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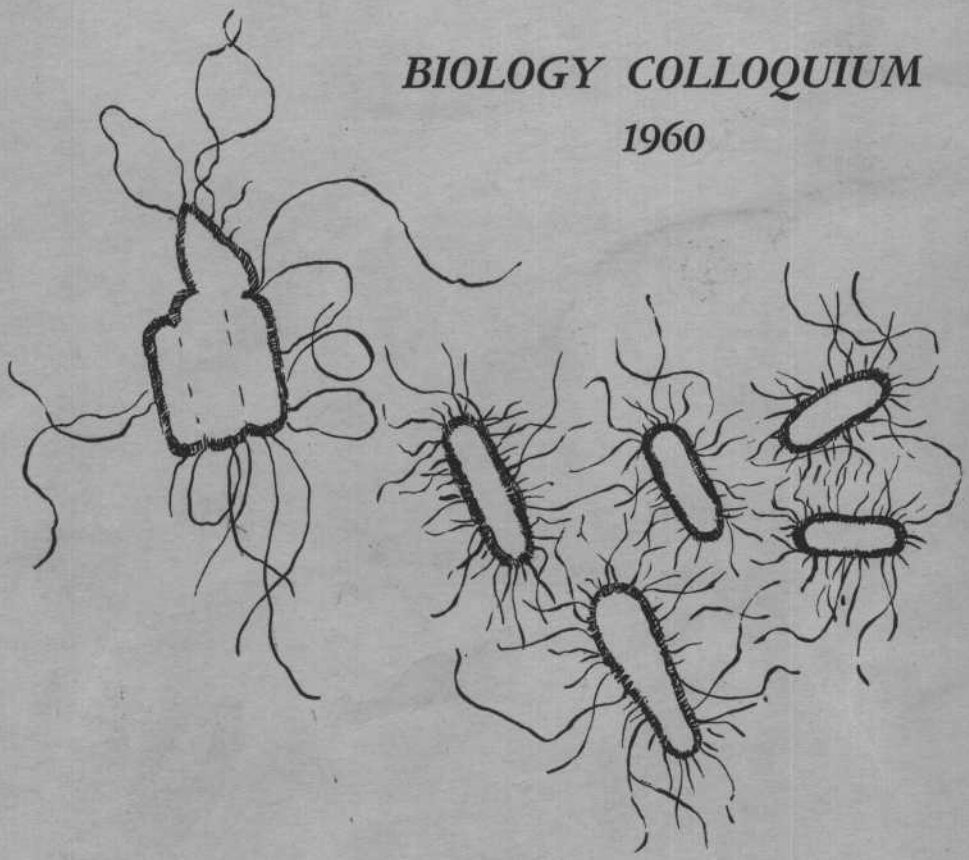
Microbial Genetics



BIOLOGY COLLOQUIUM

1960

OREGON STATE CHAPTER OF PHI KAPPA PHI
OREGON STATE COLLEGE • CORVALLIS • 1960

Twenty-first Annual Biology Colloquium

April 22-23, 1960

Microbial Genetics

Edited By

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and

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OREGON STATE CHAPTER OF PHI KAPPA PHI

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Foreword

The Biology Colloquium, established in 1939, is conducted in a spirit of informal discussion and provides opportunity for participation from the floor. The Colloquium is sponsored by the Oregon State Chapter of Phi Kappa Phi with the collaboration of Sigma Xi, Phi Sigma, Omicron Nu, and Phi Lambda Upsilon.

Grateful acknowledgment is made of the support of the National Science Foundation in a grant to Oregon State College and of the cooperation and interest of the schools of Oregon State College that are concerned with biology, of those biologists and other scientists contributing to the program, of Chancellor John R. Richards, President A. L. Strand, and other executives of Oregon State College.

Leaders and themes of previous colloquia have been as follows:

Recent Advances in Biological Science, March 4, 1939. Dr. Charles Atwood Kofoid of the University of California.

Ecology, March 9, 1940. Dr. Homer LeRoy Shantz, Chief of Division of Wildlife Management, U.S. Forest Service.

Growth and Metabolism, March 8, 1941. Dr. Cornelis Bernardus van Niel of the Hopkins Marine Station, Stanford University, in collaboration with Dr. Henrik Dam of the University of Copenhagen.

The Biologist in a World at War, March 14, 1942. Dr. William Brodbeck Herns of the University of California.

Contributions of Biological Science to Victory, April 3, 1943. Dr. August Leroy Strand, biologist and president of Oregon State College.

Genetics and the Integration of Biological Sciences, April 22, 1944. Dr. George Wells Beadle of Stanford University.

1945—Biology Colloquium omitted.

Aquatic Biology, April 27, 1946. Dr. Robert C. Miller of the California Academy of Sciences.

Biogeography, April 19, 1947. Dr. Ernst Antevs of the Carnegie Institution of Washington.

Nutrition, April 10, 1948. Dr. Robert R. Williams of the Williams-Waterman Foundation.

Radioisotopes in Biology, April 23, 1949. Dr. Eugene M. K. Geiling of the University of Chicago.

Viruses, April 29, 1950. Dr. W. M. Stanley of the University of California.

Effects of Atomic Radiations, April 21, 1951. Dr. Curt Stern of the University of California.

Conservation, April 19, 1952. Dr. Stanley A. Cain of the University of Michigan.

Antibiotics, April 18, 1953. Dr. Wayne W. Umbreit of the Merck Institute for Therapeutic Research.

Cellular Biology, May 6, 1954. Dr. Daniel Mazia of the University of California.

Biological Systematics, April 30, 1955. Dr. Ernst Mayr of the Museum of Comparative Zoology, Harvard University.

Proteins, April 6-7, 1956. Dr. Henry Borsook of the California Institute of Technology.

Arctic Biology, April 19-20, 1957. Dr. Ira Loren Wiggins of the Museum of Natural History, Stanford University.

Photobiology, April 11-12, 1958. Dr. F. W. Went of the California Institute of Technology.

Marine Biology, April 3-4, 1959. Dr. Dixy Lee Ray of the University of Washington.

Twenty-First Colloquium Participants

Colloquium Leader

AARON NOVICK, Ph.D., Professor of Biology; Director, Institute of Molecular Biology, University of Oregon.

Colloquium Speakers

L. S. BARON, Ph.D., Chief, Department of Bacterial Immunology, Walter Reed Army Institute of Research.

N. B. GROMAN, Ph.D., Associate Professor of Microbiology, University of Washington.

J. SPIZIZEN, Ph.D., Assistant Professor of Microbiology, Western Reserve University.

D. R. STADLER, Ph.D., Assistant Professor of Genetics, University of Washington.

G. S. STENT, Ph.D., Professor of Virology and of Bacteriology, University of California, Berkeley.

A. L. STRAND, Ph.D., President, Oregon State College.

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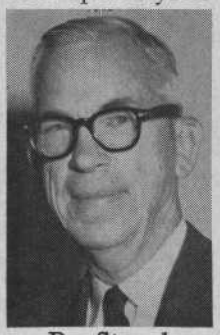
Opening of the Colloquium

DR. A. L. STRAND
President of Oregon State College

IT IS ALWAYS A PLEASURE to say a few words of welcome at these meetings. This is the 21st Colloquium. That means we have come of age. I say it is a pleasure for me because I used to look upon myself as a biologist with a taste for biochemistry but in the past 25 years things have changed.



Dr. Strand

I discovered a word several weeks ago to describe college presidents who think they are something else. They are sciolists. I do not think it refers to the town of Scio in Oregon, but a sciolist is a smatterer who just has a smattering of a lot of things. This is the kind of biologist I am at the present time.

The idea of the colloquium, which generated in the minds of members of Phi Kappa Phi some 21 years ago, has grown into an important meeting for this campus, an important scientific meeting. I may be somewhat alone in my viewpoint, now, but I think it is well to recall the earlier situation that caused so many nonbiologists to be interested enough to form an organization to discuss biological subjects.

The other evening while I was ruminating around at home I found a paragraph in one of the lectures of Helmholtz that seems to me somewhat fitting to this proposition. Indeed, although the lecture was given in the winter of 1862-63, this particular paragraph from it might have been written yesterday for this occasion.

"The natural sciences," said Helmholtz, "partly in consequence of their practical applications and partly from their intellectual influence on the last four centuries, have so profoundly and with such increasing rapidity transformed all the relations of the life of civilized nations that they have given these nations such increase in riches of enjoyment of life, of the preservation of health, of means of industrial and social intercourse, and even such increase in political power that every educated man who tries to understand the forces at work in the world in which he lives, even if he does not wish to enter upon the study of a special science, must have some interest in that particular kind of mental labor which works and acts in the sciences in question."

I was pleased to find this. It expresses better than I could the original idea behind the Colloquium for there is an interest of educated men in trying to understand the forces that work around them, even if they do not wish to enter upon the study of a special science. That's why, through the years, these meetings have been attended by engineers and geologists, physicists and teachers at all levels of education as well as biologists and biochemists and why I think it is sometimes appropriate to warn the leaders of the Colloquium that it will be appropriate, it will be appreciated by at least some in the audience, if they will do a little filling in once in a while to orient some who are unfamiliar with the scientific jargon or who, in this instance, might never have had a course in microbiology but yet, as Helmholtz says, have an interest in that peculiar kind of mental labor which works and acts in the science in question.

It is a pity, of course, if an educated person has lost out in his undergraduate years in something as fundamental to general education as bacteriology, to use what seems to be an archaic term. I realize that this may be a handicap to some scientists who are very professionally minded but, at the same time, as C. P. Snow says, scientists and nonscientists must begin to understand each other a little better.

Many notable leaders in many different fields of biology have participated in these meetings, and the published fruits of the Colloquia comprise valuable additions to the literature of biology. Some of these publications of former years are still available as perhaps you noticed when you came in. It is true, unfortunately, that we have been a little hard-pressed in some years, especially in 1943, and had to depend on some rather low-grade local talent.

This year's subject is microbial genetics. We seem to have two extremes of interest on this

campus, the genetics of unicellular organisms on the one hand and the genetics of such massive plants as the Douglas fir on the other. The cell is a wonderful common denominator.

I do not know much about microbes except as everyone knows there are the good guys and the bad guys, like all the Westerns, and as I wrote this I remembered a paper I had read a long time ago in *Science*. It might have been 20 years ago, there was an article by a bacteriologist by the name of Kendall. I have always remembered it because it was a very strange paper. He talked about bacteria that could disembody themselves and pass through Berkefeld filters and then reform on the other side.

I read that article but did not read any followup. There was never any third act of this play as far as I was concerned. I never found out anything additional. Now, however, I notice in the program there is something on conversion by Dr. Groman and maybe I will learn the sequel to Dr. Kendall's paper of long ago.

Genetics is a most pervasive study; it permeates the affairs of man and the operation of his institutions like no other biological subject, I am sure. "Woe be to the generation that lets any higher faculty in its midst be unemployed," wrote Henry Thoreau—while sitting unemployed himself by Walden pond, no doubt, or apparently so. This remark might be paraphrased: "Woe be to any generation that lets any higher faculty in the organisms associated in the life on this planet go unemployed, or does nothing to counteract those with lower faculties." It is as true of microorganisms as it is with humans.

I am sure of one thing: that the study of a cell and all its complications (from any aspect whatever) is always productive. The answer to many great human problems will be untangled by studies of the cell. One thing leads to another. You can't possibly investigate the genetics of single cell bodies without turning up things that hold significance to other matters quite distinct from inheritance.

We are very pleased for this important subject to have as leader of this year's Colloquium our neighbor from the University of Oregon, Dr. Novick, and I welcome him and other speakers, and all others who have come to join in this conversation. Thank you.

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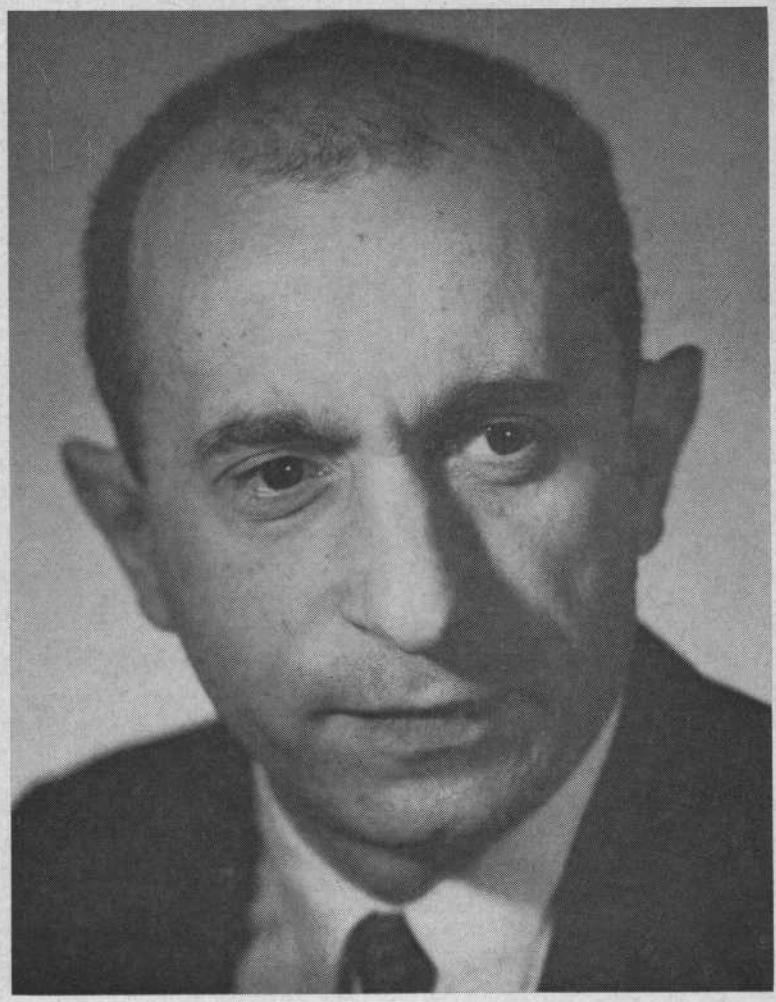

AARON NOVICK, Ph.D.

Leader of Twenty-first Annual Biology Colloquium

Microbial Genetics
Introduction to the Colloquium

AARON NOVICK
Institute of Molecular Biology, University of Oregon

It is a very great honor for me to be invited here to lead this Colloquium, especially in view of the fact that from time to time there is a minor amount of rivalry between this institution and the institution to the south from which I come. Therefore, I came here wondering what sort of trap was in store for me. When I discovered the program with my picture on the cover, I realized that this was it.

More seriously, when I look at the list of people who have been Colloquium leaders in the past and see the distinguished names on the list, I indeed do become somewhat embarrassed. My claim to fame here is not really any great contribution to the science as much as a close acquaintance with many of the people who have made great contributions to our understanding. So I come here as a kind of Boswell for people who have done really great things in a very exciting field in the last few years.

The discoveries in microbial genetics have become basic to all of biology, and an amusing aspect of this is partly illustrated by the following incident. I was at a meeting of the Society of Illinois Bacteriologists when it honored Joshua Lederberg with the Pasteur Award. A curious aspect of these proceedings was the fact that the traditional bacteriologists who compose a large fraction of the members of the society were quite puzzled as to what was going on in bacteriology.

All kinds of high-powered, strange-looking people had moved into the field, and there was a great sense of excitement and importance. Many of these people were pleased to see such activity in their field but were frankly embarrassed by not quite understanding why. That evening after the celebration, I went to a party where a distinquished physicist was present. I told him this curious thing—how these poor bacteriologists suddenly had fame thrust upon them, suddenly had their field become important, but that many of them did not quite understand what was going on. I followed this by saying, "you realize that some of the greatest advances in biology have occurred in bacteriology in the last few years." The famous physicist disagreed and replied, "Oh, no, no, no. The greatest advance in biology in the recent years was the discovery of the structure of DNA by Watson and Crick." I retorted, "But that is bacteriology." What I meant of course is that basic to the discovery of the structure and its relevance are the ideas of microbial genetics.

In one sense, microbial genetics has provided instruments for the attack on a number of the most fundamental problems in biology. Science has always depended very much upon the invention of either new techniques or new systems. As we look back at the history of genetics we see that the introduction of the fruit fly and maize were necessary for the great developments of the first half of the 20th century. Likewise, the appearance of bacteria and viruses as genetic tools has provided recently for a rapid expansion of our understanding of basic genetic mechanisms.

By finally permitting questions to be answered in terms of the structures of biological macromolecules, microbial genetics is evolving into the the field of molecular biology. Microorganisms, like all other life we know, function by means of nucleic acid and protein. Thus what is learned about the basic biology of microorganisms is useful toward the understanding of all other life.

I would like to turn back in history and trace, if I can, the principal lines of development, mentioning some of the principal names and no doubt omitting many others, mostly by ignorance or accident, and their contributions which have led to our present understanding to be described by this Colloquium.

We should go back at least as far as the development of the ideas of physiological genetics which arose from work with *Drosophila*, guinea pigs, maize, and other forms. It was realized that the genes must eventually determine some kind of chemical function.

As is now well known, the great breakthrough came when Beadle and Tatum and their collaborators began to study the genetics of *Neurospora*. Their studies led to much under-

standing of the role of genes in the control of biochemical processes, and they were led to state the great simplifying "one gene, one enzyme" principle. This principle argues that what the gene does is to determine the presence of a single enzyme.

Some of the discussions in this Colloquium, especially that by Dr. Stadler on complementation, will bear very strongly on this principle.

Back during the very early forties, bacteria and viruses were genetically enigmatic. When I was a student, about 20 years ago, we were taught that bacteria did not have nuclei, that bacteria appeared not to have genetics. As recently as the mid-forties or even up to 1950, even some biochemists would speak of training bacteria to adapt to a given sugar. One had the picture of the biochemist with his big club beating the bacteria into using this substrate or that substrate. Bacteria were deemed too primitive to have genetics. They were some sort of plastic creatures that responded by some mechanism at once mysterious and unrelated to that in higher forms. In fact, Luria found the right words some years ago in a review he wrote in which he said that bacteriology had become the last stronghold of Lamarckism. And it was Delbruck and Luria (1) who finally beat down the walls of ignorance and introduced bacteria as respectable genetic creatures when they showed that the change in a bacterial population, for example, from sensitivity to a drug to resistance to a drug, or from sensitivity to a phage to resistance to a phage, was the result of a Darwinian process, mutation and selection.

They proved, in certain cases at least, that the appearance of variant individuals in the population was by a process like mutation, as it was known in higher forms. They showed this in a very elegant and since many times repeated experiment. It became apparent in 1943 when they published their paper that bacteria possessed the same kind of genetic mechanism as higher forms —at least with respect to like begetting like, except for the rare exception that was a mutant.

During this time, the idea of a selective marker appeared, a most important idea to the development of microbial genetics. This meant that one could do experiments with enormous numbers of bacteria because in certain cases one could select the desired individual no matter how rare. For example, one can select one streptomycin-resistant bacterium out of a billion sensitive bacteria simply by putting streptomycin in the medium. Only the resistant individual can grow. With the ability to detect rare individuals, the microbial geneticist had a great advantage over other geneticists, who had to examine flies or kernels of corn directly.

A most important use of the idea of the selective marker was made by Lederberg and Tatum (2). It occurred to them that if sex occurred in bacteria, it could be detected even if the phenomenon occurred exceedingly rarely. They took two types of individuals—two strains, one requiring substance A and the other requiring substance B. The first strain would grow only if A were added to the medium; the second strain would grow only if B were added to the medium. They reasoned that if the two strains were mixed in the absence of A or B and plated, the two parent strains would be unable to grow since the medium would lack both A and B. But if by genetic recombination of some kind an individual could appear which could grow in the absence of A or B, getting the synthetic capacity for substance A from one parent and for substance B from the other parent type, such an individual could be detected even though only one were present in a million bacteria. They did such an experiment and did detect such recombinant individuals. It appeared that sex was a very rare phenomenon in bacteria, occurring only at the level of about one in a million. As many of you know, since then fertile strains have been discovered, first by Cavalli (3). Recently because of the brilliant work of Jacob and Wollman (4), it is now possible to get strains to mate with one hundred percent efficiency.

During this period, a very important parallel line of development was the growth of the so-called phage school. This included originally people like Delbruck, Luria, Demerec, Hershey, and Adams. Delbruck was a theoretical physicist who came to Cal Tech on a Rockefeller fellowship to pursue a recently acquired interest in biology. There he learned about bacteriophage and began to do some experiments. Meanwhile he met Luria, who had come as a refugee from Italy where he had begun some studies on the effects of radiation on bacteriophage. Their collaboration set a pattern of high scientific quality that was very important to the growth of microbial genetics.

Meanwhile Demerec, an outstanding *Drosophila* geneticist, and Hershey, a microbiologist, turned their attentions to bacteriophage. These workers communicated with each other and the phage school was born in the early 1940's. An important development was the creation of a

course in bacterial viruses which was first given by Delbruck. This course was responsible for the education of a wide number of people from many fields in the methods and ideas of bacteriophage. It was possible in the late 1940's and the early 1950's to take a three-week course at Cold Spring Harbor and be all set to do very interesting research.

The early investigators decided to limit themselves to a relatively small number of organisms so that what was found out about the physiology and biochemistry of one organism could be coupled with what was learned about the genetics of the same organism. It was agreed to work on seven bacterial viruses, the now famous T1 to T7.

During those days many great discoveries were made. It was discovered that bacterial viruses can suffer mutations analogous to higher forms. It was discovered that viruses can engage in genetic recombination. Great strides were made in the understanding of the nature of the life cycle of a virus using the tools of genetics and biochemistry.

During this period, in fact for some 20 years or so, people had spoken of lysogenic bacteria. Bacterial strains were known which were reputed to harbor a virus in some way. These were strains that could not be freed of a virus, strains that were afflicted somewhere deep in their souls with the virus that they carried. The attitude of most American phage workers was that this phenomenon resulted from people who were simply sloppy and did not know how to streak out their cultures and get rid of phage. Thus, it was not until about 1950 or 1951 when Lwoff (5) did his now famous experiment in Paris with *Bacillus megatherium* phage that he convinced Delbruck and the others that lysogeny did indeed exist. This most extraordinarily interesting relationship between a virus and a host cell became an active subject for study.

The understanding that a virus could maintain this intimate relationship with the cell led to the discovery of a very curious phenomenon called transduction. As you recall, when Lederberg first discovered that he could mate two strains of bacteria, questions were raised about the interpretation that this was indeed some kind of *bona fide* conjugation. One possibility was that this resulted from a transformation like that known to occur with *Pneumococcus*.

Griffith observed more than 30 years ago that it is possible to take a heat-killed virulent strain of *Pneumococcus* and mix it with an avirulent strain and convert the avirulent strain to virulence.

It was shown by the brilliant work of Avery, Macleod, and McCarty (6) in the middle 1940's, that the active agent that could be isolated from the heat-killed virulent strain was the substance DNA. DNA could be crystallized and purified from a suitably marked strain, like a virulent strain. This DNA could be added to an avirulent strain to convert it to a virulent strain. Later people were able to isolate DNA from a penicillin resistant strain and use it to transform a sensitive strain to resistance to penicillin. This phenomenon is called transformation.

Thus, some suggested to Lederberg that perhaps he was not observing conjugation but some kind of transformation. At this point Bernard Davis (7) suggested an amusing experiment that came to be known in politer circles as the bacterial bundling board experiment. A U-tube was taken, a sintered glass filter was placed to separate the two arms of the U-tube from each other. One strain of bacteria was put in one side and one in the other side. The filter would not permit the passage of bacteria from one side to the other, but would permit the fluid bathing the bacteria to pass freely from one side to the other. Thus, if it were transformation, the DNA from one side could leak through and transform the bacteria on the other side. But if it were some kind of conjugation requiring physical contact between the cells, the filter would prevent such conjugation. When this experiment was done, no recombinant individuals appeared, and this was ascribed to the prevention by the filter of cellular contacts.

Lederberg wanted to extend these results, and a student, Norton Zinder, looked for recombination in *Salmonella*. He discovered evidence of genetic recombination, but in contrast to the case studied by Lederberg, recombination occurred even when a filter was present to separate the two parental strains. He was able to show that the recombinants arose by a new kind of mechanism which they called transduction.

Zinder and Lederberg (8) soon showed that the active agent in this genetic transformation is not free DNA as in case of *Pneumococcus* but a virus carried by one of the strains. The virus carries genetic markers from one strain to the other. The phenomenon of genetic transduction as it has come to be called, the transfer of genetic material from one individual to another by means of a virus, is now well known in a wide number

of different bacteria with a wide number of different viruses.

During this period an extremely important result was obtained by Hershey and Chase (9) at Cold Spring Harbor which established that the material basis of the genetics of phage is DNA. By the early 1950's it was known that the bacterial viruses were chemically quite simple. They were composed chiefly of proteins and DNA. They were tad-pole like structures which attached to a sensitive bacterium tail first. It was already appreciated that there was some separation of function between genetics and physiological function within these viruses.

Hershey and Chase appropriately labeled the DNA and proteins of a phage with radioactive phosphorus and sulfur respectively. They showed that upon infection only the phosphorus and, therefore, the nucleic acid entered the cell. The protein remained outside and could be separated from the bacterium by treatment with a blender.

This experiment, the transformation results, and other evidence identified DNA as the unique basis of heredity. Thus, when in 1953 Watson and Crick (10) proposed their now very famous structure for DNA, it was greeted with great excitement. They showed how a wide variety of genetic phenomena could be understood in terms of the properties of the DNA molecule. Replication could be simply explained. How DNA could contain enough information to specify the complex molecules which proteins are was apparent. Moreover, a very elegant theory for mutation was proposed by Watson (11) at this time.

Not long after, Benzer (12) was able to relate the genetics of phage to the structure of DNA. With phage he could look at very rare recombination events and could thus observe recombination that might occur only in distances as small as the distance between two adjacent nucleotide pairs in the Watson-Crick structure. In these studies Benzer raised in a crystal clear way questions that have been around the fringe of genetics for a long time.

The question of the definition of the gene has always been unclear, although the notion of the gene was introduced a long time ago. By 1950, the geneticist had many different definitions for the gene. One was the definition of the gene by genetic recombination. By this definition, two mutant individuals which give wild-type progeny by recombination are said to have mutations in different genes.

Similarily, geneticists had a test called the dominance test, which was used to see whether two characters were in the same gene on the basis of the phenotype of a diploid. Benzer recognized the independence of these definitions and proceeded to introduce operational definitions of the gene which have served to clarify our understanding of the gene and its function. These included the *recon*, which is the unit defined by the recombination test, and the *cistron*, the unit defined by the dominance test. The former appears to be nothing more than a nucleotide pair, since recombination apparently can occur between adjacent base pairs. The cistron seems to define a region which is inviolate—no alteration being complementable by a cistron on another chromosome. Recent work, mostly with *Neurospora*, to be discussed by Dr. Stadler, raises questions about the cistron; and time will tell whether these difficulties are fundamental or not.

Thus has microbial genetics led to the birth of the new science of molecular biology. Because of the power of the techniques of microbial genetics and because of our improving understanding of the structures and properties of biologically important macromolecules, we are in the midst of one of the most exciting periods of discovery in the history of biology.

In this Colloquium we hope that the subject of microbial genetics will be made clear and that the pressing, exciting problems of today will emerge.

References

1. Luria, S. E., and M. Delbrück, 1943. Genetics, *28:* 491-511.
2. Lederberg, J., 1947. Genetics, *32:* 505-525.
3. Cavalli, L. L., and J. L. Jinks, 1956. Genetics, *54:* 87.
4. Jacob, F., and E. L. Wollman, 1958. Symposia of the Soc. for Exptl. Biol., *12:* 76-92.
5. Lwoff, A., and A. Gutmann, 1950. Ann. Inst. Pasteur, *78:* 711.
6. Avery, O. T., C. M. MacLeod, and M. McCarty, 1944. J. Exptl. Med., *79:* 137.
7. Davis, B. D., 1950. J. Bacteriol., *60:* 507-508.
8. Zinder, N .D., and J. Lederberg, 1952. J. Bacteriol., *64:* 679.
9. Hershey, A. D., and M. Chase, 1952. J. Gen. Physiol., *36:* 39.
10. Watson, J. D., and F. H. C. Crick, 1953. Nature, *171:* 737-738.
11. Watson, J. D., and F. H. C. Crick, 1953. Cold Spring Harbor Symposia Quant. Biol., *18:* 123-131.
12. Benzer, S., 1955. Proc. Natl. Acad. Sci. U. S., *41:* 344.

Genetic Studies on the Replication of Bacterial Viruses[1]

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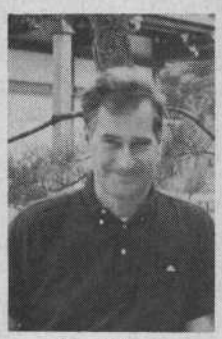

Dr. Stent

The experiments that I want to discuss here concern the replication of the viral hereditary material, i.e., of the infecting deoxyribonucleic acid (DNA), during the intracellular growth of bacterial viruses, or *bacteriophages.* This work was carried out in collaboration with my associates D. Pratt, H. Uchida, and G. Fermi. Let us first recapitulate briefly the process of intracellular bacteriophage growth. The virus particle attaches itself to the bacterial cell wall and injects its DNA complement into the cell, and then, once inside the bacterium, the viral DNA carries out both genotypic and phenotypic functions; that is to say, the DNA replicates itself several hundred times, and it induces, or presides over, the synthesis of several hundred replicas of the viral protein that the virus had shed at the moment of infection. At the end of this replication process, replica viral DNA units are encapsulated into replica viral protein units to reconstitute the infective, structurally intact virus particles of the progeny generation. The part of this reproductive process on which I now want to focus our attention is the mechanism by which the viral DNA is replicated and by which the progeny viruses are endowed with their hereditary substance. In 1953 Watson and Crick (1) made the first molecular proposal for this process; their scheme envisaged that the two complementary polynucleotide strands of the parent DNA molecule separate and that each of the two strands adds on a *de novo* complement through specific hydrogen bond pairing between purine and pyrimidine nucleotide residues. Hence after the two strands have unwound and each added on its complement, two DNA molecules have been generated, each of which is identical to the parent molecule; at this point, one cycle of replication has occurred. For the next cycle, the two daughter molecules once more unwind and give rise to four single polynucleotide chains, each of which now adds on its complement to produce finally four DNA daughter molecules, each of which is identical in its purine and pyrimidine nucleotide sequence to the original DNA molecule. In this way the DNA of the infecting parent virus would undergo successive rounds of unwinding and complement addition, ultimately building up an intrabacterial *pool* of viral replica DNA molecules identical to the DNA of the parent and thus provide the hereditary substance for the offspring virus.

A few years ago it seemed to me that this proposal did not fit some of the observations on the biochemistry and genetics of bacterial virus reproduction, and I permitted myself the heretical thought that the replication might proceed by a mechanism (2, 3) different from that of Watson and Crick. I do not want to go into the boring details of my proposal here because, as will soon be evident, I now believe it is necessary to abandon it. But perhaps I might just indicate one or two features in which this idea differed from the Watson-Crick mechanism. I imagined that the first stage in the DNA replication process consists of the growth of a ribonucleoprotein molecule on the surface of the intact parental DNA molecule; that is, instead of believing that the two polynucleotide strands of the parent DNA unwind and each serves as a direct template for the synthesis of its complementary replica strand, I thought that an intermediate substance is first constructed to which the genetic information of the parent molecule is transferred. This intermediary then, in turn, acts as the template for the synthesis of replica DNA molecules. I imagined, further, not only that this ribonucleoprotein template acts genotypically, in the sense that it provides the pattern for the synthesis of the DNA replicas, but that it can also act phenotypically, in that it is also the template for the synthesis of the proteins whose polypeptide amino acid sequence is presumably coded in the parent DNA molecules. In this way genotypic and pheno-

[1] Supported by a grant from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

typic expression of the parental DNA would have occurred in one and the same act.

Now this proposal differs from the Watson-Crick mechanism in at least two aspects. One of these is that the synthesis of an *essential protein* is necessary before the replication of the viral DNA can begin, for no replica DNA could be formed until the ribonucleoprotein template has been synthesized. The direct Watson-Crick type of replication, in contrast, would not involve any such requirement, provided all the nucleotide building blocks and polymerizing enzymes were present in the host cell. A second difference between my ill-fated proposal and the mechanism of Watson and Crick concerns the fate of the atoms of the parent molecules. Now let us imagine that the parent DNA molecule is labeled with an isotope and that replication is allowed to proceed in the absence of this isotopic label. Then under the Watson-Crick mechanism, after one replication cycle there would be two replica molecules, each of which possesses one of the isotopically labeled polynucleotide strands of the parent molecule and one strand which has been built on *de novo* and is, therefore, not isotopically labeled. After the next replication cycle, among the four progeny DNA molecules, there would be two possessing one parental strand and one *de novo* strand, and two molecules of which both strands were entirely *de novo*. This predicted distribution of parental atoms has been called *semi-conservative* (4). Under my indirect replication hypothesis, on the other hand, the distribution of the atoms would be expected to be *conservative,* because the replica molecule synthesized on the surface of the ribonucleoprotein template would have no actual physical contact with the parent DNA molecule, and would be entirely *de novo*. The original DNA molecule would, therefore, retain all of its isotopic label and the replica molecule contain none of the label. Isotopic tests of this sort have already been carried out by Meselson and Stahl (5) for the replication of bacterial DNA, and their results support the mechanism of Watson and Crick. Meselson and Stahl labeled bacterial DNA with the heavy nitrogen isotope N^{15} and then allowed the bacteria to reproduce in the presence of the normal N^{14} isotope. This experiment showed that N^{15}–N^{14} hybrid DNA molecules arise after one generation of growth in N^{14} medium, exactly in agreement with semi-conservative distribution of the parental label. However, the results of similar experiments on the fate of the atoms of bacterial virus DNA in the course of their replication are not very clear as yet.

Let us now consider some experiments relevant to the question whether transfer of the viral genetic information to a protein-containing intermediate template occurs during the replication of the bacteriophage DNA. Some years ago it was discovered that at the very outset of its intracellular growth the T4 bacteriophage, active on *Escherichia coli,* synthesizes an *essential "early" protein,* which has to be made before phage DNA replication can begin (6, 7, 8). This was demonstrated in the following way. If a bacterial culture infected with T4 bacteriophage is analyzed at various times for its content of phage specific DNA (this is possible because the DNA of T4 contains an unusual pyrimidine, 5-hydroxymethylcytosine, which is not present in the DNA of the bacterial host (9)), then phage-specific DNA synthesis is seen to start at about the 5th or 6th minute after infection and to continue at a constant rapid rate of about 5 phage equivalents per infected bacterium per minute (10, 11). (A phage equivalent is the amount of DNA contained in one single virus particle, about 2×10^{-13} mg. The time scale in all the experiments mentioned here refers to virus growth at 37° C.) However, if infection is carried out in the presence of *chloramphenicol,* an antibiotic that inhibits protein synthesis (12), no phage specific DNA is synthesized in that culture; i.e., if bacteria are infected with phage under conditions where protein synthesis is prevented, synthesis of phage DNA is blocked. But if infection is made in the absence of chloramphenicol and the antibiotic added only 6 to 8 minutes after the onset of intracellular phage growth, then one finds that viral DNA synthesis can proceed. Of course, no infective viruses are made until the antibiotic is removed, since the structural protein of the virus still has to be synthesized. It thus appeared that during the first 6 to 8 minutes of phage growth a protein necessary for the replication of the virus specific DNA has to be made within the cell. Furthermore, it was also found that during the same time a phage specific ribonucleic acid appears in the infected cell (13). Hence, this essential "early" protein and the new ribonucleic acid could represent the hypothetical ribonucleoprotein template whose formation I thought to be necessary before DNA replication could get under way.

Not long after these discoveries, however, the probable nature of this essential "early" protein was uncovered. For it was found that soon after

infection, the phage induces the formation of a set of new enzymes inside the host cell. These enzymes concern the synthesis and polymerization of hydroxylmethylcytosine and other specific constituents of the phage DNA (14, 15, 16). Thus it seems reasonable that replication of the phage DNA cannot get underway until all those enzymes are present which are necessary for making and joining its building blocks, i.e., that those enzymes represent the essential protein. Nevertheless, the discovery of phage-induced enzymes does not disprove the indirect replication hypothesis, since the mere finding of such enzymes does not necessarily *exclude* the existence of the hypothetical ribonucleoprotein molecule. A somewhat sophisticated test is necessary, therefore, to determine whether among the "early" protein there is perhaps one species which plays the role envisaged under the information transfer theory. We have attempted to use the occurrence of mutations in the course of the replication of the viral DNA for such a test, and some preliminary remarks concerning phage mutants and mutagens are necessary before we consider these experiments.

Figure 1 presents a photograph of the plaques formed by the wild type of T4 bacteriophage, designated as T4r^+, when plated on *E. coli*, strain B. Among thousands of such r^+ plaques, one generally finds a few variant plaques, which exhibit the morphology shown in Figure 2 and are due to the presence of rare r mutant viruses in the r^+ wild type population. However, when T4r^+ wild type phages are grown on bacteria in a culture medium to which the thymidine analogue 5-deoxybromouridine (BD) has been added, the proportion of r mutants is greatly increased. In other words, BD is a very potent *mutagen* for the phage genome (17). The basis of this mutagenesis is thought to be the following: the genetic information of the DNA undoubtedly derives from the specific sequence of the four possible nucleosides —adenosine, thymidine, guanosine, and cytidine— along the two polynucleotide chains. A mutation, therefore, would be that rare *copy error* which introduces an *incorrect* nucleoside at some site of a replica polynucleotide chain. On the basis of their structure of DNA, Watson and Crick (1) proposed that one copy error very likely to occur is that in which cytidine is introduced in place of thymidine, or vice versa. As can be seen in Figure 3, BD is very similar structurally to thymidine, since the size of the bromine atom of BD is very nearly that of the methyl group of thymidine. A number of theoretical considerations and experimental observations (cf. 18), which cannot be discussed here in detail, have indicated that the presence of BD in the growth medium of phage-infected bacteria exerts its mutagenic effect by greatly increasing the chance that such a thymidine-cytidine copy error occurs in the replication of the viral DNA.

The r mutants shown in Figure 2 fall into several classes, the most important of which for our discussion is the rII class, none of whose members grow on *E. coli*, strain K12(λ), on which strain the r^+ wild type phage is able to grow perfectly well (19). Another class of r mutants is made up of rI mutants, which, like rII mutants, produce the r plaque type on strain B but, unlike rII, *are* able to grow on strain K12(λ). Mutants belonging to both of these classes can be induced by growth in the presence of BD; furthermore, once isolated after BD mutagenesis, such mutants can also be induced to back mutate, or *revert*, to the r^+ wild type state by BD. One particular rII mutant mentioned in the following is the BD-induced mutant rII(5). This mutant sports r^+ reverse mutants with a spontaneous frequency of 10^{-7}; this frequency can be raised to nearly 5×10^{-4} if growth of the mutant proceeds in the presence of BD.

Among spontaneous rII mutants, Benzer has also identified individuals in which the mutated region of the phage genome does not correspond to a point, or single mutational site, but is of finite length, so as to include many mutational sites. These extended mutants, or "deletions," which may owe their mutant phenotype to the *absence* of a whole piece of the phage genome, do not sport any r^+ revertants, either spontaneously or under the influence of any mutagen. "Deletion" mutants do not, of course, produce any wild type r^+ recombinants in genetic crosses to other rII "point" mutants whose mutational sites are covered by the "deletion." One of these "deletion" mutants, rII(H23), has found application in the present experiments; it covers the BD-induced point mutant rII(5) and does not sport any r^+ reverse mutants whatsoever.

We are now ready to consider some experiments designed to examine a possible genetic role of some component of the "early" protein. These experiments are directed towards the question whether there is an *individually specific* type among these "early" proteins. Now, by individually specific, we mean the following: Suppose there are two types of infecting viral DNA in the bacterial cell, as is the case when the cell

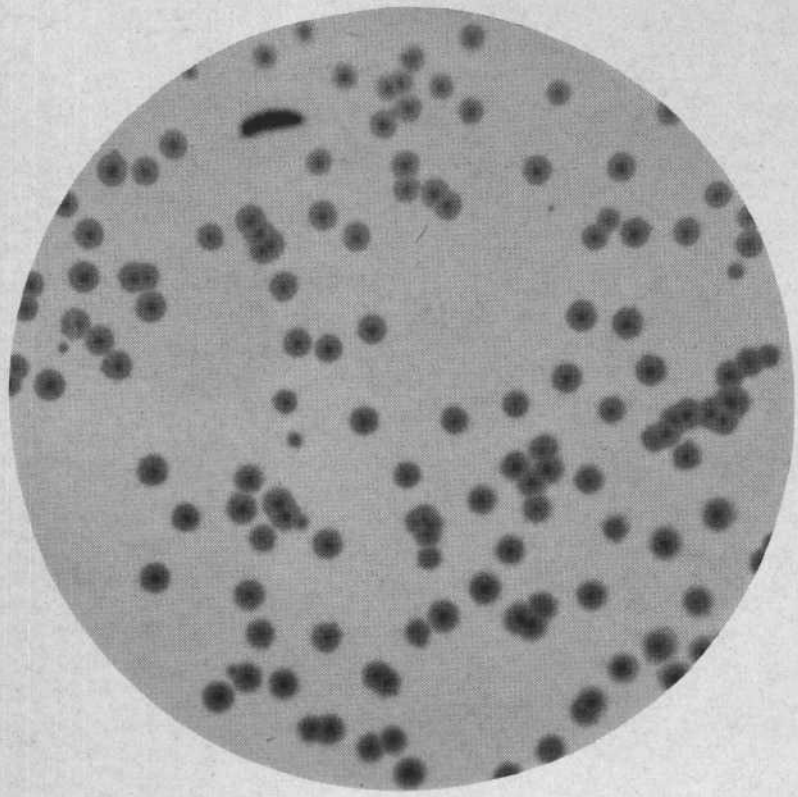

Figure 1. Plaques of the T4*r*⁺ wild type on *E. coli*, strain B.

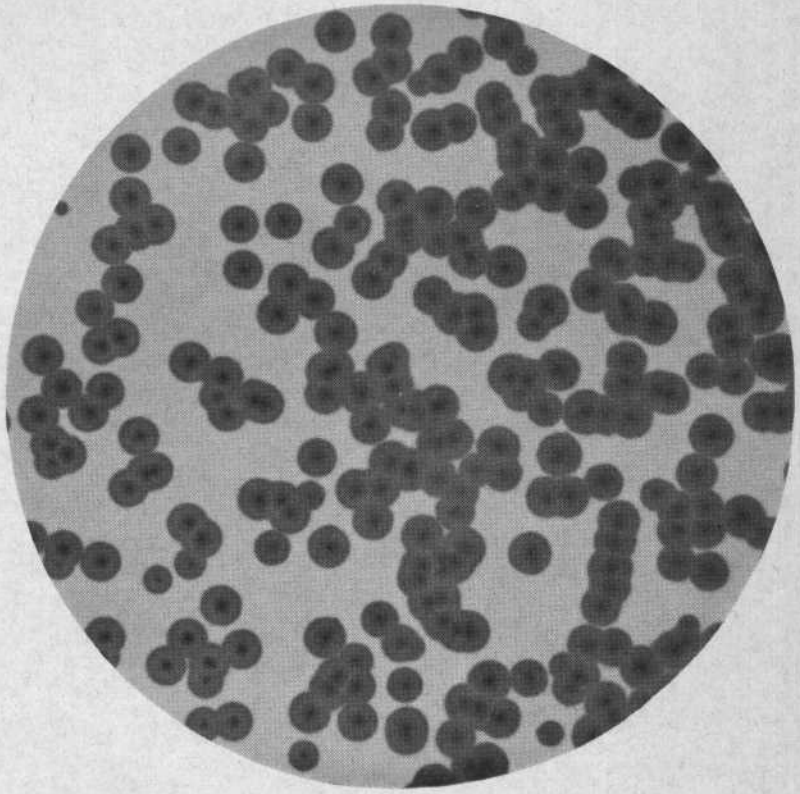

Figure 2. Plaques of the T4*r* mutant on *E. coli*, strain B.

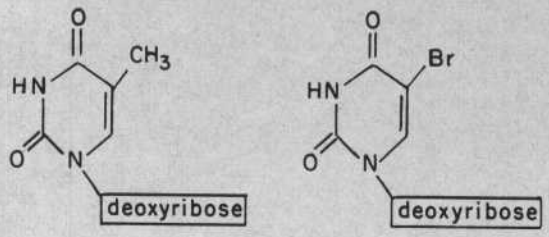

Figure 3. Structure of thymidine and its structural analogue, 5-deoxybromouridine (BD).

is infected with not one, but two or more virus particles of different genetic types. Then it may be asked whether the molecules of the "early" protein synthesized under the influence of one of the infecting DNA's are competent to aid in the replication of the other, genetically different DNA type. If they *are* competent to do so, then the essential protein would *not* be individually specific. On the other hand, if it is not competent to do so and if every viral DNA in the infected cell has to synthesize its own protein before it can begin its replication, then the essential protein would be individually specific. Now under my indirect replication hypothesis, it would be expected that every DNA moiety in the cell has to synthesize its own ribonucleoprotein template before the genotype corresponding to that DNA molecule could begin replication. On the other hand, under the direct replication envisaged by Watson and Crick, the essential protein would not be expected to be individually specific, and all types of DNA could commence their replication as soon as one of the DNA's has induced the synthesis of the metabolic enzymes necessary for the formation and copolymerization of the building blocks of the viral DNA.

It would thus be very important to test whether the essential protein could serve only for the replication of the genotype that directed its synthesis. This has been done in the following way. Bacteria were infected with the *r*II(H23) "deletion" mutant of T4 and the infection allowed to progress normally for 9 minutes. At this point, chloramphenicol (CMP) was added to the culture in order to arrest protein synthesis. By this time, the essential "early" protein has already been made within the cells, and if the infected bacteria are now incubated further in the presence of the antibiotic, phage DNA synthesis proceeds at nearly the normal rate. However, shortly after the addition of CMP, the already infected bacteria were now infected *secondarily* with the *double mutant* *r*II(5)*r*I of T4, carrying both the *r*II(5) point mutation and a mutation at the *r*I locus. Under the conditions of this experiment, the secondary virus is excluded from the majority of the primarily infected cells (20), but a minor fraction of the primarily infected bacteria can, nevertheless, be successfully superinfected with the secondary phage type (21). This superinfected fraction can be readily measured by assaying for the number of bacteria which liberate both the *r*I$^+$ allele of the primary phage and the *r*I allele of the secondary phage among their virus issue. After the secondary infection the culture was divided into two aliquots *A* and *B*, and the mutagen BD added to part *A*. Both aliquots were then incubated further for 50 minutes to allow phage DNA synthesis in the presence of the antibiotic. At the end of this 50-minute period, BD and chloramphenicol were removed from aliquot *A*, and chloramphenicol was removed from part *B*, and incubation of both aliquots continued until mature phage progeny were released from the infected cells. The frequency of *r*$^+$ wild type revertants among the phage progeny of both aliquots of the superinfected culture was then assayed. The program of these manipulations is summarized in Table 1.

The idea of this experiment is that if any significantly greater number of *r*II$^+$ reversions are found in aliquot *A* than in aliquot *B*, then these additional reverse mutants can have derived only from the multiplication and BD-induced mutation of the *secondary* T4*r*II(5) "point" mutant phage, since, as we have stated before, the primary *r*II(H23) phage, being an *r*II "deletion," cannot sport any reverse mutations at the *r*II locus at all. But since the mutagen was present in aliquot *A* only while protein synthesis was inhibited, so that the secondary phage had no opportunity to synthesize any of its own protein prior to the removal of the mutagen, such *r*II$^+$ reverse mutants would testify to the fact that the secondary phage genome can, in fact, utilize for its own replication the "early" protein synthesized in that cell by the primary phage.

The result of this experiment is also presented in Table 1. It can be seen there, first of all, that 5% of the primarily infected bacteria could be successfully infected with the secondary phage genome to yield both *r*I$^+$ and *r*I alleles of the *r*I locus. Secondly, it is evident that the proportion of *r*II$^+$ revertants among the phage progeny was 38 times greater in aliquot *A* than in aliquot *B*, i.e., the presence of BD in aliquot *A*

Table 1. BROMOURIDINE-INDUCED MUTATION OF A SECONDARY PHAGE GENOME

Program	
Minutes after infection	*Operation*
0	Infect culture with an average of 3 T4 *r*II(H23) phages per bacterium.
9	Add 300 μg/ml chloramphenicol (CMP). Infect with an average of 5 T4 *r*II(5)*r*I phages per bacterium.
10	Divide culture into aliquots *A* and *B*.
12	Add 200 μg/ml 5-deoxybromouridine (BD) to aliquot *A*.
60	Centrifuge aliquots *A* and *B* to remove BD and CMP. Resuspend bacterial pellets in fresh medium and continue incubation.
100	Induce lysis of aliquots *A* and *B* with chloroform. Assay progeny on plates seeded with strain K12(λ) and on plates seeded with strain B.

Results			
	Fraction of bacteria yielding both *r*I$^+$ and *r*I	Average burst size at time of chloroform lysis	Fraction of *r*II$^+$ revertants relative to total *r*I phage in progeny
Aliquot *A* (BD present)	0.05	10	38×10^{-7}
Aliquot *B* (BD absent)	0.05	10	1×10^{-7}

exerted a considerable mutagenic effect. Thus it may be inferred that the DNA of the secondary phage is able to replicate without synthesizing any "early" protein of its own. Some controls are actually required before this conclusion can be accepted. For instance, it is necessary to show that in this experiment the BD really induced mutation in the secondary genome while protein synthesis was arrested. It could be supposed, for example, that the secondary genome does not, after all, replicate in the presence of the antibiotic and that the mutagen lingers on in the cell after removal of all BD from the medium and thus exerts a delayed mutagenic effect only after protein synthesis has been allowed to resume. We have carried out a number of such control experiments, which time does not permit me to mention here in detail. All of these controls point to the fact, however, that the BD-induced *r*II$^+$ reversions in aliquot *A* do derive from the replication of the secondary genome in the absence of protein synthesis. It thus appears that the "early" protein synthesized by the primary phage can also serve for the replication of the DNA of the secondary phage, and hence it would follow that transfer of the hereditary in formation to a protein-containing macromolecule, such as my hypothetical ribonucleoprotein template, cannot be an obligatory feature of DNA replication. This conclusion is in agreement with the observations of Thomas (22) on the "early" protein synthesized by phage λ. It is, however, in contradiction with our own earlier inferences based on the finding that the "early" protein of phage T2 does play an individually specific role in the reactions leading to the stabilization of the intracellular phage against P^{32} decay inactivation (23). We are at present attempting to understand why the two methods—BD mutagenesis and P^{32} decay—lead us to apparently contradictory conclusions.

We may now proceed to the second aspect of DNA replication I mentioned at the outset of my remarks, namely the nature of the distribution of the material of the parent phage DNA molecule over its replicas. We have attempted to study this problem by using genetic instead of isotopic tracers, basing our approach on the following consideration. Now if under the semiconservative Watson-Crick replication scheme a rare copy-error mutation occurs at the time of the DNA replication act, then this copy error would, of course, be present only in the newly synthesized polynucleotide strand. Hence a mutational event would result in a *hybrid* double-stranded DNA replica molecule, made up of a *de novo* polynucleotide strand which is mutant at some site and an old parental polynucleotide strand which is still non-mutant at the same site. Such a hybrid DNA molecule corresponds to a genetic *heterozygote*, a genome which harbors two

different alleles of the same gene. At the next replication cycle, however, the two strands of such a mutational heterozygote DNA molecule would again unwind and separate from one another, and each add on its proper complementary strand, giving rise to two *homozygous* DNA molecules, one carrying the mutant site and the other carrying the non-mutant site in both complementary polynucleotide strands. In subsequent replications, each of these homozygous *segregants* would in turn give rise only to homozygous mutant and non-mutant DNA replicas.

If the Watson-Crick replication-mutation concept is correct, it should be possible to find such mutational heterozygote DNA structures among the phage particles issuing from phage-infected bacteria. For if a newly arisen heterozygous mutant DNA molecule present in the intracellular pool of phage DNA happens to be encapsulated into phage protein units before it has had a chance to undergo segregation into two homozygous molecules in a further replication cycle, a mature, genetically heterozygous phage particle should be produced. This phage will manifest its heterozygosity by giving rise to both mutant and non-mutant offspring upon infection of a sensitive bacterial cell. Now in BD mutagenesis experiments, similar to those already mentioned, in which the mutation $r\text{II} \rightarrow r\text{II}^+$ is studied, a few % of the induced r^+ revertants are generally found in *mottled* plaques. These mottled plaques owe their characteristic morphology to the joint growth of both r and r^+ genotypes within the same focus of infection. Such mottled plaques could very well represent the putative mutational heterozygotes, but their low frequency also admits alternative explanations concerning the origin of these mottled plaques. We have, therefore, endeavored to raise the frequency of such mottled plaques in mutagen-treated phage stocks in the following manner.

A culture of bacteria was infected with the BD-revertible T4rII(5) mutant and phage growth allowed to progress for 10.5 minutes. At this point, which precedes the intracellular appearance of the first mature progeny viruses by about a minute or two, the BD mutagen was added to the infected culture to initiate mutagenesis. After 3 to 4 minutes' further incubation in the presence of the mutagen, the first intracellular progeny phage were released from the infected bacteria by shaking the culture with chloroform and the genetic character of these progeny examined. The trick of adding the mutagen only a very short time before harvesting mature progeny phage should render it more likely that all those phage DNA units which happened to be mutated and encapsulated into intact virus particles within this short period had undergone only a *single* replication cycle during that same time. That is to say, any mutational heterozygotes formed would not have had time to segregate into homozygous DNA units.

The result of this experiment (24) was that more than 80% of the r^+ reverse mutants induced by such brief exposures to the BD mutagen are indeed of the r/r^+ heterozygote type, in that they form mottled plaques which contain both the r^+ revertants and the original r mutant type. Now, two or three control experiments are still required before even this very high proportion of mottled plaque formers among the induced mutants can really be accepted as definitive evidence for the occurrence of mutational heterozygotes. A discussion of these controls, which actually constituted by far the greater part of the work connected with this experiment, would require more time than is available here; these controls are described in some detail in the original paper in which the occurrence of mutational heterozygotes was reported (24). Suffice it to say that the results of these control experiments gave what appeared to us to be convincing proof that the elementary mutational event responsible for reverting the mutant rII(5) site to its wild r^+ state produces a heterozygote phage genome carrying both mutant and mutant alleles.

Of course, we cannot be certain that this mutational heterozygosity does indeed reflect two complementary polynucleotide strands which are not exactly complementary at a single site in the rII region. But the fact that *in vitro* deamination by nitrous acid of purines and pyrimidines in the DNA produces mutants a considerable proportion of which are similarly heterozygous (25, 26) makes it very likely that the two alleles are indeed carried within a single DNA duplex. Hence, a further conclusion may be drawn: *both* polynucleotide strands of an infecting viral DNA ultimately find genetic expression among the progeny genotypes. This inference, though probable to start with, was not *a priori* certain before these experiments. Thus the occurrence of mutational heterozygotes lends support completely independent of isotopic tracer experiments to the semiconservative nature of the mechanism of viral DNA replication. In contrast, the indirect conservative replication scheme I envisaged, though

actually not completely incompatible with the occurrence of mutational heterozygotes, would, it is only fair to say, have hardly ever led one to the prediction that virus mutants do not arise as homozygous structures.

In conclusion, therefore, I would like to state that since the replication of the viral genome seems neither to require the synthesis of a genetically specific ribonucleoprotein intermediate nor to follow a conservative route in the distribution of the parental genetic substance, but instead appears to be direct and semi-conservative, it is highly probable that the phage DNA is replicated either by the mechanism of Watson and Crick or by some other mechanism which is exactly like it.

References

1. Watson, J. D. and F. H. C. Crick, 1953. Cold Spring Harbor Symposia Quant. Biol., *18*:123.
2. Stent, G. S., 1958. Advances in Virus Research, *5*:95.
3. Stent, G. S., 1959. Proc. IVth Inter. Cong. Biochem., *7*:200.
4. Delbrück, M. and G. S. Stent, 1957. "On the Mechanism of DNA Replication." In *The Chemical Basis of Heredity*, W. D. McElroy and B. Glass, eds., p. 699, The Johns Hopkins Press, Baltimore.
5. Meselson, M. and F. W. Stahl, 1958. Proc. Natl. Acad. Sci. U. S., *44*:671.
6. Burton, K., 1955. Biochem J., *61*:473.
7. Tomizawa, J. and S. Sunakawa, 1956. J. Gen. Physiol., *39*:553.
8. Hershey, A. D. and N. E. Melechen, 1957. Virology, *3*:207.
9. Wyatt, G. R. and S. S. Cohen, 1953. Biochem J., *55*:774.
10. Hershey, A. D., 1953. J. Gen. Physiol., *37*:1.
11. Vidaver, G. A. and L. M. Kozloff, 1957. J. Biol. Chem., *225*:335.
12. Wisseman, C. L., Jr., J. E. Smadel, F. E. Hahn, and R. E. Hopps, 1954. J. Bacteriol., *67*:662.
13. Volkin, E. and V. Astrachan, 1956. Virology, *2*:149, 594.
14. Flaks, J. G. and S. S. Cohen, 1957. Biochim. Biophys. Acta, *25*:667.
15. Kornberg, A., S. B. Zimmerman, S. R. Kornberg, and J. Josse, 1959. Proc. Natl. Acad. Sci. U. S., *45*:772.
16. Keck, K., H. R. Mahler, and D. Fraser, 1960. Arch. Biochem. Biophys., *86*:85.
17. Litman, R. M. and A. B. Pardee, 1956. Nature, *178*:529.
18. Freese, E., 1959. In "Structure and Function of Genetic Elements," Brookhaven Symposia in Biology, No. 12, p. 63.
19. Benzer, S., 1955. Proc. Natl. Acad. Sci. U. S., *41*:344. In *The Chemical Basis of Heredity*, W. D. McElroy and B. Glass, eds., p. 70, The Johns Hopkins Press, Baltimore, 1957.
20. Dulbecco, R., 1952. J. Bacteriol., *63*:209.
21. Visconti, N. and A. Garen, 1953. Proc. Natl. Acad. Sci. U. S., *39*:620.
22. Thomas, R., 1959. Virology, *9*:275.
23. Uchida, H. and G. S. Stent, in press 1960. J. Molecular Biol.
24. Pratt, D. and G. S. Stent, 1959. Proc. Natl. Acad. Sci. U. S., *45*:1507.
25. Vielmetter, W. and C. M. Wieder, 1959. Z. Naturforsch, *14b*:312.
26. Tessman, I., 1959. Virology, *9*:375.

Genetic Fine Structure in *Neurospora*[1]

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Nearly 50 years ago Sturtevant and Morgan, working with *Drosophila,* reasoned that they could determine the relative positions of different genes on the chromosome by observing the frequencies of various combinations of the genes in the progeny of certain crosses. A fly which received the linked genes for yellow body color (*y*) and white eyes (*w*) from one parent and the genes for wild-type gray body color (*y*+) and wild-type red eyes (*w*+) from the other parent would produce gametes most of which carried parental combinations (*y w* or *y*+ *w*+). Only a smaller number of the gametes carried *y w*+ or *y*+ *w*.

They concluded that these recombinants, these new combinations, resulted from a chromosome exchange or crossover which occurred somewhere along the parent chromosomes between the locus of the genes for body color and the locus of the genes for eye color. Crossing over might occur at any point along a chromosome, and thus the frequency of these crossover types became a measure of the distance along the chromosome between the two loci. By this method the genes which no one had ever seen could be mapped.

Five years ago Seymour Benzer (1) pointed out that the same methods might now be used to study genetic structure at a finer level. Earlier studies had been confined to measurements or recombination frequencies of the order of one percent or more. Such studies had given useful pictures of the gross structural relationships of genes along a chromosome.

Benzer observed that selective methods with microorganisms would permit accurate measurement of recombination frequencies of one in ten thousand or even less. He reasoned that if these rare recombinations are measures of distances between genes, they should allow us to map the genetic structure down to the molecular level. Benzer carried out such an experiment himself using the rII mutants of the bacteriophage T4. (This is the same system that Dr. Stent has described for us.)

Benzer infected the same bacterium with two phage particles carrying different rII mutant genes and counted the frequency of recombinants. He was able to select the *r*+ recombinants on bacterial strain K because neither of the parent mutants could grow on it. By this method he could count these recombinants even though they occurred very rarely, some of them as rarely as once or twice in ten thousand. By the frequencies of these recombinations he attempted to make a map of the mutant genes.

Figure 1 shows a portion of one of the Benzer maps of the rII region. The vertical lines represent the positions of the different mutant sites. The map is constructed by assuming that the frequencies represent distances along a one-dimensional structure; the mutants are placed in the order most compatible with additivity.

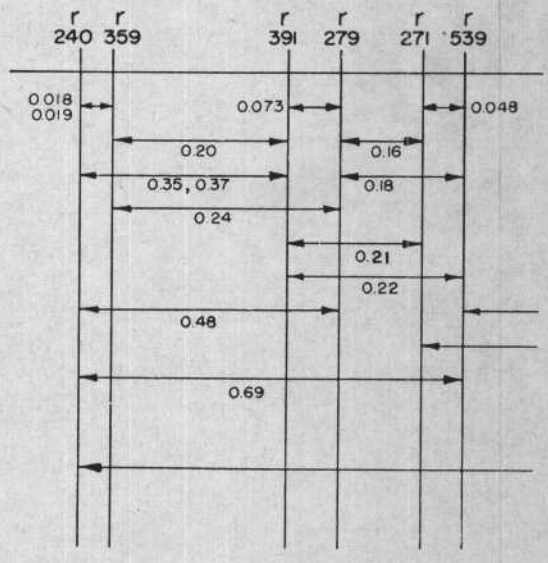


Figure 1. Recombination map of rII mutants of bacteriophage T4. The map distances are expressed in percentage frequencies of recombinants. (From Benzer.)

[1] This work was supported by a grant from the National Science Foundation and by State of Washington Initiative 171 Funds for Research in Biology and Medicine.

How well do the results fit the linear model? Are successive segments additive? If we are mapping three points along a straight line A B C, the distance AB plus the distance BC is equal to the distance AC. In the observed data AB + BC is somewhat greater or less than AC. Thus 0.018 plus 0.20 is less than 0.35; 0.20 plus 0.073 is greater than 0.24. It is difficult to say how precise the additivity should be to confirm the model. There are credible ways of explaining away either kind of discrepancy. It is possible that there are sometimes recombinations in both regions (AB and BC). This results in no detected recombination between A and C, whereas if those recombinations had taken place in the cross of A × B, or B × C, they would have been scored. Thus double crossovers would tend to give this sort of result: AB + BC greater than AC.

Another possibility is that the genes are not really along the linear axis, but on side chains. It is perhaps not so likely with bacteriophage, but you can see that on the model in figure 2 the distance AB plus the distance BC would be greater than the distance AC.

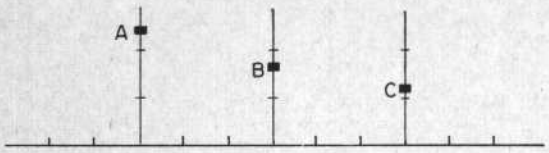


Figure 2. A hypothetical genetic structure which could account for nonadditivity of recombination frequencies.

Benzer anticipated the reverse kind of failure of additivity and had an explanation ready, even though he did not run into so much of it in his data. He pointed out that if AB + BC is less than AC, this might give us a measure of the length of the mutant region in B. Figure 3 shows how this works. It is assumed that a wild-type recombinant will be recovered only if the exchange takes place at such a point as to completely exclude the mutant regions. The *effective* recombination distances AB and BC would add up to less than AC by an amount equal to the mutant region in B. This type of disparity was not common in Benzer's results, and since he got the reverse kind frequently, he was unable to measure the length of mutant regions by this method.

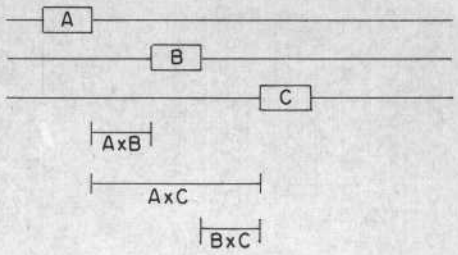


Figure 3. This diagram illustrates how the length of the altered region in mutant B might be calculated from a comparison of the frequencies of wild-type recombinants in the crosses AxB, BxC, and AxC. (From Benzer.)

Benzer attempted to translate the distances on his map into molecular distances by making some rather broad assumptions about the form in which the DNA is present in the phage particles and about the total number of recombination events which take place during the phage duplication in the bacterium. He assumed that the phage DNA is in the form of a double string of nucleotides and that an exchange can take place at any level along the string and is equally likely at any point. This enabled him to calculate the exchange frequency per nucleotide pair. According to these calculations, the shortest intervals on the Benzer map (0.02% recombination) represented mutant sites only one or two nucleotide pairs apart.

I have attempted to make a map of this sort for a series of mutants of *Neurospora* which all require the amino acid cysteine for growth. (Four of the cysteine mutant strains used in this study were kindly provided by Dr. Brooke B. Webber of the Oak Ridge National Laboratory.) These are all single gene mutants in the sense that when one of them is crossed to wild type, half the progeny are mutant and half are wild type, the pattern expected for a single pair of segregating genes. Crosses between two of the mutants yield wild-type progeny at a low rate of the order of one per 1,000. It is this recombination that I would like to talk about today. What kind of event is it and what kind of structure of the genetic material can we imagine to explain this recombination event?

Figure 4 shows a map of six of these cysteine mutants based on the same sort of approach that Benzer used. It is assumed that each cysteine mutant results from an alteration at some point along a linear genetic structure and that the dis-

tance separating two mutant loci is proportional to the frequency of cysteine-independent progeny in the cross between the two. The units in figure 4 are cysteine-independent spores per hundred thousand. The frequencies shown in the upper part of the figure are all fairly compatible with each other. The ones below do not seem to fit in.

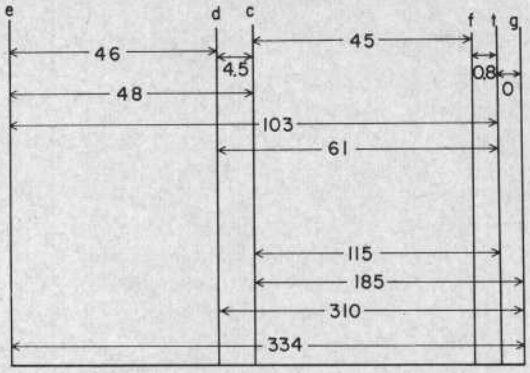


Figure 4. Recombination map of cysteine-requiring mutants of *Neurospora*.

The question arises whether this map has any meaning at all in regard to the structure of that region of the chromosome in which the cysteine mutations took place. Can we carry over the method developed by Sturtevant and Morgan to the study of structure at this much finer level? I think that Benzer is much more justified than we are in assuming a one-dimensional structure. The genetic structure in higher organisms is chemically more complex than that of phage, which is all or nearly all DNA. Benzer has some confirmation of his one-dimensional model in studies of overlapping deletions (2). He finds that some of his mutants behave as though a considerable region is altered at the rII locus, and he can make a meaningful arrangement of these along a one-dimensional scale. This tends to substantiate the model—the idea that the genetic material is a string of DNA.

In dealing with a chromosome of *Neurospora*, we apparently have a more complicated structure. It is made of both DNA and protein, apparently more than one kind of protein. On the other hand, we have some comfort in the fact that we can get more information about these recombination events in *Neurospora*. Marker genes are used in the study of the cysteine mutants. The locus of a mutant gene *lys* for lysine requirement is to the left of the cysteine region as is the locus of *un*, a temperature mutant with unknown nutritional requirements. On the other side of the cysteine region is the locus of a mutant gene *ylo* causing yellow conidiospores (instead of the wild-type orange). In a cross between two cysteine mutants, one of the parents carries *lys* and the other carries its wild-type allele; the same is true for *ylo*. By examining the cysteine-independent recombinants to see which markers they carry, we get more information about the recombination event.

A second valuable tool we have for analyzing this recombination event is tetrad analysis. In *Neurospora* we can isolate all four products of the same meiosis, and if we have enough patience we can isolate the four products of that rare meiosis in which the recombination event occurred and see what other products came out of the same event. This could give us added information about the nature of the event and about the nature of the structure involved in this event.

Our problem is to see something that is beyond the limits of the microscope, even the finest electron microscope. We want to work out the structure of the genes, and we are trying to do it by approaches like the one I have described: analyzing frequencies of these recombination events. This is a very crude approach, and perhaps I should give you a crude analogy to try to impress you with how blindly we are groping at this structure.

Let us suppose for a minute that we are very large organisms looking at the earth, which to us is about the size a basketball is to a human being. We can just see the small towns on the surface of the earth with a low power microscope, and we are interested in these towns and how they work. Using our highest power microscope we can even see little specks moving around inside the towns. By carefully tabulating the movement of specks we might find that there were certain times that there were different movements than other times. We might pick out a particular town in Kansas and note that on Friday there was unusual activity, people going out to the grocery store, the clothing store, the movie theater, and sometimes the ball park. We observe this extra activity every Friday, but not always the same. One Friday there is much movement at Jones' department store, and the next Friday there is more over at Brown's Drug Store, and then some other Friday the ball park is very busy. We try to figure out what determines these movements. We might have a tool with which we could bombard particular points in the town with fine

particles (which would be like boulders to the town), and we would try hitting different places in the town and see if that interrupted this special movement. Actually the reason for the special movement is that Friday is the day the weekly newspaper comes out, and the people go by the ads of the stores that have the best sales; they go to the ball park when there is a game or to the theater when there is a good movie. We would find this out by our research. That is, we would find that when we had bombarded the printing house, this would disrupt the special activity. So we would conclude that this is the center where the information is—the nucleus of the town. Then we would want to know more about the structure of the things inside this printing house and how the information is transferred out. Some rather dubious investigators would say they could see little specks on Friday mornings coming out of this printing house and maybe this is the information coming out of the nucleus, but nobody would believe them. Perhaps we would have even finer particles or some kind of electric shocks which could hit points inside the printing house, and if we hit just one press that was printing one page, we would find certain correlations. Maybe by knocking out the information on the grocery store, we would at the same time knock out the information on the drug store, so that the same event that prevented people from showing unusual activities toward one would prevent it toward another. We would see correlations between certain categories of information. Maybe we would detect negative correlations also. When there was a movie with Brigitte Bardot, the people seeing that ad might not see the sporting goods ad on the same page. And we might describe this as interference between these two types of information. We could measure the amount of this interference or correlation. And so we could make accurate measurements of these correlations and failures of correlation, but the thing I want to impress you with is the extreme difficulty of translating these measurements, accurate and consistent though they be, into any kind of picture of how this printing press works or how it is built. This would be an immense job, and we might come out with a very distorted picture. This is the sort of job we face, I think, in getting a picture of the genetic material out of the kind of information we can gather. Still we try it, and we are hopeful that a gene is not as complicated as a printing press. Perhaps this is just a blind hope —I do not know.

The hypothesis of Sturtevant and Morgan is now established fact. It has been demonstrated cytologically by ingenious experiments with *Drosophila* (3) and with corn (4) that a genetic crossover does truly represent an exchange of material between two chromosomes. Furthermore, the crossover mapping method is supported by studies of flies in which a short region of a chromosome has been deleted (5). These flies exhibit a mutant phenotype characteristic of the missing genes. One can look at the salivary gland chromosomes of these flies and see what region in the visible chromosomes is missing. This must be the site of the gene associated with the phenotype we observed in these flies. By this method one can determine the locations on the visible chromosomes of the genes which were previously known genetically. When this is done, we find that these locations correspond to those previously determined from crossover mapping. This substantiates the mapping method and tells us that it is in fact a measure of the spatial arrangement of the genes along the chromosomes.

Regarding the rare recombinations in *Neurospora*, we would be more confident that they are a measure of gene structure if we could satisfy ourselves that these events are in fact crossovers. Here are three characteristics of crossing over which we can check in these rare recombination events:

1) Crossing over is reciprocal. In a cross between two linked mutants m_1 and m_2 (see figure 5), the meiosis which yields the crossover product $m+$ (m_1+ m_2+) will also yield the reciprocal product m_1m_2.
2) Crossing over gives a consistent array of linked markers. In the cross illustrated in figure 5, if we selected the $m+$ crossover products, we should expect most of them to carry the linked markers $a+$ and b.
3) Crossing over interferes with crossing over in a neighboring region of the same chromosome pair. In figure 5, among the progeny with crossing over in the a-b interval, the frequency of crossing over in the b-c interval will be lower than in the general population.

Rare recombination events in *Neurospora* were first tested for the characteristics of crossing over (the first two of the three listed above) by Mary Mitchell (6). I will illustrate her findings with my own experiments (7), as the results are essentially the same.

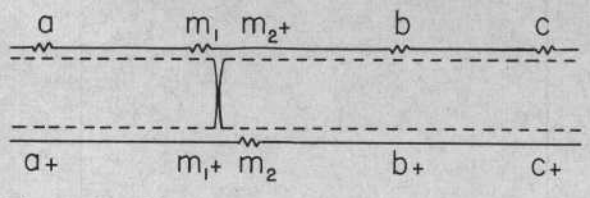


Figure 5. Diagram of crossing over between linked, segregating genes in the cross *a* m_1 *b c* x m_2.

One hundred and fifty-three asci of the cross *un cys-c ylo*+ x *un*+ *cys-t ylo* were dissected and germinated on medium deficient in cysteine. Three of these asci each contained one cysteine-independent spore pair. These asci were grown and fully classified. (The two cysteine mutants can be distinguished in nutritional tests by their response to thiosulfate, a precursor of cysteine.) The results are shown in figure 6. The crucial

ASCI CONTAINING CYS+ PROGENY

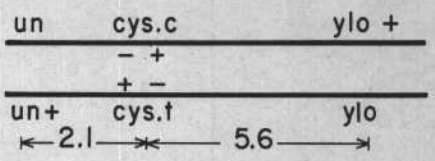
un	cys*	ylo	Mating type	Backcross test (prod. of cys+ progeny in cross to) cys-c	cys-t
+	+	+	a		
un	cys-c	+	a	–	+
un	cys-t	ylo	A	+	–
+	cys-t	ylo	A	+	–
un	cys-c	+	A	–	+
un	cys-c	+	A	–	+
+	cys-t	ylo	a	+	–
+	+	ylo	a		
+	+	ylo	A		
+	cys-t	ylo	A	+	–
un	cys-c	+	a	–	+
un	cys-c	+	a	–	+

* The cys classifications in this column are based on nutritional tests at 25° C

Figure 6. Classification of three asci containing *cys*+ recombinants. The four rows between successive horizontal lines represent the four spore pairs of a single ascus.

question was whether an ascus containing a *cys*+ product also contained a reciprocal product. This should be a cysteine double mutant (*cys-c cys-t*) if these mutants represent two loci separable by crossing over. No such double mutant was found in any of the three asci. (These cultures were tested for double mutants by backcrossing each to each of the two parent cysteine mutants. A cross of a cysteine mutant to another strain carrying the *identical* mutation yields no *cys*+ progeny, while crosses between the two mutants yield about one *cys*+ per 1,000 spores. The double mutant, if it occurred, should produce no *cys*+ progeny in crosses to either of the parent mutants.)

The same cross was examined to see whether the event resulting in a *cys*+ recombinant gave the consistent array of markers which might be expected from crossing over. The upper part of figure 7 represents the two homologous chromosomes with which we are concerned in this cross. The mutant region (-) of *cyc-c* has been arbitrarily placed to the left of *cys-t*. If the cysteine mutants were at two separate loci in the order shown, it might be expected that the *cys*+ crossover products would usually carry the markers *un*+ and *ylo*+. If the order of the cysteine mutants were reversed, most of the *cys*+ progeny should carry the markers *un* and *ylo*. The tabulation at the bottom of figure 7 shows that neither of these presumptive crossover types occurs in excess of the other marker combinations. The result does not suggest a crossover basis for the production of *cys*+ progeny.



cys + progeny among 72,545 germinated spores:

68	un	cys +	ylo +
26	un +	cys +	ylo
18	un	cys +	ylo
25	un +	cys +	ylo +
137	Total		

Figure 7. Classification of 137 *cys*+ recombinants for linked markers *un* and *ylo*.

The recombination event which produces the *cys*+ spores does not yield a reciprocal product and is not associated with a consistent marker array. It does not appear to be a crossover in the classical sense. However, it is important to note that this event, whatever it is, is correlated with crossing over. Among the 137 *cys*+ spores classified in figure 7, 43 (over 30%) have crossover

combinations of genes at the *un* and *ylo* loci. Among random spores from this cross, less than 8% have crossing over between *un* and *ylo*.

There is another type of aberrant event which has been studied more in yeast than in *Neurospora*, involving the cross of a mutant gene to its wild-type allele. If we examine dissected asci of such a cross, we expect 2:2 segregations—two of the spores (spore pairs in *Neurospora*) carry the wild-type gene and two carry the mutant. But an occasional ascus has one wild-type and three mutants or three wild-types and one mutant. These aberrant asci are like the *Neurospora* recombinations I have been discussing in that they are not reciprocal (anything reciprocal should add up to 2:2). We like to think that both of these processes derive from the same unknown mechanism, and we call them by the same name, gene conversion. This term was originally used by Lindegren (8) to label his hypothesis of a mechanism for aberrant segregation in yeast. As used here, gene conversion implies no mechanism. It refers to those recombination events which do not have the characteristics of classical crossing over.

In studying the mechanism of gene conversion, we have two inseparable problems: what kind of event is it, and what is the structure of the genetic material in which it takes place?

One possibility which must be considered is that gene conversion is a mutational type of event rather than a recombination. It cannot be mutation in the simplest sense, because it does not occur in selfed crosses. That is, the crosses *cys-c* x *cys-c* and *cys-t* x *cys-t* yield no *cys*+ progeny. Lindegren has suggested that gene conversion is mutational even so and that one gene is stimulated to mutate by the presence of a different allele in the homologous chromosome.

There have been several studies in yeast and *Neurospora* in which a large number of detectably different alleles were shown to occur at a given locus. Such a case can help to answer the question of whether gene conversion is a mutational event or a recombinational one. When a gene can exist in many different forms, we expect that mutation might result in any of these. Recombination, on the other hand, is limited to a reshuffling of the parts present in the two parents. There have been two studies relating to this point, and both suggest that gene conversion is the result of recombination.

Giles (9) and his associates have studied a pair of allelic *Neurospora* mutants which require adenine for growth and fail to produce the enzyme adenylo-succinase (see figure 8). By plating vegetative cells of the mutants on medium lacking adenine, they selected revertants—products of reverse mutation. They were assayed for adenylo-succinase activity, and only one out of 72 had an activity approaching that of wild type, indicating the diverse range of enzyme activities determined by this locus. Under the same selective conditions they isolated gene convertants from a cross between the two mutants. These were uniformly wild type in their enzyme level. Strains with intermediate levels of enzyme activity were not produced by gene conversion, even though they dominated the yield of the mutation experiment.

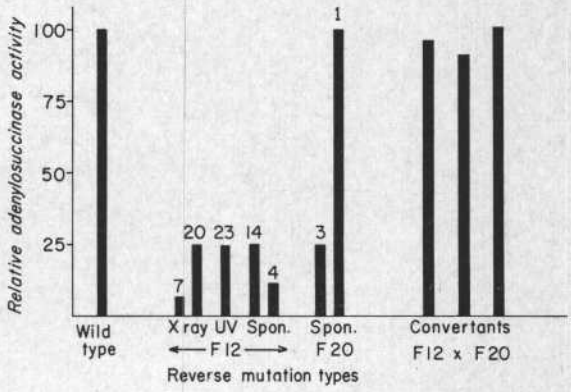


Figure 8. Enzymatic activity of revertants of adenine-requiring mutants in *Neurospora*. Comparison of vegetative back mutants and meiotic convertants. A number above a bar indicates how many revertants reached this level of enzyme activity. (From Giles.)

Another piece of evidence that indicates gene conversion is not a mutation event comes from the work of Roman (10) on adenine mutants in yeast. He found that a diploid containing two allelic but different adenine mutants would yield vegetative reversions. This enabled him to study a series of allelic mutants and find out how many differed from each other. This study revealed that mutation at a given adenine locus can result in any of about 100 detectably different mutant alleles. He applied this technique to the products of gene conversion in meiosis. Aberrant asci with one wild-type spore and three mutants were selected from a cross of a mutant to wild type. The three mutant spores were checked for identity by the reversion test. In each of eight such asci tested, the third mutant spore proved to have the

same adenine allele as the other two (and the same as the mutant parent). Thus gene conversion in this situation appears always to yield the one gene form in a hundred which happened to be present in the parent.

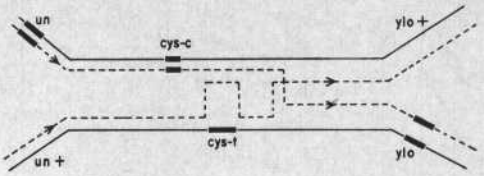
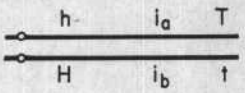


Figure 9. Diagram of paired homologous chromosomes in meiosis illustrating recombination by the Switch Hypothesis of Freese.

Freese (11) has attempted to explain the mechanism of gene conversion with his Switch Hypothesis (see figure 9). He suggests that both gene conversion and crossing over result from the same event—conversion taking place at the site of the event which may result in crossing over for markers embracing this site. This is a nice economy of mechanisms to account for both kinds of recombination. He says that when one of the two new chromosome strands is formed in meiosis, it copies along one of the two templates (the old strands) until it comes to a region of switching. This may be a point at which the two homologous chromosomes lie especially close together. The new strand switches to the other template to copy a short length and then may switch back to the first template. It may switch several times in a very short distance. The synthesis of the second new strand (which may be separate in time from the first) does not have to reciprocate each of the switches; it is only required to enter the region of switching and to leave it on the template not occupied by the first new strand. This hypothesis seeks to explain the two differences between gene conversion and crossing over which were demonstrated by Mitchell. The two new strands are not reciprocal for the conversion locus (the region of switching), even though they are reciprocal for the surrounding markers. The occurrence of two or more switches in a switching region accounts for the randomizing of markers accompanying conversion.

The switch hypothesis is an attractive one and with slight modifications may be correct. Case and Giles (12) have done the most extensive study to date of gene conversion in complete asci of *Neurospora,* and their results show impressive parallels to the switch hypothesis. However, a number of other experiments tend to cast doubt on this hypothesis. None is a crucial test, but in each case the outcome is different from what might have been predicted on the basis of the switch hypothesis.

Roman (10) studying vegetative gene conversion of adenine mutants of yeast, found no evidence of crossing over among the convertants. In another experiment (13) he studied conversion between a pair of isoleucine mutants (see figure 10), and here he did find evidence of recombination (presumably crossing over) involving the linked markers in 41% of the convertants. The rate of conversion was greatly stimulated by ultraviolet treatment, but the UV-induced conversion was not accompanied by crossing over. In each of these experiments the switch hypothesis would lead to the prediction of a correlation between conversion and crossing over which was not observed.



UV	Number tested	Revertants with requirement for			
		h	t	ht	%
0	144	8	24	27	41.0
45"	93	1	6	2	9.7
90"	134	2	4	2	6.0

Figure 10. Recombination of linked markers among vegetative convertants to isoleucine-independence in yeast. (From Roman and Jacob.)

The frequency of crossing over between two loci in *Neurospora* may vary in different crosses, depending on unknown genetic factors. Figure 11 shows the results of five crosses between two cysteine mutants (7). The marker loci *a* and *b* were the same in all five crosses, but the genetic background was varied in such a way as to give a threefold difference in crossing over. It can be seen that the frequency of *cys+* convertants did not show any parallel fluctuations.

A cys.c B

a cys.t b

cross	Map distance a–cys	Map distance cys–b	cys + per 10^5 germinated spores A + B	a + b	A + b	a + B	Total
1	2.8	3.8	164	16	4	100	284
2	2.1	5.6	94	36	25	34	189
3	4.9	6.2	136	14	29	58	237
4	8.1	13.0	68	41	8	62	179
5	5.1	17.5	90	25	3	106	224

Figure 11. Recombination of linked markers among *cys*+ convertants in crosses with different frequencies of recombination between the outside markers.

The third characteristic of crossing over, listed earlier, is interference with crossing over in neighboring regions. An experiment was performed in *Neurospora* to see whether gene conversion showed any interference (14). If, as postulated by the switch hypothesis, conversion and crossing over are two observable results of the same recombination event, we might expect this event to cause interference regularly. This should be true whether we detected the recombination event by crossing over or by gene conversion.

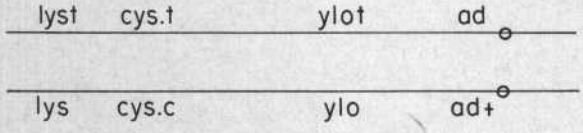


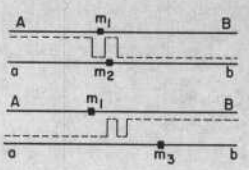
Figure 12. Diagram of the homologous chromosomes containing the segregating genes in the cross in which *cys*+ conversion was tested for interference with crossing over in the *ylo-ad* region.

The relationship of gene conversion to crossing over interference was studied in *Neurospora* in the cross represented in figure 12. Gene conversion at the *cys* locus was compared with crossing over in this region (between *lys* and *ylo*) for effects on interference of crossing over between *ylo* and *ad*. Figure 13 shows the results of this experiment (the results at 25° are more simply interpreted than those at 18°). Crossing over in the neighboring region causes *ylo-ad* crossing over to drop from 13.8% to 0.7%. This is interference. But with gene conversion in the neighboring region, the rate is 13.3%. This indicates no interference. Thus gene conversion, which had previously been shown to fail the first two criteria for crossing over, is seen to lack the third property as well. To salvage the switch hypothesis, we could postulate that an event involving an even number of switches does not cause interference, while an odd number event does.

	ylo-ad recombination frequency 25°	18°
***cys* + convertants**		
parental combination of *lys* and *ylo*	13.3% (48/361)	17.1% (41/240)
non-parental combination of *lys* and *ylo*	3.9% (11/282)	8.5% (16/188)
random spores		
parental combination of *lys* and *ylo*	13.8% (79/572)	11.7% (43/367)
non-parental combination of *lys* and *ylo*	0.7% (1/152)	9.4% (9/96)

Figure 13. Effect of *cys*+ conversion on recombination in the neighboring *ylo-ad* region.

One further type of check of the switch hypothesis is illustrated in figure 14. If we are dealing with recombination between pairs of mutant loci which may be different distances apart, then in a cross between two which are very close together most of the switching will take place *around* them and not *between* them. This will result in a randomizing of marker genes in the convertants. This situation is illustrated in the upper diagram of figure 14. If, on the other hand, the distance separating the two mutant loci is large compared to the length of a region of switching, most of the switching will take place between the two mutants. This is shown in the lower diagram of figure 14, and it can be seen that such a cross should yield convertants, most of which carry the markers *a B*. The switch hypothesis would predict that increasing distance between the mutants (as revealed by increasing conversion frequency) should be accompanied by a rise in the proportion of the convertants carrying the markers *a B* to a level approaching 100%. The data in figure 14 show no such rise. (It may be that even the most



Cross	Conversion frequency x10^5	Percent of a + B convertants
d x c	4.5	6
f x c	45	27
e x d	46	14
e x c	48	20
d x f	61	27
e x f	103	16
g x c	190	25
d x g	310	17
g x e	334	23

Figure 14. Diagram illustrating the prediction of the Switch Hypothesis that the distance between mutant sites in the conversion locus will affect the relative frequencies of outside marker combinations.

distantly spaced of these cysteine mutants are close together compared to the length of a switch region, in which case the data are not in conflict with the switch hypothesis. An analysis of this type by deSerres (15), on recombination between *Neurospora* adenine mutants, did tend to substantiate the switch hypothesis).

The switch hypothesis assumes a one-dimensional genetic structure. Let us consider briefly an alternative model. Figure 15 represents a possible two-dimensional structure of the genetic material. Let us assume that the chromosome consists of a protein backbone with the actual genetic material on side chains of DNA. A number of people have suggested such a model (7, 13, 15, 16, 17). We will propose that reciprocal crossing over takes place by exchange along the backbones of the homologous chromosomes, while gene conversion is a nonreciprocal, copy-choice event which occurs along the side chains. In the upper part of figure 15 the two mutants are pictured as alterations at different points along the same pair of side chains. The newly forming chromatid (dotted line) is an $m+$ convertant and carries the parental combination of markers A B. In order to account for other marker combinations we could postulate that conversion and crossing over frequently occur together because both are favored by the same localized conditions (like especially close pairing of the homologous chromosomes). The conversion might be accompanied by crossing over along the backbone to the left of the m side chain, yielding a convertant with the nonparental marker combination a B. If the coincident crossover took place to the right of m we would get the other nonparental combination A b. By this scheme, we could only get convertants with the fourth marker combination (a b) by a more complicated set of events. So we might predict that one of the two parental combinations of markers should be rare among convertants. There is no indication of a consistent pattern of this kind in my data.

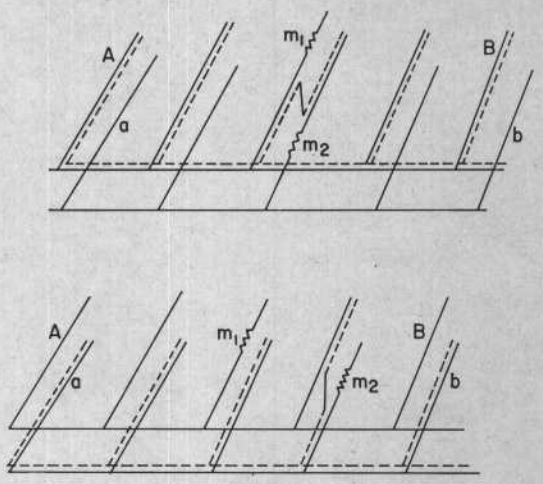


Figure 15. Two dimensional models of chromosome structure with mutant loci on side chains.

The lower part of figure 15 shows the same model with the mutants on separate pairs of side chains. In this case it is possible to get $m+$ recombinants either by conversion or crossing over. Three of the marker combinations accompanying the recombinant can be accounted for without having to postulate any fortuitous correlation of conversion and crossing over. Conversion along the m_1 side chains gives $m+$ with the markers A B. Conversion along the m_2 side chains (shown in the illustration) gives a b. Crossing over in the section of backbone between m_1 and m_2 can yield an $m+$ recombinant with a B. The other nonparental combination (A b) would require a more complicated event and should be less common among the $m+$ recombinants. The model would also predict that the frequency of conversions carrying a parental combination of markers would be determined by the length along the side chain from the backbone attachment to the mutant region. Thus, if the m_2 mutant region were very close to the attached end of the side chain, a b convertants should be rare. Also, this rate should be constant for m_2 in differ-

ent crosses to different *m* mutants on other side chains. The data on crosses between cysteine mutants do not appear to fit this model very well. However, it must be remembered that some of the cysteine mutants could be on the same side chain and some on others. Much more data involving more cysteine mutants will be required for an adequate test of this model.

Recombination and genetic fine structure in *Neurospora* is presently in a confused state which makes it a difficult subject to treat in a discussion of this type. New insight and perhaps new ways of thinking about structure at the molecular level will be required to fit the kind of observations reported here into a meaningful picture of the gene.

References

1. Benzer, S., 1957. "The Elementary Units of Heredity." In *The Chemical Basis of Heredity,* W. D. McElroy and B. Glass, eds., p. 70, The Johns Hopkins Press, Baltimore.
2. Benzer, S., 1959. Proc. Natl. Acad. Sci. U. S., *45:*1607.
3. Stern, C., 1931. Biol. Zentr., *51:*586.
4. Creighton, H. B. and B. McClintock, 1931. Proc. Natl. Acad. Sci. U. S., *17:*492.
5. Painter, T. S., 1934. J. Heredity, *25:*464.
6. Mitchell, M. B., 1955. Proc. Natl. Acad. Sci. U .S., *41:*215.
7. Stadler, D. R., 1959. Genetics, *44:*647.
8. Lindegren, C. C., 1953. J. Genet., *51:*625.
9. Giles, N. H., 1958. Proc. X Intern. Cong. Genetics, *1:*261.
10. Roman, H., 1956. Cold Spring Harbor Symposia Quant. Biol., *21:*175.
11. Freese, E., 1957. Genetics, *42:*671.
12. Case, M. E. and N. H. Giles, 1958. Proc. Natl. Acad. Sci. U. S., *44:*378.
13. Roman, H. and F. Jacob, 1958. Cold Spring Harbor Symposia Quant. Biol., *23:*155.
14. Stadler, D. R., 1959. Proc. Natl. Acad. Sci. U. S., *45:*1625.
15. de Serres, F. J., 1958. Cold Spring Harbor Symposia Quant. Biol., *23:*111.
16. Schwartz, D., 1955. J. Cellular Comp. Physiol., Suppl. 2, *45:*171.
17. Taylor, J. H., 1957. Am. Naturalist, *91:*209.

Recent Studies of Transformation in *Bacillus subtilis*

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The term "transformation" has been coined for the transfer of genetic information produced with isolated DNA. The biological functioning of DNA has been of great interest in a number of different areas and the current advances in the problem of the gene-enzyme relationship must involve a knowledge of the structure and function of the DNA itself.

Dr. Spizizen

Two main lines of work have demonstrated the role of DNA as genetic material. The classical studies of Avery and co-workers (1) on the transformation of capsular characters in *Pneumococcus* first demonstrated that DNA as such could in fact promote genetic changes. The more recent experiments of Hershey and Chase (2) in which DNA was shown to be involved in the replication of a bacterial virus T2, was another indication of the biological role of the DNA of the bacterial virus. Of course, it was inferred that DNA was the genetic material from earlier studies in genetics but these direct demonstrations were more convincing.

Our interest in transformation originated from a desire to study the mechanism by which virus replicates. And in attempts to determine more directly perhaps than the Hershey-Chase experiments that the DNA was in fact the replicating moiety of the viral particle, we isolated the DNA from T2 phage to see whether this would have infecting properties.

The experiments which we performed will be mentioned to illustrate some of the principles upon which we based our subsequent studies. We made spheroplasts or protoplasts of *E. coli* by lysozyme treatment in hypertonic sucrose. In this way one removes specific receptors for the T2 virus. If one mixes intact T2 virus with properly prepared spheroplasts one gets no infection. That is, the T2 virus cannot attach and replicate. The reaction between the tail receptor of the virus with the receptor of wall is no longer possible because the wall receptors were removed during the formation of spheroplasts. However, we took the T2 virus which has a property of osmotic sensitivity, shocked and further inactivated it, so that now it could not infect intact cells of *E. coli* B.

When we mixed the spheroplasts with these shocked T2 particles an increase in phage particles occurred. This experiment can be readily reproduced. The experiment suggested that we were dealing with isolated DNA and so we had perhaps a direct way of demonstrating the role of DNA in phage replication.

The infecting material was found to be deoxyribonuclease sensitive. This is a criterion of free DNA for biological studies which we will discuss in some detail later. But as often happens, enzymes of this kind when employed turn out not to be pure. Deoxyribonuclease, a magnesium activated enzyme, is often contaminated with proteolytic enzymes.

The inactivation of the T2 shockate was also found to occur in the presence of citrate which binds magnesium and prevents the deoxyribonuclease from acting. It was found that the components that inactivated the infecting material were in fact proteolytic contaminants of the enzyme. This in itself is a very interesting point. It would appear, therefore, that some degraded phage particle was thus prepared but this was not simply DNA.

We attempted to isolate infective DNA but all our attempts failed. That is, as soon as DNA was separated from protein all viral activity was removed. The spheroplast experiment did, however, demonstrate one way in which we might allow DNA to penetrate a microbial cell.

These experiments taught us a number of things. First of all, we became aware that perhaps the experiment was ill-conceived. In the first place, the viral particle is a very complex entity in the sense that it contains many genic components. For instance, we now know that upon infection with the T2 phage in *E. coli* B, before DNA can be replicated, a large number of new

enzymes are synthesized. These new enzymes are undoubtedly being formed as a result of the directed activity of some of the genetic components of the viral particle, presumably the DNA.

If each enzyme requires a separate gene many genes must be involved. Thus it is unlikely that all the separate genes would penetrate the same cell in order to effect viral replication. It would have been more logical to look for more specific effects of DNA as single gene actions.

Since the discovery by Avery and co-workers of pneumococcal transformation by pure DNA, very few other organisms in nature have been transformed. Table 1 illustrates some of these studies which have been made.

Table 1. GENETIC ACTIVITY OF DNA IN MICROBIAL SYSTEMS

Character transformed	Microorganism
Capsule containing specific polysaccharides	D. pneumoniae H. influenzae N. intracellularis (E. coli)
Resistance to drugs	D. pneumoniae H. influenzae B. subtilis
Nutritional independence	B. subtilis
Enzyme-forming ability	D. pneumoniae B. subtilis
Virulence in plants (tumor-inducing ability)	A. tumefaciens

The reason we selected *Bacillus subtilis* was that the organism during germination from the spore presents very peculiar morphologic tendencies. That is, the germinating tube appears in bizzare shapes under certain conditions of growth and this suggested to us at least the possibility that it was protoplast-like, presumably since it does not have a complete cell wall. Under these circumstances in analogy with spheroplasts of *E. coli*, it was possible that the DNA might be able to penetrate and produce genetic effects.

We performed experiments with germinating spores and adding DNA. We used an organism initially which required indole for growth, strain 168, which I will continue to refer to because it is the one strain with which most of the studies were performed. DNA isolated from wild-type strains which do not require indole were able to convert indole-requiring strains to indole independence and one could detect this very readily on minimal plates that contained no additives, just the ammonia, glucose, and salts required for minimal growth. And the frequency appeared to be very high.

The use of germinating spores as recipients for DNA was found to be impractical since the process could not be syncronized. Vegetative cells could be made to take up DNA if they were subjected to very specific physiological conditions which will be discussed.

The transformation of *Bacillus subtilis* will be discussed in some detail, partly because I think we can illustrate some principles of transformation with this system. First let us look at some of the strains we employed and some of the marker systems. Table 2 indicates some of the strains which were obtained from Burkholder and Giles' collection at Yale.

Strain 168 requires indole for growth and we have a number of other strains available from this collection which were blocked in other parts of the biosynthetic pathway of tryptophan; the 166 and 160 which were slightly transformable. The 122 strain, nicotinic minus mutant was also slightly transformable.

Table 2. *B. subtilis* STRAINS GENETICALLY ALTERED TO NUTRITIONAL INDEPENDENCE BY DNA
(Donor DNA isolated from "wild type" 23)

Strain	Nutritional block
166 (Yale)	anthranilic acid
168 (Yale)	indole
160 (Yale)	L.tryptophan
122 (Yale)	nicotinic acid
Pi	isoleucine and
	indole

None of these strains are as transformable as the 168 strain. And the bottom line is simply to illustrate that one can make mutants of the indole-requiring strain 168 in addition to this requirement and it turns out that every one of these are as transformable as the indole marker in this strain. Consequently, we have here a situation where there seems to be something selective about the strain with regard to transformability. We will see presently that there may be some genetic basis for transformability.

Table 3 shows some enzyme markers which we have employed in transformation studies in *Bacillus subtilis*. We can make mutants of our indole strain which cannot grow on sucrose media and lack the enzyme levan-sucrase. This enzyme not only catalyses the hydrolysis of sucrose but also synthesizes levans. This is an inducible enzyme and the transformation can be achieved with DNA from sucrose-positive organisms. We can detect these transformants as large colonies on sucrose agar and subsequent enzyme tests determine that they have in fact the wild-type amount of enzyme.

Table 3. TRANSFORMATION OF SPECIFIC ENZYMES IN *B. subtilis* 168

Enzyme deficiency in mutants	Transformants detected in colonies by
Sucrase (inducible)	Large colony on sucrose-minimal agar (not on glucose-minimal)
Alkaline phosphatase (repressible)	P-nitrophenyl phosphate treated plates after growth on phosphate-deficient minimal agar (yellow colonies)
α-Amylase (inducible) ..	Colorless zones on starch-minimal agar treated with iodine-KI after growth
Threonine dehydrase	Colony growth on glucose-minimal agar (isoleucine independence)
Diaminopimelic acid decarboxylase (+ other decarboxylases associated with asporogenic physiology)	Large asporogenous colony on glucose-minimal agar

Alkaline phosphatase, a repressible enzyme in *B. subtilis*, is another marker. We have a series of mutants lacking the ability to form alkaline phosphatase which are transformable. Amylase is another good enzyme in *B. subtilis* and can be detected very readily on starch agar plates by using iodine staining. Amylase-negative mutants can be transformed.

We have studied another enzyme, threonine dehydrase, and this can be detected by nutritional methods: isoleucine-requiring organisms lack this enzyme and the enzyme formation occurs when transformation to prototrophy is effected. Our interest was aroused as to the possibilities of employing biochemical markers because the utilization of simple markers of this type makes it feasible to investigate the nature of the information required and some of the processes involved in enzyme formation.

Our first studies were aimed at determining the optimal conditions required for maximal transformation in order to evaluate the physiological basis of competence.

One of the requirements for transformation was very early noted, namely some component in yeast extract which was subsequently found to be L-tryptophan or indole, Table 4 shows the effect of L-tryptophan in promoting transformation.

Table 4. REQUIREMENT OF L-TRYPTOPHAN FOR COMPETENCE IN *B. subtilis* 168
(0.8 μg DNA from wild type)

L-Tryptophan μg/ml.	Transformation for indole
	Percent
0	0
2	0.001
20	0.27
50	0.56

The transformation is performed in a minimal medium. Now the question is, why do it that way? Why not put it in a good medium and grow it for a short time and then just follow transformation? It turns out that if you use a complete medium, one that contains high levels of amino acids and particularly amino acids which are cell wall precursors you get little or no transformation. This is either due to the fact that the competence period has been gone through very rapidly, or more likely that cell wall components once formed in large amounts prevent the DNA from being taken up for transformation.

It is necessary, therefore, to put the cells in a state of semi-starvation, perhaps in an unbalanced growth without making much of their cell wall components. Under these circumstances they become competent. Now L-tryptophan is the first requirement we found. The second requirement for transformability in these strains is illustrated in Table 5.

Table 5. CUPRIC INHIBITION OF COMPETENCE (90-minute period) B.S. 168 indole-; DNA (wild) 8 μg/ml.

Addition to minimal + 5 μg Tryptophan/ml.	Transformation
	Percent
None	0.05
Cu^{++} 10^{-5} M	0.005
L-histidine 50 μg/ml.	0.24
" + Cu^{++} 10^{-5} M	0.23
Fe^{+++} NaEDTA (10^{-4}M) + Cu^{++} 10^{-5} M	0.20

It was found that copper inhibits and copper chelators permit transformation. The requirement for a low concentration of casein hydrolysate for competence was found to be due to the L-histidine which can be replaced by other compounds which chelate copper. Thus, if one uses small amounts of L-histidine and larger amounts, like 10^{-3} M of copper ions, one can of course show competition and inhibition of transformation. Table 6 shows a number of compounds which were found to replace the casein hydrolysate required for competence. Thus, it would appear that some metal chelator is required. However, if you add the metal chelator just prior to transformation, you get no effect. It turns out that the chelator has to be in the presence of the cell for a minimum of about 60 minutes.

Table 6. COMPOUNDS ACTIVE IN COMPETENCE (90-minute period) *B. subtilis* 168 indole-; DNA (wild) 8 μg/ml.

Compound 20 μg/ml.	Transformation
	Percent
L-histidine	0.55
D-histidine	0.57
Histamine	0.64
EDTA, Na	0.32
Histidyl-histidine	0.82
Carnosine	0.32
Histidine methyl ester	0.39
2, 2′ bipyridyl	1.66
β-mercaptoethanol	0.72

Table 7 illustrates an experiment to demonstrate this point. If L-histidine is added at 60 minutes, DNA at 85 minutes, incubation continued until 100 minutes, you get only .05% transformation. However, if the chelator is added at 30 minutes and the DNA added at 85, and the system is incubated for 15 minutes longer under aerobic conditions at 37° one obtains more efficient transformations (1.2%). This suggests that possibly the chelator is required to be present in order for the formation of some component which is necessary for competence.

Table 7. TIME OF ADDITION OF L-HISTIDINE (20 μg/ml.) DNA 0.8 μg/ml.

Time of addition		Transformation (100 minutes)
Histidine	DNA	
Minutes	*Minutes*	*Percent*
0	85	1.2
30	85	0.4
60	85	0.05
75	85	0.05

An examination of the growth curves of strain 168 under the special conditions described indicates that competency begins to become apparent near the end of the logarithmic phase of growth. The cells can be transformed with the same optimal efficiency at any time during a 3- to 4-hour period in this medium following the initial appearance of competency. In fact, cells once prepared can be washed free of medium and transformed with the same efficiency in a minimal medium. They can be frozen in 10% glycerol at –20° C. and remain active for weeks.

Once these conditions were established, we were surprised to find that other strains of *B. subtilis* were either poorly transformed or were completely nontransformable for many different markers. That this was probably not due to the marker itself was evident when similar mutants were produced in strain 168 and found to be transformable.

We did, however, note that in all strains whose DNA would transform 168 for indole and which could not themselves be transformed, the nonsporulating or SP– character was present. These include a number of aterrimus varieties of *B. subtilis*. Furthermore, we had the experience

of occasionally losing transformability in passing strain 168 on complete media, and on examination found that such organisms had reverted to SP–. Normally transformable 168 is SP+ (sporulating on minimal medium).

We, therefore, proceeded to transform 168 to SP– still retaining the I– character. We isolated a dozen strains thus by transformation after segregation was complete.

The growth of the I– SP– strains is identical for 10 hours with the original I– SP+ strain of 168. However, cells of the SP– removed at various times of the growth period and placed in contact with DNA from an I+ organism were poorly or not at all transformed to prototrophy whereas SP+ organisms were transformed with high efficiency during the competence period.

We, therefore, conclude that a sporulating trait in *B. subtilis* controls the ability of the cells to be transformed. This may be due to alterations in the cell wall which precede sporulation, and in fact, our growth studies suggest that the optimal time for transformation occurs at a stage of presporulation.

Experiments with P_{32} labeled DNA indicate that there is a markedly greater uptake of DNA by the SP+ cells at this time, whereas nonsporulating organisms are impaired in their ability to take in DNA. The genetic trait might also control some physiological changes involving nuclear modifications associated with sporulation. In any case, the inability to sporulate in minimal medium prevents transformation.

Now if all of this is true and there is a direct relationship between the sporulating character and the ability to be transformed, then it should be possible to convert nontransformable strains to transformability. Now it is not possible to do this readily by transformation, that is to convert SP– organisms to SP+, but we have attempted to do this by mutagenic agents.

Our preliminary results do show that in fact former nontransformable strains become transformable with the acquisition of the SP+ trait. Now we have only done this with strains that were poorly transformable and we have not as yet done this with strains that were completely nontransformable.

I would like to close with a few remarks about what we consider to be criteria of transformation, because many experiments are considered to demonstrate transformation without adequate controls. First of all it is rather apparent that DNA must be isolated to demonstrate transformation. No other compound has so far been found to transform.

In the second place, one ought to show that the DNA isolated from recipient, that is from the homologous strain, is inactive. A third control is to test DNA from genetically unrelated cells which should be nontransforming but at high enough concentrations should compete with transforming DNA. Homologous DNA will also compete. In fact, one can use this method to detect highly polmerized DNA simply because only highly polmerized DNA will actually compete in this system.

DNA depolymerized by desoxyribonuclease or other DNA specific reagents, for instance mustard, should be inactive. In fact DNA is very sensitive to desoxyribonuclease; as little as 10^{-4} or 10^{-5} gamma crystalline enzyme will inactivate under proper conditions. Finally we have a typical dose response curve which must be done under conditions where you pulse the DNA in for short periods of time.

This is, in brief, some of the data and some of the thinking that we have done on the problem up to now. I think that transformation in many organisms is a distinct possibility in the future. In the case of *Bacillus subtilis*, it is now possible to examine the transformation process under defined conditions. *B. subtilis* is an organism which could not be examined genetically up to now. The genetics of sporulation, for instance, can now be investigated.

I wish to acknowledge the collaboration of the following in the studies reported above: Dr. C. Anagnostopoulos, Dr. F. Young, and B. Reilly.

References

1. Avery, O. T., C. M. MacLeod, and M. McCarty, 1944. J. Exptl. Med., *79:* 137.
2. Hershey, A. D. and M. Chase, 1952. J. Gen. Physiol., *36:* 39.

Genetic Recombination Between Genera of the Enterobacteriaceae

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The detection of infrequent recombinants by Tatum and Lederberg (1), in 1947, by means of selective methods which eliminated the parental types, established the phenomenon of genetic recombination in *Escherichia coli* K-12. Parental strains with different nutritional requirements were mixed and plated on a minimal medium lacking the required growth factors. Progeny independent of these growth factors were obtained at a relatively low frequency (10^{-6}). Unselected properties such as fermentative abilities, susceptibility or resistance to bacterial viruses or antibiotics could be transferred to these hybrids as well.

Since intact cells were proven to be required for the mating process, as opposed to transformations which are brought about by desoxyribonucleic acid extracts of cells, a sexual process which related recombination to the formation of paired bacterial cells was postulated by Lederberg et al. (2). This sexual mechanism was thought to involve the transfer of a genetic element (in the form of many linked markers) from one intact cell to another in a manner similar to fertilization.

The compatibility scheme of Cavalli et al. (3) and the work of Hayes (4) have demonstrated further that sexuality in bacteria is, in actuality, unilateral conjugation specifically requiring a donor, or male cell, and a recipient, or female cell. Although strain K-12 was self compatible and was also, in retrospect, a male or F^+ culture, a mutant, self-sterile type referred to as an F^- or female strain has also been isolated from K-12. While F^- x F^- crosses were sterile, F^+ x F^- crosses, in addition to being fertile, also produced an efficient conversion of the F^- female population into F^+ male cells. In spite of its infectious, virus-like behavior, the F agent was not considered to be a virus, since contact between cells was required for transfer of the F^+ property as well as for recombination.

A notable observation, which incidentally helped to make possible the direct microscopic observation of bacterial conjugation (5, 6), was the discovery by Cavalli (7) of an exceptionally fertile mutant of K-12, which was designated as a high frequency of recombination (Hfr) strain. The Hfr strain was a male strain which was 100 to 1,000 times more fertile than its F^+ predecessor strain, but was *not* able to transfer the F male fertility factor to the progeny of Hfr x F^- crosses.

Perhaps the most meaningful analysis of the sexual process in bacteria, however, has occurred as a result of the remarkable experiments of Wollman and Jacob (8). These workers were able to interrupt the conjugal process at intervals during the transfer of genetic material by means of a Waring blender. The findings of these time sequence studies have indicated that an oriented transfer of genetic material takes place from the Hfr strain to the recipient organism. Mapping of the known markers by genetic analysis of relative frequencies of transfer gave results which were, in fact, corroborated by these studies of the order and time of entry of the markers into the recipient cell. The data obtained on linkage relationships of the known genetic characters using a number of different Hfr mutants have enabled Wollman and Jacob to locate these markers on a circle. Presumably, after rupture of the "circle," the transmission of a chromosomal segment would be determined by a point of origin which would characterize the frequency and order of transfer of the markers with the Hfr character probably located at the end of the particular group of determinants since it is transferred least frequently. Failure of appearance of all possible markers in any hybrid could be attributed as being due to spontaneous breaks occurring during transfer.

The occurrence of fertility in *E. coli* strains other than strain K-12 was surveyed by Lederberg (9) by examining approximately 2,000 strains for their ability to act as males (F^+) with

an F^- tester strain of K-12. A relatively small number of other strains were observed to be fertile by this procedure. A more concerted effort was made by Lederberg and Zinder (10) to detect recombination among *Salmonella* serotypes. These results were unsuccessful, but hardly fruitless, since their efforts led them to the discovery of another mechanism of genetic transfer. This process involved the use of bacteriophage particles as vectors of genetic material and has been termed genetic transduction. In contrast to bacterial recombination, a bacteria-free phage lysate was found to be competent in the transfer of markers from the bacterial host (donor) to the recipient bacterial cell with the phage particles acting as vectors of genetic material.

Although the findings by Lederberg and Edwards (11) had established genetic transduction between different bacterial species, an extension of this process to organisms belonging to different genera depended upon the availability of a suitable phage. Transduction experiments performed by Lennox (12) using a phage which could either lyse or lysogenize both species of the Genus *Escherichia* and the Genus *Shigella* accomplished this result. In addition, Lennox was able to verify the linkage of certain markers, as determined previously by recombinational analysis, by showing that these closely-linked markers could be transduced simultaneously as well.

The actual mating of bacteria classified in different genera, however, was first demonstrated by Luria and Burrous (13) in 1957, approximately ten years after the first report by Lederberg and Tatum of sexuality in *E. coli*. Evidence was obtained which indicated clearly that many of the species of the Genus *Shigella* were able to act as recipient (F^-) organisms in crosses with Hfr or F^+ *E. coli* strains. A simple selective method based on the fact that *Shigella* strains are generally stable lactose negative (Lac^-) cultures was employed. Streptomycin-resistant (S^r) mutants of the *Shigella* cultures were isolated for use as recipients, and these were plated directly with the streptomycin-sensitive (S^s) *E. coli* Hfr or F^+ parent on Eosin-Methylene Blue (EMB) lactose agar containing streptomycin. The addition of streptomycin to this medium served to eliminate the streptomycin-sensitive male parent by inhibiting its growth, but did not interfere with its ability to mate with the recipient strain. Lactose-utilizing hybrids could easily be detected as darkly-pigmented papilli growing out as individual clones from the background of the recipient strain.

As was expected, the Hfr cultures gave much higher frequencies of recombination than did the F^+ cultures, although in both instances the frequencies obtained were lower than those resulting when F^- *E. coli* strains are employed as recipients. Hybrids obtained from the *E. coli* x *Shigella* crosses acquired various combinations of the genetic characteristics of the male *E. coli* parent, but still retained most of the genome of the F^- female *Shigella* parent. A number of markers from the *E. coli* parent evidently were not transferred to the *Shigella* cultures; these included motility, gas production, the utilization of certain carbohydrates, and the production of indol.

Studies to determine whether *Shigella* hybrids from crosses with F^+ *E. coli* had acquired the ability to act as males yielded negative results. These *Shigella* hybrids, however, had acquired the F^+ agent, since they were able to transfer this infectious factor to F^- strains of *E. coli* by contact. These *E. coli strains* could now function as typical males.

A number of attempts in various laboratories to demonstrate genetic recombination between *Salmonella* species or between *E. coli* and *Salmonella* were reported as yielding negative results (3, 13). Aside from the genetic interest in *Salmonella* aroused by the transduction experiments of Zinder and Lederberg (10), the Genus *Salmonella* provides a useful tool for studies on host-parasite relationships as well. Certain *Salmonella* species are known to cause a natural infection in the mouse, as well as in man, whereas *Shigella* species, although able to produce dysentery in man, are relatively avirulent for the mouse.

For a number of years, our interest has been centered on the study of immunity to enteric disease. The basis for the virulence and host specificity of the etiologic agents of the various *Salmonella* fevers or for the protective factors, if any, in the vaccines produced from these microorganisms, remains in dispute. It was felt, therefore, that studies using the mechanism of genetic recombination might provide a useful approach to these problems.

Experimental Results

The crossability of *Salmonella* with *E. coli* was re-investigated using a number of species of *Salmonella* consisting of representative serotypes from the various groups of the Kaufmann-White classification scheme. In preliminary experiments, the *E. coli* male culture was grown together in nutrient broth with each of the *Salmonella* species

to be tested for behavior as potential recipient strains. The cells were harvested by centrifugation, washed thoroughly, and then plated on minimal agar plates containing lactose (Lac) as sole source of carbon. The minimal lactose agar medium used was unable to support the growth of either parental strain alone, since the *E. coli* Hfr and F^+ strains require methionine (M^-) for growth, while all the *Salmonella* strains tested were stable lactose negatives (Lac^-).

The initial experiment revealed that a smooth culture of *S. typhimurium* strain TM-9 had mated with the Hfr strain of *E. coli* at a relatively low frequency; no recombinants of this strain, however, were detected with the *E. coli* F^+ strain. The failure to observe recombinants in crosses with the F^+ strain was not unexpected, in view of the low frequency obtained using the best available Hfr strain, that isolated by Cavalli and characterized as a high frequency of recombination strain for lactose and closely linked markers.

The hybrid clones which appeared on the minimal lactose plates were purified from the background by restreaking on selective media (either minimal or EMB lactose agar plates) and subjected to antigenic and biochemical analysis. The results established that these recombinants were lactose positive progeny which still retained the antigenic structure of the *S. typhimurium* parent strain.

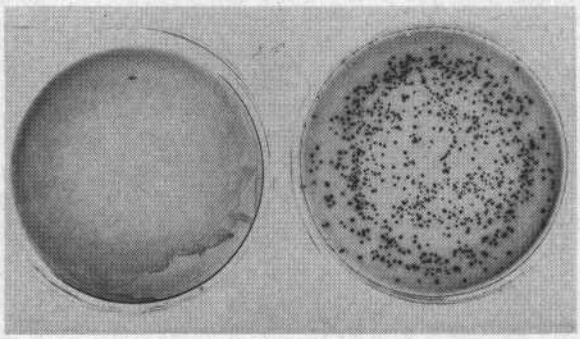


Figure 1. Mating between *E. coli* Hfr and *S. typhimurium* TM-9S^r-2 on EMB lactose agar containing streptomycin. The darkly-pigmented colonies on the experimental plate are lactose positive hybrids of *S. typhimurium*.

In order to simplify the selection and further purification of the hybrids, a number of streptomycin-resistant mutants of strain TM-9 were isolated by plating dense suspensions of cells on nutrient agar containing streptomycin. The resistant clones obtained were then tested on EMB lactose agar containing streptomycin for the ability to cross with the Hfr and F^+ strains of *E. coli*. A number of these streptomycin-resistant mutants were able to mate with the Hfr strain at a considerably higher frequency than the streptomycin-sensitive strain TM-9 from which they were derived. Figure 1 shows the results of a cross between the *E. coli* Hfr strain and a streptomycin-resistant isolate, *S. typhimurium* TM-9S^r-2 on EMB lactose streptomycin agar. The darkly-pigmented colonies seen on the experimental plate are the lactose positive *S. typhimurium* hybrids resulting from a mixed plating of *E. coli* and *S. typhimurium*. The control plate shows the lactose negative *S. typhimurium* culture plated by itself. *S. typhimurium* strain TM-9S^r-2 was now able to yield recombinants in crosses performed with the F^+ strain of *E. coli* as well. The frequencies of recombination of several strains of *S. typhimurium* and other *Salmonella* species expressed as the ratio of recombinants to the number of Hfr or F^+ parent cells can be seen in Table 1.

Table 1. FREQUENCY OF RECOMBINATION OF *Salmonella* SPECIES (Selection for Lac + hybrids)

Recipient	*E. coli* Hfr	*E. coli* F^+
S. typhimurium 9	4×10^{-8}	0
" 9S^r-2	1×10^{-4}	1×10^{-7}
" LT-7	$<1\times10^{-8}$	0
" HB	"	0
S. paratyphi B	"	0
S. paratyphi C	"	0

The greatly increased frequency of recombination displayed by *S. typhimurium* TM-9S^r-2 also made possible the microscopic visualization of the conjugation process. Figure 2 illustrates a mating between photoplasts of the *E. coli* Hfr strain prepared by the penicillin method described by Lederberg (14) and rods of strain TM-9S^r-2, as observed by means of phase microscopy. The nonmotile protoplasts of *E. coli* employed to distinguish the parental strains, were seen attached to the motile rods, the resulting combination with strain TM-9S^r-2 being motile and suggestive of conjugation.

The higher rate of recombination exhibited by TM-9S^r-2 appeared to be associated only coinci-

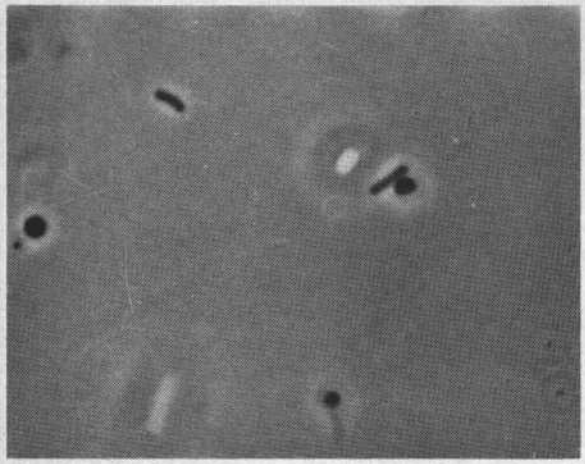


Figure 2. Pairing of protoplast of the *E. coli* Hfr strain with rod of *S. typhimurium* TM-9S^r-2 suggestive of mating.

dentally with streptomycin resistance, since a large number of additional S^r clones which were tested in further experiments behaved similarly to TM-9 with regard to recombination ability, being low frequency recipients (15). It was postulated, therefore, that strains of *S. typhimurium* and possibly other species of *Salmonella* as well were not F$^-$ as are *Shigella* species, but might best be described by the symbol F^0, i.e., unable to act as recipients or donors (sterile). Such strains could mutate, however, to the F$^-$ state at low frequency (10^{-8}), and it would be these rare F$^-$ cells in the population which are able to recombine with the *E. coli* Hfr parent. Recent observations by Wollman and Jacob (8) have indicated that the fertility of F$^+$ *E. coli* strains is actually due to the appearance in the cell population of a small number of F$^+$ to Hfr mutants. The mechanism of this change is thought to involve a transfer of the plasmagene-like F$^+$ factor from the cytoplasm of the cell to a fixed position on the bacterial chromosome. This postulate and the assumption of infrequent F^0 to F$^-$ mutations in potential recipient strains may readily explain the inability to detect recombinants from an *E. coli* F$^+$ cross with *Salmonella*. The possibility of an F^0 to F$^-$ mutational event in *Salmonella* coinciding with an F$^+$ to Hfr change in *E. coli* would be very unlikely in view of the frequencies at which these events occur.

With the availability of a strain of *S. typhimurium* that acts as a recipient at high frequency, further genetic study of the hybrid progeny became feasible. The frequency of transfer of various unselected markers were thereby determined with a view towards establishing linkage relationships. The presence of recombinants capable of producing indol also was demonstrated in these experiments, as well as the ability to utilize various carbohydrates. Table 2 lists the selected and unselected characters employed and the percent frequency of transfer of the unselected markers from the *E. coli* parent to the *S. typhimurium* hybrids.

Table 2. FREQUENCY OF UNSELECTED CHARACTERS FROM *Escherichia coli* PARENT TO *Salmonella* HYBRIDS

Selected characters	Unselected characters		
	Inositol negative	Lactose positive	Indol
	Percent	*Percent*	
Lactose positive	4	---	Negative
Arabinose positive	32	68	Negative
Rhamnose positive	53	7	8% Positive

HYBRIDS OF S. TYPHOSA

In addition to strains of *S. typhimurium*, a number of strains of *Salmonella typhosa* were found to be fertile at low frequency (16). The concept that recombination is due to occasional F$^-$ cells which appear in the F^0 *Salmonella* population predicts that a hybrid isolated from a mating between an *E. coli* Hfr strain and *Salmonella* should be F$^-$, and therefore able to recombine in further crosses at the frequency expected of a typical F$^-$ culture. Such a strain should also be able to cross with the lower-frequency *E. coli* F$^+$ donor strains.

The presence of a number of selective markers in *S. typhosa* strain 643 allowed the testing of this hypothesis. Strain 643 was crossed with the *E. coli* Hfr and a lactose positive (Lac$^+$) hybrid, strain 643 Lac$^+_3$, was isolated which retained the antigenic structure and biochemical characteristics of the parent typhoid culture except its acquisition of lactose-utilizing ability. Since strain 643 Lac$^+_3$ was still unable to utilize l-arabinose, this factor could be employed as a selective marker.

The presumptive F$^-$ strain, 643 Lac$^+_3$ and the previously unmated F^0 strain 643 were both used as recipients in matings with the *E. coli* Hfr and also with an *E. coli* F$^+$ strain on EMB medium containing l-arabinose. The l-arabinose positive hybrids appeared at a relatively high frequency in

the Hfr cross and could be detected at lower frequency in the F^+ cross, only with the postulated F^- strain, 643 Lac^+_3. It was also possible to determine the frequency of recombination for the lactose marker by use of lactose negative (Lac^-) hybrids which could be obtained by crossing a Lac^- Hfr strain of *E. coli* with *S. typhosa* strain 643 Lac^+_3 and selecting for l-arabinose positive hybrids. The Lac^- negative hybrids of 643 Lac^+_3 which were observed among the progeny from this cross were now capable of mating at a high frequency for lactose when crossed with the usual Lac^+ *E. coli* Hfr. The frequencies determined in these crosses were of the order of 2×10^{-4}, which was typical of the recombination frequency observed in the case of the F^- *S. typhimurium* strain TM-9S^r-2. The fertility of the *S. typhosa* strains examined in these experiments is depicted in Table 3.

Table 3. FERTILITY OF *Salmonella typhosa* STRAINS

Salmonella typhosa strain	Antigenic formula	Recombinants from crosses with *E. coli* Hfr (Cavalli)	
Ty-2	Vi,9,12,: d	+	$<10^{-8}$
643	Vi,9,12,: d	+	$<10^{-8}$
H-901	–,9,12,: d	+	$<10^{-8}$
0-901	–,9,12,: –	+	$<10^{-8}$
Ty-2W	–,9,12,: d	+	$<10^{-8}$
643 Lac^+_3	Vi,9,12,: d	Lac^+ hybrid of 643	
643 Lac^-_3	Vi,9,12,: d	+	$2x10^{-4}$

Since strain 643 Lac^+_3 possessed a number of negative markers in addition to inability to utilize l-aribinose, a series of further crosses with the *E. coli* Hfr strain was performed using these factors as selective markers. Table 4 shows the parental characteristics of the two strains, and the selected markers used were the differences in carbohydrate utilization between the strains.

The number of recombinants obtained in these matings depended upon the selective sugar used. The l-arabinose selected hybrids occurred at the highest frequency 1×10^{-5}, and 85 to 90% of these hybrids were found to be stable both biochemically and colonially. These stable hybrids, however, had acquired only the ability to utilize l-arabinose from the *E. coli* parent. Recombinants selected on d-xylose, l-rhamnose, or l-fucose containing medium were highly unstable colonially.

Table 4. PARENTAL CHARACTERISTICS OF MATING STRAINS

E. coli Hfr W1895	Markers	*S. typhosa* 643Lac^+_3
+	Lactose	+
+	l-Arabinose	–
+	d-Xylose	–
+	l-Rhamnose	–
+	l-Fucose	–
+	d-Arabinose	–
+	Cystine	–
+	Tryptophan	–
–	Methionine	+
+	Indol	–
+	λ	–

When a recombinant clone was restreaked on nutrient agar, two colonial forms were observed. These two types consisted of a small, dense colony which was highly unstable giving rise to translucent segments and large stable translucent colonies. The remaining 10 to 15% of the hybrids selected on l-arabinose plates were also unstable and behaved in the above manner.

In addition to the colonial appearance of these hybrids which can be seen in figure 3, these forms could not be purified as stable positives on EMB media containing the selective carbohydrate on which they were isolated. Thus, no matter how many times positive clones were restreaked from transfers on EMB carbohydrate media, stable negative forms were segregated continuously. These negative colonies on EMB media were identified as the translucents observed on nutrient agar and were unable to utilize either the selected or unselected markers. The dense colonies observed on nutrient agar were positive on EMB media containing the carbohydrate on which they were selected (excepting the segregated negative clones identified as large translucent forms on nutrient agar plates) and could also ferment the unselected carbohydrates as well as being able to produce indol (17).

In addition, the dense colonies cross-reacted with rabbit antiserum to *E. coli* strain K-12, while the translucent colonies failed to show this serological reaction. Antigenic analysis of the dense and translucent colonies revealed that both forms had retained the somatic, flagellar, and Vi antigens

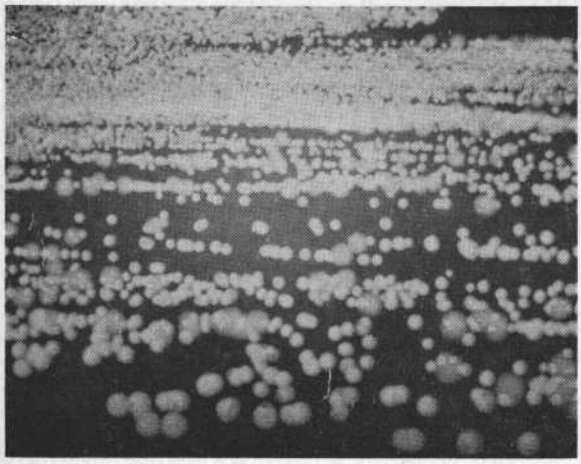


Figure 3. Unstable diploid (dense colony) and stable translucent segregants of *E. coli* W1895 x *S. typhosa* 643 Lac$^+_3$ hybrids.

Table 5. RECOMBINATION FREQUENCIES AND CHARACTERISTICS OF HYBRIDS FROM MATINGS BETWEEN *E. coli* Hfr and *S. typhosa* 643 Lac$^+_3$

Selective agent	Frequency	Characteristics
L-Arabinose	1 x 10^{-5}	10-15% "diploids" highly unstable
D-Xylose	1 x 10^{-6}	entirely "diploids" highly unstable Strain X30D, relatively stable
L-Rhamnose	6 x 10^{-7}	entirely "diploids" and unstable
L-Fucose	5 x 10^{-8}	entirely "diploids" and unstable
D-Arabinose	0	

typical of *S. typhosa*. It was also found that a cell suspension of the dense colonies which was boiled for one hour at 100° C. was no longer agglutinated by antiserum to *E. coli*. Thus, the dense form, in addition to the acquisition of a number of the biochemical capabilities of *E. coli*, also appeared to have acquired a heat-labile antigen from the *E. coli* parent. Neither the original culture of *S. typhosa* strain 643, nor the hybrid from the initial cross with *E. coli*, strain 643 Lac$^+_3$ was cross-reactive with antiserum to *E. coli*. These unstable hybrids were considered to be very similar to the diploid heterozygotes which were described by Lederberg in *E. coli* (18, 19). Due to the marked instability exhibited by the diploid form, a complete study of its characteristics was rather difficult. Many of these diploids could not be maintained in this condition for more than a few transfers.

STABLE DIPLOID HETEROZYGOTES

A considerably more stable diploid colony was isolated, however, from a mating on minimal d-xylose medium. This hybrid will be referred to as strain X30D. Most of the work to be discussed is concerned with studies of strain X30D, although other strains of this type with some differences in behavior have also been examined in detail. The recombination frequencies and nature of the hybrids in these matings is presented in Table 5.

A fairly uniform culture of X30D could be retained by careful selection of typical colonies for transfer. The typical colony form of X30D did not resemble either the *E. coli* or *S. typhosa* parent strain in colonial morphology, but appeared to be very similar to the highly unstable colonial forms seen previously. The cells of X30D could also be differentiated from its parents microscopically by virtue of its distinctly shorter and wider appearance. Upon transfer of a typical dense clone of X30D, a considerably larger, translucent form, X30T, was observed occasionally. A smaller colony type, X30P, was observed when transfers were made from slants which were about a week old. These two forms, X30T and X30P, were found to be completely stable colonially. The three colonial forms can be seen in figure 4. The medium-sized dense colony is the diploid form X30D, the smaller less dense colonies are those of the segregant X30P, while the large, crinkled colonies are the segregant, X30T. More recently, a number of other segregant types have been isolated from X30D which have also demonstrated distinct differences in antigenic and biochemical patterns. These patterns for X30D and its segregant forms X30T and X30P are described in figure 5. The symbol P$^+$ represents the presence of an *E. coli* antigen responsible for a serological cross-reaction of a hybrid with *E. coli*, while P$^-$ indicates the absence of this antigen. Strain X30D, in addition to having all the typhoid antigens, Vi, O, and H, also cross-reacted serologically with *E. coli* antiserum. Strain X30T, the large translucent form segregated from X30D, was antigenically distinct from *E. coli*, possessing only the typical typhoid antigens. Strain X30P retained the somatic (O) and flagellar (H) antigens of *S. typhosa* as well as the antigenic cross-reaction

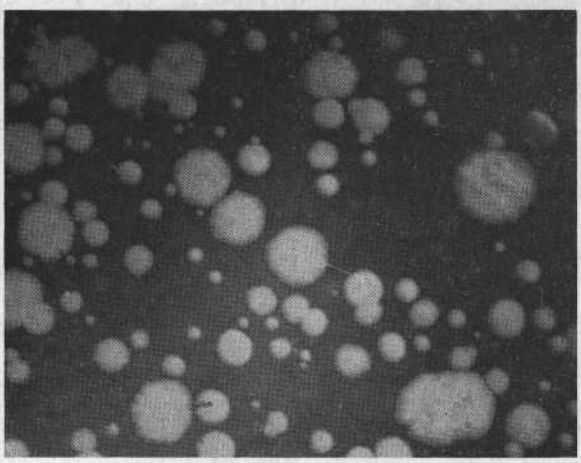
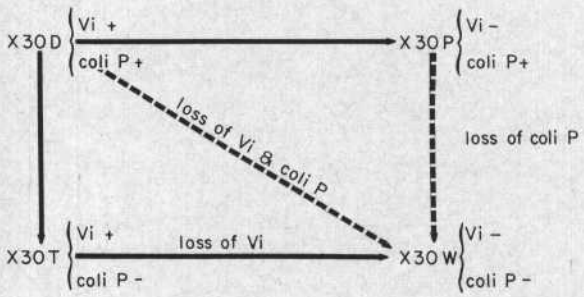


Figure 4. Diploid strain of *S. typhosa*, X30D (medium-sized dense clones) and its haploid segregants, X30T (large crinkled clones), and X30P (small dense clones).

with *E. coli*, but lost the Vi or virulence antigen of *S. typhosa*. Both X30D and X30P were identical in biochemical behavior having acquired the ability to utilize l-arabinose, d-xylose, l-rhamnose, and indol production from the *E. coli* donor parent. In addition to the absence of a cross-reaction with *E. coli*, X30T differed biochemically as well, being unable to utilize l-arabinose. This result was observed consistently for all isolates of the X30T colonial type which were obtained from transfers of single clones of X30D.



BIOCHEMICAL CHARACTERISTICS

X 30D lac +, l-ara +, d-xyl +, rha +, indol +
X 30T lac +, l-ara -, d-xyl +, rha +, indol +
X 30P lac +, l-ara +, d-xyl +, rha +, indol +

Figure 5. Antigenic and biochemical characteristics of relatively stable diploid strain of *S. typhosa*, X30D.

SEROLOGICAL STUDIES

In order to determine the antigenic component transferred from the *E. coli* parent to the recombinant, X30D, a series of agglutination tests were performed employing sera prepared from live and heat-killed cells. Live and heat-killed cells were also both used as antigens, and separate tests were incubated at 37° C. and 52° C. The purpose of this procedure was to distinguish between agglutination due to either somatic, flagellar, or surface components. These studies established that the strongest agglutination reaction took place at 37° C. with the antiserum prepared from living cells of *E. coli*, thus indicating that the serological cross-reaction between X30D and *E. coli* was due to a thermolabile antigen acquired from the *E. coli* parent.

Since the heat-labile nature of the unknown antigen excluded the transfer of a somatic component, the posibility of transfer of a flagellar antigen through recombination was considered next. The modified Wassen-bard technique of Lederberg and Edwards (11) was used whereby immobilization of the bacterial growth occurs on semi-solid agar plates containing homologous anti-flagellar serum. The results indicated that a dilution of antiserum able to immobilize the homologous culture was unable to affect the motility of the heterologous strains. Thus, the cross-reaction could *not* be due to acquisition of a flagellar antigen from *E. coli*, although these structures are thermolabile. These results were supported also by microscopic observation of the clumping pattern which was not typical of flagellar or somatic agglutination, but was rather a fine, granular type of flocculation.

The possibility that a transfer of the ability to produce pili, or fimbria, the non-flagellar appendages present in some bacteria (20, 21), had occurred was suggested to us by Dr. C. C. Brinton, Jr., of the University of Pittsburgh. The known antigenicity of pili and the remarkable resemblance of the colonial variation of the cross-reacting *Salmonella* hybrids to that of a piliated form of *E. coli* in which smaller dense colonies break down to yield larger, translucent colonies (20) resulted in a collaborative study (22).

In order to detect visually the presence or absence of pili, a series of preparations were examined by means of electron microscopy. Cells of the *E. coli* Hfr parent were found to be almost all piliated, as seen in figure 6. None of the cells of the *S. typhosa* 643 Lac^{+}_{3} parent examined were

piliated, nor were there any fragments of pili present on the supporting collodion film (figure 7). Pili were completely absent in cells of the original strain, *S. typhosa* 643. Practically all cells of the hybrid X30D, however, were richly piliated, as were cells of X30P; piliated X30D cells can be seen in figure 8. The X30T segregant, which does not cross-react with *E. coli*, showed a total absence of pili as well. These results indicated that pili, now present as structures in the *Salmonella* hybrids X30D and X30P through genetic recombination with *E. coli*, were responsible for the serological cross-reaction observed.

The pili of these strains were similar to each other and the pili of other strains of enteric bacteria in size, shape, and distribution. They were about 80A in diameter with a maximum length of about 3.0 microns and were uniformly distributed. Confirmation of the presence of the pilial antigen in the parent and hybrid strains was achieved by means of agglutination tests using an antiserum prepared against highly purified pili from *E. coli* (21). Since piliated strains are able to agglutinate red blood cells, this procedure also provided further confirmation of their presence or absence among the parents and hybrids. The biochemical, antigenic, and other characteristics of the strains examined are listed in Table 6.

Another point to be mentioned is the apparent linkage of l-arabinose utilization and pili production, at least as far as segregation of the X30T form from the diploid, X30D, is concerned. A

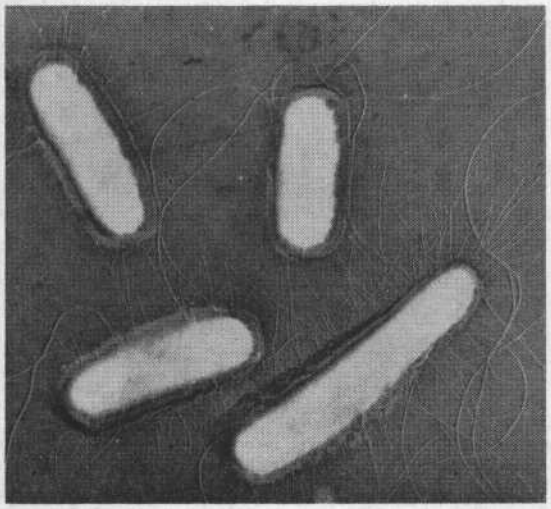


Figure 6. Electron micrograph of piliated (P^+) male parent, *E. coli* Hfr.

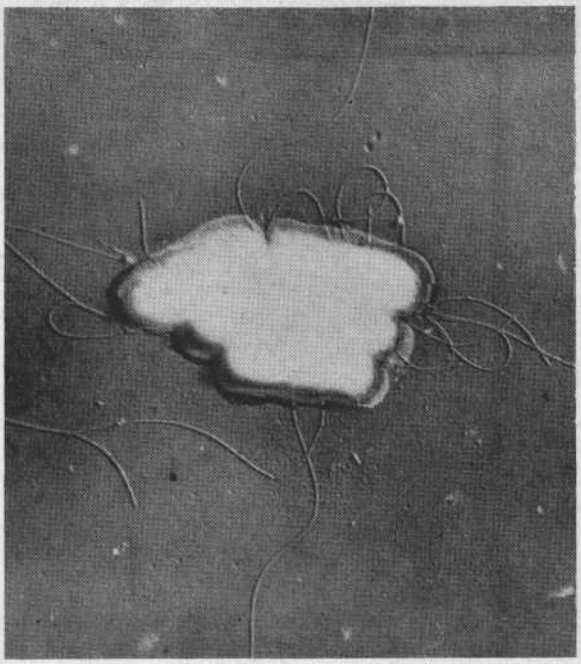


Figure 7. Electron micrograph of nonpiliated (P^-) female parent, *S. typhosa* 643 Lac^+_3.

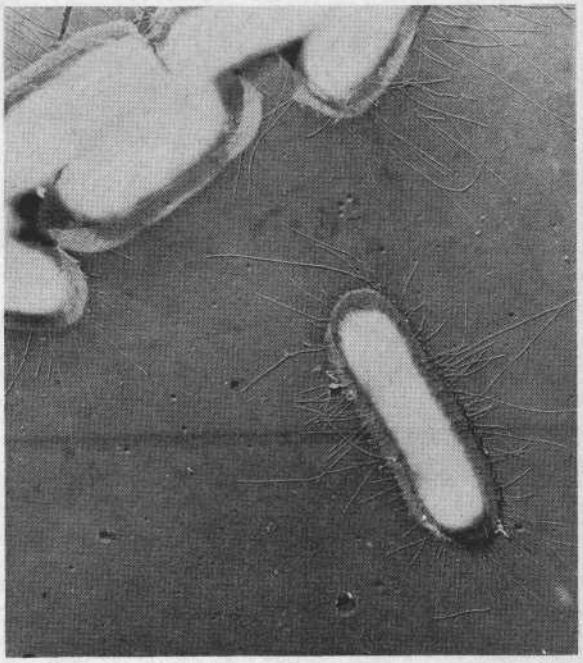


Figure 8. Electron micrograph of piliated (P^+) hybrid X30D, from cross between *E. coli* Hfr (P^+) and *S. typhosa* 643 Lac^+_3 (P^-).

number of X30T l-arabinose positive reversions which were isolated remained pili negative, indicating that pili production and arabinose utilization are not always associated, as might be expected in the case of pleiotropy.

PHAGE STUDIES

The behavior of the parents and hybrids towards various phages was examined in further studies. The original *S. typhosa* strain 643 has been classified as a phage C2 susceptible type by the Vi phage typing procedure of Craigie and Felix (23). The hybrid strain 643 Lac^{+}_{3}, the diploid hybrid X30D, and its segregant strain X30T all retained the Vi phage-type specificity of strain 643. The *E. coli* parent and the Vi negative segregant were not lysed by any Vi phages. The T series of bacteriophages capable of lysing the *E. coli* parent were also examined for possible lytic action on the *Salmonella* parent or hybrid strains. Lysis was not observed with phages T1 through T6 when plated on any of the Vi containing parent or hybrid strains, *S. typhosa* 643, 643 Lac^{+}_{3}, X30D, or X30T. Strain X30P, the Vi negative segregant from X30D, however, was lysed by phage T1, T2, T4, T5, and T6, but not by T3. Transfer of the lysogenic bacteriophage lambda (24), harbored by the *E. coli* parent, to any of the hybrids was not detected in these experiments.

VIRULENCE STUDIES

Preliminary experiments designed to detect any changes in the virulence of the hybrids were also undertaken using the mouse virulence test described by Felix and Pitt (25). A significant decrease in virulence of the piliated diploid form, X30D, was observed in spite of the presence of the usual typhoid antigens in this strain. The nonpiliated strain X30T, derived as a segregant from the diploid strain, was fully virulent. An important factor to be considered in these results, however, is the observation that the diploid strain, X30D, has a slower growth rate than does its segregant X 30T (26). Whether there is a role directly or indirectly attributable to the presence of pili here has not been established, although a report has been published which has indicated greater mortality in mice infected with nonpiliated strains of *S. typhimurium* than was observed in piliated strains of *S. typhimurium* (27).

Table 6. CHARACTERISTICS OF PARENTAL AND HYBRID STRAINS

	Male parent		Female parent	Hybrid selected on xylose	Segregants from hybrid X30D	
Characteristics	*E. coli* K-12 Hfr W1895	*S. typhosa* 643	*S. typhosa* 643Lac^{+}_{3}	X30D	X30T	X30P
Lactose	+	−	+	+	+	+
L-Arabinose	+	−	−	+	−	+
D-Xylose	+	−	−	+	+	+
L-Rhamnose	+	−	−	+	+	+
L-Fucose	+	−	−	−	−	−
D-Arabinose	+	−	−	−	−	−
L-Cystine	+	−	−	−	−	−
L-Tryptophan	+	−	−	−	−	−
Methionine	−	+	+	+	−	−
Indol	+	−	−	+	+	+
Electron microscopy	piliated	non-piliated	non-piliated	piliated	non-piliated	piliated
Purified pili antiserum	+	−	−	+	−	+
Morphology	large rough and smooth	large smooth translucent	large smooth translucent	medium dense	large smooth translucent	small dense

FERTILITY OF THE HYBRIDS

Studies of the fertility characteristics of the diploid form, X30D, and its stable segregants, X30T and X30P, demonstrated that they were unable to act as donor strains in crosses using various species of *Escherichia, Salmonella,* or *Shigella.*

RECENT OBSERVATIONS

The occurrence of a Lac$^+$ donor strain of *S. typhosa,* however, which is compatible with many species and strains of *Salmonella, Shigella,* and *Escherichia* was recently observed in our laboratory (28). This strain, isolated from a case of typhoid fever, was also able to confer donor ability on all hybrids which were selected for lactose utilization. Recent results have indicated that this strain behaves in a manner similar to the F^1 mutants which convey the Hfr character in *E. coli* as described by Adelberg and Burns (29) and Hirota (30). The experiments of these workers suggest that the F^1 factor is released from the Hfr chromosomal site and enters the cytoplasm of the cell from whence it can be transferred by contact to a recipient cell as is the usual F$^+$ factor. However, in the case of F^1, genetic markers such as lactose utilization, for instance, are carried along with the sex factor without further transfer of chromsomal genes. The marker or markers transferred would be due, presumably, to the retention of specific chromosomal segments by the F^1 factor.

Under proper conditions, the lactose positive donor strain of *S. typhosa,* ST-2, was also able to act as a recipient in crosses with the *E. coli* Hfr parent yielding diploid progeny similar in behavior to those described previously. In addition, strains of *Salmonella* previously sterile as recipients in crosses with *E. coli* became fertile in such crosses after acquisition of F^1 ability following a mating with ST-2.

Discussion

The experiments which have been discussed indicate that some species of the Genus *Salmonella* were capable of mating with *E. coli* strains. In addition, the chance isolation of a recipient strain of *S. typhimurium* exhibiting the frequency of a typical F$^-$ culture prior to selective recombination techniques has provided a useful tool for the study of *Salmonella* genetics.

In further studies, an extensive transfer of genetic material was observed in the case of intergeneric hybrids resulting from crosses between *E. coli* and *S. typhosa.* Such hybrids possessed the characteristics of unstable segregating diploid heterozygotes. This assumption was supported by the fact that the diploid form could not be purified to yield stable types of both parents, but segregated various forms which exhibited a loss of certain characteristics typical of *E. coli.* The results also are in agreement with the finding of Lederberg (18) that utilization of substrates is dominant to non-utilization since the heterozygotes were positive for arabinose, xylose, rhamnose, fucose, and indol production prior to segregation and reduction to the haploid form. Further confirmation of Lederberg's studies on the dominance of streptomycin-sensitivity (19) occurred when it was noticed that some diploids were not recovered when selections were made on media containing streptomycin in crosses with streptomycin-resistant recipient strains of *S. typhosa* and streptomycin-sensitive *E. coli.* These results demonstrated that the streptomycin-sensitivity of the *E. coli* Hfr parent was dominant to the streptomycin-resistance of the *S. typhosa* recipient in diploid hybrids.

If an oriented transfer of genetic material has taken place in these crosses, the order of entry of the markers would most likely be lactose (10^{-4}), then arabinose (10^{-5}), followed by the markers for xylose, rhamnose, indol, and fucose at lower frequency and only in the diploid forms. This result was determined from a consideration of the frequencies observed. Since only these *partial* diploids (not all dominant *E. coli* characteristics appear in the diploid form) are observed when matings are carried out on l-xylose and l-rhamnose (or l-fucose) containing media, utilization of these substrates can serve as a selective device for isolation of the diploid forms. Thus, probably as a lower limit, about 1% of the total recombinants which are selected on minimal lactose medium would be diploids and these are recovered selectively on xylose or rhamnose plates. The relatively stable variety as typified by strain X30D would occur at a considerably lower frequency.

It seems likely that unstable forms of enteric bacteria isolated from natural sources could be transient diploid forms or may possess extrachromosomal aberrations as a consequence of recombination. In nature, these types would probably arise most frequently from homozygous matings and, as a result, would be exceedingly difficult to study in the laboratory, in contrast to the diploid forms of *S. typhosa* considered here.

Experiments conducted recently in accord with this point of view have indicated that genetic recombination *in vivo* between *E. coli* and *S. typhimurium* can take place (31). With the mouse as the experimental animal, carrier states have been established in these mice from which various types of hybrids of *S. typhimurium* have been isolated consistently over reasonably long periods.

It has been suggested that genetic recombination and other similar processes of genetic exchange may be the means whereby intermediate groups of the Enterobacteriaceae have evolved (13). Genetic transfers between genera have been considered remarkable, unusual, or troublesome. These attitudes continue to prevail mainly because of the great faith of microbiologists who are concerned primarily with diagnostic or taxonomic procedures in the bacterial classification system. The value of these bacterial classification schemes presently in use is unquestioned, but the continual addition of new species of *Salmonella* and other enteric organisms perhaps should be re-evaluated, in terms of whether or not sexual mechanisms play a major role in the evolution of micro-organisms in nature or whether other mechanisms may be responsible. It is hoped that results of genetic studies, at any event, will be considered in the future classification schemes proposed for the Enterobacteriaceae.

It is possible, of course, and even likely that genetic studies of sexuality and hybridization in bacteria represent a sort of artificial and unnatural result in some instances. The observation that sexual compatibility occurs between *Salmonella* and *Serratia* (32), as well as a reported cross between *E. coli* and *Serratia* (33), may be a case in point. Nevertheless, the usefulness of such matings for continued genetic study, as well as the impetus exerted by previous results towards the discovery of other genetic phenomena among the microbes, is undoubted.

References

1. Tatum, E. L. and J. Lederberg, 1947. J. Bacteriol., *53:*673.
2. Lederberg, J., E. M. Lederberg, N. D. Zinder, and E. R. Lively, 1951. Cold Spring Harbor Symposia Quant. Biol., *16:*159.
3. Cavalli, L. L., J. Lederberg, and E. M. Lederberg, 1953. J. Gen. Microbiol., *8:*89.
4. Hayes, W., 1953. Cold Spring Harbor Symposia Quant. Biol., *18:*75.
5. Lederberg, J., 1956. J. Bacteriol., *71:*497.
6. Anderson, T., E. L. Wollman, and F. Jacob, 1957. Ann. Inst. Pasteur, *93:*450.
7. Cavalli, L. L., 1950. Boll. Ist. Sierotera, Milano, *29:*281.
8. Wollman, E. L. and F. Jacob, 1958. Symp. Soc. Exptl. Biol., *12:*75.
9. Lederberg, J., 1951. Science, *114:*68.
10. Zinder, N. D. and J. Lederberg, 1952. J. Bacteriol., *64:*679.
11. Lederberg, J. and P. R. Edwards, 1953. J. Immunol., *71:*232.
12. Lennox, E. S., 1955. Virology, *1:*190.
13. Luria, S. E. and J. W. Burrous, 1957. J. Bacteriol., *74:*461.
14. Lederberg, J., 1956. Proc. Nat. Acad. Sci. U. S., *42:*574.
15. Baron, L. S., W. F. Carey, and W. M. Spilman, 1959. Proc. Nat. Acad. Sci. U. S., *45:*976.
16. Baron, L. S., W. F. Carey, and W. M. Spilman, 1959. Science, *130:*566.
17. Baron, L. S., W. M. Spilman, and W. F. Carey, 1959. Bacteriol. Proc., pg. 29.
18. Lederberg, J., 1949. Proc. Nat. Acad. Sci. U. S., *35:*178.
19. Lederberg, J., 1951. J. Bacteriol., *61:*549.
20. Brinton, C. C., Jr., A. Buzzell, and M. A. Lauffer, 1954. Biochim. Biophys. Acta, *15:*452.
21. Brinton, C. C., Jr., 1959. Nature, *183:*782.
22. Brinton, C. C., Jr. and L. S. Baron, 1960. Biochim. Biophys. Acta, in press.
23. Craigie, J. and A. Felix, 1947. Lancet, *1:*824.
24. Lederberg, E. M. and J. Lederberg, 1953. Genetics, *38:*51.
25. Felix, A. and R. M. Pitt, 1934. J. Pathol. and Bacteriol., *38:*409.
26. Brinton, C. C., Jr., 1960. Personal Communication.
27. Dotti, F. and R. Casetta, 1959. Igiene Moderna, *52:*21.
28. Baron, L. S., W. F. Carey, and W. M. Spilman, 1959. Proc. Nat. Acad. Sci. U. S., *45:*1752.
29. Adelberg, E. A. and S. N. Burns, 1959. Genetics, *28:*57.
30. Hirota, Y., 1959. Genetics, *28:*75.
31. Schneider, H., S. B. Formal, and L. S. Baron, 1960. Bacteriol. Proc., pg. 73.
32. Carey, W. F., W. M. Spilman, and L. S. Baron, 1960. Bacteriol. Proc., pg. 73.
33. Mandel, M., L. Morton, A. D. Der Sarkisian, and F. A. Mohn, 1960. Bacteriol. Proc., pg. 187.

Conversion by Bacteriophage— A Factor in Bacterial Variation

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In recent years the role of bacteriophage in bacterial variation has become increasingly evident as reports of phage-mediated modifications termed transductions, special transductions, and conversions have accumulated. The purpose of the present paper is to give a brief account of one aspect of this area, that termed *conversion*. In this account emphasis has been placed on certain general features of the phenomenon, and the presentation has been oriented to view conversion in terms of the maintenance and expression of these phage-induced traits in a bacterial species. An intensive discussion of the relationship of conversion to other phage-mediated modifications has not been included since nothing can be added at this time to the efforts of Luria (1, 2) to unify these observations conceptually.

History and Definition

It has long been recognized that a bacterium lysogenized with a particular phage is endowed with new properties specifically related to the presence of that phage. Two such properties, which in fact define lysogeny, are the potential of a lysogenic bacterium to produce phage and the immunity of the bacterium to superinfection by homologous phage. The fact that these characteristics are apparently linked to maintenance of phage probably delayed general recognition of the potential role of phage in bacterial variation. However, Wollman's formulation of "phage as gene" (3) clearly pointed out the intimate nature of the phage-host association in lysogeny, and in 1936, Burnet and Lush (4), in assessing the significance of the specific immunity of a lysogenic bacterium, cited it as "a clear-cut example of the direct positive induction of change in bacterial character by the action of bacteriophage."

Craigie (5) reported that when *Salmonella typhosa* type A or E was grown in the presence of gamma phage, which he had isolated from a D1 strain, bacteria of the D1 type could be isolated from the resistant growth. These in turn were lysogenic for gamma phage. Craigie went on to say, "If the latter (gamma phage) can promote serological variation, either directly by infecting and modifying the bacterial cell, or indirectly by eliminating the serologically normal type of bacteria and thus permitting the emergence of a variant, salmonella phage must be studied systematically in relation to salmonella variation." Thus Craigie raised the question of phage-induced modifications once again, but his alternative hypothesis of mutant selection by phage was apparently a satisfactory hypothetical explanation of his observations and the first hypothesis of a phage-induced change was not tested.

Freeman (6) reported the isolation of toxigenic *Corynebacterium diphtheriae* following exposure of a nontoxigenic strain to a diphtheria phage and noted a one-to-one correlation between toxigenicity and lysogeny. Evidence accumulated (7, 8, 9, 10) which made it clear that toxigenicity was an induced property, causally related to the presence of specific phage in the lysogenic bacterium (11, 12) and more particularly to a specific portion of the phage genome (13). Because the property of toxin production bore no apparent relationship to phage survival, the correlation between toxigenicity and phage was generally accepted as an example of a specific and heritable modification of the host by the phage genome.

The term "conversion" or "conversion to toxigenicity" was applied to the *C. diphtheriae* phenomenon (8) as a means of avoiding continued repetition of the phrase "the change from the nontoxigenic to the toxigenic state." The term itself was intended to convey no other meaning than that given in the dictionary, *i.e.*, "an act which involves a change of state or character or form to another." However, the hypothesis relating to the process by which the change oc-

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curred soon became associated with the term, and in a general sense conversion has now come to mean (14) all modifications of the host cell resulting from infection by phage. For purposes of the present discussion conversion will be defined as the specific and heritable modification of a bacterium due to lysogenization with specific phage, the temperate phage involved in this process being described as a *converting* phage.

With conversion defined in this general manner, we may now turn to the specific features of the process (9, 15). 1) Conversion can be carried out by complete phage, *i.e.*, phage capable of lysogenizing the host strain amenable to conversion or reproducing in it via the lytic cycle. 2) Each plaque-forming unit of a converting phage strain has the potential of converting a sensitive host cell to the particular trait under study. 3) The ability of the phage to convert is inseparable from phage *per se* as evidenced by the simultaneous gain or loss of lysogeny (most sensitively tested as gain or loss of immunity) and the converted trait in the modified bacterium. 4) The cell in which the converting phage is produced need not differ genotypically from the cell which undergoes conversion. Thus converting phage can be produced directly through the lytic cycle in the same strain which can be converted by lysogenization. 5) Modification of the host appears to be the result of the addition of information to the host genome rather than a substitution, since the loss of phage returns the cell to its prelysogenic state.

General Considerations

Two general points will now be discussed. Formulated as questions, they are as follows: 1) What evidence is required to establish the phage-induced nature of a modification? 2) When are such modifications phenotypically expressed in relation to the phage life cycle?

Before it can be claimed that conversion has occurred, it is necessary to exclude the mechanism of mutant selection by phage. A one-to-one relationship between lysogeny and a given modification is a prerequisite, but is not in itself sufficient to establish the causal relationship between the two events. Modification might precede lysogenization, but in some manner correlate with the ability of the modified cell to be lysogenized. In this case, phage would not be converting, but rather selecting for the modification through its ability to lysogenize.

A decision on mechanism can be reached by determining what proportion of cells is simultaneously modified and lysogenized in a population exposed to phage. If the proportion of modified cells is high or if selective methods are available, it is simple to demonstrate that the modification is *not* present in the same proportion of the population prior to lysogenization and therefore the modification is phage-induced. There is a second more definite approach which also shows whether the modification is dependent on the continued presence of phage. This is to isolate from a modified, lysogenic strain clones which have either lost the modification or the phage and then determine whether the other characteristic has been lost simultaneously. The simultaneous loss of both phage and the modification followed by the demonstration that the reverted cell line can again be modified following lysogenization with the same phage is evidence for the occurrence of conversion. It should be pointed out in connection with the use of this method that a one-to-one correlation between loss of the modification and phage is characteristic of conversion. A one-to-one correlation between retention of the modification and loss of phage is suggestive of selection of a mutant by phage, while a correlation of less than one in either of the two situations is suggestive of transduction. Other criteria, for example the role of the genome of the phage-propagating strain, may also have to be employed in selecting between these mechanisms.

The second general point to be discussed is the phase of the phage life cycle in which the modification is expressed, as reflected by the state or form of phage at the time of expression. At the present time two forms of phage have been defined (16): *prophage*, the form in which phages are perpetuated in lysogenic bacteria, and *vegetative* phage, the form in which phage (or phage genetic material) multiplies during the lytic cycle of phage growth. Let us consider a strain of bacteria which has been converted. For such a strain three possibilities exist relative to the phenotypic expression of the phage-induced trait. First, it may be expressed when phage is in the prophage state, *i.e.*, by the lysogenic bacterium, the situation to which the term "lysogenic conversion" (17) is usually applied. Second, the trait may be expressed only in those cells in which vegetative phage appears, *i.e.*, those cells in which the process of induction has occurred leading to the transition from prophage to vegetative phage and eventual lysis. Finally it may be expressed in both states of phage development. It is clear that where

phenotypic expression of a converted trait occurs exclusively during the vegetative state, the modification exists only as a potential in a stable, lysogenic bacterium. In this case the potential is maintained by prophage, but expression of the trait demands a set of environmental conditions leading to induction and vegetative development of phage. It is important to stress that while in this instance the phenotypic expression of the trait could lead to the destruction of a large fraction of the converted population it would probably not lead to the extinction of the strain. The survivors, still lysogenic, would continue to transmit the same potential for expression to their progeny.

Specific Examples

SURFACE ALTERATIONS

The most revealing studies concerning conversion are those in which a specific antigenic modification has been detected following lysogenization. As a result of the work of a number of investigators (15,18,19,20,21) it has been established that the presence of some of the surface (0) antigens in the E and B groups of *Salmonella* is a consequence of lysogenization with specific phages. The study of Uetake, Luria, and Burrous (22) on the conversion of the somatic antigens of *Salmonella anatum* from 3, 10 to 3, 15 by phage ε^{15} is of particular interest in that the conversion can be detected whether phage is in the vegetative or in the prophage state. The observation that a virulent mutant of ε^{15} rapidly induces the formation of antigen 15 is convincing proof that phage in the vegetative state can also effect this modification. More recent observations by Uetake and Uchida (23) suggest that expression of this phage-induced trait may occur in still a third phage state, a nonreplicating state referred to by these authors as the prelysogenic state. Finally these authors have observed that phage states may not be equivalent in their conversion of surface antigens. They found that cells recently infected with phage $\varepsilon^{15}a$, a mutant of ε^{15}, are sensitive to phage $\varepsilon^{34}vir$, while stable, lysogenic cells carrying $\varepsilon^{15}a$ are resistant to the virulent phage. Clearly the cell with phage in the prelysogenic state differs from one which carries prophage.

A second type of modification in which a surface change probably occurs is that in which the ability to adsorb homologous phage is lost following lysogenization. This type of relationship was reported by Burnet and Lush (4) working with a staphylococcal phage-host system and more recently by Boyd (24), who employed the A3 and A4 phages of *Salmonella typhimurium*. Stocker (25), employing Boyd's phages, has shown that the adsorption rate for phage PLT22, a member of another group of phages, is also reduced following lysogenization. However, in none of the studies cited did the authors indicate whether this alternation in phage adsorption was accompanied by antigenic changes.

The study by Uetake, Luria, and Burrous (22) cited above is unique in demonstrating still another point. By utilizing the fact that a second phage, $\varepsilon 34$, adsorbs to and subsequently kills every cell which develops antigen 15, these authors were able to follow the development of phage ε^{15}-induced antigen 15. This permitted the detection of conversion at the level of the individual cell. As will be pointed out in the following section, the fact that a phage-induced modification must often be observed in populations of cells rather than in individual cells can complicate the interpretation of how the modification is produced.

MODIFICATION OF COLONIAL MORPHOLOGY

Phage-induced modifications of colonial morphology have been reported by several authors for strains of *Bacillus megatherium* (26,27). While the interpretation is that these changes are prophage controlled, *i.e.*, expressed while phage is in the prophage state, an alternative explanation has not been excluded. For example, induction and lysis of a portion of the cells in a lysogenic clone might result in the production of an enzyme which could alter colonial morphology by acting on a surface component of the stable, lysogenic cells. Two points emerge from consideration of the alternative hypothesis. The first is that a modification may reflect phage activity in only a portion of the total population, particularly if expression of the modification is initiated by phage in the vegetative state. The second is that the observed modification may be far removed from the primary events influenced by the activity of phage.

MODIFICATION OF NUTRITIONAL AND METABOLIC PATTERNS

Ehrlich and Watson (26) and De Carlo, Sarles, and Knight (28) observed that following lysogenization a strain of *B. megatherium* lost the ability to grow in a synthetic medium in which the sole nitrogen source was either glycine or serine. When grown on a complex medium containing 1% glucose or other carbohydrates, the

cultures rapidly reverted to the nonlysogenic state and recovered their ability to grow on the synthetic medium. This reversion favors the hypothesis that the nutritional modification was phage-induced, but the critical experiment, *i.e.*, reinfection of a reverted strain with simultaneous restoration of lysogeny and the modification, was not performed. It seems surprising that the phenomenon just described has been neglected, since this is one of the rare instances in which a nutritional modification with some accessible metabolic consequences may be prophage controlled.

A specific change in the biochemical potentialities of strains of *Corynebacterium belfanti* following lysogenization with a diphtheria phage was recently reported by Gundersen and Henriksen (29) and a similar change has been observed in our laboratory (unpublished results). Strains of *C. belfanti* do not produce diphtheria toxin, nor do they reduce nitrate to nitrite. However, when some were exposed to a phage capable of converting nontoxigenic *mitis* strains of *C. diphtheriae* to the toxigenic state, clones were isolated which had become lysogenic and had acquired the ability to produce toxin and reduce nitrate to nitrite. There was a perfect correlation between toxin production, lysogeny, and ability to reduce nitrate. The data are such that it is not possible to decide with certainty whether a conversion has occurred. However, since ability to produce toxin is known to be phage-induced, the experiments suggest that reduction of nitrate is similarly induced.

INTERFERENCE WITH HETEROLOGOUS PHAGE

It has often been observed that lysogenization with a specific phage leads to a modified response to heterologous phage. On rare occasions modifications may lead to true immunity to heterologous phage, but more often they lead to interference with the reproduction of the superinfecting phage followed by death of the host cell. Bertani (30) has recently reviewed the characteristics of heterologous interference. As background for this discussion it is only necessary to keep in mind that these modifications are not only dependent on the nature of prophage (31), but are also affected by the genotype and phenotype of the host (32) and the genotype (33,34) and phenotype of the superinfecting phage.

The relation of heterologous interference to phage typing illustrates the significance of this type of phage-induced modification. Some time ago Craigie suggested that type determination in *Salmonella*, that is, the response of a bacterial strain to a series of typing phages, might be related to the phages carried by that strain. In recent years (35) it has been demonstrated that phages serologically unrelated to the Vi II typing phages do determine, in a number of instances, the phage sensitivity of the bacterium carrying them. The isolation of what have been termed "degraded" strains of *Salmonella*, some of which have lost their phage and have reverted to the phage type they exhibited prior to lysogenization, supports the notion that type specificity can be phage-induced. It is reasonably certain that the phage-induced modification which interferes with the reproduction of some of the Vi typing phages is not due to a surface alteration of the cell. This conclusion is based on the observation that all typing phages adsorb to all strains and that no antigenic changes have been detected in any of the Vi cell types. Those cells which fail to produce phage are killed.

Other cases of modifications affecting heterologous phage which are almost certainly phage-induced are as follows: 1) the immunity to phage P2 conferred on a *Shigella* strain lysogenized with phage P1 (31), 2) interference with coliphage reproduction following lysogenization of a *Shigella* strain with *Shigella* phages (31,32), and 3) interference with the ability of the rII mutants of coliphages T2, T4, and T6 to reproduce in *Escherichia coli* K12 following its lysogenization with lambda phage (33).

HOST-INDUCED MODIFICATIONS

Brief consideration will now be given to the relation of conversion to the phenomenon of host-induced modification. Host-induced modification has been defined (16) as a variant property of phage strictly determined by the kind of bacterium in which it last grew. Since the variant property is lost after one cycle of growth in some other host, these modifications are considered phenotypic variations imposed on the phage by the host.

Two examples in which the host-induced modification of the infecting phage may be controlled by the phage already resident in the host were provided by Lederberg (32). In the first example *E. coli* B and a strain of *Shigella dysenteriae*, both sensitive to coliphage T1, became largely immune to T1 when lysogenized with phage P1. However, a small proportion of the lysogenic cells (10^{-3}) were able to produce some phage which could now reproduce in the lysogenic as well as the nonlysogenic strains. This "unre-

stricted" phage returned to its restricted state on passage through *E. coli* B. Thus lysogenization with P1 seems in some way to be related to the production of unrestricted phage. The fact that induction of the P1-carrying host cells failed to increase the frequency of cells which produced the modified phage suggests that the effect is prophage mediated.

In the second case studied by Lederberg, phage P2 was modified by its passage in *Shigella* lysogenized with phage P1 in a manner similar to T1. The interesting feature of this study was that while P2 also undergoes a recognizable modification in the nonlysogenic *Shigella* this is different from the modification it undergoes in the same strain lysogenized with P1. Thus it seems likely that lysogenization with P1 altered the modification which the host could impose on the infecting phage.

CONVERSIONS CORRELATED ONLY WITH THE VEGETATIVE STATE OF PHAGE

Exclusive of the events related directly to phage synthesis as such, there are two phage-induced modifications which have been described for established lysogenic strains which are exhibited *only* when phage is in the vegetative state, or at least some state which is not prophage, *i.e.*, following induction. It should be reiterated that in these instances cells lysogenized with the appropriate converting phage perpetuate the potential for the modification in the prophage.

The first of these has already been discussed in some detail, that of conversion to toxigenicity of *C. diphtheriae*. The work of Barksdale (14) strongly supports the position that diphtheria toxin is produced only when a converted strain of *C. diphtheriae* is induced and lyses. The second example is the production of a lysin (or lysins) following the induction of *E. coli* K12 carrying phage lambda (36,37). It seems likely that many other modifications which have been recently described will eventually be included in this group. These include the polysaccharide depolymerase of *Klebsiella pneumonia* (38), the lysins of streptococci (39,40), staphylococci (41) and *B. megatherium* (42), and the hyaluronidases of streptococci (43), all produced concurrently with phage reproduction. These latter modifications have been observed only following infection from without and usually with a virulent phage. It is reasonable to expect that temperate phages will be found which will produce the same modification following induction of a bacterium lysogenized with them. However, it would be well to keep the reservation in mind that infection from without may not always be equivalent to induction and lysis in terms of conversion of the infected cell.

Summation

When a given bacterium is infected by a temperate phage, one of two events will transpire. First the phage may reproduce vegetatively, leading to lysis of the bacterium and the release of new phage. Alternatively the phage may stabilize in the prophage state and produce a lysogenic bacterium. From the viewpoint of bacterial variation the first event is a selective event, one in which the phage acts to destroy sensitive bacteria. The second event is one in which a new genetic entity, the lysogenic bacterium, has been formed. In this case a bacterial variant has been produced and therefore a conversion has occurred.

In the simplest case of conversion, lysogenization leads to two recognizable changes in the bacterium, immunity to homologous phage and the potential to produce phage. In addition to this it is recognized that other modifications, some not obviously related to phage maintenance or reproduction may be causally related to the presence of the phage. These modifications may be expressed in the stable, lysogenic cell or may be observed only after induction leading to lysis, or both. The possible relationships between the phage state and an observed modification have already been discussed in detail.

It is important to know whether the frequency with which conversions occur in nature is high enough to be considered significant in bacterial variation. If we take frequency of lysogeny as a crude measure of conversion, then we would admit to an impressive role for phage in variation. In one specific case studied, that of conversion to toxigenicity, an examination of 56 toxigenic strains revealed that 25 of them (46%) were lysogenic for a converting phage (44). It seems reasonable to assume that this represents the minimum number in which toxigenicity was governed by converting phage in this particular sample. This type of analysis strengthens the concept of conversion as an important factor in bacterial variation; however, it fails us in one sense. If we assume that phage is endogenous in origin, then it is not surprising to find that many strains of bacteria are lysogenic and polylysogenic and that phage contributes to variation both at the time of its origin and throughout the history of the bacterial strain. However, we would also like to

know how frequently these infective bearers of heredity actually cross from one bacterial strain to another under natural conditions. Data are so limited (45) that an assessment of this point cannot be made at the present time. Until this information is obtained, our evaluation of converting phage as a factor in bacterial variation will remain incomplete.

References

1. Luria, S. E., 1958. Cold Spring Harbor Symposia Quant. Biol., *23:*71.
2. Luria, S. E., 1959. Brookhaven Symposia in Biology, *12:*95.
3. Wollman, E., 1928. Bull. Inst. Pasteur, *26:*1.
4. Burnet, F. M. and D. Lush, 1936. Austral. J. Exp. Biol. and Med. Sci., *14:*27.
5. Craigie, J., 1946. Bact. Rev., *10:*73.
6. Freeman, V. J., 1951. J. Bacteriol., *61:*675.
7. Groman, N. B., 1953. Science, *117:*297.
8. Groman, N. B., 1953. J. Bacteriol., *66:*184.
9. Groman, N. B., 1955. J. Bacteriol., *69:*9.
10. Barksdale, L. W. and A. M. Pappenheimer, Jr., 1954. J. Bacteriol., *67:*220.
11. Barksdale, L. W., 1955. Comptes rendus, *240:*1831.
12. Groman, N. B. and M. Eaton, 1955. J. Bacteriol., *70:*637.
13. Groman, N. B., M. Eaton, and Z. Booher, 1958. J. Bacteriol., *75:*320.
14. Barksdale, L. W., 1959. Bact. Rev., *23:*202.
15. Uetake, H., T. Nakagawa, and T. Akiba, 1955. J. Bacteriol., *69:*571.
16. Adams, M., 1959. *Bacteriophages.* Interscience, New York, 592 pp.
17. Lederberg, J., 1955. J. Cellular Comp. Physiol. Suppl. 2, *45:*75.
18. Iseki, S. and T. Sakai, 1953. Proc. Japan. Acad., *29:*121.
19. Terada, M., T. Tomu, and K. Kurosaka, 1956. Virus (Osaka), *6:*274.
20. Baron, L. S., S. B. Formal, and O. Washington, 1957. Virology, *3:*417.
21. Harada, K., 1959. Japan. J. Microbiol., *3:*61.
22. Uetake, H., S. E. Luria, and J. W. Burrous, 1958. Virology, *5:*68.
23. Uetake, H. and T. Uchida, 1959. Virology, *9:*495.
24. Boyd, J. S. K., 1954. Nature, *173:*1050.
25. Stocker, B. A. D., 1958. J. Gen. Microbiol., *18:*IX, *Abst.*
26. Erlich, H. L. and D. W. Watson, 1949. J. Bacteriol., *58:*627.
27. Ionesco, H., 1953. Comptes rendus, *237:*1794.
28. De Carlo, M. R., W. B. Sarles, and S. G. Knight, 1953. J. Bacteriol., *65:*53.
29. Gundersen, W. B. and S. D. Henriksen, 1959. Acta Pathol. Microbiol. Scand., *47:*173.
30. Bertani, G., 1958. Advances in Virus Research, *5:*151.
31. Bertani, G., 1953. Cold Spring Harbor Symposia Quant. Biol., *18:*65.
32. Lederberg, S., 1957. Virology, *3:*496.
33. Benzer, S., 1955. Proc. Nat. Acad. Sci. U. S., *41:*344.
34. Zinder, N. D., 1958. Virology, *5:*291.
35. Andersen, E. S. and R. E. O. Williams, 1956. J. Clinical Pathol., *9:*94.
36. Panijel, J. and J. Hupert, 1957. Comptes rendus, *245:*240.
37. Jacob, F. and C. Fuerst, 1958. J. Gen. Microbiol., *18:*518.
38. Adams, M. and B. Park, 1956. Virology, *2:*719.
39. Krause, R. M., 1957. J. Exp. Med., *106:*365.
40. Maxted, W. R., 1957. J. Gen. Microbiol., *16:*584.
41. Ralston, D. J., M. Lieberman, B. Baer, and A. P. Krueger, 1957. J. Gen. Physiol., *40:*791.
42. Murphy, J. S., 1957. Virology, *4:*563.
43. Kjems, E., 1958. Acta Pathol. Microbiol. Scand., *44:*429.
44. Groman, N. B. and R. Memmer, 1958. J. Gen. Microbiol., *19:*634.
45. Anderson, E. S., 1957. "The Relations of Bacteriophages to Bacterial Ecology." In *Microbial Ecology.* Seventh Symposium of Soc. for Gen'l. Microbiol., pp. 187-217. Cambridge University Press, Cambridge, England.

Microbial Genetics and the Synthesis of Proteins

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One of the charming features of the development of microbial genetics is the informality of the meetings that have been held. The fields covered include not only phage and microbial genetics, but also such subjects as adaptive enzyme formation.

These topics reflect two major problems of great interest in biology these days: (1) How does DNA make more DNA? (2) How does DNA bring about the synthesis of protein?

I will summarize my opinions on the status of research on the second of these questions with special emphasis on enzymes that have been called "adaptive," "inducible," or "repressible." It is these enzyme systems that have furnished a key to our understanding of how DNA might make protein.

For some time it has been acknowledged that bacterial variation has its origin in mutation and selection. But this is only a first approximation of the truth. It has also been known that bacteria can adapt in a physiological sense to a changed medium. That is to say, they can alter their enzymatic composition. One of the classic cases is that of the formation of the enzyme β-galactosidase that *E. coli* bacteria need in order to use lactose.

Normally if lactose is not present in the medium, the bacteria do not make the enzyme. When lactose is added to the medium, they are stimulated somehow to make the enzyme that enables them to use lactose as a substrate. There are other enzyme systems which seem to be controlled in another kind of way. For example, normal *E. coli* bacteria can make all the amino acids they need. But if you feed them an amino acid, they stop making that amino acid and utilize instead the supplied amino acid.

Two mechanisms seem to operate in the cessation of the manufacture of the amino acids. In the first place the added (exogenous) amino acid appears to suppress or inhibit the *functioning* of the enzyme already present in the cells. Secondly, the *formation* of the enzyme is also repressed. We therefore see that an exogenously added substrate, for instance the sugar lactose or the amino acid arginine, can change the enzymatic composition of a bacterium. That is, a bacterium may or may not manufacture β-galactosidase and still be genetically the same bacterium, or a bacterium may or may not have the enzymes needed to make arginine and still be genetically the same bacterium. In one case it depends upon the presence or absence of lactose in the medium, in the second case the presence or absence of the amino acid arginine in the medium.

For the last 10 or 15 years phenomena like these have posed important questions: How can these bacteria regulate the rate at which they form these enzymes? How can an added nutrient like lactose act as an inducer to stimulate the formation of the enzyme β-galactosidase? Or, on the other hand, how can an added amino acid like arginine act as a repressor to inhibit the formation of the enzymes ordinarily involved in its manufacture?

A classic example of a repressible enzyme is that studied by Maas and Gorini (1). Here an enzyme, involved ultimately in the synthesis of arginine, converts ornithine and carbamyl phosphate into citrulline. But the rate of formation of this enzyme, called ornithine transcarbamylase, is controlled not by the presence of citrulline, but by the end product of this biosynthetic pathway, arginine.

In fact, it has been shown that arginine controls the formation of each of the enzymes involved in its own formation. An experiment performed by Maas and Gorini demonstrates this repression very nicely. They grow bacteria in medium containing arginine, whereupon the bacteria stop making a particular enzyme involved in the manufacture of arginine. These bacteria are then transferred to medium not containing arginine. The amount of enzyme in the cells is plotted as a function of time after transfer to the medium without arginine. Immediately after this transfer the amount of enzyme in the cells starting at zero begins to rise very rapidly and then falls downward to a plateau.

We understand this curve as follows: The bacteria before transfer, having grown in the presence of arginine, do not contain arginine. After transfer to argininelessmedium, the bacteria start making arginine. This arginine soon fulfills the arginine requirement of the cell and a slight concentration of arginine builds up within the cell. This then suppresses the rate of formation of the enzyme. So the rate of formation falls off and the enzyme concentration, plotted per cell, falls as the cells grow, since cell growth dilutes any enzyme that is present. The concentration of the enzyme falls to just that level which supplies arginine at the rate needed by the bacteria in this particular medium. This regulatory mechanism for the control of the rate of arginine formation is a feedback type, preventing the cell from making excess arginine.

The mechanism is also of interest to us because we would like to know how an end product like arginine can interfere with the production of an enzyme. Many enzymes involved in the production of a wide variety of amino acids and pyrimidines have been studied, and many cases are now known where the end product in a biosynthetic pathway represses the formation of the enzymes needed in that particular pathway.

For the last 20 years another kind of enzyme has been extensively studied, the so-called adaptive or inducible enzyme. β-galactosidase, an enzyme needed by bacteria to consume the sugar lactose, is one of the best studied cases. Bacteria placed in lactose-containing media will form β-galactosidase, while in the absence of lactose they will not normally form the enzyme. The work of Monod and Melvin Cohn in Paris and more recently of Jacob and Pardee (2) has given an even deeper understanding of how the synthesis of this enzyme is controlled. Rather early, Monod and his group discovered that not only did lactose induce the formation of β-galactosidase, but a wide variety of synthetic galactosides could also serve as inducers. For example, a now well-known analogue of lactose is the sugar thiomethyl-β-galactoside, having the thiomethyl group in place of glucose. This substance is not a substrate of β-galactosidase but is a very potent inducer of its formation. That is, bacteria placed in a medium containing this substance, TMG for short, immediately begin to synthesize β-galactosidase. Monod, Cohn, and Hogness (3) showed that the induction of the formation of this enzyme is not the conversion of some pre-existent protein or precursor into β-galactosidase but represents the *de novo* formation of the complete protein from amino acids following the addition of inducer to the medium.

Let me describe a typical experiment. A flask of growing bacteria is taken at some predetermined time, TMG is added to the medium, and the enzyme concentration is measured. This enzyme turns out to be a very easy one to measure because of a synthetic substrate o-nitrophenyl galactoside (ONPG) which yields o-nitrophenol upon hydrolysis, the color of which is measured and the rate of the reaction thus determined.

The kinetics of the formation of β-galactosidase can be plotted in many ways. I will plot the enzyme per cell as a function of time after the addition of inducer. From time to time, samples are removed from the flasks, their enzyme content measured as well as the number of bacteria present at that time. The enzyme content is divided by the number of bacteria to get a measure of the enzyme per cell. It is observed that this rises linearly to begin with and then more slowly as it approaches a constant value.

We can calculate from this curve what happens to the rate of formation of enzyme per cell, *i.e.*, the rate at which each cell makes enzyme. How does this change with time? Prior to zero time the rate is almost zero, barely detectable; but upon addition of inducer the rate rises abruptly to a constant value. One might say that the addition of TMG at zero time opened a valve all the way, permitting enzyme to be made at a constant rate thereafter.

It was discovered, however, that the system is a little more complicated than I have described. Jacques Monod and his collaborators in Paris (4) discovered that the metabolism of lactose involves not only the enzyme β-galactosidase, but also a specific transport mechanism for bringing lactose into the cell. This mechanism, called permease by its discoverers, is also inducible, being made only when lactose is present in the medium.

The existence of the inducible permease produces some curious effects on the kinetics of β-galactosidase induction. One aspect is seen in an observation we once made which seemed very puzzling in that it appeared to provide an example of induced inheritance (5).

Let me describe the experiment and then explain how it can be understood on the basis of the presence of permease. The experiment is this: We take a culture of a strain of bacteria and divide it into two parts. To one part we add TMG, to the other nothing. The cultures are in-

cubated for 20 minutes or so, which for this particular medium is only a third of the doubling time. Subsequently they are both transferred to medium with a very low concentration of TMG. Thus, both cultures are grown under identical conditions, and except for the twenty-minute treatment both have had identical histories.

We measure the β-galactosidase in these cultures and we observe a strange thing. In the culture which was exposed briefly to high TMG, the enzyme quickly rises to some maximum value and remains high and constant. The enzyme in the other culture remains very low. We continue to make transfers every day for some 200 bacterial generations. The difference in level of β-galactosidase is maintained with no sign of change throughout this time.

It would appear that some alteration in the heredity of the cultures has been induced by the pretreatment. In fact, one could well say that a mutation has been induced. But, as I said earlier, this strange phenomenon is a consequence of the presence in these bacteria of an inducible permease for the transport of galactosides into the cell.

In the one case the short exposure to TMG is of sufficient duration to stimulate formation of galactoside permease. Enough permease is formed by these cells to effect the build up of a high internal concentration of TMG despite the low external concentration. These bacteria maintain a high internal concentration and may therefore continue permease production at a high rate. In the other case the bacteria do not have sufficient permease to maintain the concentration of inducer high enough to induce significant quantities of permease and β-galactosidase. Apparently there is a critical level of permease, which if exceeded will forever maintain a high level of permease at the low concentration of inducer.

Of course the experiment the bacterial geneticist would have demanded at this point would have been to transfer the two cultures from the low TMG to the absence of TMG for a few generations and then return to the low concentration of TMG. When this is done, in both cases the enzyme level comes to the same low value. Once again the bacteria are the same.

But now, let us leave this case of apparent inducible alteration of heredity and return to the central problem of the control of protein synthesis. There are genes that control the ability of *E. coli* bacteria to utilize lactose. Our understanding of the nature of this control comes from the brilliant work of the group around J. Monod and F. Jacob at the Institut Pasteur in Paris (6).

There appear to be a number of genes involved in lactose utilization. First, there is a gene which is given the symbol z, which seems to be responsible for the formation of β-galactosidase. Second, there is a closely linked gene, called y, which is responsible for the formation of galactoside permease. Normally the wild-type bacterium is by convention indicated by z^+y^+. It can make β-galactosidase and permease. There are mutants, for example z^-y^+ or z^+y^-. The first cannot make β-galactosidase although it can make galactoside permease. The latter can make β-galactosidase but it cannot make galactoside permease. It was discovered that there is a third gene in this system, called i, which controls the need for inducer. The wild type, i^+, makes enzyme only in the presence of inducer, and there are mutants, i^-, which make β-galactosidase even in the absence of inducer. These mutants are said to be constitutive. Very recently a fourth gene in this system has been discovered by the French group. This gene, called o, is also very closely linked to z, y, and i, and I will discuss its function shortly.

The fact that these genes are closely linked is remindful of the discovery of Demerec and his colleagues of several similar cases where a group of genes controlling the various steps in a given biosynthetic pathway are closely linked. Using transduction as a technique for genetic mapping, Demerec (7) discovered in *Salmonella* that the genes controlling four enzymatic steps in tryptophan synthesis are arranged in a close group. Likewise, Hartman (8) discovered the same phenomenon for histidine, and Lederberg and Kalckar (9) found the same phenomenon for the enzymes involved in galactose metabolism. By now there are probably many other cases where a set of genes controlling enzymes involved in the same biosynthetic pathway are for some curious reason all closely linked.

Paralleling the studies of induced enzyme synthesis like β-galactosidase were studies of other enzymes apparently under the control of a completely different kind of mechanism. In these cases enzyme synthesis is controlled by a repressor. In the presence of repressor enzyme synthesis is inhibited; in its absence synthesis is maximal. One case, described earlier, is that of ornithine transcarbamylase, an enzyme catalyzing one of the steps in arginine synthesis. This enzyme is not formed in the presence of appreciable con-

centrations of arginine, but it is formed at a high rate at very low concentrations of arginine.

Thus, there appeared to be two kinds of enzymes, inducible and repressible. A number of people, Werner Maas particularly, began to wonder whether both kinds really exist. Perhaps there are only inducible enzymes, and in the case of repressible enzymes, repressor inhibits synthesis by antagonizing a normally present inducer. Alternatively, it was thought that possibly inducible enzymes are really the same as repressible enzymes in that synthesis is normally blocked by a normally present repressor and inducers stimulate synthesis by antagonizing the action of the repressor.

It was possible finally to distinguish between these two alternatives. With a brilliant experiment—now referred to as the Pa-Ja-Mo or "Pajama" experiment—Pardee, Monod, and Jacob (2) were able to show that in the case of β-galactosidase, in the absence of inducer, synthesis is blocked by a repressor. Thus, inducer acts by antagonizing this normally present repressor. Hence the synthesis of both β-galactosidase and arginine transcarbamylase are "repressible." The β-galactosidase constitutive mutant, i^-, is apparently unable to make the repressor and as a result makes enzyme in the absence of inducer.

The experiment performed by Pardee, Monod, and Jacob which led to this conclusion is briefly the following: They took advantage of the fact, discovered by Wollman and Jacob (10), that during bacterial conjugation the genetic material donated by the "male" (Hfr) parent appears to enter the "female" (F^-) cell in some linear order, the time of entry being directly related to the position on the genetic map. They were able to show this by disrupting mating at various times by placing the mating bacteria in a Waring Blender. On the basis of these discoveries it is possible to ascertain the time at which a given gene enters the F^- cell. Thus, they knew for a particular pair of strains at which time the z gene entered the F^- strain.

They proposed and did the following experiment. It should be recalled that in a cross the Hfr parent donates only genetic material, and the F^- parent provides all of the cytoplasm as well as its own genetic material. They made the following arguments: Consider a cross between an Hfr bacterium which is i^+z^+ and an F^- which is i^-z^-. The Hfr does not make β-galactosidase in the absence of inducer since it is i^+. The F^- parent would make enzyme in the absence of inducer but does not, since being z^- it is unable to make β-galactosidase under any condition. So neither of these bacteria have β-galactosidase. When a zygote is formed from these two strains, what should one expect on the basis of the two hypotheses? That the inducible fails to make enzyme because it lacks the inducer or that the inducible strain fails to make enzyme because of the presence of a repressor? When the z^+ gene enters the F^- "constitutive" cytoplasm, it should begin to function and make β-galactosidase on either hypothesis since in one case an inducer would be present and in the other a repressor would be absent. This is what is observed. Apparently from the moment of entry of the z^+ gene, synthesis of β-galactosidase occurs at constant rate. This can be conveniently seen from a plot of the enzyme per zygote present as a function of time since entry of the gene. In such a plot the enzyme per zygote rises from zero time along a straight line. However, after a half-hour or so, there is a fall in the rate of synthesis until synthesis essentially ceases. This cessation of enzyme synthesis, they argued, could be explained by the hypothesis that synthesis in the wild type (i^+) does not occur in the absence of added inducer because of the presence of a repressor normally made. According to this view the z^+ gene when it enters the F^- cytoplasm brings about the synthesis of β-galactosidase because of the absence of a repressor in this i^- cytoplasm. However, along with the z^+ gene the i^+ gene is also introduced, and it leads to the formation of repressor. Presumably, by thirty minutes sufficient repressor is formed to inhibit synthesis. The alternative hypothesis cannot so readily explain these results. Another experiment which supports this interpretation is the reciprocal cross where an i^-z^- Hfr is mated with i^+z^+ F^- parent. Again both hypotheses have the same initial expectation; both predict that upon entry of the i^-z^- genes into the i^+z^+ cytoplasm no β-galactosidase should be made. However, if the i^- gene made an inducer, then after a while enzyme should be synthesized. Since no such synthesis is observed, this was taken as support for the view that the i^+ gene determines the formation of repressor and that the i^- gene does not give rise to repressor. It is, of course, much more satisfying to imagine that the wild type loses an ability when it mutates to i^- than to assume it gains a new ability, as would have to be assumed if i^- determined an inducer.

Recently a paper was published by the Monod-Jacob group in the Comptes rendus (6) which describes the discovery of still another gene which

enters into the control of β-galactosidase formation. These observations have led them to propose a model for the control of protein synthesis which not only accounts for all of the observations seen with the β-galactosidase system, but also explains many other observations. I described earlier that frequently genes concerned with related biochemical functions are closely linked on the chromosomes. A control for these is also explained by the Monod-Jacob mechanism.

Let me first give their model and then I will describe the experimental evidence. The new gene they call the operator or *o* gene. This gene controls the operation of a block of genes which they call the operon. In the present case the operon would include the *z* and *y* genes, whose functions are to supply the information for the structure of β-galactosidase and galactoside permease, respectively. The operator gene is like a switch—it either turns on the synthesis of β-galactosidase or galactoside permease or turns their syntheses off. Since both *z* and *y* are in the same operon, they are turned off or on together. They propose that the gene is itself controlled through the action of the *i* gene. That is, the *i* gene makes the repressor which acts upon the *o* gene. In the presence of repressor, the *o* gene is turned to the off position and no enzyme is made. In the absense of repressor the *o* gene is turned on and enzyme synthesis proceeds at maximum rate. Hence, the *o* gene is controlled by the *i* gene, but through the action of an agent in the cytoplasm, the repressor. The *o* gene, however, seems to act directly on the operon and not via an agent in the cytoplasm. Thus, in a heterozygote the behavior of a given operon is determined by the *o* gene adjacent to it and not by the *o* gene on the other chromosome.

To explain the action of inducer they suggest that it acts somewhere along the path of action of repressor, either at the point of action on the *o* gene or somewhere earlier in the chain of events leading to *o* gene function. This could take place actually at the gene or at some product of the gene which carries out its function.

They offered as evidence for this picture the fact that two kinds of mutations are known which can be ascribed to changes in the *o* gene. One is called o^c and the other o^o. The action of the former mutation seems to cause the switch (*o* gene) to be stuck in the closed ("on") position, while the latter acts as though the switch is stuck in the open ("off") position. When the operator is locked in the closed position, the operon functions independently of the presence of inducer or repressor. Thus an o^c mutant is a constitutive mutant. But it is a different kind of constitutive mutant than the i^- mutant because it is now a dominant mutant.

The mutant allele i^- is recessive to i^+ because the *i* gene acts through the cytoplasm. But the *o* gene acting directly on the adjacent operon is not affected by the state of the *o* gene on another chromosome in a heterozygote. Similarly, the o^o mutant is one in which the operon is rendered nonfunctional independent of the presence of repressor or inducer. Thus an o^o mutant appears as double negative, being unable to make β-galactosidase or permease. Such a mutant is thought not to be a deletion since it does mutate back to wild type. Also offered as support is the fact that this interpretation provides a simple explanation for a number of puzzling cases of pleiotropism. For example, there are point mutations which at once result in the loss of ability to make a number of the enzymes involved in galactose metabolism. Such mutations would thus be explained as operator mutations.

Much of the evidence supporting the picture proposed by Monod and Jacob comes from studies on the properties of certain kinds of diploids made by introducing genes associated with a transferable fertility factor. It was discovered by Jacob and Adelberg (11) that genes can be transferred not only by transformation, by transduction, and by conjugation but by even another method which has been called sexduction, or F^- duction. There is a fertility factor, F^+, in *E. coli* bacteria which can act as infective agent. In certain types of bacteria the *F* factor has associated with it a group of bacterial genes and transfers these genes with it when it enters a cell. The suitable diploids were prepared by using a strain in which the *F* factor carried the genes *i o z y*. An F^- bacterium infected by the *F* factor from this strain is then diploid for the genes *i o z y*. Such diploids were prepared using various combinations of the alleles of these genes and their enzyme content compared with theoretical expectations. In the present discussion I will omit discussion of the gene *y* since *z* will illustrate the argument. Use was made of an allele of *z*, which I will call z^m, characterized by making a modified form of β-galactosidase which lacks enzyme activity but which is recognizable serologically.

All of the diploids constructed had the properties predicted by the model. Note, for example, the results given in Table 1, which I have pre-

pared from the Jacob, Perrin, Sanchez, and Monod paper. In the first line it is seen that the haploid wild type makes enzyme only when inducer is present and makes no detectable cross reacting (CR) protein. In the second case, in the absence of inducer neither enzyme nor CR protein is made since the presence of the i^+ gene represses both o^+ genes, and both are made when inducer is present. In the third line, in the absence of inducer enzyme is made because there is an o^c gene adjacent to a z^+, but no CR protein is made because the o^+ gene adjacent to the z^m gene is repressed by the i^+ gene on the other chromosome. Both again are made when inducer is present. The last two lines also show that the genes adjacent to o^c function whether inducer is present or not, while those adjacent to o^+ function only if the cytoplasm lacks repressor or inducer is present.

Returning to the Pajama experiment, we find there is one puzzling aspect that is not easy to reconcile with current ideas of how proteins are made. This is the fact that in the first cross discussed, i^+z^+(Hfr) $\times$ $i^-z^-(F^-)$, the rate of enzyme formation reaches its maximum value very soon —within 3 or 4 minutes—after entry of the z gene. What does this mean? Well, it could mean that the gene makes the enzyme and no new apparatus need be made, since once the gene is in it would make the enzyme at maximum rate. Of course, this leaves unexplained what all the other things in the cell are doing. What about all the ribosomes, so frequently implicated as the site of protein synthesis? But if we try to invoke some kind of intermediate like the ribosome in the formation of the enzyme then we have to understand why the rate of formation reached its maximum value so quickly. In other words, if the rate of synthesis increases so rapidly for three minutes, why does the increase stop so soon; why does it not go on rising? Well, it would tend to stop, it would come to a steady state value, if the enzyme-forming apparatus were unstable for one reason or another. If it had a mean life of something like about two or three minutes, one would expect, despite the rapid formation of these units, that since it is unstable, finally a balance would be reached, a steady state where the number of active units remains constant.

It is a little unpleasant to imagine that it is the ribosomes that are so unstable because this would mean that most ribosomes in the cell would be inactive. For example, if the generation time were 100 minutes and the ribosomes lived only three minutes, 97% of the ribosomes present would be inactive. The cell can hardly afford to be that wasteful because there are only about five or ten thousand ribosomes altogether in a cell. If 97% were no good, only 300 would be good ones, and there must be more than 300 different enzymes in the cell which can be made at any time. But it presents an interesting question to be studied, whether at any given instant a single cell can make all possible enzymes.

Thus, there is a really major puzzle as to how this enzyme is formed. A number of theories can be made to account for the paradoxical kinetics just discussed, but none of them are very happy theories. With the very powerful tools provided by bacteria, by viruses, and by modern biochemi-

Table 1. AMOUNT OF ACTIVE AND INACTIVE (CR) PROTEIN PRESENT IN UNINDUCED AND INDUCED STRAINS OF *E. coli* OF DIFFERENT GENOTYPES

Genotype	*Uninduced bacteria*		*Induced bacteria*	
	β-galactosidase	CR protein	β-galactosidase	CR protein
$i^+o^+z^+$	<1	.....	100	.....
$i^-o^+z^m/i^+o^+z^+$	<1	*nd*	320	100
$i^-o^+z^m/i^+o^cz^+$	36	*nd*	270	100
$i^+o^+z^m/i^+o^cz^+$	110	*nd*	330	100
$i^+o^+z^+/i^+o^cz^m$	<1	30	100	400

The β-galactosidase content and the amount of inactive β-galactosidase (CR protein) determined serologically are given in arbitrary units for a number of different genotypes when grown in the absence of inducer (uninduced) and when grown in the presence of inducer (induced). *Nd* means not detectable. Data are taken from Jacob, Perrin, Sanchez, and Monod (6).

cal and physical chemical techniques, I suspect it will not be long before we have an explanation of this paradox and possibly gain an understanding of what intermediates are actually involved in the synthesis of proteins and how the rates of synthesis are regulated.

Note added in proof:

The paradoxical kinetics are explained by recent discoveries by Jacob, Brenner, and Meselson (personal communication) and by Gros (personal communication). Their experiments lead them to propose that the gene (DNA) makes an RNA molecule which contains the information for the structure of the enzyme. This "messenger" or "tape" RNA affixes itself to a ribosome, where protein synthesis occurs. The tape RNA is destroyed in the process of synthesizing a relatively small number of protein molecules (quite possibly only one). In this view, the ribosome is nonspecific and is the vessel where any protein can be made, depending on which tape RNA is present. On the basis of this picture, the rate of enzyme synthesis would reach its maximum value in a time given by the lifetime of the tape RNA. Since the rate reaches its maximum in a short time, it is assumed that the tape RNA is inactive after making a relatively small number of enzyme molecules.

References

1. Gorini, L., and W. K. Maas, 1957. Biochim. Biophys. Acta, *25:* 208-209.
2. Pardee, A. B., F. Jacob, and J. Monod, 1959. J. Molecular Biol., *1:* 165.
3. Hogness, D. S., M. Cohn, and J. Monod, 1955. Biochim. Biophys. Acta, *16:* 99.
4. Cohen, G. N., and J. Monod, 1957. Bact. Rev., *21:* 169-194.
5. Novick, A., and M. Weiner, 1957. Proc. Natl. Acad. Sci. U. S., *43:* 553-566.
6. Jacob, F., D. Perrin, C. Sanchez, and J. Monod, 1960. Comptes rendus, *250:* 1727-1729.
7. Demerec, M., 1956. Cold Spring Harbor Symposia Quant. Biol., *21:* 113-121.
8. Hartman, P. E., 1956. *Genetic Studies with Bacteria,* Carnegie Institute of Washington Pub. 612, pp. 35-61.
9. Kalckar, H. M., K. Kurahashi, and E. Jordan, 1959. Proc. Natl. Acad. Sci. U. S., *45:* 1776.
10. Wollman, E. L., F. Jacob, and W. Hayes, 1956. Cold Spring Harbor Symposia Quant. Biol., *21:* 141-162.
11. Jacob, F., and E. A. Adelberg, 1959. Comptes rendus, *249:* 189.

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