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# Genetics and Chemistry of Bacterial Flagella[1]

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## INTRODUCTION

Bacterial flagella are attracting the attention of investigators in various fields of biology. Physiologists who are concerned with cellular motility are interested in them as the simplest locomotive organelle in living organisms. To biochemists, bacterial flagella provide a rich source of an isoprotein reflecting the evolution of a biologically active macromolecule. The formation of bacterial flagella is becoming a unique object for studies of both cellular regulatory mechanisms and the molecular basis of morphogenesis. Immunologists have been using bacterial flagella as highly specific and effective protein antigens, for chemical characterization of antigen determinants, and for the studies of the specificity of antigen-antibody reactions. Because of the wide interest in bacterial flagella, several review articles on them have already been published. This review, therefore, does not intend to cover past studies on bacterial flagella comprehensively, but rather to focus on their chemistry and genetics, especially in relation to their biosynthesis and morphogenesis. For more comprehensive references to earlier work, readers may consult the articles by Stocker (182), Weibull (198), and Joys (96); in connection with motility, the articles by Jahn and Bovee (94) and Doetch and Hageage (36); and for the experimental techniques, Iino and Enomoto (81) and Koffler (114).

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## STRUCTURE OF BACTERIAL FLAGELLA

### Flagellin as the Chemical Component of Bacterial Flagella

The fundamental procedure employed for the isolation of bacterial flagella is the mechanical breaking of flagella from cell bodies followed by their separation by centrifugation. Either a shaking machine or a blendor has been usually used for the breaking (49, 103, 113, 194). The conditions for breaking off flagella from bacterial cells with minimal destruction and without a decrease in the viability of the cells were investigated by Stocker and Campbell (183, 185). The purification of the isolated flagella is accomplished by repeated differential centrifugations (57, 113, 194). Further purification is attained by ammonium sulfate precipitation (113, 115, 195), by low-temperature ethyl alcohol precipitation (103), or, more efficiently, by ion-exchange chromatography on diethylaminoethyl cellulose (144).

The protein nature of highly purified flagella has been demonstrated in various bacterial strains (113, 196), and it is now believed that in most bacteria flagellar fibers are composed of pure protein. The possibility has not been definitely excluded, however, that minor nonprotein entities which are loosely bound to the native structure are eliminated during the course of purification. An exceptionally tight binding of nonprotein substance to the flagellar protein was reported on the flagella of *Bacillus stearothermophilus* (2)

and *Spirillum serpens* (145). In these bacteria, carbohydrates are bound to flagellar fibers and they are disaggregated only after acid dissociation of the flagella. Poglazov (164) reported the release of lipid from purified flagella of unspecified source upon ether extraction. Almost nothing is known about the chemical composition of the sheaths of sheathed flagella. In *Vibrio metchnikovii*, Follett and Gordon (53) reported that the flagellar sheath was easily autolyzed by 6 M urea or 0.01 N HCl, whereas the core filament was resistant to these reagents. On the basis of these findings, they suggested that the sheath is an extension of the cell wall. The sheath of *Spirochaeta* seems to be clearly distinct from that of *Vibrio*. It appears as a component layer of macromolecules on the surface of the flagellar filaments (65).

Flagella can be dissociated by a variety of agents, including acid (193, 194), alkali (162), detergents, phenol, sonic oscillation (117), heat (117, 150), and compounds capable of breaking hydrogen bonds, such as urea (117). Acid dissociation of flagella of a bacterial strain gives rise to a homogeneous solution of the flagellar protein *flagellin*, a term originally used by Astbury and his associates (21).

In *Proteus vulgaris*, the dissociated flagellin exists as a monomer below *p*H 3.8 in the absence of salt and as a dimer above *p*H 4.5 (52). The change in optical rotation of a flagellin solution brought about by lowering its *p*H below 3.8 was interpreted as an unfolding of the monomer accompanied by considerable loss of secondary and tertiary structure (52). Such a monomer-dimer transition has not been observed for other flagella. In acidophilic bacteria such as *Thiobacillus thiooxidans*, flagella as well as other cellular entities are resistant to acid, and lowering the *p*H to 0.6 does not destroy the structural integrity and function of the flagella (35). The homogeneity of flagellin molecules obtained by acid dissociation of a single kind of flagella from several different bacterial strains has been demonstrated by both physical and chemical analyses, i.e., sedimentation (6, 7, 52, 111, 147, 194), ammonium precipitation (113), chromatography (49, 51), and electrophoresis (7, 147). A highly homogeneous suspension of flagellin monomers was obtained by thermal dissociation of *Salmonella* flagella (18). Kinetic studies of the thermal transition of flagella to flagellin in *S. serpens* was studied by Martinez and Rosenberg (150). They found that the thermal transition exhibited by flagella closely follows the process of transition reported for many soluble proteins and is similarly affected by low ionic strength, *p*H, and urea. The effective temperature for the dissociation of flagella differs in different bacteria. Generally, flagella of thermophilic bacteria are more resistant to high temperature than those of mesophilic bacteria, the difference being attributed to molecular differences in the flagellin of mesophiles and thermophiles (10, 117, 143).

The molecular weight of flagellin was first estimated to be about 40,000. This figure was suggested by Weibull (194, 196) for flagellin in *P. vulgaris*, *Bacillus subtilis*, and *Salmonella paratyphi* B obtained by employing Archibald's method with acid-dissociated preparations. Later studies by Koffler and his co-workers (52, 113) on acid-dissociated preparations of flagellins of *P. vulgaris*, *Serratia marcescens*, *B. subtilis*, and three additional *Bacillus* species gave molecular weights of 14,000 to 20,000 among the different species. These values were obtained by use of ultracentrifugation and N-terminal amino acid measurement. The authors ascribed the higher value for the same species obtained in earlier investigations to the dimer formation of flagellin molecules under the experimental conditions employed in the earlier studies. A molecular weight of about 25,000 for *B. subtilis* flagellin reported more recently was obtained when the sedimentation constant measured at *p*H 2 or 3 was used for the calculation of molecular weight; a value of 40,000 was obtained when an alkali-dissociated preparation was dialyzed against triethylammonium-NaCl and the molecular weight was calculated from sedimentation equilibrium data (147). The validity of the higher value was supported by amino acid analysis and fingerprinting analysis of the flagellin as described below. The lower value was attributed to the low sedimentation constant of flagellin denatured under the experimental conditions employed for the centrifugation (149). In *Salmonella typhimurium*, molecular weight determinations calculated from ultracentrifugation data on flagellin preparations obtained by various dissociation procedures gave a consistent value of about 40,000 (18, 111, 141). The amino acid composition of flagellin was analyzed in *P. vulgaris* (113), several different serotypes of *Salmonella* (12, 141), *B. subtilis*, and *S. serpens* (147). Although the composition of flagellins from different organisms differs, some common features can be recognized by a comparison of these reports. The most striking feature is that cysteine is absent in all flagellin preparations examined. This is quite a contrast to the contractile proteins of muscles responsible for the movement of animals. The presence of cysteine in the flagellin of *P. vulgaris* and *B. subtilis* reported earlier (113, 116, 194) was later disclaimed by the same investigators (cited in 141). Tryptophan is also absent, whereas aspartic acid, alanine, glutamic acid, threonine,

glycine, leucine, valine, lysine, serine, isoleucine, arginine, tyrosine, phenylalanine, and methionine are present in all flagellins analyzed. The relative amount of each amino acid decreases approximately in the order indicated above; the first four are remarkably abundant and their sum exceeds half of the total amino acid residues of the molecule. Proline and histidine are present in small amounts or are absent.

An unusual amino acid, $\epsilon$-*N*-methyllysine, is present in *Salmonella* flagellins of some but not all serotypes (12). This amino acid has hitherto been found only in *Salmonella* flagella and nuclear histone (11, 156).

The N-terminal amino acid of flagellin polypeptides is alanine in *P. vulgaris* (113, 116, 197), *B. subtilis* and three other species of *Bacillus*, *S. marcescens* (113), and two species of *Salmonella* (Yamaguchi, *personal communication*). The total number of amino acid residues per flagellin molecule was calculated to be 156 or 208 for *P. vulgaris* (113), about 380 for several serotypes of *Salmonella* (141), 364 for *B. subtilis*, and 372 for *S. serpens* (147). By fingerprinting analyses of tryptic digests, 30 to 35 peptides were distinguished in several different antigenic types of *Salmonella* flagellin (51, 140, 141, 204), and in *B. subtilis* flagellin (147); 36 to 37 peptides were distinguished in *S. serpens* flagellin (147). These numbers are consistent with the number of lysine and arginine residues per flagellin molecule estimated by taking the molecular weight of these flagellins to be about 40,000.

### Flagellin as the Unit of Flagellar Antigen

The presence of flagella-specific antigens was first recognized in *Salmonella choleraesuis* by Smith and Reagh (177). Weil and Felix (199) gave the symbols O and H to nonflagellated and flagellated types of bacteria, respectively, based on the form of their colonies, namely, spreading (Hauch) and nonspreading (Ohne Hauch). The use of O and H has been extended to indicate the somatic and flagellar antigens. The specificity of flagellar antigen type has been extensively surveyed in *Salmonella* on a worldwide scale, and an abundance of well-determined antigen types have been synthesized in the Kauffman-White scheme (101, 104), which has provided the standard for the identification of *Salmonella* serotypes. Antigenic specificity has also been observed in flagella of different species of *Spirillum* and of different strains of *B. subtilis* (147).

The antigenic type of a particular kind of flagellin is a complex of a number of subunits (antigen type determinants), although for descriptive convenience they are often indicated by a simplified symbol (101). This determinant complex is carried as a unit not only by a single flagellum but also by a single molecule of flagellin. The former was first shown by Nakaya, Uchida, and Fukumi (157), who demonstrated that the antiserum for antigen subunit *g* or *m* of *Salmonella enteritidis* agglutinated all isolated flagella of this bacterium. Ada and his co-workers (6) obtained rabbit antisera for flagellin, flagellar fibers, and flagella-like filaments produced by reaggregation of the flagellin of *Salmonella adelaide*. These antisera had a complex flagellar antigen *f*, *g*. Comparison of their serological properties showed that their immunogenicities were roughly equal; each antigen was effective in neutralizing antibody prepared against any one of the antigens, and gel diffusion and immunoelectrophoresis showed one main common antigen with sometimes a second minor antigen. The significance of the second minor antigen in their experiment was not clarified. In *B. subtilis* and *S. serpens*, only one precipitin band has ever been observed when homologous flagellins are used as antigens in gel-duffusion tests (147). Thus, the flagellin molecule appears to constitute a unit of antigen carrying the common specificity of the flagella.

Comparative amino acid analysis of flagellins of antigenically distinct *Salmonella* flagella showed that the antigenic difference is associated with the difference in amino acid composition between flagellin molecules (141, 204). This finding led to the conclusion that the specificity of a flagellar antigen is a reflection of the surface conformation of a flagellin molecule, which is primarily characterized by specific amino acid sequence. Precipitation-inhibition tests with the polypeptides obtained by breaking flagellin with cyanogen bromide further indicated that the polypeptide region responsible for antigenic specificity is localized on a flagellin molecule (8). Although the specificity of flagellar antigen is attributed to the specific conformation of the flagellin molecule, some evidence has been presented that the organization of flagellins in a flagellum causes a certain degree of quantitative difference in antigenicity between a flagellum and its component flagellins (6, 111).

The binding of the homologous antibody molecules on the surface of flagella was observed under an electron microscope with negatively stained preparations (19, 43, 59). The antibody molecules seemed to carry the specific reaction sites at the ends of the long axis and to become attached radially to the surface of the flagella, resembling the bristles of a bottle brush.

### Arrangement of Flagellin Molecules in Flagella

Electron micrographs of shadowed flagellar specimens sometimes show helical fine structure (118, 119, 151, 169, 179), although in most cases a flagellum is seen as a smooth thread. The molecular architecture of a flagellum was revealed by the application of negative staining techniques in electron microscopy. Kerridge and his coworkers (111) observed spherical subunits with an estimated diameter of 4.5 nm, arranged in accordance with hexagonal packing, in negatively stained preparations of *Salmonella* flagella partially degraded by ultrasonic vibration, by sodium dodecyl sulfate, or by heat treatment. In thin-sectioned preparations, they also observed a hollow in the cross section of a flagellum posessing fivefold symmetry. A spherical unit with a diameter of 4.5 nm was estimated to correspond to a protein molecule having a molecular weight of 37,000 to 40,000. The value was in good agreement with the molecular weight of *Salmonella* flagellin determined by ultracentrifugation. Hence, molecular models of a flagellar fiber were proposed in which one, three, or five chains of flagellin molecules are helically wound and hexagonally packed. From geometric considerations of the electron microscopic figures of negatively stained flagella, Lowy and Hanson (135) proposed a modified model for *Salmonella* flagella in which eight longitudinal rows of helically connected flagellin molecules align in such a way that the number per turn of each helix equals the number of longitudinal rows and the molecules form a cylinder. The model agrees better with the data of X-ray diffraction analyses (21, 22) than the former model. However, the flagellar fiber constructed from any of the above models is not helical, as seen in the native flagellum, but straight. To make the filament helical, Asakura and his collaborators (19) proposed that flagellin molecules constructing a flagellum are differentiated into two different *meta*-stable states without a change in primary structure.

In flagella of *Pseudomonas fluorescens*, *P. rhodos*, and *P. vulgaris*, Lowy and Hanson (134, 135) observed two types of flagellar fine structure. One, called type A, is similar to *Salmonella* flagella and consists of helically connected globules aligned in logitudinal rows. The number of the rows was estimated to be 8 in the latter two species and 10 in the former one. The other type of structure, called type B, shows neither globules nor helices, but has thick longitudinal lines. In *P. rhodos*, sometimes two types of structure were seen along a single flagellum. In *B. subtilis*, only the type B structure was found. Whether or not the two types of structure have anything to do with flagellar movement is unknown.

As for the hollow in a flagellum, observed by Kerridge and his collaborators (111), succeeding investigations showed that it is demonstrable only after disruption of flagella by ultrasonic treatment or by some other means (28, 60, 170). Thus, in native flagella, hollows may be filled with a substance or substances easily removed upon disruption.

Surrounding the main part of the *P. rhodos* flagellum, a single thin line with periodic undulations with a spacing of about 10 nm was observed by Lowy and Hanson (135). They called it a sheath and presumed that the hilical structure observed in shadowed preparations of the same strain by earlier investigators (e.g., 151) corresponds to the sheath.

### Reaggregation of Flagellin into Flagella

When flagellin is exposed to a certain concentration of salt, it precipitates and forms a stable aggregate polymer (52). In *P. vulgaris*, flagellin aggregation was observed to occur slowly at 4 C in the presence of low concentrations of salt, indicating that this polymer is a more stable form than the flagellin monomer (52).

Electon microscopy of flagellin aggregates from *Bacillus pumilus* revealed three forms (2). At high temperature (namely, at 38 C) and below *p*H 5.4, amorphous material is formed, which was presumed to be a precipitate of denatured protein. At temperatures below 26 C, straight reaggregates are formed within a *p*H range of about 4.0 to 4.9, and flagellum-like filaments are formed between *p*H 5.3 and 6.2. The latter are morphologically indistinguishable from native flagella. Straight filaments can be transformed to flagellum-like filaments if they are resuspended at an appropriate *p*H; this transformation is irreversible. Because the reaggregation occurs at low temperatures, such as 2 C, it was thought that the process is nonenzymatic. Thus, it was inferred that the ability to aggregate and form a specific filamentous polymer is a property of flagellin monomers, which are capable of self-assembly; the structure of the native flagellum may constitute the most stable organized state in which flagellin can aggregate. The spontaneous reaggregation of flagellin to flagellum-like filaments was also observed on *Bacillus stearothermophilus* (2) and on *B. subtilis* (149).

In contrast to *Bacillus* flagellins, monomers of *Salmonella* flagellin can reaggregate and form flagellum-like filaments at high concentrations of an appropriate salt, e.g., $(NH_4)_2SO_4$, at neutral *p*H (136; Asakura, *personal communication*). At

the salt concentration employed with *B. pumilus*, e.g., 0.05 M phosphate buffer, the monomer solution remains in a state of super saturation (18). To polymerize the monomers in such solutions, it was found necessary to add fragmented flagella. Then the ends of added fragments act as nuclei, resulting in the rapid formation of long flagellar filaments; the process is characteristic of crystallization (18). The reconstituted flagellar fibers are indistinguishable from native flagella not only in their gross structure but also in electron microscopic fine structure (136). Serologically, native flagella and reconstituted flagella react identically with the specific antisera, but the former are more immunogenic than the latter (6).

The parallel measurement of the rate of polymerization and the volume change of the solution associated with the polymerization of flagellin to flagella indicated that in *Salmonella* flagellin the protein is reversibly transformed to a state which lacks the capacity to reconstitute flagella with the increase of temperature (58); above 28 C, this transformation becomes rate-limiting for polymerization. The overall rate of polymerization is largely dependent on the nature of monomer, and polymerization of flagellin takes place at a rate less than first order with respect to the concentration of monomer (19). Copolymerization of two different type of monomers, namely, normal and curly, proceeds at a rate markedly less than the sum of the polymerization rates of the constituent monomers (19).

By using flagellin monomers and seed segments antigenically distinct from each other, and staining the reconstituted filaments with antiserum specific for monomer or fragment, it was demonstrated that the reconsitution proceeds from one end of the fragmented flagella (20). The detailed observation of flagellar fibers by high-magnification electron microscopy showed that each filament has polarity, and only its distal end appeared to be frayed (4). This distal end was found to correspond to the end where in vitro flagellar growth takes place (20). The polarity of the in vitro reconstitution, together with the information obtained from kinetic studies mentioned above, was explained by the transformation of flagellin upon polymerization (19, 20). This explanation received support from the observation by K. Imabori (*personal communication*) that flagellin monomers and flagella differ markedly in their optical dichromism. Asakura (17) assumed that polymerization consists of two steps: the first is the reversible binding of a monomer to an end of an existing filament, and the second is the incorporation of the bound monomer into the filament. Only after incorporation can the bound monomer act as a new end for further polymerization. Asakura (17) calculated the speed of incorporation of a monomer to be 0.34 sec.

## GENETICS OF FLAGELLAR FORMATION

### Genetic Mechanism of Flagellar Phase Variation

Genetic studies on bacterial flagella were primarily motivated by the interests in the remarkable variation of their antigenicity in *Salmonella*. Not only the extensive polymorphism present among the different serotypes in the genus, but also a characteristic intraclonal variation, called *phase variation*, has attracted the attention of both microbiologists and geneticists. Phase variation in flagellar antigen is characterized by the appearance of two alternative types of flagellar antigen in a bacterial clone. The one is called the "specific phase" or "phase 1" and the other is the "nonspecific phase" or "phase 2." When a mass culture of such a strain is plated, it dissociates into colonies of phase 1 and phase 2. During successive cultures of the two types, the population of each type gives rise to cells in the alternative phase. A bacterial strain expressing two alternative antigenic phases is called a diphasic strain; a strain which expresses only one is called a monophasic strain. The phenomenon of phase variation was discovered by Andrews (13) in *S. typhimurium*. Later, the presence of phase variation in many *Salmonella* strains was recongnized by various investigators, as reviewed by Kauffmann (102). In *Salmonella*, each serotype has its own specific type of phase 1 and phase 2 antigens. Besides *Salmonella*, phase variation of flagellar antigens was detected in some strains of *Arizona* (40), which is most closely related to *Salmonella* among coliform bacteria. Bernstein and Lederberg (24) observed that the cells of smooth-type motile *Salmonella* are agglutinable by acridine dyes, phase 2 cells reacting at distinctly lower dye concentration than phase 1 cells.

The population dynamics of phase variation were studied by Stocker (180) in *S. typhimurium*. He noted that the interchange between two phases occurs with a small probability of $10^{-2}$ to $10^{-3}$ per bacterial division. The value differs in different strains and also between opposite directions in a given strain. The phenomenon resembles the occurrence of forward and back mutation, but is distinguished from it by its extraordinarily high frequency, and also by its oscillation between two fixed alternatives which are characteristic of the serotype.

A genetic approach to the mechanism of phase variation was initiated soon after the discovery of phage-mediated transduction in *Salmonella*.

Transductions between different diphasic serotypes disclosed two series of multiple alleles at loci designated *H1* and *H2* (39, 122). *H1* and *H2* determine the antigenic specificities of phase 1 and phase 2 (39, 122, 171). As discussed above, the specificity of a flagellar antigen is a reflection of the surface conformation of a flagellin molecule, and is primarily determined by the amino acid composition of the flagellin. It is therefore now believed that *H1* and *H2* are the structural genes for the flagellin of phase 1 and phase 2, respectively.

In *Salmonella*, *H1* and *H2* are located far enough apart on the chromosome so as not to be transduced simultaneously by a phage particle. Their chromosomal locations were mapped by Col-mediated transfer (176) and by F-mediated transfer (47, 142). Recombination, carried out by conjugation between *S. abony* Hfr and *E. coli* $F^-$, showed that *Escherichia* has only one *H*-gene, which is allelic to *H1* of *Salmonella*, and lacks the counterpart of *H2*; if *H2* is introduced into *E. coli*, diphasic strains are produced which undergo phase variation at the same rates as those in the *Salmonella* parent (142).

The knowledge that the antigenic specificities of the two phases are determined by separate loci led to the conclusion that phase variation is the alternative manifestation of each of the two separate loci, namely, *H1* and *H2*. Transduction experiments between single-phase cultures of diphasic strains showed that *H1* can be expressed only when transduced into phase 1 cells, regardless of the phase of the donor, whereas *H2* can be expressed in any phase of the recipient, but only when the donor is in phase 2 (123). These results, together with other supporting data (123), indicate that the *H2* gene plays a decisive role in the expression of the phases. The process of phase variation is thus explained as follows: *H2* can exist in two different states, active and inactive; when *H2* is in the active state, the production of the phase 1 antigen by *H1* is repressed, while *H2* carries out the production of phase 2 antigen. When *H2* changes to the inactive state, corresponding to the change from phase 2 to phase 1, the production of phase 1 antigen, specified by *H1*, proceeds. The apparent process of phase variation raises two questions, both associated with fundamental problems of genetics. The first is by what mechanism *H2* changes its oscillatory state, and the second is how the production of phase 1 antigen is repressed in the *H2*-active state.

Information useful in understanding of the state change of *H2* has been obtained. First came the discovery of an abnormal flagellar antigen type recombinant which alternatively expresses phase 1 antigens of both donor and recipient in transductions between *S. typhimurium* and *S. abony* (73). The recombinant was presumed to be originated by an unequal recombination: namely, the *H2* locus of the recipient chromosome was replaced by *H1* of the donor in transduction. The *H1* gene thus translocated to the position of the *H2* locus became unstable and changed its activity at the frequency typical of the *H2* locus of the parent strain. This means that the instability of the *H2* state is not intrinsic to the antigen type determinant of *H2* but is regulated by a factor closely linked to it.

This concept has been further extended by the studies of *Salmonella* mutants which produce nonflagellate cells as an expression in phase 1 and flagellate cells in phase 2 (71). Phase variation, equivalent to O-H variation, occurs in these mutants as frequently as in the diphasic parent strains (71). The inactivation of their *H1* is indifferent to the activity of *H2*. The phenomenon also shows that the change in the *H2* state is not influenced by the state of *H1*. Transduction analysis of these mutants demonstrated that a region called *ah1* (*H1*-activity controller) adjoins *H1*, and switches on or off the genic activity of the latter (71). When *ah1* is in a state designated as $ah1^+$, the adjoining *H1* is active; when *ah1* changes to the $ah1^-$ state, *H1* is inactivated, and the cells become nonflagellate in phase 1. When the $ah1^-$-*H1* fragment is introduced from the mutant of *S. typhimurium* to diphasic *S. abony* by transduction, the resulting abortive transductants, which are partially diploid in the *ah1-H1* region, produce flagella of the recipient antigen type (76). Thus, *ah1* and *H1* behave as two component parts of a genetic transcription unit analogous to the operator-structural gene system. The $ah1^-$ allele may correspond to an operator-negative state or to nonsense chain-terminating mutations in *H1* (83, 160).

Transductional analysis of phase 1 monophasic mutants of *S. typhimurium* and *S. paratyphi* B disclosed an activity controller, *ah2*, which is adjacent to *H2* (75). Although the investigations on *ah2* have not proceeded as intensively as those on *ah1*, the relationship of *ah2* to *H2* was found to parallel that of *ah1* to *H1*. Diphasic strains carry an allele, $ah2^+$, which switches on the production of phase 2 antigen. A mutant allele, $ah2^-$, switches off the production. Consequently, the mutant carrying $ah2^-$ remains stable in phase 1. The controller of the *H2* state in a diphasic strain is functionally indistinguishable from *ah2* except for the high frequency of state change in the former. Thus, as a simplified hypothesis, it was proposed that *ah2* and the phase determinant are identical (83).

Investigations of another type of monophasic

*Salmonella* strains, represented by *S. abortusequi*, indicated further that the instability of the phase determinant is not intrinsic but is under the control of a chromosomal factor termed *vh2* (72). The *vh2* factor is closely linked to *H2* but separable from it. Replacement of $vh2^+$ in a diphasic clone by $vh2^-$ of a monophasic clone causes the stabilization of the state below $10^{-7}$ per bacterial division in its existing state. A type of monophasic behavior similar to that of *S. abortusequi* has been reported in *S. paratyphi* A (26, 37). Whether the genetic factor which stabilizes the antigenic phase in *S. paratyphi* A is identical to that of *S. abortusequi* is not known.

The working hypotheses which have been proposed on the mechanism of regulation of the *H2* state by the *vh2-ah2* system are classified into the following three categories.

(*1*) *The existence of a regulator-operator system.* This hypothesis identifies $ah2^+$ as a repressible operator. Then, in phase 1 *ah2* is repressed and *H1* is active, whereas in phase 2 *ah2* is unrepressed and *H2* is active (83). Transduction experiments (123) suggest that the regulator gene which produces the hypothetical repressor of *ah2* must be closely linked to *H2*. Essentially the same model was proposed by Klein (112). Formally, it is possible to identify *vh2* as the regulator gene: in the $vh2^+$ cell, repressor may be produced in such an amount that in a certain fraction of the cells $ah2^+$ is repressed, and in the $ah2^+$-$vh2^-$ cell repressor may not be produced and must be stable in phase 2 as observed in *S. abortusequi* (77). A low frequency of phase variation in such a strain may occur by mutational change of $ah2^+$ to $ah2^-$ and vice versa.

A difficulty of the repressor-operator hypothesis for the explanation of the *H2* state change is evident from the studies of Pearce and Stocker (160), who demonstrated that, when an active *H2* allele is introduced into a nonflagellate phase 1 cell of the $ah1^-$ type, the partially diploid cell expresses the donor *H2* allele but the recipient *H2* allele in the chromosome remains unexpressed. This result showed that the expression of an *H2* gene depends on the state of a phase determinant on the same chromosome and is unaffected by the state of another *H2* region on a different genetic element in the same cell. Thus, a phase-control mechanism by means of a cytoplasmic repressor seems experimentally unsupported.

(*2*) *The existence of a structural anomaly in the chromosome.* This hypothesis assumes that the *vh2* region in diphasic strains resulted from a structural anomaly in the chromosome (77, 83). This hypothesis has an advantage in explaining the position effect of phase determinants mentioned above. In *Salmonella*, the following data have been presented to support the hypothesis that phylogenetically the *H2* gene originated by the duplication of *H1* and its translocation followed by structural differentiation. (i) Among the naturally occurring monophasic serotypes, many of them having *g*-complex antigens are deficient in *H2* (102, 122, 123), whereas phase 2 monophasic strains deficient in *H1* have not been detected. (ii) *Escherichia*, the genus most closely related to *Salmonella*, possesses a single *H* locus allelic to *H1* of *Salmonella* but does not carry an allele of *H2* (55, 142, 158). (iii) The rare replacement of an *H2* gene by *H1* as a result of unequal recombination has been observed, and partial structural homology between *H1* and *H2* has been suggested (73, 76). (iv) Occasionally, *Salmonella* strains having three or four phases are isolated from nature (41), and in one such triphasic strain duplication of the *H1* locus has been demonstrated (121). (v) Persistent partial heterozygotes involving both *H1* and *H2* have been obtained by transduction (178). (vi) Finally, antigen types appearing in phase 1 are more numerous than those in phase 2, and several antigen types appear commonly in both phase 1 and phase 2 (102). If the *H2* locus had been originated by translocation of the *H1* gene, it may well have resulted in a certain structural anomaly at the junction of the chromosome and the translocated segment to the *H2* region. This phenomenon would be analogous to the variegated-type position effect described in higher organisms (132). The *vh2* region would then represent a structural anomaly that causes the instability of the adjacent region. Consequently, *ah2* may frequently change its activity independently of any repressor substance. Both the constancy of the frequency and the randomness of phase variation at different stages in the development of a bacterial clone under various cultural conditions (142, 180) also favor this hypothesis rather than that of the formation and dissociation of an *ah2*-repressor complex.

(*3*) *The presence of a superimposing element.* This hypothesis assumes the presence of either an episome (93) or a controlling element (77, 83) in a diphasic cell. For example, $vh2^-$ cells are presumed to have no phase-controlling episome; in $vh2^+$ cells, the episome switches on and off the activity of *ah2* depending on its intracellular state. The presence of a controlling factor which causes the unstabilization of certain specific genes or gene activities has been demonstrated both in plants (138, 139) and in bacteria (32). However so far we have no direct experimental evidence to support this hypothesis in preference to the former ones.

Consider now the repression of *H1* when *H2* is

active. It was shown that the active *H2* in a transduced chromosomal fragment can repress the recipient *H1* without being integrated into the recipient chromosome (160). This means that the repression is effective through the cytoplasm. The simplified hypothesis to explain this is that a product of *H2* acts as the repressor of the *ah1-H1* operon (83). A difficulty of this hypothesis was discussed by Pearce and Stocker (160). A strain of *S. paratyphi B* of exceptional antigenic type which has an *H1* allele determining flagellar antigen *1.2* was detected by Lederberg and Edwards (122). Antigen *1.2* in other strains of *S. paratyphi* B and many other *Salmonella* serotypes is a phase 2 antigen, determined by the *H2* locus. The *1.2* flagellin determined by the exceptional *H1* allele cannot be distinguished from the *1.2* flagellin of phase 2 cells, either serologically (122) or in amino acid composition (141). Nevertheless, the *H1-1.2* locus when present on a transduced fragment does not repress the expression of an *H1-b* allele in the chromosome, although *H2-1.2* can repress, when similarly introduced by abortive transduction (160). Therefore, it was thought unlikely that, in the ordinary diphasic strain, phase 2 flagellin itself is the repressor substance for *H1*; a modified hypothesis was presented which proposes that there is a special repressor substance coded for at an *H1* repressor locus closely linked to *H2* and forming a part of the same operon as *H2* (160). Evolutionarily, such a regulator gene might have been originally adjacent to *H1* and then involved in the duplication and translocation which produced *H2*. Later, it might have changed in such a way that its product continued to repress *H1* but no longer to repress *H2* (160). The presence of the *H1* regulator gene in the *H2* operon was also suggested by Klein (112) without any critical discussion of its possible identity with *H2*. However, the experimental evidence which has now been obtained for the identity of *1.2* flagellin determined by *H1* with that determined by *H2* does not exclude the possibility of a minor difference in the amino acid sequence which might be responsible for the repressor activity. The intensive studies of the amino acid sequence of phase 1 and phase 2 flagellin with identical antigenicity, and a search for repressor-negative mutants or repressor-positive phase 2 nonflagellate mutants will lead to further clarification of this problem. It may be also worth noting that we have no experimental evidence for presuming that the reaction site of the repressor is *ah1* deoxyribonucleic acid (DNA): the possibility of interaction of the repressor with the expression of phase 1 flagella at the level of translation in flagellin synthesis or at the step of polymerization of flagellin cannot be a priori excluded from consideration (83).

### Regulator Genes Controlling Flagellar Formation and Motility

Besides the phase-specific regulator genes discussed in the foregoing section, a group of phase-nonspecific regulator genes called *fla* have been found to regulate flagellar formation. These genes were first disclosed by studies of nonmotile mutants of diphasic *Salmonella* strains (187). The characteristic of *fla* is that a mutation of any of the *fla* genes from $fla^+$ to $fla^-$ results in the loss of the ability to produce flagella in both phase 1 and phase 2. The production of a trial, an array of minute colonies extending into semisolid media, upon P22-mediated transduction of $fla^+$ genes into a $fla^-$ recipient was the first example of abortive transduction (120, 181, 187). The production of a trail also indicates that $fla^+$ is dominant over $fla^-$ (187). The appearance of trails in transduction between two different $fla^-$ mutants was taken as the evidence of complementation between $fla^+$ alleles of two $fla^-$ genes, and the grouping of $fla^-$ mutants by complementation test has been carried out. Thirty-seven mutants of *S. typhimurium* were classified into eight complementation groups, namely, the cistrons *flaA, B, C, D, E, F, J,* and *K* (68, 79, 80, 99). Such complementation tests have also been applied to interserotypic combinations, at least between *S. typhimurium*, *S. paratyphi* B, and *S. abortusequi*. A $fla^-$ mutant of *S. paratyphi* B was found to belong to group *A* (80, 99), and, among 11 $fla^-$ mutants of *S. abortusequi*, 10 were assigned as *flaA, C,* or *F* (80). The remaining one was distinct from any cistrons detected in *S. typhimurium*, and was given the symbol *flaG* (80).

A common feature of the complementation between two $fla^-$ mutants is that partial complementation is frequently observed between two mutants in a cistron. The trails produced by partial complementation are not only smaller in number but are also shorter in comparison with those produced by transduction from $fla^+$ to the same recipient $fla^-$. In *flaA*, colinearity was observed between the map position of $fla^-$ mutations estimated from the yield of stable transductional clones and those estimated from the degree of complementation in abortive transduction (80). Depending on the degree of complementation, *flaA* was divided into five subunits (99). The mechanism of partial complementation in the *fla* genes is still unknown. Extensive occurrence of partial complementation in *fla* leaves the possibility that the minor group, for example, *flaJ* and *flaK*, will be placed in another known group when nonflagellate mutants are surveyed more extensively in the future.

Among the nine *fla* cistrons mentioned above, *flaA, B, C, D, E, J,* and *K* were found to be cotrans-

duced with *H1* by P22 phage; that is, they are closely linked to *H1* (48, 80, 99, 187). Those *H1*-linked *fla* cistrons were termed the *flaI* group (79). Recently, an additional cistron, *flaL*, was detected among the *fla*⁻ mutants *S. abortusequi* given *H1-g* from *S. derby* by transduction (203, 204). *FlaL* is most closely linked to *H1* among the *fla* cistrons cotransducible with it. So far as they have been studied, except for one mutant of *flaG*, all *fla*⁻ mutants which are not cotransduced with *H1* have been assigned to *flaF*. The chromosomal location of *flaF* was mapped by Col-mediated conjugation between the *gal* and *trp* operons (176). The *fla*⁻ mutant of *flaG* shows unusual behavior in transduction: transduction from *flaG* to any other *fla*⁻ mutant gives swarms and trails comparable to the transduction from *fla*⁺, but when *flaG*⁻ is used as the recipient no transductants are obtained even when *fla*⁺ is used as a donor (80).

The presence of the *fla* genes was also demonstrated in *E. coli* (152, 158). These *fla* genes are located near *H*, which is homologous to *H1* of *Salmonella*, and also between *trp* and *gal* (16). The former group may correspond to *flaI* group and the latter to *flaF* of *Salmonella*.

With nonflagellate (*fla*⁻) mutants of *B. subtilis*, transformation was carried out from *fla*⁺ donors (184). Unlike transduction of a *fla* gene in *Salmonella*, no pedigrees indicating unilinear transmission of an unincorporated *fla*⁺ gene were observed among the phenotypically transformed bacteria of *B. subtilis*, but in a few clones some partial heterozygotes were present even 10 generations after DNA uptake. By the use of two strains differing in their major flagellar antigen, a gene *H*, which controls the specificity of flagellar antigen, and a gene *fla* were identified (97). They were unlinked in transformation tests. Preliminary transduction experiments were also carried out on the flagellation and motility characters of *Proteus mirabilis* (29).

As regards functional characteristics, the *fla* cistrons were divided into two types. One is represented only by *flaG*, and the other includes all *fla* genes other than *flaG*. Strains carrying *flaG* are characterized by the production of flagellin which is antigenically indistinguishable from normal flagellin (80, 82) and which can be incorporated into flagellar fibers in an in vitro reconstitution process (188). It was thus inferred that the *flaG*⁻ mutant is deficient in the ability to organize its flagellin into flagella rather than being deficient in flagellin synthesis (188). As will be discussed in a later section, the initiation of in vivo flagellar formation requires a starter for the polymerization of flagellin, but once the polymerization starts the growth of flagella can proceed by the self-assembly of flagellin molecules. Therefore, the most plausible function of *flaG* might be the control of the polymerization starter of flagellin. An alternative is that *flaG* is responsible for the formation of a special structure in the bacterial cell wall which permits flagella to penetrate through it.

The second group of *fla*⁻ mutants is characterized by the absence of the substance which cross-reacts with H antiserum. This means that the mutants cannot synthesize detectable amounts of flagellin. It was therefore assumed that the *fla* genes may regulate the amount of flagellin synthesis by controlling the activity of the flagellin structural genes (80, 83). On the other hand, it was indicated that leaky mutants of this type of *fla* produce a smaller number of flagella of normal length (T. Iino, *unpublished data*); that is, the *fla* genes seem to control the number of active flagella-forming sites in the cell. To unify these alternative concepts, it must be assumed that synthesis of flagellin molecules occurs at the base of each flagellum and is coupled with their polymerization reactions. Structurally, a ribosome system specialized for flagellin synthesis, namely *flagellosomes* (83), may be associated with a polymerization starter of flagellin and construct a flagella-forming apparatus.

Let us reconsider the hypothesis hitherto proposed on the function of the *fla* genes in the light of these observations. The alternative hypotheses proposed by Iino and Lederberg (83) are (i) that *fla* controls the production of flagellosomes and (ii) that *fla* produces an internal inducer of flagellin synthesis. When the synthesis of flagellin molecules and their polymerization are coupled, *fla*⁻ is not necessarily defective in flagellosomes but may be defective in the polymerization starter or in other components composing the flagella-forming apparatus. Therefore, for the present we must express the first hypothesis more broadly: that is, *fla* controls the production of a component of the flagella-forming apparatus.

The second hypothesis still survives without modification. We may add an annotation that the internal inducer may regulate the production of messenger ribonucleic acid (RNA) by the flagellin structural genes; in leaky *fla* mutants, a limited amount of the messenger RNA is produced so that the number of active flagellosomes, and consequently the number of active flagella-forming sites, is decreased. Regardless of which hypothesis is correct, suppressibility of some of the *fla*⁻ mutants of this type by an amber suppressor gene suggests that the defective product of *fla*⁻ is not RNA but protein (P. Vary and B. A. D. Stocker, *personal communication*). For the present we have no experimental evidence on which to base a

preference for one of these alternatives. It is also possible that some *fla* genes function as in the first hypothesis and others as in the second hypothesis.

Among the nonmotile mutants, paralyzed mutants have been studied the most intensively in *Salmonella*, next to nonflagellate (*fla*$^-$) mutants. Paralyzed mutants are nonmotile, although they produce flagella indistinguishable from those of the motile parent in shape, number per bacterium, configuration by X-ray diffraction (23), and antigenicity (46). The paralyzed mutants of diphasic strains show flagellar phase variation as frequently as the motile parent strain (46). Therefore, the paralysis is assumed not to be due to any defect of flagella themselves but to a defect of the flagellum-activating mechanism within the bacterium (46).

Neither nonflagellate nor paralyzed mutants can spread to form swarms on semisolid media (14, 80, 98, 187), and both are resistant to flagellotropic phages, e.g., chi phage in *Salmonella* (153). Therefore, by using both semisolid medium and flagellotropic phage for selection, both type of nonmotile mutants are very efficiently isolated from a culture of a motile strain (80, 98, 99). Conveniently enough, it was further found that, in smooth-type *Salmonella* and *Escherichia*, the colonial type formed on semisolid medium is different for a nonflagellate clone and a paralyzed clone: the former produces an LP-type colony which is large, translucent, and pale yellowish-gray, whereas the latter produces an SD-type colony which is small, opaque, dense, and yellowish-gray (50). Paralyzed mutants are thus easily distinguished from nonflagellate ones immediately on the selective media. Genetic analysis of the paralyzed mutants of *Salmonella* was started by Stocker and his co-workers (187), who reported that two paralyzed strains of *S. typhimurium* can produce motile recombinants by P22 phage-mediated transduction from other motile strains or with each other. Succeeding transduction experiments by Iino (68) showed that the mutational sites of four paralyzed mutants are contained within a single cistron distinct from the known *fla* cistrons; the new cistron was given the symbol *mot*. Further extensive studies were carried out by Enomoto, who isolated nearly 100 paralyzed mutants from *S. typhimurium* and analyzed the fine structure and the chromosomal localization of *mot* by means of both transduction (44, 46) and sexual recombination (47). The paralyzed mutants were classified into three cistrons on the basis of abortive transduction tests: namely, *motA*, *motB*, and *motC*. The order of the representative mutational sites in these *mot* cistrons was determined by deletion mapping and two-factor transduction tests. In all *mot* cistrons the wild-type allele, *mot*$^+$, is dominant over *mot*$^-$. Studies by cotransduction and sexual recombination further show that the chromosomal order of these three *mot* cistrons in relation to other markers investigated is *met-ser-H2-his-H1-motB-motA-leu*, and that *motB* and *motA* constitute adjoining functional units (46, 47).

Complementation tests among some *mot*$^-$ mutants of *motA* or *motB* suggest that *motA* and *motB* or their products constitute a functional complex: a group of *mot*$^-$ mutants which belong to *motA* complement weakly with the *mot*$^-$ mutants of *motB* and vice versa, but complement normally with *flaC*$^-$. As with the partial complementation of *fla*$^-$ mutants (80), the decrease in the number of trails in complementation tests on semisolid medium is often accompanied by a decrease in the number of minute colonies forming each trail. Mutational sites of the mutants which complement weakly with the mutants of the adjoining *mot* cistron are not restricted to a specific region but are distributed over all regions of the cistron.

Among bacteria other than *Salmonella*, the existence of the *mot* genes was shown in *E. coli* by P1 phage-mediated transduction (14) and in *B. subtilis* by transformation (97). In *B. subtilis*, only one *mot* gene which is not linked to either *H* or *fla* was detected in a preliminary study. In *E. coli*, *mot*$^-$ mutants were classified into two complementation groups, group I and group II, probably adjoining each other (14). Although allelism tests between the *mot* genes of *Salmonella* and *E. coli* have not yet been carried out, it is most likely that groups I and II of *E. coli* are homologous with *motA* and *motB* of *Salmonella*. Mapping by conjugation has located the *mot* genes of *E. coli* between *H-uvrC* and *aroD* (16).

Weak complementation occurs commonly in *mot*$^-$ of *E. coli* as it does between *motA*$^-$ and *motB*$^-$ of *S. typhimurium* (14). In addition, nonreciprocal complementation is more commonly observed in *E. coli*. Armstrong and Adler (14) proposed two hypotheses to explain the existence of nonreciprocal complementation. The first attributes the nonreciprocity to a gene dosage effect, in assuming that the partial heterozygote contains only one chromosomal fragment received from the donor, whereas more than two recipient-type chromosomes are present in a cell. The second is that complementation may be inhibited if defective subunits, presumably produced by the *mot*$^-$ gene, are incorporated into a structural component of the cell. For the present, there are no experimental data on which to base a preference for two hypotheses; furthermore, they are not necessarily mutually exclusive.

In a previous discussion, it was mentioned that the paralysis caused by a *mot*$^-$ mutation may be not a defect in flagellar structure per se, but a defect in the flagella-activating mechanism. Because it has been suggested that the bound energy of adenosine triphosphate (ATP) is involved in flagellar motility (33, 34, 175), the correlation between ATP activity and the type of the *mot*$^-$ mutants was examined (46). The ATP content in the wild-type cell, however, was not significantly different from that of representative *motA*$^-$ or *motB*$^-$ mutants. Moreover, ATP and adenosine triphosphatase activity were not detected in flagella detached from either motile or paralyzed mutant cells (46).

The ability of a bacterial clone to swarm on semisolid medium is usually associated with the motility of the component bacteria in liquid medium. A group of mutants isolated by Armstrong and his co-workers (16) from *E. coli* behave exceptionally: those mutants are fully motile under the microscope but fail to swarm on semisolid tryptone plates. They have flagella normal in shape and antigenicity, they are sensitive to the flagellotropic phage chi, their growth rate is normal under several different conditions, and their growth requirements are unchanged from the parent strain, but they do not make bands, which are characteristic of the chemotactic response, in capillary tubes containing tryptone broth (9). On a mutant of the group, more detailed investigations were carried out and it was found that it fails to show chemotaxis toward oxygen, glucose, serine, threonine, or aspartic acid. These mutants were inferred to be chemotaxis-negative mutants, and the symbol *che* was given to the corresponding gene (16). It is not known, at present, what biochemical functions are affected by the mutation. Three *che* cistrons were mapped by transduction and conjugation; one, *cheC*, is located between *his* and *H*, and the other two, *cheA* and *cheB*, are located between *mot* and *aroD* (15).

### Genetic Determination of Flagellar Shape

Flagellar shape mutants of various bacterial strains have been isolated from nature (reviewed in 127). The change in flagellar shape is very often associated with an alteration in cellular motility, so that it is possible to isolate several kinds of flagellar shape mutants by using a semisolid nutrient gelatin-agar plate as the selective medium (85).

The most commonly occurring mutant type is curly (14, 74, 125, 127). Curly flagella are characterized by a wavelength which is about half that of normal flagella. Their amplitude is a little smaller than that of normal ones. Mutant bacteria possessing curly flagella move rotationally and have a tendency to aggregate in liquid medium. They cannot form swarms on semisolid media and their growth is restricted at the area of inoculation. Phenocopy of curly flagella is obtained by addition of *p*-fluorophenylalanine to a culture medium of *S. typhimurium* producing normal flagella (105).

Five different types of flagellar shape mutants, namely, hooked-curly, para-curly, heteromorphous, small-amplitude, and short, have been isolated from a curly flagellar strain of *S. abortusequi* (85). Hooked-curly flagella have a wavelength similar to that of curly flagella, but their amplitude is 1.5-fold that of curly flagella; therefore, their waves are more curled than those of curly flagella. Para-curly flagella have approximately the same wavelength and amplitude as hooked-curly flagella. They differ from hooked-curly flagella, however, in that the flagella of the para-curly mutant transform to normal waves when they form a bundle. Heteromorphous mutants are characterized by their occasional production of heteromorphous cells having both normal and curly flagella among a normal population; cells having only curly flagella have never been found among them. Therefore, dimorphism occurs in the mutant without a mutational change from normal to curly flagella.

Flagella of small-amplitude mutants have a wavelength and an amplitude both of which are smaller than those of the normal type, and the shape of the wave is flattened in appearance. The mutants called short produce flagella shorter than the normal ones. Because of their short length, the shape of their wave has not been identified. Not only the length of the flagella but also the number of flagella per bacterium is decreased in this mutant as compared with the normal type.

A more distinguished type of flagellar shape mutant is a straight mutant obtained as a natural isolate (127) and also detected among nonmotile mutants from normal strains of *Salmonella* (86) and *B. subtilis* (149).

Genetic analyses by P22 phage-mediated transduction in *Salmonella* indicated that all mutant sites responsible for flagellar shape are inseparable from a structural gene of flagellin, *H1* or *H2*; depending on which gene mutated, the mutant shape appear in either phase 1 or phase 2 (74, 85, 86). The peptides of the flagellin from a curly mutant of *S. abortusequi* were analyzed by the fingerprinting method, and a difference in 1 peptide among 35 was shown between normal and curly flagellin (51). A more detailed comparison of chemical compositions was carried out on the flagellin of normal and straight flagella of *B. subtilis* (149), and it was shown that in flagellin of

straight mutants an alanine molecule in normal flagellin is replaced by valine in the altered peptide. Thus, the genetic change of flagellar shape is attributed to a mutation at a site in a structural gene of flagellin which results in the replacement of a certain amino acid in flagellin polypeptide by another specific one. The flagellin polypeptide with the altered amino acid sequence may then undergo folding to form an altered conformation of monomer, and consequently the mode of polymerization of the flagellin may be changed. The primacy of the type of monomer for flagellar shape was also shown by reconstitution of flagellar fibers from flagellin: the overall shape of the reconstituted flagellar fibers was found to be predominantly determined by the nature of the monomer (19). For example, when fragments of flagella from a normal strain are mixed with flagellin monomers from a curly mutant, the resulting filaments are usually curly.

### Sensitivity to Flagellotropic Phages

As mentioned in an earlier section, there is a group of bacteriophages which can attack only flagellate-motile bacteria, such as chi phage of *Salmonella* (42, 174) and phage PBS1 of *B. subtilis* (95). By electron microscopy, the receptor sites for these phages were shown to be located on the flagellum (153, 173). Upon adsorption, the helical fibers of the phage are wrapped around the flagellum and the tip of the tail attaches to a flagellar fiber (173). The mutation of a bacterium which is sensitive to a flagellotropic phage from flagellate to nonflagellate always results in the concomitant change to resistance to the phage (95, 153). The resistance to the flagellotropic phage is acquired not only by deflagellation but also by paralysis, either genetic or physiological (54, 153, 166). Therefore, the presence of flagella is not enough for the susceptibility of bacteria to the phage: the flagella must be active. Flagellotropic phage cannot adsorb to isolated flagella (153, 166, 173). They rarely attach to the flagella of paralyzed mutants (173), and they attach at a reduced rate to flagella of bacteria paralyzed by low temperature, anaerobic conditions (173), cyanide treatment, or protoplasting (166). Infection by the flagellotropic phages of entirely nonmotile bacteria was observed on straight flagellar mutants of both *S. typhimurium* (86) and *B. subtilis* (166). This may mean that cellular locomotion per se is not a prerequisite for infection by the phage; rather, the presence of active flagella is the requirement. In straight flagellar mutants, the flagella themselves may have retained activity, but, because of loss of the helical structure, this movement may not be capable of effecting locomotion of the bacterial cells. In the chi phage-*E. coli* system, Schade and his co-workers (173) observed that empty phages, that have already injected phage DNA into the host cells, increased with time at the bases of flagella after phage infection but not on the flagella themselves; they inferred that the ultimate receptor site for the phage is located at the base of the bacterial flagellum and that a flagellotropic phage slides along the filament of the flagellum to the base.

The host range of a flagellotropic phage, chi, has been studied in relation to the specificity of flagella. Chi phage can infect not only various serotypes of *Salmonella* but also certain strains of *Arizona* (153), *E. coli* (87, 173, 190), and *Serratia marcescens* (87), suggesting that the flagella of these bacteria have common phage receptor structures. On the other hand, *Salmonella* serotypes with flagellar antigens of the *g*-complex, *l*, or *e,h* are generally resistant to chi phage.

The introduction of *g*-complex antigen into a sensitive strain by transduction of *H1-g* makes the strain resistant to chi phage in phase 1 but not in phase 2. Thus, the resistance of *Salmonella* strains carrying the *g*-complex antigen was inferred to be due to the presence of this antigen in their flagella (153). The resistance of *Salmonella* serotypes having *g*-complex antigen cannot be attributed solely to their *g*-antigenic flagella, however, because the introduction of *H1-i* or *H1-a* from a sensitive strain to a serotype having a *g*-complex antigen sometimes does not influence the resistance of the recipient strain (153). Host-range mutants of chi phage which attack serotypes with H antigens *l*, *e,h* (153), or *g* (172) have been obtained. A host-range mutant, M8, of the latter serotype was shown to be adsorbed to all of the *g*-complex antigenic flagella tested at about the same efficiency, and the differences in M8 sensitivity among *g*-complex antigenic strains were attributed to some factors other than their flagellar antigenic characters, factors which control the infection process after adsorption (201).

A mutant which is resistant to chi phage but still flagellated and motile was isolated from *S. typhimurium* (172). The mutant is resistant in phase 2 (*1.2*-antigen) and sensitive in phase 1 (*i*-antigen). Although the mutant flagella do not immunologically cross-react with *g*-antigenic flagella, cross-sensitivity to the host range mutants of chi was present between the mutant and the *g*-antigenic strains (172).

In reviewing these results, we may summarize by saying that the specificity of the flagellar receptor to chi phage is not directly associated with the antigenic specificity of the flagella, but it is determined by a structure of flagellin which is common to the related bacterial strains. This

common structure may be altered in *g*-complex flagella. Genetically, it is determined by a region in the structural gene *H* of flagellin. It is worth noting that *g*-complex flagella have a distinct amino acid composition as compared with other antigenic types of flagella in *Salmonella* [e.g., the absence of histidine (141)].

### H Antigen Specificity and Fine Structure of the Flagellin Genes

The genetic approach to the determination of H antigen specificity has so far been confined to the *Salmonella* group. Transduction analyses between different serotypes of *Salmonella* have demonstrated that the antigenic specificity of their flagella is determined wholly by the structural gene of flagellin (83, 122). Genetic and biochemical studies of the antigenic mutants have further confirmed this conclusion. Using homologous antiserum as the selective agent, Joys and Stocker (98, 100) isolated mutants of *S. typhimurium* which are altered in a minor antigenic component from phase 1 (*i* antigen) and found that all of them occurred by mutation in *H1*. Differences in the fingerprints of their flagellin from that of the wild type were shown for some of these mutants (140).

An exception to the genetic determination of H antigen specificity by the *H* gene is the genetic modification of antigenic specificity by a gene termed *nml*, which is closely linked to *H1* and located between *H1* and *flaA* (186). In *nml*$^+$ cells, regardless of whether they are in phase 1 or phase 2, about half of the lysine residues present in a flagellin molecule are methylated, probably because of the production of a specific methylation enzyme by the *nml* gene (186). The data favor, but not conclusively, the concept that the methylation of lysine occurs after the flagellin molecules are synthesized (110). Antigenically, the methylation of lysine in the flagellin molecule by *nml* often results in the production of a unique H antigen. For example, the phase 2 antigen of *nml*$^-$-*S. typhimurium* is *1.2*, whereas it changes to *1.2.3* when *nml*$^-$ is replaced by *nml*$^+$ (B. A. D. Stocker, *personal communication*). Thus, the *nml* gene acts as a modifier of H antigen specificity.

The distribution of the sites of antigenic determinants in the *H* genes was investigated by both mutation and recombination studies. Mutants expressing H antigens altered from the parental type have been selected from many *Salmonella* serotypes by growing them in the presence of homologous anti-H serum (25, 27, 38). Systematic genetic mapping was first undertaken on phase 1 antigen *i* of *S. typhimurium* (98, 100). The altered *i* antigens of the mutants were not only deficient in one or more antigenic factors (that is, antigenic subunits identified by absorption-agglutination tests) of the wild type but also generally acquired a unique factor(s) absent in the wild type. Therefore, the wild-type recombinants formed by crossing two such mutants could be selected on semisolid medium supplemented with antisera against the mutant-specific antigens. For three-point crosses, the recipient was marked by *fla*$^-$ closely linked to *H1*. From the transduction analyses on this system, five mutant sites within *H1-i* were mapped linearly (98). A further extensive absorption-agglutination test on the altered *i* antigens of these mutants showed that at least 13 antigenic factors exist in the wild-type *i* antigen, and that each of the mutant antigens obtained lacked a different combination of these factors (100). Attempts to infer the linear order of the presumed sites of amino acid substitution in the polypeptide chain of flagellin from the serological data were unsuccessful, probably because some species of antibody molecules have specificities for pairs of chemical features which are close together on the surface of the flagellum but far apart along the flagellin polypeptide chain; also, substitutions of different amino acids in a flagellin molecule may sometimes cause a similar change in the tertiary structure of the flagellin molecule, and result in a similar alteration in antigenicity (100).

The possibility that the determinant sites of antigenic factors can be assigned linearly each as a unit in the *H* gene was suggested by Iino (69), who, considering the factor composition of naturally occurring *g*-complex antigens, proposed a preliminary model dividing *H1* into five sections, each of which is responsible for an antigenic specificity. An experimental approach to the mapping of the component factors of *g*-complex antigens was made by P22 phage-mediated transduction between different pairs of *H1*-linked *fla*$^-$ mutants, whose *H1* alleles were different and whose *fla*$^-$ sites were on opposite sides of *H1* (204). Among the *fla*$^+$ transductants developed as swarms in semisolid medium, recombinant antigens carrying some factors of one or both parental type antigens were detected. Fingerprinting analysis proved that flagellin of an antigen recombinant is a chimeric molecule in which a part of the flagellin polypeptide of one parent is replaced by the homologous region of the flagellin polypeptide of another parent. On the assumption that the antigen recombinant has resulted from a single crossover within *H1*, determinant sites of antigenic factors involved in the antigens *fg*, *gt*, *mg* were mapped each as a unit and as a whole arranged in a linear array within *H1* (204). This, however, may not necessarily mean that the specificity of an antigenic determinant of flagellin

depends exclusively on the primary structure specified by the corresponding section. As in the case of the *i* antigen mutants (100), examples were found for *g*-complex antigens in which several antigenic factors changed simultaneously, presumably as a consequence of only one or a few amino acid replacements (202). The amino acid sequence of an antigenic specificity-determining section in the *H* gene may manifest the specific antigenicity only when it has a specific tertiary structure in the overall conformation of the flagellin molecule.

An attempt to obtain intra-*H1* recombinants between non-cross-reacting *a* and *g*-complex antigens was unsuccessful, probably because the high degree of heterogeneity between these alleles prevents intragenic recombination (204). In the *H2* alleles, only a preliminary study of intragenic recombination between *H2-1.2* and *H2-enx* was reported (70).

As discussed in earlier sections, not only antigen specificity but also flagellar shape and sensitivity to flagellotropic phage are controlled by the flagellin genes. Mapping of the sites responsible for these latter characters in the flagellin genes has not yet been carried out, except in the case of a mutant site determining curly flagella. By transductions between a phase 1 curly mutant and *fla*$^-$ forms of *i* antigen mutants of *S. typhimurium*, the curly mutant site was mapped between two mutant sites for antigenicity (98). On a curly mutant of *g*-complex antigen type, the mutant site has been mapped outside of the cluster of the determinants of antigen factors in *H1* (T. Iino, *unpublished data*).

## MORPHOGENESIS OF BACTERIAL FLAGELLA

### Biosynthesis of Flagellin

The accumulated genetic and biochemical data lead to the conclusion that the *H* gene determines the complete amino acid sequence in the polypeptide of the corresponding flagellin. The basic process of flagellin biosynthesis is believed to be the same as that of other proteins: namely, transcription of the nucleotide sequence of the structural gene to messenger RNA, its translation to polypeptide on the ribosome, and the folding of the polypeptide to form the protein molecule. To perform flagellin synthesis in vitro, a cell-free system incorporating radioactive amino acids into protein was constructed (F. H. Gaertner and H. Koffler, Bacteriol. Proc., p. 81, 1966). The reaction mixture contained an S-15 extract from lysozyme-treated cells of *Bacillus pumilus*, certain $^{14}$C-L-amino acids, and the usual other components, and was incubated at 37 C for 30 min. Radioactive flagellin was purified together with carrier flagellin, digested with trypsin, and chromatographed; the coincidence of resulting ninhydrin and radioactive profiles was demonstrated.

Interest in flagellin biosynthesis has been mainly focused on the stability of messenger RNA specific for flagellin. Martinez (146) used a mutant of *B. subtilis* (*trp*$^-$, *ura*$^-$) stringent for RNA synthesis. Under metabolite starvation of the mutant, he observed a marked suppression (80 to 90%) of total RNA synthesis and a complete block in the synthesis of $\alpha$-galactosidase and in $^{14}$C-valine incorporation; the incorporation of labeled amino acid into flagella appeared to be unaffected. He inferred from these results that messenger RNA for flagellin is stable for at least 60 min. McClatchy and his collaborators (137) drew the same conclusion concerning the flagellin of *S. typhimurium*. Their conclusion was based on the fact that actinomycin D treatment severely inhibited synthesis of RNA and $\beta$-galactosidase in ethylenediaminetetraacetate-treated bacteria, whereas flagellin synthesis was unaffected. Contrary to the above reports, Aamodt and Eisenstadt (1) observed the absence of flagellin synthesis in a tryptophanless mutant of *S. typhimurium* when RNA synthesis was reduced to less than 1% after tryptophan starvation and actinomycin D treatment. They concluded that the production of flagellar protein requires the concomitant synthesis of RNA, and found no evidence of a stable messenger RNA specific for flagellin synthesis. The authors attributed the difference from the earlier report to the difference in the degree of inhibition by actinomycin D. They agree with the earlier investigators that the synthesis of cellular protein as a whole has a greater sensitivity than that of flagellar protein to partial inhibition of net RNA synthesis by the drug.

The second point of interest regarding flagellin synthesis is its site in the cell. It is generally believed that flagellin is synthesized at the base of each flagellum (146). To explain the function of the *fla* genes, Iino and Lederberg (83) proposed that flagellin molecules are synthesized on a specialized ribosome system termed a *flagellosome* at the base of each flagellum. Direct biochemical evidence for such a structure, however, has not been presented so far. A preliminary report that the hook structure at the base of flagella contains RNA (145) is still not conclusive; it is more likely that the RNA concerned attaches to the cell membrane near hooks rather than to hooks themselves (R. J. Martinez, *personal communication*).

### Initiation of Polymerization of Flagellin

Although flagellin molecules can polymerize by a self-assembly process, as reviewed in an earlier section, the initiation of their polymerization in vivo must be under control of a regulatory mechanism localized in the cell. For example, polarly flagellated bacterial strains usually produce a flagellum or a tuft of flagella only once during a cell division cycle, and the new flagellum produced is localized at the opposite pole of a nonflagellate daughter cell (127). In peritrichously flagellate bacteria, the distribution of flagellar number per cell is characteristic for each bacterial strain, and new flagella appear not simultaneously but sequentially during cell growth (78, 124). In a plate culture of *Proteus mirabilis*, the number of flagella per cell changes during multiplication with the change of cell form from coccoid to rod-shaped to elongated, increasing to a peak at about 6 hr and then decreasing; the number increases from a few to several thousand per cell (62, 63). The change of flagellation during growth was also observed in broth cultures of *Aeromonas*, in which a cell of an earlier growth stage tends to produce several lateral flagella whereas that of a latter stage has predominantly a single polar flagellum (130).

The number of flagella per bacterium is under genetic control. Mutants which produce a smaller number of flagella of normal length have been isolated from *S. typhimurium* (45). A nonflagellate mutant of *S. abortus-equi* was found to produce flagellin which can polymerize in vitro; thus, the mutant was inferred to be deficient in the initiation of polymerization in vivo (80, 188).

Electron microscopy of partially disrupted or autolyzed bacterial cells has permitted the observation of the basal structure of the flagellum in various genera of bacteria, namely, *Bacillus* (5), *Proteus* (3, 64, 67, 88, 133), *Pseudomonas* (133), *Salmonella* (111, 133), *Spirillum* (61, 66), *Vibrio* (60, 61, 167, 189), and *Rhodospirillum* (30). A common feature in these bacteria is that a flagellar fiber initiates from an area of cytoplasm just inside the cell membrane and appears to be anchored in the cell by means of a structure called a hook, which is structurally an extension of the flagellum but differs from the flagellum in fine structure. Its end is tapered and rounded. In some organisms, the hooked region is seen to be greater in diameter than the flagellum proper and the hook-shaped bend is not clear (133, 164). A hook is sometimes seen to be connected to a round or disc-shaped basal body, 10 to 20 nm in diameter (3, 5, 30, 63). The clearest electron micrograph reveals it as two pairs of discs separated by a thin connecting strand. Cohen-Bazire and London (30) observed in *Rhodospirillum* that a disc was connected by a short, narrow collar to the hook, and interpreted the narrow collar as the region of the flagellum which traverses the wall and the membrane, and which is anchored immediately within the membrane to the basal organelle. Abram and her collaborators (3) also inferred that this structure is associated with the cytoplasmic membrane and presumed that it consists at least partly of a fragment of the cytoplasmic membrane. Flagella associated with cell walls free from cytoplasmic membrane frequently have larger bodies, 20 to 70 nm in diameter, associated with their base. A portion of the larger basal structure is frequently seen folding around the smaller disc structure. Interpretation of this larger basal structure is still controversial. Abram and her co-workers (3) suggest that it may not be a real structural entity, but perhaps is an artifact resulting from the persistence of a part of the membrane after the rupture of the wall. On the other hand, Hoeniger and her collaborators (64) believe that it is an organelle corresponding to the basal body. Van Iterson and her co-workers (89, 92) observed electron microscopically in both gram-positive and gram-negative bacteria that tellurite is reduced and deposited in bodies contiguous with the plasma membrane, and they presumed that the bodies might function as the basal granules of the flagella. This presumption, however, was later found not to be true (5).

Although direct evidence is still lacking, it is the current belief that a basal structure must be formed before de novo formation of a flagellum, and a part of it, presumably a part of the hook, may act as a starter for the polymerization of flagellin in vivo. In mesophilic bacteria, a temperature range of 42 to 44 C permits bacterial multiplication but inhibits the de novo formation of flagella; therefore, flagella preexistent on a cell are diminished in number through growth and cell division at that temperature, ultimately yielding nonflagellate cells (165). Kerridge compared the process of flagellar regeneration after deflagellation by either mechanical shaking or cultivation at 44 C (106–108). Mutants of *S. typhimurium* requiring for growth an amino acid present in flagellin were unable to regenerate flagella in the absence of this amino acid, but those requiring an amino acid that is absent (or present in small amounts) in flagellin were able to regenerate flagella without this amino acid. Their regeneration was completely inhibited by 2,4-dinitrophenol but not by sodium azide or by sodium arsenate. None of the purine or pyrimidine analogues tested prevented the regeneration. The bacteria deflagellated by mechanical shaking

could regenerate their flagella even at 44 C. Unlike mechanically deflagellated bacteria, nonflagellate bacteria derived by cultivation at 44 C showed inhibition of flagellar regeneration at 37 C by immediate application of thiouracil or 8-azaguanine. However, when the addition of 8-azaguanine was delayed for 1 hr after transfer of the cells from 44 to 37 C, there was no inhibition. These data were interpreted to mean that the systems responsible for the formation of flagella involve RNA and were absent from cultures grown at 44 C. Results suggesting the involvement of RNA in the flagella-forming system were also obtained from the regeneration experiment on an uracil-less mutant of *S. typhimurium* deflagellated by phenol treatment (84). It must be noted that the experimental procedures employed for flagellar regeneration cannot distinguish between flagellin-synthesizing systems and flagellin-polymerizing systems; therefore the flagellar-synthesizing system discussed above may correspond to either one of them.

The correspondence between the flagellar-synthesizing system deduced from the regeneration experiments and the basal structures observed by electron microscopy is inferred but not yet experimentally proven (108). The presence of a functional flagellin pool in bacterial cells was inferred from Ouchterlony or quantitative precipitation tests on cell lysates of *P. vulgaris* and *B. subtilis* (D. Nasser and H. Koffler, Bacteriol Proc., p. 35, 1963; D. Weinstein, H. Koffler, and M. Moskowitz, Bacteriol. Proc., p. 63, 1960) and also from the regeneration experiment on flagella of *B. subtilis* (148). However, Kerridge (109) studied the kinetics of isotope incorporation into flagella, and concluded that a functional pool of flagellin is not present in *S. typhimurium*. Attempts to demonstrate the association of intracellular flagellin with a specific intracellular structure have been unsuccessful (108, 109). The report of Quadling and Stocker (165) that chloramphenicol inhibits flagellar regeneration in *S. typhimurium* also supports the absence of a flagellin pool. In several monotrichous bacteria, on the contrary, Roberts and Doetsch (168) could observe the regeneration of flagella in the presence of chloramphenicol at a concentration 100 times that required to inhibit cell multiplication.

### Growth of a Flagellum

For a flagellum to start growing, it must extrude from the cytoplasm through the cell wall. We have little information on the fine structure of the cell wall area where flagella pierce. However, observations on spheroplasts suggests that flagella formation is dependent on prior synthesis of the normal cell wall: flagella of penicillin-induced spheroplasts of *S. typhimurium* were confined to those areas of the spheroplast where cell wall fragments remained; spheroplasts produced from nonflagellate cells were incapable of forming flagella; upon inactivation of the penicillin, flagella again were formed during reversion of the cells to their original rod form (192).

As discussed in an earlier section, reconstitution experiments in vitro on *Salmonella* flagella have shown that the flagellar filaments have structural polarity, and polymerizatior of flagellin molecules occurs at a structurally defined end corresponding to the distal end of a flagellum when it has been attached to the cell (20). Therefore, if flagellar growth in vivo is homologous with that in vitro, it must take place at the tip. The phenomenon whereby *Salmonella* cells produce curly flagella in media containing *p*-fluorophenylalanine was applied to investigate this question (78). Cells of *S. typhimurium* having normal flagella were cultivated in a medium containing *p*-fluorophenylalanine for 2 to 3 hr, and the distribution of newly grown curly waves among their flagella was observed: curly waves appeared at the distal portion of flagella, indicating that the growth of flagella in vivo occurs at their tip. From the absence of the production of heteromorphous flagella upon mixed cultivation of a normal flagellar strain and a curly mutant strain it was inferred that flagellin molecules reach the tip of a growing flagellum without being excreted into the culture medium, probably moving through the hollow core of the flagellum (78).

In the same series of experiments, Iino (78) further observed that the growth rate of a flagellum declined as its length increased, reaching zero at approximately 15 $\mu$m. The growth rate of flagella shortened by mechanical breaking was not less than that of intact ones of similar length. Therefore, the decline was attributed to a decrease in transport efficiency with increase in length, rather than to ageing of the flagella-forming apparatus. The length of flagella grown on bacterial bodies has an upper limit. In *Salmonella*, the maximal length is approximately the same as that at which the growth rate of flagella reached zero in the above experiment. It is quite plausible that the mechanism determining the decline in growth rate of flagella also plays an important role in determining flagellar length in growing bacteria. Such a postulate does not necessarily exclude the possibility of functional ageing of the flagella-forming apparatus. In a study of the process of reappearance of flagellate bacteria in deflagellated suspensions of *Salmonella* cells, Stocker and Campbell (185) observed that a flagellum gradually elongates for a limited period

and thereafter persists without further elongation, and also that the rate of growth of flagella is unaffected by deflagellation. They also observed that up to one generation after deflagellation the mean number of flagella per bacterium was less than before deflagellation, and they inferred that a proportion of the original flagella-forming apparatus was damaged and could not regenerate flagella any more. Kerridge (108) observed the same phenomenon and proposed two alternative explanations: either the flagella-forming system has a limited life, or the synthesizing system is liable to be damaged as a result of ripping out the flagellum.

An interesting phenomenon as regards the length of bacterial flagella was reported by Weinberg and Brooks (200), who examined the effect of various metal ions on flagellation. Among the metal ions they examined, $6 \times 10^{-4}$ M manganese or aluminum caused less than a 10% decrease in the number of flagellated cells, but resulted in an increase in their length as much as 10-fold over that of normal flagella. The mechanism of this effect is not yet explained.

### Polymorphism and Structural Conversion

Clonal polymorphism of flagella has been observed in various bacterial strains (130, 131). In diphasic *Salmonella* strains, polymorphic alternation of flagellar shape is associated with phase variation, as already discussed (74, 85, 86). Polymorphism has been observed also among the flagella on a single cell or among different regions of a single flagellum on certain bacterial strains (62, 85, 126, 129). In a strain of *Lophomonas*, polar flagella and nonpolar ones show different shapes on a single cell: the former are undulant, i.e., of long wavelength, and the latter are curly, i.e., of short wavelength (56).

As for the cause of the appearance of such polymorphism, the following alternative possibilities may be considered: (i) it may be caused by the production of flagellin molecules different in amino acid composition, and (ii) it may be caused by the formation of flagellin molecules different in three-dimensional conformation even though the primary structure (that is, the amino acid sequence) is identical. Polymorphism associated with flagellar phase variation in *Salmonella* belongs to the first category. Polymorphism brought about in *Proteus* (62, 129) by a change in *p*H may represent the second type. More direct evidence for the occurrence of the second type of polymorphism is provided by the conversion of preformed flagella or isolated flagellar fibers. Leifson (128) reported that the addition of Formalin causes a change of flagellar shape in some bacterial strains: for example, from normal to coiled or small amplitude to normal. Normal-shaped flagella isolated from the cells of a *Salmonella* strain were found capable of conversion to curly, straight, or coiled (18). The change is reversible, and the curly flagella thus formed were found to revert to normal ones in a buffer solution containing ATP or pyrophosphate (19, 159).

Observing bacteria including *Sarcina*, *Salmonella* and *Bacillus* under a dark-field microscope, Pijper and his co-workers (161–163) observed the sudden change of a flagellar bundle from normal to curly and vice versa. They called the phenomenon *biplicity*. Observations of flagellar bundles of various flagellar shape mutants of *Salmonella* showed that biplicity involves specific flagellar types, namely, changes between small-amplitude and curly and para-curly and normal, when flagella are bundled (85, 154, 155). It was inferred that the tight association of the component flagella enhances the stress among the flagella, thus causing the conversion.

A remarkable feature of flagellar polymorphism and structural conversion is that the shapes which occur are confined to two or more discrete types; no intermediate types have been detected, even where polymorphism (or "transconformation") occurs in a single flagellar fiber. This is also true in flagellar fibers reconstituted by the copolymerization of two different types of flagellins. For example the copolymer of normal and curly flagellin molecules is curly through a wide range of proportions of the two types of flagellin (19), and the copolymer of normal and straight flagellin shows one of three distinct shapes through a gradual change in the proportion of the two types of flagellin (S. Asakura, *personal communication*). These phenomena suggest that a flagellin molecule with a definite primary polypeptide structure can take more than one but a limited number of stable conformations in flagellar fibers; the type of conformation selected by a flagellum depends on the physicochemical conditions under which the polymerization of flagellin molecules takes place. The flagellar shape characteristic for each flagellar shape mutant may represent the type of polymer of the component flagellin molecules having the most stable conformation under ordinary physiological conditions. The absence of flagellar shape mutants which arise from the modification of the flagellar-forming environment may reflect the presence of complex and elaborate physicochemical homeostatic regulatory systems in bacterial cells.

## CONCLUDING REMARKS

Knowledge of the structure and biosynthesis of bacterial flagella has advanced in the past 20 years through the collaboration of the bacteri-

ologist, immunologist, geneticist, biochemist, biophysicist, and electron microscopist. The goal is, however, only half completed. Despite the relative ease of their purification and chemical analysis, and their importance for the study of biochemical evolution, amino acid sequence analysis has been accomplished in none of the flagellins. The flagellins analyzed so far are limited to a small number of bacteria. We know the molecular size of the representative flagellins, and something about their molecular arrangement in a flagellar fiber. However, we still have no more than hypotheses to explain the appearance of the regular helical structures on the basis of flagellin conformation and arrangement. The in vitro reconstitution experiments permit us to describe the process of flagellar growth in terms of polymerization of flagellin. Nevertheless, the ordered sequential formation and distribution of flagella on bacterial cells must involve additional mechanisms not yet reconstructed in vitro. Although the flagellar basal structure which might be responsible for these phenomena has been studied electron microscopically, unfortunately such studies have not been paralleled by chemical studies. Genetic analyses of bacterial flagellation have disclosed unique regulatory systems at the level of both flagellin synthesis and flagellar morphogenesis. Several hypotheses on their mechanism have been proposed, but the experimental approach to choose among the alternatives is left to the future. Opinions on the stability of flagellin messenger RNA still conflict with each other. In view of these unsolved problems, bacterial flagellation will continue to attract the attention of pioneering investigators in every field of biology and will need their collaborated contributions. These contributions will not only lead to an understanding of bacterial flagella per se, but will extend our fundamental knowledge of the structure, function, and formation of the macromolecular architecture of living organisms.

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