CHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF THE FLAGELLA OF *PROTEUS VULGARIS* AND *BACILLUS SUBTILIS* A COMPARISON

by

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The preparation of flagella from *Proteus vulgaris* in a highly purified state has been previously described[1]. In order to find out possible differences in the structures of flagella from different species, the same scheme of preparation has now been applied to a strain of *Bacillus subtilis*.

As has been already mentioned[1], the flagella are reversibly precipitable with ammonium sulphate. Further chemical and serological tests, described below, have not shown it probable that any damaging effect of this salt precipitation is likely. In addition, sulphur analyses have not revealed that any appreciable amount of sulphate is absorbed during the precipitation. Therefore, in order to remove possible impurities, a repeated precipitation with ammonium sulphate to half saturation has been inserted in the preparation scheme after the first two centrifugations at 27000 r.p.m. (*c.f.*[1]). The sulphate was brought to p_H 7 with ammonia. As will be shown below, the precipitation has a small but distinct purification effect on the flagellar preparations, and the procedure is now applied in the routine preparations. The analytical figures in this paper refer, unless otherwise mentioned, to salt precipitated flagella.

On the flagella from the two species analyses for nitrogen, phosphorus, amino acids, carbohydrate and fatty material have been performed. Moreover, some serological and physicochemical observations have been made.

EXPERIMENTAL

The methods for determining dry weight, nitrogen and phosphorus have been described previously[1]. The nitrogen content of the salt precipitated *Proteus* flagella have been determined to 16.3–16.5%, which with regard to the used micro-methods can be regarded as a variation within the experimental errors. The value found is somewhat higher and more constant than for non-salt-precipitated flagella. This difference will be discussed later in the present paper in connection with other analytical results.

The nitrogen content of the *Subtilis* flagella was determined to 15.8–16.0%.

Only traces of phosphorous have been found in the *Proteus* flagella[1]. The same was true for *Subtilis* preparations.

In order to estimate carbohydrate in a semi-quantitative way the MOLISCH test

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has been used in the following way: To 1 ml of the sample or of a known sugar solution was added 0.1 ml, 1% α-naphtol in 50% ethanol. 1 ml conc. sulphuric acid was run under the fluid, and the solution was gently mixed. In this way 1 μg of carbohydrate was detectable. The colour given by about 1 mg of protein in the test was compared with that of freshly prepared solutions containing 1–20 μg saccharose.

The carbohydrate content of the *Proteus* flagella was found to be less than 0.2% whereas the *Subtilis* flagella contained 1–2% carbohydrate.

The estimation of fatty material has followed the micro-method given by BLOOR[2], using ether-ethanol and petroleum ether as extracting agents. Carefully distilled solvents were used and the blank values obtained from the evaporation of the pure solvents were determined. Both *Subtilis* and *Proteus* flagella gave only 0.7–0.8% extractable material. As less than 0.1 mg of this material was obtained, it was impossible to judge its true nature.

The qualitative amino acid composition of the flagella was determined according to the paper chromatography method[3]. The Swedish filter paper Munktell OB was used with phenol-collidine and n-butanol-collidine as developing agents for the two-dimensional chromatograms. A few μg of individual amino acids, except histidine, were easily detectable in artificial mixtures after spraying with 0.2% ninhydrin in butanol and drying at 100°. Histidine sometimes entirely disappeared in the chromatograms or gave only weak spots. This has also been found to be true by other investigators[4].

In the phenol-collidine diagrams all amino acids were separated with the exception of methioniue and the leucines. In the butanol-collidine diagrams, however, the methionine forms a quite distinct spot. The leucines were not separated altogether here, but enough for detecting the separate components in a mixture. The other amino acids except the basic and dicarboxylic ones formed distinct spots in the butanol diagrams and therefore the two types of chromatograms could be checked against each others.

The flagellar preparations were hydrolysed with 6–n HCl for 24 hours at 105°. After completed hydrolysis the acid and water was evaporated over soda lime and sulphuric acid. 100–500 μg of the hydrolysate were applied on the filter paper, ammonia vapour was allowed to act on the dry spot for a short time and the chromatogram was made.

In the *Proteus* diagrams strong spots were obtained indicating the following amino acids: Arginine, lysine, aspartic and glumatic acid, glycine, serine, alanine, threonine, valine, leucine, isoleucine, phenylalanine and tyrosine (a somewhat weaker spot). A very weak but constantly appearing methionine spot was obtained. With 500 μg of amino acids a somewhat doubtful proline spot also appeared. Histidine, tryptophane, cystine, hydroxyproline and norleucine could not be detected in the diagrams, indicating in any case a very low content of this acids. Special, still more sensitive tests for these acids (except norleucine) were applied (see below). The *Subtilis* diagrams appeared quite the same, except for a stronger methionine spot and a weak but constantly appearing proline spot.

No spots of unknown origin were obtained in the diagrams.

Determination of the separate amino acids

The spectrophotometric method of determining tyrosine and tryptophane, according to GOODWIN AND MORTON, has already been referred to [1, 5]. As has already been mentioned, no tryptophane could be detected in the *Proteus* flagella, and the same case was found to be true for the *Subtilis* preparations. In this connection it may also be

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mentioned that no absorption, indicating nucleic acids, has been found in these preparations.

The results of the tyrosine determinations are given in Table I.

TABLE I

Prep.	% tyrosine-N/total N
Proteus I	0.842
,, II	0.813
,, III	0.847
Subtilis I	0.569
,, II	0.586

Cystine and cysteine. The phosphotungstic acid method according to SCHÖBERL AND RAMBACHER[6] was used. Less than 0.05% (limit sensitivity of the method) was found to be present in both *Proteus* and *Subtilis* flagella.

Hydroxyproline. The method of MCFARLANE AND GUEST[7] was employed. Less than 0.1% was present in the preparations investigated.

Histidine. The Sakaguchi test performed according to MCPHERSON[8] showed less than 0.2% histidine in the flagella (a yellow colour appearing in the test made it less sensitive than expected).

Methionine. The colorimetric method of MCCARTHY AND SULLIVAN[9] was employed. The *Proteus* flagella showed a low but constant methionine content of 0.3%, whereas the *Subtilis* preparations gave values between 0.4–1%.

Serological tests

Only *Proteus* preparations have been investigated so far. In order to get rabbit antisera, 0.5–1 mg of the flagella were inoculated four times every five days. Five days after the last inoculation, heart puncture was performed.

The flagella gave antisera, which agglutinated the corresponding bacterial suspension to as high a titer as an antiserum, produced by the bacterial suspension itself. The flagella were precipitated by the corresponding H-antiserum.

DISCUSSION

The question of purity. As for *Proteus*, the precipitation with ammonium sulphate has resulted in a somewhat higher and more reproducible N-content than has been reported previously[1]. Moreover the carbohydrate content has diminished from 1% to less than 0.2%. Also, after disintegrating the flagella at a p_H of 3 and centrifuging the solution at 27000 r.p.m. for 60 minutes a deposit was obtained that after washing amounted to only about 1% of the dry weight of the flagella. For preparations, not precipitated with ammonium sulphate, a value of about 5% was obtained[1]. Moreover since the chemical analyses referred to above have given reproducible values, it seems justified to regard the preparations of the *Proteus* flagella, especially after salt precipitation, as of a high degree of purity. At the lowest estimate more than 95% of flagellar material can be judged to be present in the preparations.

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where M is the molecular weight of the protein, $\bar{v}$ is the partial specific volume and N the AVOGADRO Number. Thus again for the parent $S_{14.6}$ component (assumed an unsolvated sphere)

$$\gamma = 22.5\, u.$$

The experimentally determined frictional ratio f/f_0 of 1.24 for this component leads to an axial ratio $\left(\frac{a}{b}\right)$ of 4.7 if the molecule is assumed unsolvated. It is then possible from calculations of GORIN[7] to determine the valency of this unsolvated cylinder.

Thus

$$\gamma = \frac{22.5}{0.64}\, u = 35.1\, u$$

where 0.64 (= R) is the ratio of the mobilities of the unsolvated cylinder of axial ratio 4.7 and the unsolvated sphere of radius a_0 at an ionic strength of 0.10. The values of valency for this model are found in columns 4 and 7 and it is to be noted that, although larger, these do not differ fundamentally from those for the previous model.

It is most probable that the actual valency of the parent molecule lies between those determined for the solvated sphere (3 and 6) and the unsolvated cylinder (4 and 7). Of considerable interest are the increases in valency of the parent molecule due to adsorption of sulphate ion (columns 8 and 9). The decrease observed in these values at the lower p_H's is probably not significant being dependent on the relative slopes of the mobility-p_H plots. Thus 3 or 4 sulphate ions are bound per molecule of the $S_{14.6}$ species over the p_H range 6.90–8.00 and these are not removed by dialysis or precipitation of the parent molecule at the isoelectric point.

No attempt has been made to repeat these calculations for the sub-molecule ($S_{9.0}$). The mobility of the slower component of a two component mixture when calculated in the normal manner is incorrect by a factor dependent on the magnitude of the boundary anomalies. Although some calculations are made later of the effect of boundary anomalies, a reliable value for the mobility of the $S_{9.0}$ species cannot be obtained simply from present experimental data.

d. *The equilibrium reaction as a function of the charge of the molecules*

From the ultracentrifuge results it is most probable that the parent molecule dissociates into half molecules of greater asymmetry. The influence of the two factors a. shape and b. size on the migration of the half molecules may be briefly discussed.

a. The mobilities of both parent (u_{A_2}) and half molecules (u_A) may be compared with the mobilities of spheres of equal molecular volume and charge ($u^0_{A_2}$ and u^0_A resp.) with the aid of the calculations of GORIN. Thus at an ionic strength of 0.10 and assuming axial ratios of 2.6 and 4.6

$$\frac{u_{A_2}}{u^0_{A_2}} = 0.75 \text{ and } \frac{u_A}{u^0_A} = 0.65$$

The more asymmetric sub molecule suffers the greater retardation.

Combining we have

$$\frac{u_A}{u_{A_2}} = \frac{0.65}{0.75} \times \frac{u^0_A}{u_{A_2}}$$

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b. On the other hand the smaller spherical molecule will (for a charge equal to that of the parent molecule) move faster than the parent molecule by a factor given by eqn. (1) and equal to

$$\frac{u_A^0}{u_{A_2}^0} = \frac{(1 + \varkappa a + \varkappa\nu_i)_{A_2}}{(1 + \varkappa a - \varkappa\nu_i)_A} \cdot \frac{f(\varkappa a)_A}{f(\varkappa a)_{A_2}} \approx 1.47$$

Thus

$$\frac{u_A}{u_{A_2}} = \frac{u_A^0}{u_{A_2}^0} \times \frac{0.65}{0.75} \approx 1.47 \times \frac{0.65}{0.75} \approx 1.26$$

represents the ratio of the mobilities of the half and parent molecules assuming equal charge, only changes in size and shape occurring in the dissociation having been taken into consideration. If the charge on A is half that on A_2 and no other factor intervenes, the mobility of the half molecule would therefore be expected to be just over half the mobility of the parent molecule and a very good separation of these two components would be achieved in the electrophoresis experiments.

The experimental observation that the mobility of the sub-molecule is very considerably higher than half the mobility of the parent molecule may arise from either or both the following:

a. inadequate theoretical treatment of the effect of size and asymmetry on electrophoretic migration

b. an increase in the net negative charge of the half molecule after dissociation *i.e.* the appearance of negative or disappearance of positive groups.

It is probable that a. is only of secondary importance since it has already been mentioned that good agreement has been found for valencies calculated independently from electrophoretic and titration data.

The second consideration implies that the reverse process, association, must then involve a disappearance of negative or appearance of positive groups. However, the presence of sulphate ions which increase the net negative charge, tends to retard dissociation (or significantly alter the equilibrium position) and the role played by sulphate ions and the importance of charge effects in general on the equilibrium processes is not at all clear.

III. EXAMINATION OF THE GROUND NUT FRACTIONS IN BARBITURATE BUFFERS (I = 0.04)

The incomplete electrophoretic separations in phosphate buffers made quantitative measurements on the dissociation of the $S_{14.6}$ species impossible. It was hoped, however, that the known property of the barbiturate ion in allowing better electrophoretic separations (*e.g.* SVENSSON[8]) would also operate in the ground nut system. In addition (for negatively charged protein molecules) boundary anomalies are smaller in this particular buffer and the final interpretation of the schlieren diagrams is much simplified.

a. Solutions of fraction I

The electrophoretic diagrams for these solutions (p_H's 7.00–9.50) are given in Fig. 4 and the corresponding mobility graphs in Fig. 5 (open circles).

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