GENETICS OF SALMONELLA

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(with 2 figs.)

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I. OUTLOOK

The genetics of *Salmonella* can be traced back to the discoveries and descriptions of several remarkable phenomena of antigenic variation in this genus, namely O-H variation, S-R variation, form variation and phase variation (reviewed by KAUFFMANN, 1954).

The discovery of phage-mediated transduction in 1952 (ZINDER & LEDERBERG, 1952) introduced the possibility of the detailed genetic analysis of these intriguing phenomena. Antigenicities and antigenic variations of *Salmonella* were re-examined with transductional technique, and detailed schemes on the genetic determination of antigenicity (Section IV & V) and on the cellular regulatory system of gene expression (Section VII) have been constructed. Further, an evolutionary pattern of *Salmonella* serotypes has been postulated, based on their genotypic constitutions (Section VI).

Transductional analysis was not confined only to the antigenic characters, but extended also to many other bacterial characters. In relation to the genetics of flagellar antigen, the genetic study of motility has been undertaken with paralyzed mutants (STOCKER *et al.*, 1953; ENOMOTO, 1962). Various nutritional mutants including auxotrophs for amino acids or nucleic acid bases, and mutants deficient in sugar fermentations were isolated by the application of the selection techniques established on *Escherichia coli* (LEDER-

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BERG, 1950). Detailed fine structure analyses have been carried out with these mutants (Section III). The mutants well analyzed for their mutational sites have been used for the investigation on the mutagenesis of base analogues (RUDNER, 1961a, b; MARGOLIN & MUKAI, 1961). A mutator gene which influences the frequency of various auxotrophic mutations has been discovered in a strain of *S. typhi-murium* (MIYAKE, 1960).

The second epoch of *Salmonella* genetics came with the success in transfer of the fertility factor from *E. coli* to *Salmonella* and the hybrid formation between them (Section III). It has led to the establishment of the sexual recombination system in *Salmonella* and has been used for the construction of the chromosome map of *Salmonella*. It also opened a way to investigate phylogenetic relations of *Salmonella* with other genera in Enterobacteriae.

The studies on the episomal multi-drug resistant factor *R*, which confers on a bacterial cell simultaneous resistance to sulfanilamide, streptomycin, chloramphenicol, and tetracycline, are under active investigation, with *Salmonella* as well as other Enterobacteria. (Section II). Among the chromosomal genes which control drug resistance, a streptomycin-resistant mutant showing pleiotropism has been studied (WATANABE, 1960). This requires thiamine and nicotinic acid, as well as being streptomycin indifferent. Transductional analysis indicated that all of these changes are caused by one step recessive mutation of a single gene. The pleiotropism of drug-resistant mutants was observed also on a chloramphenicol resistant mutant (WEINER & SWANSON, 1960). The mutant differs from the sensitive parent in morphological and cultural characteristics and in somatic antigens.

Together with the problem of drug-resistance, the problems of pathogenicity and host specificity are important subjects that *Salmonella* genetics may contribute to epidemiology as well as basic bacteriology. It has been known that nutritional factors influence the virulence of the pathogen (GOWEN, 1951) and that certain auxotrophic mutations of *S. typhi-murium* accompany the loss of virulence (BARON, 1953). The abundant accumulations of auxotrophic mutants in *Salmonella* provide a rich source for systematic study in this direction. The well analyzed antigen types in this group also favor the genetic investigation of the host-parasitic relationships. The genetic approach has already been started (FURNESS & ROWLEY, 1956). The development of this field in future is expected.

It is not the purpose of this article to review the entire field of *Salmonella* genetics comprehensively. Rather, the subjects will be focused on a few topics, especially on the immunogenetic aspects, which are specific to *Salmonella* and have not been systematically reviewed elsewhere.

II. GENETIC TRANSFER SYSTEM

Transduction

Salmonella is the prototype of phage-mediated transduction (ZINDER & LEDERBERG, 1952). At first, the combination of the competent bacteria was restricted to those in group A, B, and D, because phage *PLT22*, a type of A1-group phage, can attack only those bacteria which have somatic antigen *12*. Later, transduction was demonstrated with other *Salmonella* phages having host ranges different from A1-group, and the range of the competent group is being extended (BARON *et al.*, 1953; SAKAI & ISEKI, 1954; EDWARDS *et al.*, 1955). Several techniques have been invented to use virulent phages for transduction: namely the use of lysogenic recipient (ZINDER, 1955), lowering of multiplicity of infection (STARLINGER, 1958) or pretreatment of the lysate with ultraviolet light (GOLDSCHMIDT & LANDMAN, 1962). The last method is based on the information that the plaque-forming ability of a lysate is more sensitive to ultraviolet light than its transducing ability. These methods have been successfully extended to the heterogenetic transduction of nutritional markers between *Salmonella* and *E. coli* with the virulent phage "chi" which can infect certain motile strains of both genera (TSUJI & IINO, unpublished data).

The transductions in *Salmonella* so far studied are of the generalized type. Each gene of the donor bacteria has approximately an equal chance, about one to ten per 10^6 phage particles, to be incorporated into transducing phage and transferred to the recipient bacteria. The size of genetic fragment transduced by a phage particle at one time corresponds to a small fraction of the bacterial chromosome; chemically it is around 10^4 nucleotide pairs, less than one hundredth of the whole chromosome length. Therefore, only very closely linked genes are transduced simultaneously. The transduced fragments may have predeterminate broken ends (OZEKI, 1959).

The incorporation of a donor chromosome fragment in a recipient cell results in the formation of a hybrid cell which is heterozygous for a small fraction of the chromosome. Such cell is termed "heterogenote", and the donor chromosome fragment is called "exogenote", in contrast to "endogenote", which is a part of the intact recipient chromosome homologous to a given exogenote (MORSE et al., 1956). Recombination between exogenote and endogenote occurs in a fraction of heterogenotes, and consequently stable transductional recombinants are produced from time to time. The rest of the transductional clones remains as persistent heterogenotes. In generalized transduction as seen in *Salmonella*, the exogenotes of the transductional cells cannot multiply and the heterogenotic state is maintained only in a single cell in each cell generation of a transductional

clone until the exogenote is either lost or integrated into recipient chromosomes. The genetic characters expressed by such heterogenotes are inherited linearly through cell generations. The linear inheritance of motility appears as a trail of compact colonies on a semi-solid medium (LEDERBERG, 1956; STOCKER, 1956a) and that of nutritional markers as a minute colony on a minimal medium (OZEKI, 1956). The phenomenon of linear inheritance in transductional heterogenote has been used as a main criterion of "dominance" and "gene complementation". For example, the production of trails on transduction from a flagellated strain to a non-flagellated one indicated that the flagellation is dominant to non-flagellation (STOCKER et al., 1953). The production of trails in combination between two non-flagellated mutants indicates that the flagellation alleles of these two mutants are complementary to each other and presumably belong to different genetic functional units.

There is another related phenomenon of the hereditary change caused by infection with bacteriophage: lysogenic conversion. In *transduction* a phage particle carries a genetic fragment of the host bacteria. Contrasting with it, in *conversion*, phage genes function as part of the bacterial organism. Such modification occurs soon after phage infection and lasts until lysis occurs. When a bacterium is lysogenized by a conversion phage, the modified character is inherited through cell generations to the offspring as long as the lysogenic state persists. The conversion of somatic antigen type in *Salmonella* is one of the most intensively studied examples (Section IV).

Conjugation

As well as phage-mediated genetic transfer system, sexual mating system has been recently established in *Salmonella* for the genetic recombination analysis. The sex factor *F* is transferred from *E. coli* to various *Salmonella* strains (BARON et al., 1959a; ZINDER, 1960; MIYAKE, 1962) and from the latter to other *Salmonella* (MÄKELA et al., 1962). Both the ability to be infected by *F* and the degree of fertility of male (F+) *Salmonella* differ between different combinations of the strains. Generally homologous strains show the highest efficiency. The conjugation was also demonstrated between *E. coli Hfr* and certain strains of *Salmonella* (MIYAKE & DEMEREC, 1959; ZINDER, 1960; FALKOW et al., 1962). They yield hybrids between *E. coli* and *Salmonella*.

There are three other systems of conjugation in *Salmonella:* namely F-duction (or sex-duction), colicinoduction, and R-duction. They are mediated by different episomal factors: sex factor *F*, colicinogenic factor *C*, and multidrug resistant factor *R* respectively. These systems are common with the phage-mediated transduction to the point that an episome is a vector of the transmission of a piece

of genetic fragment, and also akin to the sexual conjugation in that both require cell contact for the transfer.

In "F-duction", a chromosome fragment is attached to *F*, replicates synchronously with *F*, and can be transferred to competent cells in high frequency, one per 10^2 to 10^4 cells in 1: 1 mixture of donor and recipient cells in homologous combinations. Such an autonomously replicating complex of *F* and a chromosome fragment, designated *F-prime (F')*, was first obtained from *E. coli* and introduced to *Salmonella* (BARON et al., 1959a; ØRSKOV et al., 1961). Later, an *F'* which is combined with lactose fermentation gene *(lac)* was demonstrated in a lactose positive *Salmonella* isolated from nature (BARON et al., 1959b). The *F'* introduced to *Salmonella* can be retransferred to the more distantly related bacteria such as *Shigella* (BARON *et al.*, 1959b), *Vibrio* (BARON & FALKOW, 1961), *Serratia* (FALKOW *et al.*, 1961) or *Klebsiella* (MÄKELA *et al.*, 1962) at lower frequencies, as well as to other *Salmonella* or *Escherichia*. The genes introduced to other genera by F-duction are usually not integrated into the chromosome of recipient cell; the cell multiplies as persistent heterogenote.

In "cin-duction", a bacterial chromosome fragment is transferred along with the transmission of certain colicinogenic factors (OZEKI & HOWARTH, 1961; SMITH & STOCKER, 1962). The sizes of chromosome fragments transferred by F-duction or cin-duction vary in different systems, but they are usually far longer than those in phage-mediated transduction.

Like *F'*, "multi-drug resistant factor R," originally detected from *Shigella*, has a diverse range of transferability among *Salmonella*, *Escherichia*, *Shigella* and even *Serratia* (reviewed by WATANABE, 1963), R-factor interferes with the genetic transfer system by *F;* however, the joint transfer of a chromosomal fragment with it has not been demonstrated.

Owing to the establishment of the two major recombination systems, i.e. transduction and conjugation, it is now possible to study both micro- and macro-topology of the *Salmonella* chromosome. The former is applied for the fine structure analysis of a gene or genes closely linked to each other, but it is not adequate for the mapping of the various genes distributed through the entire chromosome. Sexual conjugation and cin-duction cover the gaps of transduction analysis, and they have started to be used for the mapping of *Salmonella* chromosome (Section III). Because of its wide range and high efficiency of transfer of a particular genetic fragment, F-duction promises to be a unique system for genetic and chemical investigations of the localized chromosomal regions. It will supplement the absence of specialized transduction in *Salmonella*.

Several consequences of possible DNA-transformation in *Sal-*

monella have been reported by some investigators, but they are still ambiguous.

The detail on these genetic transfer processes, as well as the nature of the mediatory factors, will not be discussed here, as they were covered in a recently published book by JACOB & WOOLMAN (1961) and in two review articles by LURIA (1962) and by CLARK et al. (1962).

III. CHROMOSOME MAP

Microtopology

In *Salmonella*, recombination analysis has been focused mainly on the fine structure analysis in a gene or a short segment of its chromosome by means of transduction. Mapping of the various genes on a chromosome had been left behind until recently, as the conjugation system was established on the organism.

The genetic fine structure analysis on *Salmonella* raised several interesting problems on the gene structure in relation to its function. For example, (1) clustering of the genes with phenotypically similar effect, (2) coincidence of the order of the genes in a gene cluster with their sequence in respect to biochemical blocks in the chain of reaction leading to the synthesis of a certain final product, (3) heterogeneity among mutant sites in a genetic functional unit in respect to mutation rate, phenotypic expression or competence to suppressor. We will not discuss these problems here; they have been reviewed in detail by DEMEREC & HARTMAN (1959), and since then work with *Salmonella* has mainly elaborated on the analysis of individual genes. It may be worth noting, however, that the concept of "operon" as a unit of gene expression is being extended for the understanding of the phenomenon of gene clustering in *E. coli* and several other organisms, and it is interesting to examine to what extent this concept can be applied to the gene clusters found in *Salmonella* (Section VII).

Macrotopology

Mapping of the various genes on *Salmonella typhi-murium* strains LT2 and LT7 are summarized in Fig. 1 (DEMEREC & HARTMAN, 1959; FALKOW et al., 1962; MIYAKE, 1962; SMITH & STOCKER, 1962, personal comm.; MÄKELÄ, personal comm.; IINO, unpublished data). It is represented by a ring as revealed in *E. coli* K-12. The sequence and map distance of each marker on the chromosome is roughly the same as in *E. coli* (c.f. chromosome map of *E. coli* illustrated by FALKOW et al., 1962). Together with the successful hybridization between these two genera, the parallelism of their linkage maps may reflect their phylogenetic intimacy. On detailed structure, however, their chromosomes might differ in many respects, as

already suggested by preferential transduction of *Salmonella* markers from *E. coli-Salmonella* hybrids by *Salmonella* phage (ZINDER, 1960).

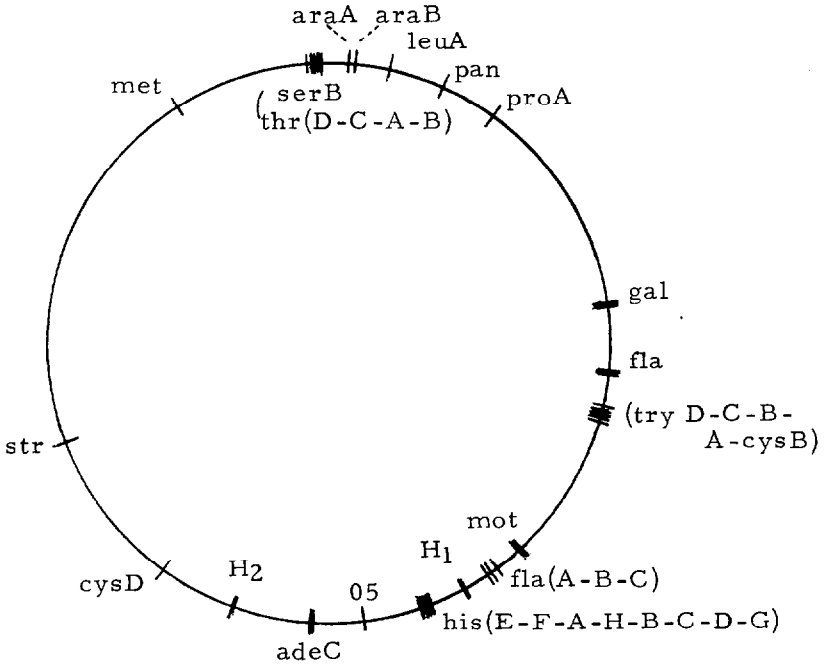

Fig. 1. Linkage map of *Salmonella typhi-murium*.
met: methionine, cys: cysteine, ade: adenine, pro: proline, pan: pantothenic acid, leu: leucine, ser: serine, thr: threonine, gal: galactose, ara: arabinose, str: streptomycin, O5: O-antigen 5, H_1: phase-1 flagellin, H_2: phase-2 flagellin, fla: flagellation, mot: motility.
A capital letter after a gene symbol represents a cistron.

Information obtained from molecular biology endorses these situations: the overall base composition of chromosome DNA is similar between *Escherichia* and *Salmonella*, but DNA molecules from each genus are imperfectly complementary as tested by their capacity for annealing with one another (FALKOW et al., 1962). Further comparative studies on the chromosome structures of various *Salmonella* species and the related bacteria from both genetic and molecular standpoints will throw light on the evolutionary history of *Salmonella*.

A distinguished difference between *Salmonella* and *Escherichia* is that the former cannot ferment lactose. In *E. coli*, a gene *lac*, which

is an operon controlling lactose fermentation (Section VII; PARDEE et al., 1959), is present near the *pro* gene. These two loci are three units apart. By introduction of a chromosome fragment of *E. coli* of this region to *Salmonella* by F-duction, the recipient *Salmonella* cell acquires the ability to ferment lactose. In *E. coli Hfr-Salmonella* conjugation, stable hybrids carrying *lac* are obtained by transfer of *pro-lac* region from the former. The recombination between *pro* and *lac* genes is never detected among the hybrids, and the recombination value between *lac* and such markers as *asa* or *gla*, which are more distal than *pro* from *lac*, is also very low compared with homospecific conjugation in *E. coli* (MIYAKE, 1962). These results indicate that the incapability of lactose fermentation in *Salmonella* is caused by deletion of *lac* region on its chromosome. As well as the *lac*-locus, the *P*-locus, which controls the production and antigenic specificity of fimbriae, is present in *E. coli* K-12 strain but absent in a certain strain of *S. typhi* (BRINTON & BARON, 1960). Conversely, *Salmonella* has a duplication of H-antigen type determinants, only one of which is present in *Escherichia*. This will be discussed in detail later.

IV. GENETICS OF SOMATIC ANTIGENS

O-antigen is a heat stable antigen of microcapsule on the surface of bacterial cell wall. Its specificity is determined by sugar terminals of lipo-polysaccharide chains. Chemistry of the antigens is described by STAUB et al. (1964) in this book. The variation of O-antigen type has been known as "Form variation" (KAUFFMANN, 1940, 1954).

Phage conversion

The genetic determination of certain O-antigen types is an excellent example of conversion by bacteriophage. Since the conversion of *3, 10* type *Salmonella* (E_1-group) to *3, 15* type (E_2-group) by infection of phage ε was demonstrated (ISEKI & SAKAI, 1953), several antigen types have been shown to be converted by infection of certain bacteriophages (Table I). A clear-cut scheme on the correlations between antigen type, its chemical structure and infecting phage was constructed on the antigenic conversions among E-group *Salmonella* (Fig. 2, UCHIDA et al., 1963). E_1-group *(3, 10) Salmonella* converts to E_2 type *(3, 15)* by infection of phage ε^{15}. The new antigen type is detected in a few minutes (UETAKE et al., 1958). Naturally occurring E_2-group *Salmonella* are lysogenic for ε^{15} or the phages relating to it. It is possible to isolate E_1 *(3, 10)* type *Salmonella* from E_2 type through anti-15 serum selection. The E_1 type cells thus obtained had lost the ε^{15} type phage and be sensitive to it. The antigenic change caused by the infection of

Table I.

Antigenic conversion caused by phage infection in Salmonella.

conversion phage	host bacteria	changes in O-antigen	reference
ε, ε^{15}	E_1 group	3,10→3,15	ISEKI & SAKAI (1953)
ε^{34}	E_2 group	3,15→(3,15)34	UETAKE & HAGIWARA (1960)
ε^{15} + ε^{34}	E_1 group	3,10→(3,15)34	UETAKE & HAGIWARA (1960)
ι, P22 Al-grop phage	4, 12 type group B Salmonella	4,12→1,4,12	ISEKI & KASHIWAGI (1957)
phage 27	1,4,12 type group B Salmonella	1,4,12→1,4,12,27	LEMINOR et al. (1961)

ε^{15} to E_1 *Salmonella* is expressed biochemically as the replacement of the terminal sugar acetyl-galactose by galactose. The change of E_2 *(3, 15)* to E_3 *(3, 15, 34)* by infection of ε^{34} is the addition of a sugar, glucose, to the terminal. The addition of glucose occurs when the terminal sugar is galactose but not acetyl-galactose. Consequently, E_1 *(3, 10) Salmonella* does not produce antigen-34 after infection of ε^{34}, but E_1 cells lysogenic for ε^{34} produce *34* antigen after double lysogenization with ε^{15}. The production of antigen-1 by A1-group phage in A, B, D, group *Salmonella* is also an additive reaction of glucose to a terminal of the antigenic chain by 1:6 glucosidic linkage (STOCKER et al., 1961). Thus, several O-antigen factors were found to be determined each by a specific bacteriophage genome. It may control directly or indirectly the production of an enzyme which catalyzes, modifies or inhibits the addition of a specific sugar to the terminal of polysaccharide chains. Phage mutants which differ in their conversion abilities have been obtained (TERADA et al., 1956; UETAKE & UCHIDA, 1959).

It is interesting to note that an episome, *F*, also confers to the carrier cell a new surface substance involved in male fertility, possibly a surface polysaccharide (SNEATH & LEDERBERG, 1961). Immunologically, the *f*+ antigen was demonstrated on *F*+ and *Hfr* clones of *E. coli* (ØRSKOV & ØRSKOV, 1960) and *S. abony* (MÄKELÄ et al., 1962).

The determinant of O-antigen type in bacterial chromosome is not disclosed yet, except the approximate position of the antigen-*5* determinant in *S. typhi-murium* (Fig. 1). We also have no direct

information on the mode of gene control of the backbone structure of O-antigen and type specific polysaccharides which are not determined by conversion phage.

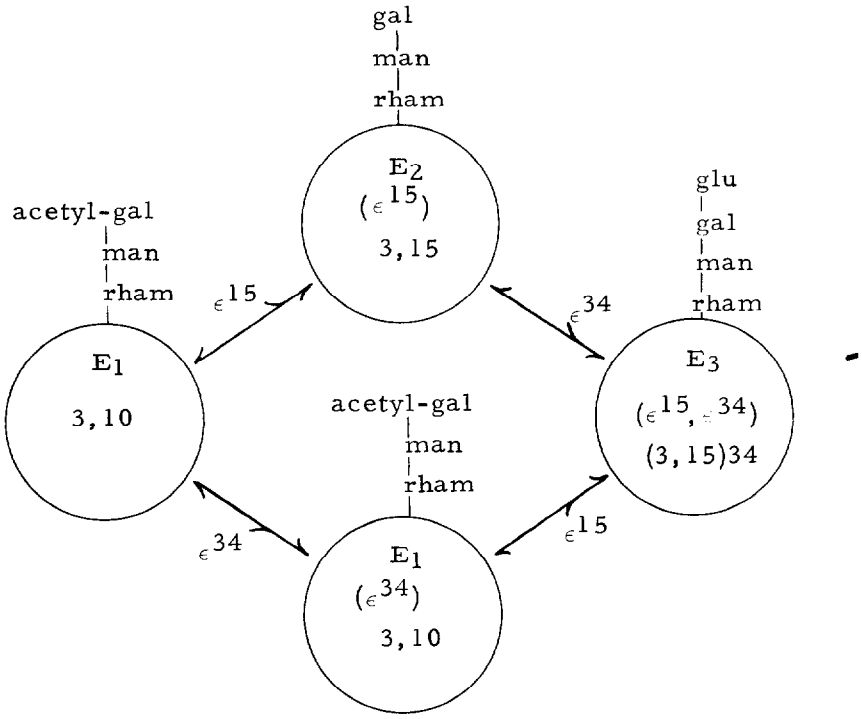

Fig. 2. Antigenic conversion by phage ε^{15} and ε^{34} in E-group Salmonella (constructed from UCHIDA et al., 1963). rham: rhamnose, man: mannose, gal: galactose, acetyl-gal: acetyl galactose.

S-R Variation

The variant cells which had lost O-antigens manifest another type of antigen called R-antigen. R refers to the roughness of the colonies in contrast with the smoothness (S) of those colonies which have O-antigens. Their type specificity has not been worked out as extensively as the O and H antigens. The changes from S to R type, called S-R variation, were first noted by ARKWRIGHT (1921). Since then the phenomenon has been reported by many investigators. S and R types are not uniquely distinct types, for there are many intermediate types. The frequency of S-R variation has been stu-

died by several workers (ZELLE, 1942; PAGE et al., 1951). ZELLE found an extraordinarily high value in one unstable strain of *S. typhi-murium*, amounting to one R mutation per 14 cell divisions in an S culture.

A suggestive result concerning the genetic basis of S-R variation and the gene control of polysaccharide structure on cell surface was obtained with a galactose sensitive mutant of *S. typhi-murium* (FUKASAWA & NIKAIDO, 1961a). The mutant, called M-mutant, has a single defect in the uridine-diphospho (UDP)-galactose-epimerase gene and was not only unable to ferment galactose but also showed galactose-induced bacteriolysis. On ordinary media, devoid of galactose, they form rough colonies, and the sugar composition of their cell walls is only glucose; while the wild type is smooth and contains galactose, mannose, rhamnose and 3,6-didesoxyhexose in addition to glucose. The mutant also acquired the resistance to phage *PLT22*. These pleiotropic effects of the epimerase mutant are explained as follows. The polysaccharide structure at the surface of the wild type cell is composed of three layers. The inner core consists of polyglucose. Galactose links to the glucose and forms the second core. The sugars of the outer layer, which is responsible for O-antigenicity, connect to these galactose molecules of the second core. The accumulated chemical evidence (STAUB et al., 1964) has shown that nucleotide-phosphate-sugars, for example uridine-diphospho-(UDP)-galactose for galactose, are generally used as the donors of the sugar component in polysaccharide synthesis. The defect of UDP-galactose epimerase in the mutant blocks the conversion of UDP-glucose to UDP-galactose. Then, the reaction of the polysaccharide synthesis must stop at the step of the second core formation. The loss of outer layer results in the simultaneous loss of O-antigenicity, including antigen-12, which is the receptor of phage *PLT22*, and the change of colonial type occurs from smooth to rough. On the mechanism of bacteriolysis in galactose-containing media, several possibilities were discussed in relation to the enormous accumulation of UDP-galactose (FUKASAWA & NIKAIDO, 1961a). The most plausible one is that the compound interferes with the incorporation of other nucleotide-phosphate-sugar compounds into the cell surface structure. These results and the accumulated information on the chemical changes which are accompanied with the S-R variation of various bacteria (reviewed by BEALE & WILKERSON, 1961) together with the antigen conversion by phage, lead to the speculation that the biosynthesis of polysaccharide in the cell surface consists of stepwise reactions: a series of genes function by bringing about an orderly conversion of the precursor, by the addition of a new terminal sugar together with adjacent groups.

V. GENETICS OF H-ANTIGENS

Flagella and flagellin as H-antigen

Flagella are long filamentous appendages, having the length of several times that of the bacterial cell. They originate in the cytoplasm and project through the cell membrane. The number and distribution of flagella differ in different bacterial strains and cultural conditions. In *Salmonella*, like other flagellated enterobacteria, generally 5 to 10 flagella are observed around a cell. Their diameter, measured on electron-micrographs, is 10 to 15 mμ. As can be seen on dark-field microscope under very powerful illumination, the shape of each flagellum is helical, with a pitch and diameter characteristic of the strain. Flagella can be isolated easily by mechanical vibration of the cell suspension, and purified by high-speed centrifugation and chromatography (WEIBULL, 1950; KOBAYASHI et al., 1959; ENOMOTO & IINO, 1962). A purified flagellum is a fibrous polymer of a unit protein. X-ray diffraction analysis indicated that it belongs to the keratin-myosine-epidermin-fibrinogen type. In acid solution below pH 3.5, a flagellum dissociates into monomers called "flagellin". Molecular weight of a *Salmonella* flagellin is about 38,000. The particle size is 45 Å in diameter. From the electron microscopy, a three stranded complex helical structure has been proposed for the arrangement of flagellin in a flagellum (KERRIDGE et al., 1962). Amino acid composition of flagellin has been studied with several *Salmonella* serotypes (AMBLER & REES, 1959; AMBLER & STOCKER, personal comm.). They contain only 15 to 17 kinds of amino acids, 373 in number (Table II). Their N-terminal is lysine. In certain *Salmonella* flagellin, an unusual amino acid "N-methyl lysine" is detected (AMBLER & REES, 1959). The details and references on the studies of general structure and function of flagella are found in a review by STOCKER (1956b).

There is abundant evidence that flagella are responsible for the H-antigenicity among the cell surface structures. The non-flagellated mutants or natural isolations, called O-type, are without exception H-agglutination negative. The reversion of these O-type strain to flagellated type (H-type) always restores the H-agglutinability. The parallelism between flagellation and H-agglutination is observed when flagellated cells lose flagella by growing in a medium containing certain concentration of phenol or at the higher temperature. Although the specificity of H-antigen is usually examined by H-agglutination of the flagellated bacterial cells for diagnostic purposes, H-antigenicity is demonstrated with flagella which have been isolated from the cells or with purified flagellins. For the latter, precipitation test, such as gel-diffusion technique, has been successfully applied (KERRIDGE et al., 1962). Both isolated flagella and flagellins are also able to induce corresponding H-antibody by

Table II.

Amino Acid Content of Bacterial Flagellins.
moles amino acid/mole flagellin

amino acid	*S. typhi-murium* SW 1061, 1.2 type[1]	*Proteus vulgaris* P[2]
glycine	22	14
alanine	42	18
valine	17	11
leucine	21	14
isoleucine	14	9
glutamic acid	27	16
aspartic acid	44	27
serine	17	11
threonine	29	14
tyrosine	6	2
phenylalanine	4	4
tryptophan	0	0
proline	4	0
cystine/2	0	1
methionine	1	1
lysine	10	8
ε-N-methyl-lysine	7	0
arginine	1	6
histidine	7	0
total	373	156
molecular weight	38,000	17,300

1. AMBLER & REES, 1959 & personal comm.
2. KOBAYASHI et al., 1959.

immunization of rabbit. Their H-antigen type specificity is not qualitatively distinguishable from that of the whole cell. Though the possibility exists that the antigenicities of a flagellum and its component flagellin are quantitatively different (KOBAYASHI et al., 1959), these results strongly support the idea that a flagellin molecule is the unit of H-antigens of a *Salmonella* cell. In other words, H-antigen specificity is a mirror of the tertiary structure of a flagellin molecule.

H-genes as the structural determinants of flagellin

Before starting the discussion on the genetics of H-antigen determinants, it is convenient to introduce a unique phenomenon of antigenic variation. A culture of many *Salmonella* strains manifests two alternative types of H-antigen. The one is called the "specific phase", or "phase-1", and another is the "non-specific phase", or "phase-2". Each *Salmonella* serotype has its own specific type of phase-1 and phase-2 H-antigens manifested by flagellar protein.

When a mass culture of such strain is plated it dissociates into the colonies of phase-1 and phase-2. During successive cultures of the two types, the population of each type give rise to cells of an alternative phase. The phenomenon was first studied by ANDREWS (1922) and has been known as antigenic "phase variation". The frequency of phase variation differ in different strains ranging from 10^{-5} to 10^{-3} per bacterial division (STOCKER, 1949). The *Salmonella* strains expressing phase-1 and phase-2 alternatively are called diphasic ones.

H-antigen determinants were disclosed by transductional analysis (LEDERBERG & EDWARDS, 1953). The transduction between different diphasic serotypes, for example from *S. typhi-murium i : 1.2* to *S. abony b : enx* gave the recombinants *i : enx* and *b : 1.2* but none of the *b : i, enx : 1.2* types (an exceptional type will be described in Section VI). This means that the antigenic specificities of phase-1 and phase-2 are determined by independent loci in each, which are symbolized by H_1 and H_2. They are on the same bacterial chromosome but located far enough from each other not to be transduced simultaneously by a phage particle (Fig. 1). Further transductional experiments in reverse direction and between different serotypes proved the generality of this conclusion and led to the establishment of two series of multiple alleles of H_1 and H_2. The genotype of H-antigens of *S. typhi-murium* is described by $H_1{}^{i}$ $H_2{}^{1.2}$, and that of *S. abony* is $H_1{}^{b}$ $H_2{}^{enx}$.

It must be emphasized that the antigen of each phase is a complex of at least several subunits, for example *e*, *n*, and *x* in phase-2 of *S. abony*, though they are often listed by a simplified symbol for descriptive convenience, such as *b* in phase-1 of *S. abony*. Many subunits, if not all, can mutate independently from the other (IINO, 1959). In genetic recombination, however, they are transferred as a unit by one of the *H*-genes.

H_1 and H_2 have been defined first as phase-1 and phase-2 antigen type determinants. Later, certain flagellar shapes were found to be controlled by the *H*-genes (IINO, 1962a). The most common mutant type in flagellar shape of *Salmonella* is "curly". Curly flagellum is characterized by a tighter pitch, half that found in the normal type (LEIFSON & HUGH, 1953). In serum agglutination test, H-antigens of normal and its curly mutant flagella behave identically. The curly character is phase specific and its expression accompanies phase variation. For example, a curly mutant of *S. typhi-murium* is curly in phase-1 while normal in phase-2. In transduction from a normal flagella strain to such a curly phase-1 strain, transductional clones with normal flagella were isolated. The transductional clones thus produced showed the antigen of the donor in phase-1 and that of the recipient in phase-2. The parallel results were obtained on curly phase-2 mutants. The replacement of the phase-2 curly deter-

minant by normal one was always accompanied by the replacement of the phase-2 antigen type by that of the donor. From these results it was shown that genetic determinant of curly phase-1 is at H_1 and curly phase-2 is at H_2.

The curly flagella are formed not only by curly mutants but also by normal cells when flagella are synthesized in a medium containing para-fluorophenylalanine in place of phenylalanine. This fact was demonstrated by flagellar regeneration experiment (KERRIDGE, 1959). Para-fluorophenylalanine is an amino acid analogue which is incorporated into proteins, replacing phenylalanine (reviewed by RICHMOND, 1962). When the proteins have certain biological roles, their function may be altered or lost by the replacement. In the synthesis of flagella, replacement of phenylalanine by the analogue in flagellin molecules may cause alteration of their molecular configuration, and consequently the change in the mode of their polymerization, resulting in the production of flagella with changed curvature. The biochemical process of flagellar formation in curly mutants might be analogous with that described above: the replacement of phenylalanine in flagellin by certain other amino acid might occur by curly mutation.

Just like the curly flagellar character, one type of resistance to a motility-specific phage has been demonstrated to be phase-specific (MEYNELL, 1961). Motility-specific phage attacks motile, not nonmotile, bacteria. Flagellins or part of them are presumably the receptor of this phage. Among *Salmonella* serotypes, those which have g . . . antigens are excluded from this rule. Even if motile they are resistant to a motility-phage, *chi*. When *g* . . . antigen is introduced into diphasic strains by transduction of $H_1{}^g$ . . ., the resulting transductional clones are resistant to *chi*-phage in phase-1 while sensitive in phase-2. The antigenic phase specificity of the resistance is also observed on a motile *chi*-resistant mutant of *S. typhi-murium*. The mutant is resistant in phase-2, *1.2*-type, while sensitive in phase-1, *i*-type (SASAKI, 1961). The determinant of the phage resistance and 1.2-type determinant, H_2, are always co-transduced.

The primacies of H_1 and H_2 as shown in the determination of the specificity of flagella strongly support the idea that H_1 and H_2 are the primary structural determinants, structure genes, of flagellin in phase-1 and phase-2 respectively. A mutation in one of these genes produces an altered configuration of the corresponding flagellin, resulting in a change in antigen type, a modification of the flagella shape or an alteration of the receptor site to certain bacteriophage.

As can be seen the excellent examples in the genetic and chemical analysis of human hemoglobin (INGRAM, 1957), and certain bacterial enzymes (YANOFSKY et al., 1961), it is the current view that the structural gene carries the genetic code of amino acid sequence in

the form of base sequence in its DNA chain, and that the amino acid sequence in polypeptide primarily determines the tertiary configuration of the protein. An alternative mechanism was proposed on the genetic determination of immunological specificity of insulin (BERSON & YALOW, 1961), and enzymatic and immunological specificities of tyrosinase (FOX & BURNETT, 1962). That is, certain genetic determinants of the protein specificity may control the type of folding of polypeptide; thus they may provide the chance of producing the proteins of different specificity with the same amino acid sequence under different genetic background (KARUSH, 1960). However, there is no definite evidence so far for the second mechanism, and the folding of a protein may be completely predetermined by its amino acid sequence.

Though the amino acid sequence is not known yet, the comparative amino acid analysis and the analysis of tryptic peptides of *Salmonella* flagellin (AMBLER & STOCKER, personal comm.; ENOMOTO & IINO, unpublished data) strongly suggest that the *H*-gene determines the actual amino acid sequence in the polypeptide of flagellin. The relative amount of each component amino acid differed among the flagellins of different antigen type. Differences are noticed even between two antigen types which represent phase-1 and phase-2 of the same strain. The finger prints of tryptic digests of *i*-flagellin and *1.2* flagellin, which represent the phase-1 and phase-2 antigen of *S. typhi-murium*, respectively, differ in 15 among 35 spots (STOCKER & AMBLER, personal comm.). The difference of certain amino acid composition between flagellins of normal and curly mutant has been suggested by preliminary experiment but is not conclusive yet. For the clear understanding of the determination of flagellin structure by the *H*-genes, we need the detailed fine structure analysis of the *H*-genes, especially the localization of the antigenic subunit determinants in *H*, and in parallel the analysis of the amino acid sequence of flagellin. Some hopeful steps have been taken in this direction. The comparative studies on the subunits of *g* . . . groups antigens (IINO, 1959) and on the mutants of *i*-antigens (JOYS, 1961) have shown that in H_1 there are sequential mutational subunits which correspond to the antigenic subunits. The intra-H_2 recombination was obtained between $H_2{}^{1.2}$ and $H_2{}^{enx}$ (IINO, 1960, unpublished data).

Genetic modification of H-antigen

Besides the specificity in the relative amount of each amino acid species, *Salmonella* flagellin has a remarkable chemical feature in that some of them contain N-methyl-lysin (NML), an amino acid that has not been previously found to occur naturally (AMBLER & REES, 1959). The amount of NML is about equal with that of lysine. Among the serotypes tested, *S. typhi-murium (i:1.2.3)* were NML-

positive in both antigenic phases. In certain other serotypes, for example *S. derby (gp: -)*, both NML-positive and -negative strains were found.

The analysis with transduction has shown that a gene which determines the presence or absence of NML is linked to H_1 (STOCKER et al., 1961). Unlike H_1 and H_2, this one gene determines the presence or absence of NML in both phase-1 and phase-2 flagellin. The immunological study of the recombinants between NML$^+$ and NML$^-$ strain disclosed an interesting correlation between H-antigen type and the amino acid: when *1.2* type NML-negative cells are changed to NML-positive by introduction of NML$^+$-gene, antigen *3* as well as *1* and *2* appear, and vice versa (STOCKER, personal comm.). The correlation between NML-positive and antigen-*3* positive is observed, only when the antigen type of the flagella is *1.2.(3)*. The H-antigen of *S. abony (b:e.n.x.)* is not altered by the presence or absence of NML.

In the foregoing chapter we came to the conclusion that H_1 and H_2 genes determine respectively the complete amino acid sequences of the phase-1 and phase-2 flagellins. Consequently, the NML-gene, which is phase non-specific and at a different locus from H_1 and H_2, is thought to modify the product of the H_1 gene, by engendering an enzyme which methylates some lysine radicals of already formed flagellin. The methylation of lysine, either in the form of free lysine or S-RNA bound lysine before its incorporation into flagellin is less likely, under our present understanding of protein synthesis.

Genetics of antigenic phases

In diphasic strains, two antigenic phases are determined at the separate loci. Therefore phase variation cannot be construed as the mutation of an antigen type determinant from one specific allele to another. Instead, it must be the *alternative manifestation* of each of the two antigenic specificities already inherent in the genotype. The transduction between single phase cultures of diphasic strains in all possible combinations showed that H_1 can be expressed only when transduced into phase-1 cells regardless of the phase of the donor, whereas H_2 can be expressed in any phases of the recipient but only when donor is in phase-2. This result, together with other supporting data (LEDERBERG & IINO, 1956), indicates that the H_2 gene plays a decisive role in the expression of the antigenic phases. The process of phase variation is thus explained as follows: "H_2 takes two different states, active and inactive. Active H_2 is epistatic to H_1 and inhibits the production of the phase-1 antigen, while it carries out the production of phase-2 antigen. When H_2 changes to the inactive state, corresponding to the change from phase-2 to phase-1, the production of phase-2 antigen stops and alternatively the production of phase-1, specified by H_1, proceeds."

Occasionally serotypic variants which express three or four antigenic phases have been isolated from nature (EDWARDS et al., 1962). As far as has been analyzed genetically, such tri- or tetra-phasic strains are duplications of H_1 and/or H_2 (LEDERBERG, 1961). They might have been produced by either translocation or by heterozygosis. The persistent heterozygotes of H_1 and H_2 are obtained either by transduction (Spicer and Datta, 1959) or by sexual recombination (HIROKAWA & IINO, 1961). In every case only one antigen type among three or four is expressed at a time.

As well as *Salmonella* having more than two antigenic phases, there are *Salmonella* serotypes and certain mutants of diphasic strains which express only one antigenic phase, either phase-1 or phase-2. They are called monophasic-1 or monophasic-2 type respectively. The serotypes carrying antigen *g* and the related antigens, such as *S. enteritidis gm:-*, are almost always monophasic-1 types, and H_2 could not be transferred from any diphasic strains to them, though the diphasic combinations of these factors are readily synthesized by transduction of the H_1 to any other diphasic strain. The genotype of monophasic-1 *(g)* type is represented by H_1^g H_2-deficient, for no homologous locus with H_2 of other strains can be detected by genetic recombination. *S. typhi d: -*, and some monophasic mutants of *S. paratyphi B b: -*, appear to belong to the same category.

Another group of monophasic-1 types, which have been isolated as variants of some diphasic serotypes, especially of *S. typhi-murium* and *S. paratypi B*, is the H_2 inactive type. They can become diphasic type by reversion or by transduction of H_2 from a diphasic donor strain. In some mutants, the phase-2 antigen type recovered by the transduction is consistently the same as that of the donor, indicating that the inactivation of H_2 is caused by mutation of a site not separable from the antigen type determinants; while in other mutants, diphasic transductional types with concealed phase-2 antigen of the recipient appear as well as the donor phase-2 type. Linkage analysis of such mutants disclosed a factor termed ah_2, which is adjoined to the phase-2 antigen type determinant, H_2, and controls the activity of H_2 (IINO, 1958a, 1962b): that is, the mutation of ah_2^+ to an allele ah_2^- causes the inactivation of H_2 and consequently the change from diphasic type to monophasic-1 type.

In contrast to monophasic-1 types, H_1 deficient type has not been detected among monophasic-2 strains either from nature or as laboratory mutants. All the monophasic-2 mutants so far examined were found to be the mutants in a region, termed ah_1, adjoining to H_1 (IINO, 1961a). The function of ah_1 parallels that of ah_2 in relation to H_2. The mutation of ah_1^- causes the inactivation of H_1, but it does not concern the activity of H_2. In such mutants H_2

changes its activity as in diphasic strains. Consequently, in phase-1 both H_1 and H_2 are inactive and the production of flagella is entirely stopped, while in phase-2 normal flagella are produced: the mutants undergo oscillatory changes between flagellated phase-2 and non-flagellated phase-1.

The last group of monophasic types include those which actually carry both H_1 and H_2 genes but have a greatly reduced rate of variation. A representative of this group is *S. abortus-equi* (EDWARDS & BRUNER, 1939). *S. abortus-equi* isolated from nature is generally in phase-2, *enx*-type. The phase-2 culture of this serotype is very stable and the alternative phase, *a*-type, is occasionally isolated after anti-*enx* serum selection. The phase-1 clone thus obtained is also stable: reversion to phase-2 can be detected only with strenuous selection with anti-a serum. Though there is no detailed quantitative measurement of phase variation on these serotypes, it is estimated to be less than 10^7 per bacterial division: this value is less than one thousandth of the frequency of phase variation in ordinary diphasic strains. Thus *S. abortus-equi* carries both H_1 and H_2 and undergoes phase variation, but the frequency is so low that a culture behave as if it is either (usually) a monophasic-1 or (rarely) monophasic-2 type. The transductions between such stable phase strain and a diphasic strain demonstrated that a major factor which regulates the stability of antigenic phases is transduced simultaneously with H_2 at the frequency of 30% (IINO, 1961b). The factor is designated vh_2. An allele vh_2^- is in *S. abortus-equi* and stabilizes H_2 in its existing state, whether inactive or active, and produces monophasic-1 or monophasic-2 types respectively. The ah_1^- monophasic-2 and the vh_2^- monophasic-2 type are identified by plating the culture on semi-solid media; the former dissociates non-flagellated phase-1 cells while the latter is stable in flagellated phase-2. The distinction between ah_2^- monophasic-1 type and vh_2^- monophasic-1 type is possible only after the stability of the phase of their phase-2 derivatives is examined: the phase-2 culture originated from the former by reversion of ah_1^- to ah_1^+ is diphasic, whereas those from the latter is monophasic-2. The genetic change from vh_2^- to vh_2^+ or the opposite direction has not been reported since. A similar type of monophasic behavior as that of *S. abortus-equi* has been reported in *S. paratyphi A* (BRUNER & EDWARDS, 1941; EDWARDS et al., 1950). The monophasic property of this serotype may be caused by stabilization of H_2 as in *S. abortus-equi*. Whether the genetic factor which stabilizes the antigenic phase in *S. paratyphi A* is identical to that of *S. abortus-equi* or not awaits further genetic analysis.

An extreme type of mutation in flagellar antigenic phase is that from flagellated to non-flagellated (O) type. The change has been called O-H variation. The mapping of flagellation genes has been

carried out in parallel with transduction (IINO, 1958b and unpublished data) and with cin-duction (SMITH & STOCKER, 1962 and unpublished data). The results are consistent with each other, and two chromosomal regions responsible for the flagellation both in phase-1 and phase-2 are assigned. The one is adjacent to H_1 and composed from at least three cistrons, *fla-A*, *fla-B* and *fla-C*. The other is located between try and gal loci, and the mutants in this region so far examined belong to one cistron. *Fla*$^-$ mutation of any one of these genes causes the loss of flagella.

Among the *Salmonella* serotypes, *S. gallinarum* and *S. pullorum* in group-D do not produce flagella and are H-negative. The spontaneous mutation to H-type of these serotypes has not been observed. Though the detailed genetic analysis of these serotypes has not yet been carried out, the preliminary transduction experiment suggests that they carry H_1^{gm} gene, but non-flagellated because of the deletion or multi-site mutation of a *Fla* region (LEDERBERG & EDWARDS, 1953).

The genotypes of several representative H-antigen types are summarized in Table III.

VI. EVOLUTIONARY ASPECTS OF SEROTYPES

As it can be presumed from the earlier discussion on the genetic determination of O-antigen, the differentiation of the O-antigen types may proceed by the loss, gain or modification of the ability to add a specific sugar to the polysaccharide skeleton of the microcapsule. This may be attained stepwise by either phage conversion or mutation of antigen type determinants. Actually, the differences of O-antigen types between E_1, E_2, E_3 and E_4 groups are explained by the presence or absence of symbiotic conversion phage(s) (Fig. 2). The differences of O-antigen type in group B are also explained by two conversion phages for antigens *1*, and *12*, and a mutation in a particular chromosomal gene for antigen *5*. As we have only scarce information on the chromosomal genes controlling the O-antigen production, it is not certain whether the differences in O-antigens between different groups are solely explained by accumulation of stepwise mutations or not.

The H-antigen situation is somewhat more complicated. If you look through the Kauffmann-White scheme (KAUFFMANN, 1964) you may notice that many H antigen types appear only in phase-1 while others in phase-2. However, there are several antigen types, like *e*, *l*, and *w*, which appear both in phase-1 and phase-2, indicating that the antigen type is not specific to antigen phase. Indeed, there are occasions that the same type of antigenic subunit appears in both phase-1 and phase-2 of the same strain as, in *S. salinatis deh, denz*$_{15}$. The same antigen *lw* is determined by H_2 in *S. wien b: lw*

Table III.

Genotypes of the H-antigen determinants in the representative Salmonella serotypes.

serotype	antigenic phase	antigen type	genotype						
			ah_1	H_1	ah_2	H_2	vh_2	ph_2	fla
S. typhi-murium	diphasic	i: 1.2	+	i	+	1.2	+	v	+
S. typhi-murium mutant SW1061	monophasic-1	—(i): 1.2	—	i	+	1.2	+	?	+
S. typhi-murium mutant SW1166	monophasic-2	i: (1.2)	+	i	—	1.2	+	?	+
S. abony	diphasic	b: enx	+	b	+	enx	+	v	+
S. paratyphi A	monophasic-1	a: (1.5)	+	a	+	1.5	—	—	+
S. abortus-equi	monophasic-2	(a): enx	+	a	+	enx	—	+	+
S. essen	monophasic-1	gm:	+	gm	0	0	?	0	+
S. gallinarum	nulliphasic	(gm):	+	gm	0	0	?	0	0

( ): unexpressed antigen type. 0: delection or multisite mutant. v: unstable state. ph_2: antigenic phase determinant.

while by H_1 in *S. dar-es-salaam lw: enz*$_{18}$ (EDWARDS et al., 1955). In some instances the antigenic phases classified by antigen type do not agree with the phases defined by genetic analysis. The phase-1 and phase-2 of *S. worthington* have been registered as *lw* and *z* respectively; in transduction, *lw* is found to be determined by H_2 and *z* by H_1 (EDWARDS et al., 1955). There is an isolation of *S. paratyphi B java*, which expresses *b* and *1.2* alternatively but at a lower frequency than the ordinary phase variation. Not only the *b*, but also the *1.2* factor of this strain was found to be determined by an allele of H_1 (LEDERBERG, 1961). The strain also carries a null-allele of H_2, allowing the synthesis of such types as ($H_1{}^b$) $H_1{}^{1.2}H_2{}^{enx}$.

Regardless of the seeming confusion in the correlation between antigen type and antigenic phases, there is actually a regular strict separation of H_1 and H_2 homologies in all strains above. Assignments of alleles to H_1 versus H_2 can be made unambiguously by genetic analysis, and the further behavior of each strain is perfectly consistent. They still precisely fit the rule of phase variation: Frequent state change of H_2 and epistasis of H_2 to H_1.

An unique anomaly in the homology of H-genes was, however, found on one occasion of transduction from *S. abony b: enx* to a monophasic-1 type of *S. typhi-murium-: 1.2* (IINO, 1961c). The recombinant expresses non-flagellated phase and *b*-phase, *b:-*, alternately, instead of *b: 1.2* or *-: enx*. By further transduction or reversion, *i:b* type was obtained from the recombinant. H^b of the strain behaves as an allelic locus of phase-2 antigen type determinant, $H_2{}^b$ rather than the original $H_1{}^b$. For example, the transduction from the recombinant to *a:1.5* type gave recombinants *a:b* and *i:1.5*. Phase variation in these strains occurs as frequently as the original recipient strain. Thus the anomalous recombinant is presumed to have originated by an exceptional recombination: H_2 locus is replaced by H_1 of the donor in the transduction, indicating residual structural homology between H_1 and H_2. The very rare chance of the event and the exclusive affinity of the translocated $H_2{}^b$ to other H_2 genes indicate that the barriers of the synapsis between H_1 and H_2 are not so much their own structural differences but may also be the differences of the other genes involved in a transduction fragment together with H_1 or H_2.

The genetic homology between H_1 and H_2 revealed from the studies of unequal recombination leads us to the speculation on the phylogenic relations of phase-1 and phase-2. We may infer that one of the *H* genes originated by duplication of the other. Then, which of H_1 and H_2 is the original locus? Like many other questions on evolution, one may never get the conclusive evidence on it. However, there are several indications which favor the idea that H_2 arose by duplication of H_1. For instance, there are numbers of monophasic-1 types deficient in H_2, but no H_1 deficient types have

been detected among monophasic-2 types. Clusters of the genes which control the sequence of a reaction process have been demonstrated for many nutritional characters. A similar gene cluster of flagellar formation is present at the H_1 region. On the contrary, the genetic factors known to be closely linked to H_2 are only the H_2 activity controler, ah_2 and its stability controller, vh_2. The interaction between H_2 and vh_2 is analogous to the variegated type position effect observed by translocation of certain genes to heterochromatic region (reviewed by LEWIS, 1950). Furthermore, in *E. coli* which is monophasic, only one *H* gene has been found and linked to *his*, to which H_1 of *Salmonella* is also linked (Fig. 1). Though allelism test of *E. coli H* and *Salmonella H* has not been led to a definite conclusion, it suggests that the *H* locus of *E. coli* is allelic to H_1 of *Salmonella*.

Based on these speculations, we may trace the evolutional pathway of antigenic phases as follows: The original type is primary monophasic-1 type having H_1 but not H_2. Duplication and translocation of H_1 happened in the monophasic-1 type. The synaptic homology between H_1 and duplicated H_1 was blocked by the disparity of the loci closely linked to each of H_1. The translocated *H* is now identified as a new locus, H_2, different from the original H_1. The H_2-gene was unstabilized by an influence of the adjoining chromosomal region. Then, the structural differentiation might have occurred between H_1 and H_2 and now the strain is recognized as a diphasic strain. Subsequently, a variety of further types has evolved through mutations and deletions in the diphasic system (of EDWARDS & BRUN, 1936), affecting the H_1 and H_2 genes themselves, their controllers ah_1 and ah_2, the variation factor vh_2 and the *fla* genes, respectively.

Next we will look into the combination of *H*-antigen and O-antigen of a serotype. The various *H*-antigen types are almost equally distributed to each group and it is difficult to find any correlations in the combination of O- and *H*-antigens. It might be a possibility that same types of mutations of *H* (or O) antigen have occurred in parallel in two independent strains having different O (or *H*) antigens. The more important mechanism might be, however, the formation of the new combination through genetic recombination. Several facts have been known to support this idea: Namely wide distribution of a specific genotype such as $H_1{}^gH_2{}^-$-x in various O-groups, *in vivo* occurrence of phage mediated transduction and conversion on antigenic characters (VELAUDAPILLAI, 1960) and the isolation of *Salmonella-Escherichia* hybrid (BARON et al., 1959b).

VII. REGULATION OF GENE ACTION

The mechanism of regulation of gene expression is a highlight of

modern genetics which aims to bridge between heredity and differentiation. The progress of biochemical and molecular genetics in the past decade succeeded in the establishment of the fundamental scheme of protein synthesis. The primary structure of a protein is determined by the nucleotide sequence of a chromosome region called structural gene. Messenger-RNA, which is complementary to the structural gene, is synthesized copying the later as the primer. The messenger-RNA (m-RNA) moves from nucleus to cytoplasm and fixes on a ribosome system. Transfer-RNA (t-RNA), each molecule of which carries an activated amino acid, comes to the ribosome and arranges itself along the m-RNA. A t-RNA has both amino acid specificity and pairing specificity to a coding unit of m-RNA. The activated amino acids, which arranged together with t-RNAs on the m-RNA, combine with polypeptide linkage side by side and leave from the ribosome system. The sequential polypeptide formation and stripping from the ribosome allow the synthesized polypeptide to fold in proper order and to form a protein with a specific tertiary structure. The mechanism of genetic regulation is being discussed in connection with these biochemical steps of gene expression (FRISCH, 1961).

A prevailing concept on the regulation of gene function is the regulator-operator theory formalized by JACOB & MONOD (1961). There is a unit segment of chromosome called operon. An operon is composed from an operator-gene and one or more functionally related structural genes linked to it. The operator-gene switches on and off the expression of the structural gene(s) directly, without mediation of cytoplasmic factor. Its function is presumably the initiation of m-RNA synthesis on the template of the following segment of DNA. The function of the operator-gene is managed by the product, termed repressor, of another gene, regulator-gene. The attachment of the repressor to the operator-gene prevents m-RNA initiation in that region and consequently inactivates the structural genes in an operon. The regulator gene may or may not be linked to the operon. In enzyme synthesis, the "inducer" is explained by its combination with the repressor, to inhibit the latter from attaching to the operator-gene. The repressor has been presumed to be non-protein substance; for example RNA. The possibility still remains, however, that certain kinds of protein might be a repressor (HORIUCHI & NOVIK, 1961), e.g. histone, which combines with chromosomal DNA and suppresses the synthesis of chromosomal RNA (HUANG & BONNER, 1962).

In *Salmonella*, a cluster of genes controlling the related function has been found on various auxotrophic mutants and fermentation mutants (DEMEREC & HARTMAN, 1959). In two groups among them, results have been reported which seem to suggest that the cluster fit to the concept of operon. They are histidine mutants (AMES et al.,

1960) and galactose mutants (FUKASAWA & NIKAIDO, 1961b). In each of these groups, a type of mutants show pleiotropic effect to block the function of all structure genes in the cluster (LEDERBERG, E.M., 1961). The mutants are not complemented by any of the structural gene mutants. Their mutant sites map at one extremity of the cluster. They were explained as the mutants in the operator gene.

The genetic system controlling the flagellar formation provides an interesting model of gene interaction. In the flagellar forming system, the genes H_1 and H_2 are assigned as the structural genes. The expression of the *H*-genes are regulated by several other genes: namely ah_1, ah_2, vh_2 and *flas*, as well as the interactions between H_1 and H_2 (section V). The *ah*-genes are phase specific regulators. An allele ah^+ switches on the production of flagellin controlled by adjoining structural gene, ah_1 for H_1 and ah_2 for H_2 respectively. A mutant allele, ah_1^-, or ah_2^- switches off the reaction in the corresponding phase. Ah^+ is dominant to ah^-, but the function of *ah* appears only in cis-position with the adjacent *H* (IINO, 1962b). It means that H_1 (or H_2) and ah_1 (or ah_2) behave as two component parts of a genetic functional unit. In chemical terms, an *ah-H* complex may be a unit of transcription of genetic code carried by a chromosomal DNA. It must determine the production and structure of a m-RNA. The *ah*-region in the *ah-H* complex may be a terminal segment which codes a part of flagellin polypeptide not responsible for the antigenic specificity but important for the folding of flagellin polypeptide; or it may be a region where the synthesis of the complementary m-RNA starts; or it may correspond to a terminal of m-RNA where polypeptidation of the orderly arranged amino acid starts. Another possibility is that *ah* carries the code of a region of m-RNA which is not responsible for the amino acid sequence in flagellin but for the specific affinity of the m-RNA to ribosome. The comparative analysis of the genetic fine structure of *ah-H* region and the amino acid sequence in flagellin will give the final conclusion on these aspects in the future.

You may notice that the ah-H system is in many respects analogous to the operon. The ah_1 (or ah_2) may correspond to the operator of H_1 (or H_2), and ah_1^- and ah_2^- to the operator negative mutants (O^c), which is inactive regardless the presence or absence of repressor. Then, is the phase variation explained as the repression and derepression of the ah_2-H_2 operon? If it is the case, we may assign ah_2^+ as the repressible operator (O^+); in phase-1, O^+ is repressed and H_2 is inactive, while in phase 2, O^+ is unrepressed and H_2 is active. The phase controller in diphasic strains is functionally indistinguishable from ah_2 except for the high frequency of state change in the former. Moreover, the recombination of the phase controller has not been demonstrated with neither H_2 nor ah_2.

Therefore, it is an acceptable hypothesis that both the phase controller and the ah_2^+ gene are identical. So far we have not detected any agent which works as exogenous inducer or repressor of phase variation. Consequently, it is premature to speculate on the nature of the endogenous factors. We can infer a phase-2 specific product which accumulates in the phase-2 cell and at a certain level of concentration represses the function of *ah*, perhaps the same agent that maintains the H_2 activity. The possibility that either flagella or flagellin in phase-2 is the repressor is excluded because of the fact that the state change of H_2 occurs even in the mutant which has lost the ability to synthesize flagellin (IINO, unpublished data). It is also unlikely that vh_2 is equivalent to the regulator gene which produces a repressor because in vh_2^- cell both phase-1 and phase-2 are stable.

"Formation and dissociation of the *ah_2-repressor* complex" is an attractive hypothesis to explain the state change in phase variation but we must keep in mind that it is not the sole possible explanation. It must be recalled that the H_2 locus had been originated by translocation of H_1 gene (Section VI). Thus the translocation may result in a certain structural anomaly at the junction of the chromosome and the translocated segment of the H_2 region. The vh_2 factor would then represent such a structural anomaly which causes the instability of the adjacent region where ah_2 is located; consequently ah_2 frequently changes its activity independently of any repressor substance. Both the constancy of the frequency and the randomness of the occurrence of the phase variation in different ages of a single phase colony under various cultural conditions (STOCKER, 1949, IINO, unpublished data) favors this hypothesis rather than the hypothesis of self-reinforced repressor production in phase-2, at least for the H_2 effect.

On the expression of the H-genes, the interaction between the two homologous genes, H_1 and H_2, superimposes on the ah-H system. In di-, tri or tetra-phase strains of various origin, we have noticed that only flagella of one phase are produced at a time by a cell. It is the general rule on all of these strains that H_2 is epistatic to H_1 regardless of the antigen type. How is the epistasis of a gene, namely H_2, to another homologous one, H_1, established? Several working hypotheses may be proposed for this question. It might be the interaction at the chromosomal level; for example, the ordered transcription of the genom in m-RNA synthesis favors the preferential production of phase-2 m-RNA, or either phase-2 m-RNA or (less likely) phase-2 flagellin acts as a cross-repressor of H_1, presumably by complexing at ah_1. Still other possibilities remain that the functional competition occurs at a localized site in cytoplasm, between phase-1 and phase-2 m-RNAs on ribosomes or between phase-1 and phase-2 flagellins at the polymerization step on a

flagella-forming apparatus. For the present, we can not exclude the possibility of any one of them.

In contrast to the above regulation systems, the regulations by the *fla*-genes are non-specific with respect to antigenic phase. The *fla*-genes are in functional units distinct from both of H_1 and H_2 genes. The dominance of *fla*$^+$ to *fla*$^-$ excludes the possibility that *fla*$^-$ produces repressor substance of the *H*-genes by itself. We may assume three other mechanisms on the function of the *fla*-genes. (1) *Fla*$^+$ supports the polymerisation of flagellin monomers. (2) Flagellin molecules are synthesized on specialized "flagellosomes" and *fla*$^+$ controls the production of such specific ribosomes. (3) *Fla*$^+$ produces internal inducer of flagellin. The test on the production of immunologically cross-reacting-material (CRM) of flagellin with twenty-five *fla*$^-$ mutants of *S. typhi-murium* and *S. abortus-equi* showed that, with one exception, they do not produce flagellin CRM at all (IINO & ENOMOTO, 1952). Presumably, they belong to the category (2) or (3). The remaining one, obtained from a strain of *S. abortus-equi*, produces a flagellin antigenically indistinguishable from the parental flagellin (IINO & HARUNA, 1960); the mutant can produce flagellin molecules but cannot polymerize them into a flagellum, hypothesis (1). The mutant gene is in a region different from either any other *fla*-genes or the *H*-genes.

As for hypothesis (2), the presence of specialized flagellar-forming apparatus in the flagellated *Salmonella* cells has been predicted from the flagellar regeneration experiment (KERRIDGE, 1960) and the deflagellation experiment with phenol (IINO & MITANI, 1962). The CRM$^+$ *fla*$^-$ mutant may be an excellent material for the studies on the genetic control of such a flagellar forming apparatus. Hypotheses of type (3) are always available when other evidence is not.

The central problem of development in higher organisms is the quasi-stable switching of gene activity — for example the transition from fetal to adult hemoglobin synthesis, the differentiation of isozymes, or the formation of specialized nerve-endings in the brain. Phase variation in *Salmonella* is a prototype of endogenous switching, in a simple organism amenable to genetic analysis. (Mainly lacking is a direct criterion of the chemical state of DNA from H_2-active and -inactive states.) It has already justified continued study by the specialist for its significance in the epidermology and evolution of *Salmonella*. Even more important it embodies the contemporary tradition of bacteriological research: Its unification with general biochemistry, genetics and cell biology.

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