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ANTIGENIC STRUCTURES OF SALMONELLA FLAGELLA. I. PRESENCE OF AN ANTIGENIC DETERMINANT EXPOSED AT ONE END OF FLAGELLAR FRAGMENTS

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SUMMARY Salmonella flagellin, which is a constitutional subunit of the flagellum, was shown to have antigenic determinants distinct from its own serotypic ones. These antigenic determinants were found to be common to flagellins from the so-called *g*-complex serotypes, such as *fg*, *mt*, *gm*, *gt*, *gp* and *gmptu*, but not to those from other serotypes, such as *a*, *i* or *enx*. Rabbits immunized with flagellin of serotype "*fg*" produced anti-"*fg*" flagellin antibodies. Only about 20 percent of these corresponded to the serotype determinants of the "*fg*" on the surface of the flagella, and the remaining 80 percent reacted with the flagellin of the unrelated serotype "*mt*", and corresponded to the distinct determinants common to the flagellin molecules. These antigenic determinants were detected by the immunoferritin technique at only one, not both, terminals of the flagellar fragments, suggesting that a unidirectional arrangement of flagellin subunits in the flagella may expose the inherent conformation of the subunits at only one end of the flagellum.

INTRODUCTION

Salmonella flagella have been widely studied serologically because of their epidemiological importance for typing salmonella and more than 80 serotypic antigens of the flagella have now been reported (Kauffmann, 1961; Kelterborn, 1967). Flagella are formed from subunits of the constitutional protein flagellin (Weibull, 1950), so there must be more than 80 different kinds of naturally occurring flagellin molecules. The serotypes of the flagella are composed of several serologically defined factors, and each factor may correspond to antigenic determinants localized on the surface of the flagella. Antibodies to a factor can be detected very efficiently by immobilization of a bacterial strain which bears the factor on its flagellum. Thus salmonella flagellar antigen seems to be an ideal protein antigen to use in studies on the mechanism of antibody formation in relation with the submolecular structure of the antigen.

To analyse flagellin antigenically, we first selected *S. haelsingborg* from the Kauffmann-White schema because its flagella has the most varied serotype, *gmptu* (Kauffmann, 1961;

Dohi et al., 1969). Immunochemical analyses suggested that the flagellin of this species had specific antigenic determinants which could not be detected on the surface of the flagella.

To elucidate this, we selected 2 more species, *S. oranienburg* (6, 7: mt;—) and *S. derby* (4, 5, 12: fg;—), which do not share any common serotypic determinant in their H antigens, and which from their flagellar serotypes have distinct determinants in their flagellins. We also tested whether these determinants were exposed at the terminal ends of the flagella.

MATERIALS AND METHODS

1. *Bacterial strains*

S. oranienburg NCTC 5743 (6, 7: mt;—), *S. derby* NCTC 1729 (4, 5, 12: fg;—), and *S. abortus-equi* NCTC 5727 (1, 4, 12:—; enx) were kindly provided by Mr. S. Lapage of the National Collection of Type Cultures, London. A phase 1 stable strain of *S. abortus-equi* SJ241 (4, 12: a;—) and its P22-mediated transductant SJ874 (4, 12: i;—) were gifts from Dr. T. Iino in the Department of Botany, Faculty of Science, Tokyo University.

2. *Preparation of flagella and flagellin*

Bacteria were grown on enriched nutrient agar containing 1% peptone (Wako Co.), 0.5% meat extract (Wako Co.), 0.5% yeast extract (Daigoeiyo Co.), 0.5% meat extract (Wako Co.), 0.5% yeast extract (Daigoeiyo Co.), 0.05 M Na_2HPO_4, 0.03 M glucose, and 1.2% agar. Cultures grown for 18 hr at 37 C were harvested in chilled saline and homogenized for 10 min in a mixer. The homogenate was centrifuged at 8,500 rpm for 30 min and the supernatant, containing the flagella, was subjected to ultracentrifugation at 40,000 $\times g$ for 60 min. The precipitated flagella were resuspended in chilled saline using a Potter teflon homogenizer. After repeating the centrifugation, ultracentrifugation and homogenization treatments, the flagellar suspension was disintegrated into flagellin monomers by treatment with HCl, following the procedure of Kobayashi et al. (1959). The solution of flagellin was separated from acid-insoluble material by ultracentrifugation at 100,000 $\times g$ for 90 min. Then it was neutralized with NaOH and phosphate buffer, pH 8.0 was added to a concentration of 0.02 M. The flagellin solution was purified further by gel filtration on Sephadex G-150 and DEAE-cellulose chromatography. The protein concentration was calculated from the UV-absorption at 276 nm and from the visible absorption at 750 nm in the Folin reaction (Lowry et al., 1951).

3. *Polyacrylamide gel electrophoresis*

The original method of Davis (1964) was followed. The gel contained 7% acrylamide, 0.2% *N, N'*-methylene-bis-acrylamide, deionized 8 M urea, and 0.375 M Tris-HCl buffer, pH 8.6. Polymerization was catalyzed with 0.08% *N, N, N', N'*-tetramethyl ethylenediamine and 0.025% ammonium persulfate. Electrophoresis was run at a constant current of 3 ma per tube for 150 min. The gels were stained with 0.25% amidoblack 10B in 7% acetic acid and 0.25% $HgCl_2$ solution for at least 30 min and destained with 7% acetic acid solution.

4. *Sedimentation analysis*

The sedimentations of flagellins were examined in a Hitachi, model UCA-II ultracentrifuge using flagellin solutions in 0.15 M NaCl containing 0.02 M phosphate buffer, pH 8. Centrifugation was performed at 60,000 rpm at 20 C.

5. *Preparation of antisera*

For preparation of anti-flagellin sera, several rabbits were injected subcutaneously into all four food pads with 2 mg of chromatographically purified flagellin emulsified with Freund complete adjuvant. Boosted injections of the same dose of antigen were given in the same way 5 weeks later. Blood was taken 7 days after the last injection. For preparation of anti-bacterial sera, several rabbits were injected intravenously 8 times with 10^9 cells of formalin-killed bacteria, giving injections twice a week. Five days after the last injection the animals were bled. All rabbit sera were heated at 56 C for 30 min. Anti-flagellin sera were absorbed to remove antibodies to the serotype antigen using bacteria which had been killed in 0.5% formalin and washed twice with phosphate buffered saline (PBS) with centrifugation to remove the formalin. For absorption, the killed bacteria were mixed with the test anti-flagellin serum and incubated at 37 C for 30 min. The mixture was then centrifuged and the supernatant was again absorbed. This treatment was usually repeated about 8 times until the supernatant showed no immobilizing activity on the bacteria. The final supernatant was subjected

to ultracentrifugation at 100,000×g for 90 min to remove insoluble material.

Goat anti-rabbit IgG serum was a gift from Dr. H. Fujio of this department.

6. *^{125}I-Labelling of the IgG of anti-flagellin sera*

IgG fractions were prepared from rabbit anti-flagellin sera by DEAE-cellulose chromatography, following the method of Fahey (1962). Then they were labeled with ^{125}I by direct oxidation of chloramin-T (McConahey and Dixon, 1966). The specific activity of the IgG derived from the anti-"*mt*" flagellin serum was 3×10^7 cpm/μmole, and that of the IgG from anti-"*fg*" flagellin serum was 2×10^7 cpm/μmole.

7. *Quantitative precipitin reactions*

Quantitative precipitin reactions between flagellins and anti-flagellin sera were carried out by the method of Heidelberger et al. (1935). That is, the mixtures were incubated at 37 C for 1 hr and then at 4 C for 2 days, and then washed twice with PBS. The precipitate was dissolved in 1 ml of 0.1 N NaOH and its absorption at 280 nm was measured.

8. *Gel diffusion reactions*

Two dimensional double diffusion (Ouchterlony, 1949) was carried out in gels containing 1% agarose and PBS, pH 8. The plates were examined after incubation at 37 C for 2 days in moist chambers. Semi-solid medium for observing bacterial swarming was prepared by adding 0.33% (w/v) agar and 8% (w/v) gelatin to nutrient broth as described by Stocker et al. (1953). Double diffusions were also carried out in this semi-solid medium. The pure flagellin solutions and anti-flagellin sera, sterilized by passage through millipore filters (HA type; pore size 0.45 μ), were put into the wells and incubated at 37 C for 24 hr. A cotton thread soaked in broth culture containing highly motile bacteria was put on the gel across the precipitin line which appeared and incubation was continued at 37 C for 9 hr and then at 20 C for 5 hr.

9. *Bacterial immobilization*

The bacterial immobilization titers of antisera were determined by microscopical examination of the mobility of bacteria in a droplet in paraffin oil. Two-fold serial dilutions of antisera were incubated with an equal volume of suspension of 10^8 bacteria/ml at room temperature for 30 min. The titers were expressed as the reciprocals of the highest dilution causing 50% immobilization of the population.

10. *Immuno-ferritin technique*

To determine the localization of an antigen on the flagellum, conjugates of ferritin and goat IgG derived from anti-rabbit IgG sera were incubated with flagella, which had previously been saturated with appropriate rabbit antibodies to the antigen.

Horse spleen ferritin (cadmium free, Lot #1217) was purchased from Nutritional Biochemical Co. (U.S.A.). Ferritin solutions in PBS were subjected to ultracentrifugation at 100,000×g for 4 hr to separate ferritin from apoferritin. Then 31 mg of goat IgG fractions derived from anti-rabbit IgG were coupled to 6.4 mg of ferritin with toluene diisocynate, as described by Singer (1961). The reaction mixture was centrifuged twice at 100,000×g for 4 hr to remove unreacted IgG. This conjugate was estimated to have an average molar ratio of 1:2 of ferritin to IgG. This preparation of conjugate was used without removal of unconjugated ferritin. It was incubated first at 37 C for 30 min and then at 4 C overnight with flagella treated with ^{125}I labeled anti-flagellin antibodies. Then the mixture of antibody-treated flagella and ferritin-anti IgG conjugates was centrifuged about six times at 40,000×g for 45 min each time to remove unbound ferritin conjugates. All but the last two of these ultracentrifugations were done on a cushion of 0.1 ml of 5% sucrose solution to facilitate resuspension.

11. *Electronmicroscopy of flagella treated with ferritin-conjugates*

Samples of the treated flagella were put on collodion-coated triafol micromesh grids, washed slightly with water, dried, and negatively stained with 1% uranylacetate. They were examined in a Hitachi 11B electron microscope.

RESULTS

1. *Purification and physicochemical properties of the flagellins*

Flagellins prepared by the method of Kobayashi et al. (1959) were purified further by gel filtration on Sephadex G-150 and DEAE-cellulose chromatography. Figure 1 shows the gel filtration pattern of the flagellin (Fi^{mt}) of *S. oranienburg*. The main fraction, Fr. 3,

which had the highest ratio of OD_{750}/OD_{276}, was condensed by negative pressure dialysis and diluted about 30 times with water to adjust the ionic strength of the solution to that of the initial buffer used for subsequent DEAE-cellulose chromatography. Figure 2 shows the elution of the Fr. 3 of Fi^{mt} on DEAE-cellulose chromatography. Solutions of the flagellins of *S. derby* (Fi^{fg}), *S. abortus-equi* (Fi^{enx}), SJ241 (Fi^{a}) and SJ841 (Fi^{i}) gave similar

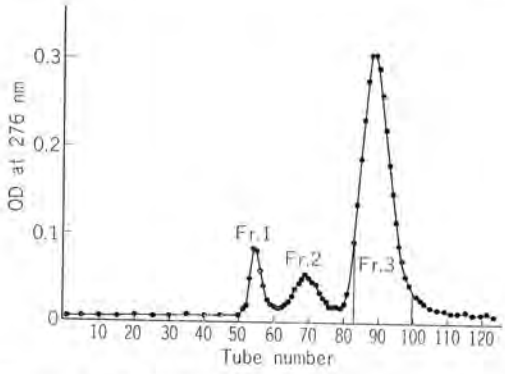

FIGURE 1. Sephadex G-150 gel filtration pattern of the flagellin of *S. oranienburg*. Crude flagellin solution neutralized with NaOH and containing 0.02 M phosphate was applied to a column (4 × 136 cm) of Sephadex G-150 and eluted with 0.15 M NaCl in 0.02 M phosphate buffer, pH 8. The flow rate was 50 ml/hr. The fraction size was 10 ml.

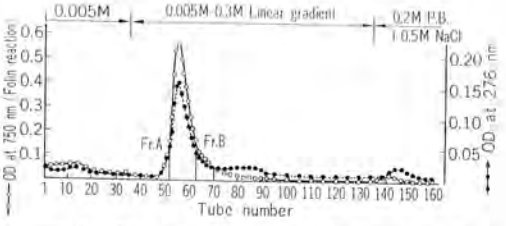

FIGURE 2. Elution pattern of the flagellin of *S. oranienburg* on DEAE-cellulose chromatography. Fr. 3 from Sephadex G-150 was condensed by negative pressure dialysis, diluted 30 times with distilled water, and applied to a column (3 × 20 cm) of DEAE-cellulose previously equilibrated with 0.005 M phosphate buffer, pH 8. The column was washed with 350 ml of 0.005 M phosphate buffer, pH 8, and eluted with 1,000 ml of a linear gradient of 0.005 M to 0.3 M phosphate buffer, pH 8. The fraction size was 10 ml.

elution patterns on gel filtration on Sephadex G-150 and DEAE-cellulose chromatography. The main fraction, Fr. A, of each strain, obtained by DEAE-cellulose chromatography, was dialysed against PBS, pH 8 and used as the purified flagellin preparation. The purity and physicochemical properties of purified Fi^{mt} and Fi^{fg} were examined as follows:

(1) Polyacrylamide gel disc electrophoresis The purities of Fi^{mt} and Fi^{fg} were tested by polyacrylamide gel disc electrophoresis in the

FIGURE 3. Polyacrylamide disc gel electrophoresis of Fi^{mt} and Fi^{fg}. The gels contained 7% acrylamide, 0.2% *N*, *N'*-methylene-bis-acrylamide, deionized 8 M urea and 0.375 M Tris-HCl buffer, pH 8.6. Electrophoresis was run at a constant current of 3 ma per tube for 150 min at 4 C.

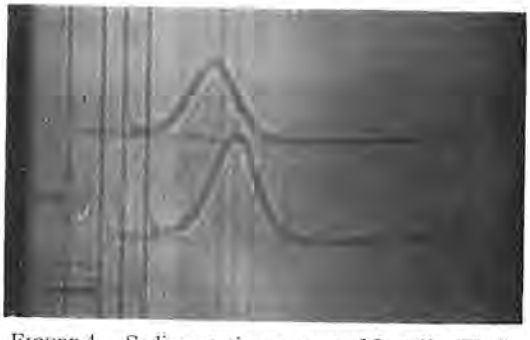

FIGURE 4. Sedimentation pattern of flagellin (Fi^{mt}). Solvent, 0.15 M NaCl in 0.02 M phosphate buffer, pH 8; Centrifugation at 60,000 rpm at 20 C; Angle, 70°; Protein concentration, 6 mg/ml (upper), 8 mg/ml (lower).

presence of 8 M urea. The Fr. A from Fi^{mt} and Fi^{fg} gave a single band in 7% gel (Fig. 3), indicating that the Fr. A was electrophoretically pure and homogeneous.

(2) Sedimentation analysis

The sedimentations of Fi^{mt} were examined with 0.15 M NaCl containing 0.02 M phosphate buffer, pH 8 as solvent. The sedimentation diagrams each showed a single peak (Fig. 4) and their S_{20} values were estimated as 2.8 (Fig. 5). These results indicate that these purified flagellins were pure monomers, in agreement with the results of others (Erlander, Koffler and Foster, 1960; Asakura, Eguchi and Iino, 1964).

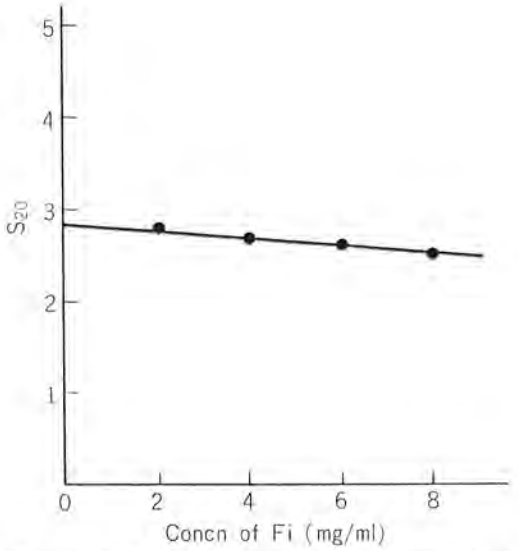

FIGURE 5. Determination of the Sedimentation coefficient of Fi^{mt}. The solvent was 0.15 M NaCl in 0.02 C phosphate buffer, pH 8. Centrifugation was carried out at 60,000 rpm at 20 C. The S_{20} value was estimated as 2.8.

2. *Immobilizing activity of the precipitating antibodies in the anti-flagellin sera*

Freshly prepared flagellin solution and anti-flagellin antiserum formed only one precipitin line in agarose gel (Fig. 7A, B). To examine whether all or part of the precipitating antibody is related to bacterial immobilization, the relationship between the precipitin line and the inhibition zone of bacterial swarming by the antiserum in a semi-solid medium, was investigated. As shown in Fig. 6A, the Fi^{fg} and the anti-Fi^{fg} serum formed one precipitin line in the semi-solid medium, and as shown in Fig. 6B, bacteria ($Bact^{fg}$) inoculated across the precipitin line, only swarmed toward the antigen side of the precipitin line, not toward the antiserum side. The boundary of the swarming zone was completely superimposable on the precipitin line (Fig. 6B), indicating that all the immobilizing antibodies in the anti-flagellin serum were involved in the precipitin reac-

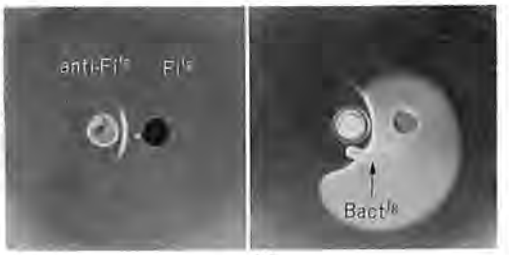

FIGURE 6. Double diffusion between the anti-Fi^{fg} serum and Fi^{fg} in semi-solid medium (A), and the inhibition zone of swarming of $Bact^{fg}$ (B). A: Double diffusion was carried out in semi-solid medium composed of nutrient broth, 0.33% (w/v) agar and 8% (w/v) gelatin. The purified Fi^{fg} solution and anti-Fi^{fg} serum were sterized by passage through millipore filters, placed in the wells and incubated at 37 C for 24 hr. A single precipitin line appeared. B: A cotton thread soaked in a broth culture of highly motile bacteria (rotatory culture at 37 C for 3 hr) was placed across the precipitin line and was incubated at 37 C for 9 hr and then at 20 C for 5 hr. Then the swarming zone was examined.

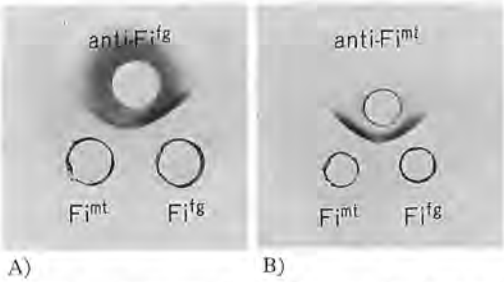

FIGURE 7. Immunodiffusion between flagellins and anti-flagellin sera in 1% agarose gel containing PBS-8 A: Double diffusion between anti-Fi^{fg} serum (R #86) and Fi^{fg} or Fi^{mt}. B: Double diffusion between anti-Fi^{mt} serum (R #83) and Fi^{mt} or Fi^{fg}.

tion. When bacteria of serotype *mt* ($Bact^{mt}$) were inoculated instead of $Bact^{fg}$, they swarmed to both sides of the precipitin line.

3. *Evidence for the presence of precipitating antibody not involved in immobilization*

1) Confirmation of lack of cross-reactivity between the flagellar serotypes of the two strains ($Bact^{fg}$ and $Bact^{mt}$)

Antisera were prepared by immunization of rabbits with Fi^{fg}, Fi^{mt}, $Bact^{fg}$ or $Bact^{mt}$, and their immobilization titers against $Bact^{fg}$ and $Bact^{mt}$ were assayed. Table 1 shows the immobilization titers of representative samples of each antiserum. These results indicate that anti- $Bact^{mt}$ and anti-Fi^{mt} sera immobilized $Bact^{mt}$, but did not immobilize $Bact^{fg}$, and that anti-$Bact^{fg}$ and anti-Fi^{fg} sera immobilized $Bact^{fg}$, but did not immobilize $Bact^{mt}$. These results show that $Bact^{fg}$ and $Bact^{mt}$ did not share any flagellar serotype antigen. This is compatible with Kauffmann-White's classification of the species.

TABLE 1. *Lack of cross-reaction between flagellar antigens ($Bact^{mt}$ and $Bact^{fg}$)*

Immunizing antigen	(Antiserum)	Immobilization titer[a] against	
		$Bact^{mt}$	$Bact^{fg}$
$Bact^{mt}$	(R #53)	12,800	8
Fi^{mt}	(R #83)	1,600	1
$Bact^{fg}$	(R #105)	8	12,800
Fi^{fg}	(R #87)	4	1,600
Non	(Pooled)	4	4

[a] The procedure used is described in the Materials and Methods.

2) Evidence for immunological cross reaction between Fi^{mt} and Fi^{fg}

The anti-Fi^{fg} serum formed a single precipitin line on immunodiffusion with Fi^{mt} as well as with Fi^{fg} and the two precipitin lines fused without spur formation (Fig. 7A). In quantitative precipitin reactions of anti-Fi^{fg} serum with either Fi^{fg} or Fi^{mt}, Fi^{fg} (the homologous antigen) precipitated 1.12 mg of the antibodies in 1 ml of the anti-Fi^{fg} serum (R#87) and the supernatants of the reaction mixtures showed scarcely any immobilizing activity (Fig. 8), whereas Fi^{mt} (the heterologous antigen) precipitated about 80 percent of the maximum amount precipitated by the homologous antigen Fi^{fg}, and almost all the immobilizing activity remained in the supernatant (Fig. 8). Similar results were obtained with anti-Fi^{mt} serum. That is, on immunodiffusion, anti-Fi^{mt} serum formed a single precipitin line with Fi^{fg} as well as with Fi^{mt} and the two precipitin lines fused without formation of a spur (Fig. 7B). In quantitative precipitin reactions of anti-Fi^{mt} with Fi^{fg} and Fi^{mt}, Fi^{fg} (the heterologous antigen) precipitated about 80 percent of the maximum amount of antibody precipitated by the homologous antigen Fi^{mt} and all

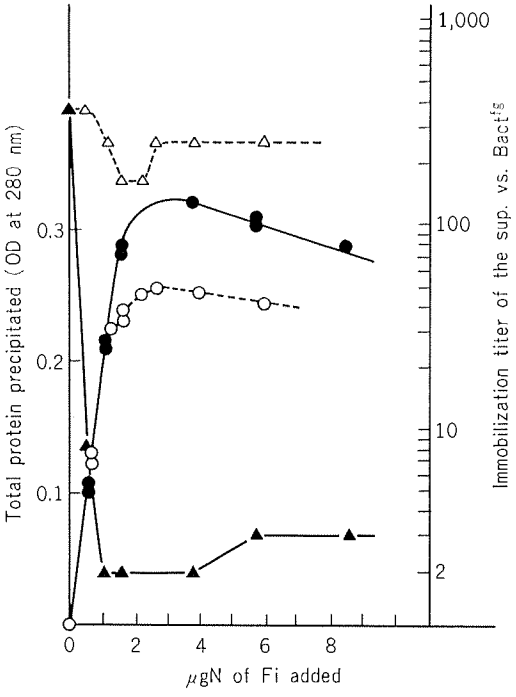

FIGURE 8. Quantitative precipitin reactions between the anti-Fi^{fg} serum (R #87) and Fi^{fg} or Fi^{mt}. Immune precipitates formed by Fi^{fg} (●——●) or Fi^{mt} (○····○) and immobilization titers of the supernatants of the reaction mixtures with Fi^{fg} (▲——▲) and with Fi^{mt} (△····△). 0.2 ml of anti-Fi^{fg} serum was used.

the immobilizing activity remained in the supernatant of the heterologous system, whereas none remained in that of the homologous system (Fig. 9). These results indicate that Fi^{fg} and Fi^{mt} share major common antigenic determinants, which are not directed to immobilizing antibodies. In other words, these determinants are not serotype determinants, suggesting that they became accessible to the corresponding antibodies when the flagella disintegrated into monomers.

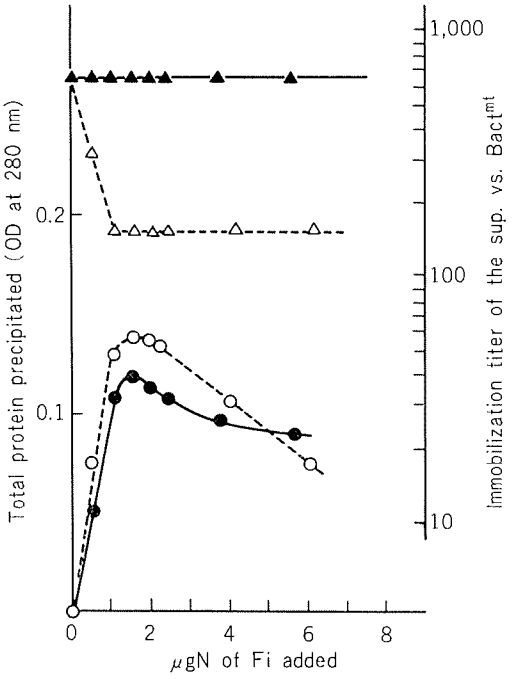

FIGURE 9. Quantitative precipitin reactions between anti-Fi^{mt} serum (R #83) and Fi^{mt} or Fi^{fg}. Immune precipitates formed by Fi^{mt} (○····○) or Fi^{fg} (●——●) and immobilization titers of supernatants of the reaction mixtures with Fi^{mt} (△····△) and with Fi^{fg} (▲——▲). 0.2 ml of anti-Fi^{mt} serum was used.

3) Identity of the distinct common determinants with all determinants other than serotype determinants

To examine the relationship between the distinct common determinants and determinants other than serotype determinants, the antibodies to the serotype determinants on the flagella were removed by absorption with killed bacteria. The anti-Fi^{fg} serum absorbed with $Bact^{fg}$ had an immobilization titer of less than 1 against $Bact^{fg}$. Quantitative precipitin reactions were carried out between this absorbed antiserum and Fi^{fg} or Fi^{mt}. The maximum amount precipitated by Fi^{fg} was reduced to one fifth of that of the origihal serum by absorption procedures, and was the same as the maximum amount precipitated by Fi^{mt} (Fig. 10). These results indicate that Fi^{mt} retains all the antigenic determinants of Fi^{fg} except the serotype "*fg*" determinants, and suggest that, among the *g*-complex serotypes, all the antigenic determinants of flagellins except the serotype determinants may be common.

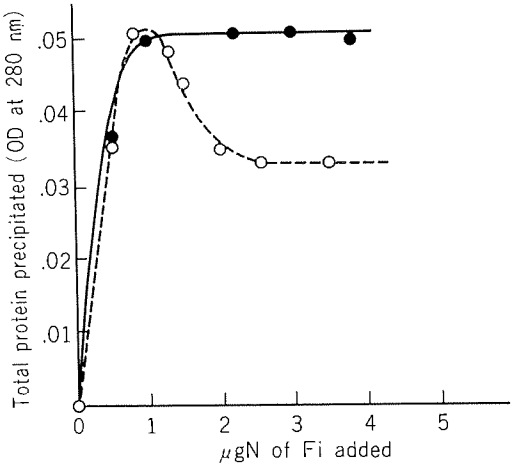

FIGURE 10. Quantitative precipitin reactions between the absorbed anti-Fi^{fg} serum and Fi^{fg} or Fi^{mt}. The anti-Fi^{fg} serum had been absorbed by formalin-killed bacteria and had no immobilizing activity. Fi^{fg} (●——●) and Fi^{mt} (○····○).

4. *Evidence for exposure of distinct common antigenic determinants of the flagellin at a terminal end of the flagellum*

Two possible ways in which the distinct common antigenic determinants of flagellin in the flagellum may appear were considered: (1) Disintegration of the flagellum may cause configurational changes of the subunits with

formation of new antigenic determinants. (2) A portion of the surface of the flagellin subunit containing antigenic determinants may normally be covered by adjacent flagellin subunits. These hiden antigenic determinants may become accessible to the corresponding antibodies on disintegration of the flagella into monomers.

It is difficult to test the former possibility, but in the latter case, at least some of the distinct common antigenic determinants may be exposed at the terminal end of flagellar fragments. The following investigations were performed to test this possibility.

First, experiments were made on the binding of labeled antibody with heterologous flagella. The IgG fraction was purified from anti-Fi^{mt} serum and trace-labelled with ^{125}I. Then increasing amounts of flagella (Fa^{fg} or Fa^{enx}) were added to 0.35 mg of ^{125}I labelled anti-Fi^{mt} IgG, and the mixtures were incubated at 37 C for 30 min and then at 4 C for 12 hr. Then the material was washed 3 times with centrifugation and the radioactivity of the precipitate was measured. As shown in Fig. 11A, the amount of radioactivity in the precipitate reached a plateau with more than 10 μgN of Fa^{fg}, but no radioactivity bound to Fa^{enx}. Quantitative precipitin reactions between the labeled IgG preparation and Fi^{fg} or Fi^{mt} were carried out to compare the plateau level with the maximum amount precipitated by Fi^{fg} or Fi^{mt}. The heterologous antigen Fi^{fg} precipitated a maximum of 2,400 cpm of radioactivity from 1.4 mg of the preparation, whereas the homologous antigen Fi^{mt} precipitated a

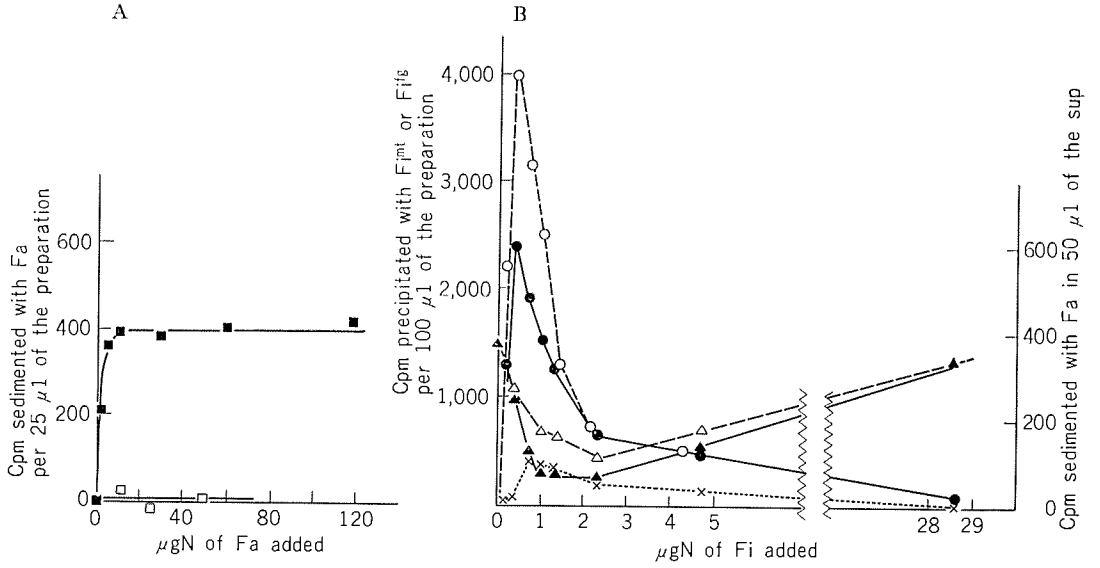

FIGURE 11. Binding of labeled anti-Fi^{mt} IgG with heterologous flagella and flagellins. A: Increasing amounts of Fa^{fg} or Fa^{enx} were added to 25 μliters of ^{125}I labeled anti-Fi^{mt} IgG (0.35 mg). The material was washed with centrifugation and the bound radioactivity was measured. Radioactivity bound to Fa^{fg}, (■—■); radioactivity bound to Fa^{enx}, (□·····□). B: Quantitative precipitin reactions between the labeled anti-Fi^{mt} IgG preparation and Fi^{mt} (○····○) or Fi^{fg} (●—●), and binding of the supernatants to Fa^{fg}. For the quantitative precipitin reactions, volumes of 0.1 ml (1.4 mg) of the anti-Fi^{mt} IgG preparation were used. For binding experiments, 118 μgN of Fa^{fg} or buffer only was allowed to react with 50 μliter volumes of the supernatants. Then the mixtures were centrifuged, and the precipitates were washed and their radioactivity was measured. Buffer only, (×·····×); total precipitate with Fa^{fg}, (△·····△). Radioactivity bound to Fa^{fg} (▲—▲) was calculated by subtracting the amount precipitated in the absence of Fa^{fg} from the total amount precipitated with Fa^{fg}. Background (52 cpm) was deduced from values precipitated.

maximum of 4,000 cpm. Thus the maximum amount bound to Fa^{fg} was equivalent to two thirds of that precipitated by Fi^{fg}. To examine the remaining antibodies capable of binding with Fa^{fg} in the supernatant of the precipitin reaction in the heterologous antigen Fi^{fg} system, 118 μgN of Fa^{fg} or buffer only were added to 50 μliter volumes of the supernatants, and after similar incubation and washing, the radio-activity precipitated on ultracentrifugation was measured (Fig. 11B). The ^{125}I precipitated in the absence of Fa^{fg} showed a small peak in the region of slight antigen excess, suggesting the presence of antigen-antibody complexes which were not precipitated by the low speed centrifugation, but were precipitated by ultracentrifugation. The amount bound to Fa^{fg} in the supernatant was obtained by subtracting the amount sedimented in the absence of Fa^{fg} from the total amount sedimented with Fa^{fg}, assuming that there was no exchange of antibody between the sedimented antigen-antibody complexes and Fa^{fg}. As shown in Figure 11B, the total amount of ^{125}I precipitated with Fa^{fg} and the amount of ^{125}I bound to Fa^{fg} had a trough in the antigen excess region, and returned to the original level in the region of large antigen excess. This suggests that the portion of antibodies which bound to Fi, was replaced by those bound to the flagella in the region of large antigen excess. These results indicate that the binding of labeled IgG to the flagella is an immunologically specific reactions, and that the flagella have other antigenic determinants besides the serotype determinants.

The immunoferritin technique was used to

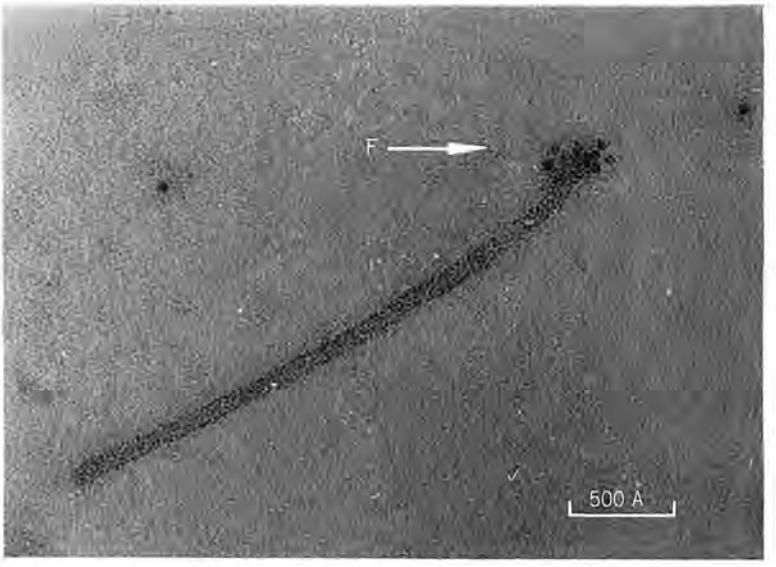

FIGURE 12. A flagellum with many ferritin granules attached to one end. Fa^{fg} was allowed to react with rabbit anti-Fi^{mt} IgG, and then treated with goat anti-rabbit IgG IgG-ferritin conjugates. The arrow indicates ferritin granules. See test for experimental details.

where the antigenic determinants, other than serotype determinants were located on the surface of the flagella.

First steps: Flagella was treated with the rabbit anti-heterologous flagellin IgG. Based on the results described above, the minimum dose of the flagella showing a plateau level, 50 μgN of the Fa^{fg} was allowed to react with 2 mg of the ^{125}I labeled anti-Fi^{mt} IgG, and 45 μgN of Fa^{mt} to react with 1 mg of the ^{125}I labeled anti-Fi^{fg} IgG. After incubation, the flagella-antibody complexes were washed with ultracentrifugation.

Second step: The above flagella-antibody complexes were mixed with 30 μg of ferritin-goat anti-rabbit IgG conjugates. After incubation at 37 C for 30 min and then at 4 C for 12 hr, the reaction mixtures were washed six times with ultracentrifugation at 40,000 $\times g$ for 45 min to remove unbound ferritin-goat IgG conjugates, and the washed flagella were observed by electronmicroscopy.

Many ferritin granules were seen at one terminal of flagella in reaction mixtures of

Fa^{fg}-anti-Fi^{mt} complexes treated with ferritin-IgG conjugates (Fig. 12). One end of about 70 percent of the flagella was covered with one to eight ferritin granules. No flagella had these clusters at both ends, and no rows of ferritin granules arranged longitudinally along the flagella were seen.

Similar results were obtained with reaction mixtures of Fa^{mt}-anti-Fi^{fg} complexes and ferritin-IgG conjugates (Fig. 13). The following experiments were performed at the same time as controls: (1) In the first step described above, 2 mg of normal rabbit IgG were used instead of rabbit anti-Fi^{mt} IgG. (2) In the second step, flagella-antibody complexes were blocked by adding 200 μg of goat anti-rabbit IgG before treatment with ferritin-IgG conjugates. No clusters of ferritin granules were seen at the ends of flagella in either of these controls. Thus immunologically, the localization of ferritin-granules at the terminal ends seems to be specific.

FIGURE 13. Flagella with many ferritin granules attached to one end. Fa^{mt} was allowed to react with anti-Fi^{fg} IgG, and then treated with goat anti-IgG IgG ferritin conjugates. Arrows indicate clusters of ferritin granules. See text for details.

Estimation of antibodies bound to the flagella and electronmicroscopical observations demonstrated that part of the distinct antigenic determinants of the flagellin from the flagellar serotypes was located at one terminal of the flagella fragments. Results suggested that at least part of these determinants of the flagellin formed the covering of the subunits in the flagellum and were exposed at one terminal of the flagella fragments.

DISCUSSION

Salmonella flagella have been widely studied

serologically and more than 80 serotype antigens have been reported (Kauffmann, 1961; Kelterborn, 1967). Each serotype may correspond to antigenic determinants located on the surface of the flagella. Ada et al. (1964), using the immobilization inhibition test, the precipitin reaction and immunodiffusion, reported the serological homology between the flagella, their subunit flagellin, and polymerized flagellin of *Salmonella adelaide* (35; fg:—). Furthermore, Parish et al. (1969a, 1969b) reported that one of the peptides cleaved with cyanogen bromide, Fragment A (MW. 18,000), carried all the antigenic determinants of the flagellin. To study the antigenic determinants of flagellin in more detail, we selected *S. haelsingborg* in Kauffmann-White's schema, because it has the richest variety of flagellar serotypes, *gmptu* (Kauffmann, 1961; Dohi et al., 1969). On immunodiffusion, antiserum against the flagellin (Fi^h) of *S. haelsingborg* ($Bact^h$) formed a precipitin line not only with Fi^h, but also with the flagellins of the follwoing *g*-complex serotypes: *mt*, *fg*, *gms*, *gp*, *gt* and *gpu*. The precipitin line formed by Fi^h and the lines formed, by the other flagellins fused with marked spur formation. Anti-Fi^h serum absorbed by formalin-killed $Bact^h$ had no immobilizing activity, but still formed a precipitin line in gel not only with Fi^h, but also with the other flagellins and these lines fused with each other and the spur which was present in the original serum was not found. These results suggest that the flagellin had distinct antigenic determinants which were not present on the surface of the flagella, and that these determinants were common to flagellins of *g*-complex serotypes.

To investigate these distinct determinants in more detail, two more species which do not share any serotype antigen on their flagella, e.g., *S. derby* and *S. oranienburg*, were chosen from Kauffmann-White's schema, and their flagellins, (Fi^{fg} and Fi^{mt}, respectively) were examined. On immunodiffusion, anti-Fi^{fg} serum formed a precipitin line with Fi^{mt} as well as with Fi^{fg} (Fig. 7A), and *vice versa* (Fig. 7B). The two precipitin lines fused with each other, although a precipitin line formed between some anti-Fi^{fg} serum and Fi^{fg} fused with a minute spur with the line formed between Fi^{mt} and the serum. These results demonstrate the presence of distinct determinants common to Fi^{fg} and Fi^{mt}. In quantitative precipitin reactions of the two antigens about 80 percent of the maximum amount of antibodies precipitated by homologous antigen was precipitated by the heterologous antigen, but even so almost all the immobilizing activity remained in the supernatant on reaction with the heterologous antigen, whereas a significant amount was lost on reaction with the homologous antigen. These results provide additional evidence that the major antibodies precipitated with heterologous antigen, did not participate in bacterial immobilization. In addition, similar maximal amounts of precipitate were formed with the two antigens in quantitative precipitin reactions with absorbed antiserum to Fi^{fg}, which had lost the antibodies to the serotypes by absorption with killed bacteria ($Bact^{fg}$). Thus the major determinants of the flagellin other than serotype determinants were identical with the distinct determinants common to Fi^{fg} and Fi^{mt}. When the absorption procedures were repeated 8 times, the maximum amount of antibodies precipitated by Fi^{fg} was reduced to 20 percent of that of the original serum. This 80 percent loss of precipitable antibody is very large, and must be due to repeating the absorption procedures 8 times. It is possible that some fragments of the flagella may have been liberated from killed bacteria during the absorption procedure and that the terminal ends of these fragments may have absorbed the antibodies specifically.

To show that immobilizing antibodies are involved in the precipitin reaction, the relationship between the precipitin line and the inhibition zone of bacterial swarming by anti-Fi^{fg} serum in semi-solid medium was investigated (Fig. 6A, B). The boundary of the swarming zone coincided with the precipitin line. This shows that all the immobilizing

antibodies in the anti-Fi^{fg} serum were involved in the precipitin reaction, and that Fi^{fg} possessed serotype determinants.

The difference (ca. 0.2 mg/ml) between the maximum amounts of antibodies precipitated by the two antigens (Fig. 8) was big enough to cause spur formation on immunodiffusion. The flagellar serotype *fg* of *S. derby* has been subdivided into g_3g_4f (Yamaguchi and Iino, 1969). Fi^{fg} also bears these three antigenic determinants at least, judging from the results of immobilization inhibition tests, and anti-Fi^{fg} sera was shown to contain anti-g_3, anti-g_4 and anti-*f* antibodies by immobilization of various strains (unpublished results). However, the precipitin lines formed by Fi^{fg} and Fi^{mt} fused without formation of a spur. These results suggested that these serotype determinants g_3g_4f were situated too close together on the flagellin to form precipitates with the corresponding antibodies. Therefore, the flagellin possesses not only the same serotype determinants as those of the flagella, but also distinct determinants, which are common to the flagellins of *g*-complex serotypes, but not to those of serotypes *a*, *i* or *enx*.

We examined the possibility that these distinct common antigenic determinants of the flagellin in the flagellum might be covered up in the intact flagellum where the flagellin subunits are in contact with each other. If this were so, at least some of these distinct antigenic determinants might be exposed at the end of the flagellum. Experiments on the binding of labeled anti-Fi^{mt} IgG to Fa^{fg} showed that some antibodies to the common determinants distinct from the serotype determinants specifically bound to the flagella, and that the amount of antibodies bound to Fa^{fg} was equivalent to two thirds of that precipitated by Fi^{fg} (Fig. 11A, B). We studied the sites where the antibodies to the distinct determinants bound to the flagella using the immuno-ferritin technique. Results showed that the antibodies to the distinct common determinants only bound to one terminal of the flagellum. Asakura et al. (1964, 1968) reported that during *in vitro* reconstitution of flagella from flagellin flagellar filaments grew at only one end in a uni-directional manner, and that growth of flagellar filaments occurred regardless of the serotypes of the flagellin and flagella. The distinct antigenic determinants at the one terminal are common to *g*-complex serotype flagella, but not to Fa^{enx}. However, the relationship between this terminal antigen and the growing terminal is still unknown.

Several studies have demonstrated differences between the configurations of subunit flagellin in the flagella and in solution. Electron microscopical observations (Kerridge et al., 1962; Lowy and Hanson, 1965) and X-ray diffraction studies (Champness, 1971) showed that the subunits of the flagellum had a globular configuration. On the other hand, measurements of the diffusion constant and the sedimentation velocity of flagellin demonstrated that in solution *Proteus* flagellin had an elongated form with an axial ratio of about 1: 15 (Weibull, 1948). Circular dichroism studies showed that the secondary structure of salmonella flagellin changed during the process of polymerization (Uratani et al., 1972). Therefore, it seems possihle that new antigenic determiants might appear on disintegration of flagella into their subunits. Whether this is so or not, some antibodies to the distinct common determinants bound to one terminal of the flagellum, and the presence of at least some of the distinct common determinants at only one terminal suggests that in the intact flagellum these antigens are covered by due to surface to surface contact of adjacent flagellins.

Our conclusions are different from those of Ada et al. (1964) and Koffler (1957), that flagella and flagellin are antigenically homologous. Assuming that there are antigenic determinants at the terminals of the flagella and that the flagellar preparations may be slightly contaminated with flagellin, our data are consistent with theirs. From studies on immobilization inhibition and DEAE-cellulose binding assay, Ichiki et al. (1969) concluded that flagella and periodate-treated flagellin of

Bacillus were antigenically heterologous. Their data are partially in agreement with ours, but their interpretation of the data is essentially different from ours. Although their DEAE-cellulose binding assay of the absorbed anti-flagellin IgG by the flagella suggested the presence of specific antibodies to the flagellin, their results on " flagellin-specific antibodies " seem to correspond with our results (Fig. 11B) on anti-Fi^{mt} IgG which reacted with Fi^{fg}, but not with Fa^{fg}, that is, the one third of the antibodies which reacted with heterologous flagellin. However, they did not detect the fraction of the antibodies which did not bind to the surface of the flagellum, but to the terminal portion of flagellar fragments.

Serological differences between orderly aggregated proteins and their homogeneous subunits have also been observed in other systems. The antigenic properties of the protein shell (TMVP) of tobacco mosaic virus (TMV) have been studies extensively. Anderer (1963) concluded from precipitation inhibition of anti-TMV sera that four peptides of TMVP (Positions 18–23, 62–68, 123–134 and 142–158) corresponded to the antigenic determinants of TMV. In contrast, Benjamini et al. (1964) from measurement of complement fixation inhibition of anti-TMVP sera concluded that a tryptic peptide (Position 93–112) of TMVP had the antigenic properties. These differences between the antigenesities of TMV and TMVP, like those seen in our experimental system, maybe because (1) new antigenic determinants may be formed on alteration of the configuration of the subunits, or (2) antigenic determinants located on covered surfaces may be exposed on disintegration into subunits.

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