

## Electrophoretic and Titrimetric Measurements on Bacterial Flagella

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By recent investigations<sup>1-3</sup> it has been demonstrated that bacterial flagella in many respects show the properties of a simple protein. The N-content is 16.0—16.4%, 14 amino acids have been found, but no nucleic acids, fatty material or carbohydrates, except as impurities. The flagella are reversibly precipitable with ammonium sulphate. Therefore it was judged worthwhile to investigate to some extent the electrochemical properties of the flagella in order to find out whether they also behave like proteins in this respect or whether characteristic deviations could be found.

### EXPERIMENTAL METHODS

For the electrophoretic experiments the Tiselius apparatus<sup>4</sup> was used together with the Philpot-Svensson optical system<sup>5</sup>. The ionic strength of the buffers was 0.05—0.20. Mostly solutions of an ionic strength of 0.10 were used, composed of 0.05 *M* NaCl and buffering substance. No appreciable boundary anomalies appeared with this system. In the range of pH 4—5.4 acetate buffers were used, between 5.6 and 7.5, veronal-acetate (Michaelis buffers) or phosphate, between 8 and 9.3, veronal and above 9.3, glycine buffers.

In order to obtain acid-base titration curves of the flagellar protein, it was found necessary to use a method which needed only about 15 mg of the substance to be investigated on account of the scarcity of the flagellar material. Therefore, the titration solutions were added in steps to the same protein solution, the pH of which was continuously read. The flagellar solution (10—20 mg in 4 ml of CO<sub>2</sub> free distilled water) was poured into a beaker and here also the glass and the calomel electrode of a direct reading pH meter (Radiometer's autojonometer, accuracy 0.02 pH units) were placed

together with a glass rod as a stirrer. For the titration micropipettes of the micrometer-syringe type were used. For the titration through the range of pH 2.5—11 0.2—0.4 ml of 0.1 *N* solutions were needed. The accuracy of the delivery of the acid and base solutions was about 0.5—1 %. — The titrations were performed with 0.1 *N* KCl present in the protein solution. By using 0.1 *N* acid and base the ionic strength of the reaction mixture was maintained approximately constant.

It was shown by the full reversibility of the curves on back titration that no significant amounts of CO<sub>2</sub> were absorbed in the alkaline region during the titration. (The solution was kept alkaline only for a few minutes.) In addition the pH-values of the protein solutions showed no drift with time.

In order to obtain values of the free H<sup>+</sup> and OH<sup>-</sup> ions present in the reaction mixtures, titration curves were made with only 0.1 *N* KCl. The values obtained were in good agreement with the theoretically expected ones and those obtained by other authors <sup>6,7</sup>.

In order to test the adopted method titrations with ovalbumin were performed. Close accordance was obtained with the results published by Cannan *et al.* Duplicates gave a difference of 2 % in the total acid base binding capacity of the protein between pH 3.0 and 10.8.

## RESULTS AND DISCUSSION

### Electrophoresis

The flagella investigated, from *Proteus vulgaris* and *Bacillus subtilis*, prepared in a highly purified state by methods described previously <sup>2</sup> migrate in the electrophoresis tube with only one, very sharp boundary. Even preparations, shown by chemical methods <sup>2</sup> to be to a certain degree inhomogeneous give rise to only one peak, showing the same mobility as the purest possible preparations. Only rather crude preparations migrate inhomogeneously. Therefore, electrophoresis does not seem to be very sensitive as a criterion of purity in this case.

At constant ionic strength reproducible values of the mobility of the flagella have been obtained. Fig. 1 shows the results obtained with an ionic strength of 0.10 at pH values between 5 and 9.5 with flagella from *Proteus vulgaris*. Below pH 5 and above pH 9.5 the flagella disintegrate and therefore no measurements could be performed here. Phosphate buffers have been avoided (Michaelis veronal-acetate buffers were used instead) since the divalent phosphate ions have been found to have a decided effect on the mobilities <sup>8</sup>. This is in accordance with our own experiences (*cf.* Table 1). The mobility curve

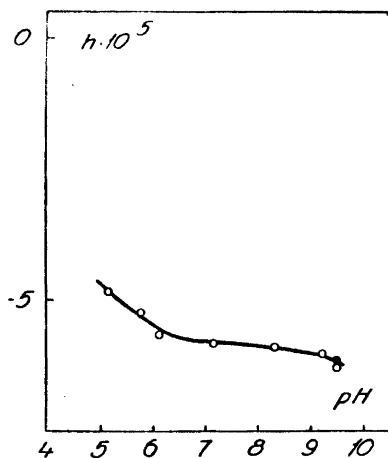


Fig. 1. Electrophoretic mobility curve for bacterial flagella (Dark dot) = *Bac. subtilis*. (Open rings) = *Proteus vulgaris*.

is of the same kind as those given by most proteins. On account of the instability of the flagella their isoelectric point can not be established.

As has been reported previously<sup>1</sup> bacterial flagella are converted into particles with fairly homogeneous molecular weight of about 40000 on hydrolysis at pH 3—4 at 20°. As these particles seem to undergo further hydrolysis when dialysed<sup>1</sup> electrophoretical measurements meet with serious difficulties. Quite reproducible values of the mobility of the particles mentioned have not been obtained. However, the mobility is always lower than that of the whole flagella (but still anodic above pH 5) and only one component has been observed in runs at pH 6.8 and 9.5. At pH 5.1, however, a tendency of separation into different components has been observed, but here the whole material seems to be electrophoretically inhomogeneous, probably on account of decomposition phenomena (*cf.* above).

At pH 4.2 the decomposed flagella showed cathodic migration.

The influence on the mobility of the undisintegrated flagella by the ionic strength and by different ion species is shown in Table 1.

The strong dependence of the mobility on the ionic strength and on the kinds of the ions present in the buffer is evident. However, the mobility of haemoglobin<sup>9</sup> and egg albumin<sup>10</sup> at different ionic strengths also show changes of the same order of magnitude as is demonstrated above.

As is shown by the figures, the bivalent ions  $Mg^{++}$  and  $Ca^{++}$  causes a somewhat greater depressing effect on the mobility than the monovalent Na-ion (at the same total ionic strength). However, the same sign of the mobility is obtained and no recharging of the flagella is obtained as has been shown to be the case with myosin<sup>11</sup>.

Table 1.

Buffer	Total ionic strength	pH	Mobility $\times 10^5$	Kind of flagella
Phosphate	0.05	6.80	- 9.35	<i>Proteus</i>
»	0.10	6.80	- 7.11	»
+ 0.05 M NaCl	0.10	6.80	- 6.35	»
+ 0.05 M »	0.10	6.80	- 6.40	<i>Bac. subtilis</i>
+ 0.15 M »	0.20	6.80	- 4.54	<i>Proteus</i>
+ 0.05 M Na <sub>2</sub> SO <sub>4</sub>	0.20	6.80	- 4.92	»
+ 0.025 M MgCl <sub>2</sub>	0.125	6.80	- 4.64	»
+ 0.05 M »	0.20	6.80	- 3.64	»
Glycine				»
+ 0.05 M CaCl <sub>2</sub>	0.20	9.05	- 4.01	
Glycine	} 0.10	9.50	- 6.14	<i>Bac. subtilis</i>
+ 0.05 M NaCl		9.50	- 6.27	<i>Proteus</i>

As is shown by the figures given, no significant differences in the mobilities of the flagella from *Proteus vulgaris* and *Bacillus subtilis*, respectively, has been observed.

#### Titration curves

The acid base binding capacity of many proteins have been investigated (for references see Cannan *et.al.* <sup>6</sup>). Mostly, in the diagrams published a quantity  $h$ , expressing the equivalents of acid bound to ( $h$  positive) or dissociated from ( $h$  negative) one gram or one mol of the protein, is plotted against pH. In this case  $h$  is expressed in eq./g since no molecular weight of the flagella or a subunit of them is exactly known.

$h$  is generally defined to be zero at the isoionic point of the protein in question. The isoionic point, at moderate ionic strengths not far away from the isoelectric point, may be determined from pH measurements on solutions containing only the protein ions. Such solutions can be prepared by exhaustive electro dialysis. Since the flagella are destroyed by this procedure and have been shown to move in electrophoresis at all pH:s within their stability range, no isoionic point can be determined in this case. Flagellar solutions, prepared by repeated ultracentrifugations from distilled water and final redissolving in CO<sub>2</sub> free water, show a pH between 6.4—7.4. Therefore pH 7.0, somewhat arbitrarily, has been chosen as a zeropoint for the quantity  $h$ .

Titration measurements on three highly purified preparations of flagella from *Proteus vulgaris* have given consistent values of the total acid base binding capacity between 3.0 and 10.5 within 4 %. Fig. 2 shows such a titration curve. First NaOH has been added to the flagellar solution (open rings on the

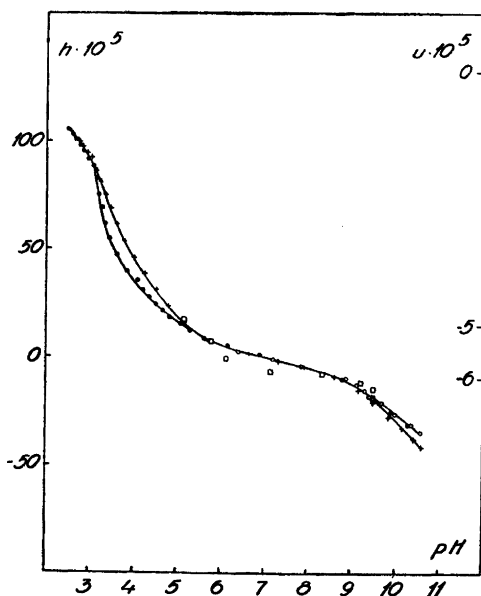


Fig. 2. Titration curve of bacterial flagella (*Proteus vulgaris*). Electrophoretic data are also included (squares). For details see the text.

curve). The pH is hereby raised from pH 6.5 to 10.6. Then HCl is added (dark dots) and pH falls from 10.6 to 2.45. Once more NaOH is added (crosses) and pH rises to 10.7.

Complete reversibility of the acid base binding capacity of the flagella within the range of their stability is shown by the curve. However, when titration is performed between pH 5.5 and 2.5 and back again, different curves are obtained. Under the experimental conditions prevailing, the disintegration of the flagella into smaller particles occurs between pH 4.5 and 3.5 (no sharp critical point has been observed as yet). The discrepancies of the titration curves in this region therefore are most easily explained by assuming that part of carboxyl anions in the undisintegrated flagella are not accessible to the acid for sterical reasons (if chemical bonds /e.g. peptide bonds/, were affected, the branches of the titration curve would not coincide above pH 5.5). After this disintegration has taken place, the consumption of acid will be abnormally high, more titrable groups being set free. This explains the very steep part of the titration curve of the flagella between pH 3.5 and 3 (dark dots). In accordance with this view, solutions of already decomposed flagella give fully reversible titration curves in this pH region.

Also in the pH range above 9 the disintegrated flagella show a higher buffering capacity than the undecomposed ones, indicating that more basic groups have been made accessible in the smaller molecules formed.

It has been shown<sup>12</sup> that essentially the acidic groups of aspartic and glutamic acid ( $pK$  about 4), the imidazole and  $\alpha$ -amino groups possibly present ( $pK$  7—8) and the  $\epsilon$ -amino groups of the lysine are responsible for the buffering capacity of the proteins in the pH range of 2—11. The titration curve of the bacterial flagella shows the expected zones of high buffering capacity between 3 and 4 and above 9, as do most other proteins<sup>6,7</sup> which is in accordance with the considerations above and with the amino acid composition of the flagella<sup>2</sup>. Since very little histidine has been shown to be present in the flagella it is somewhat difficult to explain the buffering capacity between pH 6 and 9. It may be caused by an unusually high content of free  $\alpha$ -amino groups or by some unknown component, or by impurities. Also in myosin and in  $\beta$ -lactoglobulin an unexplained buffering capacity in this region has been found<sup>13</sup>.

By extending the measurements to pH 1.5—2 and using high protein and salt concentration in the reaction mixtures in order to obtain highest possible accuracy a limiting value in the titration curves can be obtained, indicating the total positive groups present in the protein molecule<sup>6</sup>. However, for such determinations the necessary amounts of flagellar material have not been available.

A few measurements have been performed at other ionic strengths than 0.10. The deviations found have been of the same kind as those found with other proteins<sup>6</sup>.

#### COMPARISON BETWEEN THE TITRATION CURVE AND ELECTROPHORETIC DATA

As has been shown previously<sup>8,11</sup> the titration and mobility curves of a protein can be brought to coincide if a proportionality factor between  $h$  and  $u$  (mobility) is used and if  $h$  is put to zero at the isoelectric instead of the isoionic point, *i.e.* the  $h$  curve is shifted along the  $h$  and  $u$ -axis.

As no isoelectric or isoionic point of the flagella is known (*cf.* above) the shifting of the mobility curve along the  $u$ -axis must be made in another way. Let  $k$  be the proportionality factor between  $h$  and  $u$ ,  $L$  the constant used for the shifting of the mobility curve. We get the condition for the best coincidence between the mobility and titration curves:

$$\Sigma (k \cdot u_i + L - h_i)^2 = \min.$$

where  $\Sigma i = n$  is the number of electrophoretic experiments.

By differentiation and solving the equations obtained,  $k$  and  $L$  are determined. The result is plotted in Fig. 2. As can be seen, an approximative

agreement is obtained between the titrimetric and electrophoretic measurements. In any case the deviations found are not considerably greater than those found by Longworth in a similar investigation on ovalbumin.<sup>8</sup>

As is shown by the titration curve the charge of the flagella before and after disintegration at pH 3—4 must be supposed to be the same in the range of pH 5.5 to 9 as the different parts of the curve here coincide. As has been mentioned the electrophoretic mobility is lower after disintegration. However, this does not imply a contradiction since after the disintegration the frictional force per unit mass of protein may have augmented considerably.

#### SUMMARY

Electrophoretic measurements on bacterial flagella have given mobility curves characteristic for a protein. Below pH 5 and above pH 9.5 no electrophoretic measurements could be performed on account of the instability of the flagella.

Flagella from the species *Proteus vulgaris* and *Bacillus subtilis* have shown the same mobility.

The influence of ionic strength on the mobility is marked. Specific ion effects have been observed, but no reversing of the charge has been shown to be the case as with myosin.

Acid base titration curves on the flagella have the characteristic features of such curves obtained from other proteins. Comparison between curves obtained before and after the disintegration of the flagella at pH 3—4 leads one to suppose that for sterical reasons not all ionizable groups in the undecomposed flagella are accessible to the acid and base solutions used in the titration.

Between pH 5 to 9.5 the titration and mobility curves can be brought to coincide approximately.

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