SPECIFIC AGGLUTINATION OF SPIRILLUM SERPENS

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In a previous paper (Pijper et al., 1953) we described our investigations into the nature of the flagellum of Spirillum serpens. In Salmonella somatic, heat-stable or O antigens and flagellar, heat-labile or H antigens are recognized and give rise to typical agglutination patterns. H serum causes precipitates on bodies and flagella leading to fortuitous secondary entanglement of flagella, and O serum brings about polar attachment (Pijper, 1938). The purpose of this paper is to inquire into the existence of such antigens in S. serpens.

MATERIALS AND METHODS

The S. serpens used was described previously (Pijper et al., 1953). Rabbits were injected intravenously with (1) live spirilla, (2) spirilla killed at 56 C for one hour, and (3) spirilla steamed for 2 hours, respectively. Resulting sera were designated "L" (live bacteria), "56" (spirilla killed at 56 C), and "S" (spirilla steamed for 2 hours). Agglutination appeared to be specific; no serum had any effect on Spirillum volutans nor on another so far unnamed water spirillum. Agglutination was studied in tubes, and microscopically. Formolized and alcoholic suspensions were made as is customary with Salmonella. Absorbed "L" serum was "L" serum after absorption with steamed suspension.

AGGLUTINATION IN TUBES

The results are given in table 1. Obviously there were two agglutinogens, a heat-stable and a heat-labile one. The "L" serum and the "56" serum acted on both, and the absorbed "L" serum on the heat-labile antigen only. Formolized suspensions had labile antigen only. So far results resembled those with Salmonella. Differences were that alcaline suspensions hardly agglutinated and that "56" spirilla agglutinated very poorly but were excellent agglutinogens in

rabbits. A vast difference lay in the nature of the clumps, as revealed microscopically. Very little handling dispersed all clumps, those from "S" serum appearing slightly more resistant.

MICROSCOPIC AGGLUTINATION

Both ready-made clumps from tubes and clumps originating under the microscope were studied. Flagella never took an active part in agglutination, nor did polar attraction or polar attachment ever occur. The final pattern of agglutination clumps was rather similar whatever serum or suspension was used. As seen in figures 3, 4, 5, 7, 8, and 9, it was a regular irregular pattern best designated as "criss-cross".

For watching agglutination, microscopically live spirilla were best, motility assisting clump formation. In all cases the spirilla developed a "stickiness" which held them together if by chance they made contact. There were only minor differences between the effects of the "L", "56", and absorbed "L" serum on the one side and the "S" serum on the other side.

Both kinds produced a granular precipitate on bodies and flagella, but this was more pronounced with the "L" and "56" sera (figure 1). The thickened flagella could not get wound so easily round the body (figure 2). In these "L" and "56" sera in rather concentrated form motility stopped soon; in diluted form it went on for hours. Chance contacts caused adherence, not always permanent, but gradually the stickiness prevailed and produced clumps like figures 3 and 4. Figure 5 is a later stage of figure 4.

With "S" serum the precipitate was less; it is visible on the bodies of figure 6. This picture also shows the straightening of the flagella without complete loss of the original shape, a change often seen in "S" serum. Early "S" agglutination, without participation of flagella, is shown in figure 7. In corresponding dilutions motility lasted longer in "S" serum than in the others, and agglutination was slower. Figure 8 shows a final stage, exhibiting the density of "S" serum

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clumps through close adherence of the spirilla. On the whole, “S” serum clumps were somewhat more closely-knit than clumps from the other sera.

When a clump was swept along by a current, they moved with it and remained in place. Obviously there was a mass which held spirilla and granules together.

**TABLE 1**

| Agglutination of various suspensions of *Spirillum serpens* with various sera |
|-------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| SUSPENSIONS                    | Live spirilla     | “56” spirilla     | Steamed spirilla  | Formolized spirilla | Alcohol spirilla  | “S” spirilla      | Steamed spirilla  | Formolized spirilla |
| DILUTIONS OF SERUM             |                   |                   |                   |                   |                   |                   |                   |                   |
| 50                            | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |
| 100                           | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |
| 200                           | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |
| 400                           | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |
| 800                           | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |
| 1,600                         | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |
| 3,200                         | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |
| 6,400                         | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |
| 12,800                        | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |
| 25,600                        | ++                | +                 | +                 | +                 | -                 | +                 | -                 | +                 |

Microscopic examination of agglutination clumps of spirilla formed in tubes showed patterns similar to those formed under the microscope. Figure 9 is a clump of steamed spirilla from an “S” serum, very closely packed. Agglutination clumps from both kinds of sera, if the serum was used in very concentrated form, sometimes showed masses of granules, not just on bodies and flagella but in between the spirilla. Figure 10 shows a small clump with the spirilla in a cloud of granules. Figure 11 shows dense masses of granules between the spirilla. These granules did not show brownian movement, and

**WET-FILM INDIA INK METHOD**

Duguid (1948, 1951) chose the wet-film india ink method for demonstrating capsules and bacterial slime. He mixed bacteria with slightly concentrated india ink. Capsules and slime remained free from ink and left clear zones and areas. Amies (1951) in similar ways demonstrated capsules round *Pasteurella pestis*.

As the lactate medium in which *S. serpens* grew best curdled the ink, *S. serpens* was first washed in saline. Our results with the india ink method were that instead of there being ink-free zones or areas around the spirilla, ink was
Figures 1-9
found concentrated around them. When alive, spirilla swam round in a coat of ink particles from which inky tufts stuck out. Phase contrast microscopy in all experiments with india ink often was helpful to locate spirilla in the ink.

A quick method to show the avidity of the surface coat of spirilla for india ink was to pass a platinum loop charged with spirilla through a drop of half-strength india ink on a slide, and then to put the loopful on another slide where it was covered with a coverslip.

Figure 12 shows how much ink a live spirillum can collect in this way. Formalized spirilla took up about as much (figure 13) and steamed spirilla behaved similarly (figure 14). In contrast to all these, spirilla heated to 56 C took up exceptionally little ink (figure 15).

**SPECIFIC AGGLUTINATION IN INDIA INK**

By placing a small loopful of agglutinating serum on a slide and adding a large drop of spiral suspension, and also one of india ink, then covering the whole drop with a coverslip, microscopic preparations suitable for bright field examination or phase contrast microscopy were made. This technique worked well with live suspensions. With dead suspensions it was preferable to let agglutination take place in tubes and then suspend the clumps in india ink.

With both techniques it became apparent that there was a slimy sticky coat that held the spirilla together in clumps. The agglutinated spirilla always were found embedded in masses of particles of ink, and these were carried in the slimy coat of the spirilla. Figure 16 shows live spirilla agglutinated by “L” serum; figure 17 shows live spirilla in “S” serum. Figure 18 is a picture of formalized spirilla in “L” serum, and figure 19 represents steamed spirilla in “S” serum. In all cases the criss-cross pattern was well maintained and the spaces between the spirilla were filled with ink. Here again, as a rule, the “S” serum clumps were packed more closely.

With live spirilla the clumps often kept moving for some time, both with “L” and “S” type of serum, and the inky coats moved with them.

**DISCUSSION**

The india ink experiments showed that there is a slimy coat around spirilla which is instrumental in bringing about both kinds of agglutination. It follows that both antigens, the heat-stable and the heat-labile agglutinogen, must be present in this slimy coat. Heating to 56 C evidently removed this coat from the spirilla, making them inagglutinable; but the antigen was not destroyed and remained in the suspension, thus explaining that in rabbits such a heated suspension acted as a very good agglutinogen.

The granular precipitate on bodies and flagella which preceded agglutination must be regarded as the result of an interaction between agglutinins and agglutinogens which probably are present in concentrated form on bacterial surfaces. With very concentrated sera these granules naturally would be found right through the slimy coat.

The slightly more fluffy nature of the clumps arising from heat-labile antigens would indicate that these antigens were more voluminous and took up more space in the coat. The heat-stable antigen might be closer to the bacterial wall and thus give rise to denser agglutination clumps.

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**Figure 1.** *Spirillum serpens*, alive, in “L” serum, showing granular precipitate on flagella. 1,200 X.

**Figure 2.** *Spirillum serpens*, alive, in “L” serum, with thickened flagellum, not quite wound round body. 600 X.

**Figure 3.** Early agglutination of *Spirillum serpens* in “L” serum, beginning criss-cross pattern. 800 X.

**Figure 4.** Agglutination of live *Spirillum serpens* in “L” serum, note nonparticipation of flagella. 600 X.

**Figure 5.** Later stage of figure 4, note criss-cross pattern. 600 X.

**Figure 6.** *Spirillum serpens*, alive, in “S” serum, showing precipitate on body and straightening of flagella. 800 X.

**Figure 7.** *Spirillum serpens*, alive, in “S” serum, in early agglutination, flagella not taking part. 1,200 X.

**Figure 8.** *Spirillum serpens*, alive, final stage of “S” agglutination, note close packing of criss-cross pattern on right and left. 600 X.

**Figure 9.** Steamed *Spirillum serpens* in “S” serum, note close packing. 400 X.
Figures 10-19
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SUMMARY

*Spirillum serpens* has a heat-labile and a heat-stable agglutinogen, both situated in a slime layer which surrounds the microbe, and which has an avidity for India ink particles. Agglutination is due to increased stickiness of this slimy coat, and this produces a characteristic criss-cross pattern of agglutination with both kinds of agglutinogen. The flagellum takes no part in agglutination and therefore is not homologous with similar structures on *Salmonella*.

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Figure 10. *Spirillum serpens*, alive, in very concentrated serum, surrounded by granular cloud. 800 X.

Figure 11. *Spirillum serpens*, alive, in very concentrated serum, showing dense masses of granules between spirilla. 600 X.

Figure 12. Coat of India ink round live *Spirillum serpens*. 1,000 X.

Figure 13. Coat of India ink round formolized *Spirillum serpens*. 1,000 X.

Figure 14. Coat of India ink round steamed *Spirillum serpens*. 1,000 X.

Figure 15. *Spirillum serpens* killed at 56 C, in India ink, very little ink taken up. 1,000 X.

Figure 16. “L” agglutination of live *Spirillum serpens* in India ink. 600 X.

Figure 17. “S” agglutination of live *Spirillum serpens* in India ink, note close packing. 600 X.

Figure 18. “L” agglutination of formolized *Spirillum serpens* in India ink. 400 X.

Figure 19. “S” agglutination of steamed *Spirillum serpens* in India ink. 600 X.