RECENT ADVANCES IN BACTERIAL GENETICS

S. E. LURIA

Indiana University, Bloomington

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Bacterial genetics is today at a singular point of development. Scant knowledge and lack of agreement have until recently prevailed even on the most elementary facts of reproduction and character transmission in bacteria. The occurrence of sexual reproduction, although denied by most workers, was accepted by several others, mainly on the basis of suggestive but inconclusive cytological evidence. Variation in bacteria was interpreted by some as developmental, by others as genetic; and further complications resulted from attempts to explain by stricty physicochemical theories the supposed specific induction of bacterial variation (76, 58, 151), making bacteriology one of the last strongholds of Lamarckism.

Most of the sweeping generalizations that have taken the place of a genetics of bacteria are based upon interpretation of qualitative observations, often ignoring delicate population problems involved in distinguishing between cell character and culture character. In recent years, however, increasing attempts have been made to approach bacterial genetics in the same way that has been so fruitful in the study of the genetics of higher organisms, that is, by a "quantized" study of pairs or series of discrete, mutually exclusive characters. Of necessity, this approach has been limited until very recently to the analysis of variation, setting aside—though by no means ignoring—the problem of the mechanism of homologous transmission of characters, that is, the problem of genetic stability of which variation is the inseparable negation. Work has centered on the mode and mechanism of origin of new characters. Evidence for the existence of discrete unit characters and for interaction between character determinants has been collected. Attempts to compare the nature and action of these determinants with those present in higher organisms have been fruitful, thus opening the path toward an integration of the genetic systems of bacteria into a comprehensive comparative genetics. In turn, bacterial genetics has offered to the geneticist the unique proof of the possibility of specific induction of hereditary changes, in the case of induced serological type transformation (10, 22). Finally, the first genetically convincing evidence for some kind of sexual process in common bacteria has been offered within the past few months (91). Although we may find ourselves on the threshold of a deep change in our ideas of bacterial heredity, and possibly because of this, it seems useful to

bring together those results of bacteriological experimentation which satisfy the quantitative requirements of modern geneticists. This also in view of the fact that there have recently come to the fore of the genetic scene certain phenomena—particularly cytoplasmic inheritance—whose interpretation may have some bearing on the problems of bacterial genetics.

This review attempts to present recent work on bacterial genetics in a coordinated form, without claim either to complete coverage of the recent literature or to a detailed re-interpretation of the bewildering mass of observations on bacterial variability. These have repeatedly been collected in extensive reviews (102, 73, 28) and have recently been valuably discussed by Dubos (57). In the course of this review, however, we shall occasionally attempt to show how simple genetic principles may offer the lead for a correct interpretation of some of the most controversial aspects of bacterial variation.

That he may not be misunderstood, because of his choice of material, as arbitrarily excluding from the field of bacterial genetics certain observations that have previously caused much genetic speculation, this reviewer feels it desirable to put forward at this point a few statements of opinion:

1. The evidence for reproductive processes other than binary fission in bacteria is today rather suggestive; sexual, or at least fusion processes seem to take place (91, 93). Their occurrence, however, cannot at present be generalized; and most of the cytological evidence (102, 118, 119, 128) on the basis of which such processes have previously been postulated cannot be profitably discussed, since cytological observations have not been correlated with the study of transmission of well defined, mutually exclusive characters.

2. Cytological evidence for the existence of discrete chromatinic structures in bacteria, which may be described as homologous to nuclei, is very convincing (11, 137, 98, 146, 81). This agrees with the logical expectation for the existence of some structural device for equipartition of the genetic material at cell fission, in order to account for such high degree of genetic stability as is encountered in bacteria. Most of the available cytological evidence is again, however, of little help to the geneticist, since it does not yet supply either sure proof or analysis of such processes as mitosis, meiosis, or chromosomal grouping of genetic determinants.

3. It is impossible today to decide whether some cases of bacterial variation represent developmental (life cycles) rather than genotypic changes. On the one hand, proof of genotypic identity of two differing bacterial phenotypes is difficult to obtain; and cases of apparently cyclic course of variation can simply be explained on the basis of mutations, reverse mutations, and selection phenomena. On the other hand, it is on the test of accounting for the origin of permanent genotypic differences that the developmental theories of variation appear to fail. Nor does it seem profitable or justified to explain most cases of bacterial variation, as has been attempted (165), by segregation of characters from heterozygotes in some form of autogamy rather than by mutation. In fact, this only displaces the problem of the origin of the genetic differences found in the supposed heterozygote. Mutation, chased off the front porch, is read-

mitted by the kitchen door, with the added difficulty that it must now be fitted into the same household with a highly hypothetical guest.

In view of these considerations, the best approach to a discussion of bacterial genetics today seems to be an analysis of bacterial mutability in its origin, manifestations, causes, and effects on bacterial populations. We may then attempt an interpretation on the basis of the available evidence. By "mutation" we shall mean a permanent change affecting one or more properties of a bacterial cell and of its offspring. The use of this term does not imply *a priori* identification with the process of gene mutation or with any other type of hereditary change in higher organisms. Whatever similarities or differences exist should be discovered through the study of specific cases.

I. ANALYSIS OF SPONTANEOUS MUTABILITY

1. Detection and frequency of mutants

In the study of bacterial mutability we are faced with the problem of determining the differential properties of individuals within a population—which should be a pure line (36)—from the characters of the clones to which each individual gives origin (colonies, one cell cultures). Upon plating a uniparental population to obtain isolated colonies, mutants can be detected directly by colony observation, if they affect visible properties, or by testing bacteria from individual colonies for any desired property. The number of colonies that can be tested being necessarily limited, only frequent mutations can be detected by this method. The frequent mutants present special problems (41). Assuming a constant mutation rate (see below), the number of mutants increases during the growth of a culture by multiplication of previous mutants and by new mutations. In order for the parent type not to be displaced rapidly by a frequent mutant, the increase of the latter must be kept in check either by reverse mutation or by adverse selection. If reverse mutations occur, practically every sizable clone (visible colony) will contain a mixed population, often in equilibrium. The condition of equilibrium is given by the expression $M/N = a/b$, where M and N are the proportions of mutant and normal cells, and a and b the rates of forward and back mutation.

If the mutant type is handicapped by adverse selection, equilibrium will be reached at a condition defined by the equation $M/N = a/(s-a)$, where s is the "selection coefficient" defined as the difference between the growth rates of normal and mutant types (50, 41).

In most actual cases, the existence of both reverse mutation and growth rate differences makes the situation very difficult to analyze. One of the few cases in which only forward and back mutations are at play has been analyzed in the beautiful studies of Bunting (30, 31). Studying color variants of *Serratia marcescens*, Bunting found that in cultures maintained in the logarithmic phase of growth various types of mutant cells arose, each giving origin to new color types at constant rates. From the rate at which equilibrium was approached, forward and back mutation rates could be determined; for the mutation "dark

red-bright pink", these mutation rates were respectively 10^{-4} and 3×10^{-8} per bacterium per generation.

The difficulty of recognizing from the character of a colony the type of cell from which it had arisen could be overcome in Bunting's work because of the high degree of reproducibility of the pattern of variation within each clone, so that although each colony contained a mixture of types, the proportions in which these were present must have been characteristic for the type of cell from which the colony stemmed. When frequent mutations and reversions are present, the different types of cells can be better defined in terms of such mutational patterns and equilibria than of any one character of the clone stemming from each cell.

In Bunting's work, selection phenomena were encountered as soon as the bacterial cultures where studied in the ageing phase (32, 33). A case of frequent mutation apparently balanced only by adverse selection was that of the mutation R-S in *Salmonella aertrycke* (50, 154) which was found to occur at a fixed rate independently of the medium, whose only action was to alter the selection coefficient.

It is clear that, for most quantitative studies on the mutation process, frequent mutations are unsuitable, because of the difficulties encountered in determining the characters of the individual cell from those of the offspring. Rare mutations present different problems (106, 41, 105). A strongly selective environment is required to detect the presence of the mutants, and only mutants capable of growth in environments very unfavorable to the normal type can thus be detected (mutants capable of growth in media insufficient for the parent type, or resistant to inhibitory agents, or producing detectable fermentations). The problem here arises of proving the spontaneous origin of the mutations. Since the mutants can be detected only after exposure to the special environment, the hypothesis that the new hereditary character has been induced by this environment cannot easily be ruled out. The typical example is that of *Escherichia coli-mutabile* (Massini, 117): the parent strain does not ferment lactose but gives a stable lactose-positive variant. The opinion that the change is induced by exposure to lactose has been held by a number of authors. The demonstration by Lewis (96) that a fixed proportion (about 2×10^{-6}) of the cells in a negative culture grown without lactose will give positive colonies when tested in lactose has been considered by many authors as proof of the spontaneous origin of the variants. This evidence, although strong, is, however, not really conclusive: the same result would be obtained if exposure to lactose that is required for the final test produced the change in a constant, low proportion of the cells. The same can be said, for example, of acquired resistance to salts (153).

The problem of proving the spontaneous origin of rare mutations on the basis of the frequency distribution of mutants was analyzed by Luria and Delbrück (106) in relation to acquired resistance to bacteriophage. The distinguishing features of the distribution of the numbers of spontaneous mutants are those reflecting the clonal grouping of mutants in the cultures where they

originate, each clone stemming from one independent mutation. The first characteristic feature is an increase in the proportion of mutants during growth. This increase is difficult to establish for rare mutants, since the occurrence of rare mutations, obeying the laws of chance, is subject to large fluctuations, and successive samples taken from the same culture or from similar cultures give very erratic results. These fluctuations, however, are themselves a distinguishing feature of spontaneous mutations. If a change were induced by the test environment in a certain proportion of cells, this proportion should not differ from sample to sample, whether the samples come from the same culture or from different ones. If, however, the variants originate by mutation prior to the test, the chance occurrence of rare mutations will be reflected in large variation in their time of occurrence, and, therefore, in the number of individuals present in each mutant clone. This, in turn, will result in large fluctuations in the proportion of mutants in different wild-type cultures. Presence of such fluctuations in the number of mutants between cultures that have grown from one or few wild-type cells is strong evidence of clonal grouping, and hence, of mutational origin of the variants. This "fluctuation" test for spontaneous mutation was applied first (106) to proving the mutational origin of phage resistance, evidence for which had previously been offered (35), and which had been assumed by several authors (see 29). This experimental material is a most favorable one, since every single resistant cell can be isolated after quick lysis of sensitive populations of enormous sizes. Using *Escherichia coli* strain B and phage T1, enormous fluctuations were found in the proportions of resistant mutants present in series of similar cultures started from few sensitive cells. A wide distribution of the numbers of mutants was thus proved. In the absence of selection, the actual distribution of the mutants should only be a function of the mutation rate. The theoretical distribution to be expected from the hypothesis of a constant mutation rate (probability of mutation per cell per unit time) was not calculated because of mathematical difficulties, but an approximation to it (106) closely approximated the experimental distribution.

The interest of this type of analysis, besides the proof of spontaneous origin of mutation to phage resistance, is the possibility of defining and calculating mutation rates as intrinsic properties of the strains. Two methods were given by Luria and Delbrück for the determination of mutation rates, one of them based on the proportion of cultures without mutants, the other on the average number of mutants per culture. The second method can be used only when selection for or against the mutant does not occur. Both of these methods yield only rough estimates of the mutation rates; their limitations have been discussed elsewhere (106, 105).

Mutation rates were measured in probability of mutation per bacterium per generation (106). The choice of a physiological time unit was justified, at least for the case in question, by the finding that the mutation rate thus defined was the same in cultures of the same strain in different media, in spite of differences in growth rate, and by the demonstration that no new mutation occurred after multiplication in the cultures stopped. The same type of analysis was

applied by Demerec and Fano (47) to the study of the origin of resistance to other strains of phage. Mutation rates varying from 10^{-9} to 10^{-7} were found. The "fluctuation" test has also been used in the study of resistance to penicillin (44, 45) and sulfonamides (129) in Staphylococcus, resistance to radiation in *Escherichia coli* (178), and in the cases of the mutation from histidine dependence to histidine independence in *Escherichia coli* (148), and to uracil independence in *Clostridium septicum* (150). Some of these studies will be discussed later in relation to other aspects of bacterial mutability.

The demonstration that permanently acquired resistance to a number of antibacterial agents is acquired by spontaneous mutation is likely to be of general applicability to most types of resistance. It appears to contradict those theories according to which acquired resistance is explained in terms of a direct action of the antibacterial agent on the enzyme systems of the bacterial cell (76, 58), although such Lamarckian theories are often revived with rather surprising unconcern for the general outlook of modern biological thought (151).

Experiments on acquired resistance to antibiotics in Staphylococcus have recently led Abraham *et al.* (1a) to conclude that adaptation rather than mutation is the mechanism involved. Their data do not offer evidence for this conclusion, which appears to be based on misconceptions on the occurrence of mutations in pure cultures and on neglect of the population problems outlined above.

Presence of fluctuations in the number of mutants in similar cultures can be detected by inspection of the data of Lewis (96) for *Escherichia coli-mutabile*, and of Kristensen (87) for several fermentative mutations in Salmonella. The spontaneous origin of these fermentative mutations seems altogether well established.

One more result from the work on phage resistance should be noted (106): cultures were found that contained *only one mutant cell*. This was taken to indicate that a mutation can become phenotypical in the first cell in which it appears. Should the wild-type character persist in the phenotype of the mutated cell for one or more cell generations, the change might be expected to affect two or more cells at its first appearance (however, see 160).

Mutation rates for the diverse mutations listed above were found to range from 10^{-10} to 10^{-5} (105). Their values can be considered as rough estimates at best, particularly because in some cases there was evidence of selection for or against the mutant. An approximate method for estimation of mutation rates was used by Lincoln (97) in the study of colony type variants in *Phytomonas stewartii:* the ratio of the total number of mutants to the total number of cells was taken as a maximum value (assuming each mutant to arise from a separate mutation); the ratio of the number of cultures in which mutants were not found to the number of cells examined gave a minimum estimate (assuming not more than one mutation per culture). The mutation rates thus estimated were between 1×10^{-6} and 5×10^{-5}.

In all thoroughly analyzed cases, we see that bacterial variation, including apparent hereditary adaptation, is the result of sudden spontaneous mutations.

There are some cases, not yet investigated quantitatively, of apparently very slow adaptation, which might be difficult to interpret in terms of selection of one-step mutants (138). These cases, however, may be interpretable in terms of successive discrete mutational steps, as in the cases of quantitative resistance to be discussed in section I, 3, a.

2. Analysis of mutant characters

There are two approaches to an understanding of the mechanism of mutation. On the one hand, one can focus the attention on the mutational step itself, its statistical regularities, its independence of or interdependence on other mutations or on environmental conditions. On the other hand, one can analyze the effects of mutations in terms of specific physiological and biochemical changes, and attempt to retrace the primary mutational change from its end-effects. This corresponds to the study of physiological genetics in higher organisms, as examplified by work on hereditary anomalies of metabolism in man (65a), on pigment inheritance in mammals (183) and in insects (62), on flower color in plants (150a), and on biochemical syntheses in Neurospora (16, 13). The results of work on biochemical genetics have been summarized recently by Beadle (14).

The concept has become widely accepted that a gene affects a character by determining the presence and specificity of one of the enzymes whose action is necessary for the appearance of the character. Beadle and his collaborators have put forward and experimentally supported the generalization that each gene acts by controlling one specific enzyme. If the products of the reaction catalyzed by the enzyme are utilized in several chains of reactions, a gene change may affect more than one character. This "one gene, one enzyme" theory (170) has proved fruitful as a stimulus to studies in biochemical genetics, that in turn have provided a powerful tool for the analysis of biochemical syntheses. Gene mutations can produce specific metabolic blocks by suppressing the activity of specific enzymes. By studying the influence of individual gene mutations on production of nutritional requirements and on accumulation of intermediary metabolites, numerous chains of reactions have been traced in some detail, even though the corresponding enzymes have not been isolated. As to the mechanism of enzyme regulation by the gene, no conclusive evidence has appeared. Emerson (61) discussed the hypothesis that gene-enzyme relation may depend on a kind of complementary surface action—similar to that suggested by Pauling for antibody formation (135)—in which the gene would act by providing, directly or indirectly, a specific template for the synthesis of the enzyme molecule. The supposed analogy between antibody and enzyme formations has led to the suggestion that antigens may actually be primary gene products (74, 167), and that antibodies may affect the genes themselves with production of mutations, suggestion for which experiments on Neurospora, still awaiting confirmation, have offered some support (60).

It must be said that the hypothesis that each gene operates by regulating the presence and activity of one specific enzyme cannot be considered as more than

a fruitful working hypothesis. Most of the metabolic studies on Neurospora that support this hypothesis dealt with mutations causing nutritional deficiencies, bound to be due to suppression of specific enzyme systems. It is not apparent today how crucial proof for or against the hypothesis may be obtained (41a).

In bacteria, most studies on variation have dealt with characters whose interpretation in biochemical terms is still difficult. The vast field of antigenic variation and of variation in virulence (see 57) belongs in this category. An interpretation of pigment variation in terms of changes in the undoubtedly complicated synthetic reactions involved has not yet been attempted. Mutations involving changes in specific enzymatic reactions, however, have been described in recent years in increasing number.

Mutants with increased growth requirements (loss of ability to synthesize an essential cell constituent) can be detected directly by picking from individual colonies into a complete medium, and, after growth, testing the individual cultures for ability to grow in a minimal medium sufficient for the parent strain (147, 69). Mutants with nutritional deficiencies will not grow; their requirements can then be identified by determining the additions necessary to permit growth. The limited number of colonies that can be tested restricts this method to the detection of rather frequent mutants, although improved "screening" techniques can increase its efficiency (92).

Roepke, *et al.* (147) isolated by this method a number of deficient mutants of *Escherichia coli*, requiring nicotinamide, thiamine, methionine, cystine, lysine, arginine, threonine, and tryptophane, respectively. One additional mutant required either glycine or serine. Some of these mutants were obtained from x-ray treated cultures, others from non-irradiated controls. Biotin and threonine deficient mutants of *Escherichia coli*, and four mutants of *Acetobacter melanogenum* requiring glycine, serine, leucine, and adenine or adenosine, respectively, were isolated by Gray and Tatum after x-ray treatment (69). Additional deficient mutants of *Escherichia coli* were isolated later by Tatum and his collaborators (168, 169), and mutations of the same type were also discovered in *Bacillus subtilis* (see 169) and *Clostridium septicum* (150). The amounts of growth factors required in each case to produce maximum growth varied in individual cases between 10^{-4} and 5 micrograms/ml, being generally lower for vitamins than for amino acids, purines, or pyrimidines. A discussion of the individual findings in their relation to the chemistry of bacterial syntheses would be beyond the scope of this review; many of the pertinent data have been discussed by Tatum (169). All evidence indicates a basic similarity between mutation-produced deficiencies in bacteria and in Neurospora, suggesting similarity of the genetic mechanisms involved.

Synthetic deficiencies have also been detected in mutants isolated because of some other effects of the mutations. Anderson (4, 5) found that a certain mutation to phage resistance in *Escherichia coli* strain B, produced inability to grow in synthetic media, and identified the required nutrilite as tryptophane. The mutants are, moreover, unable to utilize ammonia nitrogen unless supplied with a relatively large amount of any of a number of amino acids. Deficiencies

for proline and for some other unidentified nutrilites have also been found coupled with phage resistance (181, 5). These associations were proved to be due to multiple effects of the same mutations and not to fortuitous coincidences of several mutations in the same cell.

The association of metabolic alterations with phage resistance is particularly interesting for a number of reasons. First, it offers the possibility of using phage resistance as the selective agent in isolating the mutants, so that all cells with the double mutated character present in a population can be isolated and counted. Second, it indicates the possibility of interpreting phage resistance in terms of specific metabolic changes, and hence, of deriving information on the biochemistry of phage growth.

An interesting metabolic mutation is the loss of ability to synthesize methionine in *Escherichia coli* while acquiring sulfonamide resistance upon transfers in broth containing sulfonamide (85). This case illustrates the role of selection in the establishment of a mutant type. Sulfonamide is known to interfere with the synthesis of methionine (156). Growth in the presence of methionine and sulfonamide must have selectively favored a mutant in which the sulfonamide-sensitive reaction leading to methionine synthesis was blocked. In the absence of methionine the mutation would have been lethal.

In contrast to the mutations just discussed, another group of mutations has been described which produces increase in synthetic powers (decreased growth requirements). These mutants are more easily detected, since they grow selectively in a deficient medium on which the parent strain cannot multiply. Even few mutants in a large population can be detected. Some of these mutations occur in strains which were deficient upon first isolation; others appear as reversions of mutations producing synthetic deficiencies. To this category probably belong the cases of so-called "training" to dispense with essential metabolites, for example, tryptophane adaptation in *Eberthella tyhposa* (64), nicotinamide adaptation in *Shigella paradysenteriae* (86) and in *Proteus vulgaris* (140), and thiamine independence in Propionibacterium (157). Uracil independence in *Clostridium septicum* was shown (150) to be produced by mutation. Histidine independence from a histidine deficient mutant of *Escherichia coli* (148) occurred at a rate of 10^{-8} per cell per generation.

Such cases explain the features of training experiments. Training is usually obtained either by transfer of heavy inocula to deficient media (chance of transferring at least one mutant), or by successive transfers in decreasing amounts of the nutrilite (selection for mutants that may arise during the initial growth of the normal strain in the partially deficient medium) (see also 65).

To designate a mutant which regains the ability to grow in media not containing any growth factor, Ryan and Lederberg (149) introduced the term "prototroph". When prototrophic mutants appear as reversions after mutations to deficiencies, it is important to establish if one is dealing with true reverse mutation or with a different mutation supplying an alternate pathway for the synthesis previously blocked. One possible way of deciding the question might be based on the expectation that a reverse mutation should reestablish the

status quo, restoring not only the primitive character, but also its mutability to the deficient form at the *same original rate*. This expectation may, however, be misleading, because of possible interactions with other mutations which may have accumulated in the meantime, and may act as modifiers of character expression or of mutability (see section VI, b). In cases of metabolic deficiencies associated with changes in other characters, it has occasionally been possible to prove that the return to prototrophic condition was not due to reverse mutation. For example, Wollman (181) found that proline deficiency and phage resistance could be produced by one mutation in *Escherichia coli*, but the apparent reversion to proline independence gave mutants which were still phage resistant. The apparent reversion must have been due to an independent mutation.

Other mutations producing what appear to be increased synthetic powers are those causing increased formation of some metabolite (pantothenate in pantoyltaurine resistant *Corynebacterium diphtheriae* (116), *p*-aminobenzoic acid in sulphonamide resistant staphylococci and other organisms (88, 89). Increased synthesis in these cases may be more apparent than real, since it may result from accumulation by the mutant of an intermediate metabolite more completely utilized by the parent type.

Appearance of new enzymatic functions by mutation is the feature of a whole group of phenomena involving utilization and fermentation of simple carbon sources. These phenomena include the classic examples of fermentative variation in *Escherichia coli-mutabile* and other gram negative bacteria, and the variation in utilization of various organic compounds as sole course of carbon. A typical example is that of *Moraxella lwoffi* (110, 8, 111). This bacterium can utilize alcohols but not sugars nor several fatty acids as sources of carbon. With heavy inocula of normal (N) cells in a medium containing succinate only, late growth appears, due to the presence of mutants (S+) in a proportion of about one in 10^8 normal cells. Succinate can be replaced by either fumarate or malate. The mutation involves a stabilization of the ability to decarboxylate oxaloacetic acid with the production of pyruvic acid: this ability disappears in one hour in strain N, while it persists in S+. A study of the effects of succinate, fumarate and malate on the splitting of oxaloacetic acid by S+ led Lwoff (109) to suggest that the mutation involves a change in an enzyme (or in a common precursor of a group of enzymes) catalyzing the first reaction in the attack of 4-carbon acids (phosphorylation?). It was not decided whether the mutation produced the transformation of the enzyme into a more stable form or the removal of an enzyme inhibitor. Two other mutations in *Moraxella lwoffi*, occurring independently of S+, produce ability to grow with glutamate or glutarate respectively as carbon source.

Mutational acquisition of ability to utilize citrate by *Escherichia coli* was described by Parr and his collaborators (132, 133, 134). It seems that the normal cells can undergo some degree of multiplication on citrate; growth stops early, however, whereas growth of the mutant is much more vigorous. In Parr's experiments, the citrate positive mutants appeared long after growth of the normal cells had stopped. The mutants can, however, be detected imme-

diately in a negative culture provided large enough inocula are used (184). In complete media, the mutants are specifically inhibited by some unknown product of the metabolism of the normal cells (184).

Parr and Simpson (134) described the occasional finding of some stable citrate negative variants not giving any positive mutant. The data do not encourage speculation as to the possible origin of stable citrate positive and citrate negative forms as homozygotes by segregation in a heterozygotic mutable type. Still less justified appear such speculations when applied (165, 166) to cases in which a mutable non-fermenting bacterial strain constantly throws off some apparently stable fermenting variants, but no stable non-fermenters. The classic case is that of *Escherichia coli-mutabile* (117, 96) although many similar cases have been described in the literature (see 57). *Escherichia coli-mutabile*, not fermenting lactose, gives a fixed proportion of lactose-fermenting mutants, which also can grow with lactose as sole source of carbon; the mutants appear to be stable, which only means, however, that they do not give lactose negative mutants in detectable numbers. It has been suggested (39, 40) that the difference between lactose negative and lactose positive cells consists in different permeability to lactose.

More light on the mechanism of these mutations came from Monod's studies on *Escherichia coli-mutabile* strain M.L. (122). Ability to ferment lactose is dependent on an adaptive enzyme system: the mutation L− to L+ produces adaptability. The same strain was found to give also an interesting mutation affecting galactose utilization. The normal strain G− grows slowly in galactose, while the mutant G+ grows very fast. Experiments proved that the galactozymase activity of G− is inhibited by some product of galactose utilization, whereas G+ produces less of this inhibitor and is not inhibited by it. This type of mutation in which an apparent increase in enzymatic activity is actually brought about by the overcoming of an enzyme inhibition, has been called "anaphragmic" (from ana = over, and phragmos = barrier) by Lwoff (109), who suggests that many types of apparently positive mutations, for example, the citrate positive mutation, may fall into this category. Interactions between the mutations L+ to L− and G+ to G− in the same strain has led Monod to suggest that both mutations affect a common enzyme precursor.

Altogether, biochemical studies of bacterial mutants show that many mutations affect specific enzyme systems, and it is often possible to attribute the effects of a mutation to a change in one specific enzyme. Bacteria may actually offer a most favorable material for the study of mutational enzyme changes. The relation between biochemical variation and evolutionary trends will be discussed later. We should point out here, however, that bacterial mutations suppressing synthetic ability will often act as lethals. A block of an essential enzymatic synthesis will suppress growth, and therefore be lethal, unless the organism happens to find in its environment, and absorb from it, the product or products of the missing reaction. In some cases, the missing essential metabolites may be replaceable by the products of some other enzyme reaction. A mutation lethal under the conditions of a given experiment will generally fail

to be detected unless occurring at extremely high rate, in which case it may reveal itself by reduced viability of the population as a whole.

It should be kept in mind that mutations might affect bacterial characters by mechanisms other than enzymatic changes. This may be the case, for example, for antigenic variation, including the well known cases of "phase" transformation, which, although seldom analyzed from the point of view of the mechanism of their origin, are probably caused by spontaneous mutations. The role of specific antisera in bringing forward antigenic variants has not been analyzed sufficiently, but it seems probable that in most cases the antiserum acts by inhibiting growth of the cell possessing the antigens with which they combine and allowing the variant cells to grow undisturbed or with less inhibition (57).

It is difficult to decide by which mechanism a mutation alters the antigenic pattern; the primary change may be supposed either to affect the mold or template on which the antigen is shaped (61), thus directly affecting the antigenic structure of the cell, or to alter some enzyme system involved in antigen synthesis. The frequent association of antigenic variation with metabolic changes might favor the second hypothesis. An interesting observation is that of P. Bordet (25) that growth at room temperature (20°) causes a completely reversible transformation of a smooth strain of *Escherichia coli* ϕS into a phenocopy ϕS20 of the stable rough mutant ϕR, which can originate by mutation from ϕS. Upon growth of ϕS at 20°C, the glucolipidic antigen is not formed, although the potentiality to produce it is present as can be shown by returning ϕS20 to 37°. It appears that in this case the mutation S→R causes permanent suppression of a synthetic reaction which in the S strain does not take place at 20°.

It is to be expected that any mutation altering the chemical structure of some bacterial protein or of some compound with haptenic properties may result in a change in the antigenic properties of the cell if the compound affected is located on the cell surface.

3. Relations between mutant characters

a. *Independent mutations.* Most bacterial strains can undergo changes in a variety of characters. This great variability of bacteria, often interpreted as a biological peculiarity of these organisms, can simply be explained by the relatively enormous size of bacterial populations, which offers an opportunity for occurrence and detection of even rare mutations. That various characters of the same strain can vary independently has repeatedly been observed, and interpreted as not supporting the "life-cycle" theories of variation (142, 82, 143, 144). The independent variability of different characters has been also interpreted (141) as suggesting mutations of different genes. Quantitative studies, however, on the independence and interdependence of mutations have only recently appeared. Before analyzing their results we must briefly deal again with the problem of multiple effects of mutations.

The already mentioned cases of association of synthetic deficiencies with phage resistance as a result of the same mutational step are good illustrations of such multiple effects (4, 5, 181). Other changes frequently associated with phage

resistance are variations in colony type and antigenic properties (35, 47, 175). An interesting type of variation is that of changes in growth rate: a large number of phage resistant strains, for example, has been found to grow at a slower rate than the parent type (105) in the regular media. Differences in growth rate and death rate have also been found associated with S→R variation (50, 26). In many such cases, it could be proved that the various changes resulted from the same mutational step. The simplest explanation of these cases, which we might call "pleiotropic" mutations (pleiotropic = producing more changes), in analogy to the expression "pleiotropic genes" (54), is that the mutation affects an enzymatic reaction involved in more than one chain of reactions. The phenotypic result of the mutation will be a change in all characters controlled by the affected reaction chains. Phage resistance may be associated with inability to synthesize a certain amino acid because of a block in a reaction responsible for the synthesis of a precursor of both the amino acid and the surface receptor for the phage (4). Need for two or more growth factors may arise from a mutation affecting the synthesis of a common precursor of the amino acids or of the enzymes involved in their synthesis (169).

The above interpretation is in line with the "one gene, one enzyme" theory of gene action. It is, however, possible that multiple effects of mutations may actually result from changes in multiple primary functions of the same determinant center (gene). The problem of pleiotropic gene effects, controversial in the case of higher organisms, is even less susceptible of fruitful discussion at the present stage of bacterial genetics.

Independently of their significance for the mechanism of the mutational process, multiple effects of mutations are important because, in the absence of crossing test, they facilitate the identification of a given mutational step when it occurs in strains already differing from one another by one or several mutations. Thus, it becomes possible to study the influence of various genotypes on the frequency and effects of one mutation.

In the clearer cases, the same mutation can be proved to occur in strains already differing in one or more mutant characters. This was particularly well demonstrated for mutations to phage resistance in *Escherichia coli* strain B (104, 47). Demerec and Fano, in particular, showed that a mutation to resistance to a given phage generally occurs *at the same rate* in the wild type and in a series of mutants (47). This was considered as suggesting changes at different genetic loci rather than a series of allelic changes at the same locus. Similar results were obtained (8) for three independent mutations causing ability to utilize different dicarboxylic acids in *Moraxella lwoffi*. It is known that mutations causing synthetic deficiences in bacteria also occur independently, and it has actually become common practice in their study to utilize, as a source of new mutations, strains "marked" by one or more mutant characters (genetic markers) in order to insure against misinterpretation of accidental contaminants as mutants (168). The new mutants must show the original mutant character in addition to the new ones.

The proof of independent mutability at different loci has made the study of

"mutational patterns" (41, 105) the most suitable method for an analysis of bacterial genotypes, because of the possiblity of tracing the presence of certain genetic loci in different bacterial strains.

An interesting case is that of several mutations affecting the same hereditary trait. This is well exemplified in the study of quantitative characters, for example, of resistance to various concentrations of drugs or antibiotics. Demerec's work (44, 45) on resistance of staphylococci to penicillin showed that resistance to increasing concentrations of the antibiotic is acquired by a series of successive mutations, each producing further resistance. Since the various mutations occur at comparable low rates, a sensitive strain will not directly give highly resistant mutants: these will appear only after the low grade resistants have been selected in presence of low concentrations which allow them to grow. Are we dealing with a series of mutations affecting, in different degrees, the same function or with independent mutations affecting different metabolic functions involved in penicillin sensitivity?

The question could be partially answered by the study of the genetics of sulfonamide resistance in a strain of *Staphylococcus aureus* (129). Here too, there are a number of mutations—at least five—that cause small increases in resistance; some of them can be distinguished because they produce different levels of resistance when occuring in the same strain. The study of associated effects of these mutations made it possible to single out one or two of them as causing constant increase in extracellular production of *p*-aminobenzoic acid (see also 88, 89, 152). These mutations could thus be recognized when occurring in strains already having different sulfonamide tolerance. It is apparent that resistance can be produced by alteration of a series of different sulfonamide-sensitive cell functions, each of which can be affected by one or more non-lethal mutations (see 156). Apparent increase in *p*-aminobenzoic acid production may actually be due to increased excretion because of a non-lethal block of its utilization, which, naturally enough, would result in increased sulfonamide tolerance.

Permanent, hereditary resistance to various salts, as distinguished from temporary adaptation (55) is also acquired by a stepwise mutational process (153). A similar situation, however, does not hold for all cases of quantitative resistance. Ultraviolet sensitivity in *Escherichia coli* strain B seems to be affected by one mutation only (178, 179), which produces a moderate degree of resistance, apparently by suppression of the ability of the wild type to react to small doses of ultraviolet radiation with an inordinate, semi-lethal increase in synthesis of protoplasm (elongation not followed by cell division).

b. *Non-independent mutations.* Up to now, we have discussed cases of different mutations occurring independently, possibly at different genetic loci, even when they affect the same phenotypic trait. In the study of resistance of *Escherichia coli* strain B to phages T1–T7, however, cases of complex interrelations are found, which may require additional assumptions for their interpretation (105).

Resistance to one given phage can result from any one of a number of different mutations recognisable by other effects—such as resistance to some other un-

related phage. If we indicate resistance to a phage Tn by the symbol /n, resistance to phage T1, for example, can result either from the mutation /1, also producing tryptophane requirement, or from the mutation /1,5 causing also resistance to phage T5. It is easy to prove the independence of these two mutations, which occur one after another at similar rates in the same clone, with resulting superposition of the corresponding phenotypes (104).

Another group of mutations, however, occurring more rarely, produce resistance to a number of phages, and the pattern of resistance resulting from each mutation is the exact superposition of that which can be produced by two other mutations also known to occur separately at independent rates (105). For instance, a mutation /1,5,3,4,7 produces exactly the same phenotype obtainable by successive mutations /1,5 and /3,4,7, or by the reverse series /3,4,7/1,5. This can be confirmed by examination of many other effects of the mutations involved, which are all found in the complex mutant. The complex mutation, although occurring more rarely, is too frequent to be due to chance occurrence of the two simpler ones together. Occasionally, some complex mutations are found to produce, in addition to character changes caused also by the simpler mutations, additional phenotypical effects, most of them indicative of deep metabolic disturbances (very slow growth, lack of gas production from sugars).

How are these complex mutations to be interpreted? According to the one gene, one enzyme hypothesis, one could simply assume that the two simpler mutations affect different enzymatic mechanisms, blocking separate reaction chains, while the complex mutation blocks a third reaction common to both reaction chains. This interpretation seems not only improbable—in view of the extreme complication of the reaction chains to be postulated in order to explain even a limited number of actual cases—but also rather pointless. In effect, such type of explanation, if repeated *ad infinitum*, might become a purely verbal interpretation, impossible to disprove (41a) and interfering with the recognition of other possible genetic mechanisms. Another view might be taken by assuming that the simpler mutations result from allelic changes at the same genetic locus, while the complex mutations represent a third allelic change. This interpretation seems unlikely, in view of the completely independent occurrence of the simple mutations, as discussed above.

A more likely mechanism appears to be one by which several mutations at different loci can occur together, by a deeper change in some material center carrying the hereditary determinants. This seems supported by the occasional association of deep metabolic disturbances with the complex mutations. It would be unjustifiable to debate now whether this center may be a complex molecular unit endowed with several, independently mutable specificities, or a more complex unit similar to chromosomes of higher organisms.

It could finally be suggested that complex mutations may occur because of some special conditions enhancing mutability and affecting simultaneously two or more distinct functions of the same cell.

Another type of unusual interaction between mutations to phage resistance has recently been found to involve an effect of one mutation on the rate of ap-

pearance of a different one (unpublished experiments by the reviewer). Influences of the genotype on the pattern of variability have often been described in bacteria, but in most cases they are likely to represent effects of the genotype on the rate of selection for or against the mutants, rather than effects on mutation rates. In *Escherichia coli* strain B, however, it was found that a mutation /2 (causing resistance to phage T2) does not occur with any appreciable frequency in the wild-type strain B but is found to occur regularly at rather high rate (about 10^{-7} per cell per generation) in a series of mutants B/3,4,7 distinguishable from one another by a number of minor differences. The different rate of appearance of the /2 mutation is not due to different selection; it is also unlikely, for a number of reasons, that the mutations /2 and /3,4,7 represent allelic changes. We must then consider, either an effect of the mutation /3,4,7 on mutability at a different locus—such as have been found in higher organisms (145)—or an interaction between the effects of two independent mutations. The mutation /2 might actually occur at the same rate in wild-type and B/3,4,7 mutants, but its effect may remain masked in the former because of a "suppressor" effect by the wild-type genotype, which effect is eliminated by the mutation /3,4,7.

This possibility has been mentioned because of the interest which the suggested mechanism might have for the genetics of bacteria (see Section VI). Moreover, complex mutations involving changes in a number of independently variable characters might be simulated by the occurrence of a "revealing" mutation in a cell already carrying a "suppressed" one, although this is probably not the explanation of the complex mutations discussed above.

Altogether, it appears that intensive study of the mutability patterns of some typical representative bacterial strains and of their mutants should offer a most interesting way of gaining insight in the basis of hereditary processes in bacteria. More work in this direction is greatly desirable.

II. INCREASE IN MUTATION FREQUENCY PRODUCED BY NON-SPECIFIC AGENTS

Induction of bacterial variants by a variety of environmental agents—chemicals, antisera, high temperatures—has often been claimed, and a Lamarckian belief in the inheritance of acquired characters has persisted longer among bacteriologists than among any other group of biologists. We shall see later that most of these cases may find a better explanation by the assumption of differential selection of spontaneous mutants. A number of agents, however, have been proved truly to affect mutability in bacteria, that is, to increase mutation rates and to cause the appearance of new mutations which had not yet been found to occur spontaneously.

The most useful agent of this type is radiation. Increases in the rate of dissociation ratio of mutant to normal colonies and in the rate of appearance of other variants have been described repeatedly after exposure to various types of radiation (71, 72). Most data, however, do not allow a decision as to whether the effect was due to selective killing or to increased mutability, and, in this case, whether by immediate or delayed action. Gowen (67) described a large increase

in mutation rate in *Phytomonas stewartii* after exposure to x-rays, and found rates of induced mutation of the same order for individual mutations in bacteria, viruses, and Drosophila. X-rays have been used to produce biochemical mutants in bacteria (147, 69, 168, 169), resulting in isolation of a variety of nutritionally deficient mutants. The mutations encountered were of the same types as those found to occur spontaneously, although a great many new types were also obtained. Because of the hit-or-miss mode of their detection, these various types of mutants are not very suitable for a study of the mechanism of action of radiation in inducing mutations. Changes whose spontaneous rates can be determined fairly accurately, and in which all mutant individuals can be detected, are better suited for the purpose. Typical of this are mutations to bacteriophage resistance. A very important study by Demerec (46) showed that ultraviolet radiation, as well as x-rays, increases the rate of mutation to resistance to phage T1 in *Escherichia coli* B, higher doses producing higher increases. The remarkable fact was discovered that mutations continue to occur at higher rate for a relatively long time after irradiation, and the mutation rate does not return to normal until several hours later, after the bacteria have possibly undergone as many as 13 generation cycles. Apparently all mutations thus produced belonged to the same types that also occur spontaneously. The data on delayed effect of radiation on mutability were obtained by an ingenious technique which permitted counting the number of mutations that occur in the population in a given interval of time directly rather than calculating it indirectly from the number of mutant cells present.

In further expansion of this work, the dosage effect was quantitatively studied (48). The number of mutations produced was proportional to the dose for x-rays, but increased more rapidly than the dose for ultraviolet rays. This held for both immediate and delayed mutations. The ratio "immediate/delayed" increased rapidly with the dose. Mutation frequencies as high as 2.8% were obtained with very high doses of radiation.

A delayed effect of radiation seems to be present also in the production of biochemical mutations (169). The rate of the mutation "succinate positive" in *Moraxella lwoffi* (36a) could also be increased by x-ray treatment.

Before we discuss these results, we wish to point out that induction of mutation by radiation is a completely aspecific process. The accepted theories of the mode of action of radiation (63, 90) indicate a direct action on molecules by transfer of radiation energy in elementary acts of absorption. Whether the molecule thus activated will undergo a certain change depends on the properties of the molecule and on probability considerations, but not on the nature of the radiation, provided the energy transferred in one act of absorption is greater than a given threshold. Radiation is accordingly supposed to cause mutations by raising the probability of occurrence of a multitude of mutations, and not by affecting specifically this or that mutable determinant.

An interesting case is that of a mutation to radiation resistance in *Escherichia coli*, which, besides occurring spontaneously, was found to be induced by radiation (179). In this case, there is simulation of a specific effect, but radiation is

again likely to act only by increasing the overall mutability, including mutability to radiation resistance, rather than by specifically acting on the latter.

The other group of agents, which, besides radiation, have been found to produce mutations both in higher organisms (9) and bacteria are the chemical compounds commonly known as nitrogen and sulfur mustards (β-chloroethyl amines and sulfides, 66). These also act in a strictly unspecific way, due to the high reactivity of certain groups in their molecules which allows them to react with a variety of substances. A number of biochemical mutants in bacteria have been obtained by treatment with nitrogen mustards (169). Treatment with 0.1% mustard for 30 minutes produced as much as one mutation per 100 treated cells, comprising a variety of biochemically mutated types. In this case too, most mutations seem to manifest themselves after a certain delay, indicating a mechanism basically similar to that induced by radiation.

Ark (6) reported production of some mutants in plant pathogenic bacteria by treatment with acenaphthene, a compound which has been found to be a powerful inducer of polyploidy in higher organisms. Ark's results do not prove, however, that actual induction of mutations is involved, and rather suggest selection for mutants, possibly spontaneous, in the presence of acenaphthene. It is interesting to note that attempts to produce bacterial mutations with colchicine have given negative results (176).

Interpretation of the data on non-specific induction of mutations by radiation and mustards brings us to the question of the mechanism of bacterial mutations, which will be discussed in the following section.

III. BACTERIAL MUTATION AND THE GENETIC DETERMINANTS OF BACTERIA

The mutational processes in bacteria, as described above, present so many similarities with gene mutations in higher organisms, where the existence of discrete genes can be proved by crossing-over and chromosomal break experiments (124), that a comparison of the mechanisms involved is indeed appealing. In both types of organisms, mutations occur in a random, apparently spontaneous way and at specific, generally low rates independent of physiological conditions. Once a mutation has appeared the new character has often a stability of the same order as that of the alternate character. Mutation rates spread very much over the same range of values, although the size of bacterial populations permits recognition of mutations rarer than can be detected in most higher organisms. The functions affected by mutations in bacteria belong to the same type as those affected by gene mutations in higher organisms (168): in both cases, it seems that mutations often affect enzyme specificity. These bacterial mutations can be induced by the same agents, radiations and mustards, that produce gene mutations. Specific induction of mutations by environmentally induced adaptation and inheritance of the acquired character can be disproved in almost every case. The most remarkable exceptions—type transformations induced by specific bacterial extracts (10, 22), to be discussed in a later section—belong to a separate category and, by their very nature, do not encourage a similar interpretation of the more common types of variation.

Mutations affecting different characters occur as a rule independently of one another in bacteria as well as in higher plants and animals: multiple effects of mutations may in both cases be ascribed to multiple results of a primary change. Apparently true reversion of mutant characters, a possible indication of the presence of determinants self-reproducing in the mutated form, has been observed even more frequently in bacteria than in other organisms (150).

Are we justified then, on the basis of these analogies, in assuming the existence in bacteria of discrete mutable determinants comparable to genes in higher organisms, and similarly endowed with the property of homologous reproduction both in the original and in the mutated form? That some mechanism for orderly segregation of character determinants at cell division exists in bacteria is a necessary postulate in order to explain the stability of cell characters. Although this can be visualized better in terms of distribution to the daughter cells of discrete material elements concentrated in some structural unit (nucleus?), we must admit that fairly orderly segregation might be obtained by equational partition of enzyme molecules present in large numbers; mutations could then appear when, by fluctuations in the division process, one enzyme happened either to be absent or present in amounts lower than a given threshold in one of the daughter cells. The constancy of spontaneous mutation rates, however, is hardly in favor of this hypothesis. Besides, reversion might be difficult to explain if mutation were due to the chance loss of a self-reproducing enzyme. It must be remembered that induction of specific mutations by changes of substrate was considered as the strongest evidence for the hypothesis of mutation by induced enzyme change (76). We have seen above that in all well investigated cases this type of induction has been disproved.

Interpretation of variation as due to segregation in heterozygotic diploid cells (165, 2), besides being devoid of experimental basis, would still leave open the problem of the origin of heterozygosis.

The possibility that very frequent mutations in actinomycetes appearing as sectors in colonies may be due to segregation of characters upon germination of heterozygotic conidia has been suggested by Badian (12) on the basis of cytological findings that require confirmation. This type of explanation encounters the obvious objection of failing to account either for the origin of heterozygosis, or for sector formation at stages of colonial growth when no conidia are formed.

Mechanisms of cell fusion, even if proved to be of more general occurrence than is now known (91, 93), cannot account for most instances of variation, since fusion within pure line clones should not bring about new characters. As for other "life-cycle" interpretations of mutation, we have already stated that there is no evidence in favor of them since independent and random variation is the rule; we shall see later that apparent directional series of variation may find their explanation in differential selection for certain mutant types common to a large number of bacterial species.

Besides this negative evidence, do we have any positive one for the existence of discrete genetic determinants in bacteria? The results of radiation experiments, although still of preliminary type, offer some pertinent evidence. It

has been found (48) that, at least for x-rays, the number of induced mutations to phage resistance is proportional to the dose. According to the generally accepted interpretations of radiation effects, this result should indicate a "one-hit" action in the production of a mutation. The primary mutational change must be the result of a single photochemical reaction process, involving a direct action on one or a few molecules within a limited spatial domain. Such a process could hardly produce mutations by mass inactivation of an enzyme scattered over the whole cell volume; action on a specialized center, photochemically reacting as a unit, seems indicated. The gene has been considered to be such a center (126, 171) and the one-hit interpretation of radiogenetic experiments is considered one of the main supports for the hypothesis of the gene being something like a nucleoprotein molecule (see 90). Results of radiation experiments have been considered before as supporting the hypothesis of a basic similarity between bacterial mutations and gene mutations (67).

It must be said that, although direct proportionality of the number of mutations produced to the dose is indication of a one-hit direct effect, it is not in itself sufficient proof. Only proof that the effectiveness of a given dose of radiation is independent of the temperature and of the intensity of irradiation (dose per unit time) would be completely satisfactory (90). It is to be hoped that such proof will be forthcoming.

The presence of delayed effects of radiation (46) and the non-linear relation between dose and effect in the case of mutations produced by ultraviolet light (48) indicate some complexities which the simple picture does not account for. Production of the mutational change by ultraviolet may require accumulation of a number of primary reactions, if each of these affected one of several equivalent portions of a material determinant. One act of x-ray absorption, producing a greater transfer of energy, may affect the whole structure producing the effect at once whereas each ultraviolet quantum may affect only one of the several portions. This might also explain the delayed appearance of some of the mutations. The presence of duplicated genes at the time of irradiation has already been suggested by Muller (125) and others to explain delayed genetic effects of radiation in Drosophila. Other interpretations of the delayed effect have also been suggested (46, 48). In spite of these complications, the presence of the "one-hit" type of action for induction of bacterial mutations by x-rays, if confirmed by further studies, would appear to be the strongest evidence for a direct action on discrete material units, comparable to genes, which determine the hereditary characters of the bacterial cell. The best cytological evidence available (81, 146) can be viewed as supporting this hypothesis by affording proof of the existence of discrete masses with the microchemical properties of desoxyribonucleoproteins, comparable to nuclei or chromosomes, in many and possibly all types of bacterial cells. Recent work (91, 93) demonstrating fusion with genetic recombinations in bacterial cultures (see Section V) may provide direct genetic proof for the existence of discrete heredity determinants.

Reed, one of the strongest advocates of the presence of genes in bacteria, has proposed a theory of bacterial variation (141) based on a mechanism of unequal

segregation of genes. By failure of a gene to divide simultaneously with the others, or by failure of the products of division to migrate to one of the daughter cells at the proper time, differences in genotype could arise. Transitional unstable forms would depend on successive unequal divisions in genes present in multiple copies. This hypothesis can be made to account for every type of bacterial variation, and is not clearly in opposition to the result of induction of mutations by radiation, which might affect the orderly division and segregation of genes rather than their structure. The following reservations should be made, however. If all bacterial mutations depended on irregularities of gene segregation, the independent occurrence of several mutations, some of them at very high rates, should be explained by assuming a less precise mechanism for gene segregation than is present in the chromosomal apparatus of higher organisms. We incline, however, to believe that some very precise mechanism for equal segregation of genetic determinants is necessary to explain the high degree of hereditary stability of bacteria. Moreover, the high reproducibility of the frequency of rare mutations, and its independence of physiological conditions (50, 106) do not seem to favor this interpretation. Unequal division, or loss of some segments of the hereditary material, may be responsible for the occurrence of complex mutations producing the same effects as two or more other mutations (105).

In trying to assimilate bacterial mutations to genetic changes in higher organisms we should not forget the existence in the latter of a group of phenomena which have come to the fore of the genetic scene within the past few years. These phenomena, only partially understood, involve cases of cytoplasmic inheritance and give evidence of the existence of cytoplasmic determinants of heredity, whose occurrence may be more common than has hitherto been recognized. Besides the semi-independent plastid inheritance, other types of cytoplasmic determinants ("plasmagenes") have been described, particularly in unicellular organisms. These determinants may show various degrees of dependence on nuclear genes. In certain races of *Paramecium aurelia* (158, 159) the presence of a given gene is required to insure continued production of each cytoplasmic determinant but is not sufficient to initiate its production. In yeasts, a situation has been described (99, 100, 161) in which a gene is needed to initiate production of a given enzyme but this production can then continue in the presence of substrate even after the gene is removed by appropriate crosses. The self-reproducing unit is supposed to be, not the enzyme itself, but a nucleoprotein (plasmagene) regulating enzyme production (162). It must be said that these experiments on yeast still require confirmation. Other possible examples of the role of plasmagenes in heredity have been discussed by Darlington (37).

It is thus likely that there occur various types of self-reproducing, mutable cytoplasmic determinants of heredity in plant and animal organisms. Their recognition is particularly important as they may offer a key to interpretation of differentiation in the course of development (158): character differences between cells with the same genotype might arise by differential segregation, irregularity of reproduction, or mutation of plasmagenes.

It is interesting to speculate on the possibility that bacterial mutations correspond to plasmagene changes rather than gene mutations, that is, to changes in cytoplasmic rather than in nuclear hereditary determinants. A choice, however, would be impossible to make at the present time, since we know even less about plasmagene mutations than about bacterial mutations. It may be of value, however, to suggest that plasmagene inheritance may prove less stable and more susceptible to environmental influences than gene inheritance. Cases of bacterial variation apparently caused by the environment, or regularly reversible, have been supposed (28) to be more similar to "*Dauermodifikationen*" (78), as described in protozoa, than to gene mutations. The mechanism of *Dauermodifikationen* is unknown, but it seems likely that their interpretation may lie in plasmagenic effects.

It is this reviewer's opinion that an important task of bacterial genetics today might be a critical reinvestigation with appropriate techniques of those cases of variation which appear to involve slow progressive hereditary changes under the influence of changing environment. Even if most of them should again prove, as we consider likely, to correspond simply to the ordinary type of spontaneous discontinuous mutations—selection phenomena complicating the course of variation—discovery of some new type of genetic mechanism might be forthcoming.

To conclude, we wish to suggest that a distinction between gene and plasmagene in bacteria might not be feasible. Differentiation between nuclear and cytoplasmic determinants may not have arisen in organisms which, as a rule, undergo little developmental differentiation and do not require a nuclear apparatus as elaborately organized as is needed for carrying out the meiotic process in sexual organisms. In such case, we might also envision the existence in bacteria of a more direct type of gene action than in organisms with genetic systems of higher complexity.

While this review was in press, there appeared an important article by McIlwain (116a), suggesting that a number of enzymes may be present in one or a few copies in each bacterial cell. The suggestion was based on a comparison between the number of molecules of certain vitamins per cell and the turnover number of several enzymes (number of molecules of substrate used up per second per molecule of enzyme), assuming that similar values obtain for the enzymes involved in vitamin synthesis. This suggestion leads to the hypothesis that enzyme production may be directly associated with gene reproduction, and that in bacteria some enzymes may actually be identifiable with the gene themselves, the latter having both autocatalytic (hereditary) and heterocatalytic (enzymatic) activities.

IV. SPECIFIC INDUCTION OF MUTATIONS

There is a group of phenomena in the field of bacterial genetics whose unique character makes them of paramount interest for geneticists and biologists in general as well as for bacteriologists. These are cases of true induction of hereditary changes by specific treatments which seem to reach into the very core of

the genetic make-up of the bacterial cell. The singular importance of these phenomena has not been recognized as early as desirable, first, because of confusion with many indiscriminate claims to induction of bacterial variation by practically every kind of environmental change, and second, because only recently have rapid advances been made towards the elucidation of the phenomena.

A number of cases in which bacteria appear to acquire, after growth in the presence of products of other strains, some characters of the latter have been reported (34, 180, 94). The characters affected may be virulence, pigmentation, thermoagglutinability, agglutinability by specific antisera. Some similar changes were reported as resulting from growth of two organisms in "parabiosis" in Asheshov tubes separated by collodion septa (101). To some of these results it may be objected that the changes might have resulted from selection of spontaneous mutants in the environment containing products of other bacterial types.

The phenomenon of type transformation in pneumococci is not subject to such doubts. The subject has recently been reviewed (112), and here we need only recall the most salient facts. A non-capsulated R form of Pneumococcus, derived for example from an S culture Type II, can be transformed into capsulated S forms of Type I, II, III, . . . by growth with dead pneumococci of the respective type *in vivo* (70) or *in vitro* (38) or by growth in presence of cell-free extracts of each specific type (1). The presence of serous fluids is required for the transformation to take place. Avery and his collaborators (10, 113, 114, 115) have brilliantly developed this work to prove the following facts: 1. The specific component in the inducing extract (TP = transforming principle) is a highly polymerized nucleic acid containing desoxyribose and specific for each pneumococcal type. This was confirmed by a number of methods, including inactivation of TP by purified, crystalline desoxyribonuclease. 2. The specifically active TP represents only a small fraction of the total desoxyribonucleic acid extracted from a cell, which is to be expected, since it should only consist of that fraction of the nucleic acid which is concerned with the particular character under study. Its activity must be enormous, since transformation can be produced by as little as 0.003 microgram of the total desoxyribonucleic acid fraction. 3. Since the transformed character persists in the absence of externally supplied TP, the TP must be reproduced indefinitely in the transformed cells. 4. Under optimal conditions, the R→S transformation can affect as many as 0.5 per cent of the cells of the R culture. This high proportion makes it unlikely that the transformed cells represent spontaneous mutants that only need TP for manifestation of the mutant character. It seems practically certain that the change is actively induced by the action of TP in what probably amounts to a random sample of the exposed population of R cells. 5. The role of serous fluids in the reaction has been partially clarified by recognizing the presence in them of a number of fractions involved in various phases of the transformation reaction, in particular in the sensitization of the R cells to the transforming action of the nucleic acid (115).

Recently, Boivin and his collaborators (22, 23, 24, 173) have obtained in

Escherichia coli results confirming entirely those described above for Pneumococcus. A non-capsulated R type derived from capsulated, antigenically specific type C1 (or C2) can be transformed into either of the capsulated types by growth in presence of desoxyribonucleic acid extracted from the capsulated cells of the appropriate type. Work seems to have been facilitated in this case through the circumstance that the transformation occurs in plain media without serous fluids, and that the nucleic acid appears to be more stable and, therefore, easier to extract in active form.

The significance of these results is manifold and far reaching. First of all, they prove that biological specificity of nucleoproteins can be carried not only in the protein, but also in the nucleic acid moiety. It is not known whether this nucleic acid specificity results from different proportions of certain components of individual nucleotides, or from different spatial orientation of common components. Even more important, the results show the possibility of altering the heredity of a cell by supplying an alternative form of desoxyribonucleic acid, a specific component of chromosomes, and possibly of the gene itself (121). One might speculate whether the new form of nucleic acid thus introduced is directly incorporated into the hereditary material to yield a self-reproducing nucleoprotein endowed with the new specificity; or, by its presence in the cell, causes a change in the synthesis of new nucleic acid later used in gene formation; or else, if it affects the specificity of some other determinant of heredity. It would certainly be of great interest to attempt production of other types of bacterial variation by specific bacterial extracts.

Substances causing type transformation in bacteria have been compared with viruses (see 180, 164) long before their nucleic acid composition was known. Both types of agents have in common the ability to induce new synthetic properties in a sensitive cell. How far the analogy supports the endogenous theories of virus origin can hardly be decided at the present time.

It is interesting to point out that phenomena of the same type, though not yet as thoroughly investigated, have been described in viruses. Fibroma virus can be transformed into myxoma virus by injection into rabbits of a mixture of active fibroma and inactivated myxoma virus (19, 20, 21). Exchanges in properties between different bacteriophages growing in the same host-cell (42, 75, and experiments by this reviewer, to be published), although still incompletely understood, may bear a relation to the phenomena of type transformation in bacteria. These phenomena again point to a more accessible genetic system in bacteria and viruses than has been proved to exist in higher organisms, since in the former the genetic determinants can be reached and altered by specific components of the nuclear material supplied from the outside.

V. FUSION AND SEXUALITY MECHANISMS

The occurrence of fusion and sexuality processes in bacteria has been claimed so often (and as often disputed) on the basis of controversial cytological evidence, that it would hardly be possible today for the worker without personal cytological experience either to reach a decision, or even to select reliable examples. It is, however, important to point out that most of the older material presented in

support of the hypothesis of sexuality in bacteria (102, 118) cannot be used as genetic evidence because of the lack of information on the exchange or recombination of discrete hereditary characters in the course of the supposed sexual fusion.

Cases like those described by Almquist (3) of "hybrid" forms with double serological specificity in mixed cultures of two different organisms can easily be criticized, among other reasons, because of the possibility of spontaneous variation or of induction by soluble products. The whole problem of formation of "large bodies" is controversial (52, 80, 136) and their interpretations range from sexual forms, to symbiotic growth of pleuropneumonia-like organisms with regular bacteria, to involution forms. The constancy of their formation at the line of contact between growth of different cultures of Proteus (51) might offer a suitable material on which conclusive genetic evidence for or against their origin by fusion could be obtained by working with genetically marked strains. Fusion with exchange of characters might have been involved in cases of transfer of properties between bacteria growing in mixed cultures, mentioned in the preceding section (34, 180, 94); but the mechanisms involved were not analyzed.

Strong evidence in favor of recombination of discrete unit characters in mixed cultures, although still without cytological confirmation, has recently been supplied by experiments with carefully controlled genetic material. Earlier attempts in this direction (68), although employing the correct technique of trying to hybridize mutants from the same strain differing by one or more visible characters, had given negative results, possibly because of the necessary inefficiency of the methods available for the detection of visible colonial variation.

The discovery of biochemical mutations in bacteria with production of specific growth factor deficiencies permitted Lederberg and Tatum to demonstrate by a brilliant technique the recombination of characters in mixed cultures of different mutants (91, 93). These studies, still in the preliminary stage, appear to be among the most fundamental advances in the whole history of bacteriological science.

Mutant strains deficient for two or more growth factors were produced by irradiation of a strain of *Escherichia coli*. Two strains, each carrying a different pair or group of biochemical deficiencies (double biochemical mutants), were then grown together in a complete liquid medium. After growth, large inocula were plated on minimal medium agar on which neither of the two strains could grow. Colonies appeared, consisting of cells that had permanently acquired the ability to grow on the minimal medium like the original strain of *Escherichia coli* (prototrophic cells). These cells must therefore have the ability to synthesize all four growth factors, combining the synthetic powers of the two parental strains. The frequency of prototrophs in mixed cultures was of the order of 1 in 10^6 bacteria.

Since reversion of one biochemical deficiency was never found to occur at rates as high as 10^{-6}, the chance occurrence of two reversions in the same line should be much too rare to be detected. In fact, prototrophic forms do not appear in pure cultures of each of the double biochemical mutants. This illustrates the importance of using *double mutants* for any study of recombination.

The prototrophic forms seem, therefore, to originate from true recombination

between cells of the two strains grown together. This recombination appears to involve segregation rather than formation of double cells. Experiments with triple mutants (sometimes including phage resistance as a marker) showed, in fact, that exchanges of only one or two out of three characters can occur with frequency comparable to that of prototroph formation. This also proves that prototrophic growth does not represent a symbiosis of the two parent types. Moreover, it has been found that segregation of characters is not random (personal communication from J. Lederberg). This may be an indication of some type of linkage of determinants in a material unit (chromosome?).

These experiments appear to prove the existence in bacteria of fusion followed by exchange of genetic determinants, similar to crossing-over, followed by separation of the fused cells. The possibility that the changes are produced not by fusion but by induction through the action of diffusible products has not been ruled out, but seems rather remote. Filtrates of one mutant did not cause the appearance of prototrophic forms from the other double mutant.

When fusion occurs, it may lead to the formation of heterocaryons, that is, of cells containing nuclei of two types in a common cytoplasm, as shown to be produced in a variety of fungi by hyphal fusion (15, 139). It seems unlikely, however, that the prototrophs obtained by fusion represent heterocaryons, because of the apparently independent segregation of characters, with the possible exception of cases of linkage. If the genetic determinants are concentrated in a nucleus, nuclear fusion must be postulated to explain these results.

Temporary fusion, followed by exchange of genetic determinants and separation of the fused cells, seems to be the correct interpretation; this would then represent a true form of sexuality in a very simple bacterium. The fused forms may represent a sporophyte, while the regular type of vegetative cell represents part of the gametophyte. It would be interesting to know how long the cells remain in the fused condition, whether they can divide while fused, or whether the sporophyte lasts only one cell generation.

It must be pointed out here that, independently of the tremendous importance of these results, the range of applicability of the conclusions derived from them cannot yet be evaluated. Fusion mechanisms could not be detected, for example, in another strain of *Escherichia coli*, either by using biochemical mutants (Lederberg, personal communication) or by using phage resistant mutants (unpublished experiments by this reviewer). In particular, it must be emphasized that there is as yet no evidence that fusion phenomena of this type may be responsible for the ordinary type of bacterial mutations. The phenomena of exchange of hereditary properties between phage particles growing inside the same host-cell, mentioned in the preceding section, present some analogy with the fusion phenomena in bacteria described above.

VI. SELECTION PHENOMENA AND EVOLUTIONARY CONSIDERATIONS

a. Selection phenomena. Although the evidence discussed in the preceding sections indicates that most bacterial mutations occur spontaneously rather than as a response to the environment, the latter plays an important role in determin-

ing the course of bacterial variation. This role may be twofold. On the one hand, bacterial mutations of apparently adaptive character may require the activity of the environment to render phenotypical a change that in a different environment would have remained masked. This is certainly true in the case of mutations permitting the production of adaptive enzymes, where the substrate is necessary to reveal the new potentiality brought about by mutation. It is possible that similar mechanisms are present in other cases. Mutations to phage resistance, for example, might conceivably become phenotypical only after the phage has actually attacked the mutated cell (106).

On the other hand, the environment acts by selectively favoring growth of certain phenotypes. We have already seen that bactericidal and bacteriostatic substances act as powerful selective agents permitting the detection of resistant mutants. The same is true of deficient media used in the isolation of mutants capable of dispensing with the missing nutrient.

How normal and mutant types will compete in an environment in which they both can grow depends on the effects of the mutation on metabolic processes determining growth characteristics. A bacterial mutation can bring about changes in a number of different characters (pleiotropic mutations). Changes in growth characteristics will affect the ability of the mutant to grow in competition with the normal type. Bacterial mutations may actually offer an uncommonly favorable material for the study of selection phenomena.

Mutations producing apparent increases in biochemical activities may prove unfavorable for survival under ordinary circumstances, although useful in the exceptional environment. Typical is the case of the succinate positive mutant in *Moraxella lwoffi* (110). Although capable of growth with succinate as sole carbon source, the mutant is rapidly overgrown by the normal type in media in which both of them can grow. The same is true of the phage resistant mutants of *Escherichia coli* B (105). While some of them grow at the same rate as the normal type in broth, a great many are found to grow more slowly, in some cases the growth rate being half as rapid. Unless in the presence of the specific phage, these mutations appear to be of no value to the strain, and the mutants will be more or less rapidly eliminated. A similar situation seems to obtain in the case of salt resistant mutants of Salmonella (153) and for a number of other types of bacterial variants (7, 55, 50). Also in the case of biochemically deficient mutants, it is likely that in a complete medium mutant and normal types may not show the same growth characteristics (148). Which of two phenotypes will establish itself in a mixture as the predominant one is not always predictable from the study of growth rates of the two types when growing separately (105, 148). Among the mutants from *Escherichia coli* strain B, some of the phage resistant mutants appear indeed to grow in mixtures with the wild type or with one another as they would in separate cultures, without appreciable interactions (unpublished experiments). The radiation resistant mutant, however, was found in careful studies (179) to behave differently. Growing alone in nutrient broth, the mutant has the same generation time and the same maximum viable titer as the normal, and a shorter lag phase; it might accordingly be expected, not only to hold its

ground, but to be successful if grown and subcultured in mixture with the normal. It was found, instead, that the mutant is rapidly overgrown in such mixtures, so that the proportion of mutants in mixed cultures diminishes rapidly. Such interactions indicate competition for substrates or effects of diffusible products of the metabolism of one strain on the growth of the other, and may give additional information on the biochemical effects of the mutations. Similar phenomena have been observed also in fungi, where competition occurs between nuclei carrying different alleles of one gene in heterocaryotic mycelia (149).

An interesting point is that differences in growth characteristics have been found to occur between cells of smooth and rough variants of the same strain (49, 50, 26). These differences are dependent on the medium used, and certainly play a large role in determining dissociation percentages, as has been indicated clearly in Braun's work on Brucella (26). The proportion of S and R cells in cultures at various stages of growth is the result of competition between the two types, competition that becomes very keen in the late phases of the life of the culture, when crowding brings about strong population pressure. Late growth and death proceed side by side, as shown by the increase in total cell count with constant or decreasing viable count. This situation favors the type which can grow better and survive longer under such crowded conditions. In Braun's studies, the R cells were found to fulfill these requirements, which explained their relative increase in ageing cultures. In a further study (27) the growth of S and R cells was investigated in presence of antisera against each phase, showing enormous selective advantage for the heterologous cells. An interesting metabolic difference between S and R variants in *Proteus vulgaris*, involving increased requirement for nicotinic acid, was described by Morel (123).

Growth rate differences of various cell types in a colony will give rise to sectors whose significance has been discussed by Shinn (155) and, in relation to fungi, by Pontecorvo and Gemmell (139a). A mathematical analysis of the relation between growth rates and shape of the sectors has been given by Waddell (174).

It is the opinion of this reviewer that studies of this type will provide the key to an explanation of most cases of apparently "directional" phase variation, in which different cultures appear to undergo similar series of orderly changes (73). We can imagine that in many different strains homologous mutations occur, producing similar colony types and also bringing about changes in growth characteristics which determine whether they will be favored or eliminated. The same mutation may be favorable when occurring before another and unfavorable if occurring after it; the apparent series of successive phases as in a developmental process would thus be explained. As pointed out in section I, 1, frequent mutations producing growth advantages can be expected to be checked by some degree of reversion, which is probably the cause of the apparently cyclic course of most dissociative patterns, with reappearance of the original type.

Selection phenomena probably explain most cases of supposed induction of mutations, for example, by antisera or by salts. It has recently been shown, for instance, that variants of *Chromobacterium violaceum* appearing in presence of LiCl show different viability in presence of this salt as compared with the parent strain (79); these differences can explain the apparent dissociative action of the

salt as due to its selective effect on various phenotypes. One should be particularly cautious before claiming induction of mutation by environmental agents when the change appears to affect the whole population exposed. It is very likely that in such cases a type arisen by spontaneous mutation has completely displaced the original type because of favorable selection by the special environment.

b. Bacterial mutations and evolution. The large amount of bacterial variability brought about by mutation provides ample material for natural selection to act and lead to the establishment in given environments of those biotypes whose combinations of genetic determinants represent "adaptive peaks" in the field of the available genotypes (53).

A great number of bacterial mutations involve loss of ability to perform certain metabolic tasks. Some of the mutations that appear to bring about new biochemical abilities are accompanied by associated changes which make their survival and establishment unlikely. These facts are found to be in agreement with the hypothesis of a "regressive physiological evolution", developed particularly by Knight (84) and Lwoff (107, 108), whose monographs should be consulted for a detailed account of the basis and implications of the hypothesis. According to Lwoff (108), one can trace through a number of evolutionary series, in microörganisms and also in higher plants and animals, a progressive loss of synthetic and metabolic potentialities. In bacteria, examples are seen in the transition from coliform to typhoid to dysentery bacilli, where there seem to occur successive losses of antigens, of fermentative capacity, and of synthetic powers (increased growth factor requirements). According to White (177) the antigenic evolution of the Salmonella group has taken place by successive and independent losses of antigenic components, all present in a hypothetical common ancestor. Evolution in bacteria (108) is supposed to have proceeded from autotrophic organisms, endowed with high synthetic power and ability to utilize light or inorganic compounds as energy sources, to organisms requiring some growth factors and deriving energy from the oxidation of organic carbon compounds. Further losses narrowed the range of energy sources utilizable and increased the number of required growth factors. Anaerobes seem to have originated from aerobes by loss of enzyme systems, among them those involving cytochrome and hematin. Obligate parasites finally derive from free living forms if after a number of mutational losses of synthetic power the cells find only in a living host the necessary materials for their growth. Extreme cases of loss of functions could bring to intracellular parasitism, and possibly to virus origin.

In parallel with these losses of metabolic activities, one observes increased specialization, and often increased ability to perform certain specific functions. According to Lwoff (108) this specialization can by no means be considered as a true progress, since it is accompanied by reduced adaptability, and, therefore, by reduced chance of survival. The more specialized the metabolism of a bacterium, the more dependent it will be on particular sets of environmental conditions. Even slight changes in these conditions may mean extinction for the species.

According to Lwoff (108) the changes underlying physiological evolution are

at least in part the result of *tendencies* inherent in the heredity of the bacterial cell, although the environment would play a partly active role in determining mutational changes, as also supposed by Knight (84). The discussion of bacterial mutability which forms the major part of this review suggests, however, that spontaneous mutability is the mechanism that, in bacteria as well as in higher organisms, brings about a variety of phenotypes on which the environment exerts its selective role. We feel, moreover, that the idea of special tendencies to variation in given directions may be misleading if interpreted in any other way than as the identification of each genotype with a set of independently mutable determinants. Some of the supposed "regressive orthogenetic series" described by Lwoff (108)—particularly in the case of losses of individual reactions in the same reaction chain—are probably chance directions of evolutionary change followed under the pressure of random mutability and natural selection.

One special factor is likely to operate in the apparently progressive loss of successive steps in given reaction chains. Lack of ability to synthesize an intermediary metabolite will result in a requirement for either the metabolite itself or the end product of the reaction chain in which the metabolite is involved. If the new habitat contains the end product—which may well be more readily available than the intermediary metabolite—mutations producing blocks of other steps in the same reaction chain can then be accumulated without adverse selection, and the process may lead to loss of the whole series of reactions involved in the synthesis of the end product. Thus, a mutant unable to perform any one of the steps necessary for synthesis of thiamine may undergo successive losses of the ability to perform the other reaction steps needed for thiamine synthesis.

Although the evolutionary role of biochemical loss mutations is undeniable, it is possible that this role is not nearly so unique as seems suggested by Lwoff. Mutations by acquisition of new synthetic abilities have been supposed to have played a fundamental role in the early stages of life on earth (77), as the supply of organic compounds of prebiological origin (130) began to run low. Even in the present highly complex organic environment, a number of positive biochemical mutations have chances to affect evolutionary trends. It might actually be expected, as suggested to this reviewer by Dr. R. Y. Stanier, that every time in the course of evolution a new compound was synthesized and set free in nature, some microörganism must have been present that possessed, or developed by mutation, the ability to attack the new compound. It is likely that the examples of positive mutants being at a disadvantage when competing with their parent strains (110) do not have the general significance attributed to them. It stands to reason that most mutations occurring in a well established genotype will be somewhat detrimental in the original environment to which the parent type is well adapted. They will, however, have definite survival value if a change of environment happens to require the newly acquired biochemical property.

It has been suggested (108) that synthetically deficient mutants, if properly supplemented, may draw an energetic advantage from not having to perform the missing synthesis. A situation suggesting confirmation of this possibility has been described in Neurospora (149).

A word is possibly in order concerning the relative survival value of mutations and reverse mutations, and the irreversibility of evolution. In bacteria as in higher organisms (127), we may expect that if reverse mutation occurs after a mutant has grown for a certain time, the resulting type may not restore the original situation, so far as survival capacity or even gross phenotypic effects are concerned. During the time in which the mutant type has grown in a certain environment, mutations at other genetic loci may have occurred, and been selected for, that altered the genotype in such a way as to render our mutant better suited for life, possibly by taking over some of the functions in which the mutant determinant was handicapped in comparison with its wild-type allele. After this has occurred, reversion of this determinant to the wild-type allele will not lead to the *status quo ante*, but may actually give a less favored type. We must keep in mind that natural selection is always at work on the genotype as a whole rather than on individual characters, with the result of making a strain better fitted to life in the environment in which it has grown for any length of time. Of course, if the environment is highly specialized, the increase in adaptation may result in lack of surviving ability in a less specialized milieu.

It is interesting to note that in bacteria (31), as well as in higher organisms (127), the expectation is verified that very mutable characters often present rates of reverse mutations higher than the direct mutation rates.

If bacteria are throughout all or most of their life in a haploid condition, natural selection may be expected to work rather exactly, since all mutations are likely to find immediate phenotypic expression. This in turn will tend to reduce adaptability, since immediate selection for or against one mutant character will reduce the number of genotypic combinations available in a population. This is possibly counteracted, in the case of bacteria, by the enormous size of the populations, which increases the variety of mutant types presented to the changing whim of selective forces.

It is also possible that interactions between mutations (see section I, 3, b) may provide mechanisms by which a larger variety of genotypes is available in bacterial populations. If fusion and recombination mechanisms, discussed in a later section, were proved to be of general occurrence, they would certainly play a tremendous role in increasing the range of genotypes, and therefore the evolutionary potentialities of bacteria (53).

c. Bacterial genetics and classification. A few remarks may be added concerning the bearing of genetic research on the problem of bacterial classification. As has repeatedly been pointed out (163, 172), most of the schemes of bacterial classification in current use are determinative keys rather than natural classification systems. A determinative key is meant for practical use by a certain group of specialized workers, and as such can emphasize whatever category of bacterial similarities or differences these workers are interested in. For such purposes, the best definition of a species remains that by W. Benecke (17): a species is "what the worker who defines it includes in it according to his scientific tact".

The geneticist has no direct interest in determinative keys, but only in natural

classification. For the present, genetics can contribute little to bringing under control the hornet's nest of bacterial taxonomy, but may suggest some useful precautions in approaching it.

It is first of all important to realize that a bacterial species cannot be considered the strict equivalent of the taxonomic species in organisms with recognized sexuality, since in bacteria we lack the important criterion of partial or complete sexual isolation (53). A species, a genus, a tribe, or a family can only be a larger or smaller section of a clone including certain biotypes recognizable as sufficiently stable, similar to one another, and distinguishable from representative biotypes in other groups.

In assigning taxonomic positions in a hierarchical order a given differential criterion should be considered the more fundamental, the larger the number of differences in independently variable characters it involves. Phenotypic distinctions resulting from differences in a number of individual unit characters are extremely unlikely to be erased or to merge into one another by any sudden genetic change. On the contrary, differences that can be brought about by a single mutational step, even if phenotypically striking, are of little value for classification and should not be made the basis for taxonomic differentiation. By keeping this criterion in mind, we may hope to arrive at some kind of natural classification in which the different clones receiving taxonomic rank actually represent well established biotypes. These should correspond as much as possible to "adaptive peaks" in the almost continuous array of genetic combinations on which natural selection is at work (53).

A complex metabolic process, in particular a certain "type of metabolism" involving elaborate chains of reactions should be a valuable taxonomic criterion provided the differences between phenotypes cannot be traced to a change in one single link in the reaction chain. The same may be true of important differences in cell shape.

It is interesting to notice that in many current systems of bacterial classification "species" and even "genera" or "tribes" are often separated on the basis of character differences that may be brought about by a single mutational step: for example, the tribes *Escherichiae* and *Proteae*, the genera *Salmonella* and *Eberthella*, the species *Staphylococcus aureus* and *Staphylococcus albus* in the classification of Bergey's Manual (18). Genera (for example, *Phytomonas*) are separated from closely related groups (*Pseudomonas*) on the basis of plant pathogenicity, a character that may well arise or disappear by mutation (97). It is obvious that such mutable properties can be used only in practical determinative keys without claim to any taxonomic significance. Even then, the greatest caution should be observed, since variable characters may prove too elusive to permit recognition of organisms of practical importance. In many cases, description of a variability pattern might prove a much better taxonomic criterion than description of any one or more of the variable phenotypic traits themselves (131). Similarities in the mutability patterns of different strains are likely to indicate important genetic similarities, because they must depend on the possession of a common set of mutable determinants.

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