THE RESTRICTION OF NON-GLUCOSYLATED T-EVEN BACTERIOPHAGE DNA BY *ESCHERICHIA COLI*

by

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I hereby recommend that this dissertation prepared under my direction by Martinez Joseph Hewlett

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be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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SIGNED: Martin J. Heuschel

DEDICATION

For Martin,
in whose path I have
always tried to walk.

For Carmel,
whose faith and love
have brought me this far.

For Norma and Christopher,
because they are always a
part of me and all that I do.

Jai Guru Dev

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ABSTRACT

This dissertation describes an examination of the restriction of non-glucosylated T6 bacteriophage DNA by strains of Escherichia coli B. I have not been able to identify an activity responsible for the specific nucleolytic attack on non-glucosylated hydroxymethylcytosine containing DNA that is a feature of restriction. However, I have defined some structural features of non-glucosylated T6 phage. In addition, I have shown that the restriction activity directed against non-glucosylated T6 phage is abolished by prior infection of the host cell.

In preliminary experiments, T-even tolerant (tet) mutants of E. coli B have been shown to be defective in the enzyme UDPG-pyrophosphorylase. Progeny phage produced by T6 infection of these cells contain non-glucosylated DNA.

Membrane and cytoplasmic fractions of various E. coli strains were prepared. Comparison of membrane proteins obtained from restrictive and permissive ($r6^-$) strains revealed significant differences, indicating that the genetic alteration involved in the $r6^-$ mutation may specify a membrane associated function. Similar analyses of cytoplasmic preparations revealed no differences.

Nucleolytic degradation of DNA into acid soluble fragments was observed with membrane preparations only in the presence of ATP. This degradation was equally effective against T6 or non-glucosylated T6 ($T6\alpha gt^-$) DNA. Further experiments demonstrated that this membrane

associated activity is due, in part, to the enzyme exonuclease V (the rec B, C nuclease).

The effect of incubation with membrane preparations (without added ATP) from normal and r6⁻ cells on the sedimentation pattern of T6 and T6αgt⁻ DNA on neutral sucrose gradients was analyzed. Both DNA's were observed to sediment slower than marker DNA after treatment with either membrane preparation. This effect is due to the presence of the enzyme endonuclease I in the membrane preparations, since the effect was not seen with membranes from cells lacking this enzyme.

The metabolism of nucleic acids in normal and permissive (r6⁻) cells infected with T6αgt⁻ was investigated. In normal cells, no DNA synthesis was seen after infection by T6αgt⁻. However, extensive RNA synthesis was observed in this situation. The RNA produced was analyzed by RNA/DNA hybridization and was found to be predominantly E. coli RNA.

When r6⁻ cells were infected with T6αgt⁻, RNA synthesis was essentially normal (as judged by competitive RNA/DNA hybridization analysis), while the synthesis of DNA was increased two- to three-fold over the level found after T6 infection. DNA produced at 25 minutes after infection of r6⁻ cells by T6αgt⁻ has undergone extensive single-strand nucleolytic attack, as shown by alkaline sucrose gradient centrifugation. Similar results were obtained with DNA produced during T6 infection of a UDPG⁻ host. In addition, DNA from purified T6αgt⁻ phage showed the presence of DNA molecules containing single-strand breaks. The occurrence of such breaks in replicating non-glucosylated DNA and

in the DNA of purified T6αgt^- phage may account for the reduced burst size observed in T6αgt^- infected $r6^-$ cells.

The restriction activity of E. coli B towards T6αgt^- was found to be abolished by prior infection with T4 imm mutants (T4 mutants which fail to develop immunity to superinfection) or by co-infection with T4. This effect was not dependent on transcription or translation. The observed effect is due to some event at the time of infection and may be due to the interaction of a phage structural component with the host cell membrane.

SDS-gel analysis of proteins from disaggregated phage revealed a structural difference between T6 and T6αgt^-. This difference is due to an altered capsid protein, since analysis of proteins from T6αgt^- phage ghosts revealed that the structural alteration is not lost upon osmotic shock of intact phage. The structural difference appears to be a result of the αgt mutation. This suggests that the αgt gene is involved in the synthesis or assembly of a phage structural element.

CHAPTER 1

INTRODUCTION

When a bacteriophage of the T-even series infects an Escherichia coli cell, the initial events involve recognition of and attachment to specific sites on the cell surface, followed by injection of the phage DNA into the cell. (In accordance with current practice, the terms "bacteriophage" and "phage" will be used interchangeably throughout this work.) The DNA of the T-even bacteriophage is a linear duplex molecule of about 1.3×10^8 daltons. Each phage particle contains one such molecule, which enters the host cell intact upon infection. Immediately following infection, the cessation of host macromolecular synthesis occurs and the cell's transcriptional and translational apparatus is converted for use by the phage, leading to production of progeny phage after about 30 minutes (at 37°C). A review of these processes can be found in Mathews (1971a).

DNA Glucosylation

Phage DNA contains, instead of cytosine, the unique base 5-hydroxymethylcytosine (HMC, Fig. 1a) (Wyatt and Cohen 1952). In addition, glucose residues are found attached, through carbon-1, to the 5-hydroxymethyl group of HMC (Fig. 1b-d) (Lichtenstein and Cohen 1960). These sugar moieties occur in three types of linkages; as α-glucosyl-HMC (Fig. 1b), as β-glucosyl-HMC (Fig. 1c) or as β-1,6-glucosyl-α-glucosyl-HMC (Fig. 1d). As shown in Table I, the type and distribution

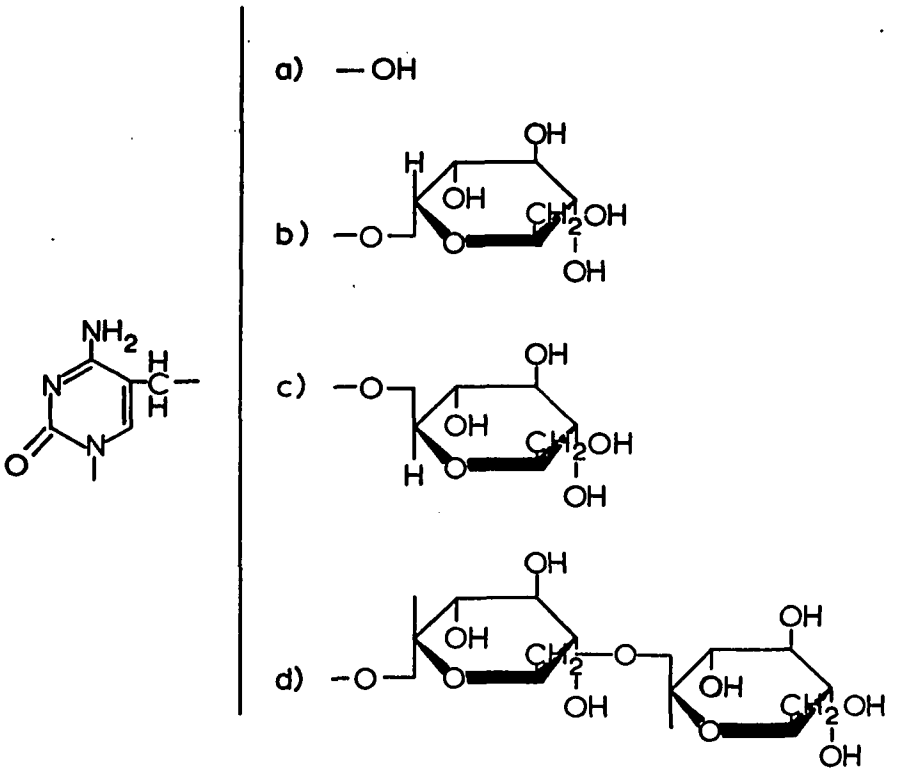

Fig. 1. Structure of 5-Hydroxymethylcytosine and its Glucosylated Derivatives.

The indicated structures are a) 5-hydroxymethylcytosine (HMC), b) α-glucosyl-HMC, c) β-glucosyl-HMC, d) β-1,6-glucosyl-α-glucosyl-HMC.

TABLE I. Distribution of Glucosyl Residues in Phage DNA[a]

	Percent Total HMC Residues			
Phage	non-glucosylated	α-glucosyl	β-glucosyl	di-glucosyl[b]
T2	25	75	0	0
T4	0	70	30	0
T6	25	3	0	72

a. Adapted from Lehman and Pratt (1960) and Revel and Luria (1970)

b. β-1,6-glucosyl-α-glucosyl-HMC

of these glucosyl-linkages is specific for each of the T-even phage, and, in some cases, not all HMC residues are glucosylated (Lehman and Pratt 1960).

The addition of glucose residues to HMC occurs after the polynucleotide chain has been formed (A. Kornberg et al. 1959). The metabolic pathways involved in this reaction are shown in Fig. 2. Glucose, from the host cell pool of uridine diphosphoglucose (UDPG), is transferred to HMC residues in newly synthesized DNA by phage glucosyl transferases synthesized de novo after infection. The specificity of the glucosyl transferases reflects the pattern of glucosylation seen in each T-even phage (Table I). Thus T2 (75% α-glucosyl-HMC) specifies an α-glucosyl transferase (Zimmerman, S. Kornberg and A. Kornberg 1962) and T4 (70% α-glucosyl-, 30% β-glucosyl-HMC) specifies both an α- and β-glucosyl transferase (Josse and A. Kornberg 1962). T6 (75% diglucosyl-HMC) produces an α-glucosyl transferase and a separate transferase specific for placing a glucose residue in β linkage to an α-glucosyl-HMC residue (S. Kornberg, Zimmerman and A. Kornberg 1961, Zimmerman et al. 1962).

The question of control of these enzymatic glucosylations is not fully understood. As seen in Table I, 25% of the HMC residues in T2 and T6 are non-glucosylated. T2 DNA, which cannot be further glucosylated by the T2 α-glucosyl transferase in vitro, can serve as a substrate for further glucosylation by the corresponding T4 or T6 enzymes (S. Kornberg et al. 1961).

In the case of T4 glucosylation, the 70:30 ratio of α to β glucosyl residues reflects a level of control, since in the absence of one

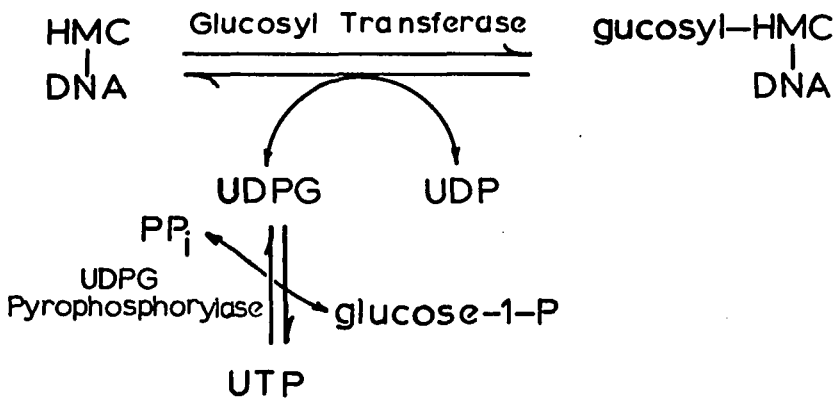

Fig. 2. Metabolic Pathways Involved in Phage DNA Glucosylation.

enzyme (α or β) the HMC residues are glucosylated (though not fully) by the remaining enzyme (Josse and A. Kornberg 1962). In vitro, the ratio of α to β glucosylation of T4 DNA can be varied by altering the Mg^{+2} concentration of the reaction mixture, suggesting that control of this ratio in vivo may be a result of the intracellular Mg^{+2} level (deWaard, Ubbink and Beukman 1967).

The kinetics of production of the glucosyl transferases and the time of onset of DNA glucosylation have been examined by several groups. S. Kornberg et al. (1961) examined the synthesis of glucosyl transferase in phage infected cells and showed that DNA replication precedes the appearance of transferase activity by about 2 minutes. Since glucosylation occurs on preformed DNA, non-glucosylated DNA might be expected to exist at early times during replication. Indeed this was observed for T6 infection, when Erikson and Szybalski (1964) found that a pool of non-glucosylated DNA (distinguished from glucosylated DNA by isopycnic banding in Cs_2SO_4 gradients) exists during the first 2 minutes of replication. However, recent evidence indicates that the T4 α-glucosyl transferase is present and active much earlier in infection, since incoming, non-glucosylated T4 becomes fully α-glucosylated within 4 minutes, when co-infected with normal T4 in a cell permissive for non-glucosylated phage (McNicol and Goldberg 1973). The evidence presented in these experiments is based on the immunological reactivity of glucosylated DNA. Since this is not an analysis of enzyme synthesis, the observed glucosylation may not necessarily reflect the de novo synthesis of the transferase at early times after infection.

"Host-controlled Modification"

Luria and Human (1952) first reported a phenomenon which they called "host-controlled modification." When T2 or T6 phage were grown on E. coli $B/4_o$ (a strain which is resistant to T3, T4 and T7 phage), the resulting progeny phage (called T*2 and T*6) were unable to grow on E. coli B. They could, however, grow on some strains of Shigella (e.g., S. dysenteriae). When T*2 or T*6 were grown for one cycle on S. dysenteriae, they regained the ability to grow normally on E. coli B.

The biochemistry of this process was found to involve defective glucosylation of T-even phage DNA. E. coli $B/4_o$ was found to lack the enzyme UDPG-pyrophosphorylase (Hattman and Fukasawa 1963). Such cells are unable to utilize galactose for growth (gal^- phenotype), since UDPG is a key molecule in the isomerization of galactose to glucose for cellular metabolism. These bacteria have no hexoses in their cell wall polysaccharide, which accounts for their altered adsorption sites for T3, T4 or T7 phage. Progeny resulting from T6 infection of $B/4_o$ have less than 5% of the normal amount of glucose in their DNA.

Shedlovsky and Brenner (1963) found that a similar UDPG negative strain (U95) produced T*6 phage. DNA extracted from these phage could be used as an acceptor of glucose from UDPG in a reaction mixture containing the T6 glucosyl transferases. When gal^- mutants that were also resistant to T3, T4 and T7 were isolated, they also produced T*2 progeny, whose DNA was non-glucosylated (Symonds et al. 1963).

Thus it is clear that host-controlled modification involves the failure of progeny phage DNA to be glucosylated normally. When such progeny subsequently infect E. coli B (or K) strains, the

non-glucosylated phage DNA is "restricted" and no progeny result. This does not happen in the case of infection of S. dysenteriae or other "permissive" hosts, which results in a normal infection including glucosylation, since S. dysenteriae has normal levels of UDPG. After a cycle of growth on S. dysenteriae, the progeny phage are again infective on E. coli B.

Host Involvement in Restriction

The principal biochemical feature of restriction is the nucleolytic degradation of the incoming (parental) non-glucosylated phage DNA. This activity is specific for DNA containing hydroxymethylcytosine, since no cytosine containing phage are restricted (Revel and Luria 1970). Phage containing non-glucosylated DNA adsorb to the cell normally, the DNA is injected (Hattman 1964) and the host cell is killed (Hattman, Revel and Luria 1966). Within the first 10 minutes after infection, the incoming DNA is incompletely degraded (30 to 50%) to trichloroacetic acid (TCA) soluble products (Hattman 1964, Fukasawa 1964).

The nucleolytic restriction activity is a function of the host cell. First, the degradation of incoming non-glucosylated DNA is not prevented by the presence of chloramphenicol (which prevents phage protein synthesis) at infection (Hattman 1964). Secondly, host mutants of E. coli B and K12 have been isolated which are permissive for the growth of non-glucosylated phage (Revel 1967).

Permissive mutants of E. coli K12 occur in two genetic loci, r6 and r2,4 (Revel 1967). Cells which are $r6^-$ permit the growth of

non-glucosylated T6 but not T2 or T4. A cell which is r2,4$^-$ is still restrictive, since the r6 locus restricts all non-glucosylated T-even phage. In order to acquire the ability to permit the growth of all three non-glucosylated T-even phage, a cell must be both r6$^-$ and r2,4$^-$.

Similar mutants of E. coli B have been isolated and characterized. In her original discussion of the work, Revel (1967) stated that the B restriction system differed from the K in that a B cell could acquire permissiveness for all non-glucosylated T-even phage in a single mutational step, that is, mutation to r6$^-$. However, in later work (Revel and Luria 1970, H. Revel, personal communication 1972) it appears that the r6 locus in B is much the same as that in K and that in B, complete permissiveness requires mutations in both loci (r6$^-$ and r2,4$^-$).

In B, the r6$^-$ mutation is found associated with a requirement for thiamine (B_1^-). Revel (1967) has stated that this association is genetic rather than functional. She concludes that all r6$^-$ mutants of E. coli B that have been isolated are deletion mutants extending into a portion of the closely linked thiamine synthesizing system. This is based on the fact that no revertants to thiamine independence (and r6$^+$) were found. In addition, E. coli K12 r6$^-$ is not thiamine dependent. However, when E. coli B r6$^-$, B_1^- cells are transduced to thiamine independence with phage P1 grown on E. coli B, all thiamine independent transductants were also r6$^+$ (Revel 1967).

The biosynthesis of thiamine proceeds via the phosphorylation of a 5-hydroxymethyl pyrimidine precursor (Fig. 3). Mutants defective in thiamine biosynthesis occur in three classes; a) those which will

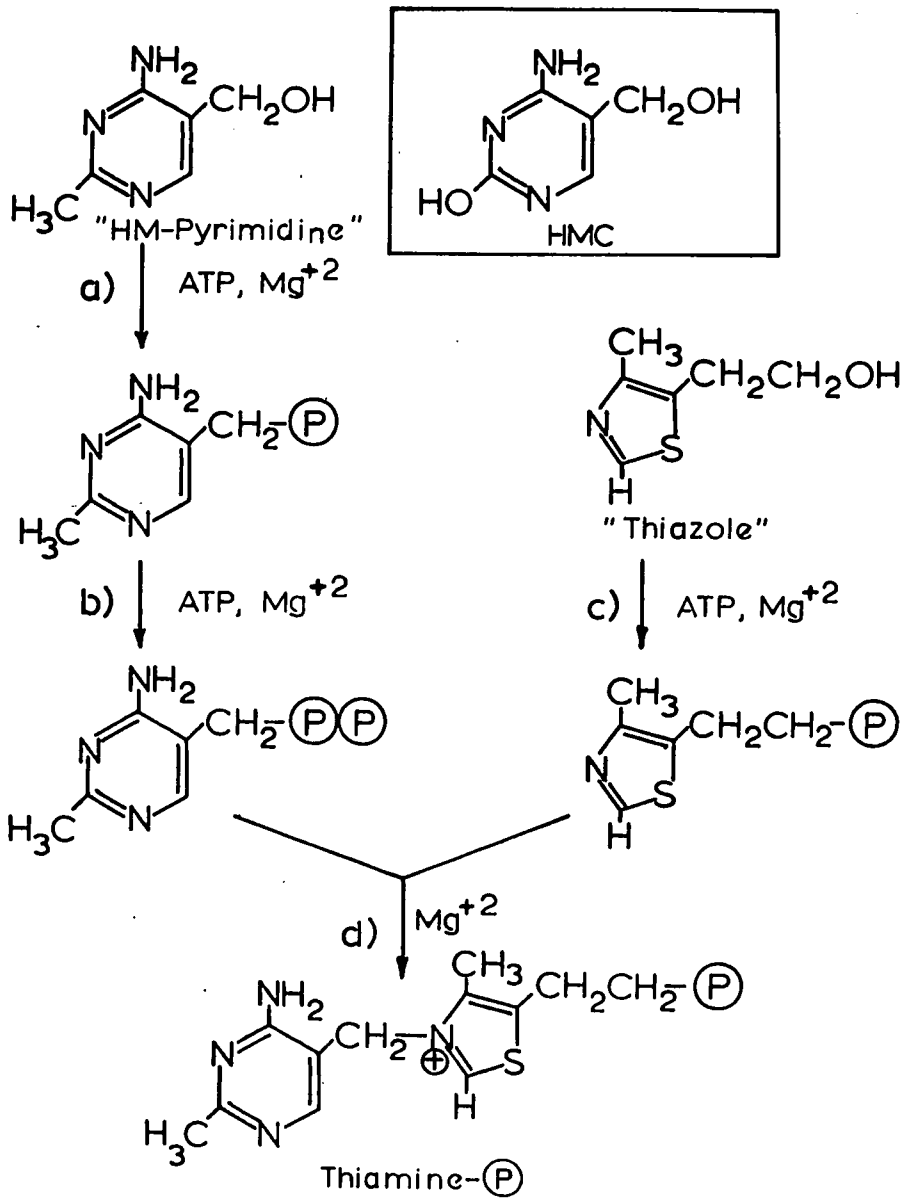

Fig. 3. Final Steps in the Biosynthesis of Thiamine.

The enzymes are a) hydroxymethylpyrimidine kinase, b) hydroxymethyl-pyrimidine phosphokinase, c) thiazole kinase and d) thiamine-P-synthetase. (Adapted from Brown 1972.) HMC (inset) is shown for comparison.

grow on thiamine or its pyrimidine precursor, b) those which will grow on thiamine or its thiazole precursor and c) those which will only grow on thiamine and not on a mixture of the pyrimidine and thiazole precursors (Brown 1972). Mutants of type a) cannot synthesize the thiazole moiety and mutants of type b) cannot synthesize the pyrimidine moiety. Mutants of type c) are deficient in some step in the joining of the two precursors to form thiamine phosphate (one of the 4 reactions shown in Fig. 3). E. coli r6$^-$ B_1^- cells will not grow on a mixture of the two thiamine precursors, but will grow only on thiamine (Revel 1967). The structural similarity between the pyrimidine moiety of thiamine and 5-hydroxymethylcytosine suggests that restriction and the thiamine system may be causally related.

Phage Mutants Defective in Glucosylation

T-even phage defective in synthesis of glucosyl transferases have been isolated. Mutants of T2 (T2αgt$^-$) are lacking the α-glucosyl transferase (Revel, Hattman and Luria 1965). Mutants of T6 (T6αgt$^-$) also lack the α-glucosyl transferase. As discussed earlier, a second glucosylation occurs in T6, adding a β-glucose to an α-glucosyl-HMC residue in DNA. Thus a single mutation of the α-transferase yields non-glucosylated phage DNA after infection (Revel et al. 1965).

For T4, however, both the α- and β-glucosyl transferases must be inactivated to produce non-glucosylated phage DNA, since either enzyme can act to glucosylate the phage DNA sufficiently to escape restriction. Such mutants (T4αgt$^-$, βgt$^-$) have been isolated (Georgopoulos 1967).

Features of Infection in Restrictive Hosts

As mentioned previously, after infection of a restrictive host by a non-glucosylated T-even phage, the incoming DNA is incompletely degraded to TCA soluble products. The size of the fragments remaining TCA insoluble is not known. However, this DNA is partially active, since synthesis of the "early" (pre-replicative) enzyme deoxycytidylate hydroxymethylase (dCMP-HMase) occurs at very low levels after infection of E. coli B with T*2 and T*6 (Hattman 1964). In vivo marker rescue was observed between T*2, T*4 and T*6 and T4 mutants in gene 42 (dCMP-HMase negative). Since the T* phage are gene 42^+, this signifies that sufficient active enzyme is synthesized under control of the non-glucosylated phage genome to rescue the defective function in the gene 42 mutant. Other work demonstrated that deoxynucleotide kinase is produced at low levels by T*4 infected E. coli W3110 (Fukasawa 1964). The kinetics of synthesis of these two enzymes was said to be abnormal since the synthesis occurs at a slower rate than normal and is dependent on the multiplicity of infection (m.o.i.), unlike normal infection. Indeed, the enzymes were being synthesized as late as 60 minutes after infection, indicating a defect in the normal termination of early enzyme synthesis. In contrast, during infection of E. coli B with T2gt^-, some early enzymes are synthesized at a low level, but are terminated at the same time as during infection with T2 (Hattman et al. 1966). The apparent regulation in the case of T2gt^- was thought to be due to the degradation of the non-glucosylated parental DNA rather than the mechanism operating during normal phage infection.

T*2 phage can be partially rescued at high m.o.i. on E. coli B. This suggests that the DNA fragments remaining after nucleolytic attack are large enough to regenerate complete genomes, possibly by recombination. However, in similar experiments performed with T*4, this effect was not seen (Hattman 1964). The explanation for this difference may be that DNA fragments remaining after T*4 restriction are too small to undergo the proposed recombination.

Although some early enzymes are produced during infection by non-glucosylated phage, no DNA synthesis occurs nor are any "late" (post-replicative) functions expressed (Hattman 1964, Fukasawa 1964).

Chloramphenicol added at the time of infection does not inhibit the degradation of non-glucosylated DNA in restrictive cells (Hattman 1964). In addition, Hattman (1964) reported that co-infection of T*2 and T2 does not inhibit the degradation of T*2 DNA. He did not present the data in the reference cited, but the experiment was performed at a high ratio of T2 to T*2 phage (presumably 20:1). This experiment was not performed with other T* phage.

The extent of glucosylation necessary to protect against restriction of phage DNA has been investigated. Only one strand of a DNA duplex need be glucosylated to protect against restriction (Boyle, Ritchie and Symonds 1965). Boyle et al. (1965) showed that the small number of T* phage which escape restriction are those which contain, in one strand of their DNA, a large amount of glucosylated (parental) DNA. Other studies indicate that it is the specificity rather than the amount of glucosylation that is important in avoiding restriction (Georgopoulos and Revel 1971). Normal T2 phage (75% α-glucosylated) are restricted by

the product of the r2,4 gene of E. coli K12. However, T6 (75% diglucosylated) or T4βgt⁻ (80% α-glucosylated) can grow on either B or K12. T4αgt⁻ (100% β-glucosylated) is partially restricted by the r2,4 gene of both B and K12, but is unrestricted by the r6 gene of K12. This data implies that both the nature of the glucosylation (α or β) and the sequences of the DNA glucosylated (e.g., T2 versus T6) are important in determining the recognition of DNA by the restriction function.

Features of Infection in Permissive Hosts

When a permissive host is infected by a non-glucosylated phage, the incoming DNA is not degraded (Hattman 1964). The infection is productive, but the number of progeny produced per infected cell (burst size) is one-fifth to one-tenth that observed in a normal infection (Georgopoulos and Revel 1971). These authors wished to determine whether the lowered burst size was due to the lack of glucose residues on the infecting DNA or to the absence of the gt product. Utilizing amber gt mutants of T4 or T6 and permissive hosts which were either su_I^+ or su_I^- (with or without the amber supressor), they observed that the decreased burst size was not a function of glucosylation of the parental DNA. For instance, T6αgt⁻ grown in a host cell CR63r6⁻ (a su_I^+ K strain) produces progeny phage with glucosylated DNA. If these progeny are used to infect a su_I^-, r6⁻ K strain, the burst size is as low as that found when the parental DNA is non-glucosylated. Therefore the lowered burst size was attributed to the lack of the gt gene product. This was substantiated by the fact that earlier reports indicated a normal burst size

after T6 infection of $B/4_o$ ($UDPG^-$) cells (Fukasawa and Saito 1964). In this case, the gt gene is functioning normally during infection.

Georgopoulos and Revel (1971) suggested other explanations for the decreased burst size. Replicating non-glucosylated DNA might be subject to nucleolytic attack within the cell. Another explanation (Georgopoulos and Revel 1971) is based on the fact that glucosylation might be considered a step in DNA maturation. If this is so, then non-glucosylated DNA may not be competent for efficient transcription of late RNA species.

Attempts to Identify the Host Restriction Mechanism

At present, the specific host functions responsible for the nucleolytic degradation of non-glucosylated DNA have not been identified. Early reports implicating either exonuclease III (Richardson 1966) or endonuclease I (Molholt and Fraser 1965) proved to be misleading. Eigner and Block (1968) demonstrated the normal restricting properties of E. coli mutants lacking endonuclease I. In addition, normal levels of the known deoxyribonucleases, including exonuclease III and endonuclease I, were reported in permissive mutants (Eigner and Block 1968, Revel and Georgopoulos 1969). However, although these studies rule out the idea that these two enzymes are the primary restriction nuclease, it is possible that they may serve some function in the further degradation of the DNA.

Attempts to detect a nucleolytic activity specific for non-glucosylated HMC-containing DNA in cell extracts have proved fruitless (Revel and Luria 1970, H. Revel, personal communication 1972). However

an activity linked to the restriction system has been demonstrated in a toluenized cell system (Fleischman and Richardson 1971). E. coli cells treated with toluene will synthesize DNA when presented with the necessary deoxyribonucleoside triphosphates and ATP. When E. coli W3110 is toluenized and provided with hydroxymethyl-dCTP instead of dCTP, no DNA synthesis is observed. If hydroxymethyl-dCTP is added to the system while DNA synthesis is in progress with dCTP, the synthesis stops. These effects are not seen with toluenized E. coli K12 $r6^{-}r2,4^{-}$. In this case, hydroxymethyl-dCTP gives 70% of the synthesis seen with dCTP. Since DNA synthesized with hydroxymethyl-dCTP would resemble non-glucosylated phage DNA, these results are interpreted as the action of the cell restricting system. The authors suggested that the inhibition of DNA synthesis is a result of a direct effect on the replication mechanism by hydroxymethyl-dCTP. However, they did not determine if newly synthesized HMC-containing DNA is degraded under these conditions. It should be emphasized that these are uninfected cells, and the DNA being made is E. coli DNA.

Toluenization presumably makes cells permeable, with the eventual loss of enzymes from the cytoplasm and the periplasmic space. The demonstration that the restriction function is unaffected by toluenization lends some support to the idea that the restriction system might be membrane associated. Fukasawa (1964) had suggested this in an attempt to explain the fact that incoming DNA is restricted while replicating DNA (temporarily non-glucosylated) is not. The idea was that the restriction function was located at some strategic point at the cell surface, and that replicating DNA exists in an area of the cell not

available to this function. If the activity is membrane associated, it could explain the failure to detect restriction activity in cell extracts.

Present Work

In this dissertation, I will show that while I have not identified the nucleolytic activity responsible for restriction, I have obtained further evidence that it is membrane associated.

I will also show that the reduced burst size of gt mutants in permissive hosts is due to the occurence of single-strand breaks in progeny phage DNA. Experiments will reveal that purified T6αgt⁻ phage contain DNA with single-strand breaks. Stocks of such phage contain a large percentage of non-viable phage particles.

I will further demonstrate that the restriction activity which degrades non-glucosylated phage DNA is abolished by prior infection with normal phage. This effect is not due to glucosylation of the phage DNA, but is due to some effect of the attachment of the phage.

A comparison of T6 and T6αgt⁻ phage structural proteins will show that a significant difference between these two phage exists. This difference is still seen after ghosts of the T6αgt⁻ phage are prepared, indicating that the altered protein is not an internal protein.

CHAPTER 2

MATERIALS AND METHODS

Materials

Bacteria and Bacteriophage Strains

Tables II and III list the strains of bacteria and phage used in the course of this work, along with their pertinent characteristics and the source from which they were obtained. Bacterial stocks were maintained on nutrient agar slants containing 0.001% thiamine. Phage stocks were prepared in M9 salts (see below) for storage. All stocks were kept at $4^{o}C$.

Media

Nutrient broth contained, per liter, 8.0 gm dehydrated nutrient broth, 5.0 gm NaCl and 1.0 mgm thiamine.

M9 medium (Adams 1959) contained, per liter, 6.0 gm Na_2HPO_4, 3.0 gm KH_2PO_4, 1.0 gm NH_4Cl, 0.01 gm $CaCl_2$, 0.005 gm $Fe(NH_4)_2SO_4$, 0.2 gm $MgSO_4 \cdot 7H_2O$ and 2.0 to 4.0 gm glucose.

Radiochemicals

The following radioactively labeled compounds (of the indicated specific activities) were used: ATP(γ-^{32}P), 16 Ci/mmole; L-leucine-1-^{14}C, 53.5 mCi/mmole; L-leucine-4,5-^{3}H, 41.2 Ci/mmole; thymidine(methyl-^{3}H), 20 Ci/mmole; UDPG(^{14}C-glucose), 246 mCi/mmole; uracil-2-^{14}C, 7.2 mCi/mmole; uracil-5-^{3}H, 20.4 Ci/mmole and uracil-6-^{3}H, 26 Ci/mmole.

TABLE II. Properties of E. coli Strains

Strain	Properties	Source
B strains		
B	normal (wild-type)	C. K. Mathews[a]
B201	low thymine requiring	"
tet-1	T-even tolerant mutant	"
tet-2	T-even tolerant mutant	"
tet-1-T$^-$	low thymine requiring derivative of tet-1	"
B/4$_o$	UDPG-PPase negative, resistant to T3, T4 and T7	H. Revel[b]
Br6$^-$B$_1^-$	permissive for non-glucosylated T6, requires B$_1$ for growth	"
ER22	endonuclease I negative	"
ER22r6$^-$B$_1^-$	derivative of ER22, permissive for non-glucosylated T6, requires B$_1$ for growth	"
K strains		
DM821	rec$^+$ (for additional properties, see Moody, Low and Mount 1973)	D. Mount[a]
DM910	recB derivative of DM821	"
DM836	recC derivative of DM821	"
Kr6$^-$r2,4$^-$	permissive for non-glucosylated T2, T4 and T6	H. Revel[b]
W3110	K strain (normal)	"
W4597	UDPG$^-$ K strain	"

a. University of Arizona, Tucson, Arizona

b. California Institute of Technology, Pasadena, California

TABLE III. Properties of Bacteriophage Strains

Strain	Properties	Source
T4D	normal (wild-type)	C. K. Mathews[a]
T6	normal (wild-type)	"
T4αgt1βgt27	lacks both glucosyl transferases, abbrev. αgt⁻ βgt⁻	H. Revel[b]
T6gt41	lacks α-glucosyl transferase, abbrev. αgt⁻	"
T6gtam16am30	double amber mutant of α-glucosyl transferase, abbrev. αgt⁻ 2xam	"
T4imm⁻2am42	immunity negative, dCMP-HMase negative	J. Cornett[a]
T4imm⁻2 s⁻	immunity negative, spackle negative	"

a. University of Arizona, Tucson, Arizona

b. California Institute of Technology, Pasadena, California

All radiochemicals were stored at -20°C until use. The compounds listed above were purchased from New England Nuclear.

Biological Materials

All enzymes used were obtained from Sigma Chemical Corp., except that Pronase was from Calbiochem. Proteins for use as molecular weight standards were also purchased from Sigma. Calf thymus DNA, *E. coli* transfer RNA and rifampicin were from Schwarz/Mann Co. Chloramphenicol (chloromycetin) was obtained from Parke, Davis and Co. Nutrient broth, nutrient agar and agar agar were products of Difco.

Other Reagents

(Tris-hydroxymethyl)aminomethane (Tris) and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Corp. Reagents for polyacrylamide disc gel electrophoresis were products of Eastman Kodak Co., except that Coomassie Brilliant Blue was purchased from Canalco. Diethyl pyrocarbonate was purchased from K and K Laboratories. Protosol, Omnifluor and Aquasol were all products of New England Nuclear. All other reagent grade chemicals were obtained from local sources.

Methods

Phage Growth, Purification and Titer

Phage stocks were routinely prepared by infecting the appropriate host bacteria (grown at 37°C in M9 to about 10^8 cells/ml) at a m.o.i. of 0.02, followed by aeration at 37°C to lysis. The lysates were treated with DNase and RNase (1 μgm/ml each) for 1 hour at 37°C, followed by 2 cycles of differential centrifugation (3,000 x g for 10

minutes, then 140,000 x g for large volumes or 111,000 x g for small volumes for 45 minutes). The final phage pellet was resuspended in M9 salts.

Phage titers were determined by observation of plaque formation in a 0.8% nutrient agar overlay on 1.5% nutrient agar plates, using an appropriate host bacterium. Titers were recorded as plaque forming units (pfu)/ml.

In some cases phage were prepared containing radioactively labeled DNA. Host cells were grown at 37°C in M9 containing 2 µgm/ml of uracil to about 10^8 cells/ml. The cells were infected with phage at a m.o.i. of 0.02 and either ^{14}C-uracil (1.25 µCi/ml) or 6-^{3}H uracil (5 µCi/ml) was added. Aeration was continued at 37°C to lysis. Purification and assay were as described above.

Centrifugation

Unless otherwise specified, all centrifugations were at 4°C. Low speed centrifugations (less than 12,000 x g) were carried out in a Sorvall RC2 centrifuge. High speed centrifugations (greater than 12,000 x g) were performed in a Beckman Preparative Ultracentrifuge.

Extraction of DNA

Intact DNA was extracted from purified phage by the method of Thomas and Abelson (1966), consisting of three phenol extractions by gentle rotation at room temperature. An equal volume of phenol (redistilled over Zn) saturated with 0.02 M phosphate buffer, pH 6.8 was added to the phage suspension. After each extraction, the phenol phase was discarded. The final aqueous phase was dialyzed extensively against

1/100 x SSC (SSC = Standard Saline Citrate; 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), and the final DNA solution was stored over chloroform. Phage DNA to be used for gradient analysis was always pipetted with minimum shear force, by using, by using a 1.0 ml plastic pipet with the constricted tip removed.

E. coli DNA was prepared by resuspending cells in 3.0 ml of M9 salts and adding 0.5 ml Tris-HCl buffer (0.1 M, pH 7.0), 0.5 ml SDS (10% w/v) and 0.5 ml Na_2EDTA (0.1 M) and incubating at 37°C for 10 minutes. After addition of 5 mgm Pronase, incubation was continued for 4 hours. 5.0 ml of phosphate buffer-saturated phenol was added and the solution was rotated slowly at room temperature for 30 minutes. The aqueous phase was removed to a separate tube and overlaid with 3 volumes of ice cold 95% ethanol and the DNA spooled out from the interface on a glass rod. The DNA was redissolved in 2 x SSC and treated with 20 µgm/ml RNase (boiled for 10 minutes in water to destroy contaminating DNase activity) at 37°C for 2 hours. The phenol extraction was repeated twice and the final aqueous phase was dialyzed extensively against 1/100 x SSC.

Protein Determination

Protein concentration was determined by the Lowry method (Lowry et al. 1951). Standard curves were obtained with 1.0 to 5.0 µgm of bovine serum albumin.

Sucrose Gradients

5.0 ml neutral and alkaline 5 to 20% (w/v) sucrose gradients were prepared as described by Murray and Mathews (1969). Linear

gradients were formed in nitrocellulose tubes (Spinco) by placing 2.4 ml 20% sucrose and 2.6 ml 5% sucrose in the chambers of a standard gradient former. Gradients were routinely overlayed with 0.2 ml of the sample to be analyzed. After centrifugation, gradient fractions were collected with a Buchler fractionator and Polystaltic pump.

SDS-Polyacrylamide Disc Gel Electrophoresis

Analysis of radioactively labeled protein samples by SDS-polyacrylamide disc gel electrophoresis was performed as described (Laemmli 1970 and R. Haselkorn, University of Chicago, personal communication 1972). Gels were prepared containing either 7.5% or 10% acrylamide in the separating gel (10 cm in length) and 3.0% acrylamide in the stacking gel (0.1 cm in length). Gels were 6 mm in diameter and were run at a constant current of 3 mA/gel. After electrophoresis, gels for radioassay were washed with acetone, frozen on dry ice and stored at -80^{o}C. For analysis the gels were sliced with a cutter (designed by C. G. Crawford of our department) consisting of a series of stainless steel razor blades, held approximately 1 mm apart by metal washers, giving slices of 1 mm thickness. Slices so obtained were placed in glass scintillation vials along with 0.5 ml Protosol/water (9:1, v/v) and incubated overnight at 55^{o}C. After cooling, 10.0 ml of Omnifluor was added and the vials were shaken and radioactivity determined.

Gels to be stained for observation of protein bands were placed in 50% TCA overnight at room temperature. Gels were stained with Coomassie Brilliant Blue as described by Laemmli (1970) and diffusion destained in 7% acetic acid.

Radioactivity Measurements

Scintillation cocktails used for counting were either Omnifluor (4 gm/liter of toluene) or Aquasol (used as supplied). All radioassays reported here were performed in a Beckman LS-250 Liquid Scintillation system. In some cases, the data output was recorded on a paper tape, using a standard (ASCII) code. The tapes were retained for data analysis.

Data Analysis

Tapes of liquid scintillation data were analyzed using a Wang Model 700 Advanced Programming Calculator, equipped with a Wang Model 702 X,Y Plotter and a Model 703 Paper Tape Reader (Wang Laboratories, Inc.). This system was used for spillover correction in double isotope experiments, computation and plotting of disc gel data, and initial plotting of sucrose gradient data.

UDPG-Pyrophosphorylase Assay

Cells were grown in 100 ml nutrient broth to 3×10^8 cells/ml, then centrifuged at 4,000 x g for 20 minutes, resuspended in 3.0 ml Tris-HCl buffer (0.1 M, pH 7.8) and disrupted by sonic oscillation for 40 seconds with a Branson Sonifier (Heat Systems, Inc.). The extracts were centrifuged at 12,000 x g for 30 minutes. A coupled enzyme assay system was modified slightly from earlier methods (Mills and Smith 1965, Fukasawa, Jokura and Kurahashi 1963). The reaction mixture contained 0.8 ml Tris-HCl buffer (0.1 M, pH 7.0), 0.1 ml cell extract, 0.01 ml $MgCl_2$ (0.5 M), 0.01 ml cysteine (0.25 M, pH 7.0), 0.02 ml NADP (12.5 mM), 0.01 ml phosphoglucomutase (12 units/ml), 0.01 ml

glucose-6-phosphate dehydrogenase (12 units/ml), 0.01 ml UDPG (40 mM) and 0.01 ml glucose-1,6-diphosphate (0.24 mM). The reaction was initiated by addition of 0.05 ml Na-pyrophosphate (0.1 M). The total reaction mixture had a volume of 1.0 ml and was contained in a quartz cuvette (1 cm light path, 1.5 ml total volume). The overall reaction results in a reduction of NADP and was observed by monitoring the increase in absorbance at 340 nm. The reaction was followed at 34°C in a Beckman DU spectrophotometer equipped with a Gilford Absorbance Indicator, Sample Changer and Multiple Sample Absorbance Recorder (Gilford Instruments). Enzyme activity (μmoles NADPH formed/mgm protein/hour at 34°C) was calculated assuming a molar extinction coefficient for NADPH at 340 nm of 6.22 $cm^2/\mu mole$.

DNA Glucosylation Assay

A crude T6 glucosyl transferase preparation was obtained by infecting a 100 ml culture of E. coli B (in M9 at 3 x 10^8 cells/ml) with T6 at a m.o.i. of 5. At 12 minutes post-infection, the culture was centrifuged at 4,000 x g for 20 minutes and the cells were resuspended in 3.0 ml glycylglycine buffer (0.05 M glycylglycine, 0.002 M Na_2EDTA, 0.002 M reduced glutathione, pH 7.0) (Revel and Georgopoulos 1969). The cells were sonicated as described in the previous section. The extracts were centrifuged at 12,000 x g for 30 minutes. The resulting supernatant served as the crude enzyme preparation.

T6 phage were grown for one cycle in various E. coli strains and purified as described earlier in this chapter. DNA was extracted by the phenol technique described earlier. For the glucosylation assay,

the reaction mixture contained 0.7 ml Tris-HCl buffer (0.1 M, pH 7.0), 0.01 ml $MgCl_2$ (0.5 M), 0.02 ml KCl (0.1 M), 10 µgm DNA, 0.005 ml UDPG (4 mM) and 1 µCi UDPG(^{14}C-glucose). The reaction was initiated by addition of 0.1 ml of the crude enzyme preparation. The mixture was incubated at 37°C and, at various times, 0.1 ml samples were transferred to Whatman #3 filters (2.4 cm diameter) containing 0.1 ml 5% TCA. The filters were dried, washed 3 times with 5.0 ml each of cold 5% TCA, and 3 times with acetone. The filters were dried and counted in 5.0 ml Omnifluor.

Preparation of Lysozyme/EDTA Spheroplasts

Spheroplasts of various E. coli strains were prepared by the method of Osborn et al. (1972). Cells were grown in nutrient broth or in M9 supplemented with 0.05 volumes of nutrient broth. A typical preparation began with a 100 ml culture of cells (at 4 to 5 x 10^8 cells per ml). Cells were centrifuged at 4,000 x g for 20 minutes. The inside of the centrifuge bottles were dried with a Kimwipe and the cells were resuspended in 6.5 ml sucrose (0.75 M in 0.01 M Tris-HCl buffer, pH 7.5) and placed in ice for plasmolysis (less than 10 minutes). Lysozyme was added to 100 µgm/ml for an additional 2 minutes. 13.0 ml Na_2EDTA (1.5 mM, pH 7.5) was added at the rate of 1.3 ml/minute with constant stirring (using a Buchler Polystaltic pump and magnetic stirrer). The effectiveness of the spheroplasting procedure was monitored by microscopic observation of the cells under phase contrast.

Preparation of Total Membrane and Cytoplasmic Fractions

Spheroplasts were disrupted by either sonication or osmotic shock (Osborn et al. 1972). A total membrane fraction was obtained by centrifuging disrupted spheroplasts at 140,000 x g for 7.5 hours. The cytoplasmic fraction (supernatant) was removed and, after one washing, the total membrane (pellet) was resuspended in 2.0 ml sucrose (25% w/v, in 5 mM Na_2EDTA, pH 7.5) as described (Osborn et al. 1972). The method of preparation of total membrane (TM) and cytoplasmic (CYTO) fractions is designated by a superscript indicating either sonication (e.g., TM^{son}) or osmotic shock (e.g., $CYTO^{osm}$).

Labeling and Disaggregation of TM and CYTO Proteins for SDS-gel Analysis

Cells were grown in M9 supplemented with 0.05 volumes of nutrient broth. Proteins were labeled with either ^{14}C-L-leucine (0.2 μCi per ml of culture) or with ^{3}H-L-leucine (0.8 μCi/ml of culture) throughout growth. TM^{osm} and $CYTO^{osm}$ fractions were prepared from mixed ^{14}C and ^{3}H labeled cells as described in the previous section. CYTO fractions were lyophilized, redissolved in a small volume of water and dialyzed against 1/10 x SSC. Disaggregation in SDS was performed as described by Laemmli (1970) at 65°C for 30 minutes. Samples for analysis contained about 0.1 mgm of protein per ml.

Nuclease Assays

^{14}C- and ^{3}H-labeled phage DNA was prepared for use as a substrate by growth of phage in the presence of ^{14}C- or ^{3}H-uracil. A typical reaction mixture contained 0.01 ml Tris-HCl buffer (0.1 M, pH 7.8,

containing 0.2 M mercaptoethanol), 0.01 ml $MgCl_2$ (0.5 M), 0.05 ml ^{14}C- or ^{3}H-DNA (about 100 µgm/ml) and 0.05 ml TM^{son} or TM^{osm}. In some cases, the mixture included 0.01 ml ATP (0.15 M). Distilled water was added to give a total volume of 0.3 to 0.5 ml. The mixture was incubated at 37°C and at various times 0.05 ml samples were transferred to 2.0 ml cold 5% TCA, along with 0.1 ml calf thymus DNA (1 mgm/ml). After 15 minutes in ice, the precipitates were collected on 0.45 µ Millipore filters (2.4 cm diameter) and washed 3 times with cold 5% TCA. The filters were dried and counted in 5.0 ml Omnifluor.

Gradient Analysis of DNA Treated with TM^{osm}

^{14}C- or ^{3}H-labeled phage DNA was extracted with minimum shear as described earlier in this chapter. The reaction mixture contained 0.02 ml Tris-HCl buffer (0.1 M, pH 7.0, containing 0.2 M mercaptoethanol), 0.02 ml $MgCl_2$ (0.5 M), 0.02 ml E. coli tRNA (2.5 mgm/ml), 0.05 ml labeled DNA (about 100 µgm/ml) and distilled water to give 0.5 to 0.6 ml total volume. The mixture was incubated at 37°C for 30 minutes, after which 0.02 ml diethyl pyrocarbonate (20%, v/v, in 95% ethanol) was added to inhibit nucleolytic action (Fedorcsak and Ehrenberg 1966). Appropriately labeled marker DNA was added at this point. A sample (0.2 ml) of the reaction mixture was layered on a 5 to 20% neutral sucrose gradient. The gradients were centrifuged at 150,000 x g (average force) for 80 minutes and fractions were collected (as described earlier) on Whatman #3 filter paper discs (2.4 cm diameter). The filters were dried, washed 3 times (15 minutes each) with cold 5% TCA, 3 times with acetone, dried and counted in 5.0 ml Omnifluor.

Measurement of DNA and RNA Synthesis in Infected Cells

E. coli strains were grown in M9 containing uracil (20 μgm/ml) at 37°C to 3 x 10^8 cells/ml. The cells were centrifuged at 4,000 x g for 20 minutes and resuspended in 100 ml M9 containing uracil (2 μgm/ml). Cells were infected at a m.o.i. of 4 and 0.2 μCi/ml ^{14}C-uracil was added. Incorporation of ^{14}C-uracil into DNA and RNA was determined as described by Mathews (1968).

When uracil-5-^{3}H was used (to label RNA only) the procedure was altered slightly. At infection, 1 μCi/ml uracil-5-^{3}H was added. At various times after infection, 1.0 ml samples were transferred to 1.0 ml ice cold 10% TCA along with 0.1 ml calf thymus DNA (1 mgm/ml). After standing in ice for 15 minutes, the precipitates were collected on 0.45 μ Millipore filters and washed 3 times with 5.0 ml cold 5% TCA. The filters were dried and counted in 5.0 ml Omnifluor.

RNA/DNA Hybridization and Competitive RNA/DNA Hybridization

Radioactively Labeled RNA. Cells were grown in 100 ml M9 containing uracil (20 μgm/ml) at 37°C to 3 x 10^8 cells/ml. The cells were centrifuged at 4,000 x g for 20 minutes and resuspended in 100 ml M9 containing uracil (2 μgm/ml). Phage were added at a m.o.i. of 4 and the culture was aerated at 37°C. Pulse-labeling (2 to 5 minutes for early RNA or 15 to 20 minutes for late RNA) was achieved by addition of 100 μCi of uracil-5-^{3}H. At the end of the pulse, the culture was poured into centrifuge bottles containing crushed ice at -20°C, centrifuged at 4,000 x g for 20 minutes and the RNA extracted as described by Mathews and Hewlett (1971).

Unlabeled RNA. Cells were grown as described above, except that no uracil was added to the medium. 500 ml cultures were infected with phage and the infection stopped at either 5 minutes (early) or 20 minutes (late) by pouring the culture into centrifuge bottles containing ice at -20°C. Centrifugation and RNA extraction were as described above.

Characterization of the RNA. Purity of RNA preparations (with regard to DNA) was measured by a) precipitation in cold 5% TCA, b) RNase digestion (0.1 mgm/ml heat-treated RNase in 2 x SSC for 3 hours at 37°C and c) KOH hydrolysis (0.3 N KOH at 37°C for 3 hours). RNA concentrations were calculated by determining the absorbancy at 260 nm (A_{260}) of a solution, and assuming that an A_{260} of 1.0 is equivalent to 40 µgm/ml RNA.

DNA Filters. T6 DNA or E. coli DNA were extracted as described earlier. Concentrations were calculated by determining the A_{260} of a solution and using the specific absorbancy of 0.0181 $cm^2/\mu gm$ for glucosylated DNA and 0.020 $cm^2/\mu gm$ for non-glucosylated DNA (Thomas and Abelson 1966).

DNA was denatured in 1/100 x SSC by adjusting the pH to 13.0 with 1.0 N NaOH and stirring at room temperature for 10 minutes. The solution was neutralized with 1.0 N HCl and the denatured DNA stored in the cold over chloroform.

DNA for binding to filters was diluted in 2 x SSC (containing 3 mM $MgCl_2$) to the appropriate concentration. Binding to 0.45 µ Millipore filters (2.4 cm diameter) and washing was carried out as described by Gillespie and Spiegelman (1965). Filters for RNA/DNA hybridization

contained 100 μgm of T6 DNA or 50 gm of E. coli DNA. Filters for competitive RNA/DNA hybridization contained 2.5 μgm of T6 DNA. All filters were dried in vacuo overnight at 80°C and stored in a desiccator until use.

Hybridizations. All hybridizations were carried out in a total volume of 2.0 ml 2 x SSC at 66°C for 6 hours. RNA/DNA hybridizations contained 1 to 2 μgm ^{3}H-RNA per assay. Competitive RNA/DNA hybridizations contained 2.5 μgm ^{3}H-RNA and up to a 60-fold excess of competing unlabeled RNA. After 6 hours, the filters were washed and treated with RNase (Gillespie and Spiegelman 1965). The filters were dried and counted in 5.0 ml Omnifluor.

Gradient Analysis of DNA from Infected Cells

Cells were grown in M9 at 37°C to 3 x 10^{8} cells/ml. For radioactive labeling of DNA with ^{14}C- or ^{3}H-uracil, the cells were grown in the presence of 20 μgm/ml uracil, centrifuged and resuspended in M9 containing 2 μgm/ml uracil. When ^{3}H-methyl-thymidine was used, the cells were grown in M9 containing 5 μgm/ml thymidine. The cells were infected with phage at a m.o.i. of 5. At 10 minutes after infection either ^{14}C-uracil (5 μCi/ml) or 6-^{3}H-uracil (20 μCi/ml) was added. In other experiments, ^{3}H-methyl-thymidine (5 μCi/ml) was added at 5 minutes after infection. At 25 minutes after infection, samples were taken (0.5 ml) and lysed at 65°C as described by Murray and Mathews (1969). The lysis mixture was 0.05 M Tris, 0.05 M NaCl, 0.05 M Na_2EDTA, 0.1 M KCN, pH 8.0 containing 200 μgm lysozome/ml. For neutral gradients, radioactively labeled marker phage DNA was added to the lysis mixture,

whereas for alkaline gradients, phage containing radioactively labeled DNA were added as a marker. Phage are ruptured and release their DNA during denaturation for alkaline gradients. Lysed samples were denatured for alkaline gradients by making the lysates 0.3 N in KOH and incubating at room temmperature for 5 minutes. 0.2 ml of the lysate was layered onto 5.0 ml gradients, prepared as described earlier. Neutral gradients were centrifuged at 96,000 x g (average force) for 60 minutes at room temmperature. Alkaline gradients were centrifuged at 150,000 x g (average force) or at 173,000 x g for 75 minutes at room temperature.

When the DNA was labeled with ^{14}C- or ^{3}H-uracil, fractions were collected into plastic culture tubes and incubated overnight in 0.3 N KOH at 37°C (to hydrolize RNA). Aliquots of each fraction were pipetted onto Whatman #3 filter paper discs (2.4 cm diameter) and dried. The filters were washed 3 times (15 minutes each) in cold 5% TCA, 3 times in acetone and dried. The filters were counted in 5.0 ml Omnifluor. When the DNA was labeled with ^{3}H-thymidine, fractions were collected directly onto filter paper discs, dried, washed as described and counted in 5.0 ml Omnifluor.

Total Progeny Measurements After Superinfection by T6αgt$^-$

Cells were grown in nutrient broth at 37°C to 3 x 10^8 cells/ml. Cultures (either E. coli ER22 or ER22r6$^-$,B_1^-) were infected with T4 imm$^-$2am 42 at a m.o.i. of 5 and incubated at 37°C. At various times after infection, 1.0 ml of the infected culture was transferred to an iced culture tube containing T6αgt$^-$2xam in 4.0 ml nutrient broth at a

concentration sufficient to give a superinfection multiplicity of 5. These secondary culture tubes (after 4 minutes in ice to allow for complete adsorption of the phage) were aerated at 37°C for 50 minutes, at which time the cultures were lysed with chloroform. Total progeny were determined by plating on E. coli ER22r6$^-$, B_1^- (permissive for T6αgt$^-$ but not for the T4 am mutant used).

Degradation of T6αgt$^-$ DNA After Infection

E. coli B, W3110 or W4597 were grown in M9 at 37°C to 3 x 10^8 cells/ml. Cells were infected with T4 imm$^-$2 s$^-$, T4D, T4αgt$^-$βgt$^-$ or T6 at a m.o.i. of 5. Superinfection experiments (with T4imm$^-$2 s$^-$ infected cells only) were carried out by transferring the infected culture to ice at 4 minutes post-infection. ^{3}H-uracil-T6 or ^{3}H-uracil-T6αgt2xam phage were added at a m.o.i. of 0.1. After 4 minutes in ice, the cultures were returned to 37°C for incubation. Co-infection experiments were carried out by mixing ^{3}H-uracil-T6αgt2xam with unlabeled phage and using such a mixture for the initial infection (^{3}H-phage, m.o.i. of 0.1; unlabeled phage, m.o.i. of 5). After infection and 4 minutes in ice for adsorption, the cultures were placed at 37°C.

DNA degradation was measured by either determining the amount remaining TCA insoluble or the amount of material made TCA soluble. For determination of the amount remaining, 0.1 ml samples of the infected culture (after addition of labeled phage) were transferred to 2.0 ml cold 5% TCA along with 0.1 ml calf thymus DNA (1 mgm/ml). After 15 minutes in ice, the precipitates were collected on 0.45 μ Millipore filters

(2.4 cm diameter). The filters were washed 3 times with 5.0 ml cold 5% TCA, dried and counted in 5.0 ml Omnifluor.

Soluble material was determined by transferring 0.5 ml samples (at various times after addition of the labeled phage) to 0.5 ml of cold 10% TCA, along with 0.1 ml calf thymus DNA (1 mgm/ml). After 15 minutes in ice, the samples were centrifuged at 1,000 x g for 15 minutes. 0.5 ml aliquots of the supernatant were placed in 10.0 ml of Aquasol and counted.

Radioactive Labeling of Phage Proteins for SDS-Gel Analysis

T6 or T6αgt$^-$2xam phage were grown in E. coli Kr6$^-$r2,4$^-$ cells in M9 at 37^{o}C in the presence of either ^{14}C- or ^{3}H-leucine (0.5 μCi/ml or 2.5 μCi/ml). After lysis, the phage were purified by a DNase and RNase treatment and 2 cycles of differential centrifugation, as described earlier in this chapter. In some cases phage ghosts were prepared by osmotic shock (Duckworth, 1970).

In another experiment, T6 phage were grown in E. coli B or B/4$_o$ by infecting 3 x 10^8 cells/ml (grown in M9) with phage at a m.o.i. of 5. The infection was carried out in the presence of ^{14}C- or ^{3}H-leucine. The cultures were superinfected with T6 at 8 minutes after infection, to produce lysis inhibition. After lysis, the phage were purified as above.

For double-label SDS-polyacrylamide disc gel electrophoresis, appropriate mixtures of ^{14}C- and ^{3}H-leucine labeled phage were disaggregated as described by Laemmli (1970) for 2 minutes in a boiling water bath. Analysis was performed on 10% acrylamide gels. Molecular

weight standards were run under the same conditions. Standards were: bovine serum albumin (m.w. 68,000), ovalbumin (m.w. 43,000), α-chymotrypsinogen (m.w. 75,700) and bovine cytochrome c (m.w. 17,500). All standards were in solution at 1 mgm/ml.

CHAPTER 3

RESULTS

These investigations concerning the nature of the restriction of non-glucosylated phage DNA by E. coli developed from earlier work involved with "T-even tolerant" (tet) mutants of B (Mathews 1970, Mathews and Hewlett 1971). These mutants, isolated by their ability to grow in the presence of all three T-even phage, are lacking the enzyme UDPG-pyrophosphorylase and thus, upon infection, yield progeny phage whose DNA is non-glucosylated. The first section of this chapter will detail work demonstrating the nature of the tet mutants. This will serve to place the phenomenon of restriction into some experimental perspective. In addition, earlier work on T6 infected tet cells can now be correlated with data obtained from experiments with phage glucosyl transferase mutants, reported in later sections of this chapter.

Characterization of tet Mutants

Progeny from T6 Infected tet Cells

Table IV shows the results of growth experiments performed by T6 infection of E. coli B, B/4$_o$, tet-1, and tet-2. The data show that all but the B infection produce progeny that can only be detected when the plating bacteria are permissive (e.g., Kr6$^-$r2,4$^-$). E. coli B/4$_o$, obtained from H. Revel (California Institute of Technology), is a sample of the original mutant strain in which host-controlled modification was first described (Luria and Human 1952). The data in Table IV are

TABLE IV. Burst Size of Phage T6 After Infection of Various Strains of *E. coli* B. -- T6 phage is grown for one cycle of infection in the indicated host strains. The burst size (number of progeny/infected cell) is determined on either *E. coli* B or *Kr6⁻ r2,4⁻*.

Strain	Burst size on B	Burst size on *Kr6⁻ r2,4⁻*
B	100	77
$B/4_o$	2	18
tet-1	4	20
tet-2	3	50

typical of this phenomenon. The results imply that the DNA of the progeny of T6 infected tet cells is non-glucosylated.

UDPG-Pyrophosphorylase Activity

Although defects in more than one host system may inhibit the glucosylation of phage DNA (Revel and Luria 1970), the fact that the tet mutants fail to adsorb T4 (Mathews and Hewlett 1971) suggested that the defect might be in the enzyme UDPG-pyrophosphorylase (UDPG-PPase), as is the case for $B/4_o$. When assays for this enzyme activity were carried out on cell extracts, the results shown in Table V were obtained. tet-1 and tet-2 have 19% and 18% respectively of the normal level of UDPG-PPase activity. ($B/4_o$ has about 10% of the normal level.)

The data in Table VI demonstrate that, when normal and mutant cell extracts are mixed, no inhibition of enzyme activity is seen. Therefore the observed decrease in UDPG-PPase activity in the tet cells is not due to a secondary inhibition of activity.

Glucosylation of Progeny Phage DNA

As a final demonstration of the tet cell defect, T6 progeny from infected B, $B/4_o$, tet-1 and tet-2 cells were purified and the DNA extracted as described in Chapter 2. The ability of this DNA to serve as a substrate for the transfer of ^{14}C-glucose from UDPG(^{14}C-glucose) catalyzed by a T6 glucosyl transferase preparation was checked. The data in Fig. 4 show that T6 phage grown in B cannot be glucosylated in such a system, while T6 phage grown in $B/4_o$, tet-1 or tet-2 can incorporate significant amounts of ^{14}C-glucose.

TABLE V. UDPG-Pyrophosphorylase Activity. -- Enzyme activity is measured (as described in Chapter 2) in sonicated cell extracts from the indicated strains.

cell extract	μmoles NADPH/hr/mgm protein[a]
B	0.540
$B/4_o$	0.052
tet-1	0.106
tet-2	0.097

a. assay performed at 34°C

TABLE VI. UDPG-Pyrophosphorylase Mixing Experiments

extract[a]	Δ A_{340}/minute expected	found
B	-	0.012
$B/4_o$	-	0.001
tet-1	-	0.008[b]
tet-2	-	0.002
B + $B/4_o$	0.013	0.011
B + tet-1	0.020	0.018
B + tet-2	0.014	0.019

a. Incubation mixtures in mixing experiments contained equal volumes of the indicated extracts in 1 ml total volume (as described in Methods, Chapter 2).

b. The relatively large value for tet-1 is due to the high protein concentration of this extract.

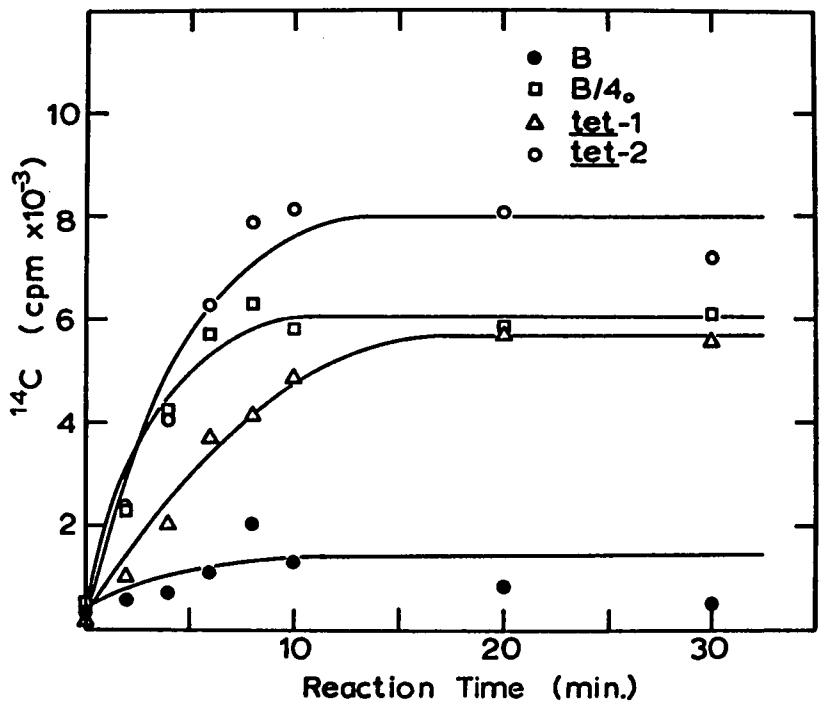

Fig. 4. Incorporation of ^{14}C-Glucose into Phage DNA.

Data represent 5% TCA-insoluble ^{14}C-cpm in 0.1 ml samples of reaction mixture, containing DNA isolated from T6 grown in the indicated bacterial strains.

Analysis of Restrictive and Permissive Host Cells

Initial experiments involving the restriction system were designed to detect the host function (or functions) responsible for the recognition of non-glucosylated HMC-containing phage DNA. As mentioned in Chapter 1, restriction involves a nucleolytic attack on the incoming phage DNA. Several lines of evidence suggest that this activity might be membrane associated: 1) the specificity for incoming DNA and not replicating DNA, implying a location away from the intracellular pool of DNA, possibly at the membrane or in the periplasmic space (Fukasawa 1964), 2) the failure to find specific nuclease activity for non-glucosylated phage DNA in cytoplasmic extracts (Revel and Luria 1970) and 3) the demonstration of the operation of the restriction system in toluenized cells (Fleischman and Richardson 1971).

Consequently, I analyzed membrane preparations from permissive and restrictive cells in several ways. Total membrane fractions (TM) were prepared by a method yielding well characterized, enzymatically active vesicles (Osborn et al. 1972).

SDS-Polyacrylamide Disc Gel Analysis of TM^{osm} and $CYTO^{osm}$

^{3}H- or ^{14}C-leucine labeled membrane preparations were obtained and the electrophoretic protein pattern was analyzed on 7.5% SDS-polyacrylamide gels. Before an analysis of the results of these experiments is begun, some comments on technique must be made. Any suspected differences in the electrophoretic patterns between two preparations were analyzed in three ways:

1. The data are plotted as percent total label (^{3}H or ^{14}C) on the gel. A difference between the $\%^{14}C$ and $\%^{3}H$ at a point in the gel indicates a protein difference between the two samples.
2. The ratio of ^{3}H to ^{14}C should remain constant over a gel in which no protein difference occurs. The ratios of each slice of a gel are subjected to statistical analysis. Significant deviations (greater than two standard deviations) of this ratio at a point in the gel were interpreted as indicating a protein difference.
3. When the labeling scheme of the two samples being analyzed is reversed, the change should be reflected in the labeling pattern of any gel fractions showing protein differences.

For the TM and CYTO gels, all three of these criteria must be met to indicate a significant difference in the pattern of the preparations being compared.

The pattern in Fig. 5 shows a control gel analysis of a TM^{osm} preparation of ^{3}H-leucine labeled ER22 and ^{14}C-leucine labeled ER22. No significant differences are seen. The pattern in Fig. 6 shows a similar analysis of TM^{osm} prepared from ^{14}C-leucine labeled ER22 and ^{3}H-leucine labeled ER22$\underline{r6}^{-}B_{1}^{-}$. Significant differences were seen at slice 32 (a peak of ^{14}C) and at slices 52 and 58 (peaks of ^{3}H). Table VII lists the $^{3}H/^{14}C$ ratios for these gel slices, along with the values for comparable fractions from a gel analysis of a sample with the labeling reversed. The same results were obtained after analysis of membrane proteins from $\underline{E.\ coli}$ B and $B\underline{r6}^{-}B_{1}^{-}$.

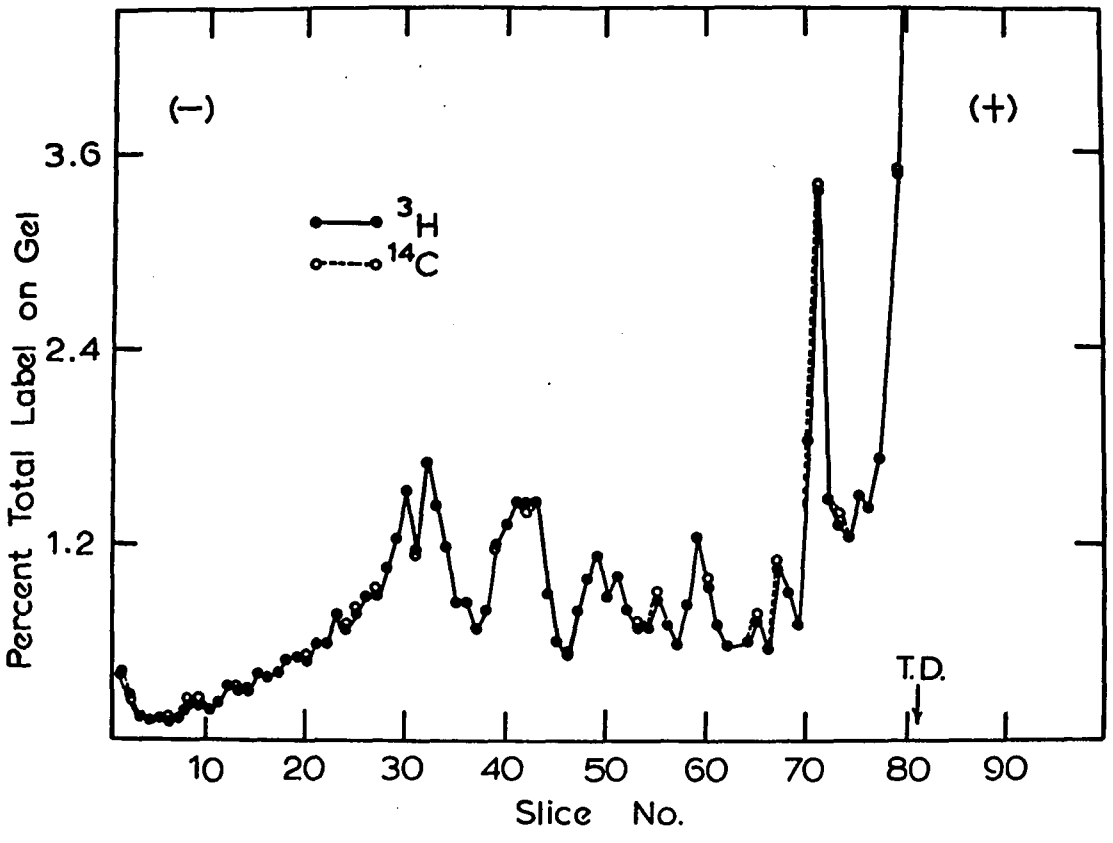

Fig. 5. SDS-Gel Electrophoresis of Membrane Proteins from Restrictive Cells.

Electrophoretic pattern of disaggregated membrane proteins from ^{14}C-leucine ER22-TM^{osm} and ^{3}H-leucine ER22-TM^{osm}. Migration is from left to right in a 7.5% acrylamide gel, at 3 mA/gel. T.D. (tracking dye) indicates the position of the front.

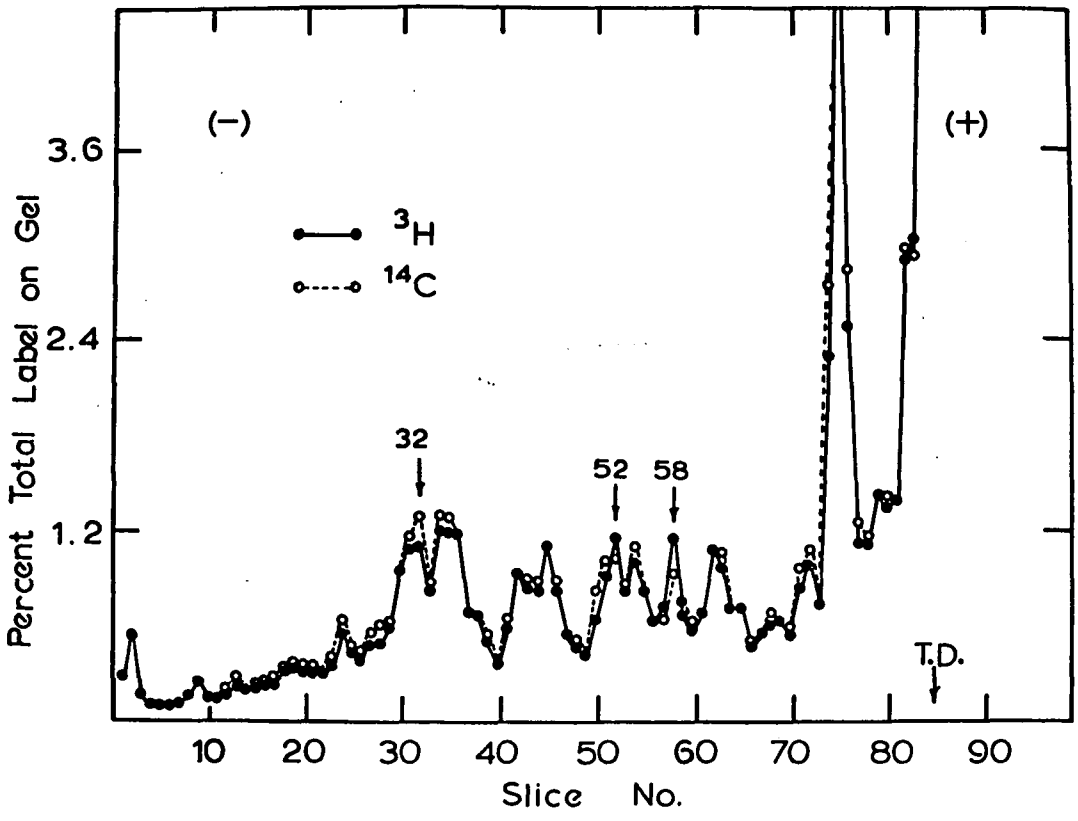

Fig. 6. Comparison of Membrane Proteins from Restrictive and Permissive Cells by SDS-Gel Electrophoresis.

The experiment is the same as Fig. 5, except that the membrane proteins are from ^{14}C-leucine ER22-TM^{osm} and ^{3}H-leucine ER22r6^{-}B$_{1}^{-}$-TM^{osm}.

TABLE VII. $^{3}H/^{14}C$ Ratios for Selected SDS-Gel Fractions

Slice No. (Fig. 6)	$^{3}H/^{14}C$[a]	Reversed Label Gel $^{3}H/^{14}C$[b]
32	1.258 (P <0.06)	1.871 (P <0.10)
52	1.657 (P <0.02)	1.511 (P <0.15)
58	1.792 (P <0.001)	1.249 (P <0.01)

a. average ratio = 1.433 ± 0.094

b. average ratio = 1.683 ± 0.119

Similar experiments were carried out with CYTO^{osm} preparations. The results of a control gel (^{14}C-ER22 versus ^{3}H-ER22) are shown in Fig. 7 and the results of an experimental gel (^{14}C-ER22 versus ^{3}H-ER22$\underline{r6}^{-}B_1^{-}$) are shown in Fig. 8. No significant differences were observed in either case.

DNase Activity in TM Preparations

The data in Fig. 9 show the results of incubating T6 or T6$\underline{\alpha gt}^{-}$ DNA with $\underline{E.\ coli}$ B-TM^{son}. In the absence of added ATP, no solubilization of either DNA occurs. However, in the presence of ATP, both DNA's are extensively degraded to TCA soluble products. Although no specificity for non-glucosylated phage DNA was seen in this experiment, the nature of the ATP-dependent activity was further investigated.

ATP-Dependent DNase Activity in TM Preparations

The $\underline{recB,C}$ gene in $\underline{E.\ coli}$ specifies an ATP dependent DNase (exonuclease V) (Goldmark and Linn 1970). To examine the possibility that the membrane associated activity observed in these studies could be exonuclease V, TM^{son} was prepared from $\underline{E.\ coli}$ K12 strains that are $\underline{rec}^{+}$, $\underline{recB}$ or $\underline{recC}$ (Moody, Low and Mount 1973). The results (Fig. 10) show that both the $\underline{recB}$ and $\underline{recC}$ TM^{son} have lower levels of this ATP-dependent DNase that does the corresponding $\underline{rec}^{+}$ preparation. The level of ATP-dependent nuclease activity in Fig. 10 is not equivalent to that seen in Fig. 9. The $\underline{rec}$ strains used were $\underline{E.\ coli}$ K strains and were more difficult to spheroplast than the B strains. The resulting membrane preparations did not have the same enzymatic activity.

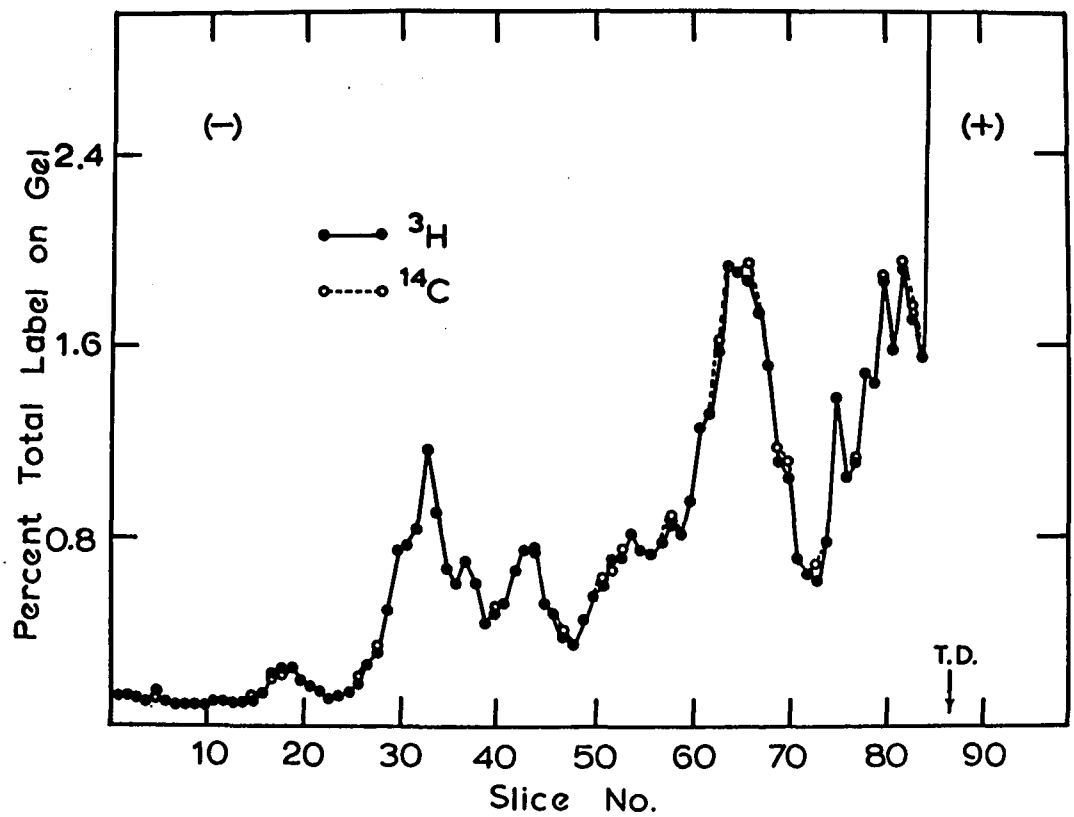

Fig. 7. SDS-Gel Electrophoresis of Cytoplasmic Proteins from Restrictive Cells.

The experiment is the same as Fig. 5, except that cytoplasmic proteins are from ^{14}C-leucine ER22-CYTO^{osm} and ^{3}H-leucine ER22-CYTO^{osm}.

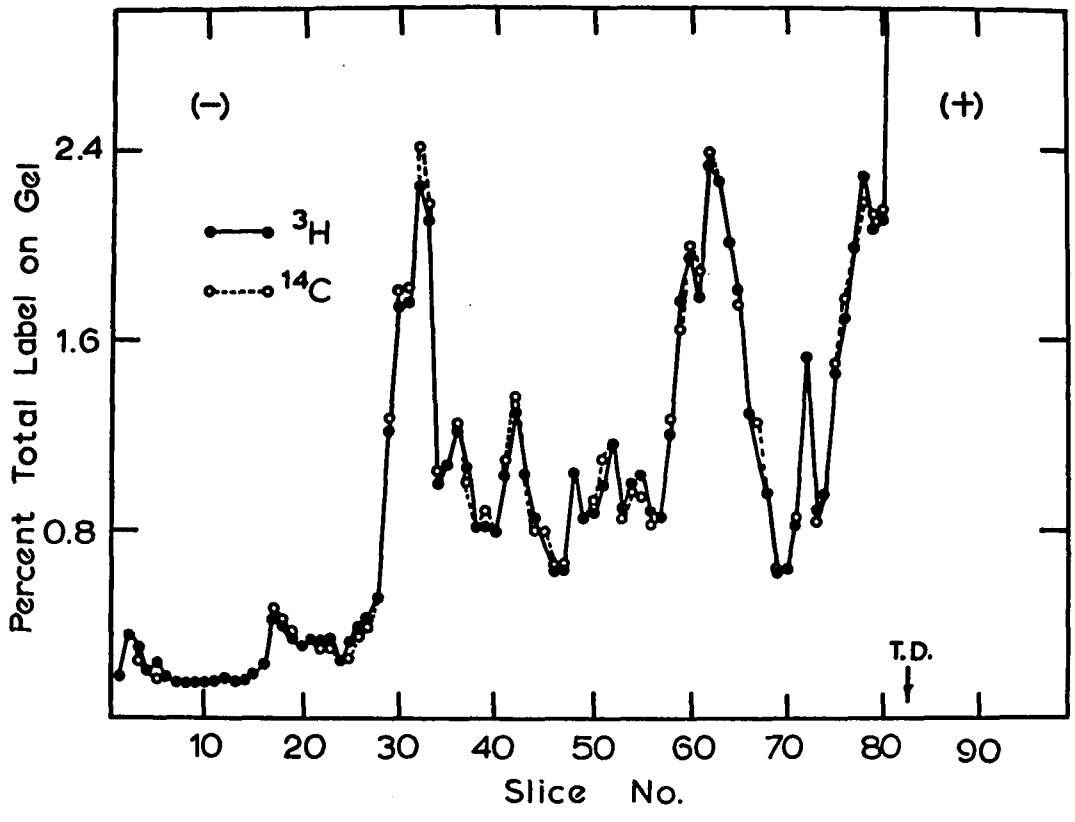

Fig. 8. Comparison of Cytoplasmic Proteins from Restrictive versus Permissive Cells by SDS-Gel Electrophoresis.

The experiment is the same as Fig. 5, except that cytoplasmic proteins are from ^{14}C-leucine ER22-CYTO^{osm} and ^{3}H-leucine ER22r6^{-}B$_1^{-}$-CYTO^{osm}.

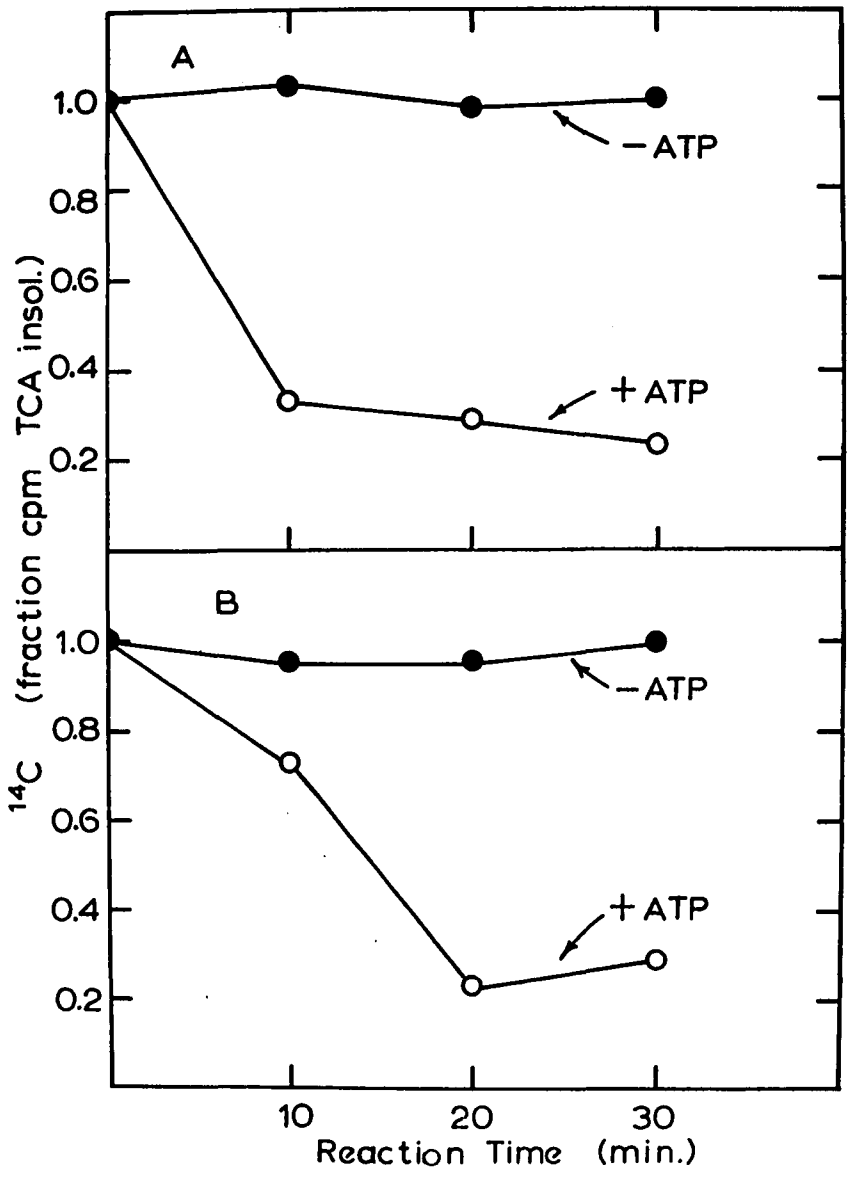

Fig. 9. Degradation of Phage DNA by Nuclease Activity in B-TM^{son}.

The data represent the fraction of initial 5% TCA insoluble cpm remaining in 0.05ml of reaction mixture at the indicated times. Panel A, ^{14}C-T6 DNA; Panel B, ^{14}C-T6αgt$^-$ DNA (plus or minus ATP, as indicated).

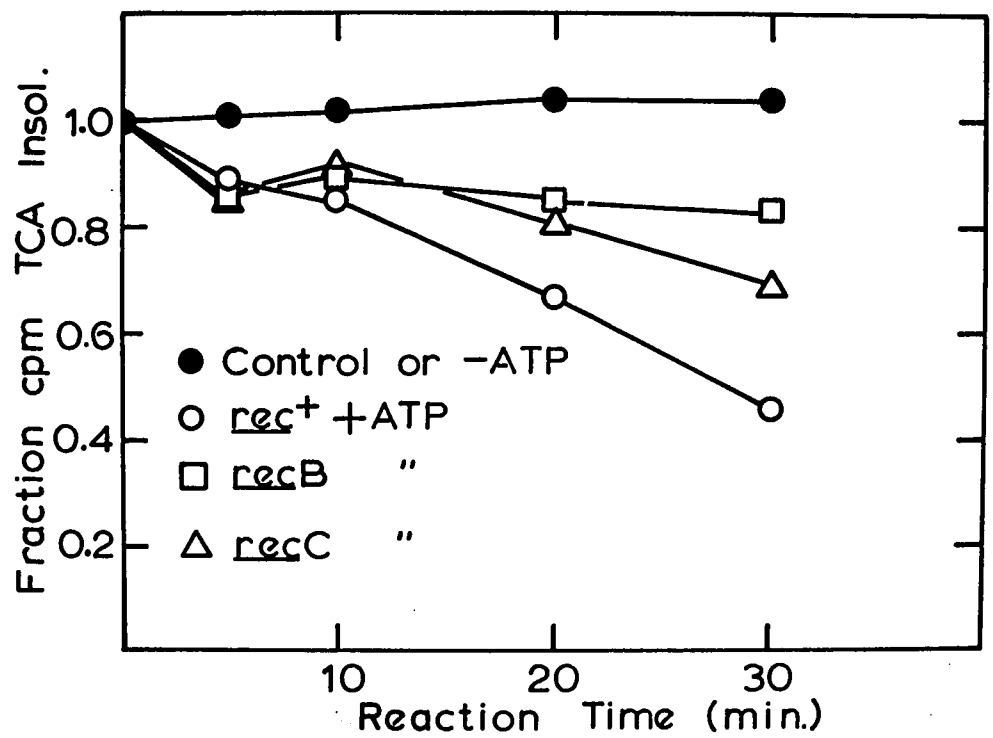

Fig. 10. ATP-Dependent DNA Nuclease Activity in TM^{son} of rec Strains.

The experiment is the same as Fig. 9. Membrane preparations are from E. coli KDM821 (rec+), DM910 (rec B) and DM836 (rec C). The substrate is ^{14}C-T6 DNA (plus or minus ATP, as indicated).

Altered Sedimentation Pattern of DNA's After Treatment with TM

The initial effect of the restriction function would probably be limited nucleolytic attack at sites containing non-glucosylated HMC residues in phage DNA (Revel and Luria 1970). Such limited attack would be expected to alter the sedimentation pattern of the DNA. No solubilization of phage DNA was observed upon incubation with B-TM^{son} in the absence of ATP (Fig. 9). I used this system to investigate changes in sedimentation patterns of T6 and T6αgt$^-$ DNA on neutral sucrose gradients, as an indication of limited nucleolytic attack. The incubation mixture contained tRNA, since Osborn et al. (1972) reported the presence of endonuclease I in membranes prepared as described in Chapter 2. It is known that tRNA inhibits the action of endonuclease I (Shortman and Lehman 1964).

After incubation with B-TM^{osm}, T6 and T6αgt$^-$ DNA undergo limited nucleolytic attack such that they sediment slower in 5 to 20% neutral sucrose than does added marker DNA (Fig. 11). The same result was obtained when the incubation was performed with Br6$^-$B$_1^-$-TM^{osm} (Fig. 12). However, when the incubation was performed with ER22-TM^{osm}, no effect on either DNA was observed (both DNA's co-sedimented with the marker (Fig. 13). Since ER22 is lacking the enzyme endonuclease I, the effect seen in Figs. 11 and 12 can be ascribed to residual activity in the membrane preparations.

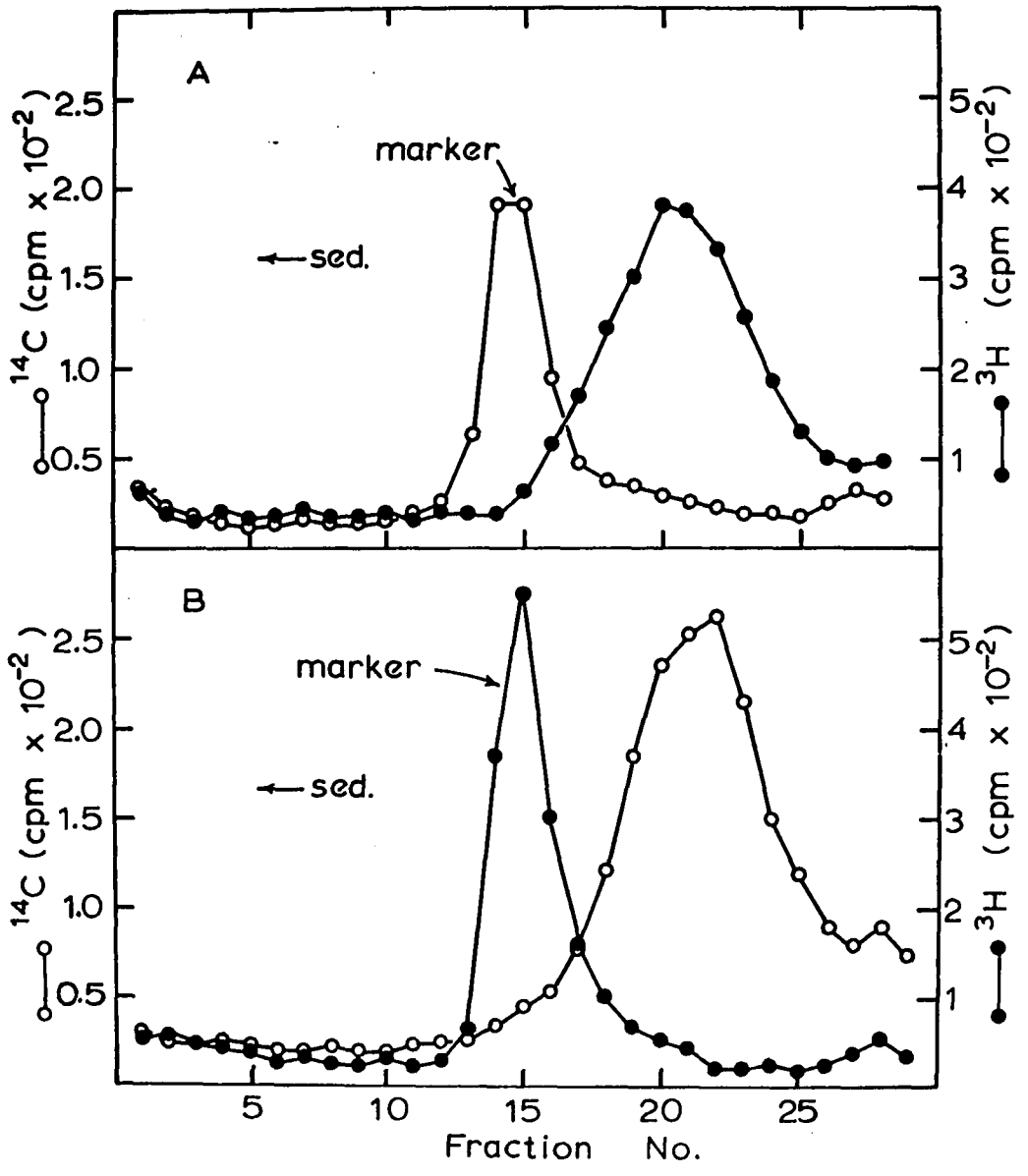

Fig. 11. Neutral Sucrose Gradient Analysis of DNA Treated with B-TM^{osm}.

Phage DNA's are incubated for 30 minutes at 37°C with B-TM^{osm}, in the presence of t-RNA and without ATP, as described in Chapter 2. Sedimentation (from right to left) is in 5 to 20% neutral sucrose. Panel A, ^{3}H-T6 DNA (marker, ^{14}C-T6αgt^{-} DNA); Panel B, ^{14}C-T6αgt^{-} DNA (marker, ^{3}H-T6 DNA).

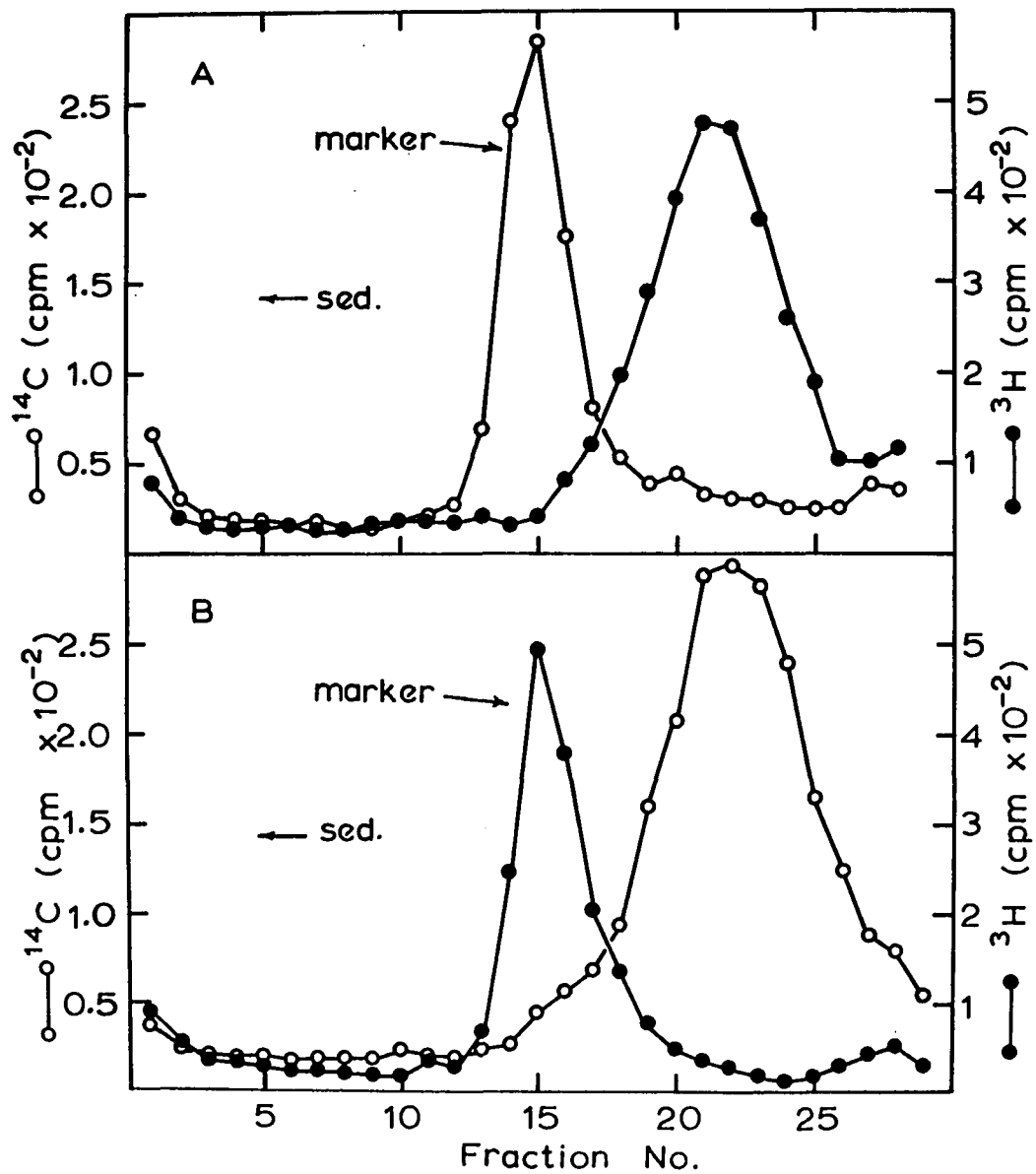

Fig. 12. Sedimentation of DNA on Neutral Sucrose Gradients After Treatment with $\underline{Br6}^{-}B_1^{-}$-TM^{osm}.

The experiment is the same as Fig. 11, except that the membrane preparation is from $\underline{Br6}^{-}B_1^{-}$. Panel A, ^{3}H-T6 DNA (marker, ^{14}C-T6$\underline{\alpha gt}^{-}$ DNA); Panel B, ^{14}C-T6$\underline{\alpha gt}^{-}$ DNA (marker, ^{3}H-T6 DNA).

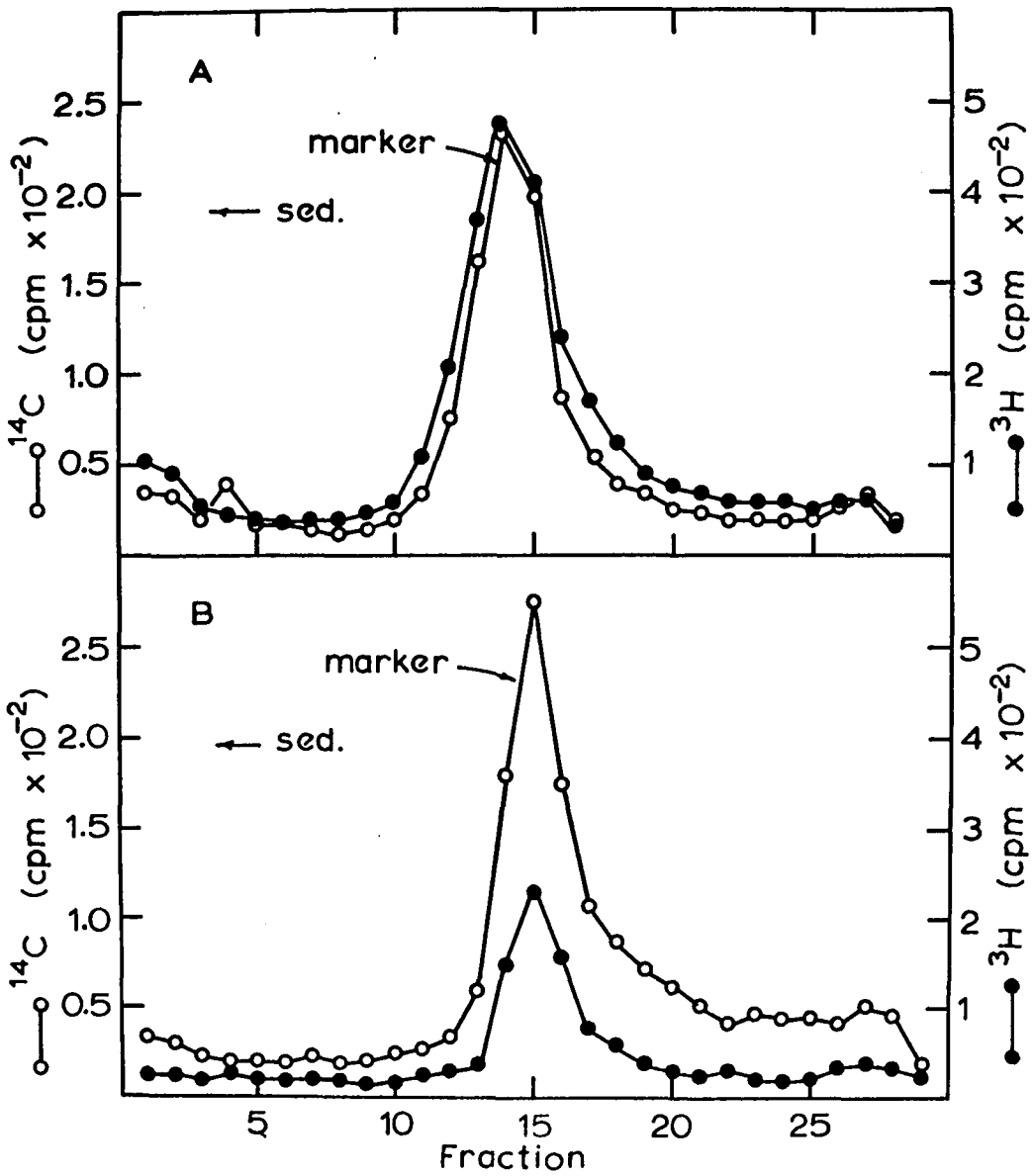

Fig. 13. Effect of Treatment with E222-TM^{osm} on the Sedimentation of DNA on Neutral Sucrose Gradients.

The experiment is the same as Fig. 11, except that the membrane preparation is from ER22. Panel A, ^{3}H-T6 DNA (marker, ^{14}C-T6$\underline{\alpha gt}^{-}$ DNA); Panel B, ^{14}C-T6$\underline{\alpha gt}^{-}$ DNA (marker, T6 DNA).

Nucleic Acid Metabolism in Phage Infected Restrictive and Permissive Cells

DNA and RNA Synthesis in T6αgt⁻ Infected E. coli B

The data in Fig. 14 demonstrate that little or no incorporation of ^{14}C-uracil into DNA occurs after infection of E. coli B by T6αgt⁻. However, extensive incorporation into RNA does occur. The apparent low level of normal T6 synthesis in Fig. 14B is due to the scale used to show the synthesis with T6αgt⁻. In another experiment (Fig. 15) ^{3}H-5-uracil was used as the precursor so that incorporation into RNA could be measured directly, rather than by difference between total counts and KOH stable counts (Mathews 1968). In addition, the results are plotted on a scale such that the level of synthesis obtained with T6 can be seen. It will be noted that rifampicin (100 μgm/ml) completely inhibits the synthesis seen in T6αgt⁻ infected B.

Competitive RNA/DNA Hybridization Analysis of RNA from T6αgt⁻ Infected E. coli B

It is known that when non-glucosylated phage infect a restrictive cell, a low level of early gene products is seen, implying some phage RNA transcription (Hattman 1964, Fukasawa 1964). In order to assess the nature and quantity of the RNA species being transcribed in T6αgt⁻ infected E. coli B, I performed competitive RNA/DNA hybridization experiments (Bolle et al. 1968). RNA isolated at 5 minutes after infection of B by T6αgt⁻ competes poorly with ^{3}H-RNA labeled from 2 to 5 minutes during T6 infection of B (Fig. 16). RNA isolated at 20 minutes from T6αgt⁻ infected B does not compete at all with ^{3}H-RNA labeled

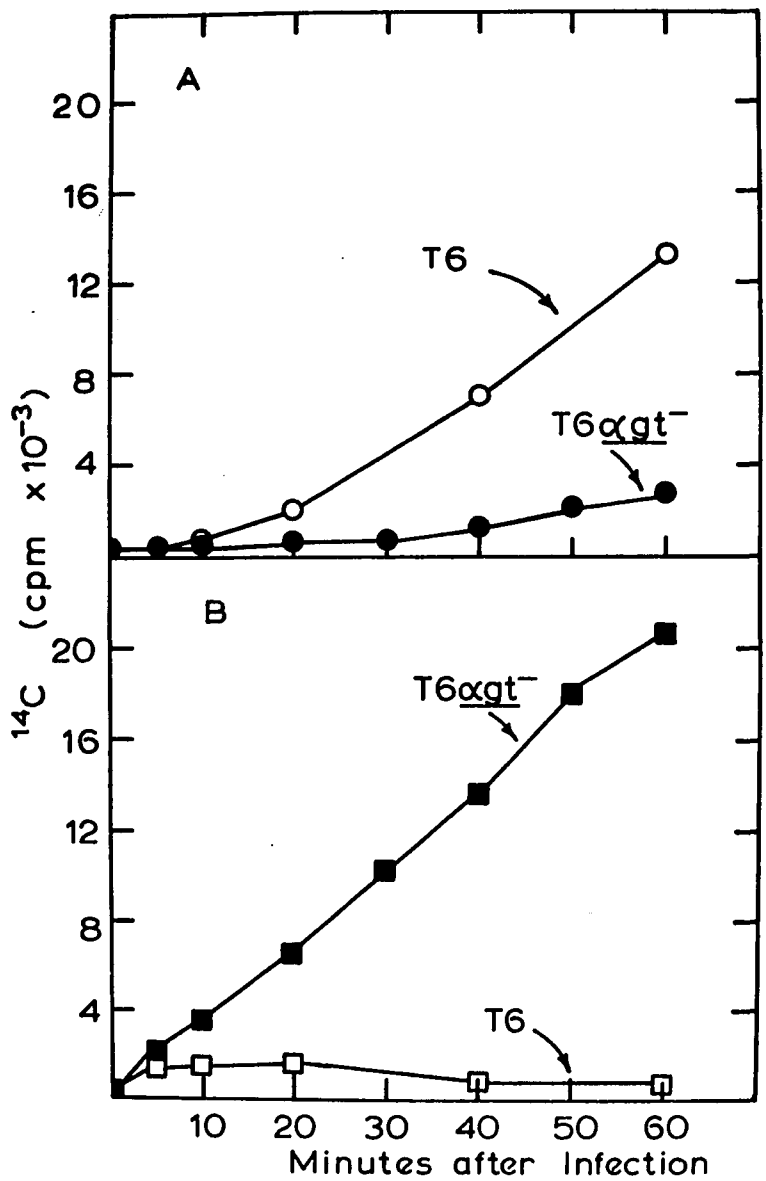

Fig. 14. Nucleic Acid Synthesis in Phage-Infected E. coli B.

The data represent ^{14}C-uracil incorporation into 5% TCA-insoluble material in 0.3 ml of culture infected with the indicated phage. Panel A, DNA synthesis (5% TCA insoluble, alkali stable cpm); Panel B, RNA synthesis (5% TCA insoluble, alkali labile cpm).

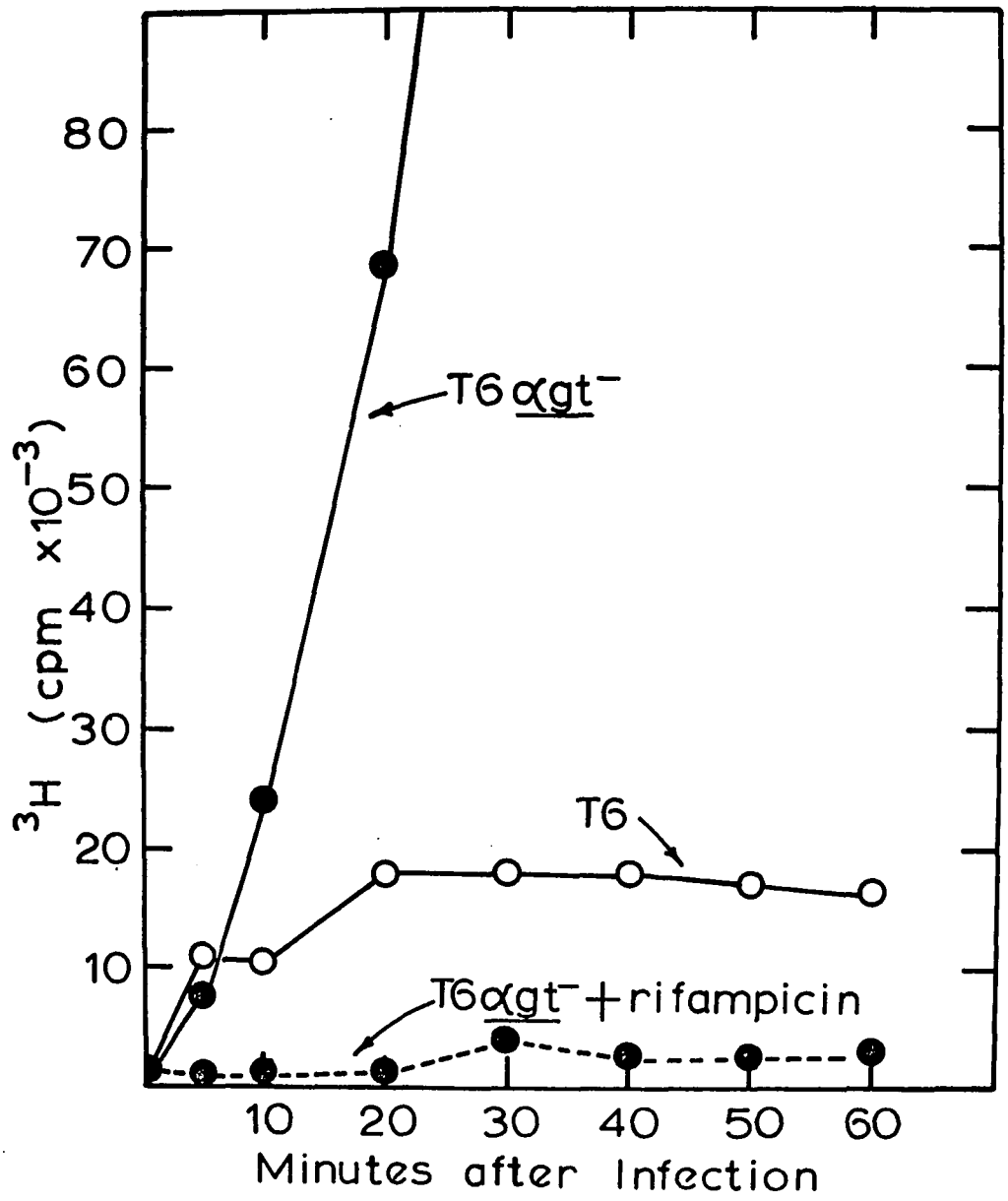

Fig. 15. RNA Synthesis in Phage-Infected E. coli B.

The data represent ^3H-5-uracil incorporation into 5% TCA insoluble material in 1.0 ml of culture, infected with the indicated phage. Rifampicin was added, as indicated, at 100 μgm/ml.

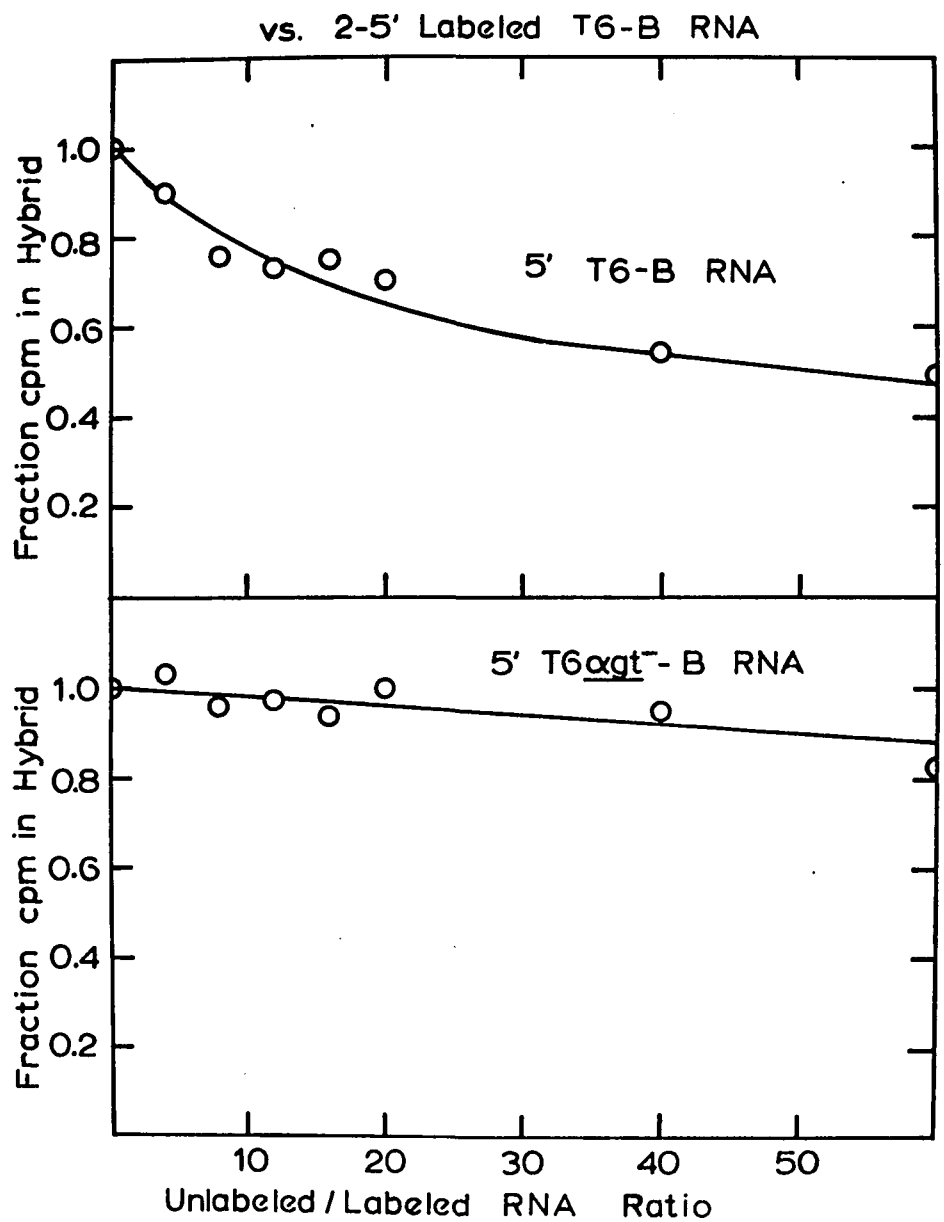

Fig. 16. Competitive RNA/DNA Hybridization with Early T6-Infected B RNA.

2 to 5 minute pulse-labeled ^{3}H-RNA from T6 infected-B (2.5 µgm) is hybridized to T6 DNA (2.5 µgm) immobilized on filters. The hybridization is carried out in the presence of the indicated amounts of unlabeled RNA, isolated 5 minutes after infection of B with T6 (top Panel) or T6αgt⁻ (bottom Panel).

from 15 to 20 minutes during T6 infection of B (Fig. 17). These data imply that little or no T6αgt⁻ specific RNA is being synthesized.

Hybridization Specificity of Late RNA from T6αgt⁻ Infected E. coli B

In light of the extensive RNA synthesis seen during T6αgt⁻ infection of B (Figs. 14 and 15) and the failure of late RNA from this infection to compete with late T6 RNA, I pursued the nature of the late T6αgt⁻ RNA further. Table VIII shows that the RNA being made late in infection of E. coli B by T6αgt⁻ contains very little phage specific RNA, but is predominantly E. coli RNA. The efficiency of hybridization to E. coli DNA is low for both T6αgt⁻ RNA and E. coli RNA. This is probably due to the short time (6 hours) used for the hybridization. For a more complex DNA such as E. coli, the experiment should have been carried out for longer periods. However, the nature of the RNA's is clear when their hybridization to T6 is considered.

DNA and RNA Synthesis in T6αgt⁻ Infected E. coli Br6⁻B$_1^-$

The data in Fig. 18 show that the incorporation of ^{14}C-uracil into DNA is abnormal in T6αgt⁻ infected Br6⁻B$_1^-$. DNA synthesis occurs at a greater rate than normal, producing 2- to 3-fold more DNA than T6. RNA synthesis, while somewhat greater than normal during the first 20 minutes of infection by T6αgt⁻, returns to normal during the later stages of infection. The nature of the DNA and RNA made after infection was investigated further.

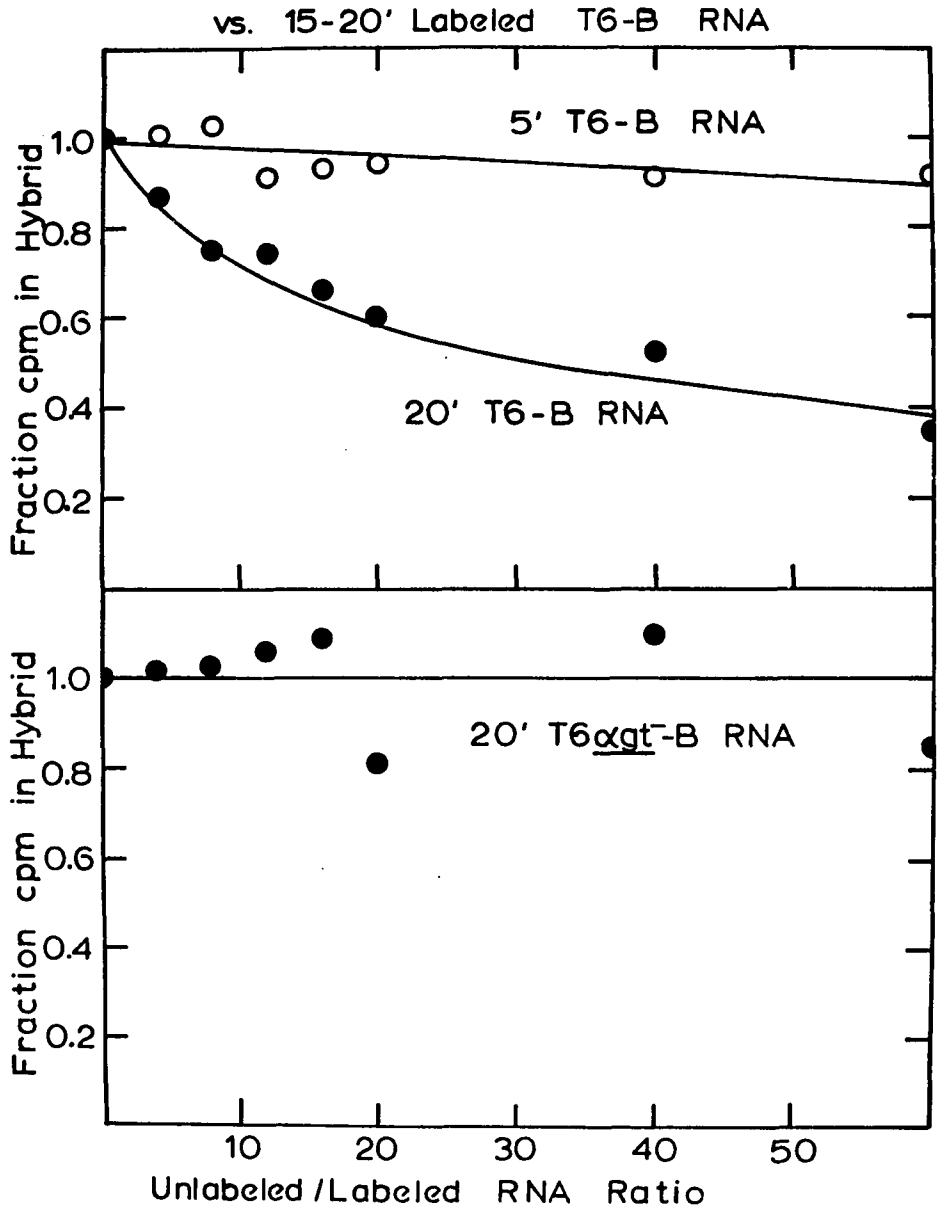

Fig. 17. Hybridization-Competition versus Late T6-Infected B RNA.

The experiment is the same as Fig. 16, except the ^{3}H-RNA is pulse-labeled from 15 to 20 minutes after T6 infection with B. The unlabeled RNA is from 5 or 20 minutes T6-infected B (top Panel) or 20 minute T6αgt⁻-infected B (bottom Panel).

TABLE VIII. Hybridization Specificity of Various RNA's

	Percent cpm hybridized to	
	E. coli DNA	T6 DNA
^{3}H-RNA		
E. coli	1.4	<0.01
late T6/b	<0.01	49.1
late T6αgt^{-}/B	1.9	1.4

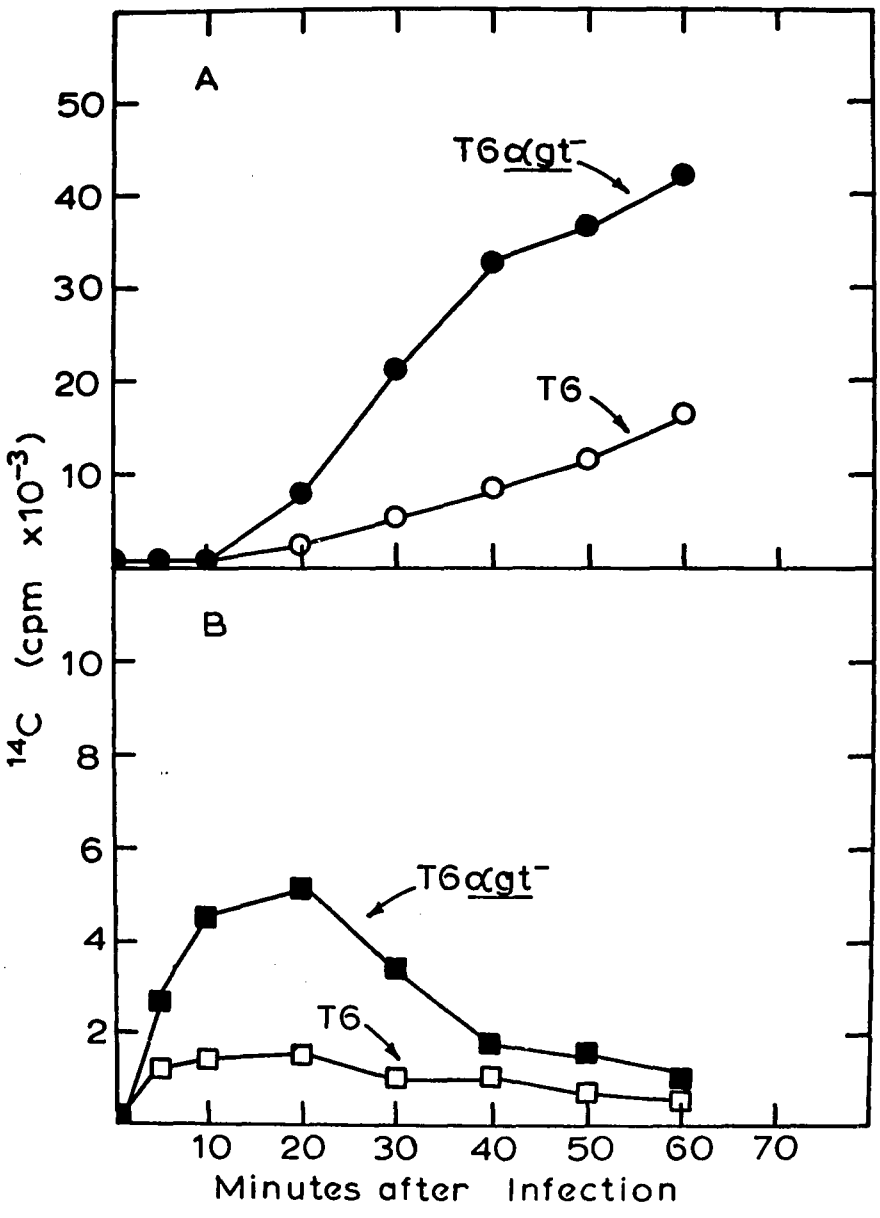

Fig. 18. Nucleic Acid Synthesis in Phage-Infected E. coli Br6⁻B$_1$⁻.

The experiment is the same as Fig. 14, except Br6⁻B$_1$⁻ cultures are infected with the indicated phage. Panel A, DNA synthesis (5% TCA insoluble, alkali stable cpm); Panel B, RNA synthesis (5% TCA insoluble, alkali labile cpm).

Competitive RNA/DNA Hybridization Analysis of RNA from T6αgt^- Infected *E. coli* $Br6^-B_1^-$

As shown in Figs. 19 and 20, the RNA isolated from T6αgt^- infected $Br6^-B_1^-$ at both early and late times competes normally with ^{3}H-RNA isolated from T6 infected $Br6^-B_1^-$. The plateau values for the competition curves (about 50%) are not as low as values reported in the literature (see, for example, Mathews and Hewlett 1971). Although this problem is unexplained, a comparison of the T6 and T6αgt^- patterns indicates that the normal species and normal relative amounts of RNA are being made at all times after T6αgt^- infection of permissive cells.

Gradient Analysis of DNA Synthesized in T6αgt^- Infected *E. coli* $Br6^-B_1^-$

As shown in Fig. 18, the amount of DNA being synthesized in T6αgt^- infected $Br6^-B_1^-$ cells is 2- to 3-fold greater than in T6 infected cells. In contrast, the burst size produced by this same infection is 1/5 to 1/10 that of T6 (Georgopoulos and Revel 1971). Consequently, the nature of the DNA being produced was examined.

The data in Fig. 21 show the sedimentation on neutral gradients of DNA obtained from 25 minute T6 or T6αgt^- infected $Br6^-B_1^-$ cells, synthesized in the presence of ^{14}C-uracil. In both cases the DNA cosediments with added marker (T6) DNA. Therefore, at 25 minutes after infection, mature-sized, molecules are being formed. This experiment would not have detected the occurrence of a small number of double strand breaks, since the DNA was not sedimented far enough down the gradient.

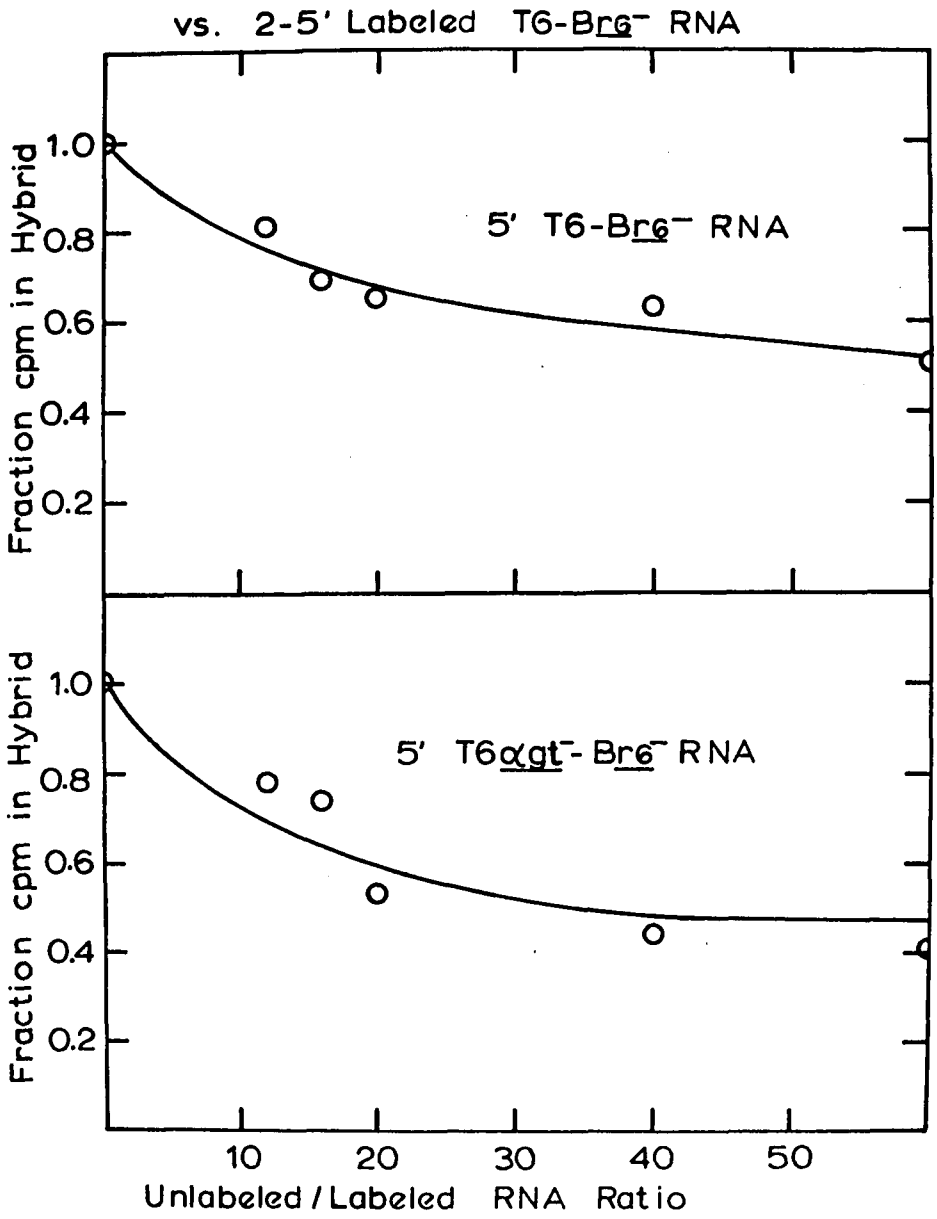

Fig. 19. Competitive RNA/DNA Hybridization with Early T6-Infected Br6⁻B$_1$⁻ RNA.

The experiment is the same as Fig. 16, except the 2 to 5 minute ^{3}H-RNA is from T6-infected Br6⁻B$_1$⁻. The unlabeled RNA is from 5 minute T6-infected Br6⁻B$_1$⁻ (top Panel) or T6αgt⁻ infected Br6⁻B$_1$⁻ (bottom Panel).

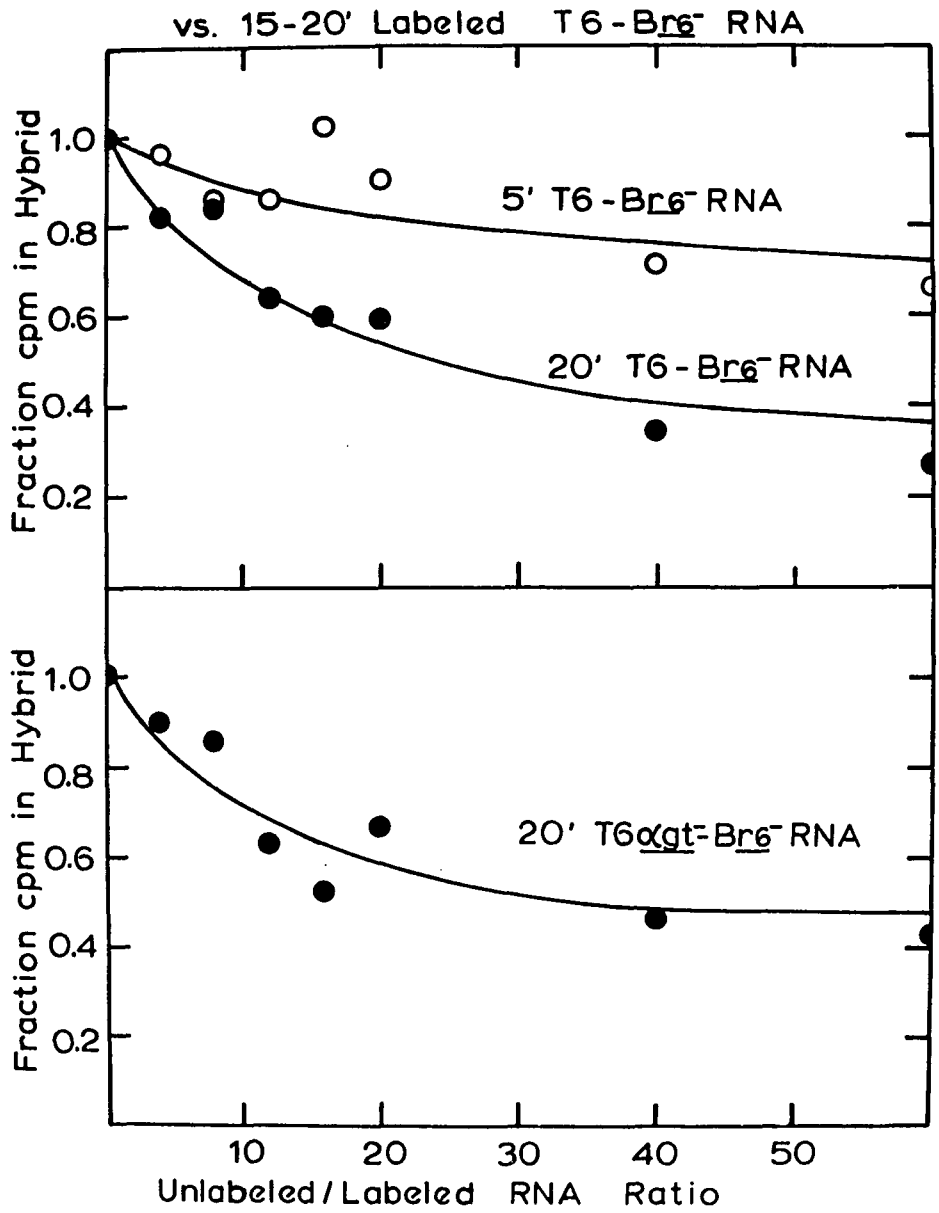

Fig. 20. Hybridization-Competition versus Late T6-Infected Br6⁻B$_1^-$ RNA.

The experiment is the same as Fig. 16, except the ^{3}H-RNA is pulse-labeled from 15 to 20 minutes after T6 infection of Br6⁻B$_1^-$. The unlabeled RNA is from 5 or 20 minute T6-infected Br6⁻B$_1^-$ (top Panel) or 20 minute T6αgt⁻ infected Br6⁻B$_1^-$ (bottom Panel).

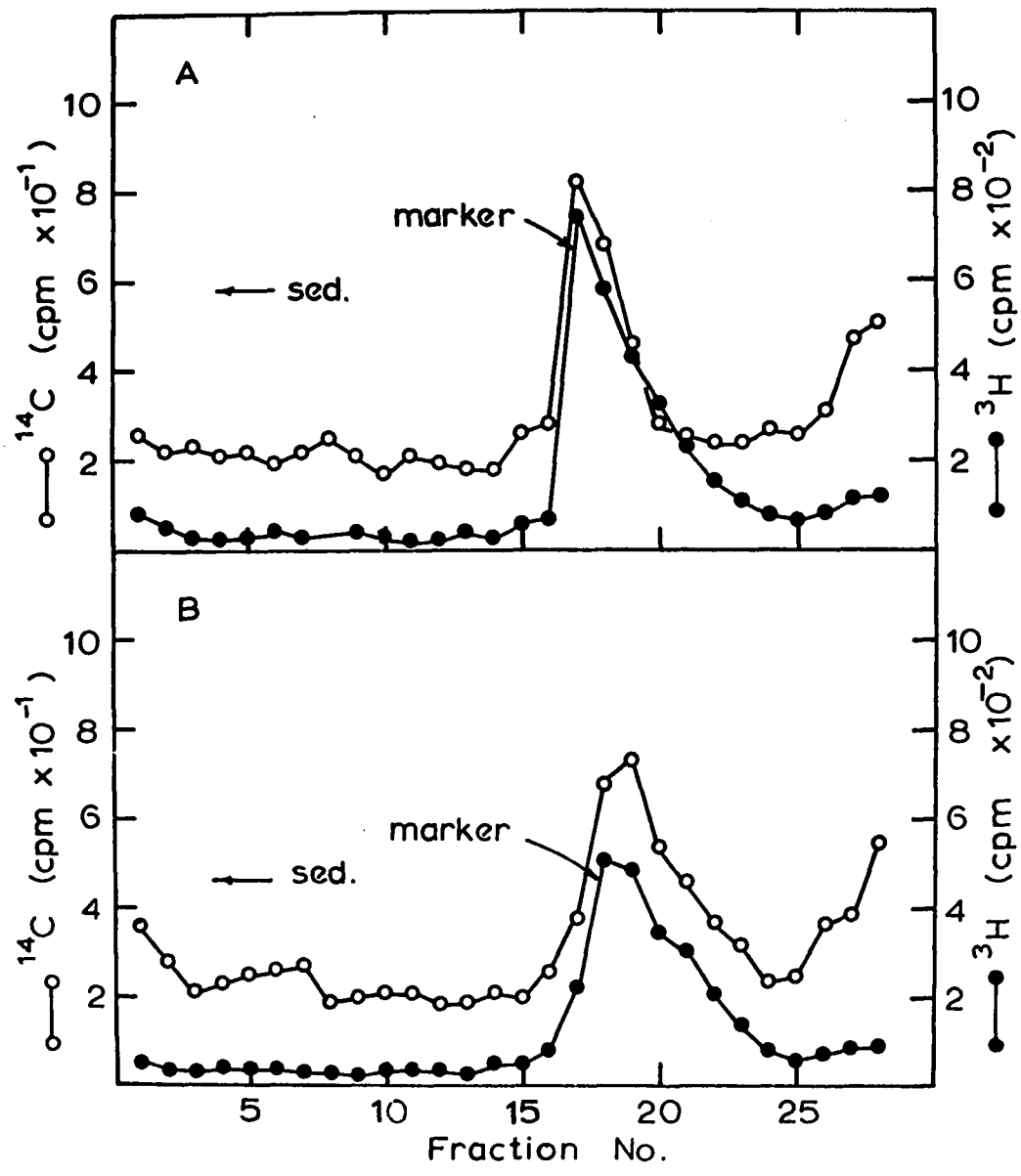

Fig. 21. Neutral Sucrose Gradient Analysis of DNA from 25 Minute Phage-Infected Br6$^-$B$_1^-$.

Samples of phage-infected cultures (labeled with ^{14}C-uracil) are lysed as described in Chapter 2. Sedimentation is from right to left in 5 to 20% neutral sucrose. The marker is ^{3}H-T6 DNA. Panel A, T6 infected Br6$^-$B$_1^-$; Panel B, T6αgt$^-$ infected Br6$^-$B$_1^-$.

The sedimentation on alkaline sucrose gradients of denatured (single-stranded) 25 minute DNA from T6 or T6αgt⁻ infected Br6⁻B$_1$⁻, synthesized in the presence of ^{3}H-6-uracil is shown in Fig. 22. T6 DNA sediments heterogeneously, with a front at the position of the marker DNA. Such a pattern is typical for intracellular T6 DNA on alkaline gradients. However, the DNA from T6αgt⁻ sediments in a heterogeneous pattern, slower than the added marker. This indicates that the replicating T6αgt⁻ DNA has undergone extensive single-strand nucleolytic attack (nicking).

In Fig. 23, the sedimentation on alkaline gradients of DNA obtained after 25 minutes of infection of either B201 or tet-1-T⁻ cells by T6, in the presence of ^{3}H-thymidine is shown. It can be seen that the T6 DNA being produced in UDPG⁻ cells (tet-1-T⁻) has also undergone single strand nicking. Thus it appears that lack of glucosylation of the replicating DNA makes it subject to limited nucleolytic attack.

The data in Fig. 24 show the sedimentation pattern obtained when T6 and T6αgt⁻ phage containing labeled DNA are placed in 0.3 N KOH and then analyzed on an alkaline sucrose gradient. It is apparent that the T6αgt⁻ phage produced during infection of permissive cells (in this case, Kr6⁻r2,4⁻) contains DNA which is also extensively nicked. It will be noted that, on neutral gradients, T6 and T6αgt⁻ DNA co-sediment (see, for example, Fig. 13).

The encapsidation of nicked phage DNA suggests that a stock of T6αgt⁻ phage may contain significant levels of non-viable particles. One method of detecting such particles is by measuring the optical cross section of the phage suspension (Thomas and Abelson 1966). This is

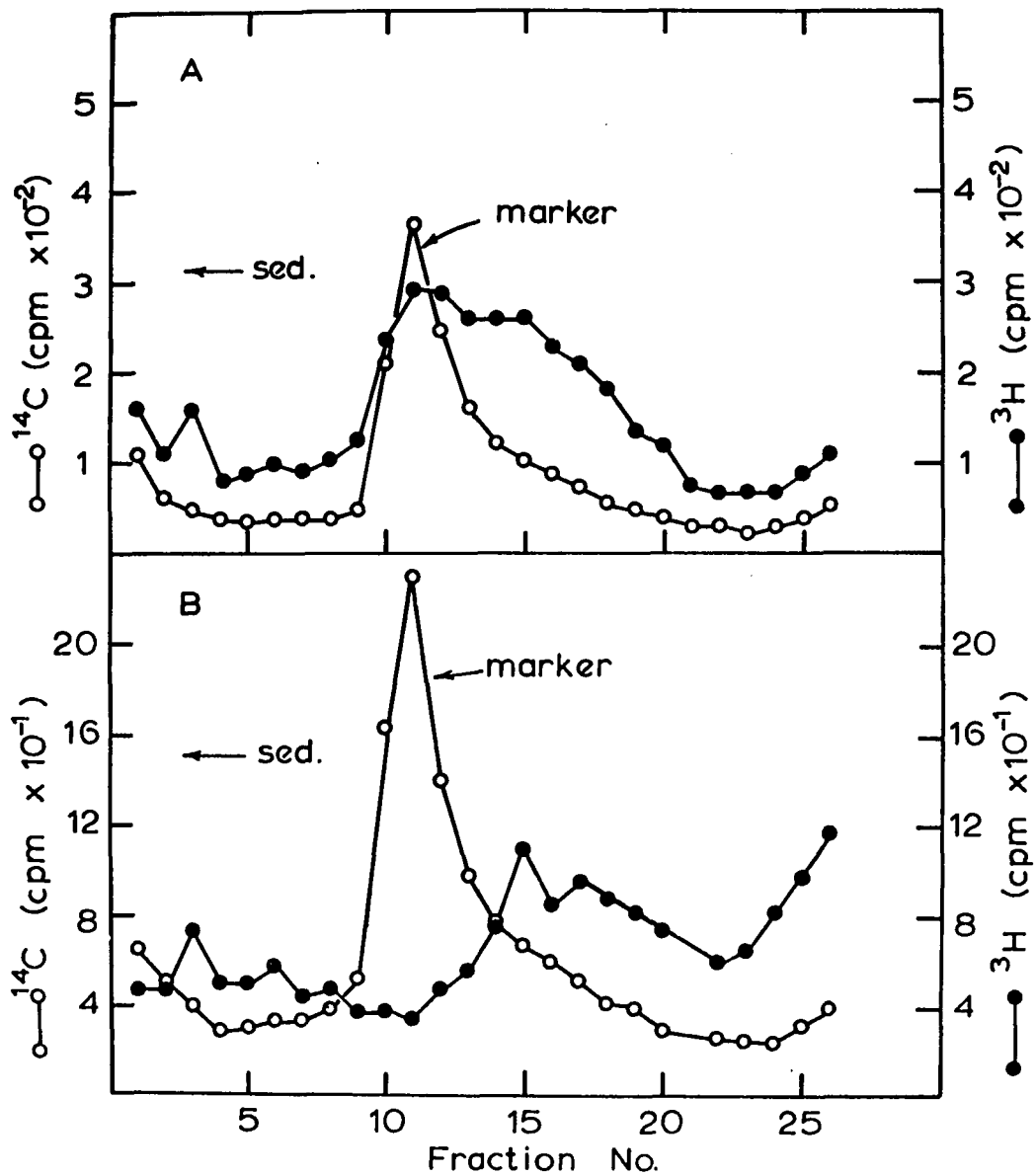

Fig. 22. Alkaline Sucrose Gradient Analysis of DNA from 25 Minute Phage-Infected Br6^{-}B$_1^{-}$.

The experiment is the same as Fig. 21, except that the ^{3}H-uracil labeled samples are denatured in 0.3 N KOH and sedimented in 5 to 20% alkaline sucrose. Marker is ^{14}C-T6 DNA. Panel A, T6-infected Br6^{-}B$_1^{-}$; Panel B, T6agt^{-}-infected Br6^{-}B$_1^{-}$.

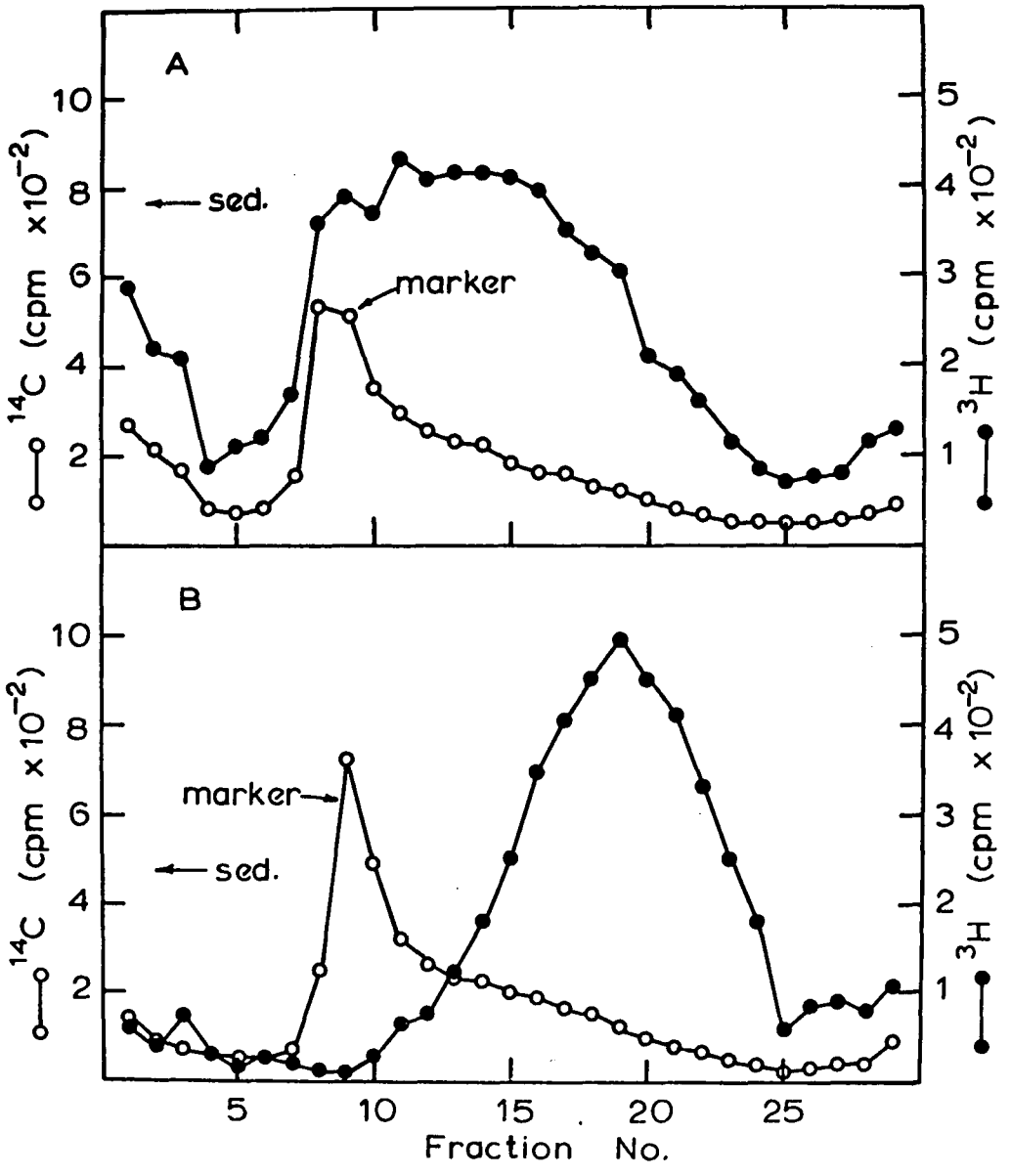

Fig. 23. Analysis of DNA from 25 Minute T6-Infected B201 or tet-1-T⁻ on Alkaline Sucrose Gradients.

The experiment is the same as Fig. 21, except that the indicated cells are infected with T6 in the presence of ^{3}H-thymidine. Lysed samples are denatured in 0.3 N KOH and sedimented in 5 to 20% alkaline sucrose. Marker is ^{14}C-T6 DNA. Panel A, T6-infected B201; Panel B, T6-infected tet-1-T⁻.

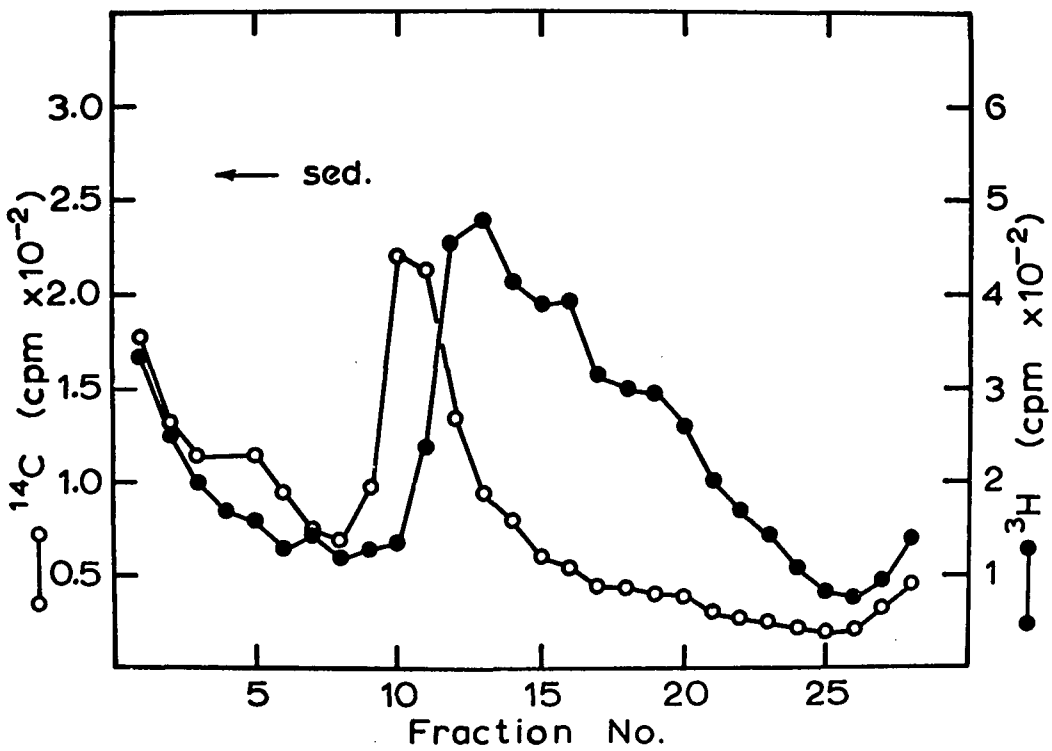

Fig. 24. Sedimentation of DNA from Purified Phage on an Alkaline Sucrose Gradient.

Purified ^{14}C-uracil T6 and ^{3}H-uracil T6αgt^{-} phage are denatured in 0.3 N KOH and the resulting DNA is sedimented in 5 to 20% alkaline sucrose. Sedimentation is from right to left.

essentially a measurement of the ratio of 260 nm absorbing material to viable phage. These measurements for T6 and T6αgt^- are shown in Table IX. The optical cross section for a purified T6αgt^- phage suspension is about 10-fold higher than for a similar T6 preparation. If one calculates the expected titer of the T6αgt^- on the basis of the T6 optical cross section, one can find that the T6αgt^- stock contains about 90% non-viable particles. This is within the range of the reduction in burst size (1/10) reported by Georgopoulos and Revel (1971).

Phage Specific Shut-off of Restriction

The data in Fig. 25 demonstrate the degradation of T6αgt^- DNA upon infection of E. coli B. The extent of degradation observed in this experiment is about 30%, in agreement with previous data (Hattman 1964) and is essentially complete after 8 minutes post-infection. However, it will be recalled that newly replicated T6 DNA is temporarily non-glucosylated and yet is not subject to this nucleolytic attack. Accordingly, I decided to ask whether prior infection could shut-off the restriction activity. It was necessary to use T4 mutants which do not develop immunity to superinfection (*imm*$^-$ mutants) (Cornett 1973, Vallée and Cornett 1972). Whereas normal T4 infection renders the cell immune to subsequent infection by other T-even phage, T4*imm*$^-$2 allows superinfection to occur. The development of immunity after phage infection results in a destruction of the superinfecting DNA (superinfection breakdown) and a failure of the superinfecting genome to be expressed (superinfection exclusion). Cells infected by T4*imm*$^-$2 can be

TABLE IX. Optical Cross Section of Phage Suspensions

Phage	Titer (pfu/ml)	A_{260} [a]	Optical Cross Section [b] (cm^2/pfu)
T6	6.65×10^{10}	9.5	1.43×10^{-10}
T6αgt2xam	3.04×10^{9}	5.2	1.7×10^{-9} [c]

a. determined in a quartz cuvette, path length = 1 cm

b. optical cross section = A_{260}/path length x titer

c. expected titer (from T6 optical cross section) = 3.6×10^{10} pfu/ml

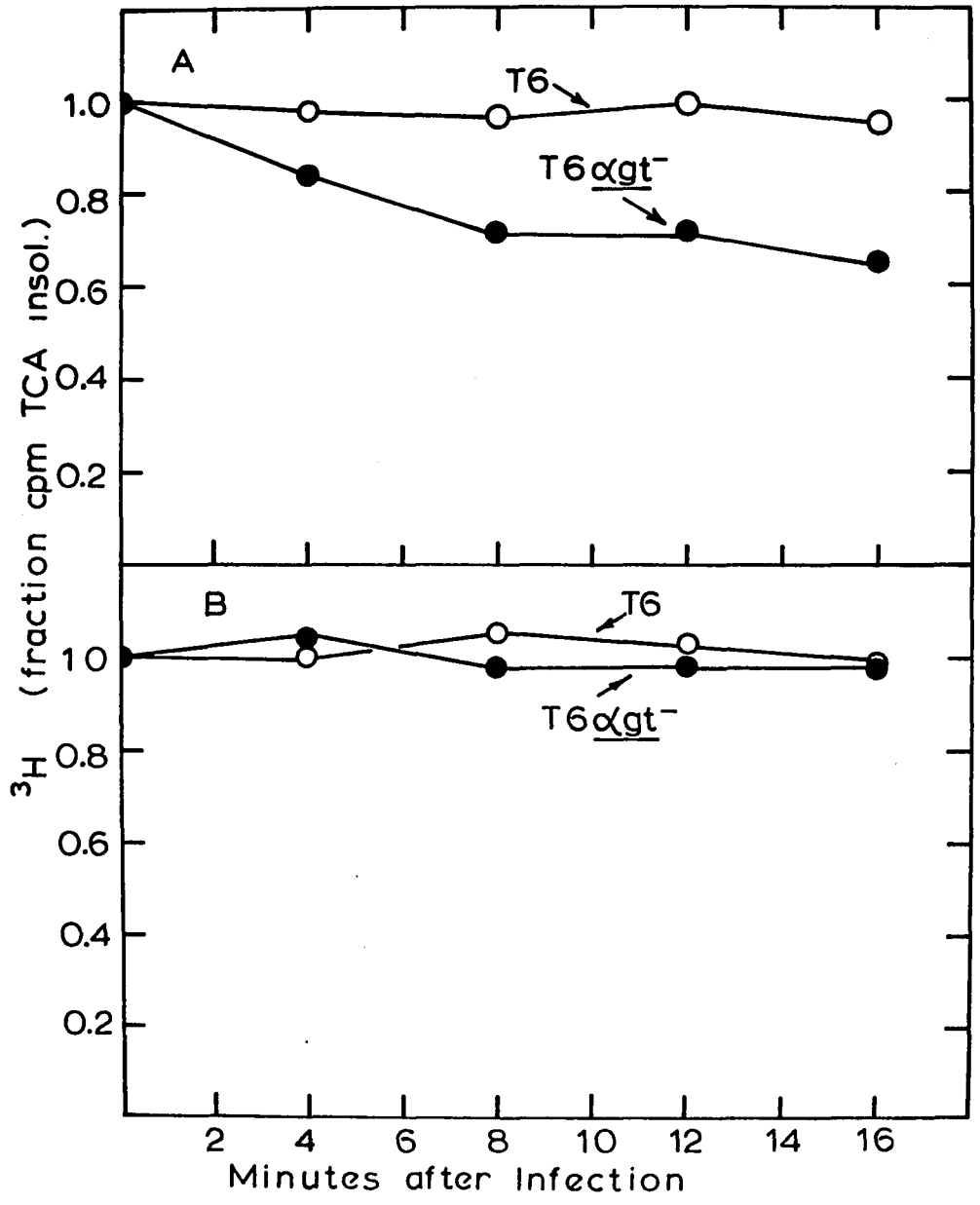

Fig. 25. Degradation of Phage DNA After Infection of E. coli Strains.

The data represent the fraction of initial 5% TCA insoluble cpm remaining in 0.1 ml of the infected culture at the indicated times. Panel A, ^{3}H-T6 or ^{3}H-T6αgt⁻2xam-infected B; Panel B, ^{3}H-T6- or ^{3}H-T6αgt⁻2xam-infected Br6⁻B$_1$⁻.

superinfected, the superinfecting DNA enters the cell intact and the superinfecting genome is expressed (Cornett 1973).

T6αgt⁻ Total Progeny from Superinfected T4imm⁻2 Infected E. coli B

E. coli ER22 and ER22r6⁻B$_1$⁻ were infected with T4imm⁻2am42 and then superinfected at various times with T6αgt⁻. The resulting total progeny were plated on ER22r6⁻B$_1$⁻ and the data are shown in Table X. Since the plating host is permissive for non-glucosylated phage but not for amber mutants, the observed progeny are due to "rescued" T6αgt⁻. A gradual increase in the total T6αgt⁻ progeny is observed, when superinfection is carried out after T4imm⁻ infection of ER22. At 8 minutes the level of progeny equals that seen in a companion experiment performed in ER22r6⁻B$_1$⁻. The difference in total progeny observed for ER22r6⁻B$_1$⁻ with and without prior infection may be due to some interference produced by the unexpressed T6imm⁻2am42.

Shut-off of T6αgt⁻ DNA Degradation by T4imm⁻2 s⁻

To investigate this effect further, I observed the degradation of T6αgt⁻ DNA in B, with or without prior infection. For these experiments, T4imm⁻2 s⁻ mutants were used. The addition of the s⁻ (spackle negative) mutation prevents the slow development of immunity seen with imm⁻ alone (Cornett 1973). In addition, the degradation was monitored as TCA soluble counts rather than as fraction remaining insoluble (as in Fig. 25). Observation of counts solubilized proved more sensitive to low levels of degradation.

TABLE X. T6αgt⁻ Total Progeny After Superinfection of T4imm⁻2am42 Infected Cells

Host	Total progeny[a] x 10^{-6} (pfu/ml) superinfection at (minutes) 0	2	4	8	16
ER22	7.7	14.5	13.0	22.3	27.0[b]
ER22r6⁻B_1⁻	23.5	-	-	22.5	27.0[c]

a. unadsorbed phage titer, 5 x 10^6 pfu/ml

b. total progeny with T6αgt alone, 5 x 10^6 pfu/ml

c. total progeny with T6αgt alone, 33.5 x 10^6 pfu/ml

As shown in Fig. 26, the degradation of T6αgt$^-$ DNA to TCA soluble products in E. coli B is inhibited by a 4 minute prior infection with T4imm$^-$2 s$^-$. The shut-off is complete, as evidenced by comparison with the data for T6 DNA in B.

I then wished to know if this shut-off of restriction requires phage transcription or translation. The shut-off of degradation of T6αgt$^-$ DNA observed with T4imm$^-$2 s$^-$ is resistant to both chloramphenicol (100 µgm/ml) or rifampicin (100 µgm/ml) (Fig. 27). In Fig. 16, it was shown that 100 µgm/ml of rifampicin was sufficient to block RNA synthesis. Thus the observed shut-off does not depend on either transcription or translation.

It could be that the shut-off phenomenon is a result of the imm mutation. Accordingly, I performed experiments to answer this objection. The data in Fig. 28 show the results of some experiments in which ^{3}H-DNA-containing T6αgt$^-$ was co-infected with other phage in E. coli B. The co-infection was necessary since superinfection exclusion and breakdown result after prior infection of B by phage not carrying an imm mutation. One can see that co-infection with T4imm$^-$2 s$^-$ does not produce the complete shut-off of DNA degradation seen in Fig. 26. While the results in Fig. 28 are not entirely straightforward, some conclusions can be drawn. Co-infection with T4D gives the same result as co-infection with T4imm$^-$2 s$^-$. This implies that the shut-off of restriction is not due to the presence of the imm or s mutation. The results seen with co-infection by T6 are in line with a statement by Hattman (1964) that co-infection of T*2 and T2 did not prevent DNA degradation. Since Hattman did not present the data on this point, it is not certain that

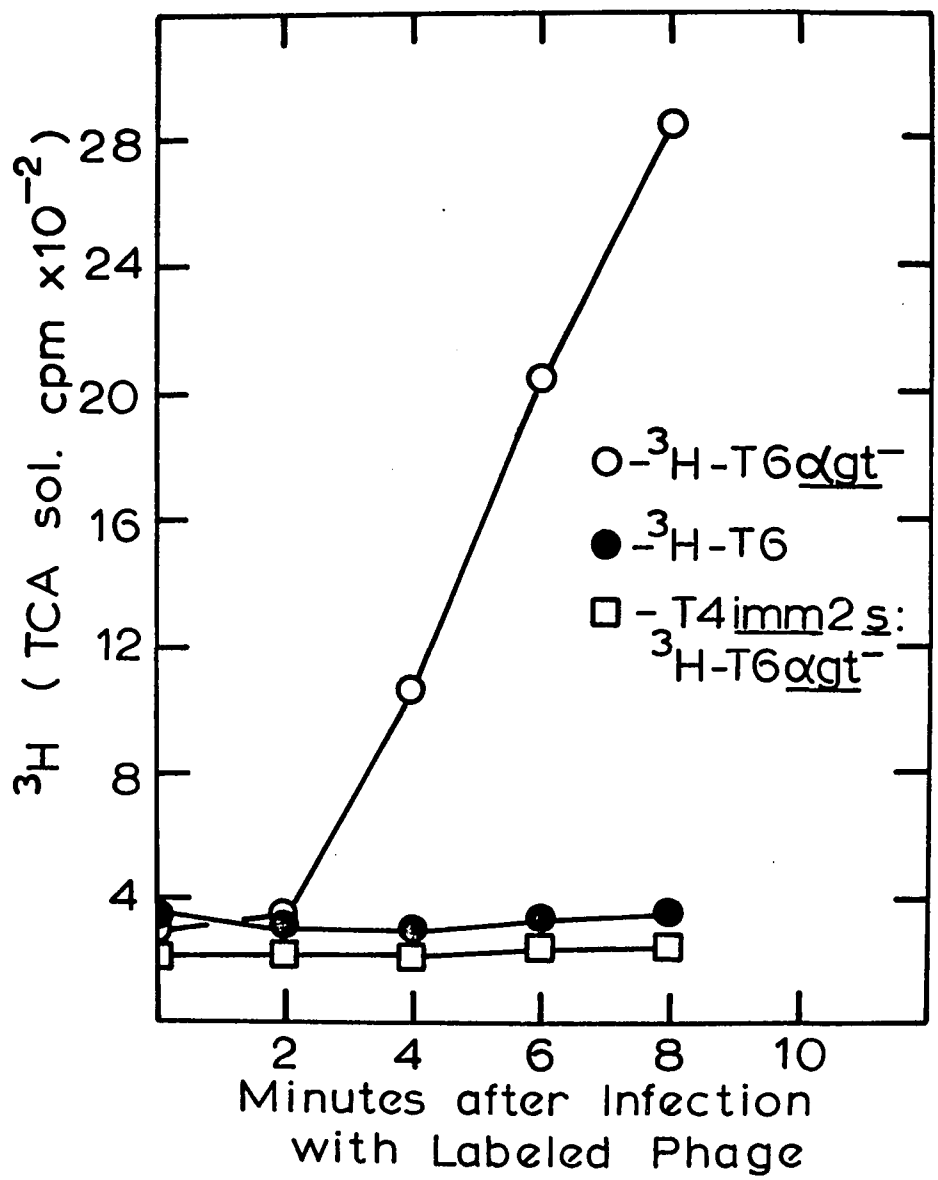

Fig. 26. Phage-Specific Shut-off of the Degradation of T6αgt$^-$ DNA in E. coli B.

The data represent the cpm made 5% TCA soluble in 0.5 ml of the infected culture at the indicated times. Prior infection with T4imm$^-$2s$^-$ is for 4 minutes.

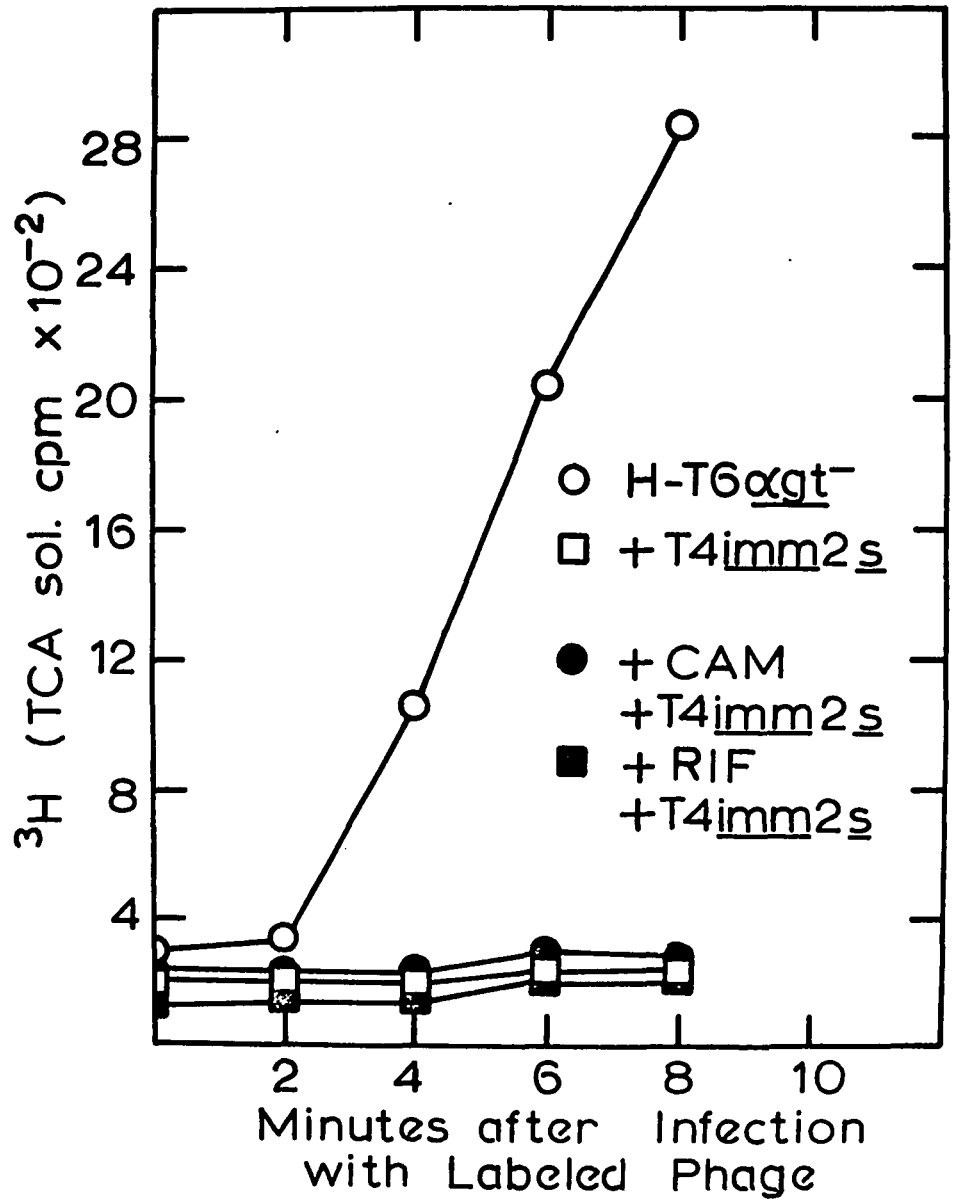

Fig. 27. Effect of Inhibitors on the Shut-off DNA Degradation in E. coli B.

The experiment is the same as Fig. 26. Rifampicin (RIF) (100 μgm/ml) or chloramphenicol (CAM) (100 μgm/ml) is added 2 minutes prior to the T4imm⁻2s⁻ infection.

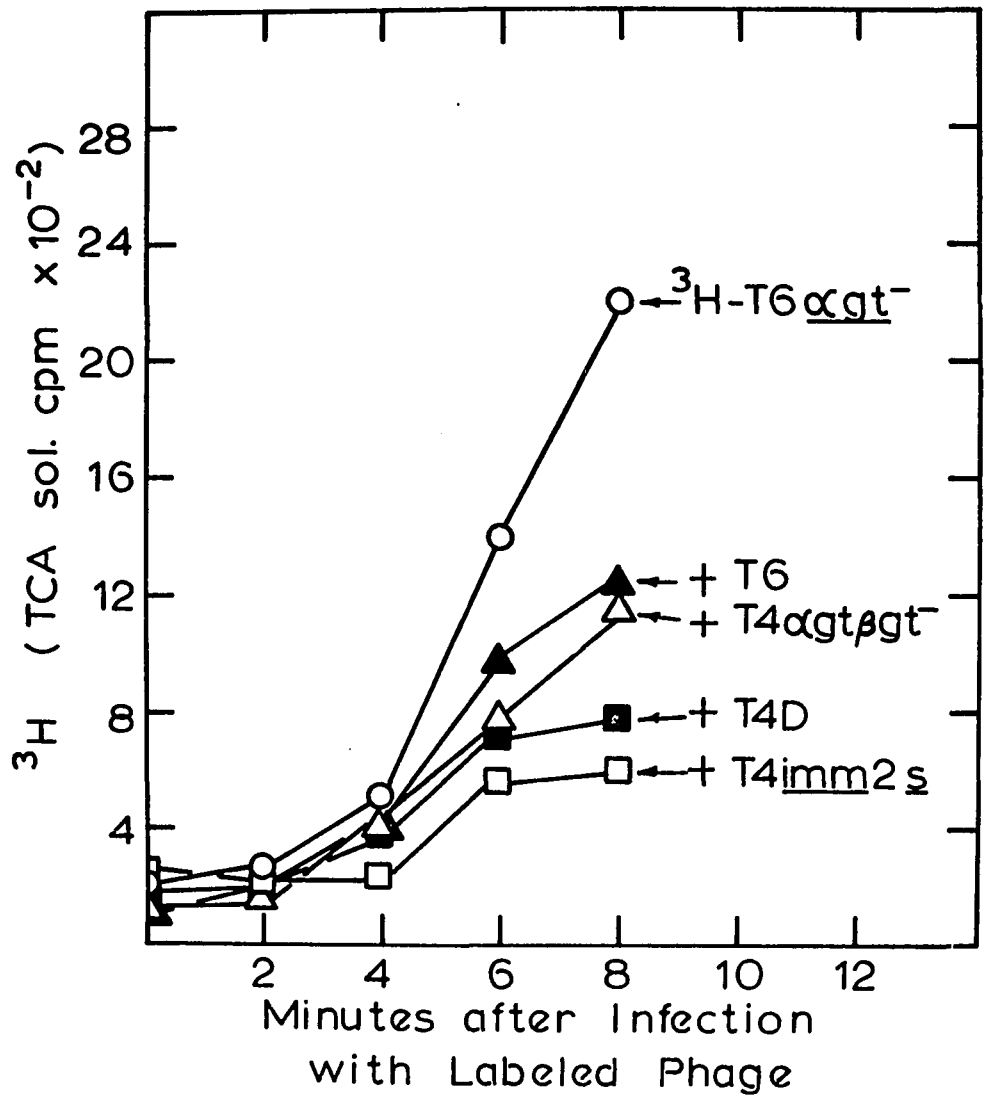

Fig. 28. Effect of Co-infection with Various Phage on the Shut-off of the Degradation of T6αgt$^-$ DNA in E. coli B.

The experiment is the same as Fig. 26. Co-infection is achieved by the addition of mixed phage suspensions.

this co-infection did not partially decrease the degradation, as seen in Fig. 28 with T6. The results in Fig. 28 also show that co-infection with T4$\alpha gt^-\beta gt^-$ is more like T6 than T4. Although the difference is only seen at 8 minutes, non-glucosylated phage may not be as efficient as normal phage in effecting this shut-off.

It is possible that the observed shut-off is due to glucosylation of the superinfecting DNA by a glucosyl transferase activity entering the cell upon infection with T6$imm^-2\ s^-$. If this is the case, the shut-off would not be seen in a $UDPG^-$ host, in which glucosylation is prevented by lack of substrate. However, the $UDPG^-$ B strain ($B/4_o$) is resistant to T4 infection, precluding the use of the T4 imm mutant. Accordingly, I performed the experiment by infecting E. coli W4597 (a $UDPG^-$ K strain which adsorbs T4) with T4$imm^-2\ s^-$ and then superinfecting with H-T6αgt^-. The results (Fig. 29) show that in the normal K strain (W3110) restriction is not abolished by prior infection with T4$imm^-2\ s^-$. Some decrease in degradation can be seen, but the K system is clearly different from the B system (Fig. 26). Thus, the effect of impaired glucosylation ($UDPG^-$ host) on the shut-off could not be observed.

Earlier work (S. Kornberg et al. 1961) had demonstrated that no glucosyl transferase activity can be seen in T4-infected cells until about 5 minutes after infection. If a transferase activity enters the cell and is responsible for the protection of superinfecting non-glucosylated DNA, its activity would have been evident before 5 minutes. Experiments performed with extracts from cells infected with T4$imm^-2\ s^-$ for 4 minutes in the presence of rifampicin (100 μgm/ml) confirmed the

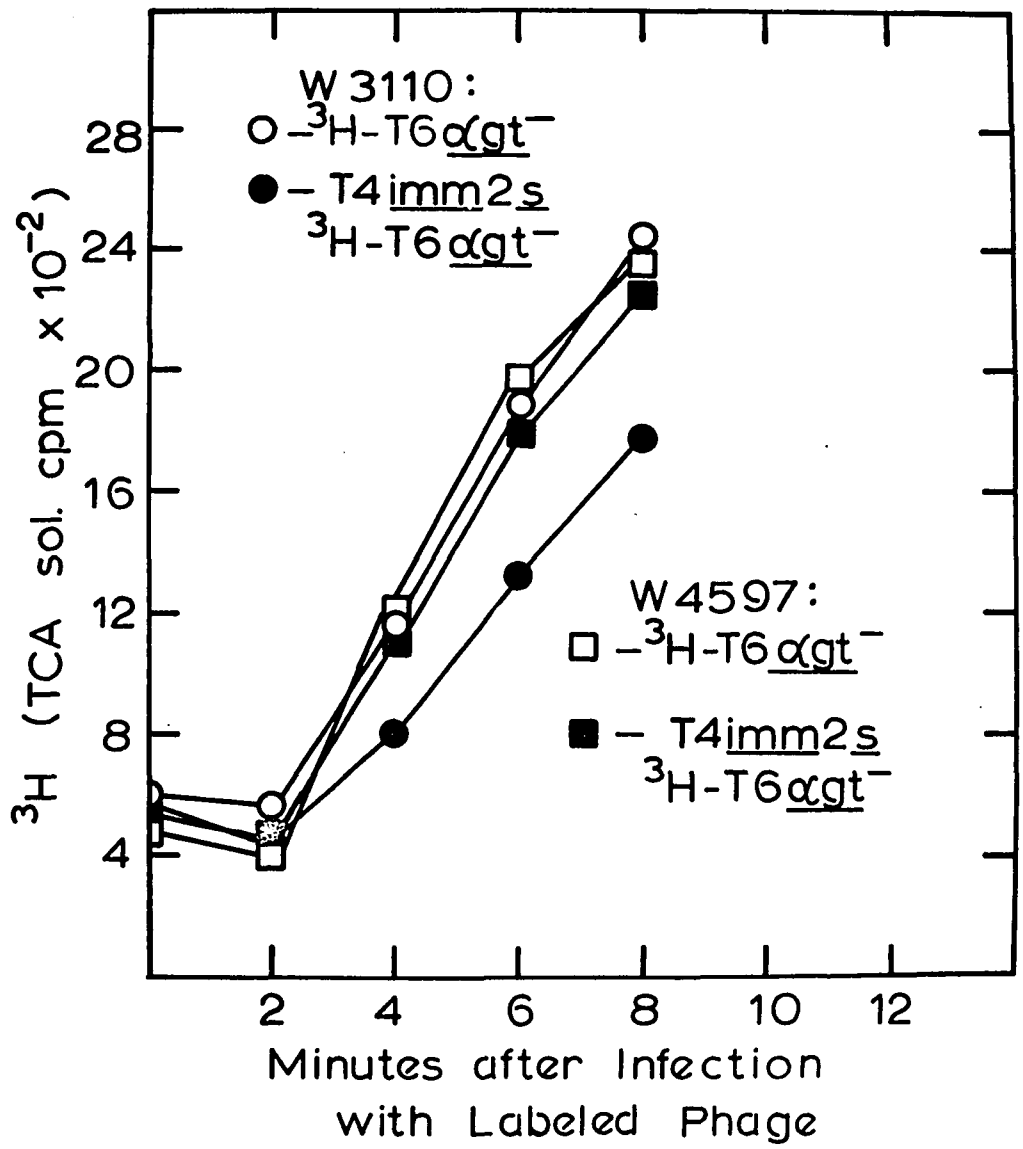

Fig. 29. Shut-off of Restriction in E. coli K Strains.

The experiment is the same as Fig. 26, except that the host cells are either E. coli W3110 or E. coli W4597 (UDPG⁻).

lack of glucosyl transferase activity. Therefore, the observed shut-off of restriction is not due to glucosylation of superinfecting DNA by a phage glucosyl transferase entering the cell upon the initial infection.

Analysis of Structural Differences Between T6 and T6αgt⁻

The shut-off of restriction, described in the previous section, is not dependent on translation or transcription of phage functions. It is possible that the shut-off is a function of the infection itself; for instance, some mechanical effect at the cell surface due to phage adsorption. It will be recalled that T6αgt⁻ phage adsorb normally to host cells, but the restriction system is not abolished. Since the abolition of the restriction activity does not involve phage transcription or translation, the effect may be due to an interaction between the cell membrane and a structural component of the phage. T6αgt⁻ is restricted, and, in addition to the lack of glucose residues, may be defective in this proposed interaction. Therefore I undertook a structural analysis of T6 and T6αgt⁻ phage using SDS-gel analysis.

T6 and T6αgt⁻ were grown in *E. coli* Kr6⁻r2,4⁻ in the presence of ^{3}H- or ^{14}C-leucine. The results (Fig. 30) of the SDS-polyacrylamide gel analysis (10% gel) of the disaggregated proteins in a mixture of ^{14}C-T6 and ^{3}H-T6 phage. The data in Fig. 31 show a similar analysis of a mixture of ^{14}C-T6 and ^{3}H-T6αgt⁻ phage. In Fig. 31, slice 49 contains a ^{3}H-labeled protein peak from T6αgt⁻ which does not appear in T6. When the labeling regime is reversed, a peak of ^{14}C can be observed (Fig. 32). An analysis of the data presented in Figs. 31 and 32

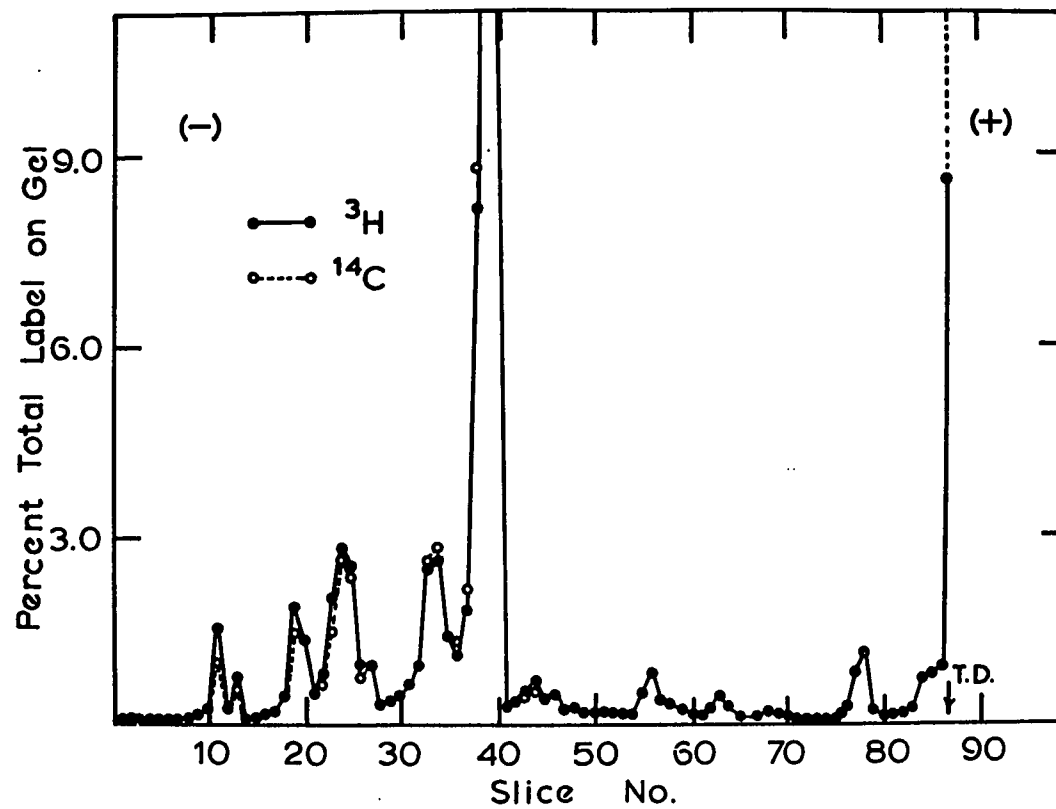

Fig. 30. SDS-Gel Electrophoresis of Proteins from T6 Phage.

The data show the electrophoretic pattern of the proteins from SDS-disaggregated ^{14}C-leucine T6 and ^{3}H-leucine T6. Both phage are grown in Kr6^{-}r2,4^{-}. Migration is from left to right, in a 10% acrylamide gel, at 3 mA/gel. T.D. (tracking dye) indicates the position of the front.

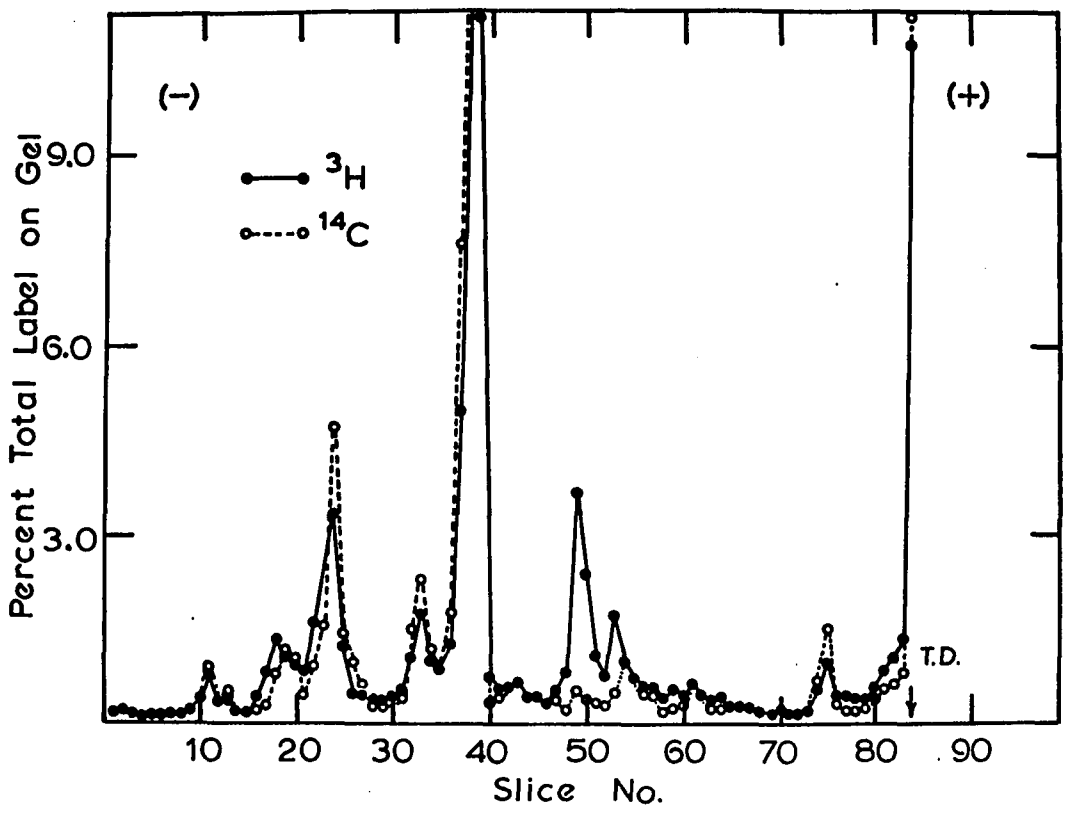

Fig. 31. Comparison of Proteins from T6 and T6αgt⁻ Phage by SDS-Gel Electrophoresis.

The experiment is the same as Fig. 29, except that the proteins are from ^{14}C-leucine T6 and ^{3}H-leucine T6αgt⁻ phage.

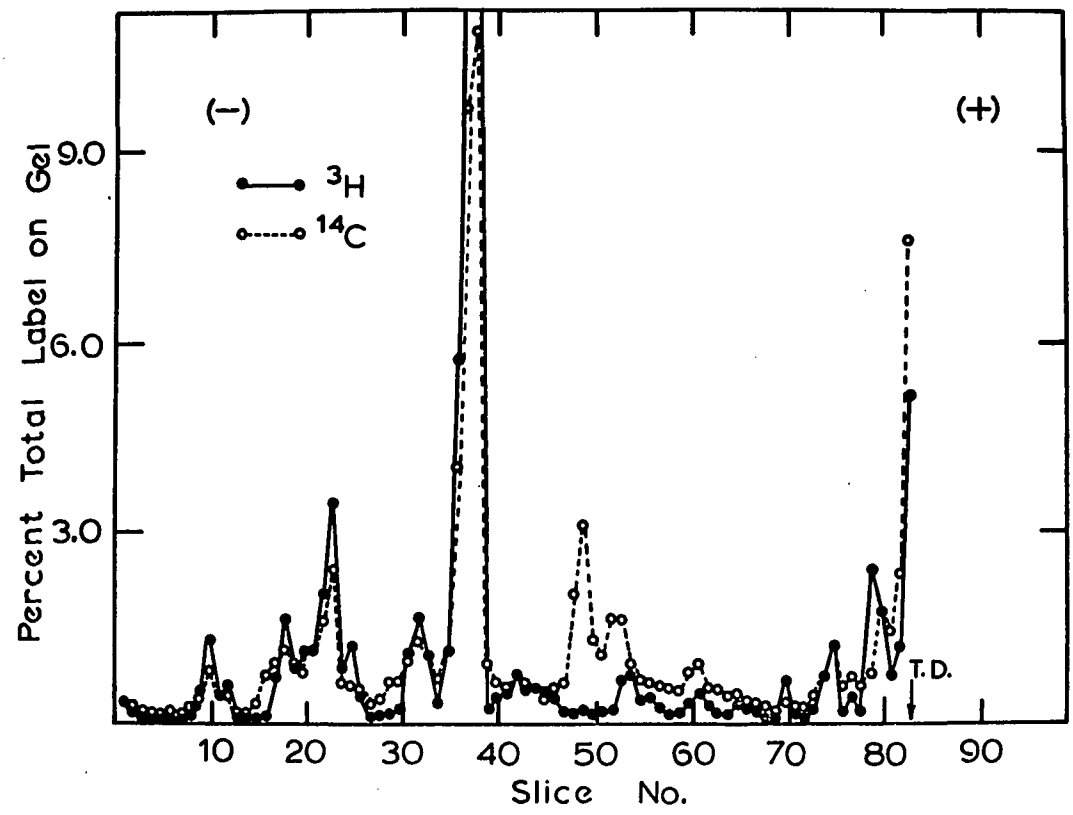

Fig. 32. Effect of Reversing the Labeling Regime on the SDS-Gel Pattern of Proteins from T6 and T6αgt⁻.

The experiment is the same as Fig. 29, except that the proteins are from ^{3}H-leucine T6 and ^{14}C-leucine T6αgt⁻ phage.

indicates that the counts occurring in the peak at slice 49 might be accounted for by a decrease in ^{3}H counts (or ^{14}C counts for Fig. 32) at slice 38 (as seen in Fig. 32, but off scale in Fig. 31).

The observed structural difference might be due to the lack of the gt gene product, which may be involved in phage assembly, possibly as a structural protein. Alternatively, the difference may be due to the lack of DNA glucosylation, resulting in a difference in the proteins bound to the DNA within the phage head. In order to distinguish between these two possibilities, SDS gels were run on proteins from T6 phage labeled during growth in either E. coli B or $B/4_o$ ($UDPG^-$). The results (Fig. 33) demonstrate that there is no difference in the pattern of T6 grown in B compared to T6 grown in $B/4_o$. However, in both cases there is a protein peak (slice 48) in the same region of the gel as the mutant protein peak observed in Fig. 31.

The data in Fig. 34 shows the results of a SDS-gel comparison of T6 grown in B with T6 grown in $Kr6^- r2,4^-$, showing that a peak at slice 50 appears when growth has been in B (but not in K).

In order to compute approximate molecular weights, protein standards were run on 10% gels under the same conditions as the disaggregated phage, and the resulting standard curve is shown in Fig. 35. Using this curve, the molecular weight of selected fractions was estimated (Table XI). In Fig. 31 the peak at slice 38 has a molecular weight of about 47,500, and the mutant peak (slice 49) has a molecular weight of about 40,000. The peak occurring in T6 grown in B (Figs. 32 and 33) has an estimated molecular weight of about 37,000.

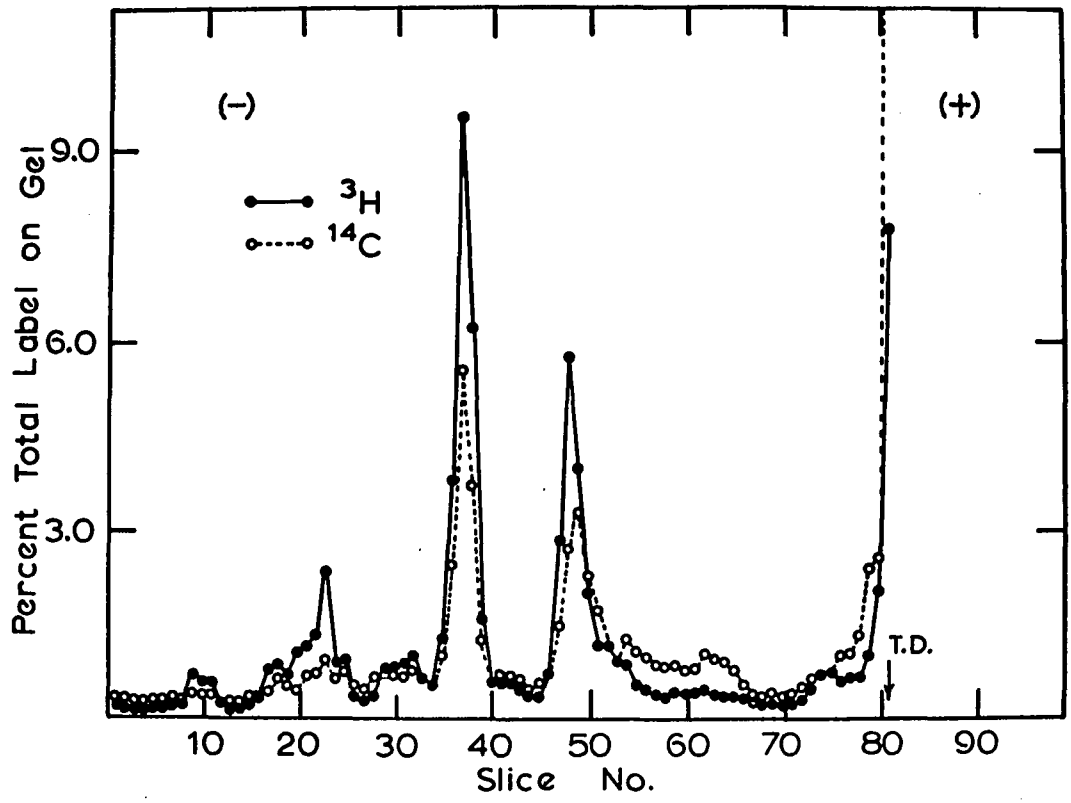

Fig. 33. SDS-Gel Electrophoresis of Proteins from T6 Phage Grown in B or B/4$_o$.

The experiment is the same as Fig. 29, except the proteins are from ^{14}C-leucine T6 (grown in B/4$_o$) and ^{3}H-leucine T6 (grown in B).

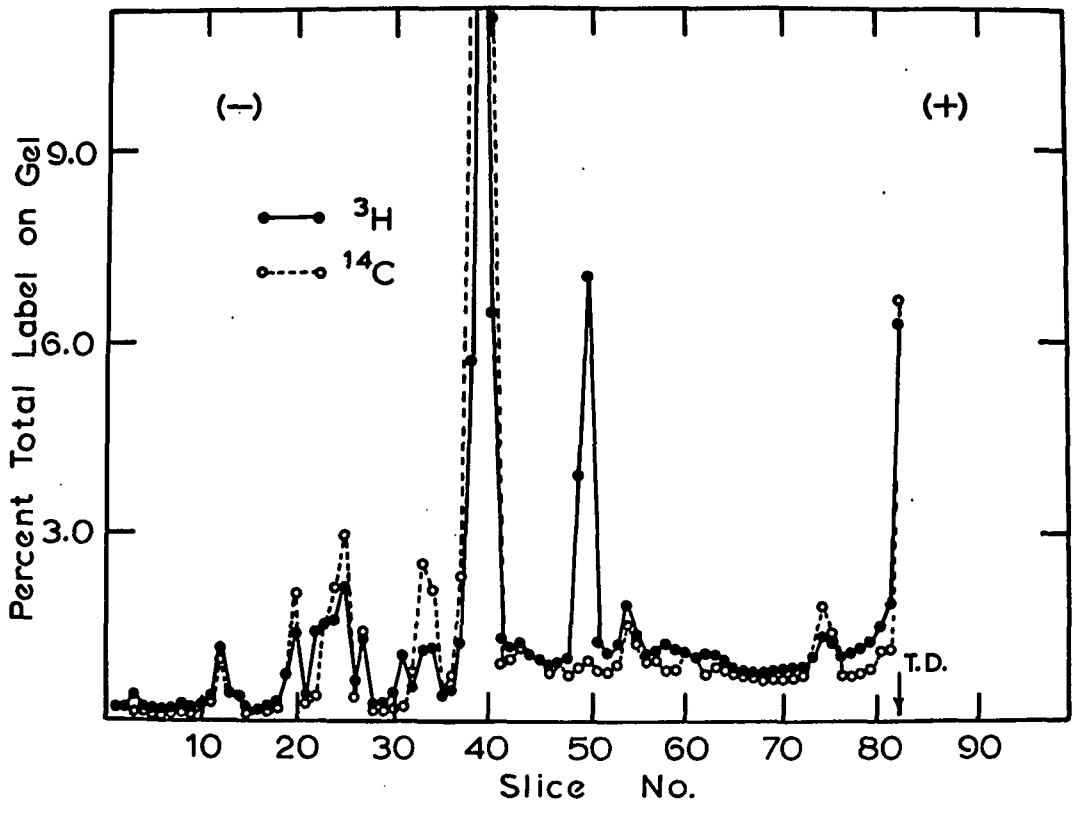

Fig. 34. Comparison of Proteins from T6 Phage Grown in B or Kr6⁻r2,4⁻ by SDS-Gel Electrophoresis.

The experiment is the same as Fig. 29, except the proteins are from ^{14}C-leucine T6 (grown in Kr6⁻r2,4⁻) and ^{3}H-leucine T6 (grown in B).

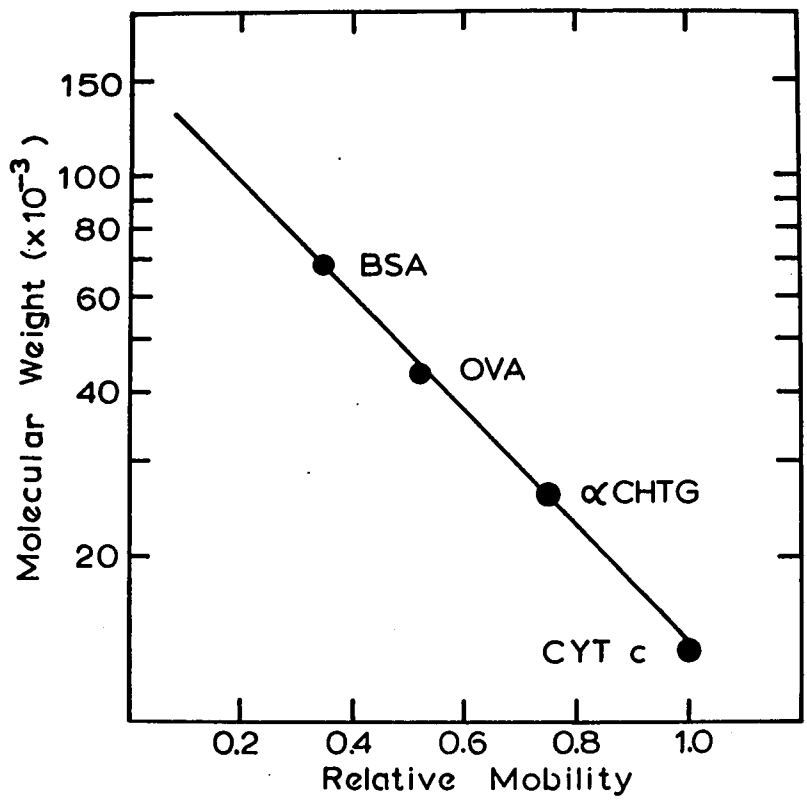

Fig. 35. Molecular Weight Standard Curve for 10% Acrylamide SDS-Gel Electrophoresis.

Samples of proteins of known molecular weight are run on 10% gels, as in Fig. 29. The gels are stained in Coomassie Brilliant Blue as described in Chapter 2. Relative mobility is the distance migrated for the protein/distance migrated for the front. BSA, bovine serum albumin; OVA, ovalbumin; αCHTG, α-chymotrypsinogen; CYTc, bovine cytochrome c.

TABLE XI. Molecular Weight Estimates of Selected SDS-Gel Fractions

Phage	Slice #	Relative Mobility[a]	Est. M.W.[b]
T6 vs. T6	39 (Fig. 29)	0.448	47,500
T6 vs. T6αgt$^-$	38 (Fig. 30)	0.452	47,000
"	49 (Fig. 30)	0.583	40,000
T6(B) vs. T6(B/4$_o$)	48 (Fig. 32)	0.592	37,500
T6(B) vs. T6(K)	50 (Fig. 33)	0.609	36,000

a. relative mobility, calculated as slice # of fraction/slice # of tracking dye

b. estimated from standard curve (Fig. 34)

It should be noted that the slicing technique used in this analysis yields only 12 to 14 protein peaks. Autoradiographic techniques (Laemmli 1970, R. Haselkorn, personal communication 1972) result in 24 or more peaks. However, the analysis of a double-labeled gel (^{3}H and ^{14}C) requires slicing and counting. Therefore, the peak observed in T6αgt$^-$ phage is not necessarily identical to that observed in T6 grown in B. However, the close proximity of these two peaks in the gel pattern makes it difficult to detect the mutant protein in B/4$_o$-grown T6. Therefore, this experiment could not resolve the question of whether the difference between T6 and T6αgt$^-$ is due to the lack of glucosylated DNA.

In order to determine if the structural difference in T6αgt$^-$ is an internal protein (and thus possibly a protein bound to the phage DNA) I performed SDS-gel analyses on T6 and T6αgt$^-$ phage ghosts. The preparation of phage ghosts results in the removal of DNA and internal proteins from the phage capsid by rupture of the phage head structure (Duckworth 1970). Phage ghosts were prepared by osmotic shock, treated with DNase and RNase and analyzed on gels as described in Chapter 2. The results (Fig. 36) show that the protein peak detected earlier in T6αgt$^-$ phage (Figs. 31 and 32) can still be seen in the pattern of T6αgt$^-$ phage ghosts. Since the viability of the phage dropped to less than 2.0% of the original value after osmotic shock, the peak is not due to contamination by intact phage. It will be noted that the positions of the various phage proteins on the gel shown in Fig. 36 do not correspond with the mobilities seen in earlier gels. The pattern in Fig. 36 was obtained on gels prepared from a separate stock of

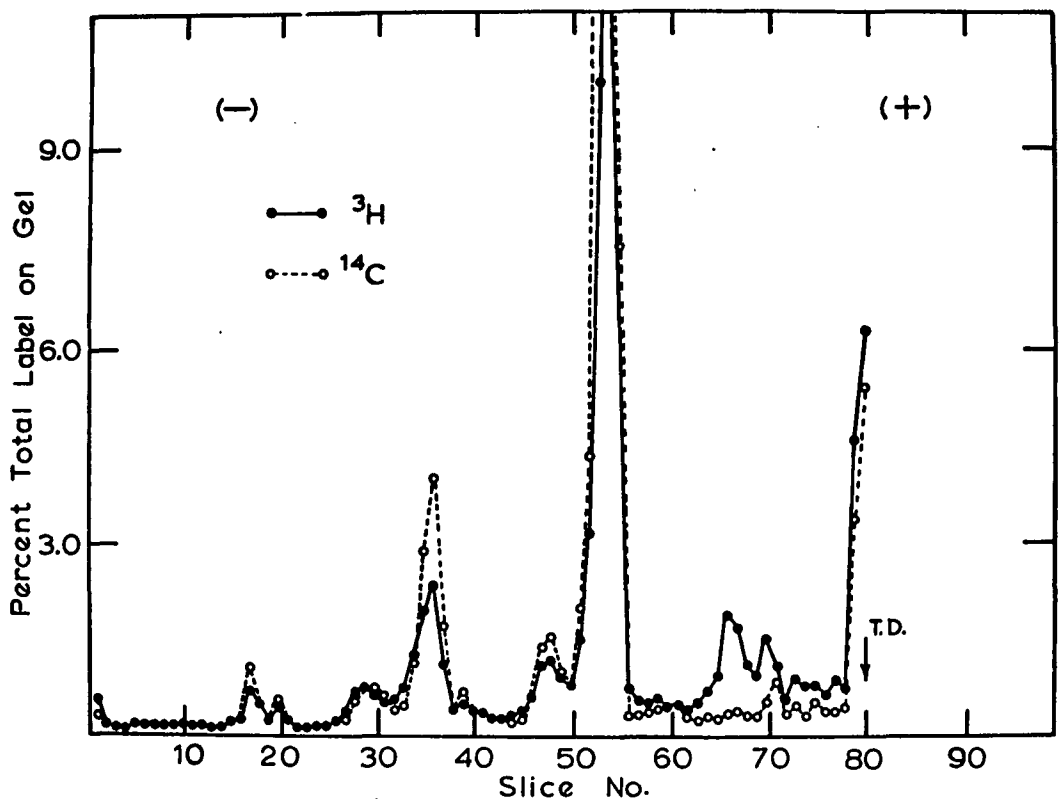

Fig. 36. Analysis of Proteins from T6 Phage Ghosts and T6αgt$^-$ Phage Ghosts by SDS-Gel Electrophoresis.

The experiment is the same as Fig. 29, except that the prctein are from ^{14}C-leucine T6 phage ghosts and ^{3}H-leucine T6αgt$^-$ phage ghosts. The viability of phage ghost preparations was <2.0% of the initial level.

acrylamide, which may account for this altered mobility. However, the mutant peak is quite distinct in Fig. 36, occurring about 15 slices after the main protein peak (slice 53). Therefore, the altered structure seen in T6αgt$^-$ phage is not osmotically sensitive and may be a capsid protein.

It appears from these data that the structural difference seen in T6αgt$^-$ phage may be due to the lack of the α-glucosyl transferase. It is possible that the αgt gene product may be a structural component of T6 phage. Further discussion of this point may be found in Chapter 4.

CHAPTER 4

DISCUSSION

The restriction of non-glucosylated T-even bacteriophage DNA represents a unique system in bacteria. In all other restriction systems, whether a property of the cell (the B and K systems), a prophage (the P1 system) or an episome (the RI and RII systems), the nucleolytic activity (restriction) and the modification (protection against restriction) are functions of the same protein or group of proteins. (For a review of these systems, see Meselson, Yuan and Heywood 1972). In all of these systems both the restriction of incoming DNA and the modification (generally by methylation of adenine residues) of replicating DNA are functions of the cell, prophage or episome.

In contrast, the restriction of non-glucosylated DNA involves a phage function for modification (the glucosyl transferases) and a cellular function for restriction (the r6 and r2,4 loci in E. coli).

Nature of the tet Mutants

Other host functions are involved in the glucosylation/restriction system, since the substrate used for the addition of glucose to phage DNA is the cellular pool of UDPG. The tet mutants are a case in point. The phenomenon of non-glucosylation of phage DNA due to lack of UDPG in the host cell was demonstrated almost ten years ago (Hattman and Fukasawa 1963, Shedlovsky and Brenner 1963, Symonds et al. 1963). However the tet mutants were isolated in an attempt to find some other

host functions involved in steps in phage maturation (such as DNA replication or phage assembly) (Mathews 1970, Mathews and Hewlett 1971). Such mutants have been described by other workers (Pulitzer and Yanagida 1972, Takano and Kakefuda 1972, Georgopoulos et al. 1972, Coppo et al. 1973, H. Revel, personal communication 1972). That the tet mutants are UDPG-negative (or more specifically, UDPG-PPase negative) is supported by the reduced activity of UDPG-PPase in cell extracts, the production of progeny phage after T6 infection which will only grow on permissive cells and the fact that the DNA in such progeny is partially non-glucosylated. Additional evidence showing that the tet mutants fail to grow on galactose, and that $B/4_o$ is similar to the tet mutants in resistance to phage infection, has been presented elsewhere (Hewlett and Mathews 1973).

Analysis of Membranes from E. coli B and Br6$^-$B$_1^-$

Attempts to identify the cellular activity responsible for the specific nucleolytic attack on non-glucosylated phage DNA had not been successful in the past (Richardson 1966, Molholt and Fraser 1965, Revel and Luria 1970). It was hoped that with a well characterized preparation of membrane vesicles (Osborn et al. 1972) and the technique of gradient analysis of DNA to detect limited nucleolytic attack, this problem could be resolved.

Membrane Associated Nuclease Activity

The set of experiments designed to assay nuclease activity (conversion of DNA to TCA soluble products) was intended to determine if any contaminating activity might be present which would interfere with

later gradient analyses of DNA. The observation of an ATP-dependent nuclease activity in these membrane preparations (Fig. 9) led to the evidence that such activity might be due in part to the recB,C gene of E. coli (Fig. 10). These data do not present a complete case for membrane association of exonuclease V (recB,C nuclease). Further studies, involving analysis of the purity of the membrane preparations (levels of cytoplasmic contaminants) and the strength of the proposed membrane association (influence of disaggregation or repeated washing) would be necessary. The suggestion that exonuclease V is a membrane protein is novel (S. Barbour, Wayne State University, personal communication 1973) and is potentially interesting, since this enzyme may be involved in DNA recombination (Willets and Mount 1969, Friedman and Smith 1973).

Sedimentation Pattern of DNA After Incubation with Membrane

Both T6 and T6αgt$^-$ DNA are not degraded by E. coli B membrane preparations in the absence of ATP (Fig. 9). This system was used to examine the effect of membranes on the sedimentation pattern of DNA. Transfer RNA was added to inhibit the endonuclease I activity reported present in these types of membrane preparations (Osborn et al. 1972). Partial degradation of either T6 or T6αgt$^-$ DNA was seen after incubation with a membrane preparation from B or Br6$^-$B$_1^-$ cells. However, no such activity was observed when membrane from ER22 (endonuclease I negative) cells was used. It may be possible to explain the failure of the tRNA in the reaction mixture to inhibit endonuclease I in the B and Br6$^-$B$_1^-$ preparations. Osborn et al. (1972) reported that ribonuclease I (RNase I) also occurs in these membrane vesicles. Although the

secondary structure of tRNA might prevent complete degradation by RNase I, sufficient damage might occur during a 30 minute incubation to render it incapable of inhibiting endonuclease I. Further investigation of this point was precluded by the data obtained using ER22 membrane, showing that endonuclease I was responsible for the initial observations.

RNA in T6αgt⁻ Infected E. coli B

Infection of the restrictive host E. coli B by T6αgt⁻ is abortive and only limited phage processes occur (Revel and Luria 1970). However, extensive RNA synthesis is seen during the course of this infection (Figs. 14 and 15). It had been shown previously (Hattman 1964, Fukasawa 1964, Hattman et al. 1966) that some early phage enzymes are synthesized after infection of B by non-glucosylated phage. Competitive RNA/DNA hybridization (Figs. 16 and 17) indicate that very little of the RNA seen in such infection is due to the transcription of these early phage functions. The evidence presented in Table VIII indicates that the RNA being synthesized late in T6αgt⁻ infection of B is, in fact, predominantly E. coli RNA. The termination of stable host RNA synthesis after infection requires the functioning of some phage genes, unlike the cessation of messenger RNA synthesis (reviewed in Mathews 1971a). In addition, the host RNA polymerase requires phage specific modification to be eventually converted to exclusive phage transcription (Travers 1969). After T6αgt⁻ infection of B, the host DNA may be incompletely degraded since the phage nucleases required for this function may not be synthesized. In addition, the phage specific

modification of RNA polymerase might not occur. Therefore some random transcription of E. coli DNA into stable RNA species may occur. This hypothesis remains to be tested in this system. This could be done by determining if the RNA produced is ribosomal or messenger. In addition, the degradation of host DNA after infection by T6αgt⁻ could be examined. However, this point is not directly involved in restriction, and was not pursued further.

T6αgt⁻ Infected E. coli Br6⁻B$_1$⁻

The infection of the permissive host Br6⁻B$_1$⁻ by T6αgt⁻ yields progeny phage, but the amount of phage produced is 1/5 to 1/10 that in a normal infection (Georgopoulos and Revel 1971). As discussed in Chapter 1, these authors presented evidence that low burst size could be correlated with the absence of the gt gene product. Several possibilities exist to explain this lowered burst size. Non-glucosylated DNA may be inefficient for late gene transcription, and there may be a limiting amount of structural protein for phage assembly. Another explanation is that non-glucosylated DNA may be subject to nucleolytic attack within the cell. A third alternative is that the glucosyl transferase might play an additional role in DNA replication (Georgopoulos and Revel 1971).

Competence of Non-glucosylated DNA for Gene Transcription

Experiments reported in Chapter 3 show that RNA metabolism is essentially normal in T6αgt⁻ infected Br6⁻B$_1$⁻ (Fig. 18). Competitive RNA/DNA hybridization experiments (Figs. 19 and 20) demonstrate that phage mRNA synthesis is normal in such an infection. The type of RNA

species being produced both late and early in T6 and T6αgt⁻ infected Br6⁻B$_1^-$ is the same, as evidenced by the similar slopes of the competition curves. The relative abundance of the RNA species is also similar, since the plateau levels of the competition curves are the same. Work reported earlier with T6 infected tet mutants (UDPG⁻) indicated that RNA synthesis at early and late times was also normal (Mathews and Hewlett 1971). Neither the lack of the gt product nor the failure of DNA to be glucosylated affects the transcription of late phage genes.

Nature of DNA from T6αgt⁻ Infected E. coli Br6⁻B$_1^-$

In light of the reduced burst size reported for T6αgt⁻ in permissive cells and the 2- to 3-fold increase in DNA synthesis observed in this infection (Fig. 18), the nature of this DNA was examined. It was found that T6αgt⁻ DNA synthesized at 25 minutes after infection in Br6⁻B$_1^-$ contains extensive single-strand breaks (Fig. 22). Furthermore, experiments performed with T6 infected tet cells yielded similar results (Fig. 23). Earlier work with tet mutants showed that parental T6 DNA (fully glucosylated), when observed at 13 minutes after infection, had undergone considerable single-strand breakage (Mathews and Hewlett 1971). The burst size in the case of T-even phage-infected UDPG⁻ cells has been reported to be normal (Georgopoulos and Revel 1971). My data for T6-infected B/4$_o$, tet-1 and tet-2 indicates a burst size of about 1/2 to 1/4 normal (Table IV). These data suggest that lack of glucosylation of the pool of mature DNA makes it susceptible to some nucleolytic attack, leading to a reduced burst size.

In a study of T4 induced nucleases, an enzyme was found which attacked non-glucosylated T6 DNA (T*4) more rapidly than T4 DNA (Ando et al. 1970). Non-glucosylated phage DNA in permissive cells may be subject to nucleolytic attack by such phage enzymes. Newly replicated phage DNA (presumed temporarily non-glucosylated) may be protected if such enzymes are synthesized late in infection.

Another experiment demonstrates that the DNA contained in T6αgt$^-$ phage, grown on a permissive host, has single-strand breaks (Fig. 24). A further interpretation of these data is that purified T6αgt$^-$ phage contain a population of molecules, some of mature size and some with extensive single-strand breaks. Note in Fig. 24 that there is material sedimenting with normal T6 DNA. Since this DNA does contain single-strand breaks, it might explain earlier work in which cell nucleases (especially exonuclease III and endonuclease I) were reported to act more rapidly on non-glucosylated DNA (Richardson 1966, Eigner and Block 1968). Nicked DNA may serve as a better substrate for these cellular nucleases in the assay systems used. The experiments to test this have not been done as yet.

The occurence of nicked DNA in purified T6αgt$^-$ phage suggests that such a phage preparation might contain non-viable particles. An analysis of the optical cross section of T6 and T6αgt$^-$ phage (Table IX) shows that this is the case. Only about 10% of the phage in the T6αgt$^-$ suspension (calculated from the A_{260}) are viable.

The reduced burst size of T6αgt$^-$ may therefore be due to two factors; 1) the extensive single-strand breakage of progeny

non-glucosylated DNA and 2) the occurrence of progeny phage containing defective DNA (non-viable phage).

Possible Involvement of the Thiamine Synthesizing System in Restriction

The similarity between the pyrimidine precursor of thiamine and HMC was pointed out in Chapter 1. The final steps involved in the synthesis of thiamine result in a phosphorylation of the HM-pyrimidine moiety (Fig. 3). One could ask if phosphorylation of non-glucosylated HMC residues in phage DNA could also be catalyzed by these enzymes. Such phosphorylation might make the phage DNA more sensitive to the putative restriction nuclease.

Experiments designed to detect this activity in crude cell extracts were unsuccessful, due to the rapid degradation of the non-glucosylated phage DNA by cellular nucleases (Richardson 1966). Further work on this hypothesis should first involve a partial purification of the enzymes of thiamine biosynthesis, to remove such nuclease activity. One could then test the specificity of the thiamine system for substrates such as HMC or non-glucosylated phage DNA.

Nature of the Restriction System

As discussed in Chapter 1, attempts to identify a specific nucleolytic activity responsible for restriction of non-glucosylated phage DNA have been unsuccessful. Experiments described in Chapter 2 also show that no such specific nuclease activity can be found in membrane preparations from E. coli B. However, two separate experimental

approaches reported in this work suggest that the restriction activity may be a membrane function.

SDS-Gel Pattern of Membrane Proteins

The restriction activity may be membrane associated by virtue of the comparison of membrane proteins from restrictive (ER22) and permissive (ER22$\underline{r6}^{-}B_1^{-}$) cells, grown in the presence of ^{3}H- or ^{14}C-leucine. Two areas of the protein pattern on SDS-gels differ between the two strains (Fig. 6). Peaks occur in the permissive cell membrane (slice 52 and 58) with a concomitant decrease in a peak of higher molecular weight (lower mobility) in the restrictive cell (slice 32).

One would like to suggest that these changes represent mutant protein fragments resulting from the altered restriction nuclease. However, it must be remembered that these peaks probably do not represent a single protein, but rather a group of proteins of about the same molecular weight (the SDS-gel technique resolves monomeric subunits of most proteins). In addition the permissive cell is lacking two functions; the ability to restrict ($\underline{r6}^{-}$) and the ability to synthesize thiamine (B_1^{-}). Revel (1967) has stated that these permissive mutants arose by a deletion of the $\underline{r6}$ locus and a portion of the thiamine locus. If this is so, either one or both of these functions might be membrane associated and could account for this altered gel pattern.

Shut-off of Restriction Activity

The activity which degrades T6$\underline{agt}^{-}$ DNA in $\underline{E.\ coli}$ B after infection is shut-off by prior infection with immunity negative T4 mutants (Fig. 26). This shut-off is not dependent on phage transcription or

translation (Fig. 27). Phage infection shuts off or alters several host functions. The inhibition of host protein synthesis does not require phage translation or transcription, since it occurs as a result of infection by phage ghosts (phage particles minus DNA) (Duckworth 1970). The activity of exonuclease V (the recB,C nuclease) is inhibited by phage infection, and this inhibition is dependent on translation of phage proteins (sensitivity to chloramphenicol) (Tanner and Oishi 1971). It may be of advantage to the phage to shut off the restriction activity, since there is a time after DNA replication begins when the newly synthesized DNA is non-glucosylated (Erikson and Szybalski 1964).

It could be argued that the observed effect is due to the protection of the superinfecting T6αgt$^-$ DNA by glucosylation via glucosyl transferases produced by prior T4 infection (McNicol and Goldberg 1973). This is precluded by the fact that the shut-off is rifampicin and chloramphenicol insensitive. However, protection by glucosylation could be the case if a glucosyl transferase activity enters the cell at infection along with the phage DNA.

Several proteins do enter the host cell at the time of infection. The phage internal proteins are among these, but their exact function is unknown. Some recent work indicates that a UV repair enzyme may enter the cell upon infection (Shames, Lorkiewicz and Kozinski 1973).

In order to test this hypothesis, I attempted to perform the shut-off experiment in a UDPG$^-$ host. A K strain (W4597) was necessary since B/4$_o$ (UDPG$^-$) is also resistant to T3, T4 and T7. However, the K and B systems are quite different. The K restriction activity against

T6αgt$^-$ DNA is not abolished by T4imm$^-$2 s$^-$ infection (Fig. 29). In Chapter 1, other differences between the K and B strain were described (for example, the association of thiamine dependence with r6$^-$ in B but not K). While the failure to observe the shut-off in K is not surprising, it does indicate that a direct test of the glucosylation hypothesis could not be easily carried out.

If glucosylation by a transferase activity entering the cell at infection were the cause of the inhibition of restriction, such activity should be present in cell extracts. S. Kornberg et al. (1961) showed that glucosyl transferase activity does not appear in cells until 5 minutes after T4 infection. Therefore, glucosylation of superinfecting DNA by such an activity is unlikely as an explanation of the observed shut-off.

Interaction Between the Phage and Host Cell Membrane

The shut-off of restriction activity may be the result of an interaction between some phage component and the cell surface. This would follow from the data showing that no phage functions other than adsorption are required to effect the shut-off. In addition, SDS-gel patterns of proteins from permissive and restrictive cell membranes suggest that this function is membrane associated. Further tests of this possibility cannot be carried out until the restriction function has been identified.

Structural Differences Between T6 and T6αgt⁻

One implication of the shut-off of restriction described in this work is that some structural properties of normal phage may be responsible, possibly a structural protein. Therefore, T6 and T6αgt⁻, grown in the presence of ^{3}H- or ^{14}C-leucine were analyzed by SDS-gel electrophoresis. Two comments should precede a discussion of these data. First, the patterns obtained do not reflect the relative abundance of the various protein species in T6 phage, since the labeling regime employs only leucine, which may not occur to the same extent in every phage structural protein. For instance, T6 tail fibers contain about half the amount of leucine found in T6 internal proteins (Cummings et al. 1970, Stone and Cummings 1972). Second, the resolution of the slicing technique used in the present study is such that about 12 to 14 bands can be observed. In contrast, using disc gels and autoradiographic techniques, Laemmli (1970) could resolve about 28 bands from purified phage. Thus the peaks of protein represented in Figs. 29-32 probably do not represent single proteins.

The gel pattern reported in Fig. 31 clearly shows a difference between the proteins of disaggregated T6 and T6αgt⁻ phage. The data shown in Figs. 32 and 34 indicate that, while there is a difference between T6 grown in B as compared to Kr6⁻r2,4⁻, the pattern of T6 grown in B versus $B/4_o$ is quite similar. Table X shows that the mutant protein peak (Fig. 31, slice 49) and the peak from T6 grown in B (Fig. 33, slice 50) have somewhat different molecular weights. The resolving power of the gel technique employed is not sufficient to state that they are, in fact, different proteins.

The occurrence of the peak in the SDS-gel pattern of proteins from T6 phage grown in B and $B/4_o$ makes it difficult to determine if the mutant protein peak is a result of the αgt mutation or is due to some altered protein binding by non-glucosylated DNA. The data of Fig. 36 indicate that the mutant protein peak can be found when phage ghosts are analyzed on SDS-gels. This implies that the alteration involves a capsid (or osmotic shock resistant) protein. It will be of interest to determine which part of the T6 particle (tail plate, fiber, head, etc.) is affected.

Glucosyl Transferase as a Phage Structural Protein

The question now arises as to whether the structural alteration seen is the mutated αgt product (i.e., an amber fragment) and therefore, whether the α-glucosyl transferase is a structural protein of T6 phage. The molecular weight of the α-glucosyl transferase is not reported in the literature so that a comparison of the estimated molecular weight of this proposed fragment cannot be made.

The idea of such dual functions for phage enzymes is not unique. T4 dihydorfolate reductase (DFR) has been shown to be a structural component of T4 phage base plates (Kozloff et al. 1970, Mathews 1971b). Capco and Mathews (1973) have implicated T4 thymidylate synthetase (TS) in a structural role. The present data are not sufficient to make such a statement about T6 α-glucosyl transferase. It could be, for instance, that the transferase plays a role in DNA synthesis. DNA synthesis has been linked to phage head morphogenesis (Luftig and Lundh 1973) and anomalies in phage structure could result from defective replication.

In order to determine that the α-glucosyl transferase is a structural protein of T6, other experiments must be performed. One such experiment might be to prepare antiserum directed against purified T6 α-glucosyl transferase and test the ability of the antiserum to inactivate T6 phage. Mathews, Crosby and Kozloff (1973) have reported this for the case of DFR and T4 phage. Another approach would involve determining the expected relative size of a series of amber fragments by genetic mapping of T6$\underline{\alpha gt}^-$ amber mutants. These data could then be correlated with the observed size of the structural protein from the same phage on SDS-gels. Finally, the occurrence of α-glucosyl transferase activity in purified T6 phage could be demonstrated, as Kozloff et al. (1970) have done with DFR in purified T4 phage.

An Alternative Hypothesis for the Occurrence of Permissive E. coli Mutants

Most workers have assumed that permissive mutants lack a nucleolytic activity specific for non-glucosylated phage DNA (reviewed by Revel and Luria 1970). Attempts to identify this activity have involved a study of permissive cells to determine if any nuclease activities are missing. However, permissive mutants could arise in another manner. As shown in this dissertation, the restriction system is shut off after infection, probably by an interaction between the phage and the host membrane. T6$\underline{\alpha gt}^-$ phage have an altered structural component which could be that portion of the phage involved in the shut-off. Thus, T6$\underline{\alpha gt}^-$ phage contain non-glucosylated DNA and, in addition, may be unable to inhibit the cellular restriction system. Permissive mutants

may be cell membrane mutants in which this altered phage structural component can interact and effectively block restriction. This is consistent with the present data and may explain the failure to detect any missing nuclease activity in permissive mutants. Thus, restriction may be a two-step process involving a defective membrane interaction and a nucleolytic attack on non-glucosylated DNA.

Functions of DNA Glucosylation--General Comments

At first glance, the system of glucosylation and restriction seems to be an elaborate series of events in the development of both the T-even phage and their host cells. It would appear that the phage and bacteria have evolved defense mechanisms against each other, resulting in restricting enzymes in the host and glucosylated HMC residues in the phage DNA.

However, DNA glucosylation and the glucosyl transferases may not only involve the phage's defense against restriction. Montgomery and Snyder (1973) have reported the isolation of T4 mutants able to grow well on a strain of E. coli containing a defective RNA polymerase. Normal T4 does not grow well on this strain. These T4 mutants are found to be lacking β-glucosyl transferase. The authors present a model wherein the β-glucosylation of specific HMC residues results in a level of control of phage transcription.

The present work concludes that the T6 α-glucosyl transferase may be involved in the production of intact phage DNA. In the absence of glucosylation, replicating phage DNA is subject to nucleolytic attack, resulting in extensive single-strand nicking. In addition, the

T6 α-glucosyl transferase is active in some step in the assembly of the T6 phage particle. This may be either indirectly during DNA encapsidation (for instance), or directly, as a structural element of the phage capsid.

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