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Investigations on Bacterial Flagella

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The bacterial flagella have already been extensively studied from the morphological and immunological points of view. So far, staining methods and serological tests largely have been used. The results of these studies will not be discussed here, especially as a short summary has already been given [1] and any textbook of bacteriology will give further information. It may only be mentioned that the flagella are generally assumed to be the actively motile organs of the bacteria, which, however, is difficult to prove by direct observation on account of the minute dimensions of the individual flagella (thickness about 120 Å [2]). — Furthermore, the flagella show antigenic properties of a rich differentiation.

The aim of the present investigations [1–8] has been to gain information about the chemical nature and the detailed structure of the flagella and also, if possible, some knowledge about their functioning. On account of their dimensions (*cf.* above) mostly methods employed for investigations of macromolecules have been used, together with analytical methods, morphological studies and serological tests. — As far as the author knows, only one paper concerning the chemical nature of the flagella has been published earlier [9].

As has been mentioned above, the type of flagellation is not quite the same for different species. For practical reasons the investigations have been limited to a few representative types. Furthermore, as the investigations were performed at an institute not equipped with facilities for handling pathogenic material, most of the investigations were carried out on harmless bacteria. As types for such bacteria two commonly occurring and easily cultivatable bacteria, *Proteus vulgaris* and *Bacillus subtilis* were chosen. They show the very common peritrichate flagellation, *i. e.* the flagella are attached all over the surface of the cell. A few observations have been made on *Salmonella paratyphi* B (the preparative work was in this case performed at the State Bacteriological Laboratory [10]).

PREPARATIVE METHODS

The success of the purification of the flagella and the obtainable yield depends above all on the conditions during the cultivation of the bacteria. As has been shown [1] a cultivation temperature of 13—15° C is essential for the purity, and the harvesting of the bacteria has to take place shortly after the logarithmic growth phase (generally 3—5 days at the temperature chosen) in order to avoid poor yields. After harvesting the bacteria with saline solution and detaching the flagella from the bacterial bodies by shaking in an ordinary shaking machine (2—3 beats a second for half an hour) the bacterial bodies are brought down with an ordinary laboratory centrifuge (about 3000 r. p. m.). The supernatant, containing the flagella, is then centrifuged at 50 000 g (27 000 r. p. m.) in the Beam's air-driven ultracentrifuge. The flagella are deposited as pellets and resuspended. Further purification involves a series of centrifugations at moderate and high speed (19 000, 27 000 r. p. m. respectively) in order to remove impurities [1]. Purification is also effected by salting out [4]. Repeated centrifugations or repeated salting out does not improve the purity of the preparations markedly. Also extractions with ether or petrol ether has no appreciable effect.

Some alterations in the cultivation and the purification process referred to above, however, have been made during the course of the work. Instead of petri dishes, aluminum plates, 50 × 50 cm, have been used for the cultivation. Furthermore, instead of the tedious ultrafiltration, the flagella are now concentrated by salting out with ammonium sulphate from the saline solution obtained after harvesting the bacteria and centrifuging down the bacterial bodies. The flagella are then resuspended in a smaller volume before the centrifugations in the Beam's centrifuge.

Cultivation of the bacteria on cellophane membranes, moistened in broth and placed on the surface of the nutrient agar has also been tried and found rather convenient.

The mentioned modifications in the cultivation of the bacteria and in the preparative process has proved to give flagellar material of the same degree of purity and posessing equal properties in other respects as the preparations earlier investigated.

CHEMICAL AND PHYSICOCHEMICAL INVESTIGATIONS AS TESTS OF PURITY OF THE OBTAINED FLAGELLAR PREPARATIONS

The obtained preparations form homogeneous, almost colourless pellets after centrifuging down in the preparative ultracentrifuge at 27 000 r. p. m. When resuspended in distilled water or dilute salt solutions or by simple swelling in the same mediums stable solutions or suspensions are formed.

No difficulty generally is encountered when the purity of an inorganic compound is to be defined. Serious difficulties, however, arise with high molecular substances, as proteins associated with other substances (*e. g.* the complex serum albumin-bilirubin [11]). Such difficulties are of course still more apparent in the case of the extremely „high molecular" particles of viruses. With regard to their dimensions the flagella may be compared with such particles.

In the author's opinion, the best way to proceed in this case is to try to get preparations of as reproducible properties as possible and then by further studies investigate these properties and try to find out to what extent the conception of a "pure substance" can be applied in the actual case.

As to the bacterial flagella, the most sensitive tests of reproducibility of the preparations have been simple chemical analyses, especially of the nitrogen and phosphorus content, carbohydrate analyses and investigations of the ultraviolet absorption. The results of such analyses have been published earlier [1,4]; however, a summary will be given here.

With preparations obtained from *Proteus vulgaris*, cultivated at 13—15° C, and salted out, the following figures have been obtained:

% N: 16.3—16.5 (3 determ.)
% P: 0.03—0.05 (4 »)
% carbohydrate: less than 0.2
% possibly fatty material: about 0.7
% tyrosin-N/total N: 0.81—0.84

The reproducibility must be regarded as good.

Flagella from *Bacillus subtilis*, prepared in the same manner as described above for *Proteus vulgaris*, have given the following values:

% N: 15.8—16.0
% P: as for *Proteus*
% possibly fatty material: as for *Proteus*
% carbohydrate: 1—2 %
% tyrosin-N/total N: 0.57—0.59

Preparations from bacterial cultures incubated at higher temperature or not submitted to salting out [1] have given less reproducible values (*e. g.* prep. from 30° cultures gave N-values of about 14 %, 0.2—0.5 % P. and so on).

Paper chromatograms of hydrolysates of different preparations have shown a constant qualitative amino acid composition of the flagella, and only small differences are found in preparations from *Proteus* and *Bac. subtilis* [4].

Analyses of the ash contents and some further elements occuring in the flagella from *Proteus vulgaris* have been performed. — The ash contents were determined by incineration at 500°, the metals sodium, potassium and calcium spectrometrically. A colorimetric method was used for the estimation of magnesium [12*]. For the determination of chlorine and total sulphur, the fla-

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gellar solution was evaporated with magnesium nitrate and the residue was incinerated at 500°. The ashes were dissolved in nitric acid. Chlorine and sulphur were approximately estimated from the turbidity of the test solution after adding $AgNO_3$ and $BaCl_2$ (comparison with known solutions); the accuracy was judged to be about 10 %.

The following analytical figures were obtained.

% ash: 1.0
% Na: 0.29
% K: 0.08
% Ca: 0.04
% Mg: 0.04
% Cl: about 0.07
% total S: about 0.2
% organic S (+ S in SO_4-ions bound to protein): 0.20

Less pure flagellar preparations have given somewhat higher values.

The related analytical figures give strong evidence that the obtained flagellar preparations, especially from *Proteus vulgaris*, are composed of protein of constant composition. Special tests after disintegration of the flagella [1, p. 880] makes it plausible that this protein makes up at least 95 % of the whole preparation. Since by investigations in the electron microscope [1] practically only flagellar threads are found in the preparations, this protein is obviously identical with the flagella. (Theoretically a protein, not detectable in the electron micrographs, *e. g.* with a particle size considerably smaller than that of the flagella (*cf.* below) could be present in the preparations. But then this protein would be bound either to have chemical properties not discernable from those of the flagella, or to be present in constant amount in relation to the flagella component. Both alternatives seem rather improbable.) — As the flagella are of uniform thickness and of quite the same appearance as before being detached from the bacterial bodies, it may be justified to talk of flagellar preparations of 95 % purity. Serological tests [4] also show no change in the properties of the flagella in this respect before and after the purification process.

Other criteria of homogeneity have been tried, but proved less sensitive than those already mentioned. Electrophoresis reveals inhomogeneity with certainty only in rather crude preparations [7]. Also in electron micrographs considerable amounts of material, especially of a size at the limit of the resolution power of the microscope, may easily be confused with the back-ground structure, which may make one overestimate the degree of purity of the preparation.

FURTHER PHYSICOCHEMICAL INVESTIGATIONS

Decomposition of the flagella in acid medium. The irreversible decomposition of the bacterial flagella by lowering the pH to 3—4 has been described [3,4]. Particles of a sedimentation constant of 2—2.5 S are obtained with flagella from the three species *Proteus vulgaris*, *Bacillus subtilis* and *Salmonella paratyphi* B. The sedimentation diagram indicates a rather homogeneous size of the particles. From the determination of the diffusion constant (upper limit 5—5.5 $\times$ 10^{-7} cm^2/sec.) and the partial specific volume (0.72—0.73) a lower limit of the molecular weight of 40 000 can be calculated for the flagellar fragments.

The cause of the decomposition phenomenon is not clear. However, it may be noted that in this region the flagellar fragments show isoelectric properties [7].

Electrophoretic and titrimetric measurements [7]. As to the acid-base binding capacity and the electrophoretic mobility, the flagella show characteristic protein properties, with the exception that some of the ionizable groups may not be accessible to the acid or base for steric reasons.

X-Ray investigations [2,8]. These investigations have shown that, on the basis of the X-ray reflections formed by the flagellar fibers, the flagella belong to the great family of elastic, fibrous proteins called the keratin-myosin-epidermis-fibrinogen group, showing the α-keratin pattern in the native state. (The other group of fibrous protein is the collagen group.) In addition, the flagella can be stretched into the so called β-form at room temperature, a property also shown by keratin and myosin. (The β-pattern is also shown by most denatured fibrillar and globular proteins.)

Therefore, at least from the X-ray point of view, the flagella may be regarded as primitive hairs or muscle fibers. The X-ray diagrams suggest a structural simplification of the flagellar fibers as compared with *e. g.* the hair keratins.

No essential difference has been found between the diagrams formed by flagella from *Proteus vulgaris* and *Bac. subtilis*.

X-Ray investigations on wet and dry flagella [8] show that the only difference is a sharpening of some reflections in the wet diagrams. Therefore, the water does not alter the arrangement of the fibrillar structure and the protein seems to be present as comparatively invariant anhydrous units, as has been found to be the case with haemoglobin [13]. The swelling of the flagella in a moist atmosphere or by direct wetting is undoubtedly caused by water forming layers of variable thickness between the protein units (*cf.* haemoglobin again).

MORPHOLOGICAL STUDIES

It has already been mentioned, that electron micrographs show the same appearance of the flagella before and after the purification process. However, the purified flagella appear somewhat shorter, on account of breakage. The micrographs published so far [1] have shown a somewhat granular appearance of the flagella. As was suggested, this appearance depends upon the crystallization process occurring in the pure gold used in the shadowing technique. When a mixture of gold and manganin (1 : 1) is used, the shadowed micrographs show flagella with a smooth surface.

By comparing the flagella with tobacco mosaic virus particles the thickness of the former has been determined to about 120 Å [2].

When the flagella are salted out from distilled water with ammonium sulphate a precipitate is obtained, consisting of spirals of a quite regular period along the spiral axis. This period has been found different for the investigated bacterial species, 2.0 μ for *Proteus vulgaris* and 2.5 μ for *Bacillus subtilis* [5].

The flagella aggregate themselves into regular, wavy structures when solutions of flagella are dried on glass slides [6]. The wavy period is slightly shorter than the spiral one.

Observations of living bacteria with dark field microscopy show that the bacteria often carry a spiral-formed tail, undoubtedly formed by individual flagella twisted together into a bundle [14–16]. The spirals are drawn out when the bacteria are in lively movement as compared to resting or slowly moving organisms. Reichert [14] gives the value 2 μ for the spiral period of the tails of resting *Proteus* bacteria (and 2.5 μ for *Salmonella*). This is quite the same value as found for the salted out spirals (*cf.* above). Somewhat puzzling is that Reichert claims the spirals to be right handed, whereas the present author always has found left handed spirals with the characteristic 2 μ period when investigating living cultures or salted out spirals. (The investigations have included 10 strains of *Proteus vulgaris* with different flagellar antigens, and have been performed with phase contrast and dark field microscopy.) As Reichert gives no photographical material it is difficult to decide whether a misinterpretation of the microscopic material is plausible or whether bacterial strains with right as well as with left handed flagellar spirals exist.

In any case, the formation of spirals of a definite spiral period is a characteristic feature of bacterial flagella. These spirals are observed in bacterial cultures on living organisms or as discarded tails [15] and in preparation of pure flagella when salting out is applied. The period of the spirals is modified when the bacteria are rapidly moving.

At least for a definite bacterial species, all spirals are wound in the same way. This proves that the spiral arrangement must depend on the molecular structure of the flagella themselves [6].

The fact that only relatively pure flagellar preparations comparatively free from foreign structural elements form spirals when salted out forms an analogy to the classical crystallization process and may thus be used as a test of the homogeneity of the preparations. However, the chemical tests mentioned above are at least as sensitive.

SOME REMARKS REGARDING THE FUNCTIONING OF THE FLAGELLA

As has been mentioned above, the flagella are generally believed to be the motile organs of the bacteria. Using dark field microscopy, Pijper has criticized this view and has argued that the flagella are only mucous threads peeled off from the surface of the bacterium [17]. This view has again been criticized [18-20].

The present investigation has not been undertaken with the aim of deciding between these two views. Combined chemical, physiological and morphological studies may solve the problem. However, Pijper is obviously wrong when he assumes the flagella of *Proteus vulgaris*, *Salmonella paratyphi* and *Bacillus subtilis* (these species form his essential investigation field) to be mucous threads, since the present investigations clearly show their protein character.

As compared to the contractile muscle protein, actomyosin and its components, myosin and actin, it may be noted that the flagella do not contain any sulphydryl groups (negative cysteine analyses) and that their qualitative amino acid composition is rather different, compared to myosin [*Cf* 4,21]. — Sulphydryl groups play an important role in the formation of the actomyosin complex [22].

An actively motile organ obviously must be furnished with a source of energy. As judged from the analyses, energy rich materials, *e. g.* adenosine triphosphate or carbohydrates, are not present in the flagella; however, such compounds may be weakly bound to the surface of the flagella when the latter are still attached to the bacterial body. They are not necessarily present in amounts big enough to influence the thickness of the flagella and are easily washed away during the purification. In this connection it may be mentioned that myosin and actomyosin can be prepared in a state containing very little phosphorylated compounds [23,24].

In any case, physicochemical and morphological studies related above strongly suggest a very specialized and elaborate structure of the flagella, indicating the nature of the organs, to be well suited to serve a special function in the bacterial organisms. It is of great interest that at the same time the

flagella can be characterized in the same manner as large protein molecules and virus particles (*e. g.* salting out from stable water solutions into ordered aggregates, electrophoresis, preparative ultracentrifugation (*cf.* above).

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