Bacterial Physiology

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in the course of which, presumably, significant properties are unaltered. That is, we postulate a uniform and conservative transmission of characters in heredity.

For some early bacteriologists, the limit of bacterial genetics was this theory of perfect stability, or "monomorphism." Unfortunately, as Dubos (1945) points out, "the blind acceptance by several generations of bacteriologists of the Cohn-Koch dogma of constancy of cell forms and immutability of cultural characteristics discouraged for many years the study of the problems of morphology, inheritance, and variation in bacteria." Monomorphism is no longer acceptable, principally because variations can no longer be dismissed as poor technique. But an equally cogent objection to monomorphism is its inability to interpret the differentiation of microbic species by evolutionary processes which leaves the incredible alternative of special creation.

We now know that bacteria are liable to a nearly unbounded array of hereditary variations that are of utmost importance in all areas of bacteriological research. During the past decade, especially, the integration of the experimental data of bacterial genetics into a unified theory that includes all living forms has rapidly approached consummation.

I. The Gene Theory

The existence of material within the cell especially concerned with genetic functions can be inferred on general grounds from the cyclical behavior of organisms (Muller, 1947). In higher animals, the life cycle is outwardly apparent, involving an intricate developmental process from the outwardly undifferentiated egg to the adult. The series of cycles, egg-adult-egg-adult, suggests some system or substance which is acyclic and whose continuity is responsible for the perpetuation of characters. This substance would also have the property of reduplication, in parallel with the proliferation of the organism at each cycle. This material is called, collectively, the "genes." The individual genetic unit or gene is recognized, or rather defined, in a number of ways, the most pertinent for the present being the control of a distinct and unique character. The definition of an individual gene as an ultimate unit is however one of the most mooted problems of contemporary genetics.

The cycles of bacteria are so much less obvious that, at first, the entire cell might be thought genetic. Closer examination shows, however, that bacteria may also show cyclical behavior analogous to ontogenetic cycles. For example, typhoid bacteria characteristically produce flagella on ordinary nutrient medium, but this potentiality is suppressed in the presence of phenol. When the bacteria are replanted into ordinary medium, flagella reappear which are serologically identical with those

originally formed. The flagellum cannot, therefore, be entirely self-determined; its potential production must depend at least partly on the genetic continuity of something outside the visible flagellum, persisting and reduplicating when the flagellum itself is suppressed. Such persistence in latent form, and subsequent reappearance, fits our definition of a gene. A similar cycle is seen in the production by cells of an adaptive enzyme formed only when they are grown in the presence of its specific substrate. The inhibition of pigment formation at elevated temperatures by *Serratia marcescens* or *Staphylococcus aureus* and its renewal at lower temperatures constitute another similar cycle.

Although these experiments justify a conception of the genes as something more restricted in quality or extent than the cell as a whole, they tell nothing of genetic variation, *i.e.*, the crucial exception to the rule of hereditary constancy.

II. Genetic Variation

A. Spontaneous Mutation

Sudden, unpredictable changes in the morphology, serology, or biochemistry of a bacterial culture are familiar to every bacteriologist (for examples, see Dubos, 1945). On a uniform medium, a "pure culture," perhaps even carefully isolated as a single cell, may give rise to a colony different in color or texture from its siblings, or to one which has lost or gained certain enzymes or antigens. Characteristically, the new variants will breed true to their new quality, although they are no more immune to variation than their parent, and may even on occasion revert to the original type. Inasmuch as these differences will be maintained between cultures propagated side by side under identical conditions, they cannot be temporary physiological modifications like those cited as cycles above, but must represent changes in the intrinsic hereditary quality of the cells, *i.e.*, in the genes. Genetic variations which are (presumably) based upon qualitative changes in single genes are called mutations. Probably, most bacterial variations are mutations (Lederberg, 1949b).

1. Autonomy of Spontaneous Mutation

The most striking characteristic of mutation in higher organisms is its "blindness," or "molar indeterminacy" (Muller, 1947). That is, it occurs without reference to the life conditions of the organism, or to its benefit or injury to the organism or species. As yet, we have no chemical or physical agent by means of which we can approach particular genes or induce their mutation in a predetermined direction.

Mutations which occur independently of consciously designed experi-

mental procedures are called spontaneous. Spontaneous gene mutations may be thought of as chemical accidents, perhaps errors in reduplication during growth, or collisions with occasional molecules with sufficient kinetic energy to activate a chemical change in the gene. The kinetic activation theory is supported by the temperature dependence of spontaneous mutation rates, whereas the reduplication hypothesis finds support in the apparent correlation of spontaneous mutation with bacterial growth, as will be subsequently discussed.

The prevalent theory of organic evolution through natural selection proposes that the environment in general has no directive influence on mutational events as they occur. Despite this autonomy, the environment must and does play a decisive role in determining whether a mutant cell, arising among millions of other offspring, will be so favored in its growth and survival that its descendants will have a discernible part in natural history or laboratory experiment. The geneticist finds the bacteria unique material for his experiments: each culture tube is literally a microcosm in which he may trace evolutionary processes on a scale otherwise out of human bounds. This advantage poses a unique dilemma. In the most cogent processes, mutations can be detected only by application of the selective environment. How can the role (or lack of it) of such an environment in the initial event of mutation be tested when it is an indispensable element of the experiment?

Since d'Herelle's classic observations, it has been common knowledge that most bacterial cultures which have been lysed by bacteriophage later give rise to a secondary growth which is resistant to the phage. Since the resistant form can be freed from phage and propagated side by side with the original sensitive form without alteration of its properties, the resistants clearly represent genetic variants or mutants. Usually, they can be distinguished from the original sensitives only by their reaction to the phage. In addition, the mutation is so infrequent that the only dependable method of isolating resistant mutants from a sensitive culture is selective removal of the sensitives by phage lysis. Since, by all appearances, untreated bacteria remain sensitive, whereas bacteria exposed to phage become immune, it might be speculated that the phage evoked the immunity (direct induction hypothesis). Alternatively, resistance mutations might be occurring sporadically but constantly at a low rate, and the phage might merely remove the preponderant, sensitive, non-mutants and allow the resistant mutants to overgrow the population (spontaneous mutation hypothesis). The second hypothesis implies that the resistance mutations occur during the growth of the culture prior to the application of the phage; according to the first, they would occur during the brief interval between the application of the phage and

the destruction of the sensitive bacteria. The discrimination between these hypotheses is complicated, however, because phage must be added to detect the mutants.

A simple experiment to settle this issue has been provided by Newcombe (1949), using the phage T_1 on *E. coli* strain B. A relatively small number of bacteria (among which no mutants were already present) was spread on the surface of a series of nutrient agar plates. After the plates had been incubated several hours, and a thin film of bacterial growth had formed, phage was applied as a spray so as not to dislocate the bacteria.

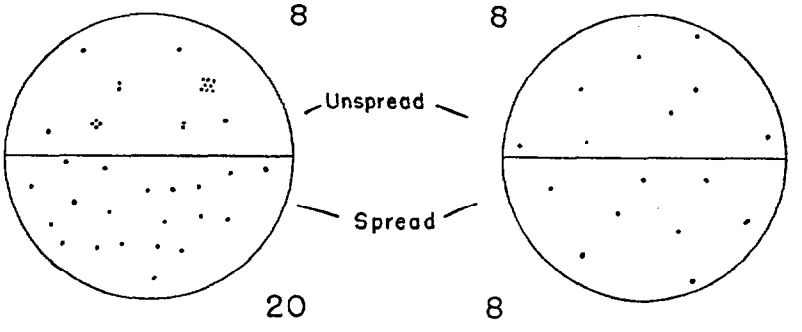

FIG. 3.1. Diagram of Newcombe's spreading experiment. The upper portion of each plate represents the number of phage resistant colonies found on unspread plates, on either the spontaneous mutation or the direct induction hypothesis of phage resistance. However, on the spontaneous mutation hypothesis, as shown on the left, the colonies originate from clones of different size, since the mutations had occurred prior to the application of the phage. On the direct induction hypothesis, it should make no difference whether the culture is respread prior to the application of the phage. The experimental result shows an increase of phage-resistant colonies after spreading, comparable to the left-hand figure, and therefore supports the spontaneous mutation hypothesis.

A series of duplicate plates was treated in the same way, except that the cells were redistributed on the plate with a glass spreading rod immediately before the phage was sprayed. The number of resistant colonies which appeared was much higher on the plates which had been respread before exposure to phage. This difference is expected if the mutations had occurred during the growth of the bacteria because mutations taking place prior to the last bacterial division would be represented by a clone of two or more resistant cells. On the undisturbed plates, each clone would be intact, and would count as a single resistant colony, whereas after redistribution of the cells, each member of the clone would be counted as a separate resistant colony. This result cannot be accounted for by the direct induction hypothesis without resort to highly implausible explanations. It may be concluded, therefore that the phage acts to select for those mutants which have occurred spontaneously prior to its application. The experiment is diagrammed in Fig. 3.1.

The conclusion had been verified less directly by Luria and Delbrück (1943) with the same bacterium-phage system. These authors at first looked for an increasing proportion of resistant cells during the growth of a culture from a sensitive inoculum as would be expected if spontaneous mutations occurred during growth. However, this experiment was hindered by an unusually large variance in the numbers of resistant cells from one replicate culture to another. Delbrück's theoretical analysis showed, however, that a large variance is expected if mutations occur throughout the preliminary growth of cultures from small inocula. The experimental variance between cultures was found, in agreement with Delbrück's theory, to be some thousand times as large as the mean, while the sampling variance, which should characterize consistent treatments of comparable cells, would be only as large as the mean itself. The null hypothesis, contradicted by the inordinately large variances, is that the cells in identical physiological condition have been exposed to identical treatments, that induce mutations conferring resistance. Therefore some variable factor not under experimental control influences the different cultures *prior* to their exposure to the bacteriophage. The spontaneous mutation hypothesis is a restatement of this conclusion. However, the argument is not as direct as that applied later by Newcombe, as has been described. In addition, it is couched in mathematical terms not readily followed by all students. Methods developed by Luria and Delbrück and others for the quantitative measurement of mutation rates are given in the Appendix. Newcombe (1949) has summarized the adaptive mutations whose spontaneous origin is suggested by variance analysis. They include mutations for resistance to streptomycin and to penicillin, for phage resistance, and for a variety of nutritional changes.

More recently, the accumulation of spontaneous mutants during prolonged cultivation has been verified (Novick and Szilard, 1950; Stocker, 1949; Atwood, Schneider, and Ryan, 1951).

Innumerable claims have been made of induced hereditary variations in bacteria directed by chemical and physical agents. None of these claims, however, has yet been supported by the detailed analysis, of the kind just discussed, which is desirable to disqualify spontaneous mutations and natural selection. It is important to emphasize that the directions of natural selection must be verified by carefully designed reconstruction experiments, rather than by *a priori* reasoning (Braun, 1947b; Lederberg, 1948, 1949b). This is not to say that bacteria, or any other organisms, are incapable of adaptive responses to their environment. However, direct adaptation, such as adaptive enzyme formation, is nonheritable, a distinction which should be clearly formulated.

2. Independent Occurrence of Spontaneous Mutations

Mutations of different genes are generally assumed to occur independently of one another. This concept is somewhat circular, however, because one of the most often used criteria for distinguishing two genes is independent mutation. In bacteria, where this may often be the sole criterion, it is impossible to test the universal applicability of this assumption. There are, however, any number of verified examples. For instance, mutation to resistance to different antibacterials, such as sulfonamides, penicillin, and streptomycin, occurs independently, so that a mutant resistant to one agent is generally unaltered in its sensitivity to any of the others. Similarly, Audureau has described independent mutations leading to the ability to utilize succinic, glutamic, or glutaric acid by *Moraxella lwoffi*, the type form of which is unable to utilize any of these compounds. References for these and similar examples are given by Luria (1947) and Lederberg (1948).

The apparent exceptions to this generalization are more difficult to interpret. For example, Luria (1946) has described certain so-called "complex-resistance mutations" which make *E. coli* resistant to phages from more than one cross-resistance group. The complex mutations occur less frequently than do the simpler mutations of which they seem to be superimpositions, but still much too frequently to be coincidences of mutation of two independently mutating genes. However, in the *E. coli* strain (B) used by Luria, there is no independent way of determining whether the complex mutants represent two interdependent mutations, or merely a change of an altogether distinct gene with complex physiological effects. Physiologically distinct characters are usually controlled by independently mutating genes. To date, the independent mutation of distinct genes is uncontradicted. However, more complex genetic phenomena are known (*viz.*, segregation, *vide infra*) which may simulate non-independent mutations.

3. Phenotypic Lag

The estimation methods summarized in the Appendix postulate that each mutation is reflected immediately in the appearance of the organism. *A priori*, a mutation would be unlikely to revise the cell phenotype instantaneously, and experiments with protozoa prove that several cell divisions may be needed for genotypic changes to become phenotypically effective. This delay is referred to as phenotypic lag.

At present, the evidence for phenotypic lag in bacteria is indirect. Newcombe (1948, Newcombe and Scott, 1949) has shown that discrepancies in the evaluation of the mutation rate of *E. coli* B to phage resist-

ance, using different methods (see Appendix), can be accounted for by phenotypic lag. If mutations became phenotypically expressed only after a few divisions, the average number of apparent resistants for each clone containing one or more resistants will be exaggerated. From Newcombe's (1949) spreading experiment (see Fig. 3.1), it could be inferred that the resistant clones were about ten times larger than expected, which speaks for a phenotypic lag of three generations or so. Luria and Delbrück had rejected the possibility of phenotypic lag in setting up their model of spontaneous mutation because they found cultures with a single cell in about the expected proportion of their experiments. To account for this, Newcombe suggests that one clonal descendent of a recently mutated bacterium may sometimes become phenotypically resistant before the others. Other inadequacies of the hypothetical model of spontaneous mutation may also contribute to discrepancies. The postulates have been discussed in some detail by Luria and Delbrück (1943), Newcombe and Scott (1949), and Lea and Coulson (1949).

Under certain conditions, where growth is contingent upon a mutation, phenotypic lag may be prolonged indefinitely. Davis (1950) has reported that certain spontaneous or radiation-induced mutations in *E. coli*, which will permit growth on a synthetic medium, may fail to come to phenotypic expression at all unless a small amount of the required growth factor is added, to allow a minimal amount of growth of the parent cells. This "phenomic barrier" is presumably the result of a vicious cycle in which phenotypic lag must be overcome to effectuate a mutation, while the mutation's effect is needed for growth to start. The phenomena of phenotypic lag are, so far, imperfectly understood, but must be allowed for in all experiments on bacterial mutation.

4. Pseudo-Mutational Processes

The genetic model of the bacterial cell that we have been using implicitly with certain reservations throughout this discussion is a uninucleate cell, carrying one set of genes, so that changes of individual genes will be reflected in phenotypic alterations and *vice versa*. This model is probably inaccurate for vegetative cells. If the current cytological evidence is interpreted correctly, even the small spherical cells of cocci may contain two nuclei, while the rods and filaments of other bacteria may be more complex. It is also by no means certain that the individual nuclei of all bacteria are haploid (*i.e.*, carry just one set of genes). Di- or polyploidy in bacteria leaves the door open to complex genetic processes that simulate mutation, but do not consist of immediate changes of individual genes.

The most prominent of these pseudo-mutational processes is segregation, the separation from a cell containing two gene forms of a nucleus in which only one form is represented. That is, a cell whose genetic constitution were *AAAaaa* might segregate a cell whose constitution is *a* or *aa*. . . . Such a segregation will be reflected in a phenotypic change if the gene form, or allel, *A* is dominant over *a* in cells where both are represented. That is, the segregation will simulate a "mutation" from *A* to *a*.

It must be emphasized that segregation does not take the place of mutation as a source of genetic variability. In our example, a mutation of the "A" gene must have occurred at some previous time to allow two forms. The immediate observation of a genetic change may represent the unmasking, by segregation, of a mutation which had occurred long before.

Very likely, segregation does not play the important role in bacterial variation that it does in the filamentous fungi (Luria, 1947). However, one verified instance of segregation, found in *E. coli*, will be discussed later (page 91).

B. Induced Mutation

In previous paragraphs, the gene was regarded as a chemical substance which was not internally modified by its commerce with the immediate environment through which it carries on its functions. This model, of course, cannot be strictly accurate, because the gene is a material substance, not immune to change. As implied earlier, the insularity of the gene means merely that at present no reagent is known which can discriminate chemically between any one gene and its neighbors. That is, even under treatments which induce mutations, the particular genes affected appear to be indeterminate, but merely because of our ignorance of the molecular differences between genes.

1. Radiations

The lack of knowledge in this field is pointed up by the fact that, until recently, the only agents for inducing mutations have been radiations—X-rays, gamma rays, neutrons, ultraviolet light (UV)—that have, at best, topographical specificity. That is, the locus of action of such radiations is determined preeminently by a topographically random quantum event, the absorption of the radiation. UV may show a higher order of specificity of absorption, but none of these agents could conceivably distinguish one gene from another, and the probability of a successful quantum hit will depend initially on whether a quantum happens to be absorbed in proximity to a gene, so that its energy can be transferred to

it. In a sense, then, we might better refer to radiations as random accelerators of the "spontaneous" mutation process.

Soon after Muller's proof of the mutagenicity of X-rays for *Drosophila* in 1928, reports began to appear of the comparable effects of X-rays and radium emanations in stimulating variations in yeasts and bacteria. However, the characters used in the earlier studies were often vague, and the results difficult to interpret. We owe our most precise information on the mutagenic effects of radiations on bacteria to the comparatively recent work of Demerec and Latarjet (1946). These authors used the same phage resistance system that has been already discussed as the test material for their work. After irradiation, they spread the bacterial suspensions on the surface of agar plates, and assayed the bacterial films for mutations by spraying phage on them. This technique has the advantage of counting each new mutation, rather than each mutant, as a single colony. Aliquots were taken from the irradiated and unirradiated samples to determine the extent of killing by the radiation, and to estimate how many mutant cells, if any, were already present before the treatment. This number, corrected for the extent of sterilization by the radiation, must be subtracted from the number of resistant colonies which are counted, to give the number of mutations induced by the radiation.

The most striking common effect of all known mutagens is that they kill cells, roughly in proportion to their mutafacient efficiency. It is usually assumed that the mutants under study are no more resistant to radiation than are the non-mutant cells, but this should be (as it was here) verified by direct experiment. Because of this pronounced killing action (see Chapter V), it may turn out that the preexistent spontaneous mutants killed by a given dose of radiation exceed the new viable mutants induced. For this reason, and assuming that the radiation does not affect the mutants differentially, the results of induced mutation experiments are usually expressed in terms of increased proportion of mutants among the surviving cells, rather than as the absolute number. Under exceptional circumstances—if the spontaneous mutants in the original culture are infrequent compared to the mutation rate, and if the proportion of cells killed has not exceeded two-thirds—increases in the absolute numbers of the mutants can be demonstrated. This type of experiment may be useful to prove conclusively that an apparent mutagen is inducing, rather than selecting, mutations (Witkin, 1950).

Demerec and Latarjet found that both X-rays and ultraviolet light acted on *E. coli* to induce mutations for phage resistance. However, the effects of these agents cannot be detected as an immediate increase in the output of phenotypically resistant cells. Although a few new mutations were detected among the bacteria sprayed with phage immediately after

irradiation, most of the induced mutants were not detectable for some time afterwards. The growth from an irradiated inoculum continued to produce additional mutations for a period equivalent to thirteen bacterial generations, although the peak rate of appearance was noticed after one or two divisions.

The delayed effect after irradiation may come from a number of causes. Firstly, as we shall see, it is possible that some of the effects of ultraviolet light may depend on the accumulation of a mediating chemical substance, so that, perhaps, the mutational chemical change may be itself delayed. Then, phenotypic lag would be expected to advance the time at which a genetically resistant cell would be scored as a mutant. The effects of phenotypic lag are probably exaggerated, in absolute time units, by the long and variable periods during which still viable cells may fail to proliferate after ultraviolet irradiation (Newcombe and Scott, 1949). Finally, since many bacterial cells contain several nuclei (Robinow, 1945), "dominance" effects become possible. That is, if only one of the several nuclei of the cell should contain a mutated "resistance gene," it might have no effect on the phenotype owing to the dominating action of unmutated "sensitive genes" in the sister nuclei (see Lederberg, 1949a, b). In this event, it would not be until resistant nuclei had been sorted out during subsequent divisions that a cell would be produced that possessed a genotype that would lead to resistance. The existence of such segregation phenomena, after irradiation, is suggested by the finding that fermentation mutants of *E. coli* are frequently found as sectors in colonies grown out from irradiated cells.

Some idea of the relative mutagenicity of X-rays and ultraviolet can be conveyed by comparing them at doses for which the killing effect is the same. In the experiments of Demerec and Latarjet 100,000 roentgens of X-rays left 10^{-5} of the original population as survivors, and induced 200 endpoint mutations per million survivors. Ultraviolet light, incident at 3550 ergs/mm.^{2}, caused the same killing, but induced 3300 endpoint mutations per million survivors. Thus, from the point of view of mutations induced per cell killed, ultraviolet light is more effective than X-rays. Demerec and Latarjet point out, however, that at these doses, each bacterial cell will have absorbed more than 200 times as much energy from ultraviolet as from X-rays. On an energetic basis, therefore, X-rays are more effective both in killing and in mutating bacteria. For most purposes, however, it is more important to conserve the biological material than radiant energy.

A quantitative comparison of X-rays and ultraviolet light is, however, hindered by differences in the shape of the response curves. The sterilizing effects of X-rays are, over a considerable range, in accord with a

"single-hit" mechanism: *i.e.*, each increment of dose kills a constant fraction of the surviving bacteria. Thus, there appears to be no cumulative effect, and if a first absorption of an X-ray quantum fails to kill a cell, the cell is not made more or less sensitive to subsequent doses. However, the quantum efficiency for X-ray sterilization is not accurately known, primarily because we do not certainly know the site of action. The kinetics of "single-hit" killing is expressed graphically by a linear plot of log S (survival ratio) against dose (usually in roentgens).

When log S is plotted against dose of ultraviolet light (in energy, or in energy/surface), however, a sigmoid rather than a linear relationship is usually observed. The initial doses of ultraviolet have a smaller effect than subsequent dose increments. This suggests that the quantum events which underly killing by ultraviolet light may be cumulative. However, the kinetic data do not tell whether the cumulative effect is due to the presence of a number of different target sites (nuclei?) each of which must be altered, or to the accumulation of some toxic material, and both may be involved.

The same comparative picture is obtained when the mutagenic effects of these two radiations are compared. Because the fraction of mutants is always small, it is more convenient to plot the number of mutations obtained (rather than its logarithum) against the dose. Whereas X-ray induced zero-point mutations show no evidence of a threshold, the ultraviolet response is again sigmoid, and there is in fact an optimum dose beyond which the fraction of mutants increases erratically if at all.

Although the kinetics of X-ray effects is simpler, it does not necessarily imply a mechanism entirely different from ultraviolet. The quantum of X-rays is much more energetic than that of ultraviolet, and it is possible that any absorption of a single X-ray quantum at a suitable site will be effective, while several ultraviolet quantum hits would be needed either to accumulate enough of a chemical, or to "destroy" a wide enough area, to have the same influence.

Research on radiation mechanisms is progressing very rapidly, partly under the impetus of the development of atomic energy. That X-rays, no less than UV, may have chemical intermediation of their biological effect is suggested by experiments carried out in air, oxygen, and other atmospheres, which were found to have profound effects on the extent of X-ray damage. A more detailed account of radiobiological advances cannot be given here, but their pertinence to bacteriology is matched only by the usefulness of bacteria as material for radiobiological investigation.

2. Photorecovery

The concept of a chemical intermediate for the effects of UV has been greatly strengthened by the recent discovery of the phenomenon of

photorecovery (Kelner, 1949; Novick and Szilard 1949). This consists of the apparent reversal of the lethal and mutagenic effects of ultraviolet light by a subsequent exposure of the irradiated organisms to longer visible wavelengths. The quantitative effect of the treatment with visible light can be regarded as a "dose-reduction" of the UV effects, for the combined responses are, under optimal conditions equivalent to those of a dose of UV alone, but reduced to 40%. Although the extent of photorecovery, expressed in these terms, is not impressive, the sigmoid response (either lethal or mutagenic) to UV exaggerates the effect when

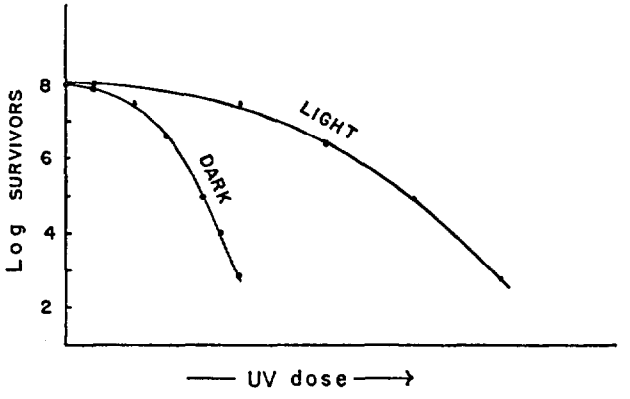

FIG. 3.2. Photorecovery of UV-inactivated cells. Adapted from Novick and Szilard (1949). *E. coli* B/r was exposed to various UV doses, and aliquots plated to determine the viable count with (light) and without (dark) exposure to an optimum of visible light. The light treatment is equivalent to a UV dose reduction by the factor 0.4, but at certain doses, *e.g.*, where the dark survival is only 10^3, this may result in a photorecovery ratio of 10^5, owing to the non-linearity of the survival curves.

expressed in terms of microbial survivors. For example, a suspension of bacteria exposed to a dose of UV that would leave only one survivor per million if left in the dark, can be photoreactivated so that 10% of the treated cells will recover in the light.

Photorecovery appears to be widespread among microorganisms, and has already been reported for actinomycetes, bacteria, fungus spores, yeast, paramecia, and bacteriophage. Bacteriophage, however, can be photoreactivated only while adsorbed on sensitive bacteria. This suggests that the chemical (?) which has altered under the influence of UV is not itself decomposed by visible light, but that the latter activates other reactive systems which in turn reverse the UV effects. A chemically distinct intermediate is hypothesized to explain UV killing and photorecovery because UV-treated cells can be photoreactivated even several hours after irradiation. Any energetically excited molecule would not be expected to remain in such a metastable state for any appreciable time unless it were somehow isolated from energy transfers associated with thermal, molecular collisions. Preliminary experiments indicate that

the mutagenic effects of UV-visible light combinations are comparable to the lethal responses. That is, the proportion of mutants in a photoreactivated population will be less than among the distinctly fewer dark survivors. The absolute number of viable mutants might be the same or more, however, owing to the much greater overall survival (Newcombe and Whitehead, 1951).

These observations raise the question whether the lethal effects of radiations may be ascribed entirely to the induction of mutations which cause irreparable damage. This problem cannot be discussed fully here, but its current status seems to be that only a small part of the lethal effect of radiations can be accounted for by simple lethal mutations. Various aspects of this problem have been discussed by Demerec and Latarjet (1946); Latarjet (1946); Lea (1947); Witkin (1947); and Lederberg *et al.* (1951).

3. Chemical Mutagens

Although radiations of various kinds have been known to be mutagenic in numerous organisms for many years, convincing evidence of chemical influences on mutation rates dates only to the time of World War II, with the work of Auerbach and collaborators on war "gases." The potency of β-chloroalkyl sulfides and amines (mustard gases and nitrogen mustards) as extremely effective mutagenic agents has been proved beyond doubt in a variety of organisms, including bacteria (reviewed by Auerbach, 1949). In general the effects of the mustard compounds are similar to those of radiations. These substances are extremely toxic, they mimic the histopathological effects of radiations and like radiations, they have little if any specificity in their mutafacient effects. These compounds will combine chemically with a great variety of functional groups found in proteins and nucleic acids—amino, carboxyl, hydroxyl, phenolic—indeed with very nearly any group carrying a reactive hydrogen atom. It would be surprising if this reactivity were not the chemical basis of mutagenicity, but this would imply that other group reagents might also be mutagenic. This notion, however, may help to understand the mutagenic activity demonstrated in *Drosophila* for allyl isothiocyanate and for formaldehyde.

In addition to the mustards, many other chemicals have been reported to have mutagenic effects for bacteria, including carcinogenic hydrocarbons, some alkaloids, dyestuffs and bile acids. The reader is referred to Auerbach's review for a detailed discussion of the present significance of this work (Auerbach, 1949). Among these newer reports, however, the finding that organic peroxides may be potent mutagens (Dickey *et al.*, 1949) is of special interest, as it may also give a clue to the mechanism of

action of ultraviolet light. Wyss *et al.* (1948) found that nutrient broth exposed to heavy doses of UV becomes mutagenic. The effects of UV could be duplicated by treating the broth with hydrogen peroxide, so that the indirect mutagenic effect of UV can be fully accounted for by the hydrogen peroxide which is liberated in UV irradiated water. The supposition that part of the "direct" effects of UV on living cells may be mediated by the same mechanism is not unreasonable. The work of Dickey *et al.* (1949) provides a tangible clue to the type of mutagenic products produced when broth is treated with hydrogen peroxide, as well as a basis for speculation concerning the intermediate involved in the photoreactivation of UV-treated bacteria.

III. Characteristics of Bacterial Mutants

Scarcely any limit can be placed on the range of physiological effects mediated by genetic changes in bacteria. Presumably, all bacterial forms, and for that matter all living things, differ from one another entirely on the basis of a finite number of differences of gene content, quality, and organization. The scope of effects of single gene changes may appear to be restricted or broad depending on the specific instance, and on the investigator's point of view. However, the presumption that even the most diverse effects of single gene changes can usually be traced back to an ultimate simple direct biochemical effect has had considerable empirical success (see Beadle, 1945). Contemporary genetic research on bacteria is concerned primarily with two types of mutants—mutations for resistance to growth inhibitors, phages, and the like, or mutations affecting the biochemistry of the organism in a direct, obvious manner, detectable as nutritional, fermentative, or similar enzymatic changes. Other mutations amenable to genetic analysis include effects on pigment formation, colonial appearance, antigenic structure, virulence, motility, antibiotic production, and vitamin excretion.

Nutritional mutants have proven to be especially useful, both for physiological and for more strictly genetic research. Many bacteria, such as *E. coli* are auxoautotrophic, *i.e.*, they have no special nutritional needs beyond a medium containing minerals, inorganic nitrogen, and an organic carbon and energy source. Such a simple nutritional pattern actually reflects an exceedingly complex biochemical apparatus, for the organism must synthesize all of the amino acids, vitamins, and other constituents of its protoplasmic and metabolic machinery. Auxoautotrophy does not mean, in general, that an organism has dispensed with growth factors, but contrariwise that it manufactures them for itself. A mutation that blocks the synthesis of an essential metabolite imposes on the cell a requirement for the substance it can longer manufacture.

The nutritional mutant is, therefore, unable to grow on the unsupplemented minimal medium.

Nutritional mutants, chiefly in the fungus *Neurospora*, but also in bacteria (Tatum, 1949; Davis, 1950), have been particularly useful in tracing the steps in the biosynthesis of various growth factors. Two types of experiment are in general application. When an amino acid, for example, is synthesized by a sequence of reactions, the response of a nutritional mutant to the intermediary compounds depends on the particular step of the reaction sequence that is blocked in the mutant. If an early step is blocked, then the mutant can utilize any added intermediate that comes later in the sequence. If a late step is blocked, however, then the mutant will be unable to use an added intermediate which has an earlier place in the sequence. Insight into a biosynthetic sequence can thus be obtained by comparing the availability of a group of postulated intermediates for a group of mutants blocked at different stages in the synthesis of a given metabolite. Many mutants, in which the further transformation of a biosynthetic intermediate is blocked by genetic change, may accumulate the compound and excrete it into the medium in far larger amounts than the original auxoautotroph. Such intermediates can then be isolated by standard chemical procedures, providing more direct evidence for their probable role in the synthesis of the growth factor required by the mutant. This supposition is strengthened if an intermediate accumulated by one mutant permits the growth of another mutant, blocked at an earlier step.

The excretion of such intermediates is the basis for syntrophism, or nutritional symbiosis, whereby mutants blocked at different steps may be able to feed each other, so that mixed cultures of two mutants will grow far better in a minimal medium than will the isolated mutants. Conversely, syntrophic growth may be a useful test to differentiate two mutants which have similar nutritional requirements, but which may be blocked at different steps in the synthesis of the growth factors (Davis, 1950; Lederberg, 1950).

IV. Interclonal Variation

Up to this point, the discussion has been concerned exclusively with genetic variations which occur within clones, and which do not involve the interaction of different bacterial strains. In recent years, increasing attention has been devoted to the ways in which different bacteria may interact genetically in mixed culture. It is likely that the classical emphasis on pure culture technique has caused many interesting aspects of interclonal variation to be neglected. On the other hand the complete control of intraclonal variations in pure cultures is a necessary preliminary

to any study of interclonal variation. The mechanisms of genetic interaction between clones may be classified: (a) "infective transmissions," in which the interaction is mediated by extracts or filtrates which can be separated from the bacterial cells; and (b) sexual phenomena which appear to require the integrity of both of the interacting clones.

A. Infective Transmission

1. Transformation

The first example of infective hereditary transmission to be widely accepted was the transformation of pneumococcal types, discovered by Griffith (1928). Pneumococci are recognized in two phases, *S* and *R* (also called *M* and *S*, respectively) depending on the presence or absence of a polysaccharide capsule. *R* Bacteria are not readily distinguished by serological methods, but the many *S* types are characterized by the serologically specific reactions of the capsular material. The *S* types are designated with a roman numeral, for example, *S-III* is one of the more prevalent types. *R* Mutants can be obtained from any of the *S* types, particularly with the help of selection with anti-*S* serums. Such *R* mutants are generally avirulent for mice, but many are unstable, and will occasionally revert to *S*, though only to the same *S* type from which they arose. Other *R* mutants are stated to be completely stable, as corroborated by their avirulence in large doses. Griffith found that certain *R* cultures, by themselves avirulent, killed mice when inoculated with heat-killed *S* cells of various types. Viable *S* cells of the same type as the heat-killed vaccine were recovered from the blood of the infected mice. Although Griffith was unable to duplicate this interaction *in vitro*, he concluded that material from the killed *S* cells had transformed the living *R* cells into the same *S* type as used for the source of the vaccine, a type that might be different from the original *S* type from which the *R* culture had been isolated.

Griffith's work was promptly confirmed and subsequently the conditions necessary for transformations *in vitro* were discovered. These researches culminated with the extraction and partial purification of the transforming principle by Avery *et al.* (1944). This and subsequent work (McCarty, 1946) demonstrated that the active principle consists principally of a highly polymerized desoxyribonucleic acid, which, contrary to earlier speculations, does not require serologically detectable amounts of the capsular polysaccharide for its transforming action. Studies with specific desoxyribonucleases established that the polymeric desoxyribonucleic acid is a necessary constituent of the transforming principle. Avery and his collaborators have claimed also that the

nucleic acid is sufficient, and that protein is not responsible for the specificity of the transforming agents. This claim has been disputed, however, chiefly on the grounds that even the most purified preparations (which still consist mostly of inert material) of the transforming principles are not entirely free from detectable protein (Mirsky and Pollister, 1946). This controversy has attracted wide interest because of the frequently stated analogy between transforming agents and genes. Although it is undisputed that nucleic acids play an important role in gene function, many workers had speculated that, by analogy with enzymes, gene specificities were ultimately determined by protein configurations.

The demonstration of type transformations *in vitro* requires a rather complex experimental system, provided by some serums, which has been analyzed as follows. In the transformation of *R* to *S*, the *R* cells must firstly be made to grow in aggregates. This is usually accomplished by the *R* agglutinins present in many normal sera. However, it is possible to replace such agglutinins with semisolid agar. Secondly, the *R* cells must be sensitized by exposure for several hours to a duplex system, consisting of a non-dialyzable component (possibly an enzyme) that occurs in serum albumin and of a dialyzable component replaceable by pyrophosphate plus neopeptone. Sensitization is apparently necessary for the fixation of the transforming principle on the cells. Presensitized cells require only a few minutes exposure to the transforming agent to produce *S* transformed cells. No estimate is yet available of the fraction of the *R* cell population which is transformed to *S*, but it is probably less than one per thousand.

Not all *R* strains are competent to be transformed under these conditions, and some attention must be given to the isolation of transformable *R* strains which are not spontaneously unstable. On the other hand, *R* strains have been found (McCarty *et al.*, 1946) which have less rigorous requirements for transformation than those just described. So far, the only characters of the pneumococcal cell which have been transformed are those which can be correlated with serological specificity: colony morphology, virulence, and the like. The polysaccharide capsular transformations have received the most emphasis, but the recently discovered *M* protein antigen can also be transformed, independently of the capsule substance (Austrian and MacLeod, 1949). Transformations have been described in a variety of other bacteria, but none of these other examples (reviewed by Luria, 1947; Lederberg, 1948, 1949b; and McCarty, 1946) has been so well documented as has the pneumococcal transformation.

At present, no definitive interpretation of this phenomenon can be

offered. The transforming principle is, in a sense, an infectious hereditary agent, since it can be transferred not only in heredity from parent to offspring, but also between cells of different descent via the medium. The newly transformed *S* cells cannot be distinguished from typical *S*, and like the latter, can be used as a source for fresh preparations of the transforming agent. Thus, in many ways, the transforming agent behaves like a symbiotic intracellular virus. Indeed, the parallelism between tranformations and symbiotic or lysogenic bacteriophages was commented on even in the more or less credible transformation work antedating Griffith.

Recent morphological and genetic investigations may require a reinterpretation of some transformation experiments. Many bacteria have been reported to produce reduced cells ("L-forms") which may pass ordinary bacterial filters and which are unusually resistant to disinfectants. Such forms may require special conditions to regenerate ordinary bacteria and may lead to a misconstrual of sterility test controls. An apparent transformation might be due to the regeneration of the bacteria from which an apparently sterile filtrate or extract had been prepared. Whether this concept can be applied to the pneumococcus experiments is doubtful, but fairly direct support for it has been found in *Salmonella typhimurium* (compare Ephrussi-Taylor, 1951 and Lederberg *et al.*, 1951; Tulasne, 1949).

The crux of the issue is the morphology of the transforming agent: is it a single nucleic acid or nucleoprotein molecule (naked gene) or an organized particle into which all of a cell's genetic material may be assimilated?

2. Virus Lysogenicity

Although the properties of actively lytic, parasitic bacterial viruses or bacteriophages are now widely appreciated, owing especially to a recent resurgence of interest in the growth and genetics of viruses, the significance of cryptic or lysogenic viruses has been generally underestimated. It has been well established (McKie, 1934; Rountree, 1949) that many bacterial cultures are infected with cryptic, symbiotic viruses. Such lysogenic bacteria show no obvious manifestation of the virus under ordinary conditions of pure culture, and other bacterial strains, susceptible to the virus must be found and exposed to the lysogenic culture to reveal the presence of the virus. Thus, it cannot be asserted that any bacterial culture is certainly uninfected without tests on unlimited numbers of potentially sensitive indicator strains for a lytic response. Many cultures, especially among the salmonellae and the micrococci, carry latent viruses which can be detected with the help of one or another

indicator strain of the same or a related species. Many latent viruses have weak lytic powers even on sensitive strains, and these viruses might easily be regarded as transforming agents, provided only that they modify the physiology of the cell in some way not obviously related to phage lysis.

A system studied in detail by Burnet and Lush (1936) may be a possible half-way station in the gradual transition between transforming agents and latent viruses. The phage, *C*, has a poor lytic effect on a micrococcus strain *SF*. After exposure to *C*, a large fraction of *SF* cells become resistant to *C*, as well as to another phage *B*, which differs from *C* in lysing almost all cells of an *SF* population. The resistance of *SF* treated with *C* was shown to result from the establishment of a symbiosis between the bacterium and the phage which, in addition to making the bacteria resistant to *C*, also appears to exclude the phage *B*. Thus filtrates of *SF/C* (*SF* bacteria once exposed to *C*), a culture resistant to phage *B*, are capable of "transforming" *SF*, typically sensitive to *B*, to a type resistant to *B*. In this sense the phage *C* is a transforming agent, but it can be recognized as a lytic phage by its action on *SF* on agar. In liquid medium, however, no bacteriolysis is evident and the virus nature of *C* would not be apparent. Inasmuch as resistance to *B* can also be conferred by a spontaneous mutation, it can be suggested that the "transforming agent" *C* mimics a gene effect.

Observations like these do not answer the question of the ultimate origin either of phages or of transforming agents, although they do suggest a remote connection between them. Some workers have proposed that viruses may have originated from cytoplasmic hereditary agents which have gone wild and escaped from the cells in which they arose, whereas others have argued that "cytogenes" may have evolved from parasitic viruses which have evolved a symbiotic relationship with their host cells, and perhaps have gradually become integrated into the genetic system of the cell. It may be suggested also, perhaps more reasonably, that both types of evolutionary change of these particles are taking place concurrently, so that it will be impossible to generalize concerning the ultimate origin of all viruses. At any rate, no experimental method nor even a widely accepted definition of the terms exists, by which we can distinguish between intracellular viruses, transforming agents, and cytogenes. The accumulated evidence, especially with higher organisms but also with microbes, suggests, however, that hereditary changes based upon differences in these virus-like agents are exceptional, and that nuclear genes bear most of the burden of heredity.

Cytogenes allow for directed "mutations" more readily than do nuclear factors. "Mutations" based upon the acquisition of such agents

(phages, transforming principles) have already been cited. The depletion of such agents in the previously infected cell which would give the converse type of hereditary change, might be accomplished by any influence on the relative rates of increase, or decrease, of the cytoplasmic agent and the cell as a whole. For example, the cytoplasmic particles might be more sensitive to heat, radiations, or other deleterious agents than is the cell, so that some of the cell survivors of such treatments would be depleted of the cytoplasmic particle. No well-authenticated examples of such a depletion or attenuation mechanism have been reported in bacteria, but effects in yeast exposed to acriflavine (Ephrussi *et al.*, 1949) and in *Euglena* and higher plants exposed to streptomycin (see Provasoli *et al.*, 1948) are best explained in this way. An attenuation mechanism is often difficult to prove; some of the methodological problems are discussed by the authors cited, and by Preer (1948), Lederberg (1948, 1949b), and Luria (1947).

B. Genetic Exchange via Sexual Fusion

Traditionally, bacteria have been supposed to multiply exclusively by fission. This concept has even been incorporated in the class name *Schizomycetes*, "fission-fungi." However, the evidence for the absence of sexuality among bacteria was entirely negative, namely, that no one had succeeded in a convincing demonstration that bacteria cells can fuse with one another with any genetically interesting consequences. Such examinations have been primarily morphological; in view of the minuteness and relatively undifferentiated structure of bacterial cells such negative results could be attributed as much to the limitations of the techniques as to the true absence of sexual mechanisms in bacteria.

Among the legacy of unconfirmed or unconvincing reports of cell fusions, very few still command the attention of contemporary bacteriologists. Perhaps the most credible of these are the observations of Stapp, confirmed by Braun and Elrod (1946), on the formation of stellate aggregates of *Phytomonas tumefaciens* by cell fusion, followed by a centripetal aggregation of the nuclear bodies. These authors themselves point out, however, that "cytological studies alone will not suffice to clarify this question. It will be necessary to bring together in a single star different strains of the same species or individuals of closely related species and determine from this cross whether a recombination of characters results."

1. Bacterial Recombination

In view of the difficulty of interpreting cytological studies, strictly genetic methods are better applied to this problem. The first clearcut experiments in this direction were reported by Sherman and Wing (1937),

who mixed various fermentative types of *E. coli*, and then examined reisolated clones for new combinations of fermentative characters. Unfortunately, the "parental" cultures were not stable enough to permit any definite conclusion as to the origin of the observed "new combinations." That is, in the terms of the previous discussion, uncontrolled intraclonal variability obscured whatever interclonal variation there might have been. Gowen and Lincoln (1942) improved the approach by using as characters that might be recombined visible differences in color and texture of colonies of *Phytomonas stewartii*, so that many more isolates could be feasibly examined than in the previous study. Some hundreds of thousands of colonies from mixed cultures were examined, but no greater variability among the mixed cultures was observed than could be accounted for by the intraclonal variability of the parents. However, in a further modification of this genetic approach, whereby any possible recombinations were automatically sieved even from very large populations of the mixed parents, Tatum and Lederberg (1947) succeeded in demonstrating genetic recombinations in strain K-12 of *E. coli*. These and subsequent observations of these authors, which have been confirmed in several laboratories, are the basis for the assertion that may now be made that a sexual stage intervenes in the life cycles of some bacteria.

The selective isolation of genetic recombinations depends on the properties of nutritional mutants already mentioned. A mutant, symbolized by A − B+, is unable to synthesize growth factor "A" but can manufacture "B." Conversely, a second mutant A + B− will require "B" for growth. Neither A − B+ nor A + B− by itself is capable of growth in minimal medium, and if the cells are carefully washed, mixtures of the two mutants may be inoculated into minimal agar without resulting in syntrophic proliferation. However, if genetic exchanges occurred between cells of the two mutants, some of the recombinations would be of the type symbolized A + B+, *i.e.*, capable of growth in medium like the original wild type. In this way, a minimal medium can be used for the selective isolation of genetic recombinants in mixed cultures. The term *prototroph* has been suggested to designate cultures like these recombinants that are nutritionally like the ancestral wild type, which is auxoautotrophism for *E. coli*, but implies a requirement for biotin for *Neurospora*.

Prototrophs appear at the rate of approximately one per million cells inoculated, in experiments with various mutants of *E. coli* K-12. This shows that recombination must occur at a comparably low rate, and thus why it has been so difficult heretofore to obtain convincing evidence of sexuality in similar bacteria. The production of prototrophs from mixtures of biochemical mutants is only one of many predicted implications

of a genetic recombination mechanism. By itself, this experiment would be merely contributory, not conclusive, evidence for a sexual mechanism. Further experiments are necessary to eliminate two possible fallacies: (a) that the prototrophs are engendered by intraclonal variation, *i.e.*, that spontaneous mutations might occur from A − B+ to A + B+; and (b) that the observed genetic exchanges are mediated by some other mechanism of interclonal variation, specifically infective transmission or transformation.

If only single nutritional mutations are used for parental stocks in these experiments, intraclonal variation is not completely controlled, since many mutants will reverse mutate at rates of the order of 10^{-7} per division. The small but inevitable syntrophic growth of the parent inocula exaggerates this effect in mixed cultures, which thus might confuse the result. This source of error can be practically eliminated, however, by the use of double rather than single mutants for the parents. That is, the cross would now be symbolized as A−B−C+D+ × A+B+C−D−, to yield a variety of progeny, including prototrophs A+B+C+D+. For a double mutant to yield a prototroph would require a coincidence of two mutations in the same clone, *e.g.* that both A− and B− revert to A+ and B+, respectively, in the same A − B− clone. The theoretical probability of such a coincidence is vanishingly small, and has never been observed experimentally with these stocks. The use of double mutants as parents has the additional advantage that syntrophic growth, which might obscure the formation of prototroph colonies in minimal agar, is even further restricted.

The most informative experiments have, however, involved the use of additional unselected markers, such as mutations affecting sugar fermentations, phage resistance, and drug resistance (see Fig. 3.3). With suitable stocks, it is possible to use nutritional differences merely for the selection of a sample of potential recombinants, and among the latter to find a representative set of recombinations of the unselected markers which should be assorted among the prototrophs willy-nilly, or else reveal the laws of the distribution or genetic segregation. Detailed studies of the segregation of unselected factors have, in the first place, provided the most critical evidence disqualifying intraclonal variation, because the unselected markers reassort in a great many different combinations, while the parents show no such variation by themselves. But more important, they have also given direct indications of a linear arrangement of the genetic factors, entirely comparable to the chromosomes of plants and animals (Lederberg, 1947). Such experiments have also shown that, unlike transformations, a wide variety of genetic factors are reassorted simultaneously, and lead to the conclusions: (a) that the

genetic factors exchanged between cells encompass the full genic constitution of the parents; and (b) that the "gametes" do not mix, *i.e.*, that no more than two parents contribute to a given prototroph. In this light, if an infective factor were responsible for recombination in *E. coli* K-12, in terms of its biological functions it would be indistinguishable from a gamete of the bacterium. Until the cytological basis of recombination has been verified, we cannot be sure of the morphology of the

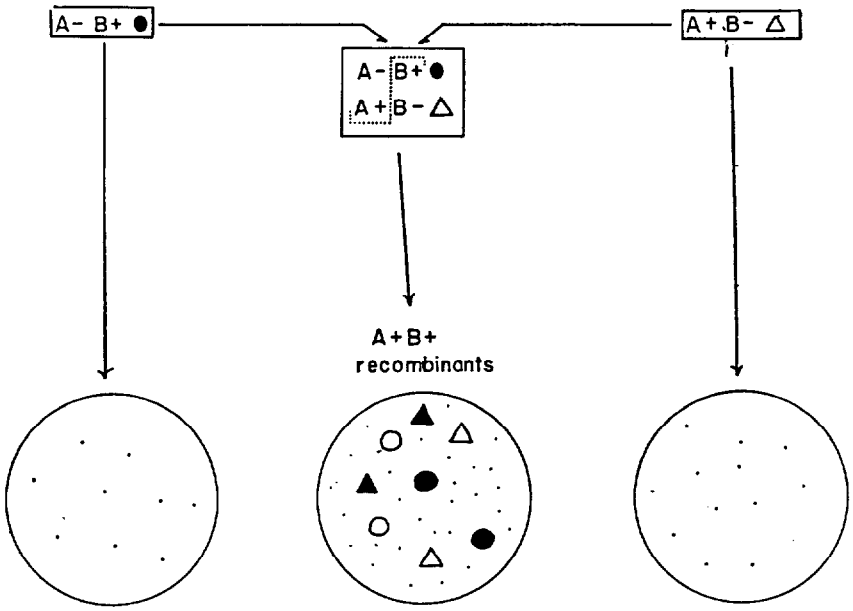

FIG. 3.3. The selection of genetic recombinants by minimal medium. Whether plated separately or together, the parent mutants (represented by dots) are unable to form visible colonies in minimal agar. However, in mixed cultures, A + B+ recombinants (prototrophs) are produced, and these are capable of forming colonies. The circle and triangle, shaded and unshaded, represent two additional pairs of factors which, being unselected by the minimal medium, are free to reassort among the prototrophs. The new combinations of unselected factors provide the strongest support for the validity of this experiment.

cells or cell products whose union results ultimately in genetic recombinations. However, many attempts (Lederberg, 1947; Davis, 1950a) to replace intact cells of one parent by extracts or filtrates in crossing experiments have been completely unsuccessful, and it seems most reasonable to suppose that, as in the simplest algae, yeasts and molds, the fusion cell results from the copulation of ordinary vegetative cells.

On the basis of the kinds of experiments just discussed, and of cytological studies of cell division (Robinow, 1945), the usual life cycle of *E. coli* K-12 would be as follows. The vegetative cell contains one or more haploid nuclei usually all alike, each containing one set of genes. The variable number of nuclei, which depends on the culture phase, is

merely the result of an incomplete synchronization between nuclear multiplication and cell division. Occasionally, haploid cells fuse, followed by fusion of one pair of nuclei to give a diploid nucleus. Ordinarily, the diploid nucleus never multiplies as such, but segregates immediately possibly to give four segregated haploid nuclei, but this is uncertain. The nutritional selective method permits the recovery, however, only of these segregants in which there has been a "crossing-over" or genetic exchange so as to result in a prototroph. This means that usually only a single prototroph can be isolated from a given fusion, and that an unknown number of fusions may remain undetected, if none of the segregated progeny happen to be prototrophic recombinants. Calculations on the probable amount of recombination, however, suggest that about half the fusions do result in detectable prototrophs.

In essence, therefore, *E. coli* exhibits what is called a "haplobiontic" life cycle, which is typical of most lower fungi and algae, and is analogous to the yeast *Zygosaccharomyces*, or to the chlorophyte *Chlamydomonas*. Many of the lower thallophytes, however, have evolved a mating type mechanism, so that fusion occurs only between clones of distinct origin, *i.e.*, which carry different forms of a mating type or, loosely, a "sex" gene. As yet, no evidence is available for the differentiation of "sexes" in bacteria. However, it may well be that the rather low frequency of recombination observed may have been due to the use of bacterial strains which are not sexually differentiated.

The details of the life cycle in *E. coli* K-12, as in other organisms, (Winge and Roberts, 1949) are under genetic control, and stocks have been found which show informative deviations from the typical pattern (Lederberg, 1949a). Here, instead of undergoing immediate segregation, the fusion nucleus is capable of indefinite proliferation in the diploid condition, although the diploid cells show a strong tendency to segregate at every division, and it is technically difficult to maintain a culture in the diploid condition. Such a life cycle would be termed "haplo-diplobiontic," and has a precedent, for example, in *Saccharomyces cerevisiae* and in higher cryptogams. The persistent diploids are especially advantageous for studying the effects of gene combinations in the same cell. Furthermore, they afford additional proof of the tangible existence of the intermediate diploid stage, in which the genes of the two parents are momentarily brought together, later to reassort and separate. This proof is particularly consolidated by experiments in which the diploid cells are allowed to proliferate under microscopic observation, and in which the isolation of the progeny with the micromanipulator leaves no doubt as to the concurrence of the genetic factors of the parents within single cells which is the essence of sexuality (Zelle and Lederberg, 1951).

This aspect of bacterial genetics is still so undeveloped that a great many important questions remain unanswered. Perhaps the most important of these is how generally sexual reproduction may occur among bacteria. The answer to this question must wait upon the application of genetic techniques to a variety of bacterial species. In *E. coli*, intensive study of perhaps half a dozen distinct strains has revealed two besides K-12 which recombine among themselves, and with K-12 (Cavalli and Heslot, 1949). More recent developments are summarized in Lederberg *et al.* (1951). A considerable number of distinct strains of *E. coli* have been found capable of crossing. Comparable methods have also been used to demonstrate a recombination mechanism in *Salmonella typhimurium*, but an agent capable of passing a filter is implicated here—see the discussion of reduced forms of bacteria on page 85.

Sexuality is generally regarded as a source of genetic variation via the many new combinations of characters that are generated. The evolutionary importance of this process depends, in large part, on how widely recombination can take place, which remains to be seen. For the moment, then, the principal biological importance of bacterial sexuality may be as a tool in the investigation of the genetics and the life cycle of bacteria, rather than as one of the underlying principles of organic evolution, which sexual reproduction is for higher forms.

2. Virus Recombination

For many years, opinions were divided on the living nature of viruses, and particularly of bacteriophages. Whatever doubts may yet linger should be dispelled by the recent discovery that bacteriophages have an elaborate genetic system, and in particular a mechanism of genetic recombination. Two converging lines of evidence have been adduced.

Luria and Dulbecco (1949) have followed up in detail an observation previously mentioned by Delbrück and Bailey that irregular plaque counts were obtained from ultraviolet inactivated phage, according to the procedure used in diluting the phage suspensions. They conclude that suspensions of UV-treated phage may contain particles which are capable to adsorption on to susceptible bacteria, but incapable of further growth and regeneration of phage, if the particle is the only one to have infected a given cell. However, if two or more such particles are adsorbed onto a single bacterial cell, they will have a chance to grow and regenerate normal phage. The probability that doubly-infected bacterial cells will produce "viable" phage is dependent on the UV dose given the phage particles. The irregular counts noted by Delbrück and Bailey depend on whether the ratio of phage to bacteria was such that multiple adsorptions per cell could take place.

The quantitative results obtained by Luria and Dulbecco in their experiments on the "reactivation" of phage by multiple infection agree with a theory of recombination of genetic units. It is postulated that each phage particle contains a number of different genetic units, such that if any of these is inactivated (as by UV), the phage particle will be unable to grow in the bacterial host. However, if two particles, carrying between them a full active complement of the different units should infect the same cell, a normal phage particle can be restituted by recombination of the active units. With increasing doses of UV, more and more of the units in each particle will have been inactivated, thus lessening the chance that a random pair of particles will be able to make up a full set of active units between them. By determining the rate at which the efficiency of reactivation decreases with UV dose, an estimate of the number of genetic units involved can be made—about 20–40 for various phages in the T group, attacking *E. coli* B. This multiplicity reactivation should be carefully distinguished from photoreactivation, which is an independent phenomenon.

Hershey and Rotman (1949), using the same phages, obtained more direct evidence of recombination of characters. The number of mutant characters available in phage is limited, but mutations affecting host range and plaque morphology have been found. These authors report that, among the output of particles from a cell infected with two kinds of mutants, new combinations of the characters of the input phages will be found. Corresponding complementary types occur with equal frequency, and they have also succeeded in mapping a number of mutations on linear linkage groups in accordance with the observed frequencies of recombination. These observations leave no doubt of the exchange of genetic characteristics between phages within the host cell. However, the details of the mechanism of exchange have not all been clarified. Both groups of workers have speculated that the phage particle within the host cell may break up into its genetic components, which are not reassembled until the cell is ready to lyse.

A similar enterprise with influenza virus has also provided evidence supporting genetic recombination in this organism. It would be difficult to overestimate the potential importance of virus recombination in medical epidemiology (Burnet and Lind, 1951).

V. Variation and Adaptation: Recapitulation

The capacity to adapt is one of the cardinal attributes of life. Students of adaptation are customarily classified either as physiologists, who deal with the adaptive responses of the individual organism, or evolutionists who deal with organic adaptation, *i.e.*, the long range responses

of the population or species. The bacterial physiologist cannot ignore populational adaptation, however, because he always works with populations, not single individuals. He must be careful to distinguish whether an adaptive response is physiological, a directed change within each bacterial cell, or populational, whereby the genetic composition of the culture is altered by differential rates of growth, death or mutations. The impression held by many bacteriologists that bacteria have unusual adaptive flexibility is doubtless based on the concurrence of these mechanisms in the bacterial economy.

Connected with this distinction is a second: whether the adaptation is hereditary. As previously emphasized, good evidence for any directed adaptive mutation in bacteria is completely lacking, from which we conclude that physiological adaptations in general are not hereditary. The converse, that populational adaptations are hereditary, holds, in general, but is not logically necessary. We can imagine, for example, that quantitative resistance to an antibiotic might be randomly distributed among the cells in a population. Populational adaptation would not result from the interaction of these cells with the antibiotic unless some correlation exists between parent and offspring in resistance, but this correlation need not be heritable in the sense that it would persist indefinitely in the absence of the antibiotic. In practice, however, the distinction between physiological and populational adaptation parallels that between the non-genetic and the genetic.

Not all workers have accepted the duality of adaptation mechanisms. Hinshelwood (1946), for example, has disregarded the selection of spontaneous mutants as an element of bacterial adaptations, apparently in order to bolster the applicability of his system of chemical kinetics to problems of bacterial growth. A more eclectic outlook seems to be justified by the evidence. In earlier paragraphs, examples of adaptive mutations have been given. Instances of physiological adaptations will be found in nearly every chapter of this book. Here, we shall discuss a case of adaptation involving both mechanisms, so that the distinction between them was confused for some time.

Perhaps the most remarkable of bacterial adaptations consists of the flexibility in amount and activity of bacterial enzymes, and of their adjustment in response to new substrates, pH change, and so forth (Monod, 1947). A lactose-fermenting strain, so-called, of *E. coli* should more strictly be re-defined as a strain that is competent to produce adaptive enzymes for lactose fermentation when exposed to lactose under appropriate conditions. Cells grown on glucose-containing substrate have virtually no capacity to ferment lactose, compared to cells harvested from a medium with lactose. After prolonged exposure to lactose, how-

ever, unadapted cells will slowly acquire fermentative competence for this carbohydrate. Since this adaptation occurs in the absence of growth—rather slowly in the present instance—and disappears very promptly when the adapted cells are transferred back to glucose substrates, this adaptation is clearly physiological and non-genetic.

Variant types of *E. coli* are known which are incompetent to adapt to lactose or to various other sugars. The designation *E. coli-"mutabile"* has been applied to lactose-negative strains which occasionally mutate to lactose-positive. Reasonably good evidence can be cited for the view that the transition from lactose-negative to lactose-positive which is observed in lactose broth cultures inoculated with *E. coli-"mutabile"* is due to the selective advantage of spontaneous lactose-positive mutants, so that they overgrow the population (see Luria, 1947). At first sight, it may seem paradoxical that the same overall adaptive process may be populational and physiological at the same time. However, the contradiction is resolved when it is realized that the genetic adaptation changes the potentialities of the cell, so that it then becomes competent to undergo physiological enzymatic adaptation.

In view of the potentially unlimited scope of physiological effects of mutations, one might predict that the course of bacterial evolution would be unerringly adaptive, and in general in the direction of an unlimited range of biochemical potentialities. That bacteria as we see them are peculiarly specialized, and that the range of their biochemical activities is (fortunately for us) often restricted, we ascribe to the microscopic character of biological evolution. That is to say, the evolution of a microculture is adaptive only in terms of the immediate local environment. The microbe's evolution is not directed by an intelligent foresight which would enable pre-adaptation to other, anticipated microenvironments. Natural selection favors those types which, at the moment, proliferate most rapidly, and pays no heed to potential changes in the environment which may turn the tables on the once dominant mutant. This principle enables us to understand experimental results which might otherwise seem paradoxical, for example, the apparently anomalous selection in minimal medium against prototrophs in mixed culture with certain biochemical mutants in *Neurospora* and in *E. coli* (Ryan, 1946; Ryan and Schneider, 1949).

These considerations also lead to a more immediate conclusion, namely that *a priori* deductions on the direction of selection in a mixed microbial population may be unsafe, even when based upon measurements of growth or killing rates on the isolated pure cultures. A number of examples are now available showing the disaccord between the simple expectations and the experimental results of mixed culture experiments.

The detailed analysis of bacterial populations may be exemplified by studies on *Brucella abortus* (Braun, 1947b; Goodlow, Mika and Braun, 1950). Cultures from smooth inocula regularly accumulate large proportions of rough variants upon aging, but this displacement is not reflected in differential growth rates of the smooth and rough types. It was ultimately shown that the amino acid alanine accumulated in aged cultures, and that this compound selectively inhibited growth of smooth cells, thus conferring a selective advantage on spontaneously occurring rough variants.

Several workers studying the increase in proportion of phage-resistant or other mutants under the influence of mutation "pressure" have been impressed by sporadic cycles in the numbers of resistants. Following a period during which the proportion of resistants increases slowly and linearly, as mutational events convert individual cells from sensitive to resistant, the resistants may suddenly disappear, and then re-accumulate at the same rate as before (Stocker, 1949; Novick and Szilard, 1950; Atwood, Schneider, and Ryan, 1951). The cycles have been ascribed to subtle, adaptive mutations which result in cells with an improved adaptation to the conditions of culture. Descendants of these cells overgrow the culture and displace the previous population. Because the proportion of resistants is usually very low, the adaptive mutation and overgrowth will almost always stem from a sensitive cell, giving rise to a new sensitive population. It has been suggested that periodic selection may protect microbial cultures from the accumulation of auxotrophic or other potentially deleterious mutations. However, the long-term equilibrium between sensitives and resistants, or other pairs of alternatives, is not likely to be affected by such non-discriminatory selective processes.

We thus conclude that our ignorance equips us very poorly for our efforts to interpret physiological evolution of bacteria except in the most general terms. We should not, however, accept the present differentiation of microbes as a *fait accompli*, but must ask how they came to be as they are. Bacterial genetics, if it has not yet solved this problem, may at least hopefully claim that its development has helped the clearer formulation of this as well as other problems of bacterial physiology.

Mathematical Appendix

The Poisson Distribution

Bacteriologists frequently wish to know the statistical distribution describing events with a low probability, p, and with a large number, n, of trials, such that $n \times p$, the expectation is of the order of unity. For example, random samples containing 10^{-9} ml. might be taken from a

population of 10^9 bacterial/ml., and the experimenter would like to know how many bacteria to expect in a given sample. The average number of bacteria per sample, $m = n \times p$, will be $10^{-9} \times 10^9$ or 1, but if the bacteria are scattered randomly throughout the suspension, some samples may contain no bacteria, while others will contain several. The probability, $P(x)$, that such a sample will contain just x bacteria, is a function of x and m (the average per sample) known as the Poisson distribution. It can be shown that $P(x)$ is closely approximated by the terms of the series

$$e^{-m}, \quad e^{-m}m, \quad \frac{e^{-m}m^2}{2}, \quad \frac{e^{-m}m^3}{3 \times 2}, \quad \frac{e^{-m}m^4}{4 \times 3 \times 2}, \quad \ldots \quad \frac{e^{-m}m^x}{x!},$$

for the probability that a sample will have 0, 1, 2, 3, 4 . . . or x bacteria. (For references and further discussion, see Eisenhart and Wilson, 1943.)

The terms of this series are not difficult to compute, but useful tables of the Poisson distribution are available (Molina, 1942).

The 0'th term of the distribution is of special interest, *i.e.*, $P_0 = e^{-m}$, as in the form $m = -\ln P_0$ it permits m to be calculated from a frequently recorded experimental datum, P_0, which may represent, for example, the fraction of replicate tubes which remain sterile. The "serial dilution code" method of enumerating viable bacteria is based upon this expression (Eisenhart and Wilson, 1943).

The Measurement of Spontaneous Mutation Rates

Spontaneous mutation rates are usually expressed as some function of the growth rate of the bacterium, rather than in chronological units. This convention is justified by the correlation of mutations with growth (Luria and Delbrück, 1943; Englesberg and Stanier, 1949), although this relationship needs further study.* It is not clear whether spontaneous mutations are thus directly related to reproduction (hypothesis of error in duplication), or whether the growth rate merely expresses the overall metabolic rate. On the latter basis, Luria and Delbrück (1943) defined

* In a further attack on problems of spontaneous mutation, Novick and Szilard (1950) made use of a device (the "chemostat") in which bacterial cultures were maintained in a steady state at a fixed growth rate by means of a continuous flow system. In agreement with previous work, they found that *E. coli* cells did not accumulate phage-resistance mutations when they were prevented from growth by deprivation of tryptophane. However, cells which were permitted to grow at different rates by limiting tryptophan concentration mutated at a constant rate per cell per unit time, independent of growth rate. The effect of temperature on mutation rate could be expressed as a $Q_{10} = 2$. These experiments suggest that spontaneous mutations are a result of metabolic activity rather than accidental errors in the copying of a gene at the time of reduplication. More decisive evidence is needed, however, to determine if this conclusion can be generalized, and to establish the particular areas of metabolic activity associated with spontaneous mutation.

a time unit equal to the mean generation time divided by ln 2. In this appendix the following symbols will be used:

a = mutation rate (probability of mutation per cell per unit time)
N = number of cells (at time t)
m = number of mutational events or mutant clones
r = number of mutant cells

As a consequence of the definition of t, the law of growth may be stated as:

(3.1) $$dN/dt = N, \text{ or } N = N_0 e^t.$$

Mutant cells (r) are augmented either by growth, at rate r, or by new mutations, at rate aN. Thus we have $dr/dt = r + aN$, which admits the solution,

(3.2) $$r = atN \text{ (for } N_0 = 1) \text{ or } r = aN \ln N.$$

Solving for a, we have

(3.3) $$a = r/(N \ln N)$$

Expression 3.3 provides a predicted value of r, mutants per culture, which represents the average over an indefinitely large number of cultures. However, early generations rarely contribute mutations (since N is relatively low at that time) but influence r disproportionately because of the considerable amount of further multiplication that such mutants enjoy when they do occur. Consequently, the application of 3.3 would *usually* result in a considerable underestimate of a if an early mutation did not occur, and *rarely* in a very great overestimate if an early mutation did occur in one of the cultures assayed.

In an attempt to correct the usual underestimation by 3.3, Luria and Delbrück (1943) formulated a derivation of what they termed a "likely average," r, which represents what might be expected as the experimental mean in a finite number of cultures, C. The fictional assumption is made that no mutations have occurred prior to an arbitrary time, $t = i$; i is chosen so that there will have been a single premature mutation, on the average, in the entire experiment of C cultures. That is, $aCN_i = 1$, or $i = -\ln aC$. Since $t = \ln N$,

(3.4) $$t - i = \ln N + \ln aC = \ln aCN.$$

If in the derivation of 3.2 the limits of integration are taken as (t, i) rather than $(t, 0)$, in accordance with our fiction that no mutations occur prior to $t = i$, we obtain

(3.2a) $$r = (t - i)aN = aN \ln aCN.$$

This equation cannot explicitly be solved algebraically, but is readily

solved numerically in the form $Cr = aCN \ln aCN$, since Cr is known (total number of mutants counted in all C cultures of the experiment).

Although relation 3.2a offers certain short-term advantages, it does not mitigate the very high variance of r, calculated by Luria and Delbrück to be: $raCN/\ln aCN$. As aCN is simply the total number of mutations in the experiment, it may be hundreds or thousands of times larger than r. Consequently, it will be difficult to obtain consistent estimates of r. A second limitation of this method is the assumption that the mutants grow at the same rate as the non-mutants, which is implicit in the "r" term of 3.2. Small deviations from equal growth rates will be reflected in large inaccuracies in the measurement of mutation rates. Although this method uses such detailed information as the number of mutants per culture, it is, therefore intrinsically inefficient and liable to error.

Rates of mutation can also be estimated by a method that may seem at first sight to be inefficient, but that is free from the defects listed above. If p_0 is defined as the fraction of replicate cultures in which no mutants are present, *i.e.*, in which no mutations have occurred, it can be related to a. Since a is defined as the probability of mutation per bacterium per time unit, $a\bar{N}t$ will give the number of mutations in a culture, where $\bar{N}$ is the mean value of N over t:

$$(3.5) \qquad \bar{N} = \frac{1}{t}\int_0^t N dt = \frac{N_0}{t}\int_0^t e^t dt = \frac{N_0}{t} e^t \Big]_0^t = \frac{1}{t}(N - N_0)$$
$$\doteq \frac{N}{t}$$
$$\therefore m \text{ (number of mutations)} = at\, N/t = aN$$

This result can also be expressed in the form "there are N chances for mutation, each with probability a." Then the probability that none of the N trials results in a mutation can be expressed:

$$(3.6) \qquad p_0 = (1 - a)^N = (1 - a)^{aN/a} \doteq e^{-aN},$$

or,

$$(3.6a) \qquad a = -\ln p_0/N.$$

Equation 3.6 is, of course, zero term of the Poisson distribution of mutations, with $\bar{m} = aN$.

The fact that some cultures will contain mutants and others will not, in accordance with 3.6, has been used fallaciously as an argument that the mutation is spontaneous. This so called fluctuation, however, simply indicates that some random variable at the time of selection

influences the result, and is by itself not incompatible with the hypothesis of induced variation. For example, the same kind of result could be obtained during bacterial disinfection: with an appropriate treatment, some cultures would be completely sterilized, while others would contain viable cells, but we could not conclude that sterilization was spontaneous. Therefore the use of 3.6a, unlike 3.2a, presupposes the hypothesis of spontaneous mutation, and is useful only for estimating its rate. Other methods for measuring mutation rate are reviewed by Newcombe (1948).

The spontaneous mutation rate is, of course, not the same for all bacterial characters, but depends on the stability of the particular genic material studied. Rates varying from 6×10^{-2} to 1×10^{-10} have been described for colonial variation in *Salmonella typhimurium* (Shapiro, 1946) and streptomycin resistance in *E. coli* (Newcombe and Hawirko, 1949), respectively. Many bacterial mutations fall within the range 10^{-6} to 10^{-8}, which is quite comparable with the frequency of spontaneous mutations in higher plants and animals.

While this chapter was being prepared, a publication appeared in which the theoretical distribution of mutants is derived using the same sort of model as above (Lea and Coulson, 1949). These authors point out that the estimation of the mutation rate, a, from the statistic, r, is very inefficient, and that the variance does not decrease with an increasing number of cultures. That is, for a given number of mutants, r, a single larger culture gives as satisfactory an estimate as a series of smaller cultures of the same aggregate size. With the help of the theoretical distribution, a rather involved method was developed for the maximum likelihood estimation of a, which uses all of the statistics most efficiently. However, Lea and Coulson show that a can be calculated from the median number of mutants, r_0, with little loss of efficiency, and with very much less computational effort. They show that

(3.7) $\quad r_0 = m(1.24 + \ln m)$, where, as before, $a = m/N$.

Like 3.2a, this expression can be solved for m numerically, or by interpolating in Lea and Coulson's Table III.

In view of the probable inaccuracies of the postulated model, such as phenotypic lag and nuclear segregation (see text), equation 3.7 seems to afford the most feasible and economic way of using data involving numbers of mutants. Inasmuch as it eliminates the necessity of using precise counts of cultures with inordinately large numbers of mutants, it is also more practicable than estimates based on the mean number of mutants. However, when p_0 is about 20%, the null fraction method is almost as efficient as any of the others, and is probably generally the method of choice.

L. Pasteur