A SHORT EPISTEMOLOGY OF BACTERIOPHAGE MULTIPLICATION

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Bacterial viruses were discovered in 1915 when Twort (49) observed a serially transmissible agent that destroys bacteria. Twort published this finding in a brief note that remained unnoticed until, two years later, d'Hérelle (20) announced *his*, probably independent, discovery of an entirely analogous filtrable virus, to which he gave the name "bacteriophage". D'Hérelle's announcement caused an immediate sensation in the world of medical microbiology, because d'Hérelle promulgated the idea that bacteriophages, rather than antibodies, are the chief agents of natural immunity against bacterial infections and should be most useful for a generalized therapy and prophylaxis. Despite holding to these, in the event quite mistaken, notions, d'Hérelle managed to recognize bacterial viruses for what we know them to be today. Within two or three years of his original discovery he had invented the method of *plaque counting* that made possible the accurate titration of phage, and by 1923 he had outlined the true life cycle of the phage (21): the virus particle first attaches itself to the surface of the bacterium, then penetrates into the interior of the cell, where it reproduces itself to generate an issue of many progeny viruses. The progeny are liberated and ready to infect further bacteria when the infected cell finally bursts open, or undergoes *lysis*. Although, now in retrospect, these ideas not only seem eminently plausible but also happen to be true, they were accepted by few of d'Hérelle's contemporaries. Especially the doctrine that the bacteriophage is a self-reproducing virus gave widespread offense, and such adversaries of d'Hérelle as Bordet (3) and Gratia (19) preferred to think of it as a self-stimulating enzyme endogenous to the bacterium. Nevertheless, as Burnet (6) observed in 1934, "however agnostic they have been in regard to the nature of a phage, all workers manipulated and in practice thought of it as an extrinsic, virus-like agent."

Phage research made a Big Leap Forward with the appearance of M. Schlesinger on the scene. From about 1930 until his death in 1935, Schlesinger was the first to train the methods of "molecular biology" on bacterial viruses. Schlesinger showed by various indirect methods, such as the adsorption capacity of the bacterial cell for phage and the sedimentation velocity of the phage, that the virus particle has a maximum linear dimension of the order of 0.1 μ and a mass of about 4×10^{-16}g.

Schlesinger also studied the adsorption mechanism of the virus to its host cell and found that the kinetics of adsorption imply that Brownian movement brings virus particles into random collisions with the bacterial surface (42). Most importantly, Schlesinger managed to purify a "weighable" amount of phage by differential centrifugation and graded filtration of crude phage suspensions, and found by direct chemical analysis of the pure virus that it consists mainly of protein and DNA, in roughly equal proportions (43, 44). Although their small size renders bacterial viruses invisible in ordinary microscopes, Schlesinger was able to estimate directly the total number of phage particles in a purified virus preparation, by counting the number of bright points produced in a dark-field microscope. In this way Schlesinger could establish that the number of physical phage particles is roughly equal to the infective titer estimated from plaque count assay. The perfection of the electron microscope shortly after Schlesinger's death made it possible for Ruska (40) and Luria and Anderson (33) to obtain direct visual images of bacterial viruses. These first electron micrographs revealed that though the phage possesses the dimensions estimated by Schlesinger, it is a particle of previously unimagined complexity: the virus consists of a head and a tail.

Unfortunately, Schlesinger left no intellectual disciples, and "modern" phage research really dates only from 1938, when M. Delbrück began to work with bacterial viruses. In collaboration with Ellis, Delbrück designed the *one-step growth experiment* (15) which showed that each phage-infected bacterium liberates some hundred progeny phage after a half-hour *latent period,* and that the end of latent period and the onset of bacterial lysis occur at the very same moment. The importance of the one-step growth experiment lay not so much in bringing radically new insights into the problem of bacterial virus mutiplication; after all, it only confirmed what d'Hérelle had asserted 15 years earlier. But it set new standards of experimental design which invalidated the criticisms, some justified and some merely specious, that had been raised against d'Hérelle's work. Delbrück, moreover, became the focus of a new school of phage workers, many of whom, like Delbrück himself, had been trained in the physical sciences. In a relatively short time, this group came to dominate not only phage research but also to exert a most important influence on the then nascent molecular biology. One reason for the rapid progress that was now made was that for more than 10 years the attention of most workers was confined to the seven strains of "T" phages, particularly to the "T-even" strains T2, T4 and T6, active on *Escherichia coli,* which had been so classified in 1945 by Demerec and Fano (12). Thus the results obtained in different laboratories could be integrated much more readily than the earlier efforts of the "classical" period of phage research, when every investigator seemed to take pains to develop his own virus-host system. In any case, the one-step growth experiment brought clearly into focus the central problem of biological replication: what is going on inside the phage-infected bacterium during the half-hour latent period, while the parental virus par-

ticle manages to effect its own hundredfold replication? It was the desire to understand this process that motivated most of the latter-day phage workers in their quest.

One of the most pressing questions pertaining to the intracellular growth of bacteriophage was how the number of phage particles increases within the infected bacterium from the moment of infection, when only the parent virus is present, until the moment of cell lysis, when the large brood of progeny viruses is liberated into the medium. This information was obtained in 1948 by Doermann (13), who broke open the phage-infected cells at various times during the latent period and assayed the infectivity of the material released by such artificial lysis. Doermann's *intracellular* growth experiment produced the surprising result that the infectivity associated with the original parental virus particle is lost at the outset of the reproductive process: no infective phages whatsoever can be found when the infected bacteria are opened within the first 10 minutes of phage growth. After more than 10 minutes have elapsed, however, an ever-increasing number of infective particles make their intracellular appearance until the final crop of progeny has been attained which would have been released by the spontaneous lysis of the infected bacteria at the end of the normal latent period. The period during which the infected host cell contains no infective material is called the *eclipse*. The unexpected discovery of the eclipse thus revealed that the virus achieves its intracellular multiplication in a non-infective, or *vegetative,* form; the increase in intracellular infective progeny observed after the end of the eclipse reflects, therefore, not the multiplication of the virus but only a terminal process of maturation of previously synthesized progeny structures into intact virus particles. The vegetative phage was thus recognized as the connecting link between parental and progeny viruses, and the elucidation of its structure and function became the central problem of phage growth (14).

The first "modern" phage worker to use the techniques of biochemistry was S. S. Cohen, who in 1947 examined the kinetics of synthesis of protein, RNA, and DNA in phage-infected bacteria (8). Cohen found that, whereas the synthesis of protein continues without interruption at its pre-infection rate, the synthesis of RNA comes to a complete and permanent halt at the moment of infection. The uninterrupted synthesis of protein did not appear to represent a continuation of formation of normal cell proteins, however, since upon phage infection synthesis of *bacterial* enzymes could be shown to cease forthwith (10, 36). Cohen also found that all synthesis of DNA stops at the outset of phage growth, but that, after an interval of a few minutes, DNA synthesis resumes in the infected cell, at a rate several times greater than that which obtained in the uninfected bacterium. The meaning of these results remained unclear for many years, except that some workers held that this apparent arrest of RNA synthesis disproves the notion advanced some years earlier by T. Caspersson and by J. Brachet that RNA synthesis has some necessary connection with protein synthesis.

Cohen (9) also introduced the use of radioactive tracer elements into phage re-

search. He presented an experiment in 1948 which demonstrated that only one-third of the phosphorus found in the DNA of the progeny phages is already present in the phosphorylated constituents of the uninfected cell; the remainder of the phosphorus is assimilated from the growth medium only after infection. This result finally laid to rest those old-time theories advanced by d'Hérelle's adversaries that envisaged that phage multiplication is nothing but the "triggering" of the metamorphosis into bacteriophage of "precursors" already present in the normal bacterium. In 1950 Putnam and Kozloff (39), adopting Cohen's use of radioactive tracers, invented an experiment directed toward the question of how many, if any, of the C^{14} or P^{32} atoms used as a label in the DNA of the parent phage infecting a bacterium reappear among the DNA of the progeny viruses. The outcome of this *transfer* experiment was that about 30–50 per cent of the radioactive atoms in the parental DNA are passed on to the progeny. The meaning of this result likewise remained unclear for many years, though Putnam and Kozloff favored the interpretation that this transfer represents a *reutilization of breakdown products* of the parental DNA in the anabolism of the progeny substance, i.e., that the dissolution of the DNA of the infecting virus is in some way connected with its reproduction.

In about 1950 there began attempts to identify the chemical nature of the vegetative phage; for this purpose phage-infected bacteria were broken open at various stages of the eclipse and a search was made for materials that possess one or another property characteristic of the extracellular bacteriophage, but which are not yet endowed with its ultimate infective power. These searches revealed that non-infective proteins possessing the antigenic specificity of and a morphological resemblance to parts of the intact infective virus do indeed make their appearance during the eclipse, but none of these materials were found to contain any DNA (34, 31). It was, therefore, thought for a time that the vegetative phage might be a protein, to which a DNA-rich viral "Cytoplasm" is added only at the moment of maturation (32). Now, in retrospect, it seems strange that although the pneumococcal transforming principle was already known to be DNA since 1944, the notion that DNA is also the germinal substance of the virus did not seem to play any important role in any of the numerous hypotheses on the nature of phage multiplication that were being considered prior to 1952. This is all the more curious in view of the circumstance that during this time the identification of DNA as the transforming principle, unlike some other important contemporaneous discoveries, was not seriously doubted by any of the leading phage workers. Possibly the idea that the DNA is the germinal substance of the virus, if it was ever proposed, had been rejected as too hopelessly naïve.

The experiments that finally revealed the true role of the different phage components took their inception in 1949 with the observation of T. F. Anderson (1) that osmotic shock releases the phage DNA from the head of the virus particle and that the proteinaceous phage "ghosts" so produced are still able to adsorb to the

bacterial host cells by means of the tail, the normal adsorption organ. These findings caused little excitement, however, until A. D. Hershey and M. Chase (24) discovered in 1952 that a formally similar separation of viral DNA and viral protein occurs at the outset of intracellular phage growth: by infecting bacteria with phages that contained a radioactive tracer either in only the DNA (e.g., P^{32}) or in only the protein (e.g., S^{35}), it could be shown that the labelled viral DNA enters the bacterial cell, whereas most of the labelled viral protein stays outside, devoid of any further function in the remainder of the intracellular growth process. It was thus revealed at last that the DNA is the carrier of the hereditary continuity of the virus and that the protein represents somatic structures that encapsulate the viral genome and facilitate its injection into the host cell. The release of the phage DNA from its protein envelope at the very moment of infection then also accounted for the eclipse phenomenon: having just been divested of its injection organs, the DNA of the infecting phage is, of course, unable to gain entrance into any further bacterial cells to which it may be presented in the infectivity test.

At about the same time that Hershey and Chase demonstrated the functional differentiation of phage DNA and phage protein, it was discovered by G. Wyatt and S. S. Cohen (52) that the DNA of the T-even phages contains instead of cytosine the unusual pyrimidine 5-hydroxymethyl cytosine. This finding made it possible to study the growth of the intracellular vegetative phage by examining infected bacteria for the presence of any phage-specific DNA. In this way, Hershey, Dixon, and Chase (25) refined Cohen's earlier studies on the synthesis of DNA in phage-infected bacteria. They showed that no further bacterial (cytosine-containing) DNA is formed after infection, and that synthesis of new phage (hydroxymethyl cytosine-containing) DNA begins about 6 minutes after infection and then proceeds so rapidly that a few minutes after the end of the eclipse enough phage DNA is present to provide the germinal substance for about 80 virus particles. The synthesis of phage DNA continues throughout the rest of the latent period, so that there is always more phage DNA present in the cell than that accounted for by the intracellular infective progeny. Kinetic studies on the flow of isotopic tracers into the vegetative phage DNA and into mature intracellular phage particles revealed that the viral precursor DNA must exist in a *pool,* from which it is withdrawn at random for encapsulation into the phage precursor protein for formation of the structurally intact, infective progeny particles (46, 22).

It was now possible to restate the basic problem of phase replication in terms of the two functions, "autocatalytic" and "heterocatalytic," "genetic" and "phenotypic," of the viral DNA complement injected into the host cell: (a) How does the DNA manage to replicate itself several hundredfold to generate the germinal substance with which the progeny virus are to be endowed? and (b) How does the viral DNA manage to induce or preside over the synthesis of as many copies of the viral protein that the infecting phage just shed *ante portam?* Within a year of the

phrasing of these questions it became possible to imagine a molecular mechanism for the first, or "autocatalytic," function, when in 1953 the double-helical structure of DNA was proposed by J. D. Watson and F. H. C. Crick (51). This replication scheme envisaged that the two complementary polynucleotide strands of the parental DNA molecule separate, and that each of the two strands serves as a template for the ordered co-polymerization of a complementary *de novo* polynucleotide chain, through specific hydrogen-bond pairing between purine and pyrimidine bases. In this way, the DNA of the infecting parental virus would undergo successive rounds of unwinding and complement addition to build up the intrabacterial pool of viral replica DNA molecules identical to the DNA of the parent, and thus provide the hereditary substance for the offspring virus. This proposal engendered a definite and previously unimagined prediction concerning the fate of the atoms of the parental DNA molecules: as soon as the first replication act has taken place, the two complementary polynucleotide chains of the parent duplex are evenly *distributed* over the two nascent DNA replicas (11). A series of investigations was, therefore, carried out to ascertain whether the atoms of the parental phage DNA really experienced such a *semiconservative* partition in the course of intracellular phage growth. In particular, the transfer experiment invented by Putnam and Kozloff was now refined to measure not only the average transmission of labelled parental DNA atoms to the progeny but also the manner of *distribution* of the transferred molecular patrimony over the progeny. The first series of these studies employed P^{32}-labelled parent phages and revealed, by methods capable of detecting the radioactivity contained in single viruses, that the parental DNA complement is not transferred intact to only one progeny individual but is, instead, dispersed over several descendants. Sufficiently large fractions of the parental DNA were found to be transferred *en bloc,* however, that the notions of Putnam and Kozloff of transfer by complete breakdown of the parental DNA and anabolic reutilization of the breakdown products could be ruled out (30, 47). But the question of the semiconservative partition of the parental polynucleotide chains was not definitively settled by these experiments. As discussed more fully in this Symposium by M. Meselson, these transfer distribution results pertain more cogently to the *fragmentation* of the parental DNA complement incidental to genetic recombination than to the elementary replication mechanism. Only after 1958, when Meselson and F. W. Stahl invented the method of detecting the presence of heavy isotopic tracers in macromolecules through density gradient sedimentation, could the manner of distribution of the parental DNA atoms be established unambiguously. Meselson and Stahl (35) showed that after bacteria grown in a medium containing the heavy isotope N^{15} as the only nitrogen source have undergone one cycle of growth in a medium containing the ordinary isotope N^{14}, all the bacterial DNA molecules have become N^{15}-N^{14} hybrids, in agreement with the semiconservative partition of the parental polynucleotide chains. Similar experiments on the fate of phage DNA

labelled with heavy isotopes finally showed that also the parental DNA experiences the semiconservative partition demanded by the Watson-Crick replication mechanism, so that after transfer to progeny phages the parental atoms show up in half-old, half-new duplex molecules (29). A proof of semiconservative replication of the phage DNA completely independent of isotopic tracers was also furnished by genetic experiments that demonstrated that mutations induced in the vegetative phage genome by exposure to a mutagen during intracellular growth first generate transient *heterozygous* structures, harboring both mutant and non-mutant alleles of the genetic site under study (38).

In 1955 it was discovered that during the first 6 to 8 minutes of intracellular phage growth there occurs the synthesis of an essential "early" protein which must be made before replication of the phage DNA can begin. Once synthesis of the "early" protein has occurred, replication of phage DNA can proceed even if synthesis of further proteins is blocked (7, 26, 48). This finding suggested to some students of the subject that this essential "early" protein might have some intimate connection with the DNA replication process itself; for instance, contrary to the proposal of Watson-Crick, the replication of viral DNA molecules might proceed *indirectly,* involving the function of an intermediate nucleoprotein template of which the "early" protein forms a part (45). More recently it has been demonstrated, however, that the "early" protein cannot form part of any such intermediate template, inasmuch as in a bacterium infected with two or more related but genetically distinct phage particles, the "early" protein synthesized under the influence of one parental genotype can serve also for the replication of the other parental genotype (16). In fact, insight into the real nature of the "early" protein was first gained in 1957, when J. Flaks, J. Lichtenstein, and S. S. Cohen showed that soon after the onset of intracellular growth, the phage induces within the host cell the formation of a new enzyme, 5-hydroxymethylase, involved in the biosynthesis of the phage-specific pyrimidine 5-hydroxymethyl cytosine (17). Soon after, other workers demonstrated similar phage-induced syntheses of a number of other enzymes foreign to the uninfected cell, enzymes that are all concerned with the synthesis or polymerization of specific constituents of the phage DNA (28). Thus it seemed plausible that replication of the phage DNA cannot get under way until all those enzymes are present that are necessary for making and joining together the nucleotide building blocks. These phage-induced enzymes illustrate an important aspect of the viral genetic material: the kind of proteins found in the extracellular infective virus, such as the polypeptides from which the phage head and tail are constructed, are by no means the only proteins whose structural information is carried in the phage genome. The synthesis of many other protein species is presided over by the viral DNA, proteins which are necessary for the process of viral growth but which are never incorporated into the mature progeny virus particles.

How does the viral DNA accomplish the second, or "heterocatalytic," of its

tasks? The development that made possible precise thought on this problem was S. Benzer's operational definition in 1955 of the *cistron* as that segment of the viral DNA macromolecule that contains the structural information for an individual polypeptide molecule (2): A cistron corresponds to a length of DNA comprising several thousand nucleotide pairs. It thus seemed all but certain that it is the exact permutation of the four possible nucleotide pairs in the cistron which uniquely specifies the exact permutation of the 20 kinds of amino acid in the protein molecule. That nucleotide sequence in the cistron and the amino acid sequence in the polypeptide must be related to one another through a *code,* in which a given sequence of three or four, or more, nucleotide pairs corresponds to a given amino acid, had already been evident in a general way for some years when in 1954 G. Gamow (18) published the first specific proposal for such a code. Other thinkers followed suit with various proposed codes of ever-increasing ingenuity, but Gamow's suggestion has the singular distinction that so far it is the only one of these proposals that has already been proven impossible (4).

In any case, it never seemed likely, from *a priori* structural as well as from physiological considerations, that the primary gene product of the cistron could be the polypeptide molecule itself; instead, it appeared much more likely that the DNA cistron serves as a template for the ordered co-polymerization of a molecule of RNA onto which the nucleotide sequence of the DNA is first transcribed; and that it is this RNA molecule which then acts as the template for synthesis of the polypeptide. Since, as was first discovered by Schachman, Pardee, and Stanier in 1952 (41), most of the bacterial RNA is contained in the small particulate *ribosomes,* and since later experiments showed these ribosomes to be the actual sites of protein synthesis in the cell, it seemed logical to think that the provenance of the RNA moiety of each ribosome is some particular cistron, i.e., that each ribosome is competent to synthesize some specific polypeptide. The finding of S. S. Cohen, already mentioned, that after phage infection the net synthesis of protein in bacteria continues at its pre-infection rate, whereas net synthesis of RNA comes to a stop, is, however, difficult to reconcile with this notion. For since the kinds of proteins made after phage infection, the new enzymes of virus metabolism and structural members of the phage progeny, are radically different from the proteins of the uninfected cell, one would have anticipated an increased rate rather than a stopping of RNA synthesis as the cytoplasm of the host bacterium is renovated for virus production. However, when, in 1953, Hershey (22) and, in 1956, Volkin and Astrachan (50) reexamined RNA synthesis in phage-infected bacteria by following the incorporation of radioactive label, it was found that there is, after all, some post-infection RNA synthesis. Since the label enters and leaves the RNA fraction at an appreciable rate, furthermore, it followed that the post-infection RNA is in a state of rapid metabolic turnover. The discovery of this RNA fraction generated considerable excitement when Volkin and Astrachan showed that its purine-pyrimidine base composition is

very different from that of the RNA of the bacterial ribosomes, in that it resembles that of the phage DNA. It thus became plausible that the phage DNA induces in the infected cell the synthesis of new ribosomal RNA, fit for viral protein synthesis, and that these virus-specific ribosomes, in contrast to the stable ribosomes of the uninfected bacterium, are in a state of rapid metabolic turnover. In fact, Nomura, Hall, and Spiegelman (37) thought that they had gained support for this idea when in 1960 they showed that most of the post-infection, phage-induced RNA possesses the sedimentation characteristics of bacterial ribosomes.

However, a few months later Brenner, Jacob, and Meselson (5) carried out an experiment in which they grew bacteria in a non-radioactive medium containing the heavy isotopes N^{15} and C^{13} as the only nitrogen and carbon sources and then infected these cells in a medium containing the ordinary isotopes N^{14} and C^{12} and radioactive phosphorus P^{32}. Sedimentation analysis of the P^{32}-labelled RNA formed upon intracellular phage growth confirmed the finding that the post-infection RNA is indeed associated with ribosomal particles, but density analysis of these particles revealed that they contain the heavy, pre-infection isotopes N^{15} and C^{13} rather than the light, post-infection isotopes N^{14} and C^{12}. In other words, the virus-induced post-infection RNA *enters old ribosomes that are already present in the cell before infection;* indeed, no ribosomes at all corresponding to the N^{14}-C^{12} density appear, showing that phage infection really brings ribosomal synthesis to term. This result led to a new image of protein synthesis, based on an idea which Jacob and Monod (27) had already reached from considerations based on the facts pertaining to the genetic regulation of enzyme formation. According to this idea, the ribosomes are not *a priori* differentiated in their capacity to synthesize this or that polypeptide; instead, each viral cistron serves as the template for the synthesis of an ephemeral *messenger* rather than a ribosomal RNA molecule. This messenger RNA molecule then enters into combination with a pre-existing bacterial ribosome, and the messenger-ribosome complex is competent to synthesize some particular polypeptide. The ribosome is, therefore, the "workshop" for protein synthesis and the messenger RNA the "blueprint". The messenger RNA has a very limited lifetime and serves for the construction of only one, or at most a few polypeptide molecules, so that during active protein synthesis the messenger RNA molecules must be in a state of rapid turnover. Virus infection arrests synthesis of all bacterial proteins by destroying the nuclear DNA of the host cell and hence the source of bacterial messengers. At the same time, the viral DNA generates its own messenger RNA molecules which then arrogate the bacterial ribosomes and thus effect synthesis of the viral proteins.

It may be said, therefore, that both autocatalytic as well as heterocatalytic functions of the viral DNA are now more or less understood, at least in their rough outlines. In fact, enzymes have already been isolated that appear to be responsible for replication of the DNA and for the synthesis of RNA messengers, and that are

capable of mimicking these reactions *in vitro*. As yet much less well understood is the nature of the code that relates nucleotide sequence in the viral polynucleotides to amino acid sequence in the viral polypeptide and the mechanism by which this translation is effected, although it is likely that the "soluble" or "acceptor" RNA of the bacteria is involved here. Perhaps still least well comprehended in the whole viral reproduction cycle is the nature of the regulation of the ordered sequence of synthesis of the different viral proteins and the mechanism by which the various structural components of the phage unite to form the mature, infective unit. But as Hershey (23) pointed out in 1957, this aspect of bacteriophage growth bears a strong resemblance to morphogenesis in general, the most likely leitmotif of molecular biology of the future.

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