NEW YORK CITY BRANCH

SOCIETY OF AMERICAN BACTERIOLOGISTS

Fifty-fourth Meeting

Hotel Statler, 7th Avenue and 33rd Street, New York City

THURSDAY, JANUARY 4th, 1951

Program

12:15 P. M. — LUNCHEON, Foyer of the Georgian Room

DAVID M. ROGER, *Presiding*

Report on the Fifth International Congress of Microbiology, held at Rio de Janeiro, August, 1950, and General Impression of a Scientific Trip through South America.—*Herald R. Cox, Director of Virus and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company.*

Dr. Benj Ruben Dr. Carl Cohen

SECTION A—2:15 P. M. Di 40500

Georgian Room

RICHARD DONOVICK, *Presiding*

A1. A Pleomorphic Organism Isolated from the Blood of Leprosy Patients. T. de
Eleanor Alexander-Jackson, Lessing J. Rosenwald Fellow, Cornell Medical College; Bureau of Biological Research, Rutgers University. (10 min.)

A2. Cessation of Bacterial Motility as a Rapid Test for Germicidal Action. T. de
Peggy Zaretsky and Leon Buchbinder, Bureau of Laboratories, New York City Department of Health. (10 min.)

A3. Recurrent Parasitemia in Chronic Toxoplasmosis Produced by the Injection of Anti-Toxoplasma Sera.—*Heinz Eichenwald, The New York Hospital and the Department of Pediatrics, Cornell University Medical College.* (15 min.)

A4. Antibiotics Active against Bacterial Viruses.—*Igor N. Asheshov and Elizabeth A. Hall, The New York Botanical Garden.* (10 min.)

A5. The Combined Action of Antimicrobial Agents against. *M. tuberculosis.*—*Tulita F. Lenert, C. Dealy, E. Schneider and Gladys Hobby, Chas. Pfizer and Company, Inc., Brooklyn.* (10 min.)

A6. A Comparison of Several Methods of Testing *M. tuberculosis* for Streptomycin Sensitivity.—*Lenore R. Peizer, Bureau of Laboratories, New York City Department of Health.* (10 min.)

A7. Rapid Testing of Sensitivity of Bacteria to Antibiotics by the Disk Method.—*A. J. Weil, Bronx Hospital.* (10 min.) T. de

A8. Studies on the Action of the Streptomycins on *Salmonellae.*—*F. Pansy, P. Kahn and R. Donovick, The Squibb Institute for Medical Research, New Brunswick.* (10 min.)

A9. A Study of the Sanitary Quality and Control of Custard Filled Bakery Products in a Large City.—*Abraham E. Abrahamson, Rubin Field, Leon Buchbinder and Anna V. Catelli, Bureaus of Food and Drugs, and Laboratories, New York City Department of Health.* (20 min.)

SECTION B—2:15 P. M.

Parlor II — VIRGINIA FISHER, *Presiding*

B1. Anaerobic Respiratory Studies on Five Rumen Organisms.—*W. M. Connors and L. S. Gall, National Dairy Research Laboratories, Inc., Oakdale, Long Island.* (10 min.)

B2. Effect of Various Levels of Glucose in Media on Morphology and Growth Characteristics of Some Rumen Bacteria.—*C. N. Huhtanen and L. S. Gall, National Dairy Research Laboratories, Inc., Oakdale, Long Island.* (10 min.)

B3. Micrococci Requiring Purines.—*S. Aaronson and S. N. Hutner, Dept. Biology, New York University, Washington Square and The Haskins Laboratories, Inc., New York.* (8 min.)

B4. What Determines the Lower pH Limit for Bacterial Growth?—*Seymour H. Hutner, The Haskins Laboratories, Inc., New York.* (8 min.)

B5. Nutritional Requirements of the Water Mold *Hyphochytrium catenoides.*—*Helen S. Reischer, The Haskins Laboratories, Inc., New York, and the Department of Biology, Queens College.* (10 min.)

B6. Sodium Chloride Requirements of Some Marine and Non-Marine Bacteria.—*Wolf Vishniac, The Haskins Laboratories, Inc., New York, and the Department of Pharmacology, New York University Bellevue Medical Center.* (12 min.)

B7. The Growth of *Torulopsis utilis* on Sodium Acetate in Shaken Culture.—*H. Christine Reilly, The Sloan-Kettering Institute for Cancer Research.* (10 min.)

B8. Bacterial Desoxypentose Nucleic Acids of Different Composition.—*Stephen Zamenhof and Erwin Chargaff, Dept. Biochemistry, College of Physicians and Surgeons, Columbia University.* (20 min.)

B9. Some Effects of X-Radiation on *Salmonella typhosa* (Hopkins Strain). —*Fanchon Hart, Bruce Allen, and William J. Wong, Columbia University, College of Pharmacy.* (10 min.)

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(OVER)

VOLUME 11 March, 1947 NUMBER 1

BACTERIOLOGICAL REVIEWS

A PUBLICATION OF THE
SOCIETY OF THE AMERICAN BACTERIOLOGISTS

EDITOR
BARNETT COHEN

It is characteristic of Science and Progress
that they continually open new fields to our vision.—PASTEUR

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BACTERIOLOGICAL REVIEWS

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Contents

Note to Contributors

Bacteriological Reviews is published under the auspices of the Society of American Bacteriologists to provide authoritative, critical surveys on the current status of subjects and problems in the diverse fields of microbiology. This includes the occasional short monograph that cannot be economically published in the Monograph series of the Society.

Suggestions for timely reviews are solicited; and prospective authors are invited to discuss with the Editor the suitability of their proposed manuscripts. The submittal of a synopsis or topical outline for an advisory opinion is recommended. In addition, the following suggestions may serve as a general guide.

Plan and Content of Review. A rational, balanced development of the topic, rather than a chronological treatment, is to be preferred. Old history, if any, may be covered usually by simple reference to earlier reviews. A mere compilation or annotated bibliography does not adequately serve the objectives of this journal. The extensive presentation of new experimental material belongs properly in the domain of the monograph or of the appropriate "experimental" journal.

References. Judicious selection of references is an important function of the reviewer. Cited references should be listed alphabetically according to author and numbered serially, the numbers being used in the text. The full title of each paper should be given. Each citation should be checked with the original publication to avoid embarrassing errors. The system used in *Chemical Abstracts* for abbreviations of journal names should be followed.

Length. The length of a paper is no measure of its quality, and it is only the latter that determines acceptability for publication. However, practical considerations make it desirable to set a provisional limit of 30 to 40 printed pages for a review, text, tables, figures and references included. (About 10,000 words go into 20 pages; 24 references per page.) A short monograph must come within the size of a single issue, about 100 pages.

The acceptability of a review cannot, of course, be finally decided until the finished product has been examined. In the case of a short monograph, acceptance is also contingent upon the advice of the Editors-in-Chief of the Monograph series and of the *Journal of Bacteriology*.

Correspondence relating to editorial matters should be addressed to the Editor:

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RECENT ADVANCES IN BACTERIAL GENETICS

S. E. LURIA

Indiana University, Bloomington

CONTENTS

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 $M/N = a/(s\text{-}a)$, 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^{-3} 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^{-3} 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 recognizable 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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THE MECHANISM OF BIOLOGICAL NITROGEN FIXATION[1]

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In late 1940 two summary reports concerned with biological nitrogen fixation were completed, the monograph, *Biochemistry of Symbiotic Nitrogen Fixation*, by Wilson and the review by Burk and Burris which appeared the following year in *Annual Review of Biochemistry*. The judgment of these three authors at that time regarding the chemical mechanism of the process is expressed in these quotations from the review,

"For many workers it has been psychologically irresistible to conclude upon finding some extracellullar nitrogenous product that it was involved in either the initial or later anabolic rather than catabolic stages. Although a considerable number of isolated and hypothetical compounds have been so regarded during the past fifty years, the main controversy during the past decade has been confined to the two rival intermediates, ammonia and hydroxylamine (or oxime). The present weight of evidence favors the latter, but by no means offers unequivocal support.

". . .the occurrence of any ammonia, observed or otherwise, as an essential intermediate in nitrogen fixation by *Azotobacter*, admittedly still possible, yet awaits sufficiently critical experiments in support."

Since these words were written, we believe that sufficient critical experiments have been completed and that their results, together with relevant data obtained in the previous decade, warrant a reversal of the opinion expressed in the last lines. Moreover, present established facts may even justify an attempt to draw a tentative blueprint of possible mechanisms, especially since there have been no frankly theoretical papers in the field since those of Burk (1934, 1937). Even though such schemes represent little more than an enlightened surmise, they should be helpful in evaluating the advances that have been made, in noting the gaps in our information, and in suggesting possible methods for filling these. Before discussing these recent studies in detail, we should, however, first consider briefly the developments that led to them.

Until about 1930 the little appreciated difficulty of insuring purity of Azotobacter cultures (Winogradsky, 1937; Burk and Burris, 1941 p. 604) was so neglected that most early papers possess only historical interest. The inherent complication of the symbiotic fixation requiring as it does two organisms, bacteria and plants, discouraged attempts to investigate the mechanism of this system. The modern studies may be said to date from the paper of Meyerhof and Burk (1928), which described a method for using the micro-respirometer to investigate the mechanism of nitrogen fixation by Azotobacter. Important as was the introduction of this technique whose usefulness already had been demonstrated in enzyme studies, their major contribution was applying the

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point of view of physical chemistry to the problem. Investigations of the mechanism by precise physical-chemical methods led to a much-needed more critical attitude toward what constituted admissible evidence for support or rejection of a particular hypothesis. Burk (1936, 1937) proposed seven criteria for examination of claims, and whether one agrees with him as to either their necessity or sufficiency, they remind the investigator that standards here as in other branches of science are essential.

While Burk and his associates continued the physical-chemical studies with Azotobacter, Wilson and his collaborators at the University of Wisconsin began analogous investigations using the symbiotic system of inoculated red clover plants. These researches by the two American groups eventually provided much necessary detailed information about the characteristics of the enzyme system responsible for fixation. By their very nature, however, they could furnish only suggestions about the chemical intermediates. Experiments on this question were made by Virtanen (1938) and his coworkers in Finland with the symbiotic systems and by Winogradsky (1930, 1935, 1938, 1939) in France using Azotobacter. The physical-chemical combined with the organic approach first yielded definite and precise information on intermediates when Burris (1942) attacked the problem by application of isotopic techniques.

IDENTIFICATION OF THE INITIAL KEY INTERMEDIATE

I. Hydroxylamine. Although numerous compounds have been proposed for the key intermediate position in biological nitrogen fixation—representing the end-product of the initial stage of fixation and the starting point for assimilation —only two, ammonia and hydroxylamine, are backed by serious experimental attempts at verification. Hydroxylamine, first suggested by Blom (1931), has received its experimental support almost entirely from the studies of Virtanen and his associates (Virtanen, 1938, 1939; Virtanen and Laine, 1939) on the excretion phenomenon in inoculated leguminous plants. The main pillars are:

1. Aspartic acid is the sole nitrogenous compound excreted in any quantity.
2. Extremely small quantities of an oxime, tentatively identified as oximinosuccinic acid, is detected in the excretory products.
3. Oxalacetic acid is found in leguminous plants.
4. Excised nodules fix more nitrogen when oxalacetic acid is supplied.

We have already examined these claims in detail and rejected their specificity (Wilson, 1939, 1940, p. 173; Burris and Wilson, 1945). Briefly, our view is that most of the evidence could be applied equally well in support of the ammonia hypothesis. The most specific—the isolation of the oxime—suffers from the fact that the quantity found is extremely small. Not only does this place the compound in the category of many other nitrogenous products traces of which are found in cultures and substrates of nitrogen-fixing organisms, but also it interposes difficulties in the essential unequivocal identification. A recent publication by Virtanen, Linkola, Hakala, and Rautanen (1946), however, reduces the entire question to an academic status as far as its significance for the hydroxylamine-ammonia controversy is concerned. They report that, contrary

to their earlier findings, the excretion products consist not entirely of aspartic acid, β-alanine and oximinosuccinic acid but of a mixture of these and *glutamic* acid. Hence, if the experiments on excretion are to be accepted as having a necessary significance for the identity of the key intermediate, a view that Wilson and Wyss (1939) questioned, the new work includes ammonia as well as hydroxylamine.

Experiments that implicate hydroxylamine with fixation of N_2 by Azotobacter are confined largely to reports that traces of oximes have been detected in cultures. Endres and his co-workers (1934, 1935, 1938) contend that they have associated specifically the occurrence of the oxime with assimilation of N_2; but Burk (1937) questions this claim on the grounds that an oxime is also found in cultures supplied with fixed nitrogen including ammonia, and that the oxime is not used for growth by Azotobacter. Finding NH_2OH in cultures of *Aspergillus niger* supplied with ammonium salts, Steinberg (1939) concluded that it arose from oxidation of the ammonia. Therefore, occurrence of hydroxylamine or its oximes can hardly be regarded as a biological rarity and therefore necessarily associated with the specific process of fixation of N_2.

II. Ammonia. Experiments with the stable nitrogen isotope N^{15} by the authors and their collaborators have supplied an array of evidence that definitely points to ammonia as the key intermediate in nitrogen fixation by Azotobacter. Four specific types of evidence can be cited, specific in the sense that the observations depend on the demonstrated functioning of the nitrogen fixation reaction.

1. In experiments of short duration N^{15} supplied as either N_2 or NH_4^+ accumulates in the same fractions, notably glutamic and aspartic acids. Later a more general distribution in other fractions appears (Burris, 1942; Burris and Wilson, 1946). Typical results from such experiments are shown in table 1. In this table the most significant data are those in which the N^{15} concentration exceeds that of the average represented by "total hydrolysate". In an extremely short-time experiment with labeled ammonium (3 minutes) the highest level of isotopic nitrogen is found in the glutamic acid fraction—"amide" nitrogen includes ammonium nitrogen and hence absorbed $N^{15}H_4^+$. After 15 minutes a more general distribution of N^{15} is noted, but by far the highest concentration of labeled nitrogen is still maintained in the glutamic acid fraction. Compare now these distributions when NH_4^+ is supplied with that observed in Azotobacter fixing N^{15}-enriched N_2 for 90 minutes when equilibrium conditions would be more closely approached. The similarities (high N^{15} levels in glutamic and aspartic acids, low levels in arginine, histidine, etc.) point unmistakably to the functioning of NH_4^+ in the fixation reaction. Additional support is provided by the fact that not only in Azotobacter but also in both plants and animals supplied N^{15} as NH_4^+ the N^{15} accumulates in glutamic acid indicative that this reaction is of general biological significance (Foster, Schoenheimer and Rittenberg, 1939; Rittenberg, Schoenheimer, and Keston, 1939; Vickery, Pucher, Schoenheimer and Rittenberg, 1940). The secondary accumulation observed in the aspartic acid fraction probably reflects the activity of the glutamic-aspartic transaminase in Azotobacter (Lichstein and Cohen, 1945). The argument that the fixation

might proceed via the NH_2OH-aspartic route and that the low concentration of this amino acid as well as its inferior N^{15} content arises from its transfer of fixed N^{15} to α-ketoglutaric acid ignores the fact that the equilibrium favors the formation of aspartate not glutamate, and that it would be impossible to pass N^{15} at a particular concentration in aspartate to build up a higher concentration in glutamate.

In an experiment such as the ones described, passing N^{15} at a particular concentration from aspartate to build up a higher concentration in glutamate would be impossible, as stated, without making the illogical assumption that during the short period when N^{15} was present the organisms suddenly shifted the ratio of aspartate to glutamate formed.

TABLE 1

Distribution of N^{15} in Amino Acids and Amino Acid Fractions

	ATOM PER CENT N^{15} EXCESS		
	15 min. NH_4^+ treatment	3 min. NH_4^+ treatment	90 min. N_2 treatment
Total hydrolysate	1.049	0.174	0.275
Humin	0.683	0.067	—
Neuberg filtrate	0.915	—	0.441
"Amide" nitrogen	0.981	0.749	0.325
Arginine	0.667	0.026	0.185
Glutamic acid	2.594	0.392	0.500
Aspartic acid	0.832	0.187	0.376
Histidine fraction	0.634	0.034	0.207
Lysine fraction	0.807	0.052	0.356
H_2O-insoluble Cu salts	0.690	0.075	0.313
H_2O-soluble MeOH-insoluble Cu salts	0.617	0.039	0.320
H_2O-soluble MeOH-soluble Cu salts	0.782	0.042	0.327
Tyrosine	0.758	—	—
Leucine	0.602	0.068	—

To illustrate, if N^{15} is supplied at 30 atom per cent excess concentration for a period during which the culture makes a tenth of its growth all nitrogenous compounds in the cell would contain 3 atom per cent excess N^{15} if the fixed nitrogen were immediately transferred directly to all of them and if all these compounds were being formed at the same rate as they had been during the period when excess N^{15} was absent. However, such is not observed; instead the concentration differs in the substances actually isolated. The high atom per cent excess N^{15} in a given compound could be explained by assuming that when the N^{15}-enriched N_2 or NH_4^+ is added, glutamic acid, for example, is suddenly produced at a more rapid rate than formerly; that is, if its rate of formation were doubled it would contain 6 rather than 3 atom per cent N^{15} excess. There is, however, no logical basis for such an assumption, since N^{15} is metabolized in the same manner as N^{14}. The high N^{15} concentration in glutamic acid can also be explained on the simple assumption that when nitrogen is fixed the product of fixation is transferred by a chain of reactions to form the nitrogenous compounds in the cell. For example, suppose that the following schematic chain mechanism of nitrogen fixation and assimilation has been in operation and has built up all the products shown:

$$N_2 \rightleftharpoons 2AN \rightleftharpoons 2BN \rightleftharpoons 2CN$$

Now if a source of N^{15}-enriched nitrogen is furnished for a short time, and if the total reaction is then stopped before equilibrium is reached, the highest level of N^{15} will be in the source supplied, for obviously no reaction could selectively effect an increase over this level. The next highest N^{15} concentration (all expressions are in terms of concentration, not absolute amount) would be in AN, for it lies closest to the original source of N^{15} and all subsequent reactions are dependent upon it. Likewise BN will have a higher N^{15} concentration than CN. *A high concentration* (atom per cent N^{15} excess) *of N^{15} indicates proximity in the reaction chain to the initial N^{15}-enriched compound supplied.* A stepwise transfer of N doubtless occurs in Azotobacter and to us presents the only logical explanation for the observed differences in the atom per cent N^{15} of isolated compounds. The high atom per cent N^{15} excess in glutamic acid noted in Azotobacter supplied N^{15}-enriched N_2 (or NH_4^+), therefore, indicates the proximity of glutamic acid to N_2 (or NH_4^+) in the nitrogenous metabolism of Azotobacter.

TABLE 2

Competition between free and combined forms of nitrogen by Azotobacter vinelandii

FIXATION IN PRESENCE OF	NON-ADAPTED CULTURES[a]	ADAPTED CULTURES[b]
	per cent	*per cent*
N_2	100	100
NH_4^+	0.0	0.7
NO_2^-	14.2	12.2
NO_3^-	20.0	1.1
Urea	—	1.2
Asparagine	73.7	48.9
Aspartate	91.4	84.2
Glutamate	96.7	89.0
Arginine	104.8	101.5

[a] Cultures previously kept on N-free medium.

[b] Cultures immediately before use transferred daily for 3 successive days on medium containing source of combined nitrogen to be tested.

Figures in table give percentage of total nitrogen in the cells that has come from N_2 in presence of combined nitrogen indicated in column 1.

Actual isotopic analyses given by Wilson, Hull and Burris (1943).

2. Ammonia is completely and immediately accepted as a source of nitrogen to the exclusion of the nitrogen fixation reaction; this occurs independently of whether the organism has been cultivated earlier on N_2, NH_4^+ or NO_3^- (Burris and Wilson, 1946; Wilson, Hull and Burris, 1943). Compounds readily converted into ammonia, as urea, are also effective in complete inhibition of fixation.

3. Compounds less readily converted into ammonia, as nitrate or nitrite, require a period of adaptation before they effectively inhibit fixation; this indicates that these compounds do not inhibit *per se* but only after the organism develops enzyme systems that convert them into an inhibitory product. Organic nitrogen compounds, which represent products somewhat removed from the assimilating system concerned with N_2 and inorganic combined sources of nitrogen, inhibit fixation only moderately.

Wilson, Hull and Burris (1943) and Burris and Wilson (1946) furnish evidence from isotopic experiments supporting points 2 and 3. Table 2 gives typical

results showing how ammonia and urea completely replace the nitrogen fixation reaction even without adaptation in contrast to all the other nitrogenous compounds tested. Figure 1 illustrates that the displacement of the fixation by the NH_4^+ assimilation is almost instantaneous. Calculations based on the recovery of N^{15} indicate that the shift from fixation of N_2 to utilization of NH_4^+ is not only rapid but total, so that ammonia completely inhibits fixation until it is exhausted (Burris and Wilson, 1946). Figure 1 also shows that a considerable period of adaptation is necessary before Azotobacter uses nitrate; its assimilation then

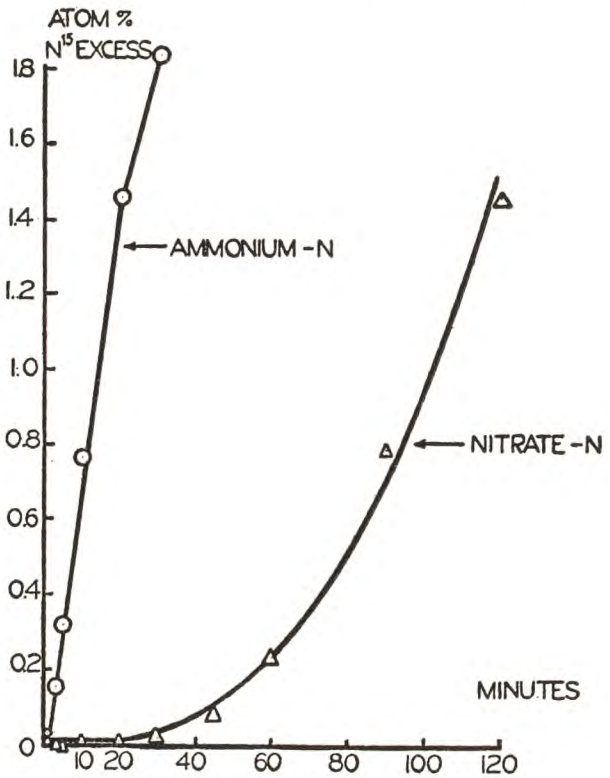

FIG. 1. Comparison of uptakes of ammonium and nitrate nitrogen by *Azotobacter vinelandii* (from Burris and Wilson, 1946)

proceeds at an increasing rate. The inhibitory product that is formed from nitrate (and presumably nitrite) after the period of adaptation is undoubtedly ammonia as was demonstrated by isotopic experiments in which Azotobacter was supplied with $N^{15}H_4NO_3$. Isotopic nitrogen ($N^{15}H_4^+$) disappeared from the medium more rapidly than did total NH_4^+ indicating that NO_3^- was being converted into NH_4^+. The residual NH_4^+ constantly decreased in its N^{15} content as can be seen in column 5 of table 3. It is also noted in this table that in the presence of both, NH_4^+ is used in preference to NO_3^-. The initial N^{15} content of the NH_4^+ was 28.90 atom per cent excess; if all the cellular nitrogen had arisen

from NH_4^+ or NO_3^-, the atom per cent excess in the cells would have been 28.90 or 0.00, respectively, whereas equal utilization of NH_4^+ and NO_3^- would give 14.45 per cent. The observed level at 8 hours (table 3, column 3), 19.62 atom per cent excess, indicates a preferential use of NH_4^+. Preferential use of NH_4^+ has also been observed with cultures previously adapted to NO_3^-.

Likewise, the limited inhibition noted with the organic compounds (table 2) probably depends on splitting off ammonia from these. Since asparagine requires only hydrolysis for liberation of ammonia, it is a fairly effective inhibitor especially with adapted cultures. Liberation of ammonia from glutamic acid, aspartic acid and arginine likely necessitates an oxidative deamination with a number of steps, so that conversion is either incomplete or at a rate too slow to compete appreciably with the fixation of nitrogen.

Although the most convincing results have been obtained with the isotopic technique, microrespirometer and macro total nitrogen experiments provide confirmatory indirect evidence. That NH_4^+ is an intermediate in the utilization of NO_3^- by Azotobacter is suggested by the observation that a lag exists in the

TABLE 3

Uptake of ammonia- and nitrate-N by Azotobacter vinelandii

HOURS AFTER INOC.	TOTAL N IN CELLS	ATOM % N^{15} EXCESS IN CELLS	NH_4^+-N IN MEDIUM	ATOM % N^{15} EXCESS IN NH_4^+	NO_3-N IN MEDIUM
	mg/100 ml		*mg/100 ml*		*mg/100 ml*
0	—	—	7.46	28.90	7.80
8	2.92	19.62	6.69	24.94	5.49
14	9.04	16.67	3.29	13.63	3.08
20	13.61	13.91	1.31	9.65	0.62
24	14.43	13.48	0.85	7.69	0.26

uptake of NO_3^-, but this lag can be eliminated if a small quantity of NH_4^+ is provided (Lind and Wilson, 1942, fig. 5). In our extensive studies (Lind and Wilson, 1941, 1942; Wilson, 1940; Wilson and Lind, 1943; Wyss and Wilson, 1941a; Wyss, Lind, Wilson and Wilson, 1941) on inhibition of the fixation of N_2 by H_2 or by small concentrations of CO, it was frequently noted that these apparently also inhibited the assimilation of some kinds of combined nitrogen when the experiments were made in the presence of N_2 (air or even helium containing only 2 per cent N_2). This was always observed with aspartic and glutamic acids, asparagine and arginine, less frequently with nitrate (nitrite) and not at all with ammonia or urea. If N_2 was removed entirely by using a $H_2 + O_2$ mixture, the inhibition disappeared. Such results demonstrate that, except for NH_3 and urea, the combined nitrogen sources had not entirely displaced fixation when N_2 was available. Inhibition of NO_3^- assimilation was not obtained in the presence of N_2 if NH_4NO_3 was used, nor was it observed in the long-time plant experiments with nitrates of calcium, potassium or sodium. These findings suggest that uptake of combined nitrogen is inhibited by H_2 or CO only if

Azotobacter or the leguminous plant had not had sufficient time to develop systems to keep available a constant supply of NH_4^+-N.

4. Azotobacter uses the combined nitrogen in surprisingly few compounds: ammonia, urea, nitrite, nitrate, aspartic and glutamic acids, asparagine, and possibly a few others (Burk and Burris, 1941; Horner and Allison, 1944; Wilson and Lind, 1943; Wilson, Hull and Burris, 1943). Of these only ammonia or compounds readily converted to ammonia are assimilated at a rate comparable to that of free N_2. In microrespirometer trials Wilson and Lind (1943) noted the following k values.[2]; for assimilation of N_2, NH_4^+ and urea, 0.30 to 0.40; NO_3^- or NO_2^-, 0.10 to 0.15 unless adapted when the values rose to 0.30 to 0.40; asparagine, about 0.10 in unadapted cultures, 0.20 to 0.25 in adapted; aspartic acid, and glutamic acid, 0.10 or less. Similar results have been observed in macro total nitrogen experiments (Lind and Wilson, 1942). Although the *ad hoc* argument might be raised that permeability difficulties restrict the rate of assimilation of the other compounds, it is significant that special pleading is unnecessary for ammonia, it behaves as a proper intermediate. It seems more likely that the rates of growth with the other sources of nitrogen reflect the ease or difficulty of conversion to the key compound of nitrogen metabolism in the Azotobacter, namely, ammonia.

Finally, a great mass of nonspecific evidence exists that is consistent with the view that ammonia is the key intermediate in biological nitrogen fixation as well as in assimilation of combined nitrogen. For example, amides, the storage form of ammonia in plants, account for a large share of the nitrogen in nodules (Wilson, 1940, p. 183); detection of ammonia liberated from detached nodules (Demolon and Dunez, 1943; Winogradsky and Winogradsky, 1936, 1941) or in the extracellular nitrogen of Azotobacter cultures is commonplace (Burk and Horner, 1936; Butkevich and Kolesnikova, 1941; Horner and Burk, 1939; Kostytschew, Ryskaltschuk and Schwezowa, 1926; Novogrudsky, 1933; Roberg, 1936; Winogradsky, 1930, 1938, 1939, 1941). But the embarrassing wealth of this seemingly essential demonstration still remains unconvincing, since no serious effort has been made to answer the argument first made by Burk and Horner in 1936 that it is of catabolic and not intermediary anabolic origin. It would add little to revive this controversy except to note that we are in accord with the view expressed by Horner and Burk (1939) that

"the extracellular ammonia observed heretofore, in our own and in all previous investigations by others, has been derived entirely from the decomposition of normal Azotobacter nitrogen upon depletion of readily available organic matter from the medium, and not, in any measurable quantity, by direct synthesis from free N_2; the ammonia observed has been liberated *after*, not *before*, a synthesis into cell nitrogen. . . ."

$$k = \frac{2.303}{t} \log \frac{\text{respiration rate after } t \text{ hours}}{\text{respiration rate at start}}.$$

A k value of 0.1 means that the rate of respiration doubles every 6.93 hours; of 0.2, every 3.46 hours.

The detailed reasons for this conclusion have been adequately discussed by Burk and Horner (1936), Horner and Burk (1939), Wilson (1940, p. 179), Burk and Burris (1941, p. 598) and others; hence they need not be repeated here. It should suffice to state that in spite of many admittedly ingenious technical approaches used by Winogradsky we have seen no paper by him or by others that has concretely met the issues first raised by Burk and Horner over 10 years ago. Until more realistic evidence is available we believe it best to avoid reliance on such shaky experimental support. Nevertheless, and without abandoning our position, if such nonspecific evidence as mere detection of a postulated intermediate in culture or plant is to be seriously considered, it is emphasized that of all postulated intermediates, NH_4^+ is most easily isolated from cultures.

Similarly, many of the other types of nonspecific evidence in favor of hydroxylamine can be matched with corresponding observations pointing to ammonia. Whether oxalacetic acid occurs in leguminous plants, and if it does, whether it implicates NH_2OH specifically is immaterial when it is realized that both α-ketoglutaric (Virtanen, Arhimo, Sundman, and Jännes, 1943) and a possible precursor, citric acid, can be found in the same plants. The alleged stimulation of fixation by excised nodules through addition of oxalacetic acid has been achieved only by Virtanen (Allison, Hoover and Minor, 1942; Virtanen, 1939; Wilson, 1940). During the past few years we have tested more than 100 cultures of excised nodules using the precise and sensitive isotope method. As is evident from typical data in table 4 that supplement data cited earlier (Burris, Eppling, Wahlin and Wilson, 1943), only occasionally have we observed fixation, and its occurrence is as likely in the culture given α-ketoglutaric acid, citric acid or no added metabolite as in that given oxalacetic acid.

ENZYME SYSTEMS ASSOCIATED WITH N_2-ASSIMILATION

In addition to providing specific information about the chemistry of nitrogen fixation, application of physical-chemical methods has furnished the first reliable details concerning the responsible enzyme systems. As will be evident in the next section, such knowledge is necessary if progress is to be made in formulating reaction mechanisms and in testing their relative validity. Burk (1934) fathered the initial attempt to describe in quantitative terms the enzyme mechanism of fixation by Azotobacter and christened the expected issue. He proposed that the entire system catalyzing the transfer of N_2 to the fixed form be called *azotase*. In addition to the enzyme *nitrogenase*, which specifically combines with N_2, azotase included several other components as calcium (strontium), molybdenum (vanadium), and hydroxyl. Later, after more detailed investigations had indicated revision, he rejected their specificity for fixation (Burk and Burris, 1941, p. 608). It appears desirable then to drop the term azotase, especially since modern enzyme chemists have discarded the concept (*cf.*, zymase) that suggested it. Nitrogenase might be retained purely as a matter of convenience. A detailed discussion of the experimental data that led to the following summary is given in the monograph by Wilson and the several reviews already cited.

1. The Michaelis constant. The most recent estimate of the apparent

Michaelis constant, the partial pressure of nitrogen (pN_2) at which the enzyme nitrogenase is half-saturated in Azotobacter, is that of Wilson, Burris and Lind

TABLE 4

Fixation of N_2 by excised nodules from leguminous plants

NODULES FROM	TREATMENT	ATOM % EXCESS N^{15} IN NODULES
Peas	6.4 g. + 200 mg. OA; 41 hrs. 25°; 1.5% N^{15} ex. atm.	0.001
Peas	Burk's soln.	0.078
Peas	Burk's soln.	0.007
	" " ; 80% O_2 in atm.	0.035
	" " + 10 mg. HDP	0.001
	" " + " ATP	0.026
Clover	Burk's soln.; 21 hrs. 22°; 32% N^{15} ex. atm.	0.724
Clover	Burk's soln.; from CO plants	0.015
	" " ; " H_2 "	0.014
	" " ; " control "	0.028
	Nodulated roots from CO plants	0.022
	" " " H_2 "	0.054
	" " " control "	0.060
Clover	H_2O; from CO plants	0.029
	" ; " H_2 "	0.050
	" ; " control "	0.031
	Nodulated roots from CO plants	0.029
	" " " H_2 "	0.082
	" " " control "	−0.001
Soybeans	Burk's soln. + 10 mg. OA; 24 hrs. 21°; 32% N^{15} ex. atm	0.189
	Burk's soln. + 10 mg. succinic acid; 24 hrs. 21°; 32% N^{15} ex. atm.	−0.002
Soybeans	Burk's soln.; 24 hrs. 25°; 30% N^{15} ex. atm.	0.007 0.002
	" " + 10 mg OA; " " " "	0.013
	" " ; nodulated roots; " " " "	0.033 0.011
Cowpeas	H_2O; 24 hrs. 25°; 32% N^{15} ex. atm.	0.013
	" + 10 mg OA; 24 hrs. 25°; 32% N^{15} ex. atm.	0.004 0.041
Cowpeas from aseptic cultures	Burk's soln.; 40 hrs. 25°; 30% N^{15} ex. atm.	0.509 1.280 0.117
	Burk's soln; nodulated roots	0.883 1.507

OA = oxalacetic acid.
CO plants = grown in presence of small quantity of CO to inhibit fixation.
H_2 plants = grown in presence of 0.6 atm H_2 to inhibit fixation.
HDP = hexose diphosphate.
ATP = adenosine triphosphate.
ex. atm. = excess of N^{15} in the atmosphere.

(1942). Four methods were used that gave values ranging from 0.01 to 0.029 atm; 0.02 ± 0.005 atm was regarded as the most probable estimate. Because

of technical difficulties (long-time experiments, equilibrium between gas and interior of nodule), determining the K_{N_2} for the symbiotic system is subject to greater error. In red clover, Wilson (1940, p. 194) secured an approximate value of 0.05 atm, *i.e.*, of the same order of magnitude as that in Azotobacter.

2. Hydrogen as a specific inhibitor. Numerous experiments, including macro total nitrogen and microrespirometer experiments, have established that molecular H_2 inhibits fixation of N_2 by the symbiotic system in red clover (Wilson, 1940, p. 197) and by Azotobacter (Wyss and Wilson, 1941a). The inhibition is specific, since uptake of combined nitrogen in the absence of N_2 is not affected. Also, it is competitive, that is, the inhibition depends on both the pH_2 and the pN_2 (Wilson, Lee and Wyss, 1941; Wyss *et al.*, 1941). As the K_{H_2} value for Azotobacter is 0.11 atm, the affinity of N_2 for nitrogenase is about 5.5 times that of H_2.

3. Carbon monoxide as an inhibitor. Carbon monoxide at extremely low partial pressures (0.0001–0.0005 atm) inhibits nitrogen fixation in red clover (Lind and Wilson, 1941) and at concentrations ten-fold greater, in Azotobacter (Lind and Wilson, 1942; Wilson and Lind, 1943). Inhibition by CO differs from that by H_2 in that uptake of combined nitrogen is likewise inhibited if a considerably higher pCO, 0.01 to 0.05 atm, is used; a quantitatively smaller inhibition is frequently observed with NO_3^- (NO_2^-) even in the ranges associated with inhibition of fixation. Thus the inhibition appears to be concerned with the reduction of nitrogen compounds rather than N_2 specifically. The inhibition is primarily non-competitive, depending only on the pCO (Ebersole, Guttentag and Wilson, 1944). Some suggestion of a quantitatively less important competitive inhibition was noted, possibly arising from the fact that CO is an isostere of N_2. Analogous results with both hydrogen and CO have been observed recently in the blue-green alga, Nostoc (Burris and Wilson, 1946a).

4. Other inhibitors. Although most of the inhibitors commonly used in enzyme research have been tested with both Azotobacter (Burk, 1934) and the symbiotic system (Wilson, 1939), none could be specifically associated with the fixation process. This means that if these inhibitors do affect the nitrogen-fixing system, the inhibition cannot be detected, since they inhibit organisms supplied with fixed nitrogen as well. Study of the nitrogen-fixing system is still restricted by the circumstance that its occurrence is growth-bound, therefore differentiating among effects on various processes is practically impossible unless a specific effect is obtained. Claims of such specificity by Kubo (1937) for hydroxylamine are probably better accounted for by the differential effect of this compound on respiration and on oxidation of H_2 by a hydrogenase (Wilson and Wilson, 1943). The recent report of Fedorov (1946) concerned with the action of narcotics on nitrogen fixation might also be regarded as implying specific inhibition.

5. Auxiliary constituents. As has been mentioned, Burk (1934) believed that the nitrogen-fixing system in Azotobacter possessed components associated with calcium, molybdenum and hydroxyl, but later rejected this idea on the grounds that further experiments had demonstrated that these ions were not uniquely required for fixation but also for growth when the organism was supplied fixed

nitrogen. Although quantitative differences were evident, he believed that these might arise from differences in strains, rates of growth, differing temperature optima and other factors. Observe that these considerations do not rule out their functioning in the nitrogen-fixing system; the restriction that we must study the reaction in growing organisms, however, makes it difficult, if not impossible, to differentiate among effects. Although we can ask the proper questions of nature, we cannot always obtain a definite answer with present techniques. Others have not had Burk's logical scruples and have not hesitated to ascribe a specific role, for example, to molybdenum and many other inorganic ions (Burk and Burris, 1941, p. 605). Bortels (1930) first demonstrated that addition of molybdenum was necessary for maximum nitrogen fixation by Azotobacter, and this has been amply confirmed (Burk, 1934) and extended to the blue green alga *Nostoc*, that fixes N_2 (Bortels, 1936), the symbiotic system (Anderson, 1946; Bortels, 1937; Jensen and Betty, 1943; Jensen, 1946; Trumble and Ferres, 1946), and to the anaerobic nitrogen-fixing bacteria (Jensen and Spencer, 1946). The Australian workers, especially Trumble and associates, have demonstrated the practicability of supplying Mo together with other trace elements such as Zn to certain types of soil intended for the culture of legumes.

That the role of molybdenum in nature is not restricted to the nitrogen-fixers was evident when Steinberg (1936, 1937) demonstrated its necessity for utilization of nitrate by *Aspergillus niger*. Higher plants, both legumes and non-legumes, also respond to it under certain conditions (Arnon and Stout, 1939; Burk and Burris, 1941, p. 605). Nevertheless the view persists that it has a special significance for nitrogen fixation. On the basis of their experiments Burema and Wieringa (1942) decided that "for the assimilation of nitrate-N Azotobacter does not need as much Mo as is required for the assimilation of atmospheric nitrogen." Jensen arrived at the same conclusion for the symbiotic system in alfalfa and white clover, since the response to Mo added to sand cultures was considerably greater for plants fixing nitrogen than for the controls supplied $NaNO_3$, and since Mo accumulated in the nodules as compared with tops and roots (Jensen and Betty, 1943; Jensen, 1946). A note of caution, however, was sounded by Horner, Burk, Allison and Sherman (1942) who made a careful study of the Mo requirements of a number of strains of Azotobacter in specially purified media. Most strains of *Azotobacter chroococcum* fixed little nitrogen unless Mo was supplied up to 1 ppm, but strains of *A. vinelandii* fixed one-half to two-thirds their maximum in the absence of added Mo. That this arose from a more efficient use of traces in the purified medium appeared unlikely since the maximum fixation with *A. vinelandii* was also obtained only when the total Mo present approached 1 ppm. They concluded that undoubtedly Mo was required for the strains of *A. chroococcum* but whether it was essential for the others was questionable. Vanadium replaced Mo, although the final total nitrogen fixed was about one-third as great.

6. Hydrogenase. Cells of Azotobacter contain a powerful hydrogenase that catalyzes the reduction of methylene blue, oxygen, and other hydrogen acceptors by molecular H_2. Its properties such as response to inhibitors and pO_2 are

similar to those noted for the enzyme in other bacteria and algae (Wilson, Lee and Wilson, 1942; Lee, Wilson and Wilson, 1942; Wilson and Wilson, 1943). Its occurrence in Azotobacter is of special interest since H_2 acts as a specific inhibitor for nitrogen fixation. Lee and Wilson (1943) found that combined nitrogen not only inhibited assimilation of N_2 by Azotobacter but also decreased markedly its hydrogenase activity. For example, when *A. vinelandii* was cultured in a $H_2 + O_2$ atmosphere on combined nitrogen, the hydrogenase activity was low. Thus its elaboration by the organism appears to depend on the presence of N_2 rather than its specific substrate, H_2. Although the occurrence and functioning of hydrogenase in Azotobacter apparently is associated with

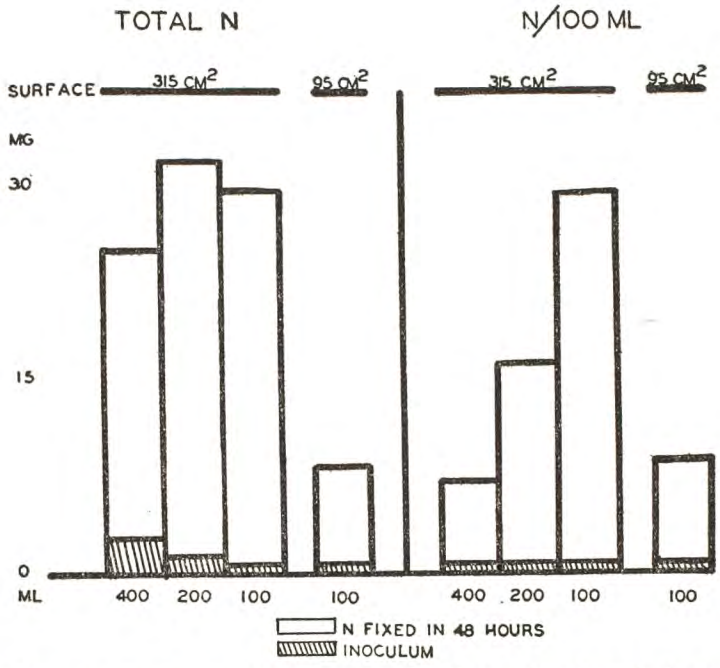

FIG. 2. Nitrogen fixation by *Azotobacter vinelandii* as a function of the surface of the medium (data of Wilson and Wilson, 1941)

nitrogen fixation, this does not appear to be true of the symbiotic fixation system in leguminous plants. The enzyme has not been found either in pure cultures of Rhizobium or in nodules from legumes (Wilson and Wilson, 1943; Wilson, Burris and Coffee, 1943).

7. Role of Oxygen. Nitrogen fixation by Azotobacter and by leguminous plants is usually stimulated by increasing the air supply. Typical results with Azotobacter shown in figure 2 illustrate that fixation is a function of the surface of the cultures, not of the volume. Similar results can be obtained by aeration (*cf.*, k values in Warburg respirometer with those in macro cultures) or even by the addition of about 0.1% of agar to the medium which apparently supports

a surface film. It is not yet established if the stimulation results from an increased supply of energy or has a more direct relationship to the fixation mechanism. Meyerhof and Burk (1928) and later Burk (1930) concluded from microrespiration experiments that the rate of nitrogen fixation reaches its maximum at a pO_2 of about 0.04 atm and that the efficiency (N_2 fixed/O_2 consumed) is maximum at about 0.01 atm. Since the effect of the pO_2 was independent of the source of nitrogen, Burk further concluded that the responses to O_2 cannot indicate the nature of the chemical mechanism of N_2 fixation. Wilson and Fred (1937) reported that, except possibly in the presence of high partial pressures of hydrogen, changes in the pO_2 caused similar effects in red clover plants whether they were using free or combined nitrogen.

The rôle of oxygen in fixation becomes of immediate interest because of the identification of the red pigment in the nodule with a hemoprotein similar to hemoglobin (Burris and Haas, 1944; Keilin and Wang, 1945; Kubo, 1939; Virtanen, 1945). Aside from the intrinsic significance of finding such a compound in plants, its function in N_2 fixation has caused varied speculation. Virtanen (1945, Virtanen and Laine, 1945a) suggests that with the accompanying methemoglobin it forms an oxidation-reduction couple based on a change of valence in iron that effects the reduction of N_2 to NH_2OH by a reversal of the reaction (Letsche, 1912):

$$\underset{\text{oxyhemoglobin}}{HbO_2\,[Fe^{++}]} + 2\,NH_2OH \longrightarrow \underset{\text{methemoglobin}}{HbO\,[Fe^{+++}]} + N_2 + 3H_2O$$

Aside from the fact that Letsche's characterization of the evolved gas was not unequivocal, this formulation is not in agreement with modern views of the reactions by which hemoglobin forms oxyhemoglobin and methemoglobin:

$$HbO_2\,[Fe^{++}] \rightleftharpoons O_2 + Hb\,[Fe^{++}] \overset{-e}{\rightleftharpoons} MetHb\,[Fe^{+++}]$$

Moreover, the reversal of Letsche's formulation implies that molecular oxygen is liberated along with the reduction of N_2 to NH_2OH. In presenting their suggestion, Virtanen and Laine, however, have substituted hemoglobin for oxyhemoglobin:

$$N_2 + \text{methemoglobin}\,[Fe^{+++}] \rightleftharpoons NH_2OH + \text{hemoglobin}\,[Fe^{++}]$$

$$\text{hemoglobin}\,[Fe^{++}] \xrightarrow{\text{oxalacetic acid}} \text{oxime} \longrightarrow l\text{-aspartic acid}$$

The rationale of this mechanism is puzzling since two oxidized compounds (N_2 and methemoglobin) are pictured as reacting to form two reduced compounds (NH_2OH and hemoglobin).[3] Finally it should be noted that the implication of

[3] Significant, perhaps, for the mechanism of the reaction studied by Letsche are the results of Warburg, Kubowitz and Christian (Biochem. Z., 1931 **242**, 170–205) dealing with the influence of phenylhydroxylamine on respiration by red blood cells. They found that this compound in the *presence of glucose* converted part of the hemoglobin (Hb) to methemoglobin (HbOH); an oxidation-reduction couple was thus formed that caused the rate of O_2

Virtanen, Laine and Linkola (1945) that the reduction of methemoglobin to hemoglobin in the nodules depends on the presence of oxalacetic acid is not supported by all the evidence. We have had no difficulty in finding the red pigment in nodules but have not been able to find detectable quantities of oxalacetic acid.

Virtanen and Laine (1945b) claimed fixation of N_2 in pure culture of *Rhizobium leguminosarum* if extracts of soybean nodules containing the pigment were supplied; the fixation was markedly increased if oxalacetic acid also was added. Later, Virtanen (1946) states that with more purified preparations of the pigment fixation was no longer obtained. Even so, if fixation in the presence of nodular extracts is confirmed, this discovery would be of great importance, as one of the most puzzling features of symbiotic nitrogen fixation is the absence of fixation by the bacteria apart from the host plant. During the summer of 1946 we tested more than 20 preparations of pigment from nodules of peas and soybeans in the presence of oxalacetic acid, α-ketoglutaric acid, citric acid and glucose but secured no fixation by the bacteria alone. Microrespirometer experiments (Little and Burris, 1947) demonstrated that addition of the pigment to cultures of bacteria including Rhizobium stimulated respiration at low pO_2 (0.01 atm). Because of the low pO_2 inside the nodule (Allison, Ludwig, Hoover and Minor,

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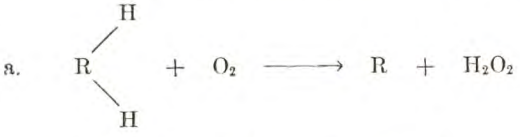
uptake to be increased 20 to 30 fold. The methemoglobin, reduced by substrate hydrogen, returned to hemoglobin but was reoxidized rather than oxygenated. From experiments in the presence and absence of CO and substrate, and from analogous reactions with phenylhydrazine and amyl nitrite, they concluded that the reaction was induced by formation of H_2O_2 (detected chemically) through auto-oxidation of the phenylhydroxylamine:



a. $R(H)_2 + O_2 \longrightarrow R + H_2O_2$

b. $2HbO_2 + H_2O_2 \longrightarrow 2HbOH + 2O_2$

c. $2HbO_2 + RH_2 \longrightarrow R + 2HbOH + O_2$

Although written as reacting directly with HbO_2, the results of trials with CO suggested that the actual reaction occurs with Hb in equilibrium with HbO_2. The net result is that the oxygen-binding capacity of the cells should be decreased twice that of the oxygen evolved. In an experiment, the observed ratio was 312/142 and the authors concluded that "Der Reaktionverlauf ist also nicht völlig klar."

If a similar reaction occurs with hydroxylamine and if, as seems probable from Letsche's results, the auto-oxidation of NH_2OH causes its decomposition, the series of reactions is:

d. $2HbO_2 \rightleftarrows 2Hb + 2O_2$

e. $2NH_2OH + O_2 \longrightarrow N_2 + 2H_2O + H_2O_2$

f. $2Hb + H_2O_2 \longrightarrow 2HbOH$

g. $2HbO_2 + 2NH_2OH \longrightarrow N_2 + 2H_2O + 2HbOH + O_2$

Although reversal of the overall reaction might appear to be possible, consideration of the individual steps suggests that this is not a plausible mechanism for fixation of N_2.

1940; Frazer, 1942), it seems that a function of the pigment might be similar to that of hemoglobin in animals.

8. Fixation Systems in Different Organisms. An important corollary of defining the properties of the responsible enzyme systems in nitrogen-fixing organisms is that it establishes their similarities and differences. In general, the aerobic organisms capable of fixing nitrogen appear to possess enzyme systems remarkably similar as evidenced by their response to the pN_2, CO, H_2 and trace elements such as Mo. Establishment of this has been helpful for development and testing of proposed mechanisms, since each organism has certain advantages for different types of studies. Assurance that we can pool the information gained in studies with all the organisms should aid in final solutions of the many problems yet unsolved. Certain differences do exist, however, and these should be kept in mind as they too should be helpful in developing hypotheses of the mechanism. Among these are:

1. Hydrogenase has been reported only in the Azotobacter.
2. The hemoglobin-like pigment occurs only in the leguminous plant; although Azotobacter contains a red pigment, its bands indicate that it corresponds entirely to the cytochromes. Some cultures of *Azotobacter vinelandii* excrete into the medium a pigment that is variously pink and green dependent to some extent on the quantities of Fe and Mo present. It shows a wide nonspecific absorption band, and yields a blue derivative with pyridine.
3. Azotobacter has an extremely active aerobic respiration and requires a large amount of O_2, whereas *Clostridium pasteurianum* fixes N_2 anaerobically.
4. The rhizobia fix nitrogen only in association with the host plant; Azotobacter, *Cl. pasteurianum* and *Nostoc muscorum* fix nitrogen as free-living organisms.

POSSIBLE MECHANISMS OF FIXATION

For primarily descriptive purposes we can summarize the overall reactions that lead to fixation of N_2 in the following, not necessarily distinct, steps:

$$\text{Step I} \quad N_2 \rightleftharpoons N_2^*$$

$$\text{II} \quad N_2^* + E \rightleftharpoons N_2^*E$$

$$\text{III} \quad N_2^*E + X \rightleftharpoons N_2^*EX$$

$$\text{IV} \quad N_2^*EX \rightarrow E + N_2^*X$$

$$\text{V} \quad N_2^*X \rightarrow N_2X \text{ (Fixation)}$$

$$\text{VI} \quad N_2X \xrightarrow[\text{(intermediates)}]{\text{(unspecified)}} NH_3$$

$$\text{VII} \quad N_2^*X \rightarrow N_2^* + X \text{ (Decomposition without fixation)}$$

N_2^* is some activated form of molecular nitrogen, for example, a molecule possessing an energy, ϵ_a, greater than some critical ϵ_0. N_2^* is adsorbed on the

enzyme surface and reacts there with some unknown molecule, X; the combination is either deactivated, step V, which constitutes fixation, or may be decomposed without fixation, step VII. Alternatively, N_2^* may not be the sole form of N_2 adsorbed but is the sole form that takes part in the subsequent reactions.

With this formulation of the overall reaction in mind we realize the eventual necessity of defining more precisely steps III to VI. The situation is analogous to that faced by investigators of photosynthesis subsequent to Warburg's fundamental studies on the quantum relationships. Once the question was raised concerning the detailed steps in the photochemical and dark reactions, no mechanism could be seriously considered that offered no more than a mere restatement of the completed photosynthetic reaction. Although many false starts were made and cul-de-sacs explored, few would deny that progress toward understanding of this reaction began when experimental work was dictated by speculations regarding the detailed mechanisms and not just the overall process. We believe that a scientific maturity has been reached now in the study of biological nitrogen fixation that not only justifies but demands that we direct our attention to exploration of steps III to VI. This is particularly desirable since it emphasizes that enumeration of the descriptive steps does not properly constitute a mechanism although phrasing these in chemical symbols may give a deceptive appearance that such is being done. As a start we shall propose three typical schemes that we regard as possible mechanisms by which Azotobacter reduces N_2 to NH_3 and to suggest variations that might cover the symbiotic system. The purpose is not so much to establish which, if any of these, has the greatest probability based on information now available but to illustrate the type of problem that we believe should concern future investigations. A second purpose is to show through example how any proposed mechanism must be tested by the touchstone of whether it agrees with facts from all types of biochemical and physical-chemical investigations, not just the ones that suggested it. The story of the blind men and the elephant still has point for the scientific investigator.

Figure 3 outlines a general scheme which can serve as a working model for possible mechanisms in Azotobacter. It includes a hydrogenase playing an active rôle in the fixation process and provides a place for specific and competitive inhibition by hydrogen in the primary reaction leading to fixation. En_N is the specific enzyme that forms a complex with N_2 to enable it to react with the specific reductant (schemes I and II, see below) or oxidant (scheme III). En_H is the hydrogen-activating portion of the hydrogenase system and En_O the oxygen-activating. Yamagata and Nakamura (1938) concluded from experiments with inhibitors that hydrogenase is a specific enzyme that transfers H_2 to a common intermediate, A_h, which further reacts with the ultimate acceptor through one or more oxidation-reduction enzyme systems. The ultimate acceptor varies with the organism, but the hydrogenase system is the same for all. As the action of an inhibitor often is concerned with the transfer of H_2 from A_hH_2 to its ultimate acceptor, a given inhibitor does not always produce the same results in different organisms. For example, CN inhibits the Knallgas reaction in *Bacterium coli formicum* and *Rhodobacillus palustris* whose respiration

is cyanide-sensitive, but not in *Bacillus delbrückii* whose respiration is unaffected by cyanide. We have accordingly included an intermediate acceptor, A_h, and similar ones, B_o and B_o', (not necessarily different) for the oxidizing side of the reactions. Both A_h and B_o may represent none or several intermediary catalysts; that is En_H and En_O may be A_h or B_o, respectively, or these might represent a series of oxidation-reduction systems before reaching the reductant (or oxidant) specific for the primary reaction with $\{N_2\}$. Braces about a compound indicate that it exists as a complex with some other molecule, for example, with a specific enzyme although *initial* complex formation need not involve the specific enzyme indicated.

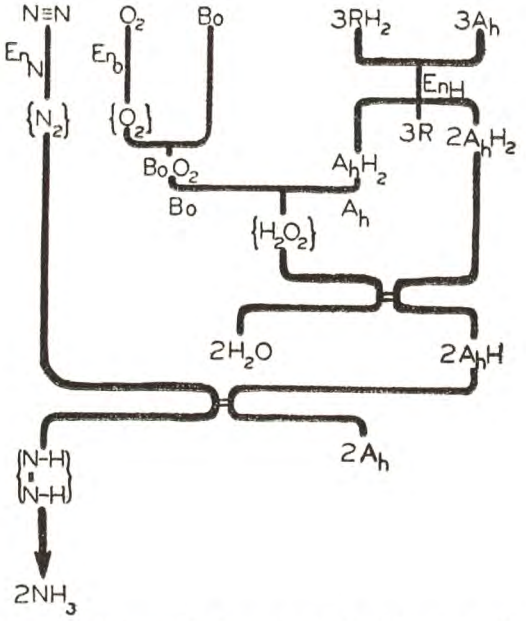


FIG. 3. Schematic representation of N_2 fixation by Azotobacter with substrate acting as hydrogen donor. See text for explanation of the symbols

Many of the symbols used in figure 3 as well as the arrangement of the reactions are based on Rabinowitch's (1945) discussion of various suggested mechanisms for reduction of CO_2 by green plants and bacteria. This was done not only because the device affords a compact summary of a great deal of information but also because we believe that evidence exists suggesting these two fundamental processes (reduction of CO_2 and N_2) may have certain points of similarity—for example, the possible rôle of hydrogenase in each. From the point of view of comparative biochemistry, then, it might be useful to employ similar basic patterns for postulated mechanisms.

Scheme I. The essential feature is that hydrogen for reduction comes from substrate (RH_2) for example, *via* the hydrogenase system. Whether hydrogenase functions in Azotobacter in this manner has not been experimentally

determined since it is not definitely assured that the organism normally evolves H_2. The older literature (Stoklasa, 1908) makes such claims, but purity of culture remains a question in these instances. Wilson and Wilson (1942) obtained evidence of the evolution of H_2 when masses of resting cells were anaerobically incubated with glucose and succinate, but the results were erratic. Production or transfer of H_2 does not necessarily require participation of hydrogenase, but because of its rôle in other organisms, it would be strange if it could not do likewise in Azotobacter. One mole of the reduced intermediate, A_hH_2, reacts with the oxidant to form the complex $\{H_2O_2\}$ which by reaction with 2 additional moles of A_hH_2 oxidizes the hydrogen of the latter to water with the formation of the free radical, A_hH. The free radical, containing the energy of the oxidation, brings about the first stage of fixation. The reactions may be summarized:

(1) $$3RH_2 + 3A_h \rightleftharpoons 3A_hH_2 + 3R;\ B_o + \{O_2\} \rightarrow B_oO_2$$

(2) $$A_hH_2 + B_oO_2 \rightarrow \{H_2O_2\} + A_h + B_o$$

(3) $$2A_hH_2 + \{H_2O_2\} \rightarrow 2A_hH + 2H_2O$$

(4) $$\{N_2\} + 2A_hH \rightleftharpoons \{N_2H_2\} + 2A_h$$

(5) $$\{N_2H_2\} \xrightarrow{\text{reduction}} 2\ NH_3$$

The key reaction is, of course, (4), and it would be here that H_2 would inhibit if competitive inhibition obtains. This could occur if molecular H_2 were to unite with the free radical, A_hH, to return it to its stable form A_hH_2, which is assumed to be incapable of reaction with $\{N_2\}$:

(6) $$H_2 + 2A_hH \rightarrow 2A_hH_2$$

After the initial fixed nitrogen compound $\{N_2H_2\}$ is formed, further reduction may occur through either A_hH or the more conventional oxidation-reduction systems (including A_hH_2) found in the organism. If we assume A_hH, steps 1 to 4 would have to be repeated twice more to effect the overall reaction for complete reduction to NH_3:

(7) $$N_2 + 3O_2 + 9RH_2 \rightarrow 2NH_3 + 6H_2O + 9R$$

If the 9 pairs of H_2 required are furnished by glucose, the reaction for fixation becomes:

(8) $$N_2 + 7.5O_2 + 1.5\ \text{glucose} \rightarrow 2NH_3 + 6H_2O + 9CO_2$$

Equation (8) suggests that the ratio, moles N_2 fixed per mole O_2 used could equal 0.133; Meyerhof and Burk (1928) from thermodynamical considerations estimated this as having a maximum value of 0.915. In their experiments the ratio varied from 0.003 to 0.008 in air to 0.10 in an atmosphere with a pO_2 of 0.0012 atm.

Scheme II. A second possibility is that the hydrogen for reduction comes from a reversal of the Knallgas reaction; although this might appear to be

difficult because of the large value of $\Delta F^\circ = 56.6$ kg cal, it could proceed readily enough if it were part of an oxidation-reduction reaction:

$$H_2O + A_h + B_o' \rightarrow A_hH + B_o'(OH) \tag{9}$$

In equation 9 the decomposition of water is written so as to provide immediately for creation of free radicals; this is mainly for simplification, as it may be more consistent to expect first the formation of the stable forms (A_hH_2 and $B_o'(OH)_2$) followed by creation of free radicals by oxidation-reduction reactions as in scheme I. Not only does this proposal provide a definite rôle for hydrogenase, but it furnishes a new unity in biochemistry through merging of photosynthesis and nitrogen fixation. Most authorities agree that the reduction of carbon dioxide by green plants is accomplished by hydrogen generated from the splitting of water (Rabinowitch, 1945, chap. 7); many would extend this concept to bacterial photosynthesis (Gaffron, 1940; Nakamura, 1937; van Niel, 1941). Gaffron's (1942) fundamental discovery that certain green algae can couple the reduction of CO_2 with the oxyhydrogen reaction, and thus dispense with the light reactions, is suggestive for nitrogen fixation.

In Azotobacter the energy for the splitting would probably come from energy-rich ~P bonds generated by respiration (*cf.*, Lipmann and Tuttle, 1945); molecular nitrogen would act as the hydrogen acceptor instead of CO_2; and the oxidizing portion of the system ("hydroxylated" enzyme), would be regenerated through reaction with substrate hydrogen. Inhibition by H_2 would occur as in *Scheme I* at reaction 4.

Scheme III. Alternatively, the primary reaction involving N_2 might be its oxidation by means of $B_o'(OH)$:

$$N_2 + 2B_o'(OH) \rightleftharpoons \begin{Bmatrix} N{-}OH \\ \| \\ N{-}OH \end{Bmatrix} + 2B_o' \tag{10}$$

If so, it suggests that inhibition of N_2 fixation by nitrate (nitrite) occurs through formation of some intermediate such as $\begin{Bmatrix} N{-}OH \\ \| \\ N{-}OH \end{Bmatrix}$ rather than the ultimate product of reduction, NH_3. That is, N_2 assimilation joins the metabolic pathway of NO_3^- assimilation at an early stage; hence fixation is inhibited by nitrate when reduction of NO_3^- proceeds at a rate sufficiently rapid to maintain a constant supply of the common intermediate. Certain similarities in the response of Azotobacter when furnished NO_2^- and NO_3^- to that obtained with N_2, *e.g.*, to CO (Lind and Wilson, 1942; Wilson and Lind, 1943) provide some support for this view. It should be noted that just as $\begin{Bmatrix} N{-}H \\ \| \\ N{-}H \end{Bmatrix}$ implies only reduced nitrogen, $\begin{Bmatrix} N{-}OH \\ \| \\ N{-}OH \end{Bmatrix}$ represents only oxidized nitrogen.

GENERAL EXAMINATION OF THE HYPOTHESIS

In the preceding section we have proposed three possible schemes for N_2 fixation suggested by certain experimental results. Others could undoubtedly be

offered, but these include interesting possibilities and, though not exhaustive, they certainly are typical. It remains to examine these in terms of all information we have on the biochemistry and biophysics of the process. This is essential not only because even tentative acceptance depends on demonstration that the implications do not conflict with available information but also because such examination suggests necessary revision and future experiments. In this section general considerations will be explored with indications of what type of experiment might provide critical data; the following section will discuss details of the reactions.

Thermodynamics. The proposed schemes appear to be thermodynamically sound. Reduction of N_2 by metabolic H_2 can be written as a first approximation:

(11) $$0.5\ N_2 + 1.5H_2 + H_2O \rightleftharpoons NH_4^+ + OH^-;\ \Delta F^\circ = 0.17 \text{ kg cal}$$

At pH 7.0 this reaction can be represented by the following energy equation based on the convention that we are dealing with an oxidation-reduction system, N_2/NH_4^+:

(12) $$E = -0.28 + \frac{RT}{3F} \ln \frac{(\sqrt{N_2})}{(NH_4^+)}$$

At a pN_2 of 0.8 atm and (NH_4^+) equal to 10^{-4} M, E becomes -0.20 v. To determine if "average" metabolic hydrogen, for example that in glucose, could bring about this reduction, we write the following series of reactions which summarize the energy changes to be calculated.

It is emphasized here, since many authors of bacteriological texts fail to make this distinction, that these and similar equations do not represent actual mechanisms but merely convenient formulations which are useful for thermodynamical calculations. Such calculations are largely independent of mechanism but are concerned only with initial and final states. Since these are primarily energy and not chemical equations, for completeness they require specification of conditions and concentrations. Some can be assigned without question, whereas others demand estimates or even guesses. Our choices in these instances have not been entirely arbitrary, however, but are based on the best information available. The pCO_2 is taken at 0.01 atm instead of the traditional 0.0003 atm as the former is more in harmony with the actual concentration of CO_2 over quiescent or even agitated cultures. The concentration of ammonium, 10^{-4} M, is based on residual levels of ammonium when this source of nitrogen is added as well as on excreted metabolic NH_4^+-N found in young cultures fixing N_2 (Horner and Burk, 1939). The CO_2 liberated will appear as both free gas and bicarbonate ion at pH 7; theoretical proportions of each are indicated in the equations. These were calculated from the pK_1 of CO_2 (aq) together with the (HCO_3^-) in equilibrium with a pCO_2 of 0.01 atm at that pH.

(13) $$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2$$

(14) $$4N_2 + 12H_2 + 8H_2O \rightarrow 8NH_4^+ + 8OH^-$$

(15) $$6CO_2 + 4.67H_2O \rightarrow 4.67HCO_3^- + 4.67H^+ + 1.33CO_2$$

(16) $$C_6H_{12}O_6\ (0.05\ M) + 4\ N_2\ (0.8\ atm) + 18.67\ H_2O\ (l) \rightarrow$$
$$8\ NH_4^+\ (10^{-4}M) + 8\ OH^-\ (10^{-7}M) + 4.67\ HCO_3^-\ (0.0012\ M) +$$
$$4.67\ H^+\ (10^{-7}M) + 1.33\ CO_2\ (0.01\ atm)$$

Although the standard free energy of this reaction ($\Delta F°$) is +5.6 kg cal per 0.5 N_2, the organism actually obtains energy by carrying out the reaction under the specified conditions, ΔF equalling −17.5 kg cal per 0.5 mole N_2 fixed. In ordinary laboratory experiments we can assume that Azotobacter will assimilate about 150 mg of 1,000 mg glucose utilized and will fix approximately 14 mg N_2 (Lee and Burris, 1943). The energy balance for 1,000 mg of sugar is:

150 mg assimilated	Small, if any, change in free energy
22.5 mg used in N_2 fixation	17.5 cal released
827.5 mg oxidized	3,200 cal released

As can be seen from these values, the energy loss is extremely small and probably undetectable in ordinary experiments if the organism substitutes N_2 for O_2 as the acceptor of part of its metabolic hydrogen. Usually, the organism has available about 98 per cent of the energy that it would obtain if NH_4^+−N were supplied so that it could oxidize completely the 850 mg glucose used in forming 150 mg of cell material.

Similar conclusions are reached if we assume that the H_2 used for reduction comes from water exclusively rather than from the substrate (*Schemes II and III*). If the splitting of water is assumed to be: $H_2O \rightleftarrows \{H\} + \{OH\}$, three moles would be required for the reduction of 0.5 mole of N_2. Since the O-H bond is equal to 110 kg cal (Rabinowitch, 1945), approximately 30 high energy ~P bonds would be necessary. These are roughly equivalent to the oxidation of one mole of glucose; hence one gram of glucose would be sufficient for fixation of about 80 mg of nitrogen, whereas actually 10 to 15 mg is usually fixed. The energy balance, however, is probably much more favorable since accompanying oxidation-reduction reactions, for example by the {OH}, could be coupled with production of ~P bonds.

Another method of approach to the energetics of the process is estimation of the energy required to form the activated complex that is assumed to govern the rate of the enzyme-catalyzed reaction (Moelwyn-Hughes, 1933, 1937; chapter 10 in Elvehjem and Wilson, 1939). Even though precise evaluation is not yet possible, an idea of its magnitude can be obtained from molecular statistics of the process. If the Azotobacter is a sphere 2 μ in diameter, it has a volume of 4.2×10^{-12} cm^3, a surface area of 12.6×10^{-8} cm^2, and 6.3×10^{-14} g organic nitrogen (1.5 per cent N) equal to 1.4×10^9 molecules of nitrogen. Assuming that En_N has a diameter comparable to that of other enzymes, about 5×10^{-7} cm, we consider that each enzyme molecule would occupy an area of 25×10^{-14} cm^2 and therefore the surface of each cell could accomodate as many as 0.5×10^6 enzyme molecules. One ml of medium containing 0.15 mg bacterial nitrogen would represent 2.5×10^9 cells. The number of effective collisions between N_2 in solution and enzyme molecules can be estimated from

$$(13) \qquad Z = n_1 n_2 \frac{(\sigma_1 + \sigma_2)}{2} [8 \pi RT (1/M_1 + 1/M_2)]^{1/2} \sigma_1^2/\sigma_2^2$$

in which

n_1 = number of molecules of N_2 in 1 ml, 7.3×10^{16} at a pN_2 of 0.2 atm
n_2 = number of enzyme molecules in 1 ml, 1.25×10^{15}
σ_1 = diameter of N_2 molecule, 3×10^{-8} cm
σ_2 = diameter of enzyme molecule, 5×10^{-7} cm
R = 8.3×10^7 ergs/degree
T = 304°K.
M_1 = 28; M_2 is probably at least 1000 times M_1, hence $1/M_2$ can be neglected
$\sigma_1^2/\sigma_2^2 = 3.6 \times 10^{-3}$ measures the probability that a collision will be effectively oriented.

When these values are substituted in (13), $Z = 3.5 \times 10^{21}$ collisions sec^{-1}. The specific rate constant k is 0.4 hr^{-1} or 1.1×10^{-4} sec^{-1} which means that in the first second of reaction the number of N_2 molecules fixed is:

$$(1.1 \times 10^{-4})\ (0.15 \times 6.06 \times 10^{23})/28{,}000 = 3.6 \times 10^{14}$$

$$\text{Then } \frac{3.6 \times 10^{14}}{3.5 \times 10^{21}} = e^{-\Delta E/RT} = e^{-16.1} = 10^{-7}$$

$$\Delta E = 10 \text{ kg cal}$$

A second method of approximation that might be applicable is to regard the reaction as bimolecular between N_2 and enzyme molecules as Moelwyn-Hughes (1937) illustrates with decomposition of H_2O_2 by catalase.

$$(14) \qquad k_{bi} = -dn_1/dt \times 1/n_1n_2 = \sigma_1^2 \sqrt{\frac{\pi k' T}{2m_s}}\ e^{-\Delta E/RT}$$

k′ = Boltzmann's constant, 1.37×10^{-16} erg/degree

$m_s = 28/(6.06 \times 10^{23})$

Since in Azotobacter the number of enzyme molecules is constantly changing unimolecularly and the number of N_2 molecules in solution may be regarded as constant, the bimolecular constant may be estimated by dividing the calculated unimolecular constant by n_1; substituting in (14), $\Delta E = 13$ kg cal. Burk (1934, p. 40) who used still another formula for calculating the number of collisions arrived at values for ΔE of 11.7 to 17.5 kg cal.

The major uncertainty, other than the pertinency of a given formula, is the number assigned for enzyme molecules per ml; the value assigned to σ_2 is relatively ineffective in (13) and disappears from (14). That the estimate of n_2 is reasonable is indicated by calculation of the *turnover number* (TN), the number of molecules of nitrogen fixed per molecule of enzyme per minute. Since 3.6×10^{14} molecules of N_2 are fixed per sec. by 1.25×10^{15} molecules of enzyme, the turnover number is 18. This is rather low in comparison with the TN of enzymes that have been studied in purified state (Green, 1940; Green, Leloir and Nocito, 1945; Sumner and Somers, 1943) so that our estimated number of enzyme molecules per cell is more likely to be too high than otherwise.

Confirmation for this view is supplied by the experiments of Lineweaver (1938) who attempted to measure directly the hyperbolic (chemical or physical) adsorption of N_2 indicated by the kinetic studies (Lineweaver and Burk, 1934; Lineweaver, Burk and Deming, 1934; Wilson, Burris and Lind, 1942). No evidence of such adsorption was obtained, the results being explicable on the basis of Henry's solubility law. It was concluded that the adsorption was beyond the limits of the method, which set an upper limit of 0.04 to 0.4 $\times$ 10^6 to the number of N_2 molecules adsorbed per cell. Every 10- fold decrease in the number of enzyme molecules means a 1.6 kg cal decrease in ΔE; therefore, an activation energy considerably more than about 12 kg cal, or about one $\sim$P, does not appear probable.

A second type of "turnover number" can be calculated if it is assumed that only those molecules react that possess sufficient energy by reason of thermal activation. Approximately one molecule in 10^7 possesses sufficient energy and since about 0.3 molecule is fixed per sec. by each molecule of enzyme, it would have to adsorb between 10^6 and 10^7 molecules of N_2 per sec. to account for the observed rate of reaction. Although not a true TN, this value is similar in that it measures the rate of "combination" and "decomposition" of N_2 and En_N. Decomposition of H_2O_2 by catalase is about the only known enzyme system with such a high TN; hence it appears likely that activation of the N_2 molecule depends on enzyme intervention rather than the fortuitous distribution summarized in the Maxwell-Boltzmann law.

Comparative Biochemistry. Probably the most important biochemical fact established by the physical-chemical studies has been the unity among the N_2 fixation processes in Azotobacter, the blue-green alga, *Nostoc muscorum*, and the symbiotic system. The mechanisms suggested in the preceding section were based on the studies with Azotobacter, and one of the most critical tests is whether they describe equally well fixation by the other organisms. Certain variations would be expected because of the differences already noted among the systems, but in general the basic pattern should be the same. The first point of consideration is whether it is essential that ammonia be the key intermediate as postulated in these schemes. Obviously this is not a critical part of any of the proposals so that decision on this point will affect their validity but little. Although recent studies definitely suggest ammonia as the key intermediate in fixation by Azotobacter, it is not claimed that these rule out NH_2OH in the symbiotic system. Our view, first stated in Wilson (1940, p. 184) and more recently affirmed (Burris and Wilson 1945, p. 700), that in the symbiotic system both intermediates may function, depending on the particular keto-acids or similar acceptor available, still appears to be valid.

But to state this possibility does not imply that it has been experimentally established. Before this could be accepted, studies similar to the ones already made with Azotobacter and the heavy nitrogen isotope would be necessary. It must be demonstrated that the system of plant plus bacteria can use NH_2OH or at least suitable oximes and that accumulation of tagged nitrogen during fixation is similar to that observed with these and not to that with NH_4^+. Similar

studies with Azotobacter should be undertaken since at present even the minimum requirement—that of utilization—is denied (Burk and Burris, 1941, p. 601). Corroboration of the excretion data (Wyss and Wilson, 1941b), especially the identity of the oxime found in small quantities (Virtanen and Laine, 1939), would be helpful although not as impressive as the metabolic tracer studies. The isotope dilution technique (Rittenberg and Foster, 1940; Rittenberg, in Green, 1946) has been useful in analogous problems and might be employed here. A better understanding of conditions necessary for occurrence of both excretion and fixation by excised nodules so that more consistent results can be obtained likewise may prove of significance for the rôle of NH_2OH. Finally, a detailed study of the rôle of organic acids in the intermediary metabolism of leguminous plants is essential.

Of interest and possibly of significance for the biological problem are the results of chemical studies on nitrogenous compounds. Nichols and Derbigny (1926) report that reduction of N_2O proceeds quantitatively to N_2, NH_2OH or NH_3 dependent on the oxidation-reduction potential of the reducing system used and that no mixture of products occurs. Joss (1926) summarizing similar work on reduction of nitrate, nitrite, and other oxides of nitrogen concluded that NH_2OH probably represents a side reaction in the formation of ammonia rather than an immediate precursor. These chemical studies emphasize that, although NH_2OH may appear in the reduction of N_2 to NH_3, its occurrence is not a necessity.

An even more serious lack of knowledge concerned with the comparative biochemistry of the process arises from the few studies of the mechanism made with Nostoc and still fewer with Clostridium. Investigations with the anaerobe are especially desirable now that the nutritional requirements of organisms are better understood so that more rapid fixation than has been obtained in the past should now be possible. The rôle of hydrogen and hydrogenase in the aerobic processes makes imperative that we learn more details of the anaerobic counterpart.

SPECIFIC EXAMINATION OF THE HYPOTHESES

Schemes I and II. The next step is to decide whether details of any of the schemes conflict with known experimental facts or reasonable assumptions about the fixation process in the various agents. The first two schemes may be considered together, as scheme II is in one sense only a special case of scheme I. Since hydrogenase has not been found in Rhizobium, nodules, or Nostoc, the reducing system for these is not hydrogenase (scheme I); and scheme II is restricted to Azotobacter. The possibility remains that, as a special case of scheme II in the plant systems, hydrogen from water split in the photosynthetic reaction rather than from reversal of the Knallgas reaction acts as the reductant. Lacking direct experimental support, this appears unlikely, and in the leguminous plant transfer of the reducing system from the chloroplasts to the nodules offers formidable difficulties. The participation of hydrogenase, however, is essential for scheme II only, as any other reducing system with the proper potential could

function in scheme I. There is no *a priori* reason why the reducing system in the various agents should be identical; in algae not only does the hydrogen-donating system for reduction of CO_2 vary among closely related species but even in the same species at different times (Gaffron, 1944). Whether hydrogenase actually is the reducing system in Azotobacter as postulated awaits further clarification of the hydrogen metabolism of the organism, but present evidence suggests it as a likely possibility.

A point much more critical is the necessity for introducing the free radical mechanism in these schemes. In photosynthesis this appears desirable to provide a system with sufficient energy to reduce CO_2 (Rabinowitch, 1945, chap. 9). Equation 12 suggests that the assumption for N_2 fixation is gratuitous as many of the conventional oxidation-reduction systems possess potentials sufficiently low to function. However, the fact that a reaction is thermodynamically feasible does not necessarily mean that it will proceed at a measurable rate. It is possible that a highly active complex is needed to surmount an initial high energy potential even though the overall reaction is downhill (see discussions by Michaelis and by Kalckar in Green, 1946). However, the estimates of the activation energy made in the preceding section are hardly suggestive of an unusually difficult energy barrier.

Apart from considerations of energetics, the chief advantage of a free radical mechanism for N_2 fixation is that it provides a definite place for *competitive* inhibition by H_2. If reduction is accomplished by metabolic hydrogen involving only normal molecules and conventional oxidation-reduction systems, A_hH_2 does not appear to be a plausible reducing system since the presence of H_2 should provide another hydrogen donator; hence inhibition by H_2 is unexpected. If one tries to surmount this difficulty by reasonable postulates, an almost inevitable consequence is that inhibition by H_2 should be non-competitive.

As an alternative we can assume that reduction is carried out by some conventional respiratory system and that H_2 inhibits primarily by mechanical blocking of the N_2 molecule from the surface of En_N. If so, van der Waals' forces might serve as a rough measure of the relative adsorption of the two molecules. For H_2 and N_2 this is $0.00277/0.000487 = 5.7$ which is unexpectedly close to the ratio of their Michaelis constants of 5.5. A corollary would be that other gases should inhibit. Of those tried CO and O_2 are ruled out because of accompanying physiological effects, and the van der Waals constant for helium, 0.00007, appears too low for detection. Argon with a constant of 0.00268 should be even more effective than H_2; but experimentally no inhibition is obtained with argon. This point should be tested further with other gases as it has attractive possibilities, though, unless H_2 has a specific action, the rôle of hydrogenase appears vague and uncertain.

Scheme III. The main advantage of this scheme is that it avoids the necessity for postulating a free radical mechanism. Although written in equation (10) as if one were involved, this is an unnecessary assumption since H_2 could compete with N_2 for the "normal" state of the oxidant, B_o' $(OH)_2$, ("hydroxylated" enzyme) taking part in the initial fixation step (*cf.* Gaffron, 1940). Its

chief disadvantage is lack of relevant experiment. Clarification of the pO_2 function of Azotobacter in the presence and absence of H_2 and the rôle of the pigment system in the nodule should be helpful in deciding whether an oxidation is a probable first step in fixation.

If an oxidation step appears likely, identification of the initial oxidized product is of interest. As written in the equations, hyponitrite is suggested. Many investigators have postulated this compound as an intermediate in reduction of nitrates (nitrites) to ammonia or free nitrogen and in oxidation of ammonia to nitrous acid by various bacteria. Sufficient experimental support exists to make some of these claims probable (Corbet, 1935; Elema, Kluyver and van Dalfsen, 1934; Lloyd and Cranston, 1930). If it is an intermediate in the reduction of nitrate by Azotobacter, it meets the first requirement of an intermediate for N_2 fixation, since obviously it is assimilable. Steinberg (1939) reports that hyponitrite gives a positive test with Blom's reagent for NH_2OH; hence the positive tests for this compound could be interpreted equally well as evidence for HNO.[4] Opposed to this evidence is the observation of Wilson and his collaborators that N_2O, supposedly the anhydride of HNO, cannot serve as a source of nitrogen for the Azotobacter. Until this very critical requirement is met, HNO must be rejected for the same reason as was NH_2OH.

SUMMARY

Ammonia emerges as the most likely key intermediate in biological nitrogen fixation, with strong experimental support based upon studies of the properties of the enzyme system and upon observations with N^{15} as a tracer. The evidence has been gathered chiefly with Azotobacter, and it might be held dangerous to attribute the same properties to all biological nitrogen-fixing systems; nevertheless, until more critical evidence is presented for the hydroxylamine hypothesis, it seems justifiable to assign a general rôle to ammonia.

A generalized scheme for nitrogen fixation has been presented in figure 3, and from it three specific routes of fixation have been proposed and examined. The first assumes reduction of nitrogen with substrate hydrogen and on the whole seems the most likely pathway of fixation. However, certain objections can be raised to it, and scheme II, with a reversal of the Knallgas reaction furnishing the hydrogen for reduction, and scheme III, with an oxidative mechanism, should be considered as possible alternative routes and subjected to experimental test.

Although cause for satisfaction exists in considering the progress made toward an understanding of the mechanism of biological nitrogen fixation during the past decade, this review emphasizes the need for additional critical information. The state of our knowledge recalls Kluyver's (1931) comment of a similar period in the development of bacterial respiratory mechanisms.

[4] Endres (1935) claimed that HNO could not be among the products giving a color with Blom's reagent in cultures of Azotobacter as he had boiled these overnight in acid solution. The evidence is equivocal, however, since he recovered only 10 per cent of the material responsible for the color after such treatment.

". . . the evidence for the occurrence of a special intermediate stage in a biochemical process *can* only be of an indirect nature. From this point of view it follows that one can only judge the probability of a supposed reaction mechanism by co-ordinating all experimental data available."

". . . either we have to accept the fact as it is and refrain from any explanation, or we have to tread the path of speculation and test the probability of different hypotheses as to the nature of this mechanism."

The special steps we have proposed in this review describe more or less accurately laboratory results based on a necessarily small sample of the totality of experimental facts to be gathered eventually. Unfortunately, by reason of the experimental approach such a sample often fails to represent the random choice desired. As suggested specifically in the text, further samplings with the methods that have proved so useful in recent work together with newly developed ones should provide a basis for choice among the suggested mechanisms, demand their modification or even rejection.

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