-
W	F	+	+	+	-	-	-	-	-
		-	-	+	-	+	+	-	-
W	S	-	-	+	+	-	-	+	-
		-	+	+	-	+	+	-	-
W	W	++	-	+	++	+	+	-	-
		++	+	+	+	+	+	-	-

In both the serum and the antigen extract: F = Flagella, S = Soma, and W = Whole cells.

mentioned rabbits sera as there is a slight reaction between the soma antigen extract and its homologous immune serum and also between the whole cell antigen extract and its homologous immune serum. But in no case do these reactions reach as high a dilution as that attained by the flagellar immune serum.

There seems to be a distinct separate precipitinogenic substance in the flagella that causes the rabbit to produce a high titer. There must also occur in the soma of the organism a corresponding substance that, although it does not have the power to incite in the rabbit the production of a high precipitin titer, does react strongly with the flagellar precipitin. In other words, the results from this experiment show that the soma of Sp. serpens contains a substance that can not incite precipitin formation but retains its power to react with the precipitin which is present from the action of the flagellar precipitinogen.

This precipitinogenic substance of the flagella could possibly be located in the plasma membrane or in a thin membrane that simulates a capsule that may completely surround the flagella and the soma of the organisms. This substance may be compared to the type-specific substance in the capsule of the pneumococcus, but while the capsule of the pneumococcus is visible under certain circumstances, the thin capsule-like film surrounding the flagellated organism is undetectable by ordinary microscopical means. When the flagella are formed,

this film containing the specific antigenic material covering the soma would also cover the flagella. Because there is a greater amount of surface on the flagella than on the soma of the organism, a serological test would reveal the presence of this antigenic material with the flagella much more strongly than with the soma. This viewpoint explains the serological difference between the flagella and the soma, not as a qualitative test but purely as a quantitative test caused by the difference in the amount of surface of the flagella and the soma. This may explain the high precipitin reaction shown in Table II between the flagellar-immune serum and the whole cell, soma, and flagella antigens. There is a much greater percentage of surface in proportion to the cytoplasmic material in the flagella and hence a greater percentage of flagellar or surface antigen bringing about a higher degree of specific immunity in the rabbit than the soma antigen is able to attain.

CYTOLOGICAL MATERIAL AND METHODS

The strain of Sp. serpens studied was obtained from the stock culture carried by Montana State College for use in bacteriology courses. The culture was transferred every day on dextrose agar slants--0.3% beef extract, 0.5% peptone, 1.2% agar, and 1.0% dextrose. It was found that this organism grew very fast and plentifully at room temperature.

Previous to actual staining, the bacteria were taken directly from the slant and suspended in a drop of water on a clean slide. The drop of suspension was then spread evenly on the slide and allowed to evaporate to dryness before proceeding with the staining reactions. This method of preparing the smear was found to be superior to the use of osmic acid fumes for killing the bacteria before drying. When these fumes were used the cells were stained lighter and with less differentiation.

In order to secure clean microscope slides for the flagella stains, new slides were allowed to remain in cleaning solution (commercial sulfuric acid saturated with potassium dichromate) for five to ten minutes, rinsed thoroughly in fresh tap water and finally placed in a beaker of distilled water covered to keep out dust. Immediately before using a slide it was taken from the beaker of distilled water, washed with distilled water and held above the flame of a bunsen burner until the water had completely evaporated. To accomplish this, and further

clean the slide it was heated quite hot. Caution was taken to be sure the slide had cooled before proceeding with the staining reactions.

Several different mordants were tried for the staining of the flagella of Sp. serpens but only one, Casares-Gil's mordant, Zinsser and Bayne-Jones (1937), was found to be completely satisfactory both in ease of handling and results obtained. Mordants are very unstable mixtures and as Wright (1928) indicated, more consistent results would probably be obtained if they were of stable composition and more was known of the factors influencing the mechanism of their action. Many of the mordants change rapidly, both chemically, and physically after preparation. The writer used Casares-Gil's mordant during the course of the investigation of the flagella and when excellent results had been obtained it was discovered that these investigations agreed with Wright, for he stated: "Preparations were mordanted at 25° C., 30° C., 35° C., and 37° C. Temperatures above 30° C. increased the rate of precipitation and caused great clots to form on the slides, even when in a vertical position. The best results were obtained at 25° C. with the Casares-Gil mordant. Loeffler's mordant gave good results at higher temperatures."

The scheme used by the writer for the Casares-Gil's mordant is given below:

Tannic acid	10.0 gms.
Aluminum chloride	18.0 gms.
Zinc chloride	10.0 gms.
Rosaniline hydrochloride	1.5 gms.
Alcohol (60%)	40.0 cc.

The solids were dissolved in the alcohol by trituration in a mortar, adding 10 cc. of the alcohol first, and then the rest slowly. It was found that this mordant acted best after allowing it to stand as this alcoholic solution in a closed vessel for about two weeks and then used. This mordant worked satisfactorily for a period of about two weeks. Immediately before using, one part of the mordant was mixed with about four parts of water, shaken or stirred well and then filtered through filter paper directly onto the freshly dried bacterial suspension.

In some processes of bacterial staining the object has been to stain heavily so that one gets a clear profile or silhouette of the bacterial cell which aids in the recognition of the species. In the writer's work the principle of understaining most strikingly illustrated by Cotner (1930) was used. In this way the bacterial cell is stained a very light shade of the color of the stain used and then, when a counterstain is added, it shows a high degree of differentiation.

In following the development of the flagella several series of stains were made from the same culture to determine the most suitable time for the observation of the actual process of formation. It was noticed from this, that the

older cultures (18-24 hours) needed an intermediary stain to differentiate the granular, vacuolated older cells from the younger ones. The stain used was a modification of the carbol fuchsin (basic) stain. Following this stain a highly diluted mixture of thionin and borax was used to bring out the granules of the soma and flagella. In the younger cultures (6-7 hours) this intermediary stain of carbol fuchsin was not needed and only obscured the clarity of the stain. The following formula was used for the carbol fuchsin:

Solution A	Alcohol (95%).....	30.0	cc.
	Basic fuchsin.....	00.009	gms.
Solution B	Distilled water.....	285.0	cc.
	Phenol.....	15.0	gms.

When the solids were dissolved the solutions were mixed and used from a dropping-bottle.

The thionin-borax solution consisted of 0.1% thionin plus 0.5% borax in distilled water and this solution diluted 1:100 for the first application.

In a typical flagella stain of a 20-30 hour culture the following time scheme was used:

Casares-Gil's mordant.....	3 minutes.
Modified Carbol fuchsin.....	12 minutes.
Thionin (+ borax) diluted 1:100.....	1 minute.
Thionin (+ borax) undiluted.....	10 seconds.
Wash with distilled water.	

Sometimes the stained slides were cleared in pure clove oil for 16-24 hours but this was found to make no great difference and was soon abandoned.

In the younger cultures of six to seven hours the following time scheme was used:

Casares-Gil's mordant..... 3-5 minutes.
Thionin (+ borax) diluted 1:100..... 10 minutes.
Wash with distilled water.

This last scheme worked very well and as can be seen, is greatly simplified.

GENERAL DISCUSSION

The writer believes that in some respects a comparison between the fungi and bacteria might be set up in regard to their cilia formation. The results of this work dealing with the formation of the flagella of Sp. serpens is in many details in agreement with Cotner's (1930) work on the formation of cilia in the Oomycetes, and in agreement with Fuhrmann (1910) and Leifson (1930).

Cotner (1930a, 1930b, 1930c) made a cytological study of the zoospores of myxomycetes, Blastocladia and Oomycetes. In his work with Reticularia lycoperdon Bull. he found that the cilia-forming organ in the zoospore is composed of several granules and as the cilium matures these granules migrate back toward the nucleus and that this complex of granules, which appears as a single large granule at the base of the cilium of the mature myxomycete zoospore, is of considerable importance. He further stated: "In the zoospores, especially of the second swimming stage of species of Achlya, Aphanomyces, and Saprolegnia, the writer has shown, however, that both poles of the nucleus, with their attached or included granules, approach very near to or actually touch the plasma membrane; they may then withdraw from the membrane leaving there one or more granules. At points near these granules we find the cilia appearing, first as pseudopod-like projections, which later are matured into active cilia. The question now arises,

are these basal granules, which seem to control the development of cilia in the zoospores of the Oomycetes, homologous with the basal apparatus of the protista and those found in the spermatogensis of some animals and plants?"

Cotner goes on further to say that it seems likely that the basal apparatus in these fungi is very definitely of nuclear origin. In the second swimming stage of these fungi it has been shown that these granules, often three in number for each cilium, are first found occupying positions near the poles of the crescent-shaped nuclear apparatus as the spore prepares for cilia formation. The granules may, however, under certain conditions remain separate from the nucleus and have complete control of the development and function of one cilium if the granules at one pole of the nucleus become completely separated from the nucleus early in the process of cilia formation, but remain in close proximity with each other.

In reference to the second swimming stage of Achlya conspicua, Aphanomyces euteiches, and Saprolegnia monica var. glomerata Cotner stated: "It is apparent, then, that even though these basal granules are shown to be of nuclear origin they may develop and control cilia after, in so far as one can see, they have been completely separated from the nucleus. This would seem to show that the cilia of the spores in these fungi may have their origin and may be controlled by differentiated parts of the nuclear material and not by the entire

organ."

At the time of staining the specimen of Sp. serpens shown in Fig. 1, the individual cell was in the stage of development in which it is just beginning to gather a nuclear or at least a collection of granular material at one pole. In almost all cases these granules seemed to be a collection of smaller ones of different size and not one huge granule. The end of the cell is pointed slightly. Whether or not this is a characteristic formation of the actual beginning of the threading out of the flagellum is hard to determine, but nevertheless the cytoplasm and the plasma membrane at the end of the cell are not rounded smoothly. The small dark granule at the opposite end may easily be the first of a group to appear, preliminary to the flagella formation. The writer believes that this single granule or goup of granules is comparable to the blepharoplast in the cilia formation of certain fungi and other motile plant and animal cells.

Since these granules are stained by nuclear stains, it seems reasonable to interpret them as being composed of nuclear material. It seems highly improbable that these granules at the base of the flagella could possibly have arisen as the result of cytoplasmic activity. If these granules had developed as a result instead of as the cause of flagella formation, it seems likely that there would be a greater concentration of granules after the flagella formation

and not before. In Fig. 1 the large group of granules has gathered preliminary to flagella formation and in Figs. 6, 7, and 8 there seems to be a general disappearance of granules, whereas if the granules were a result of cytoplasmic activity, there would be a larger collection of granules in the later stages of flagella formation.

There is no well defined nuclear body that could be connected to the possible blepharoplast, but this does not seem to be a serious objection in view of the theory that the bacterial nucleus is diffused throughout the cell. This collection of granules could still carry on nuclear activity in regard to flagella formation. After the nuclear force that moves the granules to the poles of the cells, they need not remain in close contact with nuclear material anymore to exert a force by which the flagella are formed. As soon as the granules are energized, they may be able to carry on alone and may not need the nuclear force further to develop flagella at the poles. The granules in the ends of the cell seem to be the center of energy once they have collected there. It must be admitted that this group of granules or single granule as the case may be could be attached to the diffused nucleus of the cell, but in this work no visible attachment has been observed.

In the next illustration (Fig. 2) one of the granules of the group seems to be actually pushing the plasma membrane of

the cell out in front of it. It is likely that this outermost granule or the plasma membrane which is pushed out ahead of it is the actual beginning of the flagellum itself, or is the base from which the flagellum will arise. There can also be noticed a general distribution of granular material throughout the cell. There is little doubt as to the function of the group of granules at one end of the cell because of the apparent close correlation between the granular activity at the end of the cell and the flagella formation at that end. What force is this that causes the collection of certain granules at the poles of the cell? In some of the fungi the movement of the granules and the formation of the cilia closely accompanies the nuclear or cell division.

From the conclusions reached in this study of Sp. serpens, there seems to be a natural progression beginning with the reproduction of the cell by division and ending with the complete formation of the flagella. The flagella have been observed to be forming at different stages of cell division. It is believed that after cell division and when a flagellum has started to form, the cell may begin to divide again.

In this illustration (Fig. 3) the question arises as to the source of the protoplasmic thread representing the new flagellum which projects outward from the granule. Is this a newly formed mass arising from some vital action of the granule or is it a shift or gathering of cytoplasmic material

from around the granule? The writer believes that granular activity causes a definitely new protoplasmic mass to be formed; that this energy used in granular activity is acquired from the nucleus; and that these granules act as separate, visible parts of the bacterial nucleus.

As can be noticed this cell (Fig. 3) is smaller than many of the others and may seem to be slightly out of place in the general scheme because in Fig. 2 and 4 there are several granules while in Fig. 3 there is apparently only one. The small cell was placed there because of the first appearance of the actual mass of material starting development toward a flagellum.

In Fig. 4 the cell has formed a definite beginning of the flagellum and has the characteristic group of granules at its base. This illustration also shows the first instance where granules may have either moved out into the flagellum or may have been carried along with the cytoplasm accompanying the further development of the flagellum. As can be noticed, the granules are beginning to scatter away from the end of the cell and seem to be thinning out. In all probability the flagellum is well on its way to being formed and the granules are moving back into the body of the cell where they will possibly be diffused with the other nuclear material of the cell.

The cell in Fig. 5 is shown to be in further flagella

formation with a more pronounced scattering of the granules back from the end and also a greater concentration of the granules in the flagellum. The flagella at the other end of the cell have already formed. The flagellum just forming is shown to be made up of a series of granules--which agrees very closely with the work of Leifson (1930), as he stated: "If carbol fuchsin is added to a hanging drop of Vibrios (and other organisms as well) some flagella can be seen. These flagella are not stained solidly but as a series of dots."

This illustration also shows very nicely the way in which the cell divides. Before constriction takes place, a plate or a ring of denser protoplasmic material is formed at the point of division. This phenomenon was observed many times.

Figs. 6 and 7 may be taken together because they show the same stage of development. Again these cells are younger and have not developed along with their flagella. The flagellum of each of these cells seems to have a mass of cytoplasmic material at each tip, thicker and denser than the rest of the flagellum. This tip did not appear to be as dense as the granules in the cells themselves and therefore is thought to consist not of a nuclear granule but of a denser mass of cytoplasm. This could be compared very easily and closely to the work on the cilia formation of some of the fungi. However, it would be hard to say whether or not this mass at the tip is

caused by the whipping action of the flagellum. This would seem probable if the flagella form quite fast.

In Fig. 8 the upper flagellum is fully developed as a single flagellum. As can be noticed there is still a granule remaining at the base of the flagellum which has moved out into it slightly. The flagella of Sp. serpens form as a solid mass and then split into many smaller thread-like flagella --Fig. 8A. It seems that the granules out in the flagella have some vital connection with this splitting process. No conclusive evidence has been found to state that the granules move down the flagellum as it splits into many smaller strands. Leifson (1930) and Fuhrmann (1910) have also demonstrated granules in the flagella of bacteria. The granules either recede during the splitting process or they break up into smaller parts contained in each thread as the one flagellum splits into many flagella. In Fig. 12 the flagella have already split but still contain a large proportion of granular material. Either the granule has not moved back to the cell but will in the future or it may be composed of smaller granules which may separate as the flagellum splits into smaller units. Fig. 8 is remarkable because the cell was stained just at the moment that the flagellum at one pole had just developed to the stage before splitting and at the other pole the flagellum was just beginning to split. As can be noticed, the flagella begin to split at the distal end and

split down to the cell.

In Figs. 9, 10, 11, and 13 there can be noticed a phenomenon in which the granule moves out away from the cell and remains at the base of the flagellum, forming the apex of a cone. This could possibly be caused by the granule moving away and pulling out of place a membrane from the cell. This cone could also be caused by a definite phase of growth whereas a thicker part of the flagellum was caused to form between the granule and the cell wall. This granule at the tip of the cone-shaped mass is in appearance very much like the blepharoplast of the fungi and other motile plant and animal cells. Could it be that the whole bacterial cell is the nucleus and that this highly refractive body (blepharoplast) has moved away from it? Notice that this phenomenon takes place apparently in only the older cells. In the upper portion of Fig. 11 can be seen what seemingly is a flagellum arising from the cell by two different strands and then uniting.

Fig. 13 is extremely interesting as it shows a different phenomenon which to the writer's knowledge has never been observed before. In some of the cultures approximately twenty-four hours old there could be seen small coccus-like masses with a group of flagella coming out from the central particle. After several slides of stained suspensions of organisms were made it was concluded that these small forms were tips broken off from cells with the flagella still attached. Naturally

the next move was to try to find a stage in which the tip was just breaking off and in that way confirm the theory. Fig. 13 apparently represents a cell that is just "blowing its caps". The large dark places at both ends that seem about to break off are not very much darker than the rest of the cell and are not nearly as dark as the granules found in connection with flagella formation. Apparently the cell has reached a certain stage of normal or abnormal growth, perhaps caused by environmental factors, that have brought on this phenomenon of losing its cell tips.

Figs. 1 to 8A inclusive are drawings of different bacteria from six, six and a half, and seven hour cultures and show that the flagella are developing most rapidly at that age, while Figs. 9 to 13 inclusive are drawings made of different bacteria from approximately twenty hour cultures. The younger cells stain lighter than the older ones and therefore are represented by lighter drawings.

From the observations and evidence accumulated in this investigation the sequence of events that takes place in the formation of flagella in Sp. serpens appears to be as follows: 1. An accumulation of granules occurs in one end of the cell preliminary to flagella formation at that end. This stimulus that starts the polar accumulation occuring sometime after cell division is thought to arise from nuclear activity. Although there has not been observed any apparent connection

between the diffused nucleus of the cell and the granules, these granules may actually be parts of the chromatin material of the bacterial cell.

2. The first strand of the flagellum arises directly out from one or more of the polar granules and points away from the cell. This strand becomes longer and thicker. Granules have been observed lying out in the flagellum soon after it appears.

3. While the single flagellum is developing there occurs at its tip a slightly larger mass of protoplasm. This knob at the tip is apparently not as dense as the granules previously described but seems to be a concentration of the flagellar cytoplasm.

4. After the single flagellum has reached a certain stage of growth whereby it is approximately half as long as the cell, it begins to split into many thinner strands. From the writer's observation the single flagellum splits from the distal end down to the point of attachment until it becomes a fully developed tuft of flagella.

Further studies in this field may of course lead to some revision of the statements above.

SUMMARY AND CONCLUSIONS

This study in addition to developing a new staining technic in the differentiation of the structure of the flagella of bacteria has definitely shown a number of stages in the cytological development of the flagella of Sp. serpens. Serological proof of the antigenic difference between the flagella and the soma is presented. This difference is explained on the basis of the difference in proportion of surface to cytoplasmic content which exists in the flagella and the main or "soma" part of the cell.

1. A technic has been developed by which polar granules in the cells of Sp. serpens may be stained.

2. The granules in the ends of the cells were found to accompany flagella formation.

3. Granules lying in the flagella were also stained.

4. The flagella arise closely allied with granular activity as new structures with different serological properties from the protoplasm of the cell.

5. The flagella have been found to be forming at all different stages of cell division and development.

6. The flagella arise as single stalks and then split into many thread-like flagella. From the writer's observations they usually split from the distal end down to the point of attachment.

7. Serological studies (precipitin reaction) were made

of Sp. serpens using three divisions of the cell--the flagella, the soma and the whole cells.

8. Serological tests show a difference in the antigenic structure between the soma and flagella.

9. The flagella contain a much greater amount of precipitinogenic material than the soma.

10. The soma contain a substance capable of reacting with the flagellar-precipitin in the immune serum.

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Explanation of Plate I

These drawings from stained slides of Sp. serpens were made to the same scale with India ink under a large reading glass and represent a magnification of 6,360 times the original size of the cells as measured by an ocular micrometer. In Figs. 1 to 7 inclusive a 2 mm. Zeiss apochromatic 1.40 numerical aperture objective, a pair of 10x Zeiss apochromatic oculars, and a 1.40 numerical aperture condenser were used.

Fig. 1. Cell from a 6 1/2 hour culture with a group of granules at one end preliminary to the appearance of the flagellum. Actual size 11 x 1.3 microns.

Fig. 2. Here a group of granules at one end of the cell has collected, of which one granule is actually protruding out past the natural boundary of the end of the cell and seems to be actually pushing the plasma membrane of this part of the cell out in front of it. Less dense granules are scattered throughout the cell which is from a 6 1/2 hour culture. Actual size 11 x 1.1 microns.

Fig. 3. A single granule appears at the end of the cell from which a small strand of protoplasm is protruding. This is the first appearance of the flagellum. At the other end are two small granules, possibly the first of a group to gather. The cell is from a 7 hour culture. Actual size 6.5 x 1.0 microns.

Fig. 4. This is the first cell to actually have a granule

out in the flagellum. The strand of flagellum is larger than in Fig. 3 and there are a few granules scattered throughout the cell. Stained from a 7 hour culture. Actual size 11 x 1.1 microns.

Fig. 5. A large group of granules appear at the end of the cell and many in the flagellum. The flagella at the other end of the cell have already formed. The cell is apparently in the first stage of division with a plate or ring forming near the middle. Stained from a 6 1/2 hour culture. Actual size 9.5 x 1.1 microns.

Fig. 6. A cell with a long, slender, single flagellum having a slightly larger mass of protoplasm at the tip. At the other end there is a small collection of granules. From a 7 hour culture. Actual size 5.5 x 0.9 microns.

Fig. 7. A cell with a flagellum very similar to that of Fig. 6. Stained from a 7 hour culture. Actual size of the cell in Fig. 7 is 8 x 0.9 microns.

Fig. 8. The flagella of this cell are in two distinct stages of development. In Fig. 8 the flagellum is still single with a granule in its base. In Fig. 8A the single flagellum, which has a granule at its base, has started to split into several thinner flagella. Stained from a 6 hour culture. Actual size 11 x 1.3 microns.

Fig. 9. The flagellum has a single granule at its base. This granule is out from the cell and at the apex of a cone

connecting it to the cell. This is very similar to the blepharoplast of certain fungi. This cell is highly vacuolated. Stained from an 18 hour culture. Actual size 5.3 x 1.2 microns.

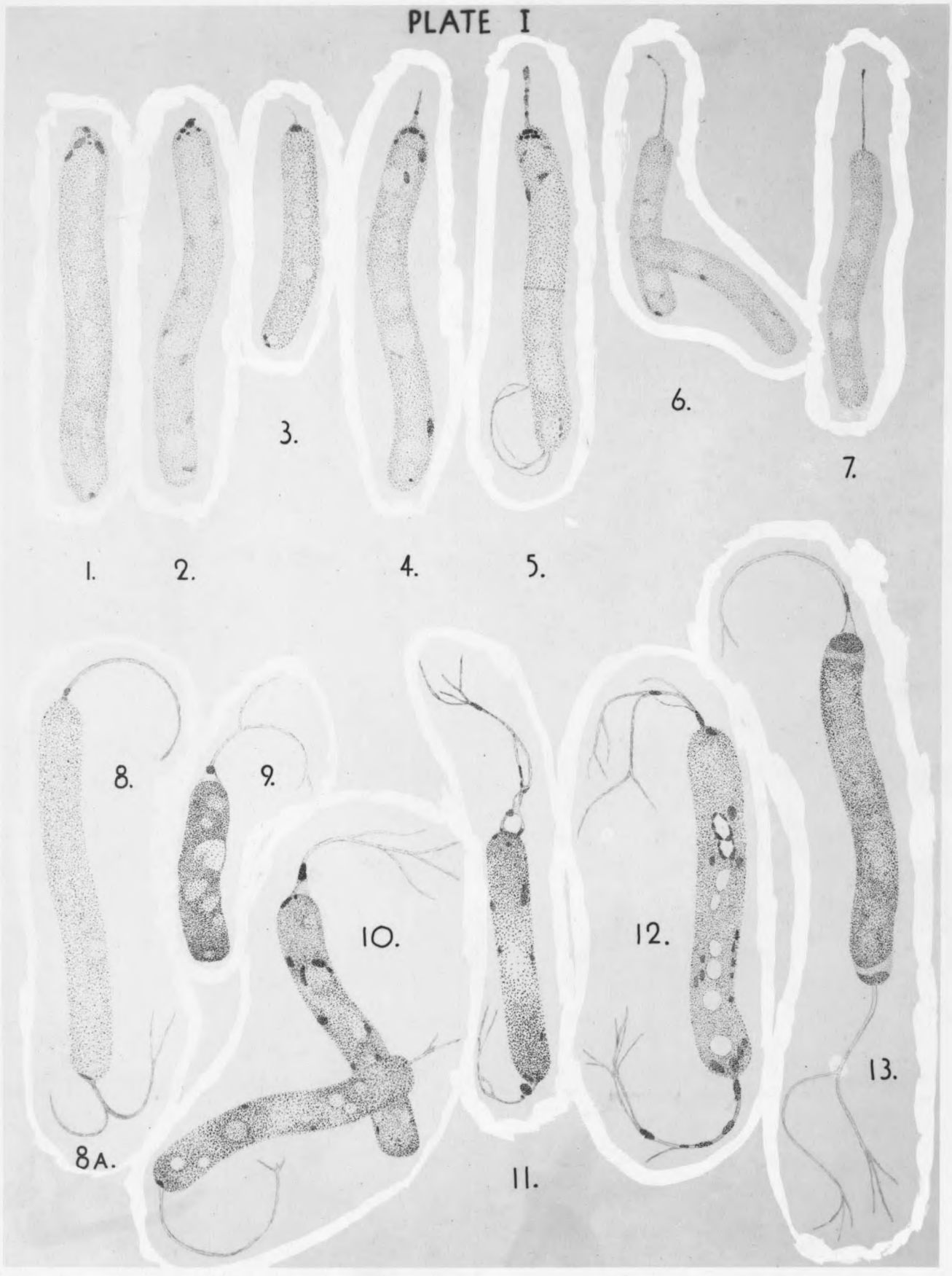
Fig. 10. The uppermost flagellum is very similar to that of Fig. 9. This cell is also highly vacuolated and has several granules scattered throughout. Stained from an 18 hour culture. Actual size 8.3 x 1.2 microns.

Fig. 11. At the upper end of this cell the flagellum is peculiarly formed. The flagellum seems to come from the cell in two separate strands which are soon joined. The flagellum at the lower end is similar to those in Figs. 9 and 10. The cell is apparently not vacuolated but has several granules scattered throughout the cell. Stained from an 18 hour culture. Actual size 7.5 x 1.0 microns.

Fig. 12. Highly vacuolated cell with many granules throughout. There are also many granules in the flagella. Stained from a 22 hour culture. Actual size 10.5 x 1.3 microns.

Fig. 13. The flagellum of the upper end has a granule similar to those in Figs. 9, 10, and 11 which forms the apex of a cone. In Fig. 13 the tips of the cell seem about to break off. Stained from an 18 hour culture. Actual size 10.5 x 1.3 microns.

PLATE I



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