A Method for the Purification of Bacterial Flagella by Ion Exchange Chromatography

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SUMMARY

A method is described for purification of bacterial flagella by elution of these organelles from a DEAE cellulose column by NaCl gradients.

INTRODUCTION

Preliminary to a study of the structure and chemistry of bacterial flagella it was essential to devise a simple and reproducible method for the purification of this organelle. Repeated attempts at purification by using the published methods of differential centrifugation (Weibull, 1950; Kobayashi, Rinker & Koffler, 1959) did not yield preparations which would satisfy rigorous criteria of purity with any degree of reproducibility. Diethylaminoethyl cellulose (DEAE cellulose) has been successfully used for purification of viruses (Wilson, 1962), and this observation tempted us to use the property of ionic charge for the purification of flagella. A new method of purification of bacterial flagella has been developed based on the observation that flagella will adsorb on to DEAE cellulose columns and may be differentially eluted by salt solutions. Attempts were also made to use the property of antigenic specificity for purification of the isolated structures. This approach was abandoned since complete dissociation of the antigen-antibody complex was not easily achieved.

METHODS

Organisms and growth conditions. *Spirillum serpens* was used in most of the experiments. *Proteus vulgaris* and *Bacillus subtilis* SB19 were also investigated. The organisms were grown in 40 l. carboys in complex media (nutrient broth or N.Z. case, an enzymic digest of casein; Sheffield Farms Co., Norwich, New York) with mild aeration. The cultures in late log phase were harvested at 2°.

Isolation of flagella. The organisms were resuspended in cold 0·01 M-phosphate buffer (pH 7·0) and deflagellated mechanically in the Lourdes Multi-Mix Homogenizer (16,000 rev./min. for 60 sec.) at 2°. The cells were removed by centrifugation at 5000 *g* for 30 min. The cell pellet was washed once with one half the initial volume of buffer, and the cells again sedimented by centrifugation. The pooled supernatant fluids were subjected to centrifugation at 105,000 *g* for 30 min. to sediment the flagella. The sedimented flagella were resuspended in 0·5 ml. buffer by allowing them to stand in the refrigerator overnight. Initial trials with water as the suspending medium for isolated flagellar suspensions of *Spirillum serpens* resulted in significant dissociation of the structures; phosphate buffer (0·01 M; pH 7·0) proved to be a suitable suspending medium.

Analytical methods. All the chemical determinations used were modifications of existing procedures. The volumes prescribed in published methods were scaled down, thus increasing the sensitivity of the analyses while simultaneously decreasing the volume of sample required. In the orcinol method (Schneider, 1955) dichromatic readings were made. All analyses were read in a Zeiss spectrophotometer. The methods used were the Lowry (Lowry, Roseborough, Farr & Randall, 1951) for protein; Fiske & SubbaRow for phosphorous (Fisk & SubbaRow, 1925); orcinol for ribonucleic acid (RNA) (Schneider, 1955) and the diphenylamine for deoxyribonucleic acid (DNA) (Schneider, 1955).

Serological methods. Anti-flagellar sera were prepared by intravenous injection of intact flagellated organisms into rabbits. The somatic antibodies were adsorbed from the antisera with deflagellated cell suspensions which had been heated at 100° for 60 min. and washed three times with buffer. Adsorption was carried out at least twice after the sera were negative for somatic antibodies.

Antisera were also prepared against purified flagella. The purified preparations (1.5 mg. protein/ml.) were injected with Freund's incomplete adjuvant into the foot pads of rabbits. Antisera prepared with highly purified flagella showed a low degree of cross-reactivity with homologous somatic antigen, indicating common antigenic sites between flagellar and somatic constituents. The somatic antibodies were removed by adsorption (see above) without a diminution of anti-flagellar titre. Agglutination inhibition (Kabat & Mayer, 1961) was used for the detection of flagellar antigen by using intact flagellated organisms in formalinized saline buffer as the agglutination indicator system. By this procedure less than 1 µg. flagellar protein/ml. could readily be detected.

Preparation of DEAE cellulose columns. The DEAE cellulose (Schleicher and Schüll Co.) was washed with several volumes of N-NaOH and then with water. Chromatography columns were packed with this DEAE cellulose slurried in 2 M-NaCl under atmospheric pressure. Preparation of the columns in high salt solutions resulted in even packing without any channelling. The columns were washed until chloride-free and were then saturated with 0.01 M-phosphate buffer (pH 7.0). The chromatography was carried out at room temperature. Crude flagellar suspensions were adsorbed on the DEAE cellulose and the columns were developed with increasing NaCl concentrations in buffer.

Electron microscopy. Cells and flagellar preparations were examined after negative staining with phosphotungstic acid (Kerridge, Horne & Glauert, 1962); a solution (1 % w/v) of uranyl acetate was also used.

RESULTS

Preliminary experiments showed that crude flagellar suspensions from *Spirillum serpens* were adsorbed by DEAE cellulose (pH 7.0) in phosphate buffer and were not eluted by 20 column volumes of this buffer. Elution of the flagella was achieved by increasing the molarity of NaCl in the buffer eluant. Figure 1 is a typical elution diagram of flagella from DEAE cellulose. In this experiment 15 ml. of a crude suspension of flagella (the pellet sedimented at 105,000 g in 30 min.) in buffer containing 3.02 mg. protein/ml. were passed through a DEAE cellulose column (1.8 × 12 cm.) previously saturated with buffer. The column was washed with 100 ml. buffer and stepwise increases in NaCl in the buffer were used as developing agent.

Elution was carried out at 1.5 ml./min.; 10 ml. fractions were collected. Absorbancy of the eluates was measured in a Zeiss spectrophotometer at 280 m μ . The optical density readings are not directly proportional to light absorption since the light scattered is very significant in the peak fractions because of the Tyndall effect of the flagellar suspensions. Protein and RNA determinations were made on all fractions. The orcinol determinations were negative with the exception of fractions 41 to 44, thus explaining the high absorbancy at 280 m μ with essentially negligible protein in these tubes. Further analyses of this 0.8 M-NaCl peak showed it to consist of RNA. Total protein recovered in the peak fractions (39.7 mg.) accounted for 88 %

Fig. 1. Elution pattern of *Spirillum serpens* flagella from a DEAE cellulose column. The column was developed by stepwise increases of NaCl concentration (0.1 M, 0.2 M, 0.4 M, 0.8 M) in 0.01 M-phosphate buffer (pH 7.0). ○, Protein; ●, optical density.

of the protein passed through the column. Electron microscopy of the samples from peak fractions showed flagellar fragments in all the three major peaks—0.1 M, 0.2 M and 0.4 M-NaCl. The 0.8 M peak showed no structures recognizable as flagella.

Three possible explanations for the presence of the discrete peaks noted in Fig. 1 were considered: (1) three different species of flagella are made by the organisms; (2) there is a discontinuous size distribution of flagella produced by the organisms or by the deflagellation method; (3) there is a continuous size distribution of flagella produced either by the organisms or by the deflagellation method.

The first hypothesis was tested serologically by using anti-flagellar antibodies prepared against the purified flagella eluted with 0.2 M-NaCl. It was found that the equivalence point for antigen-antibody reaction was identical for all three peaks. The equivalence point was determined initially for the 0.2 M-NaCl flagellar fractions with homologous antibody. The same equivalence point was obtained with the 0.1 and 0.4 M-NaCl eluates against the same serum (0.2 M-NaCl fraction antiserum).

The results of this experiment argue against the hypothesis of three species of flagella produced by *Spirillum serpens*.

Hypotheses (2) and (3) were tested by eluting the flagella from the DEAE cellulose column with a continuously increasing NaCl gradient. If hypothesis (2) were correct, one should obtain three discrete peaks even if elution were carried out by a gradient, whereas a continuous size distribution would result in a single peak. Figure 2 illustrates an experiment in which a crude *Spirillum serpens* flagellar suspension was absorbed on to a 1.8×19 cm. DEAE cellulose column, and elution carried out by using a concave gradient of NaCl (500 ml. of salt-free buffer in the

Fig. 2. Elution pattern of *Spirillum serpens* flagella from a DEAE cellulose column. The column was developed with a NaCl gradient in buffer. □, NaCl concentration; ○, protein. The solid bars denote the % inhibition of agglutination (see text).

mixing vessel and 500 ml. of 2 M-NaCl in buffer in the reservoir). Elution was carried out at 1.5 ml./min. and 10 ml. fractions were collected. The gradient was started after 200 ml. of buffer had been used to wash the column. It is evident from examination of the figure that a single peak was obtained, thus indicating that hypothesis (2) was incorrect. The data suggest, therefore, that during the deflagellation procedure a continuous distribution of flagellar sizes was produced.

The antigenic homogeneity of the flagella eluted by the gradient method was also tested by the agglutination-inhibition technique. The results of such an analysis are plotted in Fig. 2 as % inhibition of agglutination of the indicator system by the eluate fractions. It is evident that inhibition of agglutination occurred only in the peak fractions; that is where flagella were observed in the electron microscope.

These peak fractions (Fig. 2) were pooled and the flagella harvested by centrifugation. A sample of the resuspended flagella preparation was chromatographed again on a DEAE cellulose column (1.8×14 cm.) with the conditions of gradient

elution as in Fig. 2. Figure 3 illustrates the results obtained. It is evident that rechromatography resulted in a reproducible elution of the flagella by the same salt concentration as in the original column passage.

After elution from the column, the flagella may be harvested by centrifugation at 105,000 *g* for 30 min. Analyses for protein in the supernatant fluid and pellet revealed trace amounts (usually no more than 2% of total) of protein remaining in the supernatant fraction. These supernatant fractions, however, were still capable of agglutination inhibition, implying that the residual non-sedimented protein was of flagellar origin. It was sometimes necessary to de-salt the sedimented flagellar suspensions. This was readily achieved by passage of the flagellar suspension through a Sephadex (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) column.

Fig. 3

Fig. 4

Fig. 3. Elution pattern of a sample of the flagella of *Spirillum serpens* obtained in the experiment recorded in Fig. 2 from DEAE cellulose. ○, NaCl concentration; ●, optical density.

Fig. 4. Elution pattern of *Spirillum serpens* flagella from Sephadex G 50 column. Column was developed with 0.005 M-NaCl.

Figure 4 illustrates an elution diagram of such an experiment. Four ml. of a suspension of flagella recovered from DEAE cellulose eluates containing 2.65 mg. protein/ml. were passed through a Sephadex G 50 (medium) column (1.8 × 20 cm.) saturated with 0.005 M-NaCl. The column was developed with 0.005 M-NaCl; 2 ml. fractions were collected and protein determinations were made on all fractions. The flagella were eluted as a single uniform peak. In this experiment 9.65 mg. protein were recovered in fractions 3–9, indicating a 91% recovery of the total protein placed on the column. When structural integrity of the flagella is not required, water may be used as eluting agent in the Sephadex treatment. Alternatively, volatile salts such as ammonium acetate or triethylammonium acetate may be used as developer.

The DEAE cellulose NaCl-gradient elution procedure described above for *Spirillum serpens* has been tested with flagella suspensions isolated from *Bacillus subtilis* and *Proteus vulgaris*. The elution patterns obtained with the flagella from these organisms were essentially identical with those obtained for *S. serpens*, suggesting that the method may be used for the purification of flagella from any bacterial strain.

The evidence presented in this paper for the purity of the preparations obtained by the method described is the uniformity of elution pattern, from both DEAE cellulose and Sephadex, the antigenic homogeneity of the preparations, and the cleanness of the electron microscope pictures. In addition, the column peak fractions were free from nucleic acids and phosphorous (determined after elution from Sephadex with water) in contrast to the crude flagellar suspension. These data in themselves are insufficient to establish the degree of purity of the flagellar preparations; for this, extensive chemical and physical data are required. This information will appear in a subsequent communication.

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