Phage, Colicins, and Macroregulatory Phenomena

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The early work on bacteriophage growth, mutation, and recombination had the good fortune to serve as one avenue in the growth of molecular biology to its present state of an intellectually satisfying construction. It is unnecessary to recount today the story of that early work, in which it was my fortune to be engaged in friendly and exciting cooperation with Max Delbrück and Alfred Hershey. Even more difficult would be an attempt to trace here the series of developments that led from early phage work to the modern knowledge of virus reproduction, gene replication, and gene function and its regulation. My greatest satisfaction derives from the role that has been played by my students and co-workers in these developments and from the personal experience of association with many of the protagonists of this great intellectual adventure.

Phage research has branched off in many directions, each of which has contributed in some measure to the edifice of molecular biology. One of the most notable directions was that of gene function and its regulation. The major contributions of phage research in this area were made in the study of lysogeny by André Lwoff and François Jacob, which led to the formulation of the operon theory by François Jacob and Jacques Monod. The regulatory phenomena considered by this theory concerned the functions of individual genes or groups of genes. In this article I wish to deal with approaches to certain aspects of cellular regulation that involve "macroregulatory phenomena." By this I mean those phenomena in which the functional changes observed affect some of the major processes of the living cell, such as the syntheses of DNA, RNA, or protein; or the energy metabolism; or the selective permeability function of cellular membranes.

The study of antibiotics like penicillin or streptomycin, agents that act in a "molar" way on cellular processes, has played an important role in elucidating such processes as the organization of the biosynthesis of the bacterial cell wall or the mechanism of protein synthesis. When an alteration of a major cellular function is produced by the action of an agent such as a bacteriophage or some other macromolecular agent acting in a "quantal," single particle fashion, the situation is even more challenging since some mechanism of amplification must intervene between the individual unit agent and the affected elements of the responding cell. For a viral agent, the amplification mechanism may be the replication of the agent or the expression of its genetic potentials. For a protein agent, for example a bacteriocin, the amplification mechanism must be a change in the integrity of some cellular structure or of the functioning of some cellular control system. In either case, an understanding of the mode of action of such agents on major cellular processes is likely to reveal some interesting aspects of the functional organization of the cellular machinery.

In my laboratory, we are currently using bacteriophages and bacteriocins as

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probes into macroregulatory phenomena of the bacterial cell. There has not yet been much progress in this field, except in the study of the regulation of genetic transcription. Even a description of current efforts should be of value at least in illustrating what we are after.

Bacteriophage and Macroregulatory Phenomena

An early indication of the potential role of phage as a controller of cellular functions was the observation that irradiated phage T2 retained its host-killing and interfering abilities after losing its reproductive capacity (*1*). The bacteria were not grossly disrupted but died. It took years and the development of the biochemical approaches to the study of phage infection before the killing action of phage could be interpreted in terms of physiological mechanisms, that is, of specific inhibitions at the level of macromolecular syntheses. We now know that certain virulent phages, including the T-even coliphages, produce a rapid arrest of synthesis of the protein, RNA, and DNA of their host cells. Other phages have less drastic or more transient effects on these processes. But our knowledge of the mechanisms of these inhibitions has progressed surprisingly slowly.

Phage infection and host DNA synthesis. Let us take, for example, the effect of phage infection on host DNA. The case of the T-even phages would appear to be the simplest. These phages contain hydroxymethylcytosine (HMC) instead of cytosine in their DNA (*2*) and determine, among other things, the production of an enzyme, deoxycytidine triphosphatase, that destroys deoxycytidine triphosphate (dCTP), a specific precursor of host DNA [see summary by Cohen (*3*)]. The bacterial DNA is broken down rather rapidly after infection and is converted to acid-soluble fragments and ultimately to single nucleotides. That double-strand breaks in the bacterial DNA should stop its replication is understandable (*4*); but the action of phage in inducing such breaks remains unexplained. Certain mutants of phage T4 fail to convert host DNA to acid-soluble products (*5*), but the primary breaks still occur and the host DNA is broken into large fragments. The nuclease responsible for these breaks must be specific for cytosine-containing DNA; but no phage gene has yet been found whose mutations prevent the breaking of host DNA.

An even more intriguing situation is that of phage φe of *Bacillus subtilis*, which has been studied in our laboratory by David Roscoe and Menashe Marcus. This is one of several phages that contain hydroxymethyluracil (HMU) instead of thymine in their DNA and, upon infection, determine a series of enzymatic changes directed at converting the path of DNA synthesis from bacterial-type to phage-type DNA (*6*): a deoxyuridylate (dUMP) hydroxymethylase, a thymidine triphosphate nucleotidohydrolase (dTTPase), an inhibitor of thymidylate synthetase, a deoxythymidylate nucleotidase, a deoxycytidylate deaminase, and possibly also a deoxynucleotide kinase. Host DNA synthesis stops a few minutes after infection with phage φe. Roscoe (*7*) was able to show that the host DNA remains intact or, at least, that double-strand breaks do not occur in detectable numbers. The enzymatic interference with the synthesis of thymidine triphosphate (dTTP) can be bypassed by using thymine-requiring host bacteria in the presence of thymine and by using phage mutants defective in dTTPase. Under these conditions, the phage produced contains at least 10 percent and possibly 20 percent thymine in place of HMU; and yet, synthesis of host DNA is still arrested. Hence we must postulate the existence of some more specific mechanism responsible for the arrest. This mechanism is probably not an inhibition of host-DNA synthesizing enzymes by HMU nucleotides since the arrest of bacterial DNA synthesis is produced also by a phage mutant that lacks the ability to determine either deoxyuridylate hydroxymethylase or dTTPase. Yet, the arrest of host-DNA synthesis requires protein synthesis after phage infection; hence there is some specific phage function that inhibits bacterial DNA synthesis. We are currently trying to identify this phage function, which may be exerted at the level of the replication process itself or at some still unrecognized regulatory level.

Host proteins and RNA. Let me now turn to the effect of phage infection on the synthesis of RNA and proteins. At least in the case of the T-even phages the arrest in host protein synthesis appears to be secondary to the arrest of messenger RNA (mRNA) synthesis (*8*). Direct effects on translation of existing messengers may also be present, as they certainly are in some animal virus infections.

The mode of arrest of RNA synthesis remained obscure until about a year ago, when the major discovery was made (*9*) that at least some phages, including the T-even and T7 coliphages (*10*), cause an alteration in RNA polymerase that changes its specificity. A factor σ, a component of the polymerase needed for transcription of the "very early" set of phage genes—those transcribed immediately after infection (*11*)—and presumably also for the transcription of bacterial genes is altered or destroyed after phage infection. The transcription of other genes of the phage is then made possible by the appearance of some new factor or factors which confer different specificity to a persistent "core" portion of the host polymerase (*12*). The reasonable assumption is made that σ confers to the polymerase a promoter-recognizing specificity that causes it to initiate mRNA synthesis at specific DNA sites.

In this case, the "macroregulatory" phenomenon is brought about not at the level of some purely regulatory mechanism but at the level of the operational machinery itself. The phage arrests the expression of a whole set of genes by changing the specificity of an enzyme—RNA polymerase.

That this kind of regulation is not peculiar to phage infection has been shown by R. Losick of Harvard together with my student A. L. Sonenshein. Starting from the observation by Sonenshein and Roscoe (*13*) that the *subtilis* phage φe fails to grow and to express its functions when it infects bacteria in course of sporulation, Losick and Sonenshein hypothesized that, since sporulation involves an arrest of synthesis of many proteins and the appearance of several new ones, the critical step may be a change in specificity in RNA polymerase analogous to the one observed in *Escherichia coli* after T-even phage infection (*9*). They succeeded in fact in demonstrating that this was the case (*14*): A σ-like factor, part of the RNA polymerase of vegetative bacterial cells, is altered or eliminated during sporulation, and this brings about a change in the template specificity of the bacterial polymerase. Remarkably enough, in vitro addition of the σ factor from *E. coli* to the core of the *B. subtilis* polymerase restores its original activity!

Note that in this study of sporulation the phage was used not to investigate some phage-induced change in the cell but as a probe to reveal a "regulatory" phenomenon responsible for a major differentiation in the cell cycle of a bacterium—the change from vegetative to

sporulative syntheses. The possible relevance of changes in RNA polymerase, and more generally of macroregulatory changes, to problems of differentiation in higher organisms raises interesting speculations (*12*, *15*) and is likely to stimulate new approaches to the study of cellular differentiation.

Macroregulation and Colicins

Next I would like to consider another approach to macroregulation, to which we have recently turned in order to gain further insights into the functional organization of bacterial cells. This involves the study of the mode of action of certain colicins; and, although the history of colicin research is closely interwoven with the history of phage research, it may be instructive to recount the circuitous way in which my present interest in colicins came about. Again it started from a phage problem, the conversion of salmonella somatic antigens by temperate phages discovered by Iseki and Sakai (*16*). Dr. Hisao Uetake came to my laboratory in 1956, and together we studied the conversion of antigen 10 to antigen 15 by phage ε^{15} (*17*). This collaboration continued when Dr. Takahiro Uchida came from Uetake's laboratory to join me at M.I.T. in 1960, and we were fortunate to bring the problem of antigen conversion to the attention of my colleague Dr. Phillips Robbins. The story of how Robbins and his co-workers (*18*) solved the problem at the biochemical level, and in the process discovered and elucidated the role of carrier lipids in polysaccharide synthesis, need not be recounted here. My association with this work, however, roused my interest in problems of membranes, particularly in certain remarkable features of the cytoplasmic membrane of bacteria.

In bacterial cells this membrane is the only organelle. It contains enzymes and other constituents that play roles not only in permeation and active transport but also in the biosynthesis of the macromolecular components of the bacterial cell wall, such as peptidoglycan and other polysaccharides, including the lipopolysaccharide of the enteric bacteria. In addition, the cytoplasmic membrane is the site of the machinery of terminal respiration and may also play a crucial role in the process of DNA replication and in the segregation of DNA copies at cell division (*19*). And yet, the functional organization of this remarkable structure remains obscure. Each group of enzymes and carrier molecules involved in a given biochemical process must presumably be positioned in precise fashion next to each other for efficiency of function. We do not know whether such "supramolecular structures" are solely determined by the intrinsic properties of the individual components (which might be able to reform the functional structures in vitro as in the assembly of viral shells or of bacterial flagella from monomeric proteins) or whether the preexisting pattern of molecular organization plays some role in the orderly accretion of new functional elements in the membrane of a growing cell—a priming role or even a catalytic role such as a conversion of inactive precursors into active components. There is suggestive evidence for the occurrence of some such enzymatic steps in the assembly of the protein shells of certain complex viruses (*20*). An even more intriguing possibility is that the structure of the membrane may play a role not only in the positioning but also in the functioning of its active constituents, for example, by transmitting conformational signals. This might provide an additional level of regulation of cellular function.

This is where colicins come into the picture. They are protein antibiotics lethal for susceptible strains of coliform bacteria and are produced by other strains of such bacteria that harbor the corresponding genetic determinants or "colicinogenic factors." It has long been known that some colicins arrest the synthesis of macromolecular components of susceptible cells (*21*). A major advance was the discovery that different colicins cause different biochemical changes (*22*) and that the "killing" action of some colicins can be reversed by digesting away with trypsin the colicin from the cell receptors (*22*, *23*). This action from the outside, together with the one-hit kinetics of killing by colicins, suggested that a single colicin molecule sitting on some surface component of the cell envelope could exert a bacteriostatic or bactericidal effect through "amplification" mechanisms residing in the cell envelope itself. Nomura (*22*) postulated, therefore, that a colicin attached to a suitable receptor acts on a specific "biochemical target" by bringing about a functional alteration of some specific element of the cytoplasmic membrane. Nomura (*22*) and I (*24*) have considered the intriguing possibility that the amplification mechanism may be mediated by conformational changes of the cell membrane as a whole. Changeux and Thiery (*25*) put forward the same idea in a more specific way, based on consideration of allosteric interactions among membrane proteins.

The three types of actions recognized for colicins by Nomura (*22*) were (i) arrest of DNA synthesis and breakdown of DNA, typical of colicin E2 action; (ii) inhibition of protein synthesis, characteristic of colicin E3, which could be traced (*26*) to a specific alteration on some component of the 30*S* ribosomal subunit; and (iii) overall arrest of macromolecular syntheses, a mechanism common to many colicins (E1, K, A, I). In the cases of colicins E2 and E3 the magnitude of the biochemical effects is strongly dependent on multiplicity, whereas the killing action (defined by inability to grow) is strictly one-hit. Hence there is some question as to whether the effects observed, however specific, are primary or secondary. For colicin K and E1, however, the correlation between killing and inhibitory multiplicities is very good, and the biochemical phenomena observed may be more directly related to the primary effects.

How does one molecule of colicin inhibit the synthesis of all macromolecules? An important finding (F. and C. Levinthal, personal communication) was that the inhibition of protein of nucleic acid synthesis was absent when colicin E1 reacted with *E. coli* cells growing in strict anaerobiosis; admission of air brought about a prompt but reversible inhibition. This observation, and the fact that the inhibition of RNA and protein synthesis were simultaneous rather than sequential, led the Levinthals to suggest that the primary action of colicin E1 was on oxidative phosphorylation—a function of the cytoplasmic membrane. Adenosine triphosphate levels were drastically decreased although not to zero level.

Starting from this background and from our interest in macroregulatory mechanisms located in the bacterial membrane, my co-workers and I undertook to correlate colicin action with changes in membrane properties such as permeability and transport. I shall refer only to work on colicin E1 and K, where we have had some measure of success.

Kay Fields and I looked first into the effects of these colicins on the transport and accumulation of β-D-galactosides by colicin-treated *E. coli* cells (*27*). Our results indicated that the energy-dependent accumulation process was drastically inhibited, whereas the rate

of transport of orthonitrophenylgalactoside (ONPG), measured by its rate of hydrolysis by the galactosidase of intact cells, was hardly affected. Thus, the cells had not become "leaky" to ONPG. The accumulation of α-methylglucoside, which is driven by phosphoenol pyruvate rather than ATP (*28*), was insensitive to colicin E1 or K, an indication that glycolysis did proceed in colicin-inhibited cells.

When we proceeded to study the fate of glucose used by colicin-treated cells we found, unexpectedly, an indication of what we were looking for: a specific alteration of membrane permeability (*29*). The treated cells excreted into the medium almost one-third of the glucose-derived carbon as glucose-6-phosphate, fructose-1,6-diphosphate, dihydroacetone phosphate, and 3-phosphoglycerate. Other intermediates were not excreted in measurable amounts. In addition, pyruvate rather than acetate and CO_2 became the major short-term product of glucose catabolism. This was not due to a leakage of pyruvate since this substance could be converted to lactate if the colicin-treated cells had significant levels of lactate dehydrogenase. The production of pyruvate instead of acetate reflected a specific inhibition, direct or indirect, of pyruvate oxidation.

Also, the effect on energy metabolism turned out to be more complicated than just an inhibition of oxidative phosphorylation. If *E. coli* cells are growing fermentatively on glucose under conditions of adequate but not strict anaerobiosis, the synthesis of protein and nucleic acid is almost as sensitive to inhibition by colicin as in aerobic cells. Even hemin-deficient mutants, which are strongly inhibited by air, prove to be sensitive to the colicins if anaerobiosis is not complete.

These observations suggest that an early effect of these colicins may be a (reversible) alteration of the cytoplasmic membrane, requiring the presence of some oxygen and leading to a block in ATP-dependent processes by limiting ATP availability. This may result either from a reduced ATP production or from an increase in ATP destruction. The fact that biosynthetic processes are blocked despite the significant residual levels of ATP may be due in part to accumulation of AMP and the resulting rise in AMP/ATP ratios (*30*). In fact, an *E. coli* mutant with a heat-sensitive AMP kinase behaves at high temperatures very much like colicin-inhibited cells (*31*).

In a search for further effects of colicins on the membrane, David Feingold, who spent last year as a guest in our laboratory, investigated the effect of colicin E1 on proton uptake by bacteria in the presence of carbonylcyanide *m*-chlorophenylhydrazone (CCCP), a powerful uncoupler of oxidative phosphorylation which promotes H^+ permeation (*32*). By itself the colicin produced no increase in proton permeability; in fact, it prevented the slow *p*H change observed with normal washed cells. But colicin treatment, even at low multiplicities, sensitized the bacteria to CCCP so that proton equilibration occurred promptly upon the addition of as little as $10^{-6}M$ CCCP to the cell suspension. Thus the action of colicin E1 in *E. coli* mimicked the effects of valinomycin on Gram-positive bacteria (*32*). Similar findings were made independently by Hirata *et al.* (*33*). Experiments are in progress in Dr. Feingold's laboratory to decide whether this effect of colicin is secondary to the inhibition of energy metabolism or represents a specific effect on permeability, for example to K^+ ions, permitting exchange with H^+ ions when these gain access through the action of CCCP.

Colicin-tolerant "membrane" mutants. Another set of observations has made it possible to tie the response to colicins with the functional properties of the bacterial envelope. Rosa Nagel de Zwaig and I (*34*) have studied bacterial mutants of a class that is "tolerant" to certain colicins; they adsorb the colicins without being inhibited. Similar *tol* or *ref* ("refractory") mutants have also been studied in several other laboratories. In line with expectations as to the role of the membrane in the response to colicins, we were gratified to discover that all the *tol* mutants we examined exhibited some membrane defect. Some classes of mutants are fragile so that many cells lyse spontaneously during growth, as though the synthesis of the cell envelope were defective. Like other envelope-defective mutants of enteric bacteria, these *tol* mutants are very sensitive to deoxycholate, possibly because the membrane has become accessible to this surface-active agent. More interesting still, one class of *tol* mutants proves to be very sensitive to a whole series of organic dyes, mostly cations such as acridines, ethidium bromide, and methylene blue. We could show that the dye sensitivity was due to a rapid uptake of the dye by the mutant cells while the normal cells are almost impermeable. Thus this mutation to colicin tolerance was correlated with a specific change in membrane permeability (*35, 36*).

Some preliminary analytical studies of the envelopes of normal bacteria and tolerant mutants have revealed certain significant differences between them. The chemistry of the cell envelope of enteric bacteria is extremely complex and remains poorly known. Even when chemical changes are found, it is not easy to decide whether they are directly relevant to the phenomena under study. This is true, for example, of the changes in phospholipid composition reported in colicin-treated bacteria (*37*). It is encouraging, however, that both the study of response to certain colicins and those of colicin-tolerant mutants have converged to focus our attention on the relation between sites of colicin action and the functions of the bacterial membrane. For the time being, the relation is tenuous and inferential. But the observations are encouraging enough to reinforce our hope that the study of colicins may reveal, within the membrane, levels of organization at which some of the essential functions of bacterial cells are masterminded.

Epilogue

There are interesting analogies between the present state of colicin research and the state of bacteriophage research in the early 1940's. In both situations, phenomenologies described by pioneer investigators are reexamined by a small group of workers concerned with a new goal. In phage research the goal was to get at elementary phenomena of reproduction, hoping that virus reproduction would help elucidate the replication of genetic materials. In colicin research the goal is to explore the functions of the cytoplasmic membrane of bacteria, with the implicit assumption that the findings may throw light on the general problem of the functional organization of cellular membranes. In both situations, the use of simple bacterial systems represents a departure from the traditional materials of the respective disciplines, genetics and "membranology."

As in bacteriophage research 25 years ago, the practitioners of colicin research today are few, cooperative, and moderately confident of success—and somewhat fearful lest success may again transform a quiet area of research into "an elephantine academic discipline" (*38*). Again as in phage research, we know that full answers will come only

when the problems we are exploring will be ready for a rigorous biochemical approach. It may turn out to be a kind of biochemistry as novel as that of gene function and replication was in its own time. Maybe we will again turn up something meaningful and exciting.

References and Notes

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