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Molecular Bases of Parasitism of Some Bacterial Viruses

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Studies on bacterial viruses have provided many contributions to the theory and practice of virology as a whole. Among these have been the important methodological contributions that permit us to approach the numerous facets of the interactions of host cell and virus —contributions that have opened the way to the systematic study of the multiplication of animal viruses in tissue cultures. In addition to the results with bacterial viruses, which have facilitated the extension of our knowledge to other viruses, results have also been obtained that emphasize the individuality and uniqueness of different groups of viruses. Such results stress the potential pitfalls of careless analogy and extrapolation from one biological system to another. It is the purpose of this article (*1*) to consider a group of bacterial viruses that are designated the T-even bacteriophages, whose early selection for widespread intensive study had been largely a matter of historical accident. The results to be presented reveal an extreme pattern of parasitic behavior that appears to be causally related to the presence of certain unusual chemical conformations. These data are only a small portion of the very extensive biological and chemical data existing on these viruses.

The T-even bacteriophages, T2, T4, and T6, are extreme examples of virulent viruses that can parasitize the bacterium, *Escherichia coli,* prevent its division, and eventually provoke the lysis of the infected cell. It was shown almost a decade ago that infection by T2 and some other phages prevents the synthesis of bacterial enzymes (*2*) and diverts the products of host metabolism to virus synthesis. The shunting of host metabolism is most marked when it is viewed in terms of nucleic acid biosynthesis, for the formation of the host nucleic acids is almost entirely prevented by virus infection. The phosphorus that would normally enter host ribose nucleic acid (RNA) and host deoxyribose nucleic acid (DNA) is diverted to the rapid synthesis of virus nucleic acid (*3*) that is solely of the deoxyribose type.

Since 1947 it has been the major concern of this laboratory to attempt to understand the intimate mechanisms of these phenomena. The early stages of infection that lead to these metabolic events may be briefly summarized as follows. The tadpole-shaped T-even viruses contain DNA in a protein coating. They adsorb tail first to the cell that they infect (*4*) and, in some as yet unknown manner, effect the transfer of their DNA to the bacterial host, leaving the protective protein coat on the cell surface (*5*). The injected viral material is then capable of stimulating the stepwise synthesis and assembly of the important protein and nucleic acid polymers as well as the final product of intact virus. The initial steps of infection also result in the release of inhibited bacterial deoxyribonuclease (*6, 7*), which then participates in the extensive degradation of host DNA.

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Hexose and Pentose Metabolism of Growing and Virus-Infected Bacteria

To explain the change in pattern of nucleic acid metabolism on infection, it was supposed that infection might cause the inhibition of steps that lead from glucose phosphate to ribose phosphate (*3*). Since these steps were unknown at the time this hypothesis was made, the study of this postulated pathway was undertaken. Oxidative reactions were revealed that lead from glucose-6-phosphate to ribose-5-phosphate via 6-phosphogluconate (*8*). The existence of the intermediate ribulose-5-phosphate between 6-phosphogluconate and ribose-5-phosphate was described in yeast by Horecker *et al.* (*9*) and was subsequently established in *E. coli* as well (*10*). It has been demonstrated that this pathway is indeed of major importance in the formation of the ribose of RNA in growing *E. coli* (*11*).

In *E. coli,* this oxidative phosphogluconate pathway exists side by side with an anaerobic pathway of glucose degradation, the Embden-Meyerhof scheme. In the phosphogluconate route of glucose utilization leading directly from hexose to pentose, the C_1 of glucose-6-phosphate is selectively eliminated as CO_2. During normal growth, both pathways are used, as determined by following the isotope content of the CO_2 that is derived from the utilization of glucose-1-C^{14}. However, under conditions of T-even virus infection, in contrast to normal growth, it was demonstrated that considerably less glucose was metabolized by the oxidative phosphogluconate pathway and more by the Embden-Meyerhof pathway (*12*).

This result was consistent with the observed decrease in synthesis of ribose and the marked increase in synthesis of deoxyribose that was found in virus DNA. An enzymatic mechanism had been observed for the formation of deoxyribose phosphate (*13*) from triose phosphate and acetaldehyde, metabolites that might be expected to be derived more readily from the Embden-Meyerhof pathway than from oxidative phosphogluconate degradation.

However, it has now been observed in collaboration with M. Lanning (*14*) that the deoxyribose formed in host DNA in normal growth of *E. coli* is derived in a manner comparable to that of

Table 1. Origin of ribose and deoxyribose produced in *Escherichia coli* or in T6r+ phage in glucose-1-C^{14} medium. The results are shown as ratios of counts per minute of C^{14} per micromole of these sugars or isolated derivatives (*13, 14*).

Nucleic acid	Ribose/ glucose	Deoxy-ribose/ glucose
E. coli nucleic acids	0.247 ± 0.047	0.229 ± 0.023
T6r+ DNA		0.496 ± 0.078

the ribose of RNA by a route involving the selective loss of the C_1 of glucose. These results are given in Table 1. They are consistent with the possibility that in this system deoxyribose is predominantly derived from ribose, and they are apparently incompatible with the known enzymatic route for the synthesis of the deoxypentose phosphate. However, under conditions of virus infection, a new path for deoxyribose formation appears to be used, if it is assumed that the mechanism of formation of ribose via phosphogluconate is substantially unchanged. As can be seen from Table 1, the route for synthesis of deoxyribose in virus infection involves a much greater conservation of the C_1 of glucose and is consistent with a possible major utilization of the Embden-Meyerhof scheme in the formation of deoxyribose phosphate from triose phosphate and acetaldehyde.

Thus alternative paths exist in *E. coli* for the metabolism of glucose to the ribose and deoxyribose of the nucleic acids, and the degree to which each is used is profoundly altered by T-even virus infection. However, it has been possible to show that a depression of the utilization of the oxidative phosphogluconate pathway as a result of infection is not caused by an inhibition of the enzymes of this pathway (*15*). Substrates, such as gluconate, that are phosphorylated to intermediate metabolites of this route and that must be metabolized by the phosphogluconate pathway can nevertheless be used in infected bacteria for the synthesis of viral DNA. It was concluded, therefore, that the control of the balance of the pathways of glucose utilization is determined at some metabolic level apart from the enzymes that exist in the pathways themselves.

Unique Viral Constituent

A clue to the nature of this control appeared as a result of studies on the base composition of the nucleic acids of the T-even phages. In collaboration with G. R. Wyatt, it was found that a new unique pyrimidine, 5-hydroxymethyl cytosine (HMC) completely replaced cytosine, a base that had previously been observed in all other nucleic acids studied, including those of *E. coli* RNA and DNA (*16*). Furthermore, this new pyrimidine or HMC has not yet been observed in any other virus, bacterium, plant, or animal. Its distribution appears to be absolutely unique. The resulting difference in the base composition of host and virus nucleic acids may be used to distinguish the formation of virus DNA from host DNA in infected bacteria (*17, 18*).

Fragments of host DNA, which are degraded at least in part to nucleosides, can be utilized for the synthesis of virus DNA. More specifically, as shown in studies with L. L. Weed, the cytosine of host DNA can be converted to the HMC and thymine of virus DNA (*19, 20*), whereas the thymine of host DNA is used to make viral thymine but not HMC. These relationships are depicted in Fig. 1. This result and the obvious requirement of cytosine for the synthesis of host RNA and DNA have led to the hypothesis that in T-even virus infection the formation of hydroxymethyl cytosine traps cytosine (more likely cytosine derivatives), thereby limiting the availability of the latter metabolites that are essential to the synthesis of host components.

It has further been demonstrated that the β carbon of serine may serve as an important source of the hydroxymethyl group of HMC (*19*). More recently it has been observed that the hydroxymethyl group of S-hydroxymethyl homocysteine does not fulfill this function when it is added exogenously to infected bacteria (*21*). The methyl groups of thymine (*19*) and methionine (*21*) both appear to be essentially incapable of conversion to the $—CH_2OH$ level in these systems. The details of the mechanism whereby cytosine derivatives are converted to HMC are not yet known. This knowledge is crucial to an understanding of the manner in which infection initiates and compels the critical hydroxymethylation.

Additional data that support the concept that the addition of the hydroxymethyl group to form HMC is the molecular basis for the competitive winning out of viral products are derived from studies on the utilization of HMC and its derivatives (*22*). In experiments with isolated enzyme systems, growing and infected *E. coli,* and mutant organisms requiring pyrimidines for growth, it was observed that both HMC and 5-hydroxymethyl uracil are quite inert under conditions in which cytosine is deaminated, incorporated, or utilized for growth. The deoxyriboside of HMC is also relatively inert, being far more slowly deaminated by deoxycytidine deaminase or otherwise metabolized than is cytosine deoxyriboside. Thus the conversion of cytosine to HMC has the attributes of a one-way stream in which hydroxymethylation irreversibly traps the cytosine. These results are consistent with the observation that bacteria may multiply viruses that contain cytosine or HMC, but never those that contain both simultaneously. It will be of considerable interest to determine the extent of synthesis and turnover in infected cells of cytosine nucleotides, which, in addition to their structural role in the nucleic acids, have also been implicated in phospholipid metabolism.

Virus Survival and Hydroxymethyl Cytosine Glucoside

Another role for HMC was revealed as a result of an effort to isolate the HMC deoxyriboside by the usual enzymatic methods involving deoxyribonuclease and a phosphatase (*22*). In contrast to the behavior of thymus DNA, which is almost completely degraded to deoxyribosides by successive treatment with these enzymes, the DNA of the T-even viruses is degraded more slowly and less completely. Only about 70 to 75 percent of the total phosphorus is released as inorganic P, and very small amounts of HMC deoxyriboside are found among the nucleosides. Indeed, it is precisely HMC that is concentrated among the enzyme-

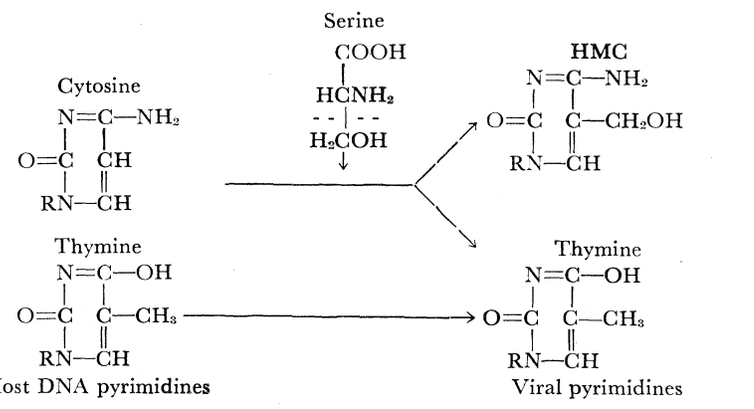

Fig. 1. Relationships of host DNA pyrimidines and viral pyrimidines.

resistant mixture of residual polynucleotides. Acid hydrolysis of viral DNA permitted the HMC deoxyriboside to be released by the action of phosphatase. The presence of HMC in viral DNA in some manner permitted an organization of the DNA that protected critical nucleotide diester linkages from enzymatic degradation. It was suggested that this resistance to enzymatic degradation, under conditions of infection in which host DNA was fragmented, is a factor that facilitates the survival of the genetic material of the virus injected into the host. Insofar as the degraded products of host DNA are further trapped after hydroxymethylation in an enzyme-resistant virus DNA, this also contributes in deciding the relative dominance of competitive nucleic acid systems in an infected cell.

The molecular nature of this relative resistance is now explicable in terms of the discovery by Volkin (*23*) and by Sinsheimer (*24*) of glucose as a glycosidic substituent of the hydroxymethyl group of HMC, as presented in Fig. 2. When the DNA of T2r+ was hydrolyzed with nuclease and diesterase, small amounts of two HMC nucleotides were obtained (*24*), only one of which contained glucose. The HMC nucleotide with a free hydroxymethyl group was readily dephosphorylated by phosphatase; that in which the hydroxymethyl group is bound to glucose is markedly resistant to this enzyme.

The presence of glucose in DNA is as unique in the T-even viral DNA as is the presence of HMC. Thus other viruses contain cytosine and therefore lack the hydroxymethyl group as a site of attachment for glucose, which helps to stabilize T-even viral DNA. How, then, do the nucleic acids of these other viruses compete for dominance in the cells that they infect? With some temperate viruses, which produce lysogeny, infection does not result in nuclear disintegration. The DNA of the virus appears to establish a symbiotic relationship with the genetic substance of its host that permits mutual survival. Nevertheless, there are some virulent cytosine-containing viruses that can kill and lyse bacteria

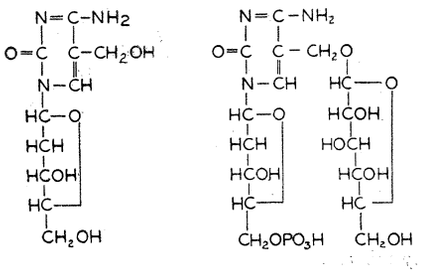

Fig. 2. (Left) 5-Hydroxymethyl cytosine deoxyriboside; (right) 5-glucosyl hydroxymethyl cytosine deoxynucleotide.

even as do the T-even phages. Since their respective deoxyribose nucleic acids must also survive and compete successfully in the cells that they infect, it must be supposed that they have different, as yet unknown, structural modifications that fulfill these functions. Indeed, the existence of any pathogenic virus implies the existence of survival and competitive mechanisms whose natures are barely beginning to be explored.

Glucose Contents of r+ and r Viruses

The T-even phages that are found most commonly in nature are designated as r+ and are distinguished by the characteristically small turbid plaques that they produce in a bacterial film on nutrient agar plates. This plaque morphology is explained by the fact that superinfection of r+-infected bacteria by a T-even phage causes temporary lysis inhibition (*25*). The r+ phages mutate to r viruses that form large clear plaques, and an apparently limitless series of such r mutants may be obtained from any r+ phage. Lysis inhibition can be accomplished as a result of superinfection with either r+ or r viruses. Although the biochemical nature of lysis inhibition is obscure, it is possible that the mechanism can be interpreted in part in terms of "superinfection breakdown." Thus it has been observed that the DNA of the superinfecting virus of the r or r+ types may be prevented from making genetic contributions to virus multiplication and may be actively degraded (*26*). It may be suggested, then, that lysis inhibition may be effected by a DNA degradation product.

A number of physiological and analytic studies had been made of mutant pairs of T-even viruses—that is, T2r+ and T2r, T4r+ and T4r, and T6r+ and T6r. Significant differences relevant to the nature of the difference between r+ and r phages were not observed in studies of the adsorption rates, of the rates of nucleic acid and protein synthesis, of the course of multiplication, and so forth (*27*). Furthermore, in detailed analyses of the various deoxyribose nucleic acids from the 6 genetically distinct phages, it was determined that the base compositions of all these nucleic acids were substantially identical (*16*). It appeared necessary to infer at that time that, if the structure of the DNA controlled the genetic bases of mutational differences among these phages, the mutational differences arose either from quantitative differences too small to be detected by existing analytic methods or from differences in the sequential order of the nucleotides.

At the present time, lysis inhibition has been observed only with the T-even viruses—that is, with viruses containing

Table 2. Glucose/deoxyribose ratios of preparations of r+ and r viruses and of DNA preparations of r viruses. A glucose to HMC ratio of 1 would yield a glucose to deoxyribose ratio of 0.17. The numbers in parentheses in column 1 indicate the number of preparations studied.

Strain	Media	Ratio
T2r+ (5)	all	0.12–0.15
T4r+ (8)	all	0.20–0.23
T6r+ (5)	all	0.17–0.25
T2r (5)	broth	0.19–0.21
T4r (3)	broth	0.31–0.61
(3)	synthetic	1.39–1.74
T4r DNA (2)	broth	0.52, 0.52
T6r (4)	broth	0.29–0.35
(7)	synthetic	0.52–1.12
T6r DNA (4)	all	0.34, 0.35, 0.37, 0.62

HMC and glucose attached to the hydroxymethyl group. As a working hypothesis, it was supposed that the presence or absence of the HMC glucoside might be directly related to the existence of lysis inhibition. The data presented in subsequent paragraphs stem from our efforts to test this hypothesis. As a consequence of this hypothesis, it appeared possible that r+ strains might differ among themselves and from r strains with respect to the average amount of glucose bound to HMC. The properties of our r+ and r strains and isolation by differential centrifugation have been described elsewhere (*27*). Lysates of each strain have been made in a single multiplication cycle in multiply infected cells in nutrient broth or in a glucose-mineral salts medium. More than 40 preparations of virus concentrates have been obtained and analyzed, of which 17 have been of the r+ type.

The anthrone reaction was modified to permit analysis of glucose in the presence of the deoxyribose in DNA. On heating glucose, DNA, and the anthrone reagent (*28*) at 70°C for 15 minutes, only purine deoxyribose and glucose react, giving absorption maxima at 475 and 625 mμ, respectively. Although reactions of mixtures of glucose and deoxyribose give spectra that are sums of the spectra of the individual components, components of the phage preparation affected the spectra at the absorption maximum of deoxyribose. This component was therefore estimated by the diphenylamine reaction, and the hexose contents (calculated as glucose) of the preparations were estimated after correcting the absorption at 625 mμ for that due to deoxyribose at that wavelength.

The glucose-to-deoxyribose ratios of the r+ strains are presented in Table 2. It was found that preparations of T2r+ contain less glucose than HMC, and it may be concluded that at least 20 per-

cent of the HMC nucleotides do not contain glucose.

This result is consistent with the observed liberation of glucose-free HMC nucleotides from T2r+ DNA as a result of diesterase action (*24*). On the other hand, all preparations of T4r+ and T6r+ contained at least one molecule of glucose per hydroxymethyl group, a result consistent with the work of Volkin (*23*) with T4r+. The existence of a G/DR somewhat in excess of 0.17 suggests the desirability of determining whether some polyglucose chains may not be present in these viruses. In any case, whereas it is not possible to distinguish preparations of T2 from T4 and T6 on the basis of HMC content, reproducible differences have been found with respect to the amount of glucose found in the r+ virus preparations (*29*).

All r phage preparations analyzed possessed G/DR ratios that considerably exceeded the highest G/DR ratio obtained for the homologous r+ strain as seen in Table 2. Two types of r preparations were found, however. When the r phages were isolated from broth lysates, the G/DR ratios were on the average about 50 percent higher than the average of all preparations of the homologous r+ phage. No preparation of r phage was obtained that had a G/DR ratio less than 20 percent higher than that of any homologous r+ phage preparation. When the r phages were isolated from glucose-containing synthetic media, a range of relatively high G/DR ratios was obtained, much higher than the ratios for the same phages obtained from broth. When T4r and T6r phages derived from broth lysates have been used to infect bacteria in glucose media, preparations of the derived isolated r phages then possessed the higher ratios noted.

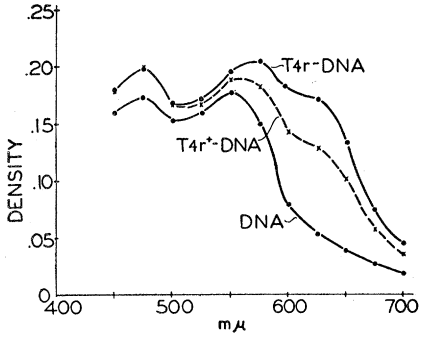

Fig. 3. Spectra of various DNA samples in the anthrone reactions: ●——● thymus DNA, 200 μg; ×——× T4r+ DNA, 234 μg, G/DR = 0.26; ○——○ T4r DNA, 194 μg, G/DR = 0.52.

In order to determine the significance of both the high G/DR ratios and their variability, two types of study were undertaken. Using the presence of RNA in the virus preparations as evidence for bacterial contamination, it was found that of 11 preparations studied, six preparations with the highest G/DR ratios were undoubtedly so contaminated. However, RNA was not detected in five preparations of r viruses in which the G/DR ratios were approximately 50 to 100 percent higher than those of the homologous r+ phages.

The analysis then turned to the study of isolated DNA to see whether the high glucose content could be obtained with these fractions of the whole phage. Preparations of r+ and r DNA were isolated by the urea method. Two have been isolated from T4r and four from T6r. The G/DR ratios of the former were 0.52 and 0.52; those of the latter were 0.62, 0.37, 0.35, and 0.34. It may be noted that the final precipitation of these polymers was accomplished by adding ethanol to the deproteinized solution containing 6*M* urea and 2*M* NaCl, the fibrous sodium nucleate being collected at 70 percent ethanol. These fibers were wound around a rod, and the adherent liquid was expressed. The fibers were then rinsed and dried. The G/DR ratios of the isolated preparations were similar to those of the preparations from which they were derived (*30*).

For example, the DNA of a T4r preparation (G/DR = 0.61) was isolated as described and found to have a G/DR ratio of 0.52, as presented in Fig. 3. Thus the DNA and hexose in excess of 1 glucose per hydroxymethyl group were coprecipitated in a fibrous strand by 70-percent ethanol. In addition, this material was partially degraded with deoxyribonuclease and caused to migrate in an electric field on paper. Sections of the single, somewhat broadened band that had moved several centimeters away from the origin were eluted and analyzed. The eluate of the front half of the material possessed a G/DR ratio of 0.42; the ratio of the back half was 0.62.

Although these data suggest a chemical relation of the extra glucose to the DNA of the r phages, it may be pointed out that the properties of the r phages increase the difficulty of their purification. Lysis of r-infected cells is more extensive than that of r+-infected cells, tending to degrade the bacterial debris to a size that may be more closely comparable to that of the phages than is debris from an r+ lysate. Such contaminating debris may be expected to liberate polysaccharide in the procedure used for the isolation of phage DNA, and conceivably such polysaccharide may precipitate with the DNA. Accordingly, experiments have been begun on the isolation of glucose-containing fragments of the DNA of the r phages in an effort to clarify the structural relationships of the extra glucose found in these preparations.

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29. We have recently found that preparations of the DNA of T2r+ are more rapidly degraded by pancreatic deoxyribonuclease than those of T6r+, although less rapidly than thymus DNA. This result parallels the observed differences in glucose content of the various virus deoxyribose nucleic acids.
30. The hexose released by hydrolysis of 10-mg samples of DNA from T6r+ and T6r has recently been found to be glucose alone, using the methods described by Volkin (*23*). The yield of glucose from T6r DNA was greater than that from T6r+ DNA, the relative yields being in essentially the same ratio as those observed by means of the anthrone reaction.

Believing, as I do, in the continuity of nature, I cannot stop abruptly where our microscopes cease to be of use. Here the vision of the mind authoritatively supplements the vision of the eye.—JOHN TYNDALL.