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Some Analytical Evidence for the Purity of Proteus Flagella Protein

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The preparation of flagella from *Proteus vulgaris* in a highly purified state has been described previously [1,2] and the properties of the flagella so obtained have been investigated in various ways (for references see Weibull [3]). It has been shown that the flagella consist almost entirely of protein material. A maximum of 0.7—0.8 % fatty material, less than 0.2 % carbohydrate and 1 % ashes are found in the flagellar preparations. There is evidence that these non protein constituents may consist of impurities from the culture medium or from damaged bacterial cells.

As to the possible occurrence of impurities of a protein nature, there are hardly any reliable criteria of purity available for particles of this size and shape besides chemical analyses. Electrophoresis and ultracentrifugation are insufficent, since even rather crude preparations of varying chemical composition show homogeneity in these tests, probably because particles of different kinds are associated and do not migrate freely. Electron microscopical observations are not quite reliable since material of a size at the limit of the resolution power of the microscope may be confused with the background structure [3].

Some chemical analyses have already been performed on the flagella [3]. Additional determinations of the constituents of the flagellar protein, namely amino acids and amide nitrogen, have now been made, in order to find out whether reproducible values could be obtained in all cases. Valuable information has been obtained as to both the nature of the flagellar protein and its homogeneity.

EXPERIMENTAL

Amide nitrogen was determined according to the method quoted by Rees [4].

For the amino acid analyses, the electrodialysis method of Theorell and Åkeson [5] was used with some modifications. The protein was hydrolyzed for 30 hours at 100°

(boiling water bath) with 6 N hydrochloric acid. After the hydrolysis, dilute sulphuric acid, equivalent to the nitrogen present in the hydrolysate, was added, and the hydrochloric acid was removed by repeated evaporation. The electrodialysis was performed in a three compartment cell of perspex of about 50 ml capacity. The cathode membrane was a cellophan sheet and the anode membrane consisted of parchment (goatskin) soaked in 2 % gelatin and hardened with formaldehyde. The cellophan was chosen because histidine did not migrate quantitatively into the cathode compartment with other membranes. The goatskin was treated with gelatin in order to make it completely water tight. Distilled water was introduced into the electrode compartments, the protein hydrolysate into the middle compartment and the electrical circuit was closed. The current reached a limiting value (about 0.5 mA at 220 V) in about 90 min. The ammonia in the cathode compartment was then determined by distillation [5], the residue neutralized with sulphuric acid to pH 5.7 and reelectrodialyzed twice. The determination of the basic amino acids was then performed according to Macpherson [6]. — The contents of the anode department were neutralized with ammonia and reelectrodialyzed. To get a quantitative migration of the acid amino acids into the anode compartment it was found necessary to replace the anolyte by distilled water towards the end of the electrodialysis and then let it proceed for another 30 min. This was done in all dialyses with acid amino acids present. In this way only 1.5 % of these acids were found in the neutral amino acid fraction, and a corresponding correction was made.

Glutamic and aspartic acid were determined in the final anolyte by separation on an ion exchange column (Dowex 50) according to a method worked out by Drake at this Institute [7]. By this method the ratio between the amounts of these two acids in a mixture of them is obtained.

Tryptophan and cystine (+ cysteine) were determined according to methods used earlier [3]. Tryptophan was also determined according to the method of Bates [8]. For the determination of cystine hydrolysis was interrupted after 6 hours in order to avoid decomposition of this acid [9]. It may be mentioned, however, that even complete hydrolysis occurred without any humin formation. Only a slight yellow colour was noted.

In an earlier paper [10] it was mentioned that the untreated flagella were decomposed during electrodialysis. This was apparently due to the fact that the electrodialysis was performed with two membranes of parchment paper. On account of electroosmosis the pH of the middle compartment drops below the stability limit of the flagella [1]. When, however, the unhydrolyzed flagella were electrodialyzed in the apparatus described above, the pH dropped to a final value of 4.8. At this pH the flagella are rather stable. In this way it was found that 0.5 — 1.5 % of the total nitrogen present in the flagella was ammonia, which migrate into the cathode compartment. This ammonia is not included in the figures given below, since it most likely originates from the ammonium sulphate used during the purification of the flagella.

RESULTS AND DISCUSSION

Tables 1 and 2 give the analytical data obtained from six different preparations of flagella from *Proteus vulgaris*.

Table 1.

Prep.	basic amino acids = I	neutral amino acids = II	acid amino acids = III	Amide, hydr. = IV	Amide, Rees = V	Arginine = VI	Lysine = VII	Histidine = VIII	Recovery I +II + III +IV
1	20.55	66.54		—	—	11.23	9.21	0.11	
2	20.87	46.64	20.14	11.62	10.91	11.02	9.71	0.14	99.27
3	20.02	47.34	20.04	11.61	10.94	10.87	9.07	0.08	98.99
4	20.43	47.44	19.78	11.43	10.92	11.21	9.11	0.11	99.02
Average:	20.47	47.14	19.99	11.55	10.92	11.08	9.28	0.11	99.07

All figures are given as % N in the specified substances (specific N)/total N. Amide hydr. = amide as found in the protein hydrolysate (30 hrs, 100°, 6 *N* HCl). Amide, Rees = amide nitrogen as determined according to Rees [4].

Table 2.

Prep.	aspartic acid	glutamic acid
5	12.58	7.41
6	12.36	7.64
6	12.30	7.70
Average:	12.41	7.58

The figures are given as % specific N/total N on the assumption that 19.99 % of the total N refers to the acid amino acids (*cf.* Table 1).

Since the individual figures given above are obtained by combining at least two determinations (total N and specific N), the accuracy of which is about ± 1 %, the deviations found for different flagellar preparations can be judged to be within the experimental errors. The difference between the values for amide nitrogen (amide hydr. and amide Rees) is due to decomposition of serine and threonine in the former case [4]. The correct value is the latter (amide, Rees).

Some analytical data concerning the amount of tryptophan and cystine (+ cysteine) have been given earlier [2]. By using greater amount of material

(cystine) and a new analytical method for tryptophan, it has now been found that less than 0.02 % cystine + cysteine and about 0.02 % tryptophan (spectrophotometric determinations as described earlier [2]; the method of Bates [8] has given still lower results) are present in the flagella.

The analytical data given above, together with earlier determinations of tyrosine and methionine [2] clearly show that the flagella consist of a protein with constant chemical composition. The fact that there is practically no histidine, tryptophan or cystine in this protein is a striking feature.

By means of X-ray analysis it has been found that the flagella belong to the keratin-myosin group of fibrous proteins [11]. Therefore, it may be of interest to compare the chemical composition of the flagella with that of keratin and myosin.

Keratins are characterized by a high content of cystine, about 7—11 % of the total N [12]. Myosin contains 1 % cystine-N/total N. — Furthermore keratins contain 2—3 % lysine N/total N, myosin 12 %. In both these cases the flagellar protein is more like myosin (9.3 % lysine-N, no cystine).

However, myosin is characterized by a high content of free acid + base groups in the side chains: 34 % of the total number of amino acid residues are of this kind [13]. From the analytical data given above the corresponding value for the flagella is 22 % *i. e.* considerably lower.

Furthermore, it is clear that the flagellar protein in other respects differs from both keratin and myosin, *e. g.* because of the absence of tryptophan, histidine (and cystine) and the glutamic: aspartic acid ratio.

The low content of tryptophan, histidine and cystine can be used as a criterium of purity for the flagellar preparations. Most other proteins contain some of these acids and then in amounts corresponding to, in terms of total nitrogen, at least 1 % cystine and tryptophan or 3 % histidine nitrogen. This is especially true for the protein and peptide material in the culture medium used for the growth of the *Proteus* bacteria (meat extract and enzymatic digests of meat), from where possible contaminations may originate. From the analytical figures given above it is obvious that such protein can only occur in a concentration of about 2 % (from cystine and tryptophan determinations) or 4 % (histidine) of the total protein present. In this connection it may be mentioned that the histidine values may be too high: the colorimetric test gave a yellowish colour not identical with the pure histidine colour.

Concerning the different tests for purity used so far (traces of non-protein material or of some amino acids, reproducible values of other amino acids, "crystallisation" tests [12] *etc.*) it has been found that the most sensitive test by far is a determination of light absorption in the ultraviolet. The values for the tyrosine and tryptophan contents obtained are very reproducible for carefully

purified preparations but much higher for crude preparations or for preparations of doubtful purity according to other tests.

In order to find out whether some material from the culture medium is adsorbed or carried down with the flagella during the ultracentrifugations in the purification process [1], pure flagella in the form of ultracentrifuge pellets were weighed and resuspended in the diluted broth, used for the growth of the bacteria. The suspended flagella were again centrifuged down and the nitrogen content of the broth determined before and after the centrifugation.

By determination of the volume of the pellets by weighing and by determination of the volume of flagellar protein (from N-analysis and specific volume) the degree of dilution of the broth during the centrifugation process could be calculated and compared with the value found experimentally. The known fact, that proteins carry about 10 times their own volume of bound water with them in ultracentrifugation experiments was taken into concideration [13]. Also the small amount of nitrogen left in the supernatent liquid when the flagella sediment in pure buffer was determined and subtracted from the values found for the broth after the centrifugations. Table 3 shows the results.

Table 3.

mg N in pellets	10 X volume of flagella in the pellets, ml	Volume of pellets, ml	Free water in pellets ml	Nitrogen in broth, mg before centr.	after centr.	calc.
1.72	0.08	0.27	0.19	1.800	1.716	1.745
1.72	0.08	0.27	0.19	1.800	1.725	1.745

Table 3 shows that a very small, if any, adsorption effect is found in these experiments. It must then be concluded that possible impurities (at the most a few per cent) consist of particles from autolyzed or damaged bacterial cells. These particles may be of a size that makes them follow the flagella during the ultracentrifugation process. Such particles are also occasionally found in electron micrographs [1].

SUMMARY

The flagella from the bacterium *Proteus vulgaris* are found to consist of a protein of constant chemical composition. The following values are found as % of total N: 10.9 % amide-N, 11.1 % arginine-N, 9.3 % lysine-N, 12.4 aspartic acid-N, 7.6 glutamic acid-N, 0.83 % tyrosine-N (earlier determinations [2]) and 47.1 % N from neutral amino acids.

According to analyses for tryptophan, cystine and histidine at most a few per cent foreign protein material is present in the flagellar preparations. By earlier determinations it had been found that a maximum of 2 % non protein material (ashes, fatty material and carbohydrate) is present.

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