SOME CHEMICAL AND PHYSICO-CHEMICAL PROPERTIES OF THE FLAGELLA OF *PROTEUS VULGARIS*

by

CLAES WEIBULL

Institute of Physical Chemistry and Biochemistry, University of Uppsala (Sweden)

As is well known, many bacteria carry flagella, long, flexible threads of uniform thickness (cf. Fig. 3). The arrangement of the flagella may differ from one species to another, so that one has the classifications of monotrichate, peritrichate, lophotrichate bacteria and so on. (For details see any textbook of bacteriology). In many cases, however, it may be difficult to determine the exact nature of the flagellation.

Most authors ascribe the motility of the bacteria to the flagella, as most flagellated bacteria show motility, and bacteria without flagella are not motile. Objections to this view, however, have been made[1].

The flagella show antigenic properties, the H-antigen of the bacteria being located in these organs. These antigens show a rich differentiation characteristic for different strains, and they are used for diagnostic purposes.

Hardly anything is known about the chemical properties of the flagella. A protein nature has been proposed, however, because of the insolubility of the H-antigen in trichloroacetic acid[2].

GARD[3] has shown the possibility of obtaining the flagella from bacterial cultures in fair yields and in a highly purified state. A more detailed chemical investigation of the flagella has thus been made possible. — With the flagellar material, prepared by GARD and obtained from *Salmonella paratyphi* B, some chemical and physico-chemical observations have already been made[4].

For the present investigation, a harmless and easily cultivated bacterium, *Proteus vulgaris*, has been used. This bacterium shows a rich flagellation.

THE PREPARATION OF THE FLAGELLA

The strain used was *Proteus vulgaris* X 19. The bacteria were cultured in 15 cm Petri-dishes on an ordinary nutrient agar medium (1 l meat infusion broth, 3 g NaCl, 2 g Na_2HPO_4, and agar-agar to 0.6%). With this somewhat low agar-agar content the Proteus bacteria easily swarmed out on the whole surface at temperatures between 10–37°.

After a convenient time (see below) the plates were harvested by means of 5–10 ml 0.9% NaCl solution. The suspension was shaken about one hour in a shaking machine, and the bacteria were then spun down in an angular centrifuge at a rate of 3000 r.p.m.

In order to decrease the volume of the supernatant to a suitable extent, ultra-

References p. 361.

filtration through a collodion membrane was performed. Thereafter the flagella were spun down in a Beams air-driven centrifuge (max. rate 27000 r.p.m.). The changes in concentration of the supernatant and deposit during the period of centrifugation of the flagella have been investigated by spinning pure flagellar suspensions or solutions (the flagella form quite stable solutions) by fractionated centrifugations and then analysing the supernatant and the deposit for nitrogen as a measure of the flagellar substance. In the graph below the percentage of deposited flagella is plotted against the area of the time-centrifugal field curve for the centrifuge. This is easily calculated, since the centrifuge accelerates linearily.

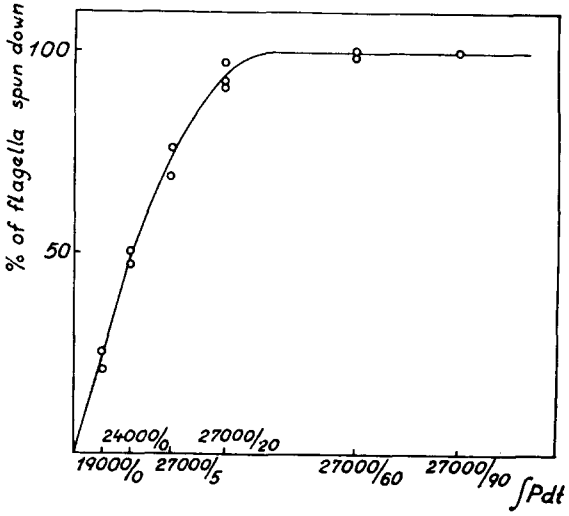


Fig. 1. The percentage of flagella spun down after different centrifugation times. p = centrifugal field. The denomination 19000/0 means a run up to 19000 r.p.m. and then stopping the centrifuge, "up and down 19000"; 27000/20 means a 20 min run at 27000 r.p.m. etc.

One can easily see that all flagella are spun down after a centrifugation time of 30 min at 27000 r.p.m. It may be mentioned that after prolonged centrifuging a small and constant amount of nitrogen was still present, probably originating from small fragments of flagella still in solution. — In the routine preparation a run of 60 min at 27000 r.p.m. was used for the sedimentation of the flagella.

The ultrafiltered preparations, however, contained other material also which settled faster then the flagella. In order to remove these contaminations, up and down runs to 19000 r.p.m. were used. Since about 25% of the flagella were then also spun down, the deposit was dispersed in solution and again run up and down to 19000 r.p.m. The new deposit was discarded.

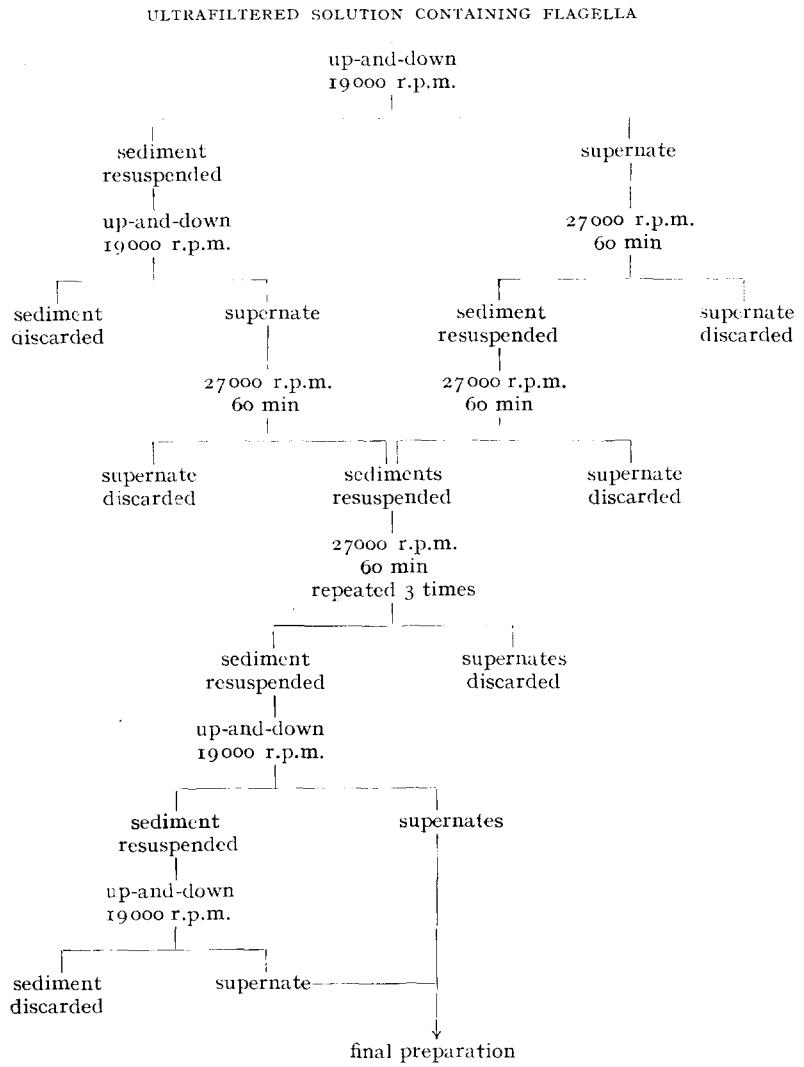
The following scheme shows the adopted method for the purification process of the flagella. For the resuspension of the deposited flagella to an aqueous solution a dilute borate buffer with a p_H of 8.5 was used as solvent, as the flagella are found to undergo decomposition at low (see below) and also at high p_H-values. The maximum stability was found to be at p_H 8.5.

The final preparation of the flagella formed a colorless stable solution with a marked TYNDALL phenomenon. Even at low concentration the solution was rather viscous and showed strong birefringence of flow. The yield can be calculated to be about 80% of the flagella originally present in the solution after centrifuging the bacteria.

It was found very important to choose the temperature and the cultivation time in a proper manner in order to obtain the flagella in pure state and in a fair yield. With incubation temperatures above 30° the flagellar preparations spun down were partly dark-coloured and apparently inhomogeneous. With an incubation temperature of about 20° the deposits were colourless and physically homogeneous. As will be discussed later chemical and physicochemical tests to determine the purity showed an appreciable lack of homogeneity in the preparations. By lowering the temperature to 10–15° preparations of

References p. 361.

fairly good chemical homogeneity were obtained. Therefore, an incubation temperature of 14–15° was chosen for the routine preparations.



The duration of incubation has also a certain importance. As the graph (Fig. 2) shows, the yields of flagella seem to be proportional to the amount of bacteria harvested. In overaged cultures (6–8 days) the yield of flagella rapidly decreases, probably because of autolytic processes.

From the graph it is clear that the bacteria ought to be harvested shortly after the end of the logarithmic growth phase. In accordance with this, the incubation time at 14° was 3–5 days.

The yields of flagella have been somewhat irregular but have been in range of 0.1–0.5 mg from one Petri-dish under the culture conditions mentioned above.

References p. 361.

The swarming phenomenon of the *Proteus bacteria* makes it rather easy to ensure pure cultures, but the stock cultures were transplanted to new plates from time to time. The stock plates were cultured at 20–27°, and from these plates the cultures for flagella production at lower temperatures were inoculated, since it became evident that the bacteria had a tendency to degenerate if cultured below 15° for a long time.

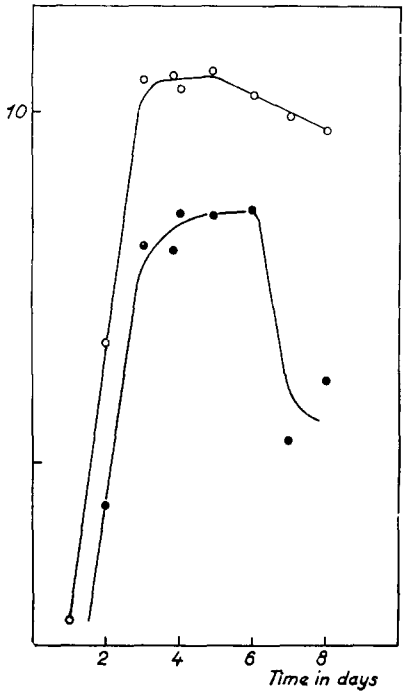


Fig. 2. Yields of bacteria and flagella from cultures at 14° and harvested at different times. o = yields of bacteria in arbitrary units (turbidimetrical measurements). • = yields of flagella (arbitrary units). The yields are plotted in a logarithmical scale.

The plates (Fig. 3 and 4) show electron micrographs of a *Proteus bacterium* from the culture medium and of the product obtained after the purification process described above. The goldshadowing technique of WILLIAMS AND WYCKOFF[5] has been used. The photograph Fig. 4 shows practically only one structural element, *i.e.*, long flexible threads of the same thickness as the flagella of the bacterium, but somewhat shorter, presumably because they were too fragile to withstand the purification process.

The product obtained also gave a precipitation reaction with a *Proteus* X 19 H rabbit antiserum.

Thus the preparation must be considered to consist of a highly purified preparation of *Proteus flagella* (cf. GARD[3]).

CHEMICAL PROPERTIES OF THE *Flagella*

For the determination of the dry weight of the flagella the preparations were, if necessary, dialysed against distilled water (the flagella disintegrate during electrodialysis) and evaporated to dryness at 105°. A microbalance was used for the weighings; the accuracy was judged to be about ± 3 gamma. The samples weighed about 1–3 mg.

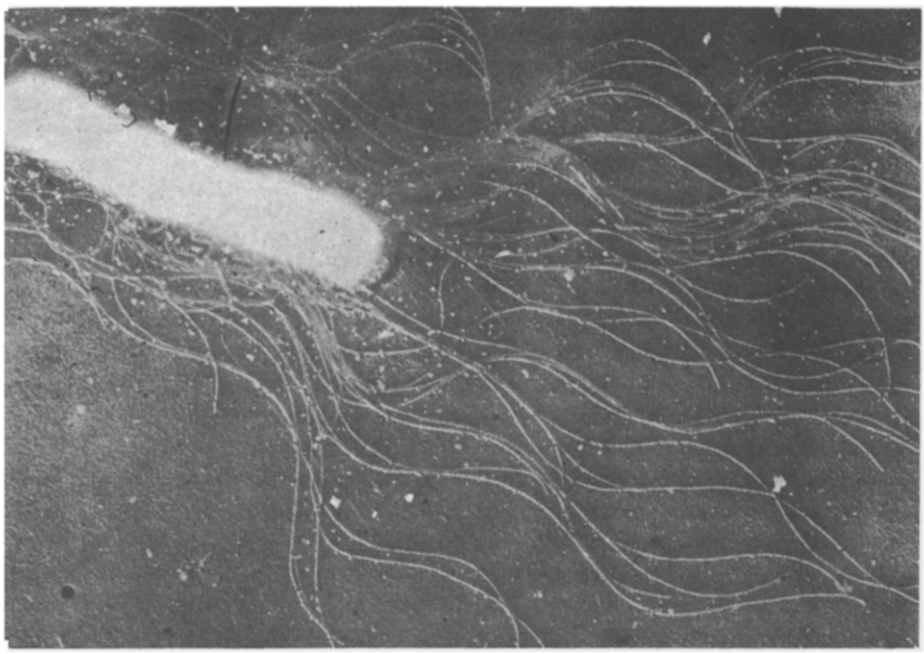
The N-content was determined according to the micro-Kjeldahl-method.

The P-content was measured according to the method of KING[7].

For flagellar preparations obtained from high temperature cultures (37–20°) the N and P contents were varying: 11–14% N and 0.2–0.5% P were found. However, from cultures at 10–15° reproducible values of the N content were obtained, namely 15.7–16.1%. In these preparations the P content was almost zero: 0.05–0.03%. As also physicochemical criteria for purity (see below) indicated homogenity for these preparations it seems very probable that the N and P content of the flagella are indicated by these last mentioned figures. Thus, there can be practically no phosphorus containing compounds in the flagella, and the N content is equivalent in amount to that of many simple proteins.

Semi-quantitative MOLISCH tests for carbohydrate were also performed. At the most, about 1% carbohydrate was found in the preparations. This carbohydrate, however, can very well be a contamination from decomposed bacteria or from the culture medium.

References p. 361.



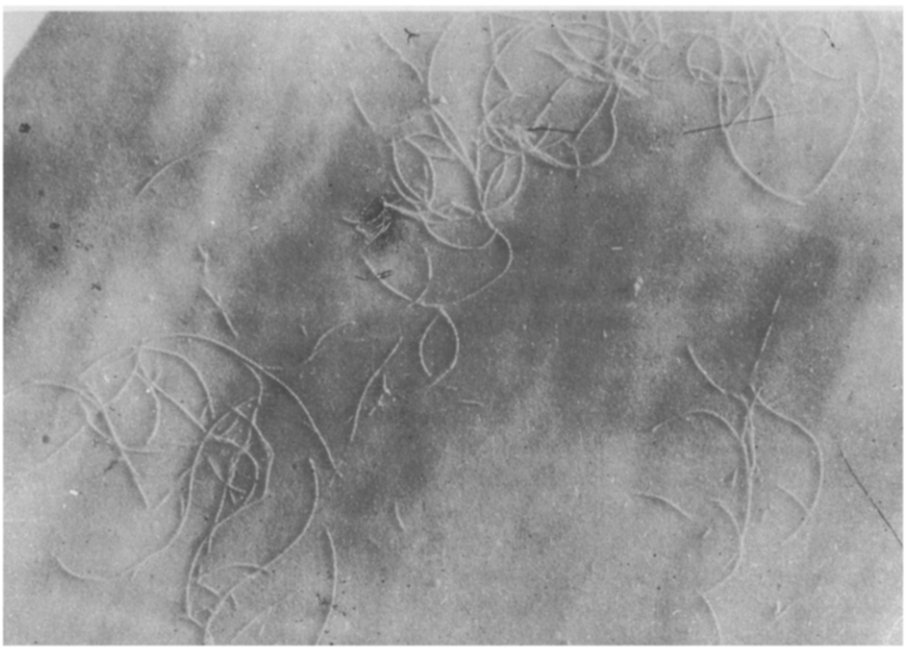


Fig. 3 and 4. Electron micrographs of a *Proteus bacterium* with flagella and of a preparation of pure flagella. Magnification about 15000 ×. — It may be mentioned that the somewhat granular appearance of the flagella probably is due to a coagulation process in the gold film used in the shadow-casting technique[6].

Some orientating qualitative determinations of amino acids according to the paper chromatography method[8] have been made. The flagella proved to contain many different amino acids: arginine, lysine, aspartic and glutamic acid, glycine, serine, alanine, threonine, tyrosine and probably some others.

LIGHT ABSORPTION MEASUREMENTS

The measurements were made with a Beckman quartz spectrophotometer between the wavelengths 4000–2400 ÅU.

As the investigated material showed a very strong TYNDALL phenomenon, it was essential to correct properly for the unspecific light dispersion (absorption). This absorption is known[9] in many cases to follow the law

$$E = k \cdot \lambda^{-n}$$

where E is the absorption coefficient, λ the wavelength of the light dispersed and k and n constants, varying with the dispersing substance. The expression can also be written

$$\log E = -n \cdot \log \lambda + \log k$$

i.e., when log E is plotted against log λ a straight line is obtained with the slope –n.

For particles small in comparison with the wavelength, n has a value of about 4. CASPERSSON[10] has used this value for protein and nucleic acid absorption curves. The present author has investigated different colloids with no specific absorption in the greater part of this range of wavelengths *e.g.*, starch, dextran and proteins, and has found good agreement with this law but with values of n in most cases lower than 4. Cf. Fig. 5.

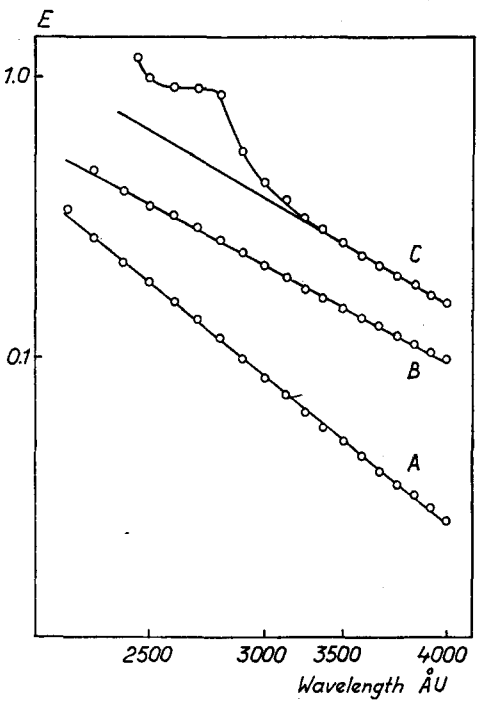


Fig. 5. Extinction values of a 3% dextran solution (A), 2.5% soluble starch (B), and a preparation of flagella, 0.25% (C). Both wavelengths and extinction values are plotted in a logarithmic scale.
For (A) n (cf. the text) = 4.14
For (B) n = 2.76
For (C) n = 3.14

Thus, in order to obtain the true specific absorption of the substances investigated the unspecific absorption has been determined in the range between 4000–3300 ÅU where proteins do not show any specific absorption. The values for the absorption have been plotted against the wavelength on a logarithmical scale and the straight line found has been extrapolated to the lower wavelengths. The specific absorption is then easily found, and the values hereby obtained show a good reproducibility for preparations of different degree of opalescence.

The absorption values mentioned below are expressed in terms of the concentration as measured by the N content of the preparations.

In the cases where chemical analyses have revealed inhomogeneity the light absorption measurements also have given inconsistent results. Preparations from low

References p. 361.

temperature cultures, however, have given reproducible values within about 5% for the maximum values of absorption. The figure shows the specific absorption of a flagellar preparation in a buffer of p_H 8.5 and in 0.1-NaOH.

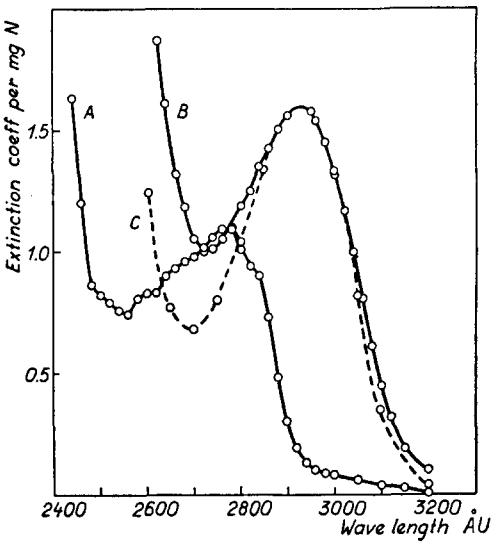


Fig. 6. Extinction values for a preparation of flagella in a buffer of pH 8.5 (A) and in 0.1-n NaOH. The measurements were made in 1 cm cuvettes. The curve (C) gives the extinction values of a tyrosine solution with the same maximum extinction as (B).

The NaOH-curve shows the maximum of tyrosine at 2930 ÅU. As the dotted line (abs. of tyrosine) shows, however, there exists an additional absorption at lower wavelengths. The curve at p_H 8.5 also shows largely the absorption of tyrosine but also an additional absorption. It can be judged that only traces of tryptophane can be present, since then the absorption maximum of the NaOH curve would have shown an appreciable deviation from 2930 ÅU.

The tyrosine content of different preparations of flagella amounts to between 1.8–1.9%.

It is evident that only traces of nucleic acids (or purin bases) can be present in the preparations, since no maximum at 2600 ÅU is found in the absorption curves. This is in agreement with the low phosphorous contents of the flagella.

Among other physicochemical properties of the flagella it can be mentioned that they are reversibly precipitable with ammonium sulphate; they also show electrophoretical mobility.

THE DECOMPOSITION OF THE *Flagella* IN ACID MEDIUM

As has been demonstrated earlier[4], the flagella of *Salmonella paratyphi* B are decomposed at about p_H 3–4; the flagellar structure was found to disappear, and the split products had a sedimentation constant of about 2.5 S.

The flagella of *Proteus vulgaris* also show the same behaviour. When one adds a dilute acid to the rather viscous flagellar solution with marked birefringence of flow, this birefringence disappears almost instantaneously (room temperature) and the viscosity of the solution drops strongly. The process is irreversible: nothing seems to happen when NaOH is added to p_H 7.

Only material of comparatively high molecular weight seems to be produced upon the decomposition of the flagella: after 2–3 days dialysis in collodion bags against distilled water and against buffers, the contents of the bags contains all the nitrogen originally present. After 5–7 days 90–95% of the nitrogen of the flagella are found in the bag.

When centrifuging the hydrolysate 60 min at 27000 r.p.m. in the Beams centrifuge a deposit is generally obtained. From preparations of flagella, shown to be inhomogeneous by chemical analysis, a rather voluminous deposit is obtained; from low tempera-

References p. 361.

ture cultures only a small deposit is obtained, corresponding to less than 5% of the total dry weight of the flagella. An investigation of the deposit in the electron microscope reveals no flagellar structure, only particles of different forms. Thus the flagella are totally destroyed and the deposit, showing variable chemical composition, may be due to impurities coming from bacterial fragments or from the culture medium.

It may be mentioned that the deposit is found to contain most of the phosphorus possibly present in the original flagellar preparation.

When investigated in the ultracentrifuge the hydrolysed solutions of the flagella give only one rather homogeneous component as shown by the figure.

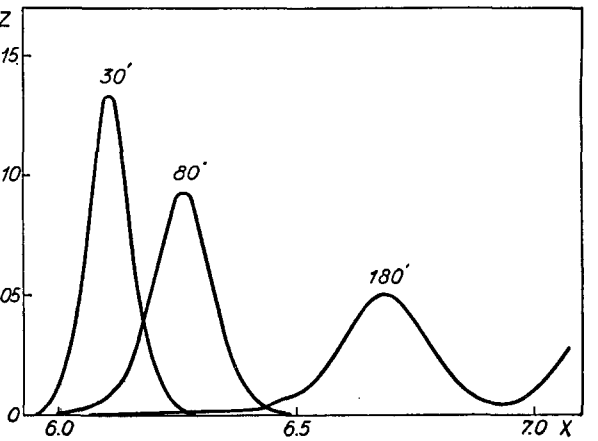


Fig. 7. Sedimentation diagram of hydrolysed flagella. Exposures at 30, 80 and 180 min after the centrifuge has reached full speed. Scale distance 1.0 cm. For details see (11).

Measurements of the refractive index of the solution and the area of the centrifuge diagrams show that, within the errors of the method[12], all or almost all of the flagellar substance is found in this component.

The table below gives the sedimentation constant at different concentrations.

TABLE I

SEDIMENTATION CONSTANTS OF HYDROLYSED FLAGELLA

Conc. %	S_{20}
1.05	2.03
0.66	2.00
0.51	2.18
0.37	2.27
0.28	2.13
0.20	2.37
0.10	2.36

The constant seems to be somewhat dependent upon the concentration. S_0 lies at about 2.4.

After dialysis for some time of the hydrolysed material against water or buffers the ultracentrifuge-diagrams show more polydispersity with a tendency to lower molecular weights. The fragments of the flagella, obtained by acid hydrolysis, thus seems to undergo further decomposition by dialysis. This is in accordance with the dialysis experiment mentioned above: after some time substance passed through the collodion membrane.

Therefore, it has been difficult to perform adequate diffusion measurements, since these measurements are performed after dialysing the material. However, values of about 5.5–5 have been obtained. (D_a-values, D_m were somewhat higher). These values can be considered as an upper limit for the true value. The diffusion curves showed little or no skewness, indicating no considerable dependence upon concentration.

References p. 361.

It is known, that one can use the frictional ratio f/f_0 which is easily calculated from the S and D values as a measure of the deviation from the spherical and unhydrated state of a large molecule. For a spherical and unhydrated molecule the ratio has a value of 1. According to the diffusion and sedimentation values of the hydrolysed flagella one obtains a frictional ratio of about 1.8. For an unhydrated particle with an assumed ellipsoidal form this means an axial ratio of about 1 : 15. In all cases the frictional ratio found strongly indicates an elongated form of the split products of the flagella.

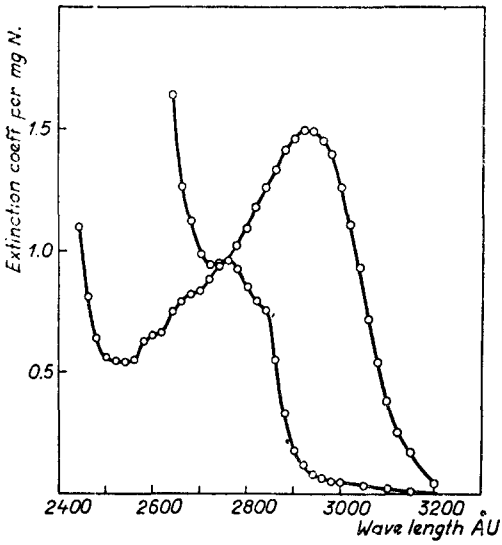


Fig. 8. Extinction values for a preparation of hydrolysed flagella at pH about 7 and in 0.1–n NaOH. The preparation was purified by running 60 min at 27000 r.p.m. Cf. Fig. 6.

The partial specific volume of hydrolysed and unhydrolysed flagella were found to have about the same value, 0.72–0.73. (For these determinations I am very indebted to Prof. C. DRUCKER).

With a sedimentation constant of 2.4 and a diffusion constant of 5.2 one obtains a molecular weight of about 41000. On account of the uncertainty of the diffusion measurements this may be considered as a lower limit for the true value.

The hydrolysed flagella have been found to contain about the same percentage of N as before hydrolysis. The ultraviolet absorption is also for the most part unchanged. After centrifuging the hydrolysate 60 min at 27000 r.p.m., however, the absorption is somewhat weaker especially at lower wavelengths. This is in accordance with the fact that less pure preparations show stronger absorption. From the absorption curves in NaOH a tyrosin content of 1.77% was calculated. Fig. below.

The author is very much indebted to Prof. ARNE TISELIUS for his kind interest in this work and for helpful discussions. The author also wishes to thank Prof. THE SVEDBERG for the privilege of working at his institute. — Some of the bacteriological work has been carried out at the Institute of Physiological Botany. For facilities put at his disposal, the author is much indebted to the head of the Institute, Prof. ELIAS MELIN.

The author is under great obligation to Prof. SVEN GARD for much advice. His great experience in the problems connected with this investigation has been of the utmost value.

The present investigation is part of a program on the structure and chemical nature of bacterial flagella which is financially supported by a grant from the Swedish Natural Science Research Council.

SUMMARY

A procedure for the preparation of flagella from *Proteus vulgaris* in a highly purified state has been described.

Investigations of the chemical composition of the flagella have shown an N content of 15.7

References p. 361.

— 16.1%; only traces of phosphorus, and at the most one per cent carbohydrate (very probably a contamination) are present. Several amina acids have been found in the flagella.

Measurements of the ultraviolet absorption show that the greater part of the absorption is due to a content of 1.8 — 1.9% tyrosine. No tryptophane, purin- or pyrimidic bases are present in any appreciable amount.

These facts strongly suggest a protein nature of the flagella. The hypothesis of the polysaccharide nature of the flagella, suggested by PIJPER[1] can not be correct, at least not for *Proteus*.

The flagella are decomposed in acid medium. The split products consist of particles of rather homogeneous size with a particle weight of about 41 000 (lower limit). These particles are probably of an elongated form.

RÉSUMÉ

Description d'un procédé pour la préparation des flagelles de *Proteus vulgaris* dans un état de pureté très poussée. L'étude de la composition chimique des flagelles montre une teneur en azote de 15.7 à 16.1%, des traces seulement de phosphore, et au plus 1% d'hydrates de carbone (constituant très probablement une impureté). Plusieurs acides aminés ont été caractérisés.

La mesure de l'absorption dans l'ultra-violet montre que la plus grande part de cette absorption est due à une teneur de 1.8 à 1.9% de tyrosine. Il n'existe pratiquement pas de tryptophane, de base purique ou pyrimidique.

Ces faits sont fortement en faveur de la nature protéique des flagelles. L'hypothèse de leur nature protéique des flagelles. L'hypothèse de leur nature polysaccharidique, émise par PIJPER[1] n'est pas valable, au moins dans le cas de *Proteus*.

Les flagelles sont décomposés en milieu acide. Les produits de décomposition consistent en particules de taille plutôt homogène, et d'un poids particulaire d'environ 41 000 (limite inférieure). Ces particules possèdent probablement une forme allongée.

ZUSAMMENFASSUNG

Ein Verfahren zur Bereitung von Flagella von *Proteus vulgaris* in hochgereinigtem Zustand wurde beschrieben.

Untersuchungen der chemischen Zusammensetzung der Flagella zeigten einen N-gehalt von 15.7-16.1%; nur Spuren Phosphor, und höchstens 1% Kohlenhydrat (wahrscheinlich eine Verunreinigung) sind vorhanden. Verschiedene Aminosäuren wurden in den Flagella gefunden.

Messungen der Absorption im Ultraviolett zeigen, dass der grössere Teil der Absorption durch einen Tyrosingehalt von 1.8–1.9% verursacht wird. Weder Tryptophan, noch Purin- oder Pyrimidinbasen sind in nennenswerten Mengen vorhanden.

Diese Tatsachen weisen sehr stark auf eine Eiweissnatur der Flagella hin. Die durch PIJPER[1] aufgestellte Hypothese der Polysaccharidnatur der Flagella kann also, jedenfalls bei *Proteus*, nicht richtig sein.

Die Flagella werden in saurem Milieu gespalten. Die Spaltprodukte bestehen aus Teilchen von beinahe homogener Grösse mit einem Teilchengewicht von ungefähr 41 000 (untere Grenze). Diese Teilchen haben wahrscheinlich eine längliche Form.

REFERENCES

1 A. PIJPER, *J. Bact.*, 53 (1947) 257.
2 A. BOIVIN, L. MESROBEAU, *Compt. rend. soc. biol.*, 129 (1938) 136.
3 S. GARD, *Arkiv Kemi, Mineral. Geol.*, 19 A, No. 21 (1944).
4 C. WEIBULL, A. TISELIUS, *Arkiv Kemi, Mineral. Geol.*, 20 B, No. 3 (1945).
5 R. C. WILLIAMS, R. W. G. WYCKOFF, *J. Applied Phys.*, 15 (1944) 712.
6 W. VAN ITERSON, *Biochim. Biophys. Acta*, 1 (1947) 257.
7 E. J. KING, *Biochem. J.*, 26 (1932) 292.
8 R. CONSDEN, A. H. GORDON, A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224.
9 W. HELLER, E. VASSY, *J. Chem. Phys.*, 14 (1946) 565.
10 T. CASPERSSON, B. THORELL, *Acta Physiol. Scand.*, 4 (1942) 97.
11 T. SVEDBERG, K. O. PEDERSON, *The Ultracentrifuge*, Oxford 1940.
12 K. O. PEDERSEN, *Dissertation*, Uppsala 1945.

Received April 22th, 1948