A METHOD OF STAINING BACTERIAL FLAGELLA AND CAPSULES TOGETHER WITH A STUDY OF THE ORIGIN OF FLAGELLA

EINAR LEIFSON

From the Department of Pathology and Bacteriology, Johns Hopkins University, Baltimore

Received for publication May 22, 1930

BACTERIAL FLAGELLA

Flagella staining is generally considered a rather difficult procedure and even with the most careful technique only a few of a great number of slides show satisfactory flagella. The main trouble with most flagella stains is that the mordant which is used is not stable. Good results may be obtained at one time and poor results the next. An important property of a good flagella stain should, then, be a stable mordant. Such a mordant was described by Gray (1). He mixed potassium alum, mercuric chloride, tannic acid, and alcoholic fuchsin and immediately poured the mixture on the smear. All these reagents are perfectly stable and the stain can always be duplicated. It is excellent as compared with other flagella stains. A counter stain of a strong solution of basic fuchsin is also necessary. This stain gives most excellent results with the sporulating organisms such as B. vulgatus, B. subtilis, B. terminalis, Cl. botulinum, Cl. tetani, etc. With Bact. typhosum, and the Vibrios, not so good results are obtained. Various modifications of the stain were tried. A distinct improvement was the addition of sufficient hydrochloric acid to the alum to make the concentration of acid 1/100 N. Ammonium alum in place of potassium alum was found to give fully a good result. However, it was not until addition of alcohol was tried that an almost perfect flagella stain was obtained. Without the alcohol in the so-called "mordant," the flagella can be seen very faintly without the counter stain. With
the alcohol, no counter stain is needed, and the flagella are intensely stained and enormously enlarged.

METHOD OF MAKING SMEARS

The slides

The slides must be as clean as possible. Our method is to put the old slides in an alkaline soap solution to remove the oil, then into good cleaning solution for a day or so. The slides are then washed carefully with distilled water and dried between paper towels. Washing with alcohol seems to have no additional cleaning effect. The slides selected should be as free from scratches as possible. Just before use they are flamed strongly.

The organisms

The organisms are best grown in bouillon for twelve to twenty-four hours from which they are centrifuged, suspended in distilled water, recentrifuged, and resuspended to give a slightly milky suspension. This amount of washing is usually sufficient but further washing may sometimes give a clearer slide. Very good results may also be obtained by making a dilute suspension of the organisms from an agar slant. A loopful of the organisms is placed on the end of the slightly warm slide and allowed to run down the slide. If the water does not run down the slide it is greasy and should be discarded. Contrary to general opinion the flagella on some bacteria at least, are not easily broken off. A suspension of typhoid bacilli in distilled water was shaken in a shaking machine for twenty minutes with only a loss of about 50 per cent of the flagella. Suspended in distilled water, the flagella on typhoid bacilli were found to remain intact for one month. No stains were made after this length of time. Bacillus terminalis flagella were still intact on the organisms after two weeks in distilled water.

The stain

Basic fuchsin is by no means the only dye which can be used in the flagella stain. Methylene blue and crystal violet have been
used with about equal success. Differences in the solubilities of these dyes in alcohol makes it necessary to add them in different amounts to obtain the best results. With basic fuchsin the stain has the following composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium or potassium alum, saturated water solution</td>
<td>20 cc.</td>
</tr>
<tr>
<td>Tannic acid, 20 per cent water solution</td>
<td>10 cc.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 cc.</td>
</tr>
<tr>
<td>95 per cent ethyl alcohol</td>
<td>15 cc.</td>
</tr>
<tr>
<td>Saturated ethyl alcoholic solution of basic fuchsin</td>
<td>3 cc.</td>
</tr>
</tbody>
</table>

Crystal violet is about twice as soluble in alcohol as is basic fuchsin. If this dye is used, only about 1.5 cc. of it should be added in place of 3 cc. of basic fuchsin. Methylene blue is soluble to only the amount of about 1.5 per cent, or one-fourth the solubility of basic fuchsin. Since considerable alcohol is thus introduced with the dye the amount of alcohol added as such should be reduced to one-half. With methylene blue the following formula is recommended:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium or potassium alum, saturated water solution</td>
<td>20 cc.</td>
</tr>
<tr>
<td>Tannic acid, 20 per cent water solution</td>
<td>10 cc.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5 cc.</td>
</tr>
<tr>
<td>95 per cent ethyl alcohol</td>
<td>5 cc.</td>
</tr>
<tr>
<td>Saturated alcoholic solution of methylene blue</td>
<td>10 cc.</td>
</tr>
</tbody>
</table>

The ingredients are mixed in the order given. A slight precipitate usually forms which may be filtered off but this is not necessary. The stain should be kept in a tightly stoppered bottle and will keep for a week or more. As the stain gets old it stains more faintly than at first but less precipitate also forms on the slides. The stain will remain in good condition longer if a greater amount of dye is added, but only after one or more days does it really become good. If the stain is to be used at the time of preparation only, the quantities of dye which are added may be reduced. Instead of 3 cc. of fuchsin 2 cc. will give somewhat better results.

On a warm day the unfixed smears may be flooded with the stain and left at room temperature for ten minutes. On a cold day, however, it is better to place them in the incubator. The
higher the temperature the more deeply stained the flagella will 
be but more precipitate will also form on the slide. After about 
ten minutes the stain is washed off with water.

Counter stain

As far as the flagella themselves are concerned no counter stain 
is needed. Very beautiful preparations can be obtained, however, 
by using a counter stain of a color different from that of the stain. 
With the flagella stained red the organisms can be stained blue by 
counter staining for five to ten minutes with an aqueous solution 
of methylene blue made as follows:

<table>
<thead>
<tr>
<th>Methylene blue (dry powder)</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borax</td>
<td>1</td>
</tr>
</tbody>
</table>

Carbol fuchsin diluted 1/10 with water applied for five to ten 
minutes makes a good counter stain in case the flagella are blue.

DISCUSSION

Very little work seems to have been done lately on the nature 
of bacterial flagella. The most complete discussion of the subject 
seems to be that of Migula in his well-known "System der Bak-
terien." From the data then at hand Migula concluded that 
flagella were out-growths of the bacterial ectoplasm. This idea 
also seems to be generally accepted at the present time. From 
the appearance of flagellar preparations of Bact. typhosum, and 
Proteus vulgaris a different conclusion may be drawn. As already 
stated, neither of these organisms stains solidly with the flagella 
stain. The flagella, the ectoplasmic zone, and a central column 
are stained red. The rest of the organism is colorless unless a 
counter stain is used in which case it takes the color of the counter 
stain. In a number of cases the flagella are seen to originate in 
the central column. Only rarely does a flagellum originate be-
tween the central column and the ectoplasmic zone. Most fre-
quently the flagella seem to originate on the surface. Figure 1 
shows drawings of a number of typhoid bacilli. Figure 2 is a
photograph of typhoid bacilli. It is possible that the central column is an artefact, and represents a shrunken endoplasm. This being the case the clear zone between it and the ectoplasm should be empty. This would not seem to be the case, however,

![Diagram of Bacterium Typhosum](image)

**Fig. 1. Bacterium Typhosum**

from the way it takes the counter stain. Even very dilute alkaline methylene blue stains it quite heavily. The alternative explanation would be that the central column is a definite structure running along the centre of the organism and having a composition similar to that of the flagella.

![Diagram of Vibrio Finklora-Prior](image)

**Fig. 3. Vibrio Finklora-Prior**
The sporulating organisms studied, both Gram positive and Gram negative, stain solidly except in the case of *B. cereus*. In the case of this organism the ectoplasm is stained only in patches. The vibrios (*V. metchnikovi*, and *V. finklor-prior*) stain evenly and somewhat faintly. When counter stained with Manson's methylene blue (5 per cent borax + 1 per cent methylene blue) many of them show the interesting structure illustrated in figure 3.

From the data obtained, with *Bact. typhosum* especially, it might be concluded that bacterial flagella are not of ectoplasmic origin but originate in the endoplasm, and perhaps in some definite structure in the endoplasm. This conclusion would find substantiation in the work of Smith and Reagh (2) where it was shown that agglutinins produced against a motile strain of *Hogcholera* organisms were not very active against a non-motile strain of the same organism. It is concluded that the agglutinins for the flagella and the cell body are different.

In the case of organisms having terminal spores, the flagella as a rule first fall off the end in which the spore is located. This perhaps may not be taken to indicate that the flagella come from the endoplasm but merely that the ectoplasm is weakened around the spore. Figures 4 and 5 are photographs of *Cl. botulinum* showing both flagella and spores. Figure 4 was especially selected to show an organism in which the flagella were still intact even on the spore end.

Flagella and capsules may be present on the same organisms. An especially beautiful demonstration of this is found in the case of *Ps. aeruginosa* (pyocyaneus) as shown in figure 6. These cells were obtained at autopsy from the peritoneal fluid of a guinea pig injected with the specific organism.

In the experiments on the effect of additions of acid (hydrochloric) to the flagella stain it was noticed that when a certain concentration (1/10 N) was reached the flagella would no longer stain evenly but as a series of dots. The dots are round and evenly spaced. If the acid concentration is increased still further the flagella do not stain at all. 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. These dots may be artefacts, of course. If they represent real structures the probability would be from their staining behavior that they are nuclear in nature.

**Fig. 2. Bacterium Typhosum**

**Fig. 4. Clostridium botulinum**

**Fig. 5. Clostridium botulinum**

**Fig. 6. Pseudomonas aeruginosa**

(Magnification 1000 X)
BACTERIAL CAPSULES

The stains generally used for bacterial capsules are not altogether satisfactory. The stain for flagella described in the preceding pages is also an excellent capsule stain. The method of making the smears and staining is exactly that described for flagella staining. The counter stain, however, is obligatory. With basic fuchsin in the stain and methylene blue in the counter stain, the capsules are stained red and the organisms blue.

Organisms such as pneumococci may be stained for capsules by smearing the slides directly from peritoneal fluid. It is, however, generally best to wash the organisms in distilled water before making the smear. The washing is performed as described for flagella staining. The stains are most conveniently made by preparing the smears from distilled water suspensions of the organisms from agar slants.

In case of organisms having both capsules and flagella, both are stained simultaneously as illustrated by figure 6. In such cases both the capsules and flagella take the color of the stain while the organisms take the counter stain. Organisms of the Bact. coli group have also been found with both capsules and flagella. The fact that flagella and capsules stain with the same stain does not necessarily indicate that they are of the same general nature. As the alcohol evaporates the dye precipitates, and in the process becomes adsorbed on the flagella, capsules, or any other material which may be on the slide.

The capsulated organisms which have been stained successfully by this stain include: pneumococci, streptococci, staphylococci, Bact. aerogenes, Bact. coli, Ps. aeruginosa, Bact. pneumoniae, and B. anthracis.

SUMMARY AND CONCLUSIONS

1. A flagella stain is described which stains in ten minutes, is simple to make, stable for a week or more, and with which no counter stain is needed.

2. The flagella and cell bodies may be stained a different color by the use of an appropriate counter stain.
3. Evidence is produced to show that flagella may not be of ectoplasmic origin, but may originate somewhere in the endoplasm.

4. Sporulating organisms may retain their flagella although fully sporulated. The flagella fall off first where the spore is formed.

5. Flagella and capsules may be co-existent.

6. There are some indications that flagella are not homogeneous but contain masses of nuclear material distributed at regular intervals.

7. The flagella stain described is also an excellent capsule stain. This does not indicate that flagella and capsules are of similar composition.

REFERENCES