An Electron Microscope Study of *Vibrio* Flagella

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SUMMARY

Electron microscopic study of vibrio flagella by using the negative staining technique revealed differences in chemical reactivity as between the sheath and core components. The sheath was easily degraded by autolysis and exposure to acid and urea, whereas the core was relatively resistant.

These differences suggest that the sheath and core are of different composition and that, whereas the core consists of the protein 'flagellin', the sheath is probably of cell-wall origin.

INTRODUCTION

Bacterial flagella have been shown by electron microscopy to be uniform sinusoidal filaments. With two organisms, *Bacillus brevis* (De Robertis & Franchi, 1951) and *Vibrio metchnikovii* (van Iterson, 1953), these filaments differ morphologically from all others examined in that they are composed of a core and surrounding sheath whose stability to autolysis (van Iterson) and trypsin treatment (De Robertis & Franchi) has been reported to differ from that of the core. Chemical analyses (Weibull, 1948, 1949, 1950, 1951; Kobayashi, Rinker & Koffler, 1959), and physical analyses (Astbury & Weibull, 1949; Beighton, Porter & Stocker, 1958) of purified non-sheathed flagella have established their protein nature, while physical analysis of the sheathed flagella of *Vibrio metchnikovii* revealed a similarity in basic structure between sheathed and non-sheathed types (Astbury, Beighton & Weibull, 1955). The application of negative staining to electron microscopy (Brenner & Horne, 1959) provided a method complementary to shadow-casting for the study of protein macromolecular organization. This technique has been used in studying the structure of flagella (Kerridge, Horne & Glauert, 1962) and fimbriae (Thornley & Horne, 1962). In the present work (preliminary results were communicated to the Fifth International Congress for Electron Microscopy, Gordon & Follett, 1962) the superiority of negative-staining over shadow-casting in the demonstration of flagellar sheaths is shown, and their response to treatment with various physico-chemical agents assessed. This has established gross differences in stability as between the sheath and core components.

METHODS

Organisms and cultivation. The organisms used were *Vibrio metchnikovii* NCTC 8848 and El Tor vibrio NCTC 5850 grown on nutrient agar at 37° for 12 hr. or at room temperature (about 20°) for 24 hr. The actively motile organisms were harvested as a saline suspension which was used fresh or allowed to autolyse for 48 hr. at 4°; they were fixed by adding 10% formalin to a final volume of 0.5% (v/v); unfixed controls were also examined to exclude the possibility of fixation artifacts. No attempt was made to separate flagella from the bacterial bodies.

Preparation of specimens. Bacteria are known to adhere to collodion film (Houwink & van Iterson, 1950) and when carbon-covered specimen mounts were floated on a suspension of organisms, sufficient bacteria and flagella adhered to the carbon film to provide a suitable specimen for microscopy. This was washed and stained by floating the grid for 10 sec. on the following solutions in series 1% (w/v) potassium iodide, distilled water (twice), 1% (w/v) ammonium molybdate, finally excess stain was removed and the remainder allowed to dry down on the film surface. By this technique, the effect of acid and urea treatment on the flagella was readily studied by interpolating appropriate solutions between the two changes of distilled water. Specimens were floated for various times on N/100 hydrochloric acid adjusted to pH 2 with sodium hydroxide or on a 6 M-urea solution. All electron micrographs were taken with a Siemens Elmiskop 1 with either single or double condenser illumination, at a plate magnification of $\times 40,000$.

RESULTS

In fresh young cultures negative staining revealed the flagella as white sinusoidal ribbons, with little or no evidence of internal structure. In older cultures, the two-component system of core and sheath became apparent, with the core outlined by two lines of stain running parallel to the flagellar axis. That this stain did delineate the core was confirmed on examination of autolysed specimens of *Vibrio metchnikovii* (Pl. 1, fig. 1) and El Tor vibrio (Pl. 1, fig. 2) where the core was observed with and without its surrounding sheath. In both cases the protruding core retained its normal wave form. Numerous specimens were observed with intact flagella demarcated into core and sheath, the degree of differentiation varying from one flagellum to another but being constant for any single flagellum.

Some autolysed specimens (Pl. 1, fig. 3) showed autolytic effects at sites other than the end of the flagellum. As well as completely removing the sheath from some flagella, autolysis merely loosened the sheath from the core from others (Pl. 1, fig. 4). A similar but more pronounced loosening was observed with flagella treated with urea for 5 min., and occasionally this was sufficient to allow a short terminal length of flagellum to escape from its enveloping sheath (Pl. 2, fig. 5). Whereas free cores—whether produced by autolysis, urea or acid treatment—always retained their wave form, empty sheaths showed no inherent rigidity and lay limply in irregular waves except where compressed against neighbouring structures by drying forces. Further urea treatment produced considerable swelling and partial removal of the sheaths (Pl. 2, fig. 6), whereas prolonged treatment completely removed the sheath, leaving an apparently intact core.

Although acid treatment also had more effect on the sheath than the core, the manner of sheath disintegration differed from that observed with autolysis and urea treatment. Instead of the loss of long reaches of sheath, characteristic of autolysis and prolonged urea treatment, exposure to acid for 10 sec. caused widespread comminution of the entire sheath (Pl. 2, fig. 7). Further exposure removed the sheath completely, leaving cores which, as after autolysis and urea treatment, retained their wave form and appeared to be intact.

Measurements of total flagellar width and core width alone were made on all control specimens and on several autolysed specimens. These measurements, a total of 150, fell into two distinct groups. In the major group (78 %) the total width was 350 ± 40 Å, with a core width of 165 ± 15 Å, while in the minor group values were greatly decreased, 270 ± 20 Å for total width and 125 ± 10 Å for core width. These latter measurements were always obtained from specimens which showed little or no demarcation of sheath and core (Pl. 2, fig. 5) and which had been prepared as fresh as possible. Autolysed specimens and suspensions left for several hours gave widths in the major grouping.

DISCUSSION

Negative staining. Negative stain is known to penetrate the hollow channel in rod shaped viruses (Brenner & Horne, 1959) and the clear demarcation of sheath and core in autolysed specimens might therefore be caused by penetration of stain from an autolysed end into a potential space between sheath and core. The observation here of a similar demarcation in intact vibrio flagella, however, indicates that a broken end is unnecessary for demonstration of the core and hence that the sheath must be permeable to the stain. This permeability would permit a thin film of stain to envelop the core with the resulting formation of areas of increased electron density at the edges of the projected image. The core would thus be outlined by two lines of stain along its edges, as was observed.

Flagellar width. The distinct division of the width measurements into two widely separate groups does not necessarily imply the presence of two types of flagella on the same organism. Young fresh organisms gave minimum width measurements and also showed a resistance to the penetration of stain. Organisms harvested and left for several hours, however, permitted ready access of stain through the sheath, indicating a considerable change in the bonding of at least the sheath component; the same flagella also gave much increased width measurements. These greater width values are therefore the result of extraneous factors introduced during preparation, and the true flagellar width is therefore 270 ± 20 Å, with a core width of 125 ± 10 Å.

In addition to the structural changes which occurred in the sheath during preparation the increased core width denotes that similar effects must occur in the core. If the structural modifications were sufficient to relax any inherent rigidity in the cylindrical flagellum and make its shape dependent on the medium surrounding it, then the removal of this medium on drying, combined with the considerable forces exerted on such a small object on drying, would result in compression of the flagellum into an ellipsoid. A much increased width related to the amount of structural relaxation would therefore be observed.

Reactivity. The clear demarcation of core and sheath afforded by negative staining has enabled us to show that the core and sheath constituents of the flagella of *Vibrio metchnikovii* react differently; identical results were obtained with El Tor vibrio. Although there is some evidence to suggest that the mode of attack on the sheath by each of the three reagents may differ, the conclusion reached from all the experiments is the same, namely that the sheath is easily removed, leaving an apparently intact core. This difference in stability indicates that the flagellum consists either of two constituents which differ chemically or of a single constituent in two sufficiently contrasting physical forms.

In the X-ray analyses of Astbury *et al.* (1955) almost identical X-ray diffraction photographs were obtained from the flagella of *Vibrio metchnikovii* and from those of a non-sheathed species, *Proteus vulgaris*. This close similarity is almost certainly produced by the core constituent of vibrios, because a substructure identical in type and periodicity has been observed both in vibrio cores and in the flagella of a paracolon bacillus, another non-sheathed species (Gordon & Follett, to be published). Since physico-chemical analyses have shown that non-sheathed bacterial flagella are composed almost entirely of the protein 'flagellin' these findings strongly suggest that the core of the vibrio flagella is also composed of the same contractile fibrous protein. Furthermore, the vibrio species of bacteria has a single flagellar antigen and thus, if their surrounding sheath is composed of flagellar protein, it must be identical antigenically with the core protein and yet have markedly differing physical properties from it. What seems more probable is that the sheath differs in chemical composition and may be of cell-wall origin.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Autolysed flagellum of *Vibrio metchnikovii*. $\times 125,000$.
- Fig. 2. Autolysed flagellum of El Tor vibrio. $\times 135,000$.
- Fig. 3. Flagellum of *V. metchnikovii* showing autolytic effects in non-terminal section. $\times 128,000$.
- Fig. 4. Loosening of sheath produced in flagellum of *V. metchnikovii* by autolysis. $\times 128,000$.

PLATE 2

- Fig. 5. Flagella of *Vibrio metchnikovii* treated with urea for 5 min. $\times 125,000$.
- Fig. 6. Flagellum of *V. metchnikovii* treated with urea for 15 min. $\times 120,000$.
- Fig. 7. Flagellum of *V. metchnikovii* treated with acid for 5 min. $\times 170,000$.