PRELIMINARY EVIDENCE THAT BACTERIAL FLAGELLA ARE NOT “POLYSACCHARIDE TWIRLS”

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Received for publication January 5, 1951

During the past few years Pijper (cf. 1949) has directed our attention again to the problem of bacterial flagellation by insisting that bacteria move by undulation and gyration rather than by the activity of their flagella. While they move in this fashion, he maintains, their slime layer is pulled out to form “tails.” If this theory were true, bacterial flagella (being merely passive appendages) should not exhibit any motion of their own and should be chemically identical with the slime layer, which in many cases consists of polysaccharides (Knaysi, 1946). That the first conclusion is incorrect has been demonstrated more or less convincingly by Migula (1904), Reichert (1909), Hutchinson and McCracken (1943), Orskov (1947), Johnson and Baker (1947), Kauffmann (1948), Rinker, Robinow, and Koffler (1950), and Mallett, Koffler, and Rinker (1951). This paper deals with preliminary evidence, already briefly described in an abstract (Rinker and Koffler, 1949), that makes the second corollary of Pijper’s views also unlikely.

METHODS

Organisms. A variety of flagellated bacteria were used, such as Achromobacter sp., Azotobacter vinelandii, Bacillus cereus, Bacillus megatherium, Bacillus polymyxa, Bacillus subtilis, Caryophanon latum, Escherichia coli, Proteus vulgaris, Rhizobium trifolii, Salmonella typhosa, and Sarcina ureae. In many experiments Sarcina lutea was used as a nonflagellated control.

Media. Practically all of the species studied can be grown on heart infusion agar or yeast extract agar, but in some instances other media were more satisfactory. Caryophanon latum was grown on a cow dung medium (Mallett, Koffler, and Rinker, 1951). For the growth of Bacillus subtilis 712, a medium having the following composition was commonly used: beef extract, 5 g; glucose, 15 g; agar, 18 g; and tap water, 1 L. For the isolation of the gum produced by this organism, a medium described by Bovarnick (1942) was used. Rhizobium trifolii was grown on a medium containing the following constituents: K₂HPO₄, 0.5 g; MgSO₄·7H₂O, 0.2 g; MgCl₂, 0.1 g; CaCO₃, 3.0 g; yeast water (pH 6.8), 300 ml; and distilled water, 700 ml. Azotobacter vinelandii was grown on Burk’s medium (Wilson and Knight, 1948). To harvest cells of Rhizobium and Azotobacter for the isolation of their gums Allison’s solution (Wilson and Knight, 1948) was used.

1 This work was done as part of a program on the biological and chemical nature of microbial flagella and was supported in part by funds from the Purdue Research Foundation and the Office of Naval Research.
Electron microscopy. Supporting films for microscope screens usually were prepared from a 0.5 to 0.75 per cent solution of formvar (an acetal derivative of formaldehyde) in ethylene dichloride. Specimens were prepared as follows: A portion of surface growth was removed from a slant or plate and mixed with enough distilled water to give a slightly cloudy suspension. Small drops were placed in the center of the mounted films with the aid of a small hypodermic syringe fitted with a no. 26 needle (the point of which had been filed flat). The screens were allowed to dry and then treated as desired. Shadow-casting was done with chromium and uranium, in essentially the same manner as was described by Williams and Wyckoff (1945). An RCA 50-kv electron microscope model EMU was used.

RESULTS AND DISCUSSION

In many micrographs flagella appear to arise from the endoplasm rather than the ectoplasm. For instance, van Iterson (1947) and Houwink and van Iterson (1950) published beautiful pictures documenting this point. At a meeting of the Society of American Bacteriologists, Lofgren (1948) showed micrographs of
Spirillum rubrum in which the flagella appeared to arise from dense masses at the end of the cells. Other examples are presented in figures 1, 2, 3, and 4.

Although cursory inspection of such micrographs may lead one to believe that flagella are of endoplasmic origin, one must be cautious in accepting such interpretations. Owing to the nature of an electron micrograph (which in a sense is analogous to an X-ray picture), it is almost impossible to determine with any degree of confidence whether a flagellum that appears to penetrate the ectoplasm actually does so. A flagellum passing over rather than through the ectoplasm would give the same impression. To be sure, shadow-casting will enable one to tell whether a flagellum lies on top or beneath the cell, but it will not settle the question of whether it penetrates the outer layers of the cell. Thus it seems that more conclusive information on the origin of bacterial flagella must be gathered by cytochemical means.

Recent data by Weibull (1948, 1949, 1950a,b), Weibull and Tiselius (1948), and Astbury and Weibull (1949), published while this research was in progress, leave little doubt that the flagella of Proteus vulgaris and Bacillus subtilis at least are protein in nature, presumably of a type similar to that of primitive muscle or hair. Demonstrating the protein character of flagella, however, is not in itself sufficient to disprove Pijper’s hypothesis, inasmuch as the slime layers of some organisms at least may also be composed of proteins or polypeptides.
Figure 3. Cells of *Bacillus cereus* from a 9-day-old culture. Drop of broth placed on screen, dried, washed in distilled water, and shadowed with chromium. Negative print. × 18,750.

Figure 4. Unidentified organism. Water suspension made from a 15-hour-old slant. Chromium shadow. Negative print. × 18,750.
This is certainly true for some strains of *Bacillus subtilis* (Bovarnick, 1942) and *Bacillus anthracis* (cf. Dubos, 1947), and may hold true also for the many members of the genus *Bacillus* that produce peptide antibiotics. Furthermore, the fact that gram-positive organisms are thought to possess Mg-ribonucleic-acid-protein complexes somewhere in the ectoplasm suggests that Pijper’s “mucous twirls” possibly could be other than polysaccharide in composition. Crucial experiments therefore must include data on the composition of both the flagella and the slime layer. Detailed work along this line is in progress in our laboratory.

To some extent the following experiments, employing phosphotungstic acid (PTA) as a selective “stain,” fulfill this criterion. These experiments involved the demonstration that PTA, a protein precipitant that reacts with basic groups, “stained” flagella of certain bacteria but did not precipitate their slime layer material, which had been isolated in relatively pure form.

Not only is it difficult to observe flagella in the light microscope, but unless they are shadow-cast they often do not show up well even in the electron microscope, in spite of the high resolving power of that instrument. However, if flagella react with PTA, they should appear dark in the electron microscope, because they will then contain tungsten, a heavy atom that is relatively opaque to electrons. To observe whether or not bacterial flagella react with PTA the following method was used: A dried preparation of bacteria, on a formvar film supported on a screen, was thoroughly washed with distilled water, dried, and observed in the electron microscope. After observation the screen was removed, dipped into a solution of PTA (0.1 to 2.5 per cent PTA in water, adjusted with H$_2$SO$_4$ or HCl to a final pH of 3.0 to 5.0), and allowed to react for a period that varied from 10 seconds to 45 minutes. The concentration of reagents and time of reaction were varied depending on the particular organism used. The screen was then removed from the solution and thoroughly washed in distilled water, dried, and again observed. Extensive use of this procedure with the various organisms mentioned has indicated fairly consistently that flagella, which without treatment showed up poorly or not at all, gave clear images after reaction with PTA. Figures 5 through 8 demonstrate some of these results. Figures 5 and 6 are of *Caryophanon latum*, a large organism, the use of which was suggested to us by Dr. C. F. Robinow. Because of its large size this organism is easily visible under the low-power objective and lends itself to a study of motility as described by Rinker, Robinow, and Koffler (1950). Occasionally, when the concentration of PTA was too high or the pH too low, cells would appear disrupted. Such extreme effects are shown in figures 9 and 10.

Interestingly enough, “staining” flagella could be brought about with such organisms as *Azotobacter vinelandii*, *Rhizobium trifolii*, and *Bacillus subtilis* 712. The slime layers of the first two organisms were reported to consist of polysaccharide complexes somewhere in the ectoplasm.
Figure 5. Caryophanon latum. Cells washed from a 24-hour-old plate culture with 5 per cent formalin, centrifuged, and washed twice with distilled water. A drop of the final suspension was then placed on a prepared screen. \( \times 18,750 \).

Figure 6. Caryophanon latum. After the micrograph in figure 5 had been taken, the screen was removed and dipped in a 1 per cent solution of PTA in \( 10^{-1} \times \text{H}_2\text{SO}_4 \) for 45 seconds, removed, washed in distilled water, and observed. The flagella are now quite distinct. \( \times 18,1750 \).
Figure 7. *Bacillus subtilis* 712, 22 hours old. A drop of water suspension was placed on a prepared screen. $\times 18,750$.

Figure 8. *Bacillus subtilis* 712. After the micrograph in figure 7 had been taken, the screen was removed and dipped in a 1 per cent solution of PTA in distilled water (pH about 3.0). After 6 seconds it was removed, washed in distilled water, and observed. Flagella are attached to the cells even at places where slimy material seems to be torn away. $\times 18,750$. 

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Figure 9. Caryophanon latum. A water suspension of a cell from a 24-hour-old plate culture. The screen was dipped in a solution of 2 per cent PTA with enough H₂SO₄ added to bring the pH to 2.0. This micrograph illustrates the harmful effect of "staining" with PTA that is occasionally noticed. × 18,750.

Figure 10. Caryophanon latum. A chromium shadowgraph of the preparation used in figure 9. Negative print × 18,750.
charides containing glucose and glucuronic acid (Hopkins, Peterson, and Fred, 1930; Cooper, Daker, and Stacey, 1938; Bray, Schlüchter, and Stacey, 1944), whereas *Bacillus subtilis* 712 produces a gum consisting of polymerized D-glutamic acid units (Bovarnick, 1942). Since neither of these gums contains any basic groups with which PTA could combine, the flagella of these organisms would not be expected to “stain” with PTA if they actually were extensions of the slime layer. To strengthen this argument we isolated, in relatively pure form, the slime materials of *Rhizobium trifolii* and *Azotobacter vinelandii* according to the method of Hopkins, Peterson, and Fred (1930). Also, through the courtesy of Dr. Bovarnick, we obtained a sample of the D-glutamic acid polymer of *Bacillus subtilis* 712. These isolates did not react with PTA in concentrations and under the conditions of pH used for the “staining” of flagella. This adds weight to our view that the composition of the flagella of these organisms at least is different from that of their slime layers.

A few experiments with crystalline trypsin are relevant in this connection, although they cannot be regarded as conclusive. Prepared screens of *Bacillus subtilis*, *Bacillus polymyxa*, *Proteus vulgaris*, and *Caryophanon latum* were placed in pH 8.0 phosphate buffer solutions containing from 2 to 4 per cent crystalline trypsin. After 20 to 30 minutes the screens were removed, washed in distilled water, shadowed, and observed. Controls were also prepared by placing the cells in phosphate buffer alone or in distilled water alone for the same lengths of time. After tryptic digestion most of the cells were devoid of flagella, and as far as one could tell the slime layers were not damaged. Were the specificity of crystalline trypsin more absolute, these experiments would indicate again that flagella are proteinaceous. Unfortunately, it has recently been demonstrated (Schwert, Neurath, Kaufman, and Snoke, 1948) that crystalline trypsin exhibits some esterase activity; our experiments therefore can be regarded only as suggestive. This caution is especially necessary because the procedure used may offer another source of error. Washing of the screens is required after tryptic digestion to remove the soluble materials that would otherwise precipitate onto the specimen films during evacuation and thus obscure the objects to be studied. Should tryptic digestion result in the breaking off of flagella rather than in complete digestion, washing may remove the loose flagella, leaving the impression that the flagella had been completely dissolved. More convincing experiments will therefore have to be done with purified preparations and slime layer materials; such experiments are in progress.

**Acknowledgments**

The electron microscopy was done by the authors in the laboratory of Dr. H. J. Yeanian of the Department of Physics, Purdue University. We wish to express our thanks for his kindness, generous counsel, and valuable co-operation. We are also grateful to Dr. K. Lark-Horovitz for directing our attention to the potentialities of selective “staining” with heavy atoms and to Dr. Dorothy M. Powelson for helpful suggestions made during the preparation of the manuscript.
Summary

Employing phosphotungstic acid (PTA) as an indicator of basic groups, we performed experiments on a variety of flagellated organisms such as *Achromobacter* sp., *Azotobacter vinelandii*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus polymyxa*, *Rhizobium trifolii*, *Caryophanon latum*, *Escherichia coli*, *Proteus vulgaris*, *Rhizobium trifolii*, *Salmonella typhosa*, and *Sarcina ureae*. Before treatment with solutions of PTA the flagella were not white in the electron microscope, but after treatment they gave clear images, probably because they had reacted with PTA. This acid contains tungsten, a heavy atom that is relatively opaque to electrons. The PTA did not react with the isolated gums of *Azotobacter vinelandii*, *Rhizobium trifolii*, or *Bacillus subtilis* 712, thus indicating a difference in the composition of the flagella and the slime material at least for these organisms. This suggests that bacterial flagella are not extensions of the slime.

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