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THE RNA BACTERIOPHAGE R23

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The RNA Bacteriophage R23" submitted by Dr. Hiroko Watanabe in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The recently isolated RNA bacteriophage R23 was characterized and shown to produce a marked inhibition of host RNA, DNA and protein synthesis in *Escherichia coli*. A comparative analysis of the biology of specific RNA phages indicated that it was duplicated closely in this and other respects by R34, but less so by R40, f2 and Qβ.

The mechanism of host suppression by R23 was investigated by examining the effects of different amber mutants on host macromolecular synthesis following their isolation and characterization, and by performing detailed studies on the inhibition of the inducible synthesis of the bacterial enzyme, β-galactosidase. R23 infection appeared to result in a coordinate repression of expression of the galactoside permease and β-galactosidase cistrons of the lactose operon which was not antagonized by cyclic 3',5'-AMP.

The effect of R23 on the functional integrity of the bacterial membrane was also assessed by documenting the changes in flux of radioactively labelled galactosides and potassium ions across the permeability barrier in infected cells.

ACKNOWLEDGEMENTS

The author is indebted to Dr. Mamoru Watanabe for invaluable advice and support throughout this study and to Miss Luisa Po for technical assistance in certain experiments.

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ABBREVIATIONS

AMP, CMP, GMP, UMP	5'-monophosphates of adenosine, cytidine, guanosine and uridine
ADP, CDP, GDP, UDP	the corresponding 5'-diphosphates
ATP, CTP, GTP, UTP	the corresponding 5'-triphosphates
cyclic 3',5'-AMP	cyclic adenosine 3',5'-monophosphate
CAM	chloramphenicol
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DS	double-stranded
2,4 DNP	2,4-dinitrophenol
EDTA	ethylenediaminetetraacetic acid
IC	infectious centre
IPTG	isopropyl-β-D-galactopyranoside
MOI	multiplicity of infection
mRNA	messenger RNA
2-MSH	mercaptoethanol
NEM	N-ethylmaleimide
ONPG	o-nitrophenyl-β-D-galactopyranoside
PEP	phosphoenolpyruvate
PFU	plaque-forming unit
PK	pyruvate kinase
pppGp	guanosine tetraphosphate
RF	replicative form
RI	replicative intermediate
RNA	ribonucleic acid

ABBREVIATIONS (continued)

RNase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SS	single-stranded
SSC	standard saline citrate
Su^+	$suppressor^+$
Su^-	$suppressor^-$
TCA	trichloroacetic acid
Tris	tris(hydroxymethylamino)methane
tRNA	transfer RNA
UV	ultraviolet
c	curie
cpm	counts per minute
g	gram
l	litre
mg	milligram
min	minute
ml	millilitre
mμ	millimicron
rpm	revolutions per minute
sec	second
A	absorbance
°C	degree Celsius (centigrade)
M	Molar (mole/litre)
S	Svedberg unit of sedimentation coefficient

CHAPTER I

GENERAL INTRODUCTION

The RNA bacteriophage contains within a protective protein coat a linear, single-stranded RNA molecule which serves as messenger for synthesis of at least 3 viral proteins and as template for replication of viral RNA. This uncomplicated morphologic and genetic structure has encouraged its rapid exploitation as a convenient tool for the investigation of phenomena such as the mechanism and control of viral RNA replication and protein synthesis and the genetic expression of a polycistronic message.

Since the discovery of the first RNA-containing bacteriophage, f2, in 1960 (Loeb), many RNA bacteriophages have been isolated. These include μ2 (Dettori, Maccaro & Piccinin, 1961), MS2 (Davis, Strauss & Sinsheimer, 1961), Qβ (Watanabe, 1964), FH5 (Fouace & Huppert, 1962), R17 (Paranchych & Graham, 1962), β (Nonoyama, Yuki & Ikeda, 1963), f_{can} (Davern, 1964a), fr (Marvin & Hoffmann-Berling, 1963), M12 (Hoffschneider, 1963a), ZIK/1 (Bishop & Bradley, 1965), R23 (Watanabe & August, 1966), and many others which remain less well characterized. Besides these *Escherichia coli* phages, RNA phages of *Pseudomonas* (Feary, Fisher & Fisher, 1964) and *Caulobacter* (Schmidt & Stanier, 1965) have been described.

This thesis primarily concerns the RNA bacteriophage R23 and, to a lesser extent, the RNA phages R34 and R40. All three phages which infect *E. coli* were isolated by Dr. Mamoru Watanabe in 1966.

A. Biophysical Properties of the RNA Bacteriophages

1. Serologic. The well characterized RNA phages have been shown

to be serologically related (Nonoyama *et al.*, 1963; Scott, 1965; Bishop & Bradley, 1965) except for Qβ (Overby *et al.*, 1966a). R23 is serologically related to f2 (Chapter III, Section A). Other unrelated phages, however, do exist. Watanabe *et al.* (1967a) separated their 30 RNA phages into 3 serologic groups: the first is related to MS2, f2 and R17, while the second and third groups which contain GA and Qβ, respectively, are not.

2. Structure. Electron microscopic studies have demonstrated polyhedral viruses of 200-250 Å diameter (De Petris & Nava, 1963; Schwartz & Zinder, 1963; Franklin & Granboulan, 1966). The phage particle has a molecular weight of 3-4 X 10^6, a sedimentation coefficient or $S_{20,w}$ of about 80 and a density in cesium chloride of 1.46 (Table 1). It is composed of the coat protein, a "maturation protein" (A protein) and viral RNA. Phage particles have been reconstituted by combining these components *in vitro* (Hohn, 1967; Roberts & Steitz, 1967; Sugiyama, Hebert & Hartman, 1967; Hung & Overby, 1969).

3. Coat Protein. The phage coat protein is composed of 150-180 monomer subunits; each has a molecular weight of 14,000-17,000 and contains about 130 amino acids. The amino acid composition is similar for phages in the same serologic group but different among groups (Table 2). Histidine is absent in the coat protein of all RNA phages. In addition, Watanabe's group 3 phages (such as Qβ) lack tryptophan and methionine and group 2 (including GA) lack cysteine and methionine (Nishihara, Haruna & Watanabe, 1968).

The sequence of amino acids in MS2 and R17 coat proteins differs

TABLE 1

CHARACTERISTICS OF RNA BACTERIOPHAGES

Phage	Molecular Weight		$S_{20,w}$		Density in CsCl	Base Composition (moles %)				Reference
	Phage	RNA	Phage	RNA	(gm/ml)	A	U	G	C	
f2	3×10^6	$0.7\text{-}1 \times 10^6$			1.46	22.2	25.1	25.9	26.8	Zinder(1963)
Qβ	4.2×10^6		84.3		1.45	22.1	29.5	23.7	24.7	Overby *et al.*(1966a,b)
MS2	3.6×10^6	1×10^6	81	27	1.46	22.4	25.6	27.5	24.5	Strauss & Sinsheimer(1963)
R17	3.6×10^6	1.1×10^6	79	27		22.6	25.5	27.1	24.8	Paranchych & Graham(1962) Gesteland & Boedtker(1964)
fr	4.4×10^6	1.2×10^6	79	21		24.3	23.7	27.1	24.9	Marvin & Hoffmann-Berling (1963)
β			79, 90			23.3	28.0	21.6	27.2	Nonoyama *et al.*(1963)
f_{can}			79.5	25	1.42					Davern(1964a)
R23		1×10^6	75	25		23.4	26.0	25.6	25.1	Watanabe & August(1967)
μ2	4×10^6		78		1.42	23.8	21.7	25.4	23.7	Ceppellini *et al.*(1963)

TABLE 2

AMINO ACID ANALYSES OF COAT PROTEINS OF RNA PHAGES

Moles / Mole	f2	MS2		R17	M12	fr	R23	GA	Qβ		A protein R17
Lys	6	6	6	6	6	7	5	7	10	7	3
His	0	0	0	0	0	0	0	0	0	0	1
Arg	4	4	4	4	4	4	4	5	9	7	5
Asp	13	16	14	14	15	17	15	13	17	15	6
Thr	8	8	9	9	9	10	8	8	15	12	4
Ser	11	12	13	13	13	13	11	13	13	10	6
Glu	10	12	11	11	12	12	10	7	15	13	6
Pro	6	6	6	6	6	5	6	5	9	9	3
Gly	9	14	9	9	10	10	12	9	10	8	6
Ala	13	14	14	14	15	17	14	15	19	15	7
Val	13	15	14	14	15	18	13	16	16	13	6
Met	1	2	2	2	2	2	2	0	1	0	1
Ile	7	8	8	8	8	7	9	9	5	5	3
Leu	8	8	7	7	7	6	7	8	13	12	7
Tyr	4	2	4	4	4	5	4	5	4	4	3
Phe	4	2	4	4	4	5	4	4	3	3	3
Trp	1	2	2	2	2	2	2	2	0	0	-
Cys/2	2	2	2	2	2	2		0	2	2	-

f2 Notani *et al.*(1965)
MS2 Overby *et al.*(1966b); Nishihara *et al.*(1968)
R17 Weber (1967); A protein: Steitz (1968)
M12 Enger & Kaesberg (1965)
fr Wittmann-Liebold (1966)
R23 Watanabe *et al.* (1968)
GA Nishihara *et al.* (1968)
Qβ Overby *et al.* (1966b); Nishihara *et al.* (1968)

from that of f2 in that residue 88 is leucine in f2 and methionine in R17 and MS2 (Weber & Konigsberg, 1967; Enger & Kaesberg, 1965). The fr coat protein (Wittmann-Liebold, 1966) differs from that of f2 in 19 amino acids (Weber & Konigsberg, 1967). The N-terminal amino residue is alanine in all phages studied, but the C terminal sequence is -ileu-tyr for group 1, (tyr, phe)-ala for group 2 and tyrosine for group 3 (Nishihara *et al.*, 1968). Besides serving to encapsulate phage RNA, the coat protein is thought to be responsible for cell lysis (Zinder and Lyons, 1968).

4. Maturation Protein. The maturation protein is a structural component of the phage particle; there is an average of 1 molecule per phage particle (Steitz, 1968). The amino acid composition of the R17 maturation protein, also called the A protein, has been established (Table 2); it contains 5 histidine residues per 38,000 molecular weight. It is highly insoluble in aqueous buffers (Roberts & Steitz, 1967). Its exact function is unknown but it is required for adsorption to the F pili (Lodish, Horiuchi & Zinder, 1965) and may be important in determining the conformation of the particle and viral RNA (Heisenberg, 1967).

5. Viral RNA. The RNA phage particle contains 25-35% RNA (by weight) with approximately equimolar proportions of adenine, guanine, uracil and cytosine (Table 1), except for Qβ RNA which has more uracil. Phage RNA forms more compact conformations than other RNAs studied (Gesteland & Boedtker, 1964). The helix content is 80% in R17 and MS2 RNA (Mitra, Enger & Kaesberg, 1963). MS2 RNA possesses no covalent configurational restraints but does form intramolecular

hydrogen bonds (Strauss & Sinsheimer, 1968). Phage RNA preparations and defective particles of maturation protein mutants often display heterogeneous sedimentation patterns as evidence of non-random fragmentation (Lodish *et al.*, 1965; Heisenberg, 1966; Overby *et al.*, 1966b; Argetsinger & Gussin, 1966; Fiers, 1967), suggesting nuclease attack of a specific tertiary structure of the RNA during isolation.

Partial analyses of pancreatic RNase digests of RNA of MS2 (Min Jou & Fiers, 1969), M12 and R17 (Thirion & Kaesberg, 1968) and μ2 (Matthews, 1968) and RNase T1 digests of MS2 RNA (Rushizky, Skavenski & Sober, 1965) have been reported. The frequency of (purine nucleotide) pyrimidine nucleotide tracts shows a regular deviation from the expected (Vandenberghe, Van Styvendaele & Fiers, 1966). The RNAs of MS2 and f2 show 75% homology by annealing techniques, while those of MS2 and Qβ show none (Weissmann, Colthart & Libonati, 1968).

The 5' end is pppGp in R23 and other RNA phage RNAs; the 3' terminus is CCA in all RNA phages studied thus far (Table 3). No chain terminating and initiating codons have yet been found in the terminal sequences of phage RNA. Two beginning sequences have been found in Qβ RNA in approximately equal amounts (De Wachter & Fiers, 1969). Modification of the 3' terminal of f2 RNA does not affect the rate of amino acid incorporation *in vitro* but causes loss of infectivity (Takanami, Yan & Jukes, 1965). The 3' terminal adenylate, but not the penultimate cytidylate, can be removed without destroying the infectivity of R17 RNA. Progeny molecules from spheroplasts infected with such RNA have the adenylate restored (Kamen, 1969).

TABLE 3

SEQUENCE OF RNA PHAGE RNA's

3' OH Terminus

Phage	Sequence	Reference
f2	..(G)UUACCACCA	Weith & Gilham (1967); Dahlberg (1968)
R17	..(G)UUACCACCA	Dahlberg (1968)
MS2	...A	Sugiyama (1965); Lee & Gilham (1965)
	..(G)UUACCACCA	De Wachter & Fiers (1967)
Qβ	..Gp(9Cp,4Up)CpA	Weith *et al.*, (1968)
	..(G)CCCUCCUCUCUCCA	Dahlberg (1968)
	..GGUUCCCC	Bishop *et al.*, (1968)[1]

5' Terminus

Phage	Sequence	Reference
R23	pppGp	Watanabe & August (1968b)
f2	pppG...	Dahlberg (1968)
R17	pppG..	Roblin (1968)
MS2	pppGGGU..	De Wachter *et al.*, (1968)
	pppGGU..	Glitz (1968)
	pppGp..	Vandenberghe *et al.*, (1969)[2]
Qβ	pppGp.	Watanabe & August (1968b)
	pppGGGGAAC..	De Wachter & Fiers (1969)
	pppGGGGGAAC..	De Wachter & Fiers (1969)
	pppGGGGAACC..	Bishop *et al.*, (1968)
	pppGGGGAACC	Bishop *et al.*, (1968)[3]
	pppGp..	Bishop *et al.*, (1968)[4]

[1] "little variant"
[2] RI and RF
[3] "little variant" plus strand
[4] "little variant" minus strand

6. Radiobiology. UV-irradiated bacteria are able to support growth of RNA phage (Zinder, 1963; Fenwick, Fenwick & Franklin, 1964; Nonoyama & Ikeda, 1964; Neubauer & Zavada, 1965; Rappaport, 1965; Gussin, 1966; Knolle, 1966). If bacteria are irradiated so that 10^{-5} cells form colonies and bacterial RNA synthesis is reduced to 5-10%, 8-10% can form infective centers (Nonoyama & Ikeda, 1964; Gussin, 1966).

f2 is 30 and 7 times more resistant to UV than T2 (Zinder, 1963) and ϕX174 (Zinder, 1965), respectively. Group 1 phages are fairly resistant and group 3 are most sensitive to UV irradiation (Watanabe *et al.*, 1967b).

B. Infection

1. Invasion. RNA phage infect only male strains of *E. coli* or other bacteria which have received the *E. coli* F^+ episome (Horiuchi & Adelberg, 1965; Brinton, Gemski & Carnahan, 1964), though RNA isolated from RNA phage is infective to spheroplasts of F^- as well as F^+ strains and to spheroplasts of *Salmonella, Shigella, Proteus* and *Aerobacter* (Davis *et al.*, 1961; Fouace & Huppert, 1962; Knolle & Kaudewitz, 1962; Paranchych, 1963; Engelhardt & Zinder, 1964; Taketa, Ono & Shibuya, 1965). The phage receptor sites are located on F pili. F pili are removed by blending or successive cycles of centrifugation and washing (Valentine, Wedel & Ippen, 1965) but are able to regenerate.

The process of phage invasion includes adsorption to F pili, release of the RNA from the protein coat which remains outside (Edgell & Ginoza, 1965), and injection and transport of the RNA via

the pilus, followed by penetration into the cell (Valentine & Wedel, 1965). Hundreds of phage can adsorb to a single sex hair but only a few inject their RNA (Ippen & Valentine, 1967). 3 types of F pili mutants of *E. coli* have been isolated: 1) those which can be infected by Qβ but not by f2 because of a block at injection, 2) those resistant to f2 and Qβ but not f1 (a single-stranded DNA phage), and 3) those sensitive to f2 and f1 at 34° C but not 42° C (Silverman, Mobach & Valentine, 1967a,b; Silverman *et al.*,1968).

A metabolically active cell is required for invasion (Knolle, 1967) or irreversible attachment; the latter is prevented by chloramphenicol, KCN, streptomycin and incubation at 0° C or 70° C (Edgell & Ginoza, 1965). At 0° C adsorption and formation of F pili-phage complexes occur but subsequent "injection stages" are prevented (Ippen & Valentine, 1965; Valentine & Wedel, 1965). Also, phage RNA is not injected into the free pilus (Valentine & Strand, 1965). According to Wendt and Mobach (1969), phenethyl alcohol, sodium cyanide, azide and iodoacetate inhibit adsorption to cells but not to detached F pili; inhibition by phenethyl alcohol is reversible in the presence of chloramphenicol. The injecting strand is degraded if RNase is present (Zinder, 1963; Valentine & Wedel, 1965), but injection is not completely prevented (Ippen & Valentine, 1967). Cold-sensitive mutants of f2 unable to inject their RNA have been isolated (Silverman & Valentine, 1969).

Penetration is the only stage which requires divalent cations (Paranchych, 1966). The DNA phage f1 and f2 adsorb to different parts of the F pilus but compete for penetration (Ippen & Valentine, 1966).

Qβ inhibits f1 penetration by 50% (Silverman & Valentine, 1969). RNA phage infection and bacterial conjugation or recombination are mutually inhibitory (Knolle, 1967; Ippen & Valentine, 1967; Novotny, Knight & Brinton, 1968).

2. Growth Cycle. The growth cycle of RNA phage differs according to the phage and growth conditions (Loeb & Zinder, 1961; Davis, Pfeiffer & Sinsheimer, 1964; Rappaport, 1965; Huppert, Blum-Emerique & Breugnon, 1967; Godson, 1968). Generally, the latent period is 30-35 min, the rise period, 15-20 min, and the eclipse, 15-25 min. Lysis begins 20-90 min after infection and releases an average of 1500-4000 phage/cell. The percentage of phage adsorbed is 80-90% of input PFU's, bacterial survivors, 10-50%, and viable phage, 5-50% of the total particle yield.

3. Fate of Infected Bacteria. Cells may respond in different ways to contact with an RNA phage. A cell infected with fr may a) become temporarily immune to fr, b) lyse, or c) become a carrier (Knolle, 1964). Multiplication and liberation of phage without lysis or inhibition of cell division has also been observed with FH5 (Huppert, Ryter & Fouace, 1963), Qβ (Watanabe, 1964), β (Tsuchida, Nonoyama & Ikeda, 1966), and fr grown at 31° C or transferred to certain hosts (Hoffmann-Berling & Maze, 1964).

4. Intracellular Events. The sequence of intracellular events after RNA phage infection is illustrated by that of R23 (Fig. 1). At 5 to 10 min is detected a viral RNA synthetase (or polymerase) activity which increases until 20-30 min after infection. RNase-resistant RNA is first detected at 6 min and phage RNA (that

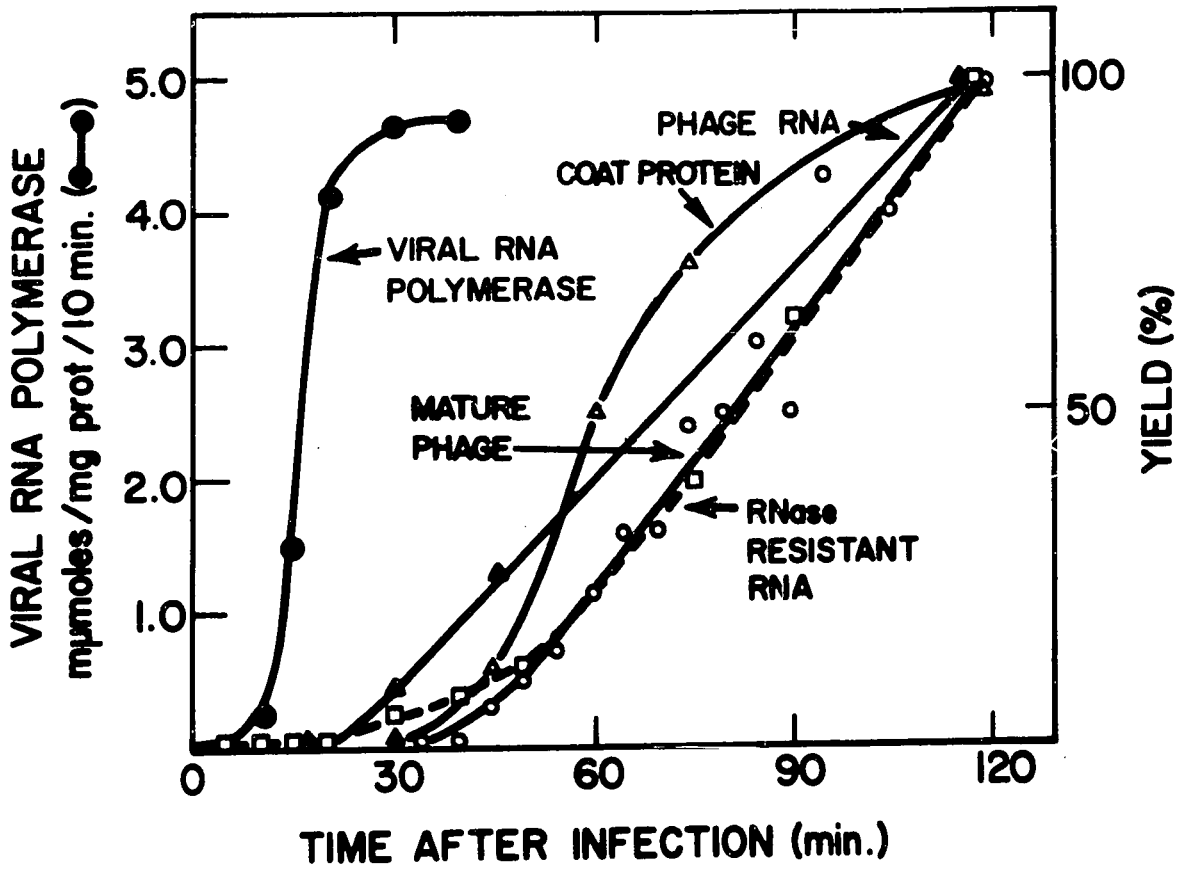

Fig. 1. Sequence of Intracellular Events Following R23 Infection. The yield of phage RNA, RNase-resistant RNA, intracellular or mature phage and phage coat protein at 120 min is represented as 100%. Viral RNA polymerase (●), mature phage particles (o), RNase-resistant RNA (□), coat protein (Δ), phage RNA (▲).

RNA encapsulated in progeny particles) is synthesized in detectable amounts by 15 min. Thereafter, phage RNA accounts for 60-70% of the total RNA synthesized while RNase-resistant RNA accounts for the remaining 30-40%. Both phage RNA and RNase-resistant RNA are synthesized throughout the infectious cycle. Synthesis of coat protein begins at 30-45 min and reaches a maximum between 45 and 75 min, when coat protein constitutes almost all of the newly synthesized protein. The first intracellular phage can be detected shortly after 30 min. The late accumulation of RNase-resistant RNA parallels the appearance of mature phage particles. Early in infection, an interval of about 5 min separates synthesis of coat protein and appearance of phage particles. Later, when phage production is maximal, this interval is 15-25 min (Watanabe *et al.*, 1968).

The third viral protein, the maturation protein, is not so easily detectable. In actinomycin-treated *E. coli* spheroplasts infected with MS2, it appears just before the coat protein (Vinuela, Algranati & Ochoa, 1967; Vinuela *et al.*, 1968).

C. Replication

1. RF and RI. The mechanism of RNA phage replication is not completely established. RNase-resistant material has been isolated from RNA phage-infected cells (Weissmann *et al.*, 1964; Fenwick *et al.*, 1964; Kaerner & Hoffmann-Berling, 1964; Nonoyama & Ikeda, 1964; Amman, Delius & Hofschneider, 1964) and implicated as an intermediate (Fig. 2). Two types can be recognized: a replicative form (RF) and a replicative intermediate (RI) (Amman *et al.*, 1964;

Erikson, Erikson & Gordon, 1966; Francke & Hofschneider, 1966; Granboulan & Franklin, 1966). Both contain an intact viral or "plus" strand which is infectious after release by denaturation (Amman *et al.*, 1964 Erikson *et al.*, 1966) and a full-length complementary or "minus" strand (Francke & Hofschneider, 1966; Weissmann & Ochoa, 1967). The RF, which sediments around 15S, is completely double-stranded. RI consists of double-stranded RNA with nascent partially completed single-stranded "plus" strands which are digestible with RNase (Erikson *et al.*, 1966; Granboulan & Franklin, 1966). But the double-strandedness found on deproteinization may not correctly reflect the structure of *in vivo* complexes since deproteinization may favour renaturation (Hofschneider & Hausen, 1968).

2. Mechanism of Replication. Replication of phage RNA may proceed via a conservative or semiconservative mechanism (Fig. 2); the latter is currently favoured (Fenwick *et al.*, 1964; Lodish & Zinder, 1966c); Weissmann & Ochoa, 1967; Kelly & Sinsheimer, 1967). Common to both theories is formation of a double-stranded duplex by synthesis of a negative strand using the parental strand as template. The second step, synthesis of viral plus strands using the complementary strand as template, is asymmetric, far less minus strands being formed than plus strands (Weissmann *et al.*, 1964). The viral strand is replaced by the newly synthesized strand (semiconservative) or the nascent strand is hydrogen-bonded to the complementary strand only for a short sequence of nucleotides at the replication point, while the original strand stays in the RI (conservative). If the conservative model is operative, the parental strand should remain quantitatively in the

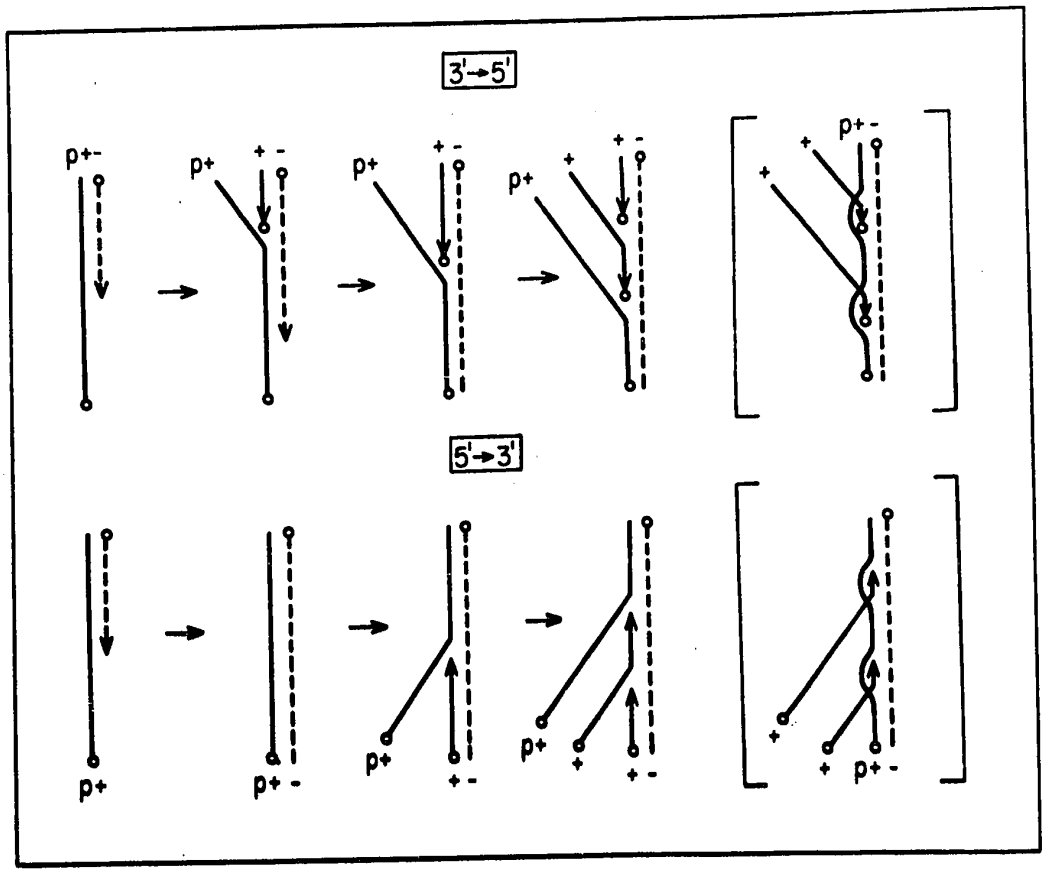

Fig. 2. Model of Replication of RNA Phage RNA. In the top part the synthesis of new plus strands is in the direction 3'→5' (a growing 5'-triphosphoryl end) and in the bottom part the synthesis is in the direction 5'→3' (a growing 3'OH end). The experimental results favour the latter mechanism. At the right, between brackets, the same polarity of synthesis is shown, but with the assumption of a conservative instead of a semi-conservative mechanism. The parental strand (p) may be either the infecting molecule, or a progeny plus strand which gives rise to a replicating complex. o indicates the position of the 5'-triphosphoryl terminus (the other end is the 3'OH). The arrow indicates the growing tip, attached to the RNA polymerase.

From Vandenberghe, Van Styvendaele & Fiers (1969).

RNase-resistant form. If the semiconservative mechanism operates, the parental plus strands should be incorporated in RF molecules then transferred via RI molecules into single-stranded RNA and appear among the products.

Parental RNA is converted into a double-stranded form and associated with ribosomes (Erikson *et al.*, 1964; Kelly & Sinsheimer, 1964) but none is incorporated into progeny particles (Doi & Spiegelman, 1962b; Davis & Sinsheimer, 1963; Kelly & Sinsheimer, 1964). In R23-infected cells, ^{14}C-uracil given as a 30 sec pulse 20 min after infection remains in RNase-resistant RNA and does not diminish or enter phage particles (Watanabe & August, 1967a). In unsynchronized infection with MS2 and f2, parental strands incorporated into double-stranded forms are only partially displaced from these forms (Weissmann *et al.*, 1964; Lodish & Zinder, 1966a; Billeter *et al.*, 1966). In synchronized infection, a periodic change in RNase-resistance of parental strands is found (Kelly & Sinsheimer, 1964). These observations may be explained by repeated incorporation (recycling) of parental plus strands into RF molecules. More recent studies of RI in MS2-infected cells labelled with short pulses of radioactivity (Kelly & Sinsheimer, 1967) show equal amounts of conservative and semiconservative types and a ratio of 1:5 throughout the replication cycle of M12 and Qβ (Francke & Hofschneider, 1969). However, the observations made by analyzing isolated RI may not correlate with the processes in the infected cells.

3. Enzyme Studies. It is not clear whether there are 2 enzymes (I and II) catalyzing synthesis of minus and plus strands, respectively,

or only one with 2 independent functions. Delius & Hofschneider (1964) suggested that 2 enzymes were involved. A temperature-sensitive mutant of f2 appears to be defective in enzyme I but not enzyme II (Lodish & Zinder, 1966a). Three enzyme preparations (labelled RNA synthetase, replicase and polymerase) have been purified to varying extents in separate laboratories and are conveniently discussed individually because of the resultant differences in their properties. It is possible that RNA replicase contains enzymes for both steps (Weissmann & Ochoa, 1967), and that RNA polymerase and RNA synthetase preparations catalyze steps 1 and 2, respectively. However, recent studies with Qβ polymerase (August *et al.*, 1968) demonstrate ability to catalyze both steps. (The label "viral RNA synthetase" will be used elsewhere in this thesis to refer non-specifically to the viral-induced enzyme).

1) The RNA synthetase, partially purified from MS2-infected cells (Weissmann, Simon & Ochoa, 1963), is associated with an endogenous template, viral "minus" strands. The product is 90% "plus" and 8% "minus" strand, but after deproteinization over 50% is RNase-resistant (Borst & Weissmann, 1965).

2) RNA replicase, purified from cells infected with MS2 and Qβ (Haruna *et al.*, 1963; Haruna & Spiegelman, 1965), specifically requires the RNA of the phage which induces the enzyme. When degraded Qβ RNA is used to prime the reaction, synthesis is slower and limited and the product largely double-stranded (Haruna & Spiegelman, 1965). With intact RNA as primer, minus strands are synthesized during the first few min followed by greater synthesis of plus strands (Weissmann &

Feix, 1966). Primer RNA enters RF (maximum at 2 min), then RI (maximum at 3 min); 28S RNA is synthesized soon thereafter (Pace, Bishop & Spiegelman, 1967, 1968).

The product is infective for spheroplasts and stimulates its own synthesis autocatalytically under non-saturated conditions (Spiegelman *et al.*, 1965). By serial transfer of diluted product for use as template and reduction of incubation periods in successive reactions, it is possible to propagate a self-duplicating RNA ("little variant") with a shortened genome and increased multiplication rate (Mills, Peterson & Spiegelman, 1967; Levisohn & Spiegelman, 1968).

Qβ replicase can be dissociated into a light and heavy component; the latter is present in uninfected cells and has poly C-dependent poly G polymerase activity (Eikhom & Spiegelman, 1967; Eikhom, Stockley & Spiegelman, 1968).

3) The RNA polymerase has been purified from *E. coli* infected with sus 11, a non-polar coat protein mutant of f2 (August *et al.*, 1963), and with Qβ (Eoyang & August, 1968). The f2 polymerase and the polymerase induced by Mu 9 (a coat protein mutant of MS2) synthesize predominantly minus strands (Weissmann & Ochoa, 1967). Qβ polymerase has a molecular weight of about 150,000; it binds 1 molecule of RNA per enzyme molecule and has a preferential affinity for the 3'OH region of Qβ RNA (August, 1969). In addition to Qβ RNA template, ribonucleoside triphosphates and Mg^{++}, Qβ polymerase requires 2 factors present in uninfected and infected cells (Franze de Fernandez, Eoyang & August, 1968). In a reaction utilizing poly C, GTP alone serves as substrate (Hori *et al.*, 1967). In more recent studies

(August *et al.*, 1968), the complementary strand has been used as template for Qβ polymerase to synthesize infectious RNA.

4. Direction of RNA Chain Growth. Synthesis of plus strands is 5' to 3' when free negative strands of Qβ "little variant" RNA are used as template for Qβ RNA polymerase (Mills, Bishop & Spiegelman, 1968). Using normal Qβ RNA as template, the direction of growth of Qβ RNA and the complementary strand is 5' to 3' (Banerjee, Kuo & August, 1969). Synthesis of nascent plus strand cannot be started until minus strands have been completed if synthesis of both minus and plus strands is 5' to 3' (Fig. 2). Synthesis of the complementary strand may be initiated at the cytidine next to the 3' terminal adenylate (Banerjee *et al.*, 1969).

D. Protein Synthesis *In Vitro* and *In Vivo*

Phage RNA, but not complementary RNA (Schwartz, Iglewski & Franklin, 1969), stimulates *in vitro* synthesis of viral-specific polypeptides, mainly coat protein (Nathans *et al.*, 1962; Ohtaka & Spiegelman, 1963; Nathans, 1965; Capecchi, 1966). Yamazaki and Kaesberg (1966) reported that the major product of *in vitro* synthesis directed by R17 RNA is a basic TCA-soluble polypeptide resistant to trypsin but its identity and significance have not been established. Moreover, a similar peptide was found by Lin in both the presence and absence of phage RNA (Weissmann & Ochoa, 1967). When MS2 RNA is used as messenger in the cell-free system, most of the product is less acidic than the viral coat protein and contains much less of the C-terminal peptide (Lin & Fraenkel-Conrat, 1967).

The R17 coat protein synthesized *in vitro* possesses an N-terminal

formylmethionine (Adams & Capecchi, 1966; Webster, Engelhardt & Zinder, 1966). F Met Ala can also be recovered from MS2 synthetase synthesized *in vitro* (Vinuela, Salas & Ochoa, 1967). F Met Ala Ser, F Met Ser and F Met Lys or F Met Arg appear to be the amino termini of the f2 coat protein, enzyme and maturation protein, respectively, synthesized *in vitro* (Lodish, 1968b).

Almost immediately after infection, MS2 parental RNA appears in association with 30S ribosomes and polysomes. After 4-5 min, 30% is associated with polysomes and 38% with 30S ribosomes; 15% is free and 16%, residual whole phage. From 4-10 min, RNase-resistant 12S RNA is present in the 50S region and in polysomes (Godson & Sinsheimer, 1967). The site of ribosomal binding of f2 RNA is not the 3' terminal (Takanami, Yan & Jukes, 1965).

E. Genetics

1. Amber Mutants. Amber mutant studies have been reported for f2 (Zinder & Cooper, 1964), R17 (Gussin, 1966; Tooze & Weber, 1967), MS2 (Fiers, 1967; Vinuela *et al.*, 1968; Nathans *et al.*, 1969) and fr (Heisenberg, 1966, 1967). Temperature-sensitive mutants of f2 (Horiuchi, Lodish & Zinder, 1966), f_{can} (Davern, 1964b), β (Tsuchida, *et al.*, 1966) and MS2 (Pfeiffer, Davis & Sinsheimer, 1964), cold-sensitive mutants of f2 (Silverman & Valentine, 1969) and azure mutants of f2 (Horiuchi & Zinder, 1967) have also been isolated.

The amber triplet (UAG) causes premature termination of a growing polypeptide chain at the site of the nonsense codon in Su^- strains, but in Su^+ bacteria harboring the suppressor gene, an altered tRNA (Goodman *et al.*, 1968) is able to recognize the triplet

and insert an amino acid. The suppressor strains (Zinder & Cooper, 1964; Garen, Garen & Wilhelm, 1965; Galucci & Garen, 1966; Horiuchi & Zinder, 1967) vary in the degree of efficiency of suppression and in the amino acid inserted (Table 4). Three cistrons have been identified by analysis of amber mutants of f2 and R17. Their properties are listed in Table 5.

2. Maturation Protein Mutants. Maturation protein mutants produce viral enzyme, coat protein and infectious RNA in normal amounts (Heisenberg, 1966; Argetsinger & Gussin, 1966), but properly assembled phage particles able to adsorb to bacteria or cellulose nitrate filters are not produced (Lodish *et al.*, 1965). The RNA in defective particles appears to be more exposed; it is precipitable with anti-RNA antibodies and subject to degradation by RNase after release from the cell (Heisenberg, 1967). Thus, defective particles lack from 50% to almost all of the normal amount of phage RNA and form a heterogeneous band in cesium chloride density gradients (Lodish *et al.*, 1965) unless they are grown in a Su^- host lacking RNase (Heisenberg, 1966; Argetsinger & Gussin, 1966).

3. Enzyme Mutants. Enzyme mutants lack the enzyme necessary to convert SS to DS RNA (Horiuchi *et al.*, 1966; Gussin, 1966; Lodish & Zinder, 1966a). None have been reported which lack enzyme II.

4. Coat Protein Mutants. Lodish and Zinder (1966b) showed that a polar effect is exhibited by coat protein mutants of f2, such as sus 3, if the mutation is close to the 5' end of the cistron, i.e., viral enzyme is not made in Su^- bacteria as usual during early infection. Late in infection, enzyme is made in excess as polarity

TABLE 4

BACTERIAL STRAINS

Strain (Zinder)	Strain (Garen)	Suppressor Type	Amino Acid Inserted	Efficiency of Suppression %
K38	S26	Su^-		
K37	S26RIe	Su-I	Serine	30
K146	S26RId	Su-II	Glutamine	14
K110	H12R8a	Su-III	Tyrosine	55
K133	u11RIE	Su-IV	Tyrosine	1-5
K134	u11RID	Su-V	Lysine?	1-5

TABLE 5

PROPERTIES OF AMBER MUTANTS OF RNA PHAGE

	MP	NP CP	P CP	ENZ	WT
Plating Efficiency (and Burst Size)					
Su-1	+ +	+ +	+ +	+ +	+ +
Su-11	+ +	±	±	+ +	+ +
Su-111	+ +	-	-	+ +	+ +
Su-IV	±	-	-	-	+ +
Su-V	-	-	-	-	+ +
Su⁻	-	-	-	-	+ +
Lysis					
Su-1	+	+	+	+	+
Su-11	+	+	+	+	+
Su-111	+	-	-	+	+
Su⁻	+	-[a]	-[b]	-[b]	+
Viral RNA Synthetase (and DS Viral RNA)					
Su-1	+ +	+ + + +	+ + + +	+	+ + +
Su-11	+ +	+ + + +	+ + + +	+	+ + +
Su-111	+ +	+ + + +	+ + + +	+	+ + +
Su⁻	+ +	+ + + +	+ + + +[c]	-	+ + +
^{14}C-Uracil Incorporation in UV-Irradiated Bacteria					
Su-1	+ +	+ +	+ +	+	+ +
Su-11	+ +	+ +	+ +	+	+ +
Su-111	+ +	+ +	+ +	+	+ +
Su⁻	+ +	+ +	+	-	+ +
Complementation					
MP	-	+	+	+	
NP CP	+	-	-	+	
P CP	+	-	-	- (+)[c]	
ENZ	+	+	- (+)[c]	-	

MP Mutants	Su⁻	Su⁺
CsCl density profile	skew	normal
Sucrose gradient: % 27S RNA	50-80	100
Infectivity of RNA	-	+

[a] growth slowed; [b] no effect; [c] late

MP, maturation protein; NP CP, nonpolar coat protein; P CP, polar coat protein; ENZ, enzyme; WT, wild type.

is lost. In Su^+ bacteria, enzyme is also hyperproduced in the absence of normal amounts of coat protein, the amount correlating inversely with the amount of coat protein made due to suppression. In nonpolar coat protein mutants excess enzyme is also made in the absence of coat protein. In the absence of coat protein, early cessation of enzyme synthesis does not occur at 20-30 min as usual (Fig. 1); excess enzyme I is synthesized under the direction of progeny RNA rather than parental RNA and converts progeny RNA into 7S DS RNA. Total RNA is apparently not increased. Possibly hyperproduction of enzyme results in imbalance in distribution of SS and DS RNA in the cell, thus restricting total RNA synthesis.

Viral proteins synthesized in actinomycin-treated *E. coli* spheroplasts infected with MS2 mutants have been analyzed by polyacrylamide gel electrophoresis (Vinuela, Algranati & Ochoa, 1967; Vinuela *et al.*, 1968; Sugiyama & Nakada, 1968; Nathans *et al.*, 1969). With a nonpolar coat protein mutant, there is a marked increase in production of enzyme, a lesser increase in maturation protein and a lag in synthesis of both. The maximum rates of synthesis of these 2 polypeptides are not greater than in wild type infection, but synthesis persists for a longer time.

The polarity is also demonstrated *in vitro*. When f2 sus 3 RNA is used as template, histidine incorporation (non-coat protein synthesis) is 15% of wild type levels. A hexapeptide, with sequence identical to the N-terminal sequence of f2 coat protein, is released free of tRNA and only monosomes are formed (Engelhardt, Webster & Zinder, 1967; Webster *et al.*, 1967).

5. Order of Cistrons. The polar properties of coat protein mutants support the location of the coat protein gene at the 5' end of the phage RNA, since polar mutations in other systems cause reduction of expression of genes distal to them in relation to the 5' or operator end of the polycistronic message or operon. The coat protein is the first and major product formed *in vitro* (Ohtaka & Spiegelman, 1963). According to the modulation theory (Ames & Hartman, 1963), the amount of product formed by the first gene is equal to or greater than that of the next genes.

Phage RNA missing the 5' end directs synthesis of non-coat proteins *in vitro*, suggesting that the coat gene is closest to the 5' end (Spahr & Gesteland, 1968; Lodish, 1968a). Fragments from 5-fluorouracil-treated MS2 consisting of 2/3 of the RNA (including the 5' end) direct synthesis of coat protein but not enzyme (Shimura, Kaizer & Nathans, 1968). On the other hand, a 2/3 fragment of Qβ RNA containing the 3' terminal directs the synthesis of coat protein (Bassel, 1968).

F. Regulation of Viral Gene Expression

There appears to be both temporal and quantitative control of phage protein synthesis. Coat protein is synthesized first *in vitro* (Ohtaka & Spiegelman, 1963), but later than non-coat proteins *in vivo* (Oeschger & Nathans, 1966; Watanabe & August, 1967a; Vinuela, Algranati & Ochoa, 1967; Watanabe *et al.*, 1968). To explain these facts various theories have been proposed.

1. Parental and Progeny RNA Strands. It is possible that parental (f2) RNA directs the synthesis of viral enzyme while progeny RNA

codes for coat protein (Lodish *et al.*, 1964; Lodish & Zinder, 1966b). But progeny RNA synthesis is required for normal yield of MS2 enzyme and coat protein (Oeschger & Nathans, 1966). In the case of R23, phage RNA synthesis is required for synthesis of coat protein, whereas viral RNA synthetase induction requires little or no RNA synthesis. In cultures inhibited by phleomycin (Watanabe & August, 1968a) so that phage RNA is reduced to less than 1%, enzyme activity is equal to that in untreated cultures (Watanabe *et al.*, 1968a).

2. Polarity. Two models have been proposed to explain the polarity of coat protein mutants (Gussin, 1966; Engelhardt *et al.*, 1967; Lodish, 1968a): 1) If each cistron possesses a site for attachment of ribosomes at the beginning of the cistron, polarity is a function of the secondary structure of the phage RNA, which makes sites other than that at the 5' end unavailable for ribosome binding. Synthesis of phage coat protein may induce relaxation of the ordered configuration, allowing translation of succeeding cistrons. 2) If ribosomes attach only at the beginning of the polycistronic message, polarity is due to the increasing probability of detachment of ribosomes in the untranslatable region beyond the amber block.

Loss of the coat gene abolishes the polar effect of sus 3 and eliminates the usual lag in synthesis of non-coat proteins. It appears that translation of other genes occurs after and is contingent on translation of at least the codon for site 6 of the coat protein gene. But the 3 genes of f2 can be translated independently *in vitro* (Lodish, 1968b). Spahr & Gesteland (1968) have also obtained *in vitro* evidence that the ribosome is not obligated to start at the original

5' end of the messenger (of R17).

3. Phage Coat Protein as Repressor of Translation. Since early cessation of enzyme synthesis does not occur when sufficient coat protein is not produced, coat protein may act as a repressor of synthesis of enzyme (Lodish & Zinder, 1966b) or non-coat proteins. Thus coat protein is associated with viral RNA (Capecchi, 1966) and specifically forms a complex with it *in vitro* (Sugiyama, Hebert & Hartman, 1967; Sugiyama & Nakada, 1968; Ward, Shive & Valentine, 1967; Ward, Strand & Valentine, 1968; Richelson & Nathans, 1968; Eggen & Nathans, 1969) which appears to repress synthesis of non-coat proteins when this complex is used to direct protein synthesis *in vitro*. Coat protein molecules may attach to the middle of the RNA and impede translation beyond the site of attachment according to this postulate.

4. Phage Coat Protein as Modulator. Coat protein may instead only act as a modulator by packaging the free RNA and preventing its translation; most of the protein synthesis late in infection may occur on partially DS messengers. When DS f2 RNA is used as messenger the only *in vitro* product is coat protein; the messenger activity is attributed to the SS regions (Engelhardt, Robertson & Zinder, 1968).

5. Phage Coat Protein as Repressor of Transcription. Control may be at the level of transcription rather than translation. Coat protein of f2 binds stably and preferentially to DS RNA rather than to SS RNA. Coat protein monomers may bind to RI in such a way that it creates a differential rate of RNA synthesis between the end of the nascent single strand containing the coat gene and the remaining

portion of this strand containing the other genes (Robertson, Webster & Zinder, 1968).

A model incorporating most of the above ideas has been depicted by Hotham-Iglewski, Phillips & Franklin (1968).

G. Mixed Infection

In simultaneous infection of RNA and DNA phage or superinfection of RNA phage-infected *E. coli* with DNA phage, RNA phage yield is inhibited (Zinder, 1963; Neubauer & Zavada, 1965; Huppert, Blum-Emerique & Breugnon, 1967; Hattman & Hofschneider, 1967, 1968). In simultaneous infection by M12 and T4, parental RNA and polysomes remain intact but no RI or viral synthetase activity is detected. In delayed T4 superinfection, synthesis of coat protein antigen is blocked. T4 may interfere with the messenger function of M12 RNA (Hattman & Hofschneider, 1968). Lambda does not exclude M12 and f2 (Hofschneider, 1963b; Groman & Suzuki, 1965; Zinder, 1963).

H. Host-Phage Relationship

1. Requirement for Host. There is no detectable homology between viral RNA and DNA from uninfected and infected cells (Doi & Spiegelman, 1962a). This implies that DNA is not an intermediate in the growth of RNA phage. Also DNA synthesis is apparently not necessary for RNA phage development. Addition of fluorodeoxyuridine and mitomycin or starvation for thymine in a thymine-less strain inhibit DNA synthesis but allow RNA phage growth (Cooper & Zinder, 1962; Hofschneider, 1963b). Mitomycin C, streptonigrin, rubidomycin and nalidixic acid, all inhibitors of DNA synthesis, are also potent inhibitors of DNA phage but not RNA phage multiplication (Watanabe & August, 1967b). However,

the yield of infectious centres and RNA phage is reduced when EDTA-treated cells are treated with actinomycin before 16 min after infection with MS2 (Haywood & Harris, 1966) or when rifampicin, an inhibitor of DNA-dependent RNA polymerase, is added before 5 min after f2 infection (Fromageot & Zinder, 1968). Full activity of the viral RNA replicating enzymes requires components (Eikhom & Spiegelman, 1967; Eikhom *et al.*, 1968) and factors (Franze de Fernandez *et al.*, 1968) present in uninfected cells.

2. Inhibition of Host. RNA phages appear to have a variable effect on the host. There is no inhibition of cell DNA and RNA synthesis in f2-infected cells (Zinder, 1965). But there is decreased synthesis of rRNA, tRNA, ribosomes and DNA 15 min after infection with ZIK/1 (Bishop, 1965, 1966) and inhibition of RNA, DNA and protein synthesis in R17-infected cells at 15, 45 and 25 min post-infection, respectively, according to radioautographic studies (Granboulan & Franklin, 1966). In R17-infected cells, rRNA is inhibited by 70-80% during the first 15 min of infection, but there is no decrease in tRNA synthesis (Ellis & Paranchych, 1963; Hudson & Paranchych, 1967, 1968). Induction of β-galactosidase is not appreciably inhibited by f2, MS2 or Qβ (Zinder, 1963; Yuki & Ikeda, 1966) but is significantly inhibited by β after 25 min of infection (Nonoyama *et al.*, 1963).

tRNA from Qβ-infected cells shows decreased ability to support translation of poly C but not poly U. Prolyl tRNA from Qβ-infected cells has 2-3 times less ability to bind to ribosomes (Hung & Overby, 1968). A decrease in polysomes greater than hexamers is evident 10 min after R17 infection. By 25 min, the polysome distribution peaks

around trimers; later, there is a progressive increase in monomers (Hotham-Iglewski & Franklin, 1967). In MS2-infected cells, the polysome pattern is identical to that in uninfected cells at 25 min, but at 40 min there is a loss of larger polysomes and an increase in 70S monosomes (Godson, 1968).

Ten to 30% of the RNA synthesized after infection with f2, R17, MS2 and Qβ (Zinder, 1963; Ellis & Paranchych, 1963; Weissmann *et al.*, 1964; Zinder, 1965) is found in phage particles. In contrast, 60% of the RNA synthesized after infection with R23 is encapsulated in progeny phage particles; 30-40% is DS phage RNA. Thus, R23 completely inhibits host RNA synthesis. Synthesis of bacterial proteins is also markedly inhibited by R23 and largely replaced by phage-directed protein synthesis. Phage RNA synthesis is predominant 15-30 min after infection, followed by inhibition of bacterial protein synthesis about 30 min after infection (Watanabe *et al.*, 1968).

I. Purpose of Thesis Investigation

The inhibitory effect of RNA phage on host function has been relatively unexplored in the case of other RNA phages. R23 is distinguished by its striking ability to inhibit host macromolecular synthesis, and appears to possess this advantageous property to a degree unequalled by most RNA phages.

The aim of the experiments recorded here has been to characterize the effects of R23 infection on its host, *E. coli*, and to elucidate the mechanisms which confer upon R23 this unique characteristic. Included are analyses of 1) the life cycle of R23 and a comparison with those of R34, R40, f2 and Qβ, 2) amber mutants of R23, newly

isolated and characterized, 3) the effect of R23 on the lactose operon and host synthesis of the enzyme, β-galactosidase, and 4) the effect of R23 on the cell membrane. Part of this work has been published (Watanabe & Watanabe, 1968a) and presented (Watanabe & Watanabe, 1968b).

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Chemicals and Materials. Ribo- and deoxyribo-nucleotides were purchased from Mann Research Laboratories, Inc., New York, N.Y.; cyclic 3',5'-AMP, IPTG and ONPG from Calbiochem, Los Angeles, Calif.; polyethylene glycol (Carbowax 6000) from Union Carbide Corp., New York, N.Y.; sodium dextran sulfate 500 from Pharmacia Fine Chemicals, Inc., New Market, N.J.; pancreatic DNase 1 (electrophoretically purified free of RNase) and pancreatic RNase A from Worthington Biochemical Corp., Freehold, N.J.; pyruvate kinase from Mann Research Laboratories, Inc., New York, N.Y.; phospho(enol)pyruvic acid from Sigma Chemical Co., St. Louis, Mo.; chloramphenicol from Parke, Davis & Co., Detroit, Mich.; fluorouracil (Roche) from Hoffmann-LaRoche Ltd., Montreal, P.Q.; and membrane filters (types HA and DA) from Millipore Corp., Bedford, Mass. Rifampicin Ba 41166E was kindly supplied by Dr. J. Gelzer of Ciba Pharmaceutical Co., Summit, N.J.

2. Isotopes. ^{14}C-Uracil (40-60 mc/mmole), ^{3}H-uracil (3-19 c/mmole), ^{3}H-thymidine (51 mc/mmole), ^{3}H-histidine (140-200 mc/mmole) and ^{14}C-labelled reconstituted protein hydrolysate were purchased from Schwarz BioResearch, Orangeburg, N.Y.; ^{14}C-methionine (12.5 mc/mmole) and ^{14}C-histidine (222 mc/mmole) from New England Nuclear Corp., Boston, Mass.; ^{14}C-IPTG (25 μc/μmole) from Calbiochem, Los Angeles, Calif.; and ^{42}KCl (1-20 μc/mg) from Charles E. Frosst & Co., Montreal, P.Q., and $^{32}P_i$ from International Chemical and Nuclear Corp., City of Industry, California.

B. Bacteria and Bacteriophages

The *E. coli* strains (originally obtained from N. Zinder) and their properties with respect to suppression are listed in Table 4 (Chapter I). *E. coli* B/1,5, the host for T2, and T2 phage were originally obtained from E. Lennox. The RNA phages R23, R34 and R40 were isolated by Dr. Mamoru Watanabe from sewage of Atlantic Beach, N.Y. The RNA phages f2 and Qβ were originally obtained from N. Zinder and S. Spiegelman, respectively.

C. Media

Tryptone medium, prepared as described by Loeb and Zinder (1961), contained, per litre (l): 10 g Difco Bacto-Tryptone, 1 g Difco yeast extract, 1 g glucose and 8 g NaCl. $CaCl_2$ was added to a final concentration of 1 mM prior to infection.

Medium A, prepared as described by Davern (1964) contained, per 1: 7 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.6 g $MgSO_4$, 0.11 g $CaCl_2$, 6 g Difco casamino acids and 10 ml of 30% glycerol.

EPG medium contained per 1: 7 g of Na_2HPO_4, 3 g KH_2PO_4, 10 mmole EDTA, and 10 ml of 30% glycerol.

GC medium contained, per 1: 1.21 g Tris, 5.0 g NaCl, 1.0 g NH_4Cl, 0.052 g $Na_2HPO_4 \cdot 7H_2O$, 0.1 g $MgSO_4$, 10 g casamino acids, 10 ml glycerol and 2.5 ml 1.0 N HCl. 1.0 ml of 1M $CaCl_2$ was added per 1 prior to infection.

The medium used for plating RNA phage contained, per 1: 10 g Difco Bacto-tryptone, 8 g NaCl, and 1 g glucose. The base layer was supplemented with 10 g of Difco agar and the top layer with 6 g of agar. After the agar had melted, 1.5 ml of

1 N NaOH and 2 ml of 1 M $CaCl_2$ were added per 1.

Lysing medium contained 0.01% lysozyme, 0.01 M EDTA and 1 M Tris, pH 8.0.

Diluent for titering RNA phage contained 0.01 M Tris, pH 7.5, 1 mM $MgCl_2$, 0.1 M NaCl and 0.001% gelatin.

D. Growth of Bacteria and Phage

1. Bacterial Growth. Bacteria were grown in medium A or Tryptone medium (unless otherwise stated) in a gyrotory shaker at 37° C. At a bacterial concentration of about 2 X 10^8 cells/ml, the culture was supplemented with $CaCl_2$, 1-2 mM, and phage added at a multiplicity of infection indicated in the text.

2. Preparation of Lysates. An early log-phase culture of bacteria (usually K37) was infected with a single plaque of phage plated on appropriate bacteria. Infected cultures were incubated at 37° C with rigorous aeration for 2-4 hours. Lysing solution, 0.1 vol., and chloroform (1%, v/v) were then added and the culture shaken for 30 min. The lysate was stored over chloroform.

3. Purification of Phage. Labelled and unlabelled RNA phage were purified by a slight modification of the liquid polymer phase technique of Albertsson (1960) as previously described (Watanabe & August, 1967b). To each litre of phage lysate, 17.5 g of NaCl, 69 g of polyethylene glycol (Carbowax 6000) and 2 g of sodium dextran sulfate 500 were added. After the ingredients were dissolved, the phases were allowed to form at 4° C and the lower dextran phase containing the bacteriophage (concentrated 50 to 100 fold) was collected after 48 hours. KCl was then added to a concentration

of 1 M and the precipitated sodium dextran sulfate was removed by centrifugation at 17,000 rpm for 5 minutes. The supernatant from this centrifugation was either dialyzed against SSC (0.15 M NaCl, 0.015 M sodium citrate) or centrifuged at 40,000 rpm for 3 hours and the pellet of bacteriophage resuspended in a small volume of SSC. The phage was then banded by centrifugation in CsCl in an International angle head rotor A321 at 50,000 rpm for 48 hours. After centrifugation, the phage layer was removed and dialyzed against SSC for 24 hours.

E. Assay of Bacteria and Phage

1. Growth Measurements. Exponentially growing cells, at an A_{660} of 0.3, were infected with phage at a multiplicity of 10 after addition of 1 mM $CaCl_2$ and incubated at 37° C with shaking. At frequent intervals before and after infection the absorbance at 660 mμ was determined on a Unicam SP800 spectrophotometer and compared to that of an uninfected control.

2. Bacterial Survivors. *E. coli* K38 was grown in Tryptone broth or medium A to a density of 2 X 10^8 cells/ml and infected with RNA phage at a MOI of 10 or 20. At intervals, aliquots of the infected culture were diluted in prewarmed medium and bacterial survivors assayed by spreading appropriate dilutions on nutrient agar plates. Colonies were counted following incubation at 37° C for about 18 hours.

3. Phage Assay. Phage were assayed as plaque-forming units by the agar overlay technique as described by Adams (1959). All phage titres were determined after lysis of infected cells. Lysis was induced by the addition of 0.1 vol of lysing media and 0.01 vol of chloroform followed by incubation of the culture at 37° C for 30 min in a gyrotory shaker.

4. Adsorption and Eclipse of Infectivity Experiment. *E. coli* K38 grown in Tryptone broth was infected at a MOI of 20 phage/cell. At various intervals, 0.1 ml samples were removed and added to 9.9 ml of EPG medium. The cells were agitated for 1 min on a Vortex mixer and harvested by centrifugation at 10,000 rpm for 10 min. The cells were resuspended and washed 3 times with fresh medium. All supernatant solutions were diluted and assayed for infective particles. Phage which were not eluted as infectious particles by washing were designated as "eclipsed".

5. Infective Centres. *E. coli* K38, grown in Tryptone broth to a density of 2 X 10^8 cells/ml, was infected with RNA phage at various multiplicities. Five min after infection, the appropriate antiserum was added to inactivate unadsorbed phage. After 5 min of incubation at 37° C, the cultures were diluted in prewarmed Tryptone broth and plated for infective centres by the agar overlay technique.

6. One-step Growth Experiment. *E. coli* K38 grown in Tryptone broth was infected at a multiplicity of 10 phage/cell. After 5 min, unadsorbed phage were inactivated by addition of homologous phage antiserum. After a further 5 min incubation, the infected cells were diluted into growth tubes containing prewarmed Tryptone broth at 37° C to yield 100 infective centre/ml. At intervals, appropriate dilutions were made from the growth tubes and plated for extracellular phage. At the same time, samples from growth tubes were lysed by addition of lysing solution and chloroform and plated for intracellular phage yield.

7. Single Burst Analysis. *E. coli* K38 grown in Tryptone broth was infected with RNA phage at a MOI of 10. After allowing 5 min for

adsorption to occur, the culture was diluted 1000-fold and 1 ml aliquots were incubated at 37° C for 5 min in the presence of homologous phage antiserum to inactivate the unadsorbed phage. The culture was further diluted to a concentration of 0.6 infected cells/ml and 0.5 ml aliquots were rapidly pipetted into 100-200 tubes. These tubes were incubated at 37° C for 120 min and 0.1 ml portions were assayed for PFU.

F. Treatment of Bacteria and Phage.

1. UV-Irradiation. Bacteria, grown in medium A supplemented with 8 μg/ml of uridine, were harvested by centrifugation at 10,000 rpm for 10 min and resuspended in 1/5 vol of fresh medium A lacking casamino acids. The cell suspension was irradiated by a GE germicidal lamp at 13 cm in 5 ml aliquots in a petri plate for 75 sec. The irradiated bacterial suspension was then diluted 5-fold in medium A supplemented with uridine. Under these conditions the number of colony-forming units was suppressed by 3×10^4 and the ability to support phage infection by 10^2.

For UV-irradiation of phage, suspensions diluted in Tris buffer to a titre of 10^{12} phage/ml were exposed for 4 min to a GE germicidal lamp at a distance of 13.5 cm. Under these conditions the titre of survivors was 5×10^5 phage/ml.

2. EDTA Treatment. Bacteria were grown in medium A to a density of 2×10^8 cells/ml and harvested by centrifugation at 10,000 rpm for 10 min. The cells were washed once at room temperature with 0.12 M Tris (pH 8.0) containing 0.5% glycerol and 1 mM sodium phosphate, and resuspended in this buffer at a density of 2×10^9 cells/ml. The suspension was incubated at 37° C for 2 min with 1 mM EDTA and

diluted 10-fold into conditioned medium A. Conditioned medium A was prepared by filtering the supernatant, obtained after centrifugation of bacteria (according to the above procedure), through Millipore HA filters. This is a modification of the method of Leive (1965) as described by Perlman and Pastan (1968).

3. Phage Mutagenesis.

Nitrous Acid. R23 mutants were obtained by treating concentrated phage lysates in 0.25 M acetate buffer, pH 4.6, with 0.5 M nitrous acid at 37° C. Samples of treated phage were removed and diluted 1:100 or more in cold RNA diluent to stop the action of nitrous acid. The rate of killing was approximately 2.5 log/hour. Samples were diluted and plated on K37. Individual plaques were stabbed, diluted in RNA diluent, and spot-tested on K38 and K37 lawns. Lysates of mutants (which had grown on K37 but not on K38) were prepared from single plaques.

Fluorouracil. *E. coli* K37 was grown in medium A, infected with R23 at a multiplicity of 5-10, and incubated at 37° C with aeration for 6-7 hours in the presence of 0.1 mM fluorouracil. Cells were then lysed with lysing solution and chloroform and diluted phage plated on K37. Individual plaques were spot tested on K38 and K37 lawns, and those growing only on K37 picked for preparation of lysates.

G. Neutralization of RNA Phage by Phage Antiserum.

1. Preparation of Phage Antiserum. Antiserum was prepared as described by Adams (1959). Rabbits were immunized with purified RNA phage by administering an intravenous injection containing about 10^{11} PFU in 1 ml of sterile saline at 3-4 day intervals for a period of 1 month.

2. Phage Neutralization by Antiserum. The reaction mixture for phage neutralization contained (in 0.2 ml) 5 mM borate buffer, pH 8.5, RNA phage (1-2 X 10^7 PFU) and 20 µl of diluted R23 antiserum. After storage at 4° C for 48 hours, aliquots were diluted in cold diluent and assayed for PFU by the agar overlay technique (Adams, 1959).

3. Antibody-Precipitable Phage. The amount of phage RNA was determined by specific binding of ^3H- or ^{14}C-uracil-labelled phage to phage antibody. The reaction mixture contained (in 0.05 ml) 20 mM borate buffer, pH 8.5, 20 µl diluted antiserum and 5 or 10 µl of radioactively-labelled lysate of phage-infected *E. coli* cells or purified phage standard. Tubes lacking phage-specific antiserum or containing non-homologous antiserum served as controls. The reaction mixture was allowed to stand at 4° C for 48 hours, filtered through Millipore HA filters, and washed 3 times with 2 ml of cold 0.01 M borate buffer, pH 8.5. The filters were dried and the radioactivity determined in a liquid scintillation spectrometer.

H. Synthesis of RNA, DNA and Protein

Synthesis of RNA, DNA and protein was determined by the incorporation of radioactively-labelled uracil, thymidine and amino acids or reconstituted protein hydrolysate, respectively, into acid-insoluble material. Thymidine incorporation was determined in the presence of 2 mM deoxyadenosine. Bacteria were grown to a concentration of about 2 X 10^8 cells/ml and labelled precursor and 1-2 mM $CaCl_2$ added. The culture was then divided and portions immediately infected. At various intervals thereafter, 0.1 ml aliquots were removed and added to 2 ml of cold 5% TCA. The samples were chilled at 4° C for 30 min for nucleic

acid assays or heated at 80° C for 30 min in the case of protein determinations. The precipitate was collected on Millipore HA filters and washed with 5% TCA. The filters were dried and the radioactivity determined in a liquid scintillation spectrometer.

I. Base Composition of RNA Bacteriophages

RNA phages were labelled with ^{32}P and purified by liquid polymer phase fractionation. RNA was prepared from the purified phages and hydrolyzed in 0.3 N KOH at 37° C for 24 hours. The hydrolysis products were separated by high voltage electrophoresis and ^{32}P radioactivity of each nucleotide fraction was determined.

J. Penetration of Phage RNA

Bacteria were grown in Tryptone broth and 100 ml samples infected with ^{3}H-uracil-labelled R23 at a multiplicity of 20 phage/cell. At 10 and 30 min, 50 ml samples were removed from each flask, EDTA was added to a concentration of 5 mM and the cells were immediately chilled. The cells were harvested by centrifugation at 10,000 rpm for 10 min and washed 3 times with fresh medium containing 5 mM EDTA. The radioactivity remaining in the cells was assayed in a liquid scintillation spectrometer.

K. Density Gradient Analysis

1. Cesium Chloride Density Gradient Centrifugation Analysis. Phage labelled with ^{3}H-uracil were prepared by infecting bacteria grown in medium A (supplemented with 8 µg/ml uridine) and adding ^{3}H-uracil, 2 µc/ml, and uridine, 50 µg/ml, at the time of infection. Similarly, phage labelled with ^{3}H-amino acids were prepared in bacteria grown in medium A with 1 mg/ml yeast extract substituted for casamino acid and

supplemented with 8 μc/ml of ^{3}H-labelled protein hydrolysate. Infected bacteria were lysed 2 hours after infection. The lysates were incubated at 37° C for 1 hour with 0.03 M $MgCl_2$, 10 μg/ml DNase and 10 μg/ml RNase A.

Aliquots of each lysate, usually 0.1-0.2 ml, were mixed with previously purified ^{14}C-labelled R23 and made up to a volume of 10 ml in SSC. CsCl (about 0.6 g/ml) was added to a final density of 1.4 and the mixture was spun in a B60 International ultracentrifuge (SB 283 rotor) at 35,000 rpm for 45-48 hours at 5° C. The tubes were pierced and 5-10 drop fractions collected. To each fraction 2 ml of 5% TCA was added and the precipitates collected on Millipore HA filters. The filters were rinsed 3 times with 5% TCA and dried, and the radioactivity assayed in a liquid scintillation spectrometer.

2. Sucrose Gradient Analysis of RNA

Labelling of Cultures. *E. coli* K38 was grown in medium A to a density of 2 X 10^8 cells/ml and $CaCl_2$ added to a concentration of 2 mM. One-half of the culture was infected with R23 at a MOI of 20 phage/cell. To the uninfected flask, ^{3}H-uracil was added to a final concentration of 2 μc/ml. Samples of R23-infected culture were removed at various times after infection and ^{14}C-uracil was added to a final concentration of 1 μc/ml. After a 5 min labelling period, all cultures were chilled immediately and sodium azide added to a final concentration of 10 mM. To each infected culture labelled with ^{14}C-uracil, an identical aliquot of the ^{3}H-uracil-labelled uninfected culture was added to serve as a marker and to allow determination of the losses incurred in the subsequent isolation

of RNA. The cells were then harvested by centrifugation at 11,000 rpm for 15 min and washed once with medium A containing sodium azide. The cells were resuspended in TSM Buffer (10 mM Tris, pH 7.0, 4 mM succinic acid and 5 mM magnesium acetate) and stored at -20° C.

Preparation of RNA. The defrosted bacterial cells were lysed by the addition of lysozyme, 300 µg/ml, DNase, 10 µg/ml, sodium lauryl sulfate, 1%, and 3 drops of chloroform, and incubated at 37° C for 30 min. The suspension was then extracted twice with an equal volume of 80% phenol (previously equilibrated with phosphate buffer, pH 6.8). Excess phenol was removed from the aqueous layer with ether. Bentonite (prepared as described by Fraenkel-Conrat, Singer & Tsugita, 1961) was added to remove RNase, and subsequently removed by centrifugation in an International angle head rotor A321 at 40,000 rpm for 60 min. RNA was precipitated from the supernatant by the addition of 2 vol of cold ethanol and sodium acetate to a concentration of 0.2 M. After 120 min at -20° C, the RNA precipitate was collected by centrifugation at 10,000 rpm for 15 min and the RNA pellet was resuspended in 1 ml of 10 mM acetate buffer, pH 5.0, and 0.1 M NaCl.

Sucrose Gradient Centrifugation. A small aliquot of the RNA was layered on top of a linear gradient of sucrose (5-30%) in 10 mM acetate buffer, pH 5.0, and centrifuged in an International swinging bucket rotor SB 283 at 37,000 rpm for 8 hrs at 4° C. Following centrifugation, the bottoms of the tubes were pierced and fractions collected and analyzed for ^{3}H and ^{14}C radioactivity in a liquid scintillation spectrometer.

L. Complementation

E. coli grown in Tryptone medium was infected at a concentration of 2 X 10^8 cells/ml with 2 mutants at a MOI of 2.5 each or with each mutant alone at a MOI of 5 (as a control). The cultures were shaken at 37° C for 10 min; R23 antiserum was then added to inactivate unadsorbed phage. After 10 min incubation, the cells were diluted and distributed as 0.5 ml portions into many tubes so that each tube contained about 5-10 cells. The tubes were shaken at 37° C and the cultures lysed at 2 ¼ hours after infection by the addition of lysing solution and chloroform. Samples from each tube were plated on K37 and K38. Those which plated equally on both strains were discarded as wild type revertants.

M. Enzyme Assays

1. Viral RNA Synthetase.

Preparation of Phage-Infected Bacteria. Bacteria were grown in GC or Tryptone medium to a concentration of 3-5 X 10^8 cells/ml, $CaCl_2$ was added to a concentration of 1 mM and bacteria in 500 ml of culture were infected at a MOI of 10 with R23 or an amber mutant. An uninfected culture served as control. After 30 min incubation at 37° C with rigorous aeration, each flask was quickly chilled in a dry-ice ethanol mixture and stored at 4° C. The bacteria were harvested by centrifugation at 11,000 rpm for 10 min and the cell pellets stored at -20° C.

Preparation of Cell-Free Extracts. Cold alumina (equal to twice the weight of the bacterial pellet) was added and the bacteria disrupted by grinding. The paste was suspended in 4 vol of a buffer solution containing 0.05 M Tris-HCl, pH 7.2, 0.005 M $MgCl_2$, 0.005 M

2-mercaptoethanol and 0.001 M EDTA in 20% (v/v) glycerol. The suspension was centrifuged at 17,500 rpm for 5 min and the supernatant (crude extract) decanted.

Enzyme Assays. The assay mixture contained 0.05 ml of 0.5 M Tris-HCl, pH 7.2, 0.025 ml of 0.1 M $MgCl_2$, 0.01 ml of 0.1 M 2-mercaptoethanol, 0.01 ml of 0.1 M phosphoenolpyruvate, 0.01 ml pyruvate kinase (100 µg/ml), 0.02 ml each of 0.01 M ATP, UTP and CTP, ^{14}C-GTP, (2-5 X 10^6 cpm/µmole, 0.01 M), 0.01 ml DNase (1 mg/ml), 0.005 or 0.01 ml enzyme and deionized water to a final volume of 0.25 ml. After incubation for 20 min at 37° C, the mixture was chilled in ice and 3 ml of cold 5% TCA containing 0.02 M sodium pyrophosphate was added to stop the reaction. Samples were collected on Millipore HA filters and washed 4 times with the cold TCA-pyrophosphate solution. The filter was dried and the radioactivity determined in a liquid scintillation spectrometer. A reaction mixture containing an extract of uninfected cells was used as a blank. A crude extract of R23 wild type-infected cells was assayed (as a control) simultaneously with extracts of mutant-infected cells.

Definition of Unit and Specific Activity. One unit of activity is defined as the incorporation of 1 mµmole of GMP per 20 min at 37° C. The specific activity of the enzyme is calculated as units per mg of protein. Protein determinations were performed by the Folin-Lowry method (Lowry *et al.*, 1951).

2. β-Galactosidase.

Growth of Bacteria. *E. coli* K38 grown overnight in medium A was diluted with fresh medium and incubated at 37° C until the culture reached

a density of 2 X 10^8 cells/ml. $CaCl_2$, 1 or 2 mM, was added and the culture divided into equal parts. Phage was then added at a MOI of 10 or 20 to one (or more) portions. An uninfected portion served as a control.

Induction and Inducer Removal. β-galactosidase was induced by addition of IPTG to a final concentration of 0.5 mM. The IPTG was added 3 min after infection (unless otherwise stated in the text) to infected and uninfected cultures.

Inducer was removed from the culture by filtration on a Millipore DA filter of pore size 0.65 μ. The cells were washed with an equal vol of phosphate buffer at room temperature and resuspended in fresh medium A by blowing liquid on the submerged membrane. The entire procedure took 2-3 min.

Enzyme Assays. For β-galactosidase assays, 0.5 or 1 ml aliquots of the culture were added to chilled tubes containing 50 μg of chloramphenicol. The tubes were shaken with a drop of toluene for 15 min at 37° C. Aliquots of 0.1 ml were then added to 4.9 ml of 0.03 M potassium phosphate buffer, pH 6.8, containing 2 mg of ONPG, and incubated at 37° C for 15 min or until the desired intensity of colour developed. The reaction was stopped by the addition of 0.5 ml of 4 M K_2CO_3 and the absorbance at 420 mμ determined with the Unicam SP800 spectrophotometer.

Definition of Unit. One unit is defined as the amount of enzyme which catalyzes the hydrolysis of 1 mμmole of ONPG/min at 37° C, pH 6.8.

N. Tracer Flux Experiments.

1. Uptake of ^{14}C-IPTG. *E. coli* was grown in medium A to a density of 2 X 10^8 cells/ml. $CaCl_2$ was added to 1 mM and the culture

divided into equal portions. Phage was then added to one or more portions at a MOI indicated in the text. Unless otherwise stated, IPTG was added to a concentration of 0.5 mM and ^{14}C-IPTG to 1 μc/ml. At intervals, 0.1 ml samples were removed, added to 2 ml of cold Tryptone broth and immediately filtered through Millipore HA filters. The filters were washed twice with cold Tryptone medium and dried and the radioactivity assayed in a liquid scintillation spectrometer.

2. Determination of $^{42}K^+$ Efflux. Efflux experiments were performed as described by Silver, Levine and Spielman (1968). *E. coli* K38 was grown in Tryptone medium at 37° C for several generations in the presence of ^{42}KCl to a density of 2 X 10^8 cells/ml. The ^{42}KCl concentration was usually 0.3 μc/ml and between 0.9 and 4 mM. The cells were centrifuged at 20° C at 10,000 rpm for 10 min, washed once with fresh Tryptone medium and resuspended at 2 X 10^8 cells/ml. $CaCl_2$ was added to a final concentration of 2 mM unless otherwise stated. Fifteen ml samples of bacteria at 37° C were infected (at time zero) at a MOI of 20 phage/cell or treated as indicated in the text. One ml samples were removed at time zero and at appropriate intervals thereafter and filtered through Millipore HA filters. The filters were dried and the radioactivity determined in a liquid scintillation spectrometer.

3. Influx of $^{42}K^+$. K38 was grown in Tryptone broth as described above to a density of 2 X 10^8 cells/ml, but in the absence of $^{42}K^+$. $^{42}K^+$ was then added to a concentration of 0.3 μc/ml. The cells were infected at a MOI of 20 phage/cell or treated as described in the text. At intervals, 1 ml samples were removed, filtered and washed

with 5 ml of Tryptone broth at room temperature. The filters were dried and the radioactivity assayed.

CHAPTER III

EXPERIMENTAL RESULTS

A. The RNA Bacteriophages R23, R34, and R40: Comparison with f2 and Qβ

Introduction

Previous experiments (Watanabe *et al.*, 1968) demonstrated: 1) a drastic reduction of host RNA and protein synthesis in *E. coli* infected with R23, 2) a lesser ability of RNA phages R34, R40, f2 and Qβ to inhibit host metabolism, compared to R23, and 3) a distinct timing of synthesis of R23-specific RNA and protein throughout the infectious cycle.

Thus, quantitation of RNA in *E. coli* infected with R23 indicated that 60% of the RNA synthesized after infection was recovered in phage particles. In contrast, the corresponding figure was 43% for R34 and 20-25% for f2, R40 and Qβ. Of the RNA synthesized after R23 infection, 30-40% was RNase-resistant. Less than 1% of the RNA eventually recovered in R23 particles was synthesized during the first 15 min of infection; this constituted about 5% of the total RNA synthesized during this period. Thereafter, between 15 and 120 min, approximately 60% of all RNA synthesized appeared in phage. In these experiments, ^{14}C-uracil was present throughout the infectious cycle.

When ^{14}C-uracil was given as a 30 second pulse 20 min after infection with f2 or Qβ, only 6% of the RNA synthesized was recovered in phage particles and a further 7% was present in RNase-resistant form. In the case of R23, however, even after a short pulse, 70% of the radioactivity was incorporated into phage RNA and 32% into RNase-resistant RNA.

Synthesis of R23 phage protein was quantitatively determined by

measuring incorporation of ^{14}C-amino acids into purified phage particles. Forty-two % of ^{14}C-labelled reconstituted protein hydrolysate, 26% of ^{14}C-methionine and 4% of ^{14}C-histidine was recovered in phage. The incorporation of histidine was considered to represent incorporation into phage maturation protein, and the incorporation of other amino acids considered to be largely into coat protein.

Coat protein (defined as protein present in phage particles) was not detected during the first 30 min. Of the total protein synthesized during 30-45 min, 38% was eventually used to package phage RNA, whereas all or nearly all of the protein synthesized between 45 and 75 min was phage coat protein. Only 10% of the eventual yield of coat protein had been synthesized by 45 min, 50% by 60 min and 72% by 75 min.

The activity of the viral RNA synthetase (the third phage protein), first detected in extracts of cells infected with R23 for 5 min, increased until 20-30 min. The enzyme activity was about twice that detected in f2-infected cells and somewhat lower than the level in Qβ-infected cells (after 20 min).

These studies indicated significant differences among the 5 phages in the host-phage relationship after infection. A more detailed investigation of the viral effects on host DNA, RNA and protein synthesis was therefore undertaken. Further characterization of R23, R34, R40, f2 and Qβ, including an analysis of their biophysical properties and life cycles, was performed in an attempt to delineate the control mechanisms involved in their interactions with *E. coli*. The process of infection by each phage was examined for possible differences in burst size, latent period, efficiency of infection,

duration of cell division following infection, and other parameters which might be implicated as cause or effect in their characteristic actions with respect to host metabolism. It was important to establish whether or not the differences in quantitation described above (which were largely based on recovery of radioactivity in purified phage particles) were caused by unequal populations of infected and uninfected cells in the cultures infected by the 5 phages.

Results

a) Effect on Host Macromolecular Synthesis

1. RNA Synthesis in *E. Coli* Infected with RNA Phages. Neither the rate nor the extent of ^{14}C-uracil incorporation was appreciably altered by R23 before the onset of cell lysis (Watanabe *et al.*, 1968). If the specific activity of the uracil pool was the same in infected and uninfected bacteria it could be assumed that total RNA synthesis was unaltered or affected only mildly by RNA phage infection. Synthesis of phage-specific RNA might then occur in place of, rather than in addition to, synthesis of bacterial RNA. Since phage RNA was synthesized in such large quantities after R23 infection (Watanabe *et al.*, 1968), it could be predicted that host RNA would be drastically reduced.

The effect of R23 on synthesis of bacterial rRNA and tRNA was therefore examined. At 15 min after infection, there was a considerable reduction in 23S, 16S and 4S RNA and an appearance of some heavier material not seen in uninfected cultures (Fig. A1). From 30-90 min, the reduction in 23S and 16S was more marked and greater quantities of 27-28S (SS viral RNA) material were synthesized. After 30 min, the material sedimenting around 16S appeared heterogeneous; it was

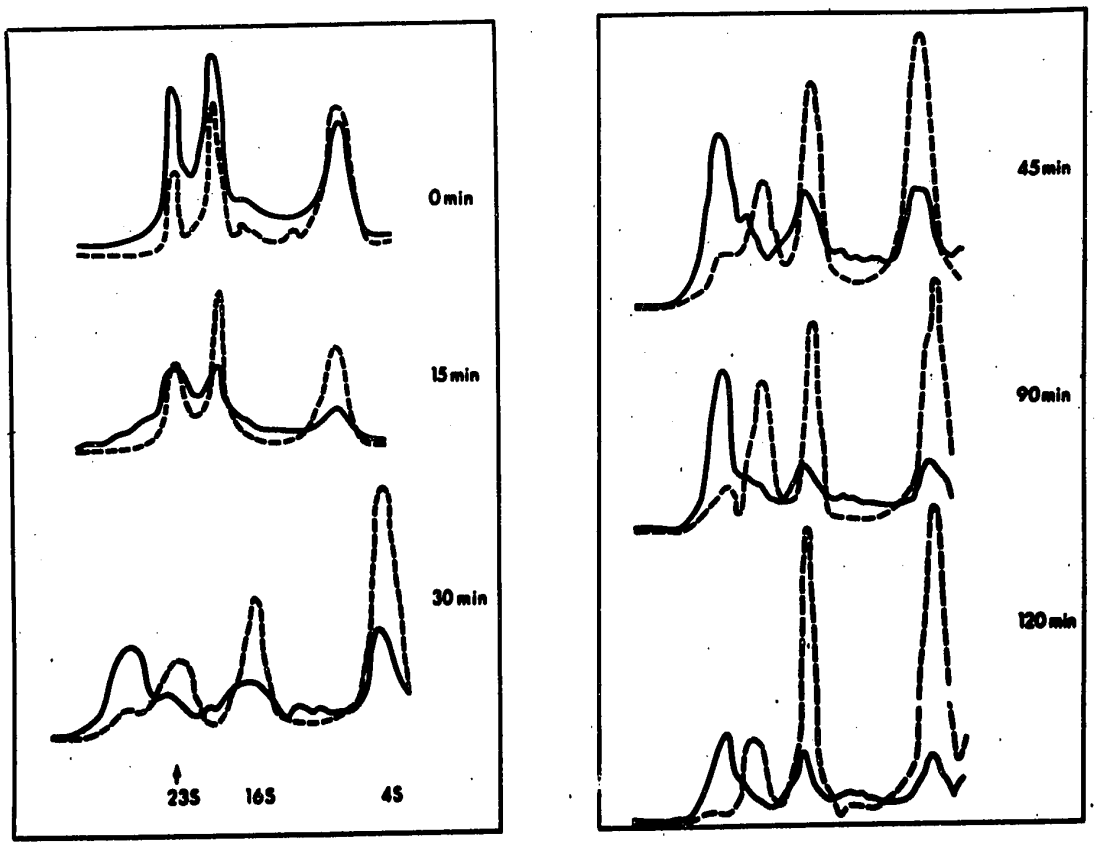

Fig. A1. Sucrose Density Analysis of RNA from R23-Infected *E. coli*. The experiment was performed as described in Materials and Methods. ___: ^{14}C-uracil, R23, ---: ^{3}H-uracil, uninfected.

considered to represent DS phage RNA which has a sedimentation constant of about 14-16S (Weissmann & Ochoa, 1967). It appeared from these studies that the inhibition of 23S and 16S rRNA was essentially complete by 30 min, and that the inhibition of 4S RNA was not as profound.

A plot of the rate of RNA synthesis as a function of time after infection (Fig. A2) confirmed that the greatest inhibition occurred during the first 15 min, and that the inhibition was maximal at 30 min with relatively little or no change thereafter. There appeared to be a residuum of (about 25%) synthesis of 23S and 16S RNA (Fig. A2), but this "residual" activity included some DS phage RNA which sedimented in this region and was therefore not separated from host RNA.

The extrapolation that the absolute amount of phage RNA synthesized was greatest in bacteria infected with R23 was predicated on the assumption that the specific activity of the newly synthesized RNA was the same in bacteria infected with the 5 phages. To test the validity of this assumption, RNA was prepared from purified phage labelled with ^{3}H-uracil and the specific activity of newly synthesized RNA determined. The specific activity was the same for all 5 phages (Table A1) indicating that the uracil pools were similar in bacteria infected with these phages. It could be concluded, therefore, that total RNA synthesis was identical for all 5 cultures and that the amounts of phage RNA synthesized varied with the phage, the greatest amount occurring in the case of R23 and R34.

The ability of RNA phage to direct RNA synthesis was also examined in UV-irradiated *E. coli* cells in which bacterial RNA synthesis was almost completely inhibited. The largest amount of RNA

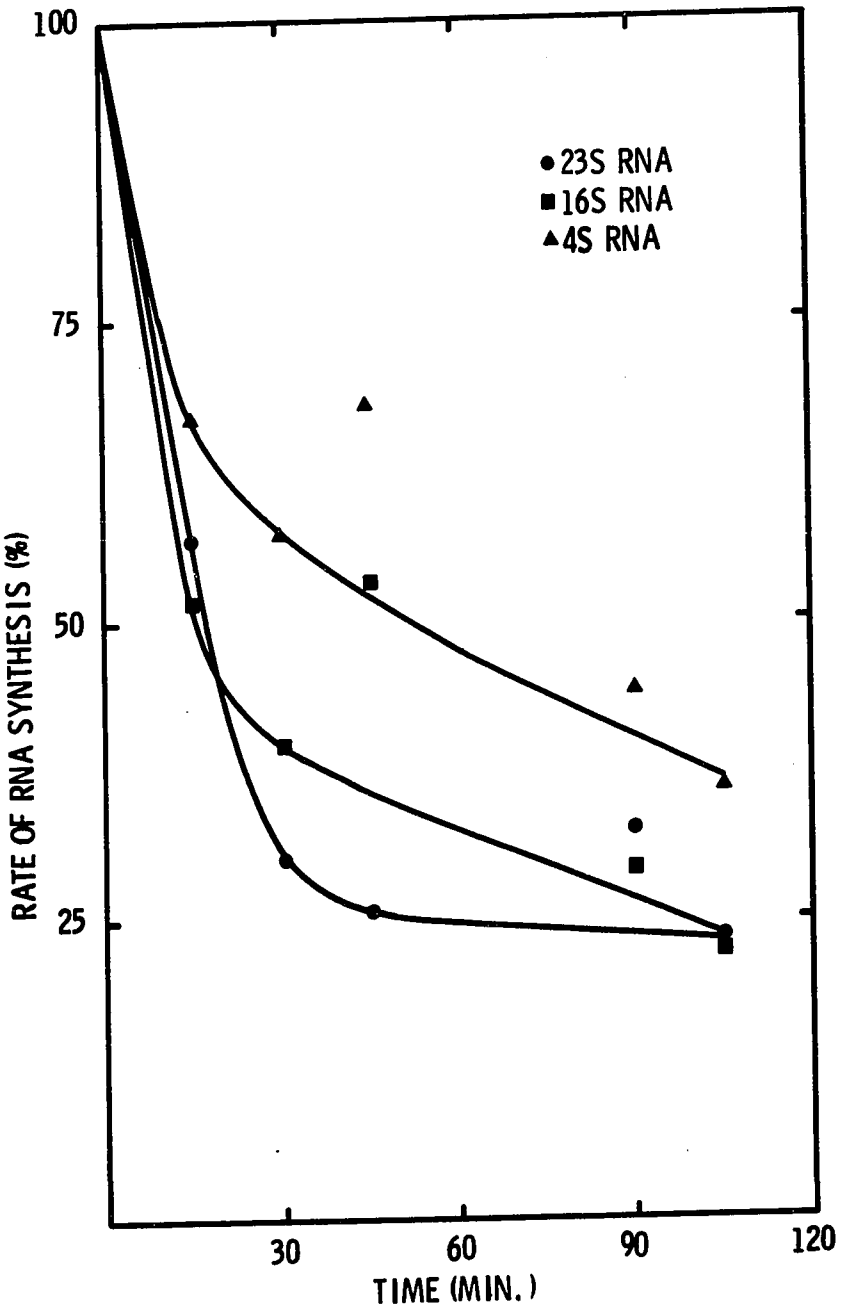

Fig. A2. Effect of R23 on rRNA and tRNA Synthesis in *E. coli*.

The data were obtained from that of Fig. A1.

Table A1. Synthesis of Phage RNA and Phage Particles. *E. coli* K38 grown in Tryptone broth was infected with R23, R34, R40, f2 and Qβ at a MOI of 30 phage/cell. ^{3}H-uracil was added to a concentration of 1 μc/ml at the time of infection. The culture was lysed 2 hours after infection and assayed for infective phage units and for radioactivity present as phage RNA (by antibody-binding techniques). The remaining lysate was used for preparation of purified phage. The purified phage was analyzed for RNA content by measuring absorbance at 260 mμ and for radioactivity. The techniques for growth and lysis of bacteria, purification of labelled phage, assay of phage and antibody-precipitable RNA have been described in Materials and Methods.

Phage RNA was calculated in μg/ml from a knowledge of radioactivity incorporated and the specific activity of phage RNA. The number of phage particles present in the lysate was then calculated according to the following equation: Number of phage particles = $\frac{\text{Radioactivity as phage RNA (cpm/ml)}}{\text{Specific activity of phage RNA (cpm/μg RNA)} \times 2 \times 10^{-12} \text{ (μg)}}$ assuming an RNA content of 2×10^{-12} μg/phage particle.

TABLE A1

SYNTHESIS OF PHAGE RNA AND PHAGE PARTICLES

Phage	Phage RNA			Phage Particles		
	Radioactivity cpm/ml	Specific Activity cpm/mg RNA	Amount µg/ml	Total Particles	Infective Centres	Particles/PFU
R23	50,400	3.2×10^6	15.7	7.8×10^{12}	2.0×10^{12}	3.9
f2	25,500	3.1×10^6	8.2	4.1×10^{12}	1.2×10^{12}	3.4
Qβ	26,300	3.2×10^6	11.4	5.7×10^{12}	4.7×10^{11}	12.1
R34	52,900	3.5×10^6	15.1	7.5×10^{12}	2.3×10^{12}	3.3
R40	26,700	3.0×10^6	8.9	4.5×10^{12}	1.4×10^{12}	3.2

was synthesized after R23 and R34 and the least amount after Qβ infection (Fig. A3). Maximum rates of RNA synthesis were obtained at a multiplicity of 10 or greater for all phages (Fig. A4). For almost all multiplicities, the initial rate of synthesis was greatest in the case of R23 infection.

2. DNA Synthesis in *E. coli* Infected with RNA Phages. DNA synthesis in RNA phage-infected *E. coli* was examined by incorporation of ^{3}H-thymidine into acid-insoluble material. R23, R34, R40, f2 and Qβ all inhibited the synthesis of DNA to some degree (Table A2). Although the differences were minimal, R23 appeared to produce the greatest inhibition of DNA synthesis.

3. Protein Synthesis in *E. coli* Infected with RNA Phages. Total protein synthesis was markedly diminished following R23 infection. A significant reduction in the rate of incorporation of ^{14}C-amino acids was apparent about 30 min after infection (Fig. A5). Synthesis of a specific bacterial protein, β-galactosidase, was inhibited most by R23, while R34, R40, f2 and Qβ had progressively lesser effects (as will be discussed in Section C).

b) Biophysical Properties

1. Serology. R23 is serologically related to f2; both were neutralized by R23 antiserum in a similar manner (Fig. A6). The rate of neutralization of R34 and R40 was 1/10 of that of R23. Qβ was unaffected by antisera prepared against R23 or the other phages.

2. Density in CsCl. R23, R34 and R40 had the same density in CsCl (Fig. A7) but differed somewhat from f2 and Qβ.

3. Base Compositions. The base compositions of R23, R34, R40 and f2 RNA were similar with almost equimolar amounts of the 4 bases (Table A3).

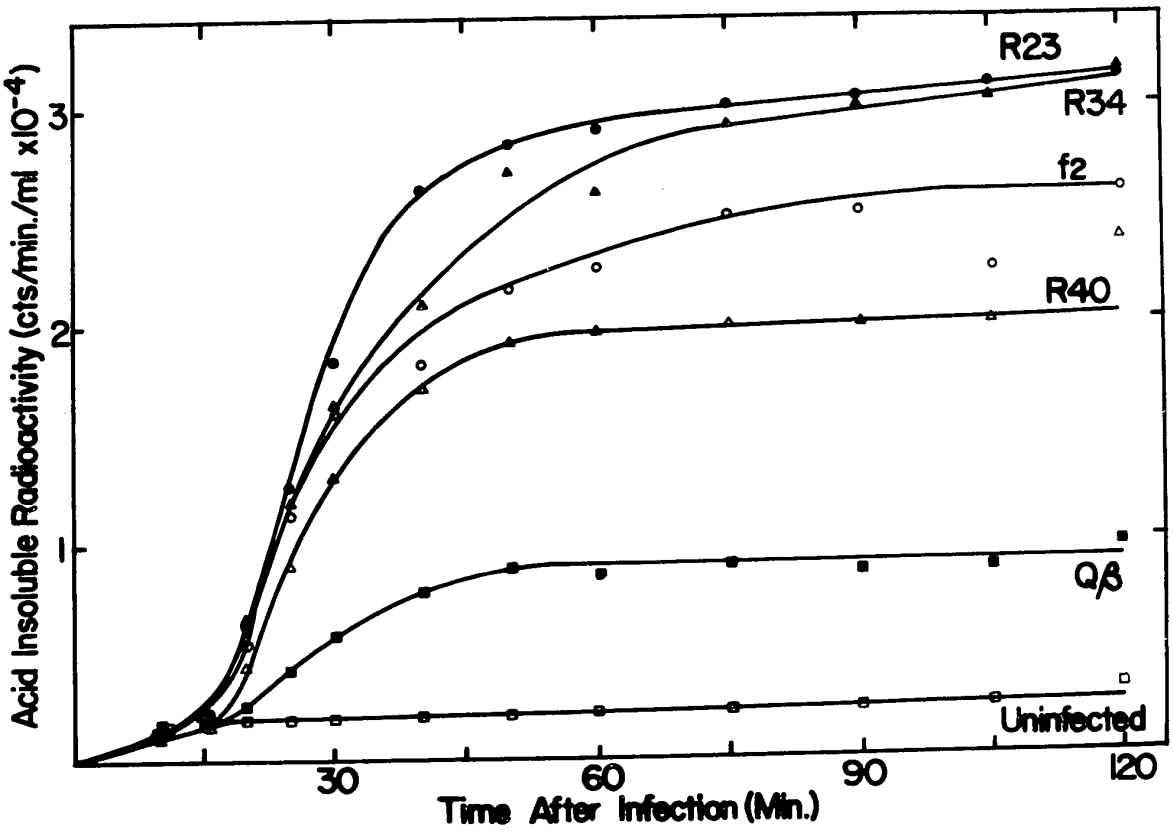

Fig. A3. RNA Synthesis in UV-Irradiated *E. Coli* K38 Infected with RNA Phages. UV-irradiated bacteria were infected with RNA phage at a MOI of 10 and ^{3}H-uracil was added to a final concentration of 1 μc/ml. UV-irradiation of bacteria and measurements of incorporation of radioactivity into RNA were performed as described in Materials and Methods.

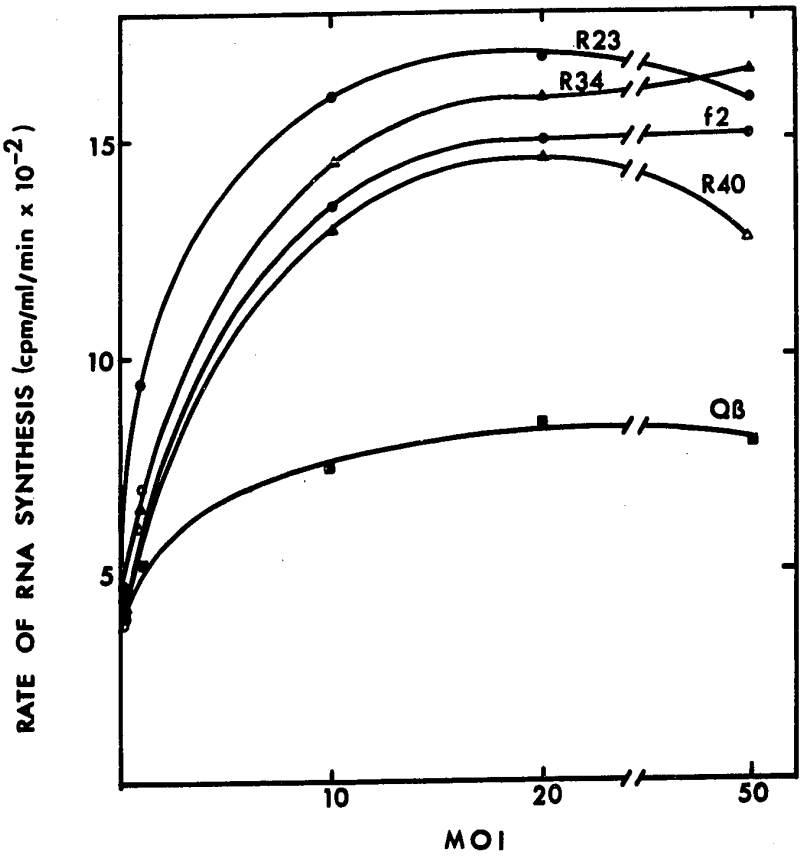

Fig. A4. Effect of MOI on RNA Synthesis in UV-Irradiated *E. coli* K38 Infected with RNA Phages. The experiment was performed as in Fig. A8 except that MOI of 0.1 to 50 were used. The rate of RNA synthesis was calculated from the radioactivity incorporated 30 and 40 min after infection.

TABLE A2

EFFECT OF RNA PHAGES ON DNA SYNTHESIS IN *E. COLI*

Phage	Radioactivity incorporated into DNA (cpm/ml) At 15 min	At 25 min
Uninfected	3320	7920
R23	2580	5500
f2	2740	6010
Qβ	3080	6500
R34	2630	5540
R40	2680	5900

The experiment was performed as described in Materials and Methods. ^{3}H-Thymidine was added to a concentration of 10 μc/ml. Samples of each culture were removed for analysis 15 and 25 min after infection.

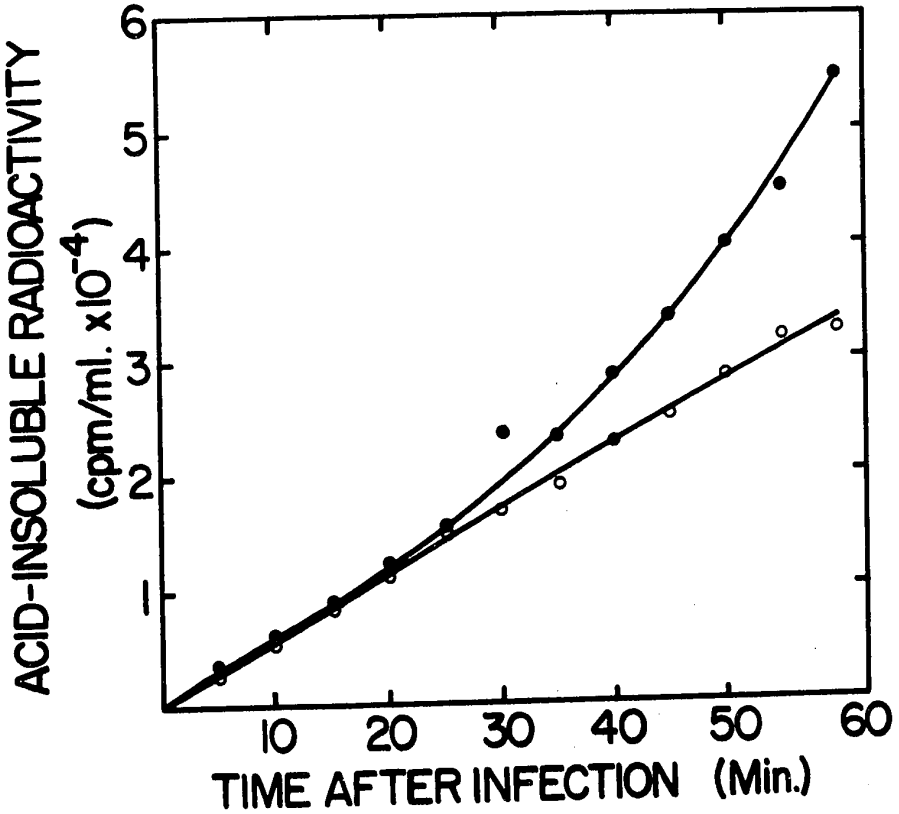

Fig. A5. Protein Synthesis in *E. Coli* Infected with R23.

E. coli K38 was grown in Tryptone broth and infected with R23 at a MOI of 10 phage/cell. ^{14}C-methionine was added at the time of infection to a concentration of 1 µc/ml. Samples from uninfected (●) and infected (0) cultures were treated as described in Materials and Methods.

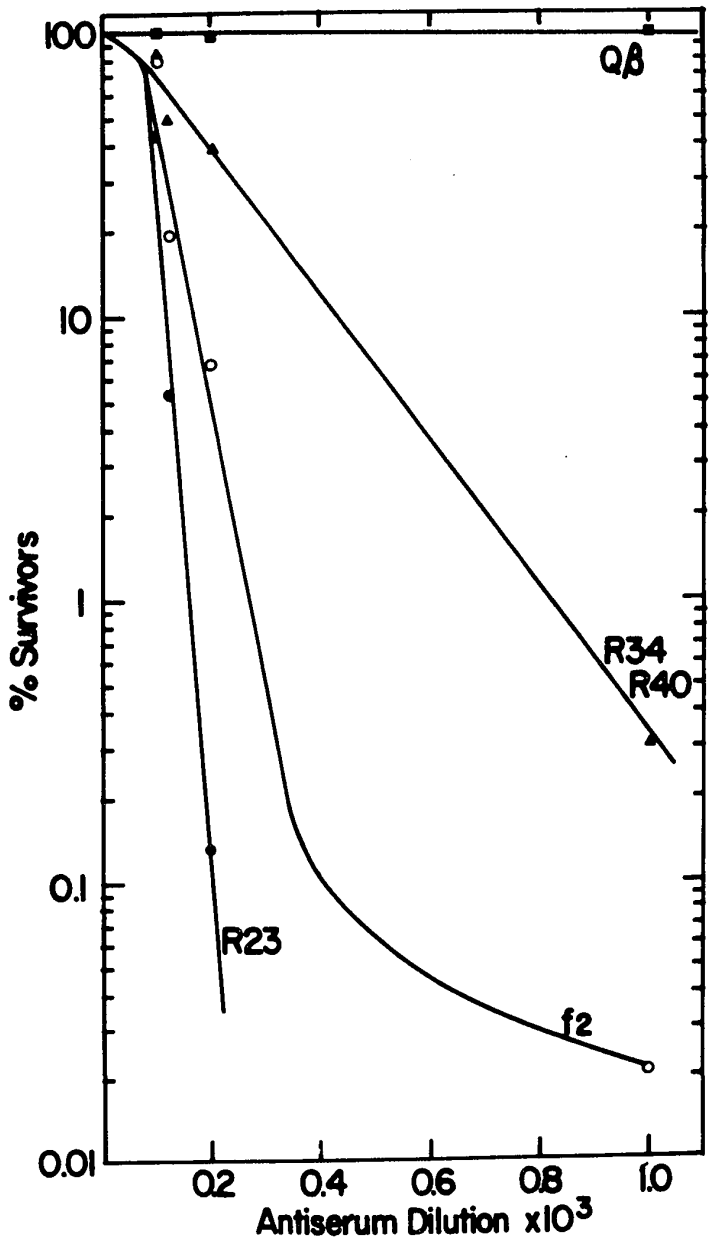

Fig. A6. Neutralization of RNA Phages by R23 Antiserum. The experiment was performed as described in Materials and Methods.

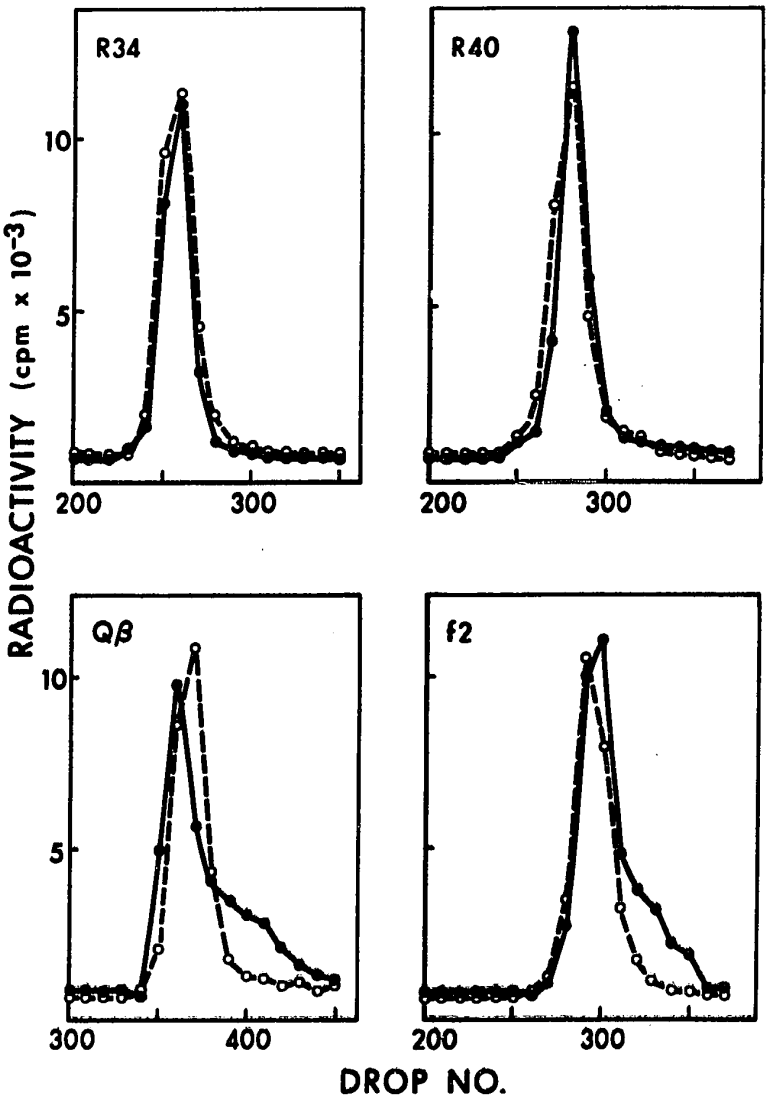

Fig. A7. CsCl Density Gradient Analysis of RNA Phages. The labelled phages were grown in K38 as described in Materials and Methods. ●: ^{3}H-labelled phage, o: ^{14}C-labelled R23.

TABLE A3

BASE COMPOSITION OF RNA PHAGES

Phage	Base Composition moles %			
	A	U	G	C
R23	23.4	26.0	25.6	25.1
R34	23.6	26.8	23.9	25.4
R40	22.4	25.5	26.9	24.9
f2	22.2	25.1	25.9	26.8
Qβ	22.1	29.1	23.7	24.7

The experiment was performed as described in Materials and Methods. Base compositions of f2 and Qβ were reported by Loeb and Zinder (1961) and Overby *et al.* (1966b).

Qβ has a higher uracil content than the other 4 phages.

c) Process of Infection.

1. Efficiency of Infection. In a culture of *E. coli*, a certain percentage of cells escapes infection by RNA phage because they are phenotypic females lacking the F pilus (Brinton & Beer, 1967). There may be other factors contributing to resistant survivors which depend on the particular virus involved. The efficiency of infection (EOI) by R23, R34, f2 and Qβ was examined by determining the number of survivors and infective centres at different times and different multiplicities of infection (MOI). For MOI up to 50 phage/cell, the EOI was similar for R23, R34 and f2, but Qβ infection produced about half as many infective centres (Fig. A8) and twice as many survivors (Figs. A9-11) as these phages.

During the first 30 min of infection, slightly more survivors were noted in cultures infected with f2 than in those infected by R23, R34 and R40 (Fig. A9). The sudden decrease in survivors at 30-45 min represented infection by progeny phage released from early bursts and occurred 10-15 min earlier with f2. The bacteria which survived the initial infection were, therefore, not resistant to subsequent infection. The number of survivors appeared to be the net result of increase due to cell division and decrease with subsequent phage infection. When adsorption was limited to 5 min and subsequent infection prevented by addition of antiserum, the bacteria surviving at 5 min continued to divide at the same rate as an uninfected culture (Fig. A10). The number of survivors was again similar for R23, R34, R40 and f2 but significantly higher for Qβ.

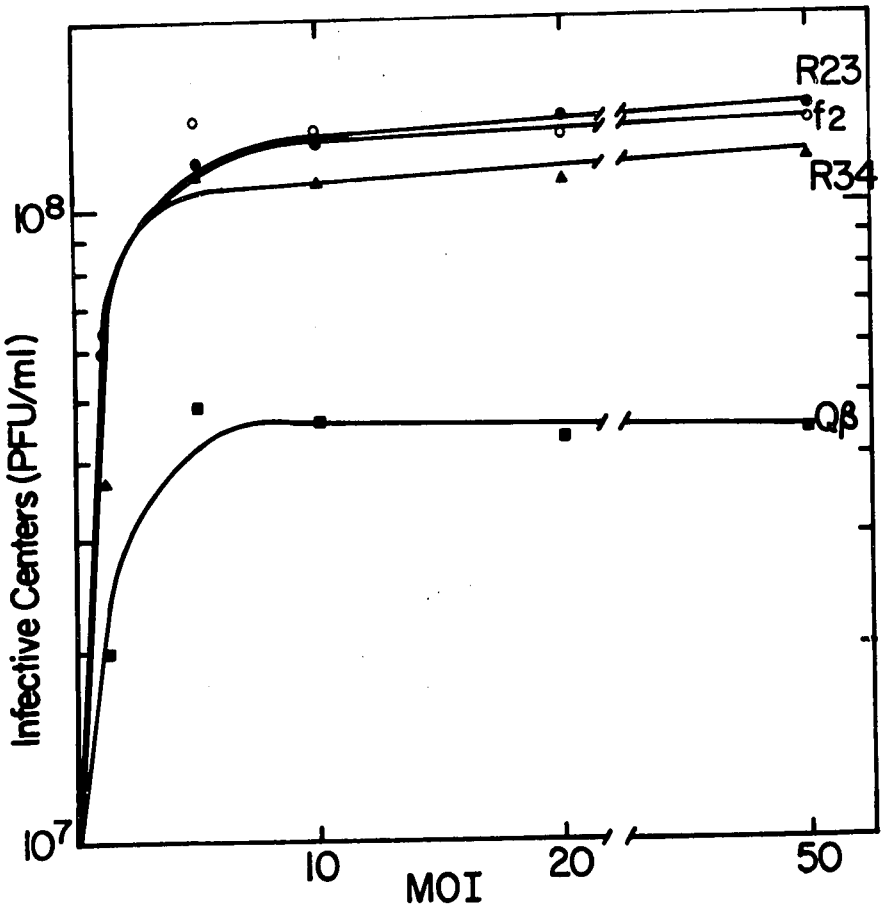

Fig. A8. Effect of Multiplicity of Infection on Infective Centres. The experiment was performed as described in Materials and Methods using MOI of 1 to 50 phage/cell.

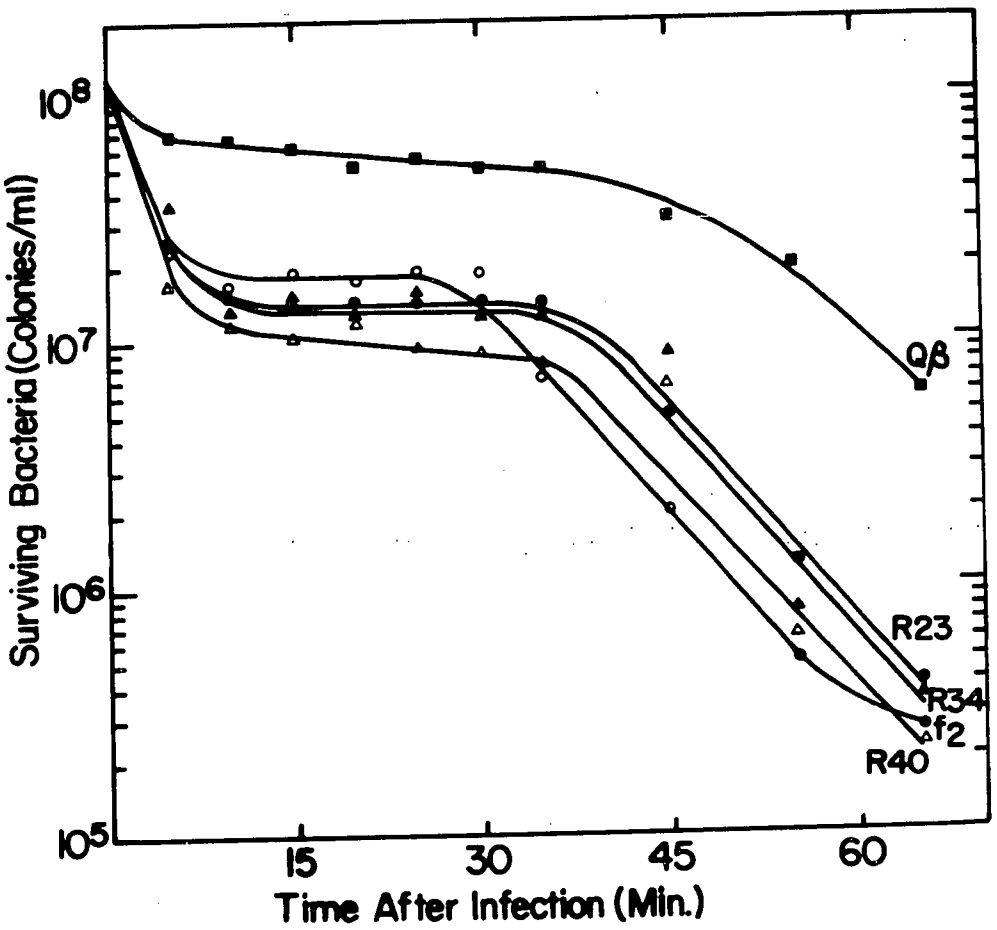

Fig. A9. Bacterial Survivors Following RNA Phage Infection. The experiment was performed as described in Materials and Methods. *E. Coli* K38 was grown in Tryptone broth and infected at a MOI of 20 phage/cell.

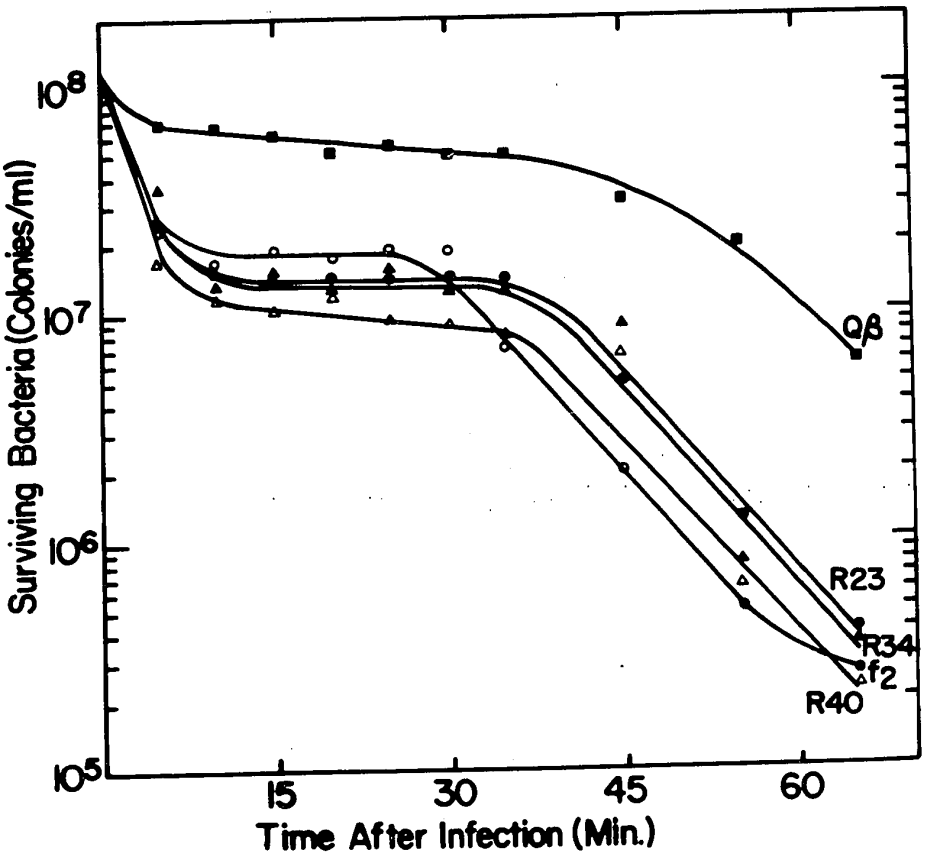

Fig. A9. Bacterial Survivors Following RNA Phage Infection. The experiment was performed as described in Materials and Methods. *E. Coli* K38 was grown in Tryptone broth and infected at a MOI of 20 phage/cell.

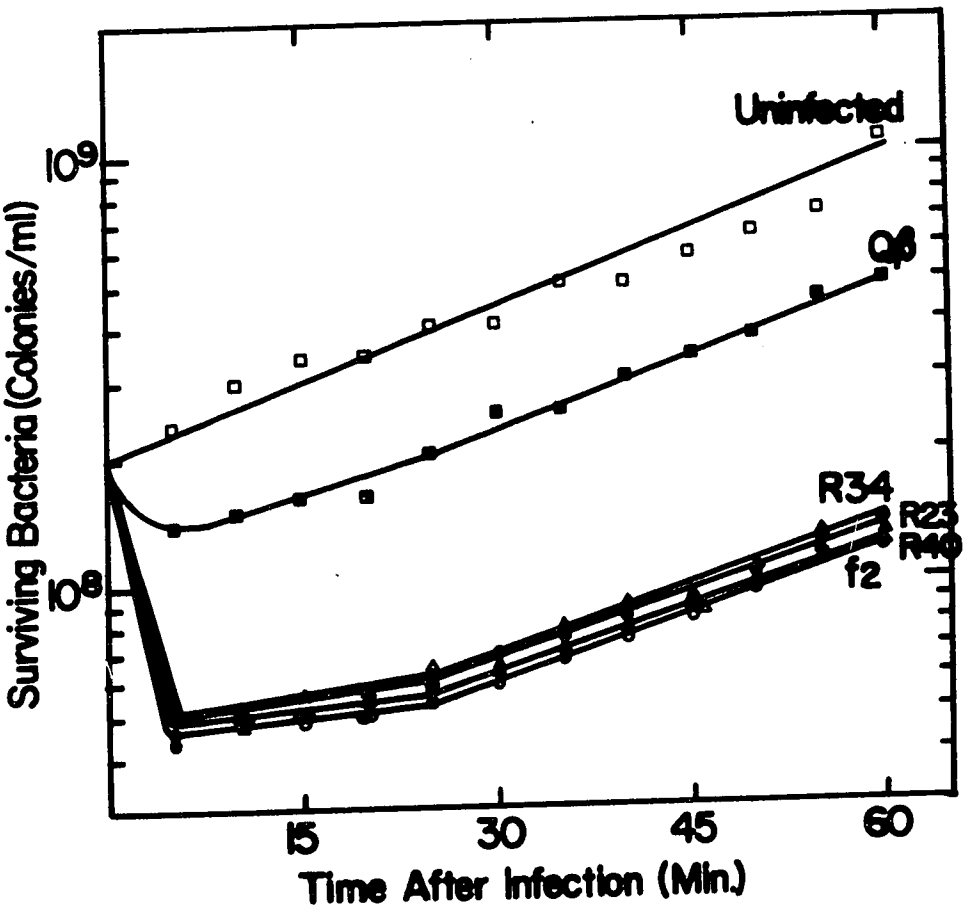

Fig. A10. Bacterial Survivors in the Presence of Phage Antiserum. The experiment was performed as in Fig. A9, except that 5 min after infection, cultures were diluted 100-fold and incubated at 37° C with appropriate phage antisera to inactivate unadsorbed phage.

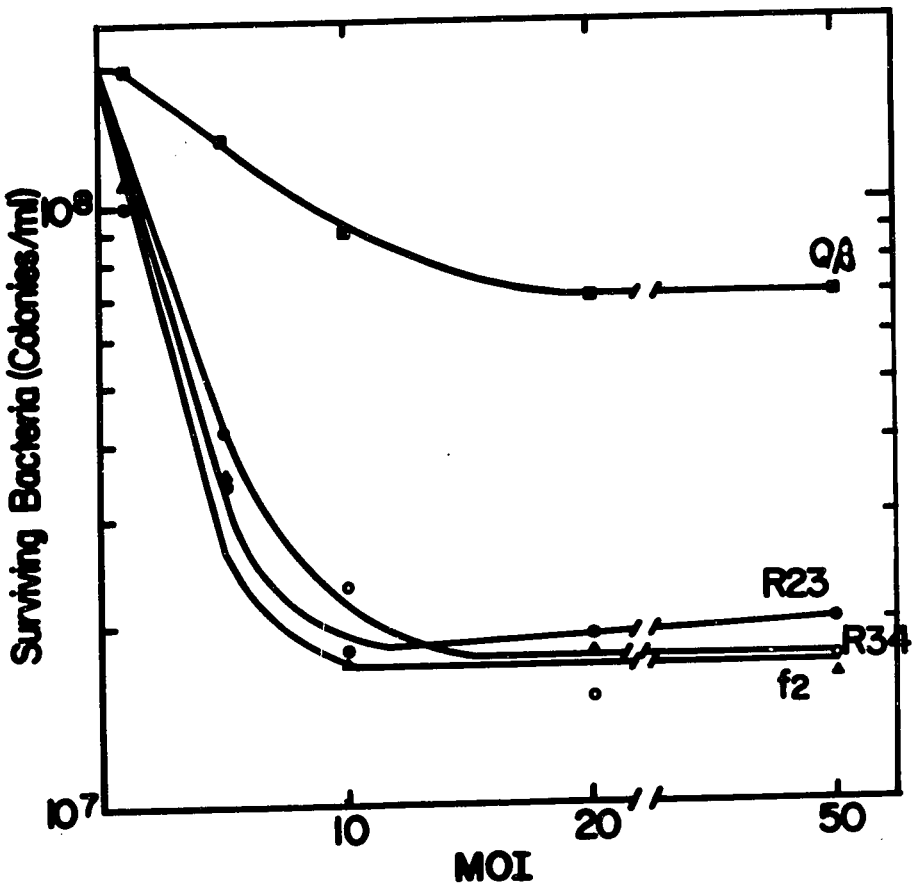

Fig. A11. Effect of MOI on Bacterial Survivors. The experiment was performed as in Fig. A4 except that phage were added at MOI of 1 to 50 and cultures were assayed for bacterial survivors after 5 min incubation in the presence of antiserum.

For each phage, maximal infective centres and minimal survivors were obtained at MOI over 10 (Figs. A8, 11). With a bacterial concentration of 2 X 10^8 cells/ml and an adsorption period of 5 min, 70-80% of the cells were infected by R23, R34 and f2, but only 30% by Qβ (Table A4). For multiplicities of 5 or over, when at least 97% of the successfully infected cells were multiply infected, similar numbers of infective centres were obtained for R23, R34 and f2. But at MOI of 0.1 or less, when the majority of cells were singly infected, the infective centre titre was approximately 60%, 50% and 30% of that expected for R23, f2 and R34, respectively. Thus, R23 was most proficient in multiplying in cells which were singly infected but the capacity to successfully infect a cell improved for all phages when the cells were multiply infected.

The relative inefficiency of Qβ infection was not readily explained. Qβ produced a larger number of non-viable particles (as discussed later), but dead (R34) particles did not reduce the ability of infectious (R34) phage to initiate infection (Table A5), even when they outnumbered viable particles by a ratio of 150 to 1. The possibility that experimental conditions such as temperature were not optimal for Qβ infection was considered, but its EOI at 32° C and 37° C were similar.

2. Phage Life Cycle. The life cycles of the 5 phages were compared. Lysis of a culture of *E. coli* K38 grown in Tryptone broth began 30, 50, 50, 60, and 90 min after infection with f2, R34, R40, R23, and Qβ, respectively (Fig. A12). The late lysis of the Qβ-infected culture was compatible with its larger population of bacterial survivors. In one-step growth experiments (Fig. A13), the more prolonged life

TABLE A4

EFFICIENCY OF INFECTION BY RNA PHAGES AT DIFFERENT MOI

Phage	Average MOI					
	0.01	0.1	1	5	10	>20
	% of expected infective centres					
R23	59	62	58	71	77	83
f2	50	44	54	83	77	83
R34	29	31	34	71	65	71
Qβ	9	23	18	29	27	29
Proportion of infected bacteria						
singly infected	100	95	58	3	<0.01	<0.01
multiply infected	0	5	42	97	>99.9	>99.9

The experiment was performed as described in Materials and Methods. Phage were added at multiplicities ranging from 0.01 to 100 phage/cell. The efficiency of infection was calculated from the observed number of infective centres divided by the expected number for each MOI, as calculated by Poisson distribution, and represents the efficiency of infection observed during 5 min of phage adsorption at 37° C.

TABLE A5

EFFECT OF NON-VIABLE PARTICLES ON EFFICIENCY OF INFECTION

Ratio of Non-Viable/Viable Particles	Bacterial Survivors %
6	17
20	15
35	13
150	18

E. coli K38 grown in Tryptone broth was infected with purified R34 at a MOI of 10 PFU/cell. The percentage of viable particles in this preparation was approximately 17%. In 3 other samples, UV-inactivated R34 was added to give non-viable to viable ratios of 20, 35 and 150. Five min after infection, antiserum was added to inactivate unadsorbed phage and 5 min later, bacterial survivors were assayed. UV-inactivation of phage and assays of survivors were performed as described in Materials and Methods.

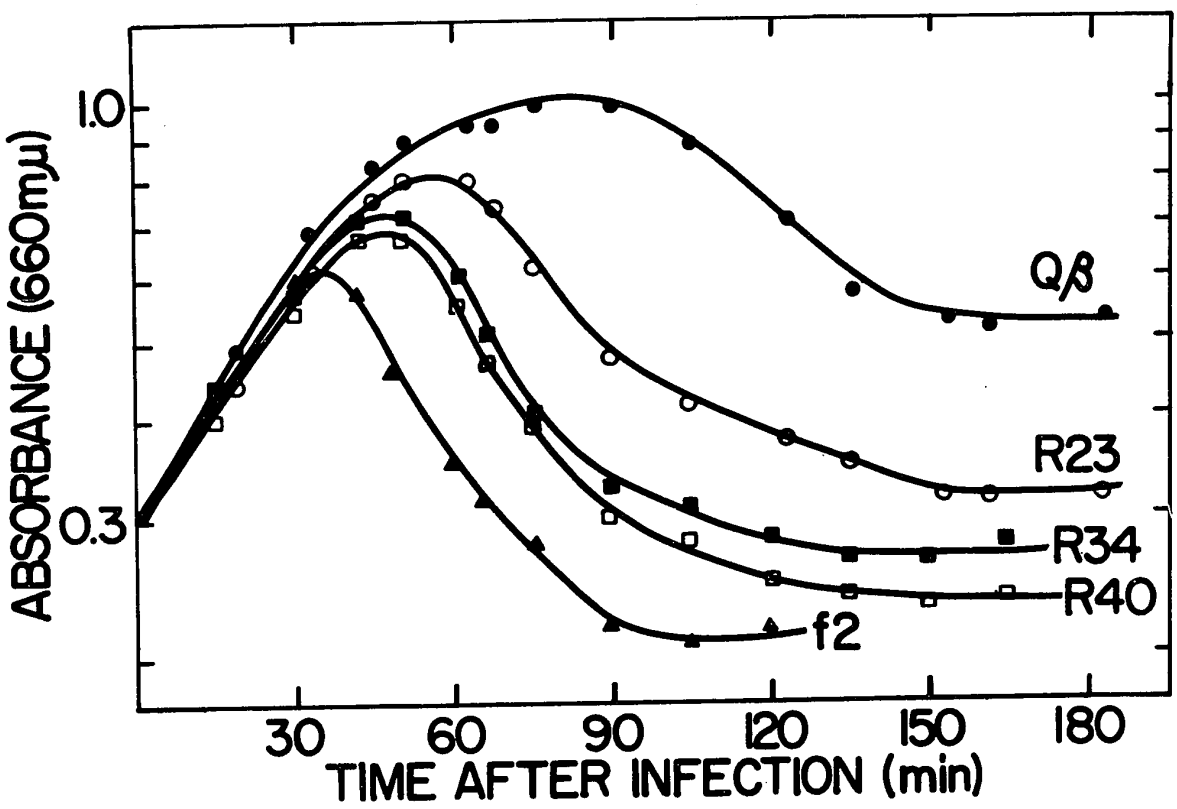

Fig. A12. Lysis of *E. coli* K38 after RNA Phage Infection. Growth measurements of infected bacteria grown in Tryptone broth were performed as described in Materials and Methods.

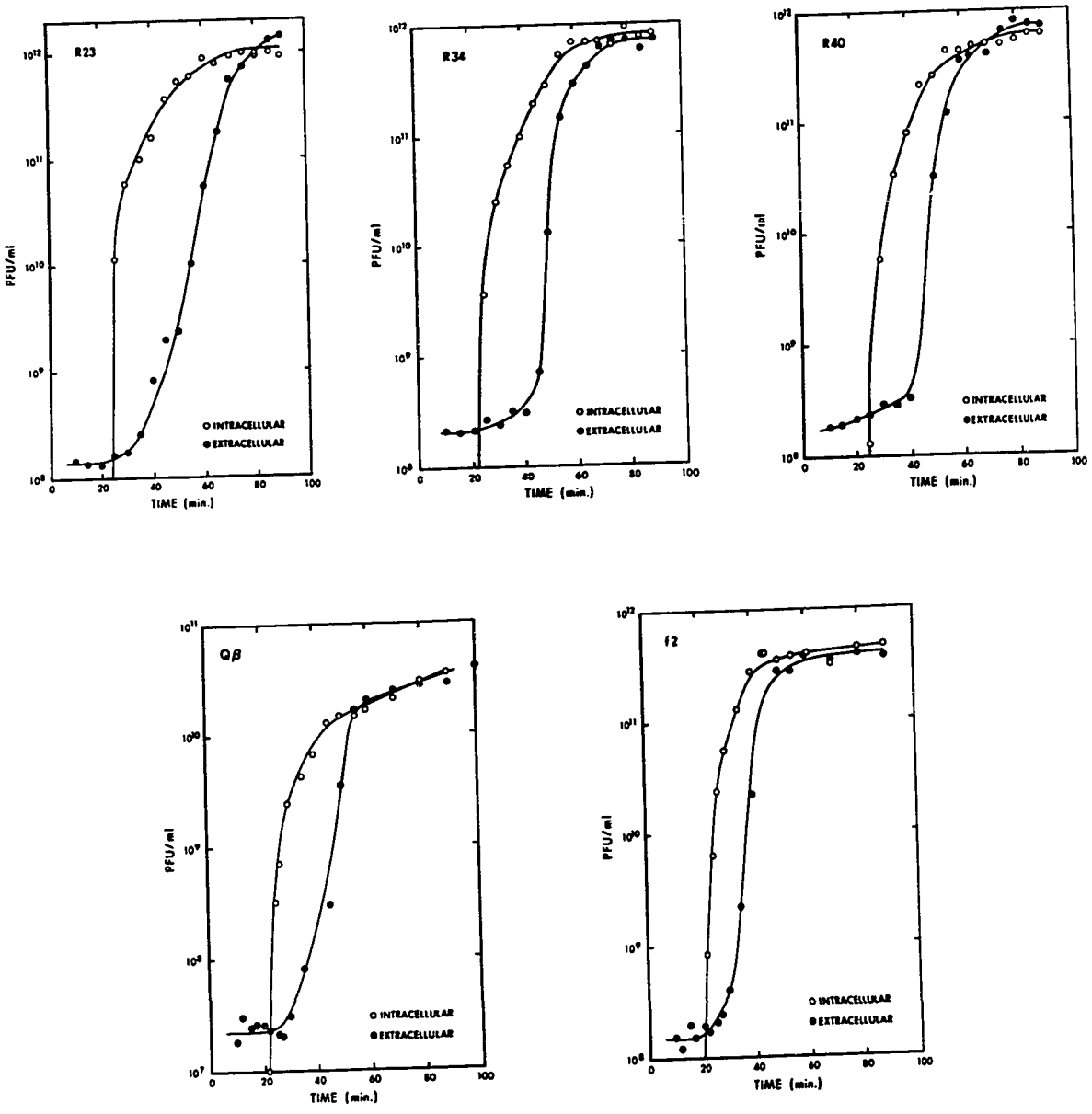

Fig. A13. One-Step Growth Curves of RNA Phages. The experiments were performed as described in Materials and Methods.

cycles of R23, R34 and R40 were reflected primarily in prolonged latent periods with little difference in the duration of the eclipse periods, compared to f2 and Qβ (Table A6). The rates of adsorption and eclipse of infectivity were similar for R23, f2, R34 and R40. Adsorption and eclipse occurred rapidly during the first 4 min, the greater portion occurring during the first minute, and 70-90% of the input phage were adsorbed within 5 min (Table A7). At all times, 90% of adsorbed phages were eclipsed. The adsorption of Qβ appeared to be more rapid, maximum adsorption and eclipse occurring within the first minute. The percentage adsorbed, however, was not significantly different.

3. Phage Yield. In one-step growth experiments, the average burst size in plaque-forming units (PFU) per infective centre was about 10,000 for R23, 4,000 for R34 and 1,500 for f2 and Qβ (Table A6). Analysis of burst sizes from single cells demonstrated a wide range for all phages (Table A8). R23 and R34 produced the largest single bursts while the lowest bursts were obtained with f2 and Qβ. Both studies (Tables A6,8) were performed using dilute bacterial concentrations. The yield of RNA phages has been reported to be greater in dense cultures with high bacterial concentrations due to lysis inhibition (Loeb & Zinder, 1961; Ellis & Paranchych, 1963). In dense cultures, R23 and R34 also produced the greatest phage yield (Table A9); the yield of R23 was not increased in dense cultures (although in other experiments a slight increase has been observed).

To determine whether the yield of PFU reflected the total particle yield, the ratio of total to viable particles was calculated.

TABLE A6

ONE-STEP GROWTH ANALYSIS OF RNA PHAGES

Phage	Average Burst Size PFU/IC	Eclipse Period min	Latent Period min
f2	1,500	20	27
Qβ	1,300	22	30
R40	3,900	25	40
R34	3,800	22	44
R23	10,000	24	40

The experiment was performed as described in Materials and Methods.

The figures represent averages of 2 experiments.

TABLE A7

ADSORPTION AND "ECLIPSE" OF RNA PHAGES

Phage	Input Phage PFU/ml	Adsorbed Phage % of input	Eclipsed Phage % of input
R23	3.8×10^9	92	81
R34	2.9×10^9	69	66
R40	3.6×10^9	78	72
f2	2.8×10^9	79	71
Qβ	3.0×10^9	86	86

The number of adsorbed and eclipsed phages were determined 5 min after infection as described in Materials and Methods.

TABLE A8

PHAGE YIELD IN SINGLE BURSTS

Phage	Number of Cells Examined	Phage Yield (PFU) Range	Average
f2	46	160 - 4,540	2,040
Qβ	58	165 - 4,760	2,045
R40	21	655 - 7,320	3,595
R34	33	230 - 11,960	5,565
R23	45	540 - 10,520	5,720

The experiment was performed as described in Materials and Methods.

TABLE A9

PHAGE YIELD IN DENSE AND DILUTE BACTERIAL CULTURES

Phage	Phage Yield (PFU/ml) in Dilute Culture	Phage Yield (PFU/ml) in Dense Culture	Ratio Dense/Dilute
f2	3×10^{11}	1.3×10^{12}	4.3
Qβ	3×10^{10}	5.8×10^{11}	19.3
R40	7×10^{11}	1.6×10^{12}	2.3
R34	8×10^{11}	2.1×10^{12}	2.6
R23	2×10^{12}	2.0×10^{12}	1.0

The experiment was performed as described for the one-step growth experiment. In dilute cultures the infective centre titre was 10^2/ml, whereas in dense cultures it was 10^8/ml. The cells were lysed after 120 min of incubation and the phage yield determined, as described in Materials and Methods.

With the exception of Qβ, this ratio was similar for all phages (Table A1). Qβ produced a much larger proportion of non-viable particles. The yield of viable Qβ particles was not increased by changing the growth temperature to 32° C. Examination of the non-viable particles of all phages by CsCl density gradient centrifugation indicated that these particles could not be separated from viable particles unlike defective particles lacking the maturation protein.

Discussion

Inhibition of host RNA synthesis by R23 appears to precede viral RNA synthesis. Thus, rRNA and tRNA were drastically inhibited within 15 min after infection, while viral RNA synthetase was induced from 5 min and viral RNA synthesized in significant amounts only after 15 min of infection by R23 (Watanabe *et al.*, 1968). Host DNA and protein syntheses (measured by incorporation of radioactive precursors) were not appreciably diminished till about 25 min after infection, but a small diminution was detectable before this time. The inhibition was more obvious in studies of the specific inducible enzyme, β-galactosidase. In this case, inhibition was also predominant after 25 min of infection, but the early period of inhibition was definite (Section C).

It is possible that 2 different mechanisms are involved in the early and late inhibition of host macromolecular synthesis. During the first 15-20 min of infection, there was a marked inhibition of host rRNA as well as a depression of bacterial tRNA and (β-galactosidase) mRNA synthesis, and a less striking inhibition of host protein and DNA synthesis. After 20-30 min, a more profound inhibition of synthesis of host DNA and proteins (including inducible enzymes) was

observed, followed by cell lysis. The mechanism for this sequential inhibition and the difference in degree of inhibition of synthesis of macromolecules will be discussed in greater detail in subsequent sections.

It is evident from comparison of the effects of R23, R34, R40, f2, and Qβ on bacterial nucleic acid and protein synthesis, as well as the quantitation of phage RNA and protein synthesis that, of these phages, R23 is most proficient at inhibiting host functions. It is closely mimicked by R34. It has been established in the present experiments that R23, R34 and R40 are related to f2, and not to Qβ, in serology and base composition. Watanabe *et al.* (1967a,b) have shown that Qβ and related phages differ from other RNA phages in serology, behaviour on Millipore filtration, RNA base ratios, UV sensitivity, buoyant density in CsCl density gradients, sedimentation constant, pH sensitivity, coat protein amino acid composition and replicase template specificity.

The present study indicates that Qβ differs from R23, R34, R40 and f2 in its efficiency of infection (as measured by infectious centres and bacterial survivors) which is inferior although adsorption and eclipse of infectivity occur most rapidly with Qβ. One possible factor is that Qβ may have a limited injection mechanism. Injection of Qβ RNA, at least, appears to proceed in a different manner from that of the f2 group of phages in that there are F pili mutants of *E. coli* which allow normal growth of Qβ but prevent injection of f2 (Silverman *et al.*, 1967a,b). Once Qβ infection occurs, however, the particle yield per infected cell is as high as in R23 infection, although the number of viable units is somewhat less. The non-viable

particles may not affect the infectivity of viable phage since a ratio of 150 non-viable to 1 viable particle did not inhibit the efficiency of infection in the case of R34.

Thus, the inability of Qβ to produce a significant effect on host metabolism correlates with its relative inefficiency of infection. But the difference in host response to R23, R34, R40 and f2 is not reflected in a similar significant disparity in efficiencies of infection. These 4 phages do not differ appreciably in rates of adsorption and eclipse of infectivity, the ratio of viable to non-viable particles, or the efficiency of infection at high MOI.

At low MOI when cells are singly infected, R23 appears to have an advantage in initiating successful infection. R23 produces the largest phage yield with or without lysis inhibition and it has the longest life cycle, the latent period being prolonged even in the absence of lysis inhibition conditions. Its long cycle may allow it to express its inhibitory capacities more completely than other phages which cause early lysis of the host; it also facilitates experimental detection and study of inhibition of host macromolecular synthesis. However, the length of the life cycle is unlikely to be the only factor involved in the difference between these phages.

The basis for the variation in virulence of these RNA phages may reside in the degree of their ability to replicate efficiently, i.e., in the extent and rate of RNA synthesis catalyzed by the viral RNA synthetase. This enzyme activity is greater in R23-infected cells than in f2-infected cultures (Watanabe *et al.*, 1968). More R23 viral RNA is synthesized and encapsulated in progeny phage particles

(Watanabe *et al.*, 1968) and R23 induces the highest level of RNA synthesis in UV-irradiated bacteria. β-galactosidase studies (Section C) indicate that R23, R34, R40 and f2 have rather similar early inhibitory effects but differ significantly in the degree of late inhibition during the phase associated with expression of the viral enzyme cistron.

The need for host proteins in viral replication may limit the degree to which a RNA phage inhibits the host. Full Qβ replicase activity requires a component (Eikhom & Spiegelman, 1967; Eikhom *et al.*, 1968) and factors (Franze de Fernandez *et al.*, 1968) present in uninfected cells. Immediate viral RNA replication may require DNA-dependent RNA polymerase or some of its subunits. Thus rifampicin, a specific inhibitor of this host enzyme, inhibits RNA phage yield if added before 4 min after infection with f2 (Fromageot & Zinder, 1968) and other RNA phages, including R23 (Watanabe & Watanabe, unpublished observations). R23 replication may be most independent of the host machinery for macromolecular synthesis. Contrarily, its inherent ability to infect and replicate rapidly and efficiently may directly result in its marked suppression of host activity by exclusion of host nucleic acid or enzyme from bacterial replication sites including the cell membrane and ribosomes.

The experiments described here do not provide a definitive answer to the problem of the mechanisms underlying the differential ability of RNA phages to suppress host processes, but they will serve as an aid to further investigation.

B. Amber Mutants of R23

Introduction

The genome of the RNA phage is a single-stranded RNA molecule of roughly 3300 nucleotides with a molecular weight of 1.1 million. It is able to code for about 1100 amino acids or 3 to 4 average-sized proteins (containing 150-300 amino acids). About 350 amino acids are accounted for by the maturation protein and 150 by the coat protein; 600 remain for the viral RNA synthetase and any other proteins which may still be unidentified. No more than 3 cistrons have been identified by complementation or other analysis of amber and temperature-sensitive mutants of RNA phages (Valentine, Engelhardt & Zinder, 1964; Pfeiffer *et al.*, 1964; Horiuchi *et al.*, 1966; Gussin, 1966; Tooze & Weber, 1967).

The tests by which they have been characterized include: relative plating efficiency on different suppressor strains, growth on the Su^- strain, incorporation of labelled uracil into acid-insoluble product in UV-irradiated bacteria, determination of RNase-resistant RNA, viral RNA synthetase assays, cesium chloride density gradient analysis of phage, sucrose density gradient analysis of phage RNA, infectivity of viral RNA to spheroplasts, protein synthesis *in vitro* and *in vivo*, analysis of tryptic digests of coat protein, and complementation analyses. Some of the results are outlined in Table 5 (Chapter 1).

The complementation test consists of infecting bacteria simultaneously with 2 mutants under conditions which are restrictive for each mutant alone. If a yield of progeny virus is obtained, the mutations must be complementary defects. If no progeny phage is

produced, the mutations must be in the same gene.

Maturation protein mutants complement strongly with nonpolar coat protein mutants, but weakly with mutants deficient in viral RNA synthetase. The result of a complementation assay for a cross of a polar coat protein and an enzyme mutant is ambiguous at 2¼ hours but positive at 4½ hours. Non-complementation at the earlier time is due to polarity which is lost later (Gussin, 1966). There is no intragenic complementation, i.e., complementation between mutants of a group. The relative yield of mutants in complementing crosses can be analyzed by their plating efficiencies on Su I and Su II strains. In a cross between maturation protein and enzyme mutants, 3/4 of the phage yield is composed of maturation protein mutants, i.e., complementation is asymmetric. In a cross of a polar coat protein and a maturation protein mutant, 2% are coat protein mutants, while in a cross of a nonpolar coat protein mutant and a maturation protein mutant, 40-50% are coat protein mutants. Thus, the genomes are preferentially replicated by their own polymerases (Tooze & Weber, 1967).

Using similar techniques, several amber mutants of R23 were isolated and characterized to determine the role of each viral gene in the effect of RNA phage on host function as well as to establish the genetic structure of R23 RNA.

Results

a) Isolation and Characterization

1. Isolation. Amber mutants were selected, after mutagenesis of whole phage with nitrous acid or infected cells with fluorouracil, for capacity to grow on *E. coli* K37 (Su-1) and failure to grow on K38 (Su^-)

strains. The reversion rates were generally 10^{-3} or 10^{-2}. Those with 10^{-4} or 10^{-1} revertants were not studied to avoid possible complications due to double mutations or excess revertants, respectively.

2. Characterization. The mutants were identified by a series of tests. Most tests were performed on K37 as well as K38 to ensure that the defect demonstrated in the Su^- host was suppressed in the Su^+ to a degree commensurate with the efficiency of suppression of that strain and that the defect was therefore attributable to the amber mutation.

Of the mutants of R23 isolated and characterized, the majority were maturation proteins mutants. Others were enzyme mutants. To date no clear polar or nonpolar coat protein mutant of R23 has been identified and the reason for its rarity remains obscure. A group of mutants, which we suspect are coat protein mutants, have been found to be very labile, mitigating against their complete characterization. From these studies we have thus far been unable to define a cistron whose sole function is the inhibition of host macromolecular synthesis.

The studies which have been performed in the characterization of R23 amber mutants are:

i. Cesium Chloride Density Gradient Analysis. Maturation protein mutants were identifiable by the characteristic pattern produced on CsCl density analysis of labelled phage (Fig. B1). When grown in the Su^- strain, defective particles lighter than normal phage were detected. In Su^+ strains, phage of normal density were produced. Enzyme mutants produced some phage of normal density in the Su^- strain; these were considered to represent revertants. Control analyses of wild type R23 lysates showed coincidence of ^3H- and ^{14}C-labelled phage peaks and

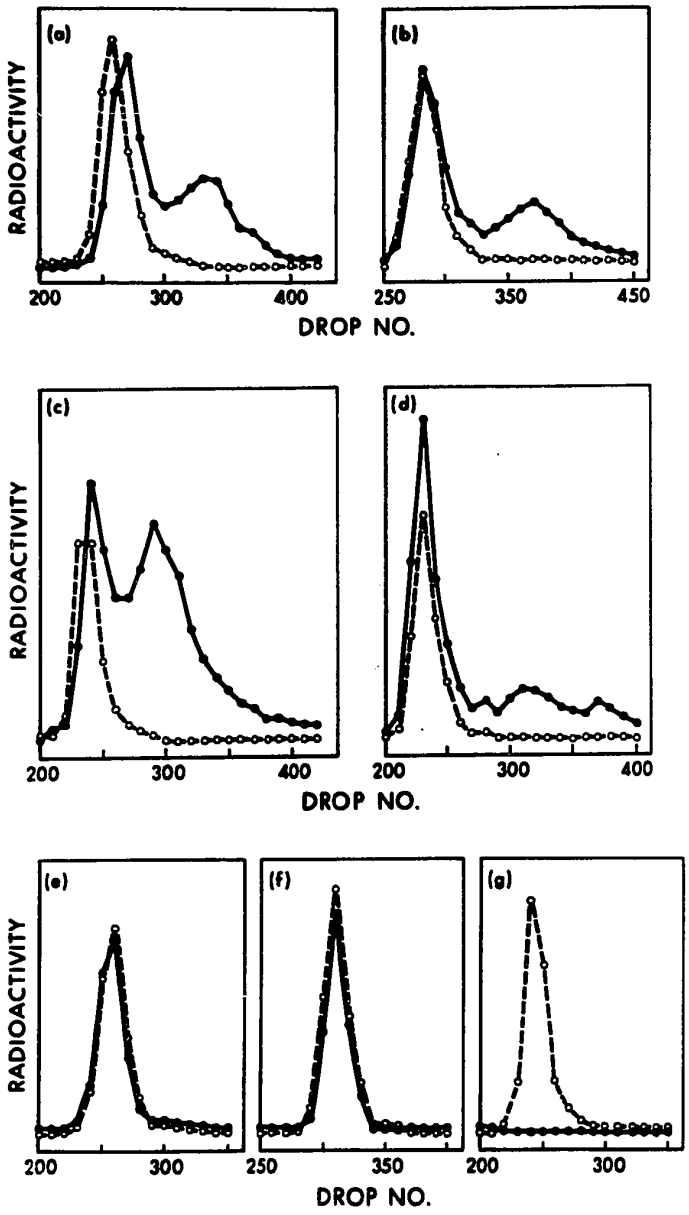

Fig. B1. CsCl Density Gradient Analysis of R23 Mutants. The experiments were performed as described in Materials and Methods. ●: ^{3}H-labelled phage. o: ^{14}C-labelled R23. (a) ^{3}H-uracil, maturation protein (MP) mutant, K38, (b) ^{3}H-uracil, MP mutant, K37, (c) ^{3}H-amino acids, MP mutant, K38, (d) ^{3}H-amino acids, MP mutant, K37, (e) ^{3}H-uracil, enzyme mutant, K38, (f) ^{3}H-uracil, R23, K37, (g) ^{3}H-uracil, uninfected, K38.

absence of phage in uninfected cultures.

ii. Viral RNA Synthetase Assays. Mutants deficient in viral RNA synthetase activity were detected by *in vitro* assays of crude extracts of infected cells. Maturation protein mutants had enzyme activities comparable to wild type R23 (Table B1). Enzyme mutants had essentially no activity when grown in the Su^- host; the levels of enzyme activity detected in the Su^+ strains infected with these mutants correlated with the degree of suppression. Polar coat protein mutants grown in the Su^- strain have been shown to induce little enzyme activity early in infection but excess enzyme beginning about 30 min after infection, as discussed in Chapter 1. Excess enzyme is synthesized also in Su^+ strains by polar mutants and in Su^- and Su^+ strains by nonpolar coat protein mutants.

iii. RNA Synthesis. Measurements *in vivo* of viral RNA synthesis corresponded to the results of *in vitro* enzyme assays. In Su^- strains in which host RNA synthesis was reduced to negligible levels by UV-irradiation (Fig. B2) or addition of rifampicin (Fig. B3), infection with viral enzyme mutants did not stimulate ^{3}H-uracil incorporation into TCA-insoluble products. In Su^+ strains, phage RNA was synthesized by these mutants and the level of ^{3}H-uracil incorporation was compatible with the efficiency of suppression in the Su^+ strain. The levels of RNA synthesis detected in UV-irradiated or rifampicin-treated K38 approached the values characteristic of wild type R23 in the case of maturation protein mutants. Coat protein mutants would be expected to stimulate wild type levels of ^{3}H-uracil incorporation except for polar mutants grown in the Su^- strain (Table 5, Chapter I).

TABLE B1

RNA SYNTHETASE ACTIVITY IN *E. COLI* K38

Phage	GMP Incorporation mμmoles/mg protein/20 min
R23	1.6 - 1.9
Maturation Protein Mutant	1.4 - 1.9
Enzyme Mutant	0.01 - 0.06

The experiment was performed as described in Materials and Methods.

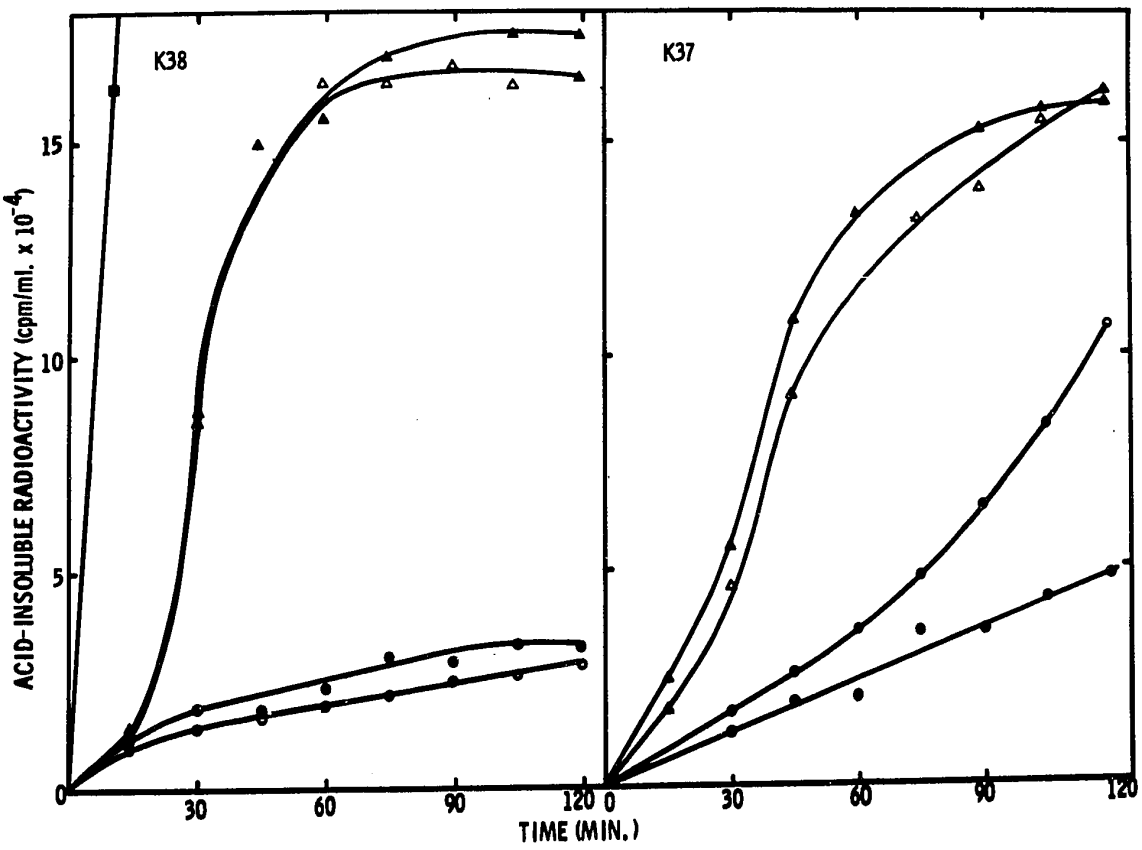

Fig. B2. RNA Synthesis in UV-Irradiated *E. coli* Infected with R23 Mutants. UV-irradiated K38 (Su^-) and K37 (Su I) were grown in Tryptone broth. ^{3}H-uracil (1 μc/ml) was added at the time of infection (0 min) with phage at a MOI of 10 phage/cell. Bacteria were irradiated and ^{3}H-uracil incorporation assayed as described in Materials and Methods. ●: uninfected, ▲: R23, o: enzyme mutant, Δ: maturation protein mutant, ■: unirradiated, uninfected.

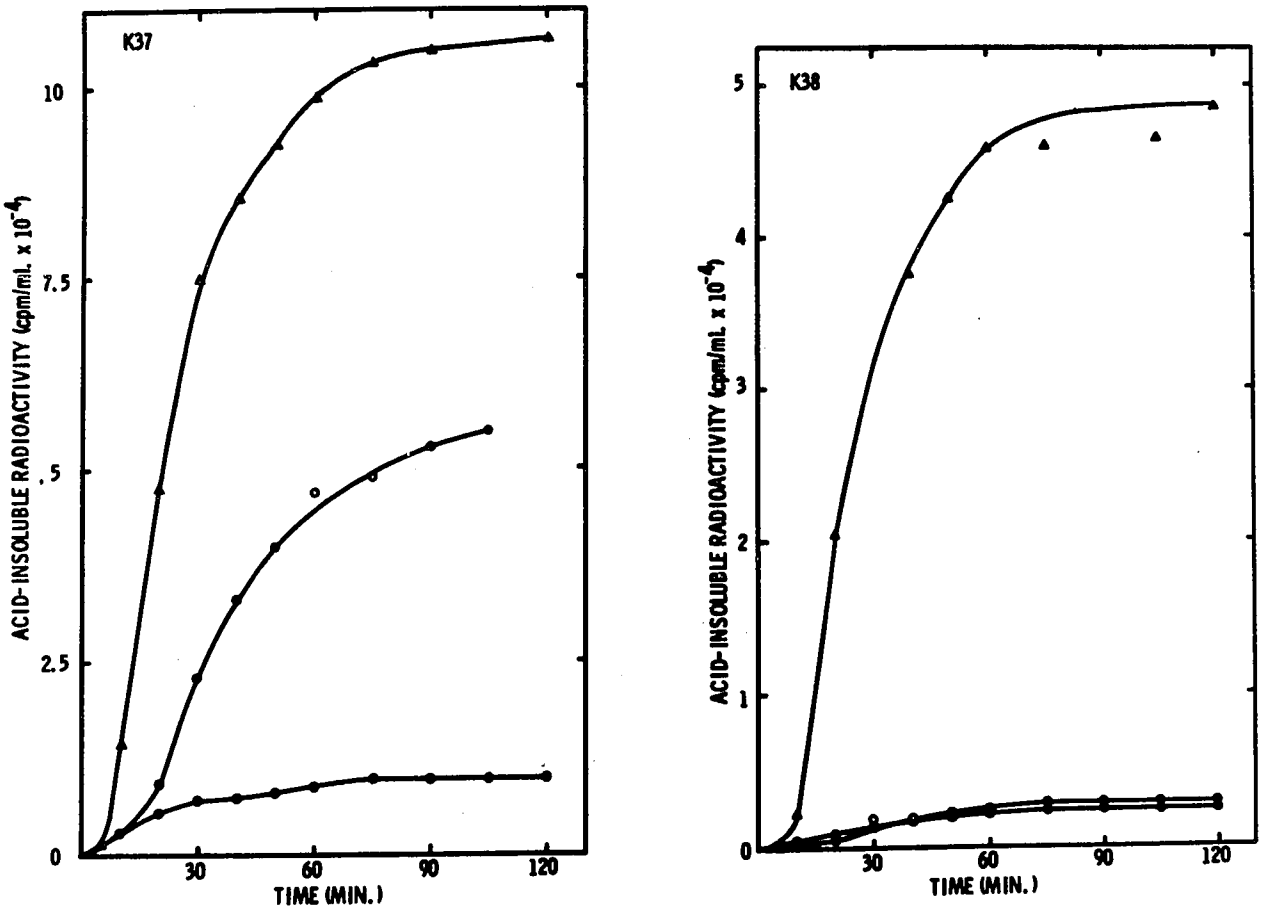

Fig. B3. RNA Synthesis in *E. Coli* Infected with RNA Synthetase Mutant in the Presence of Rifampicin. Strains K38 and K37 were grown in Tryptone broth and infected at a MOI of 10 phage/cell. Rifampicin, 50 μg/ml, was added 4 min after infection. ^{3}H-uracil was added 6 min after infection to a concentration of 5.7 μc/ml. Incorporation of ^{3}H-uracil into TCA-precipitable material at various times after addition of label was determined as described in Materials and Methods. ●: uninfected, o: enzyme mutant, ▲: R23.

The synthesis of RNA eventually encapsulated by phage coat protein was also examined by antibody-binding assays. In Su^- strains, enzyme mutants produced no phage RNA whereas maturation protein mutants produced as much phage RNA as wild type R23 (Table B2).

iv. Host Range. Maturation protein and enzyme mutants plated equally well on Su I, II and III strains, like wild type R23, and poorly on ochre suppressors and Su^- strains (Table B3). Coat protein mutants, in contrast, are known to plate efficiently only on Su I strains (Table 5, Chapter I) and can be identified, at least tentatively, by this characteristic.

v. Complementation Analysis. The mutants were classified into complementation classes by performing innumerable crosses between pairs of mutants. The yields of mutant phage in 3 types of crosses are represented in Table B4. Maturation protein mutants complemented with enzyme mutants as illustrated in Table B4. They displayed leakiness, i.e., they produced some mutant phage in the Su^- strain, while enzyme mutants were essentially not leaky.

b) Effect on the Host

1. Growth on Su^- Strain. The mutants were examined for ability to produce lysis of a culture of *E. coli* by measurement of the turbidity of the infected culture at 660 mμ (Fig. B4). Lysis induced by R23 began at about 60 min in Tryptone broth and 105 min in medium A. Maturation protein mutants lysed Su^- as well as Su^+ strains, like wild type R23; the time and degree of lysis varied to some extent with the particular mutant. In contrast, only a minimal reduction in turbidity was produced by enzyme mutants grown in K38, and this

TABLE B2

PHAGE RNA SYNTHESIS IN *E. COLI* INFECTED WITH R23 OR AMBER MUTANTS OF R23

	Phage RNA Synthesis in	
	K38 (Su^-)	K37 (Su^+)
	% of total RNA synthesis	
Uninfected	0	0
Infected with		
UV'd R23	0	0
R23	62	60
Maturation Protein Mutant	51 (42-62)	52 (42-60)
Enzyme Mutant	0.5 (0.1-1)	26 (16-40)

Synthesis of total RNA and phage RNA (measured by antibody-precipitable assay) was determined as described in Materials and Methods.

TABLE B3

HOST RANGE OF R23 AND ITS AMBER MUTANTS

Bacterial Host	R23	Maturation Protein Mutant	Enzyme Mutant
Su I	++	++	++
Su II	++	++	++
Su III	++	++	++
Su IV	++	-	-
Su V	++	-	-
Su^-	++	-	-

Phage were plated on various hosts by the agar overlay technique as described by Adams (1959).

TABLE B4

YIELD OF MUTANT PHAGE IN REPRESENTATIVE CROSSES

No Complementation No Leakage			No Complementation With Leakage			Complementation		
am E	am E	am E x am E	am A	am A	am A x am A	am A	am E	am A x am E
2	0	0	10	4	wt	2	0	20
0	0	0	6	0	5	1	0	68
0	0	1	0	0	0	1	2	11
0	0	0	4	25	0	0	0	28
0	0	0	6	5	1	0	0	48
0	1	1	6	14	2	0	0	wt
0	0	0	7	1	6	wt	0	42
0	0	0	2	0	8	1	0	27
0	0	0	5	7	3	0	wt	100
0	0	0	3	1	0	1	0	44
			13	22	3	0	0	170
			2	3	1	2	0	13
			1	0	3	2	1	36
			5	3	2	0	0	0
			1	4	7	2	0	146
a. 0.2	0.1	0.2	4.7	5.8	2.7	0.8	0.2	50.2
b.						2.0		125.5

a. Average number of phage per 0.2 ml sample
b. Average yield of mutant phage per tube

A, maturation protein mutant; E, enzyme mutant; wt, wild type.

Complementation analysis was performed as described in Materials and Methods.

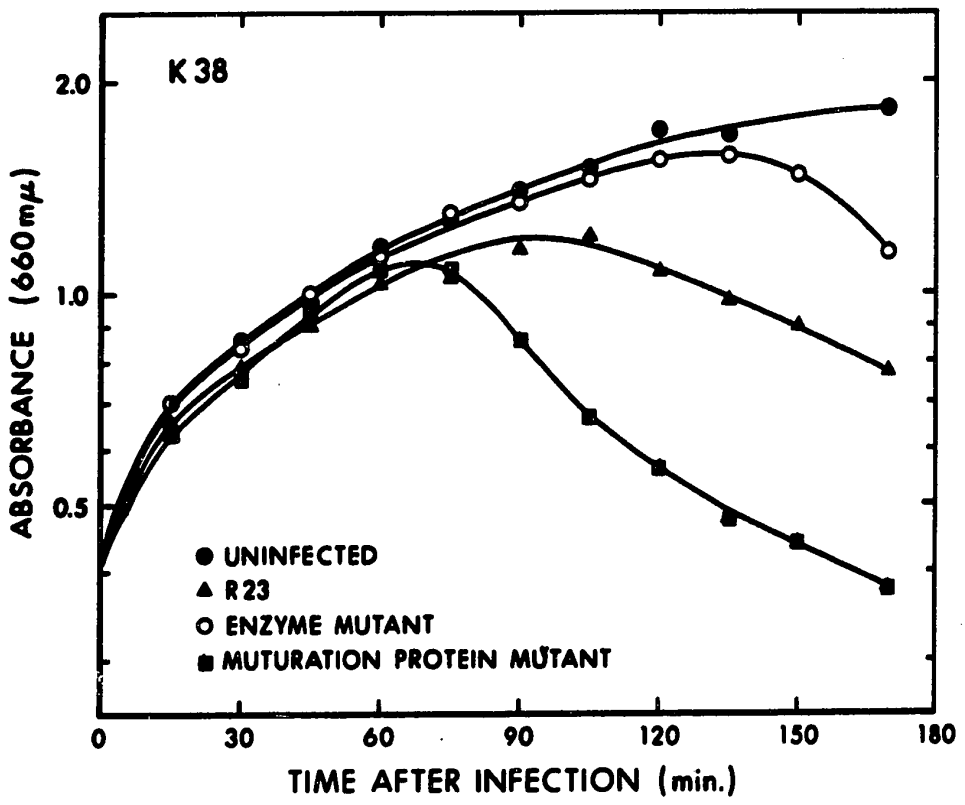

Fig. B4. Growth of R23 Mutants in *E. coli* K38. Growth measurements of infected bacteria grown in medium A were performed as described in Materials and Methods.

reduction occurred late in infection. Su^+ strains were lysed by these mutants. Coat protein mutants slow the growth of Su^- strains. Their effects in other strains have been discussed by Zinder and Lyons (1968).

2. Bacterial Survivors. The ability to kill the host was also determined by assay of the number of bacterial survivors remaining in an infected culture (Fig. B5). Maturation protein mutants were almost as efficient in killing the host as wild type R23. Enzyme mutants, on the other hand, had minimal effects on host viability.

3. Host Macromolecular Synthesis. The effect of R23 amber mutants on host RNA, DNA and protein synthesis was determined by measurement of incorporation of labelled precursors into TCA-precipitable material. Incorporation of ^{3}H-uracil (Fig. B6), ^{3}H-thymidine (Fig. B7) and ^{14}C-protein hydrolysate into acid-insoluble products in the Su^- host was inhibited by maturation protein mutants and R23 in a comparable manner, but enzyme mutants produced only a mild, transient inhibition followed by incorporation at a rate similar to that in uninfected cultures (Fig. B6,7). This differential effect of enzyme and maturation protein mutants was also observed in studies of β-galactosidase synthesis as will be discussed in Section C. UV-irradiated R23 produced a minimal reduction of the rate of ^{3}H-thymidine incorporation (Fig. B6).

Discussion

Amber mutants of R23 were isolated and characterized to determine the number of cistrons in the genome of R23, to examine the effect of mutations in each of the 3 known phage cistrons on host macromolecular synthesis, and to investigate the possibility of the existence of a

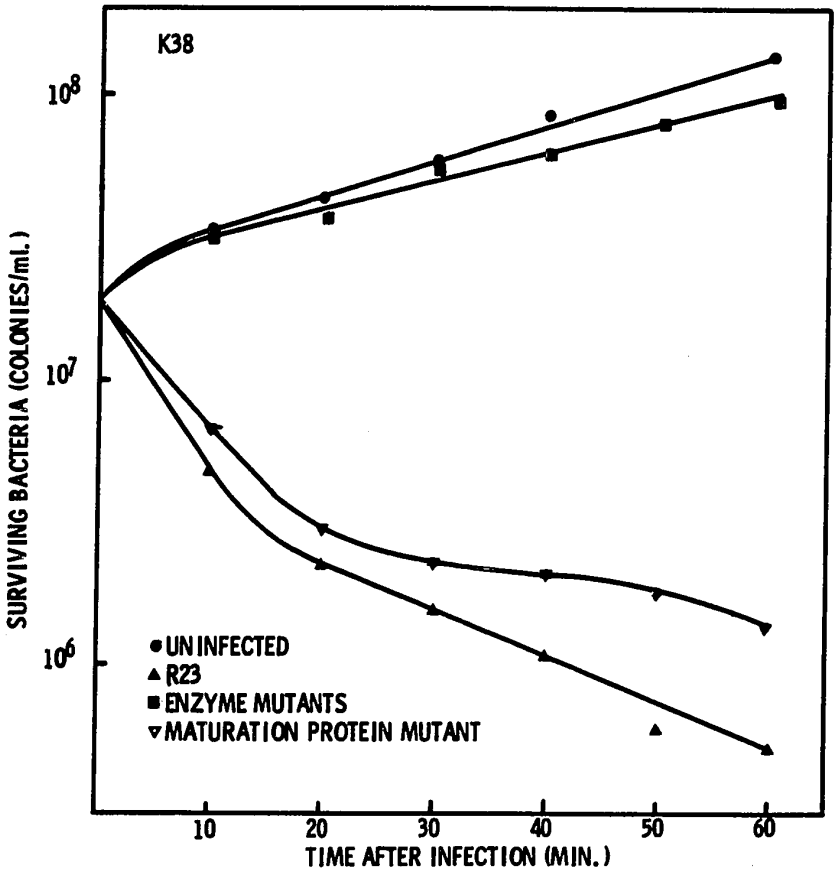

Fig. B5. Bacterial Survivors Following Infection by R23 Mutants. The experiment was performed as described in Materials and Methods. *E. coli* K38 was grown in medium A and infected at a MOI of 10 phage/cell.

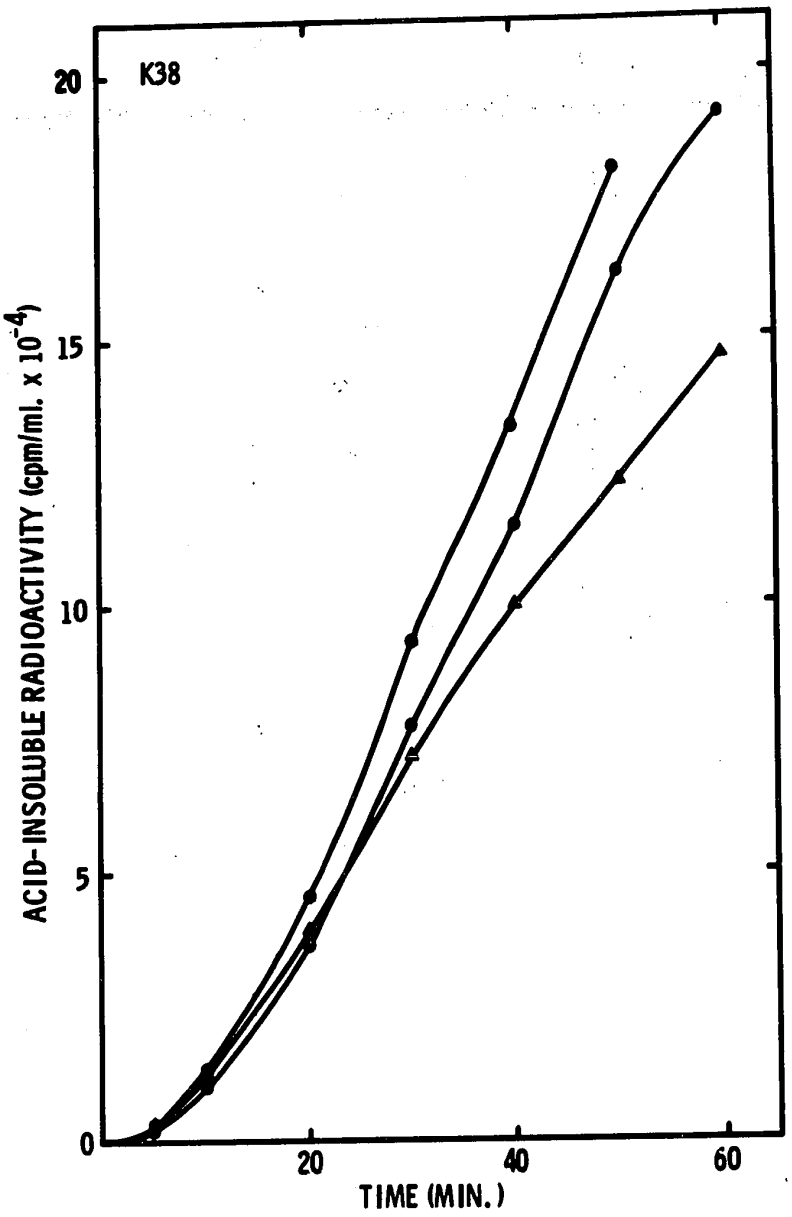

Fig. B6. RNA Synthesis in *E. Coli* K38 Infected with R23 Mutant. Bacteria grown in Tryptone broth was infected at a MOI of 10 phage/cell. ^{3}H-uracil was added 6 min after infection to a concentration of 6.2 μc/ml. Incorporation of radioactivity into TCA-precipitable material was determined at various times after addition of label as described in Materials and Methods. ●: uninfected, o: enzyme mutant, ▲: R23.

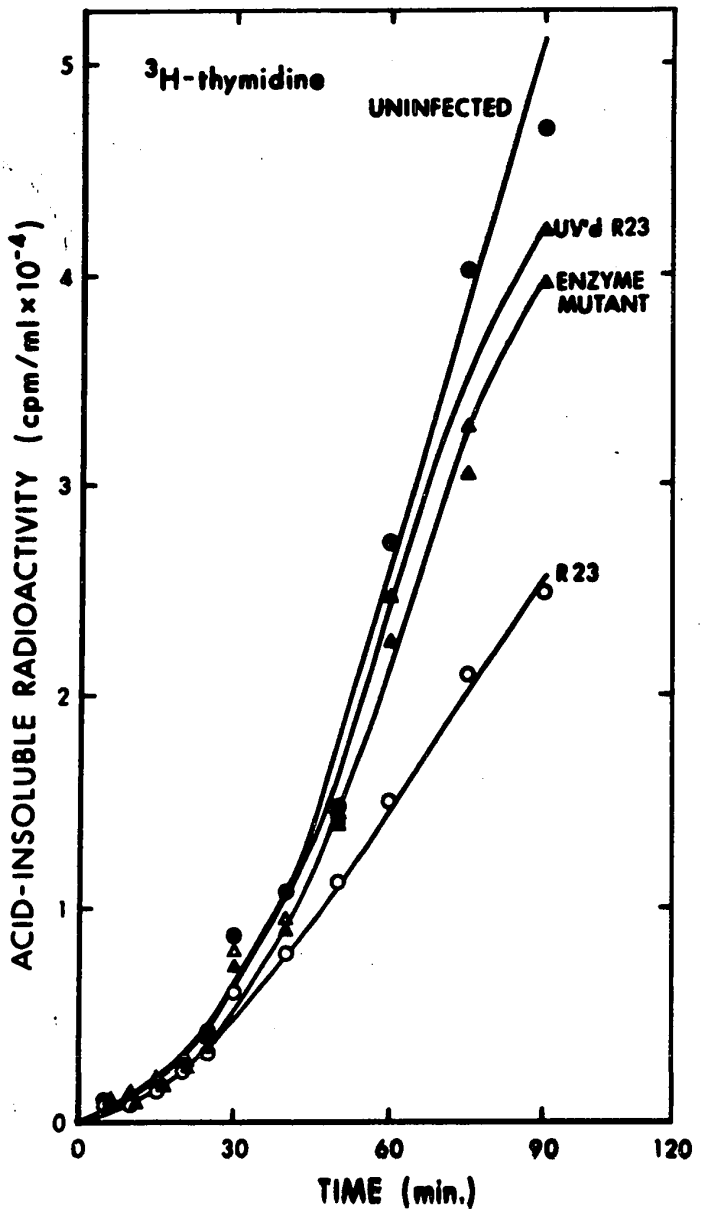

Fig. B7. DNA Synthesis in *E. Coli* Infected with R23 Mutants. Bacteria grown in medium A were infected with phage at a MOI of 10 phage/cell (time zero). Incorporation of ^{3}H-Thymidine (1 μc/ml) into acid-insoluble material was assayed as described in Materials and Methods.

new cistron responsible solely for host inhibition. Of the mutants isolated to date, maturation protein and enzyme mutants (but no coat protein mutants) have been fully characterized. Genetic studies of RNA phage mutants (isolated after nitrous acid and fluorouracil mutagenesis) are somewhat hampered by relatively high reversion rates and the frequent occurrence of double mutations. It is probable that coat protein mutants of R23 will be identified pending more exhaustive investigation.

Mutants defective only in host inhibition have not been found; this may be a reflection of the isolation procedure. The mutants which have been isolated have been valuable in clarifying the role of each gene in the effect of R23 on host functions, and will be discussed in more detail in this context in subsequent sections.

C. Effect of R23 on the Inducible Synthesis of β-Galactosidase

Introduction

Metabolism of lactose in *E. coli* involves transport into the cells by the galactoside permease or M protein followed by hydrolysis by the enzyme β-galactosidase into glucose and galactose. The structural genes for these proteins are grouped with the controlling genes (regulatory gene, promoter and operator) in the order i-p-o-z-y-a (Ippen *et al.*, 1968) to form the lactose operon (Jacob & Monod, 1961). The structural genes z, y and a code for β-galactosidase, galactoside permease and thiogalactoside transacetylase, respectively. These genes are sequentially transcribed (into a polycistronic mRNA) and translated from the promoter and starter, respectively, the starter being located probably between the o and z genes (Davies & Jacob, 1968).

The lac operon is an inducible or negative control system. The repressor binds to the operator and inhibits transcription of these genes. The inducer binds to the repressor lowering its affinity for the operator and the operon is then derepressed. Many β-galactosides are effectors, the most potent being IPTG, a gratuitous inducer.

The kinetics of β-galactosidase synthesis have been well studied (Pardee & Prestidge, 1961; Kepes, 1963; Nakada & Magasanik, 1964). Addition of inducer is followed by a lag of 3 min; the rate of enzyme synthesis then increases rapidly and becomes constant approximately 6 min later. The lag period is related to the synthesis of β-galactosidase-specific mRNA. The phase of enzyme induction can be separated from the phase of enzyme production by

removal of the inducer. Removal of the inducer at the end of the lag period by dilution or by filtration of the culture and resuspension in inducer-free medium allows the cells to express their enzyme-forming capacity, or produces an elementary wave of induced enzyme synthesis. There is a 3 min delay after inducer withdrawal before enzyme synthesis ceases. The rate of decay of β-galactosidase-forming capacity after inducer removal is a reflection of the stability of enzyme-specific mRNA. A semi-log plot of the remaining synthesizing ability versus time gives the half-life of the messenger.

Because of this elegant analysis of the lactose operon and its ready amenability to experimental manipulation, the inducible synthesis of β-galactosidase has served as an ideal model in the investigation of the mechanism of bacteriophage inhibition of bacterial protein and mRNA synthesis. Thus, in T-even phage infection, the inhibition of β-galactosidase synthesis has been shown to be due, in part, to an arrest in the initiation of synthesis of mRNA (Kaempfer & Magasanik, 1967a,b). Infection with T-even phage at the time of inducer addition completely and immediately prevents enzyme synthesis (Benzer, 1953; Kaempfer & Magasanik, 1967a). In cells carrying the F episome, the decay of enzyme-forming capacity proceeds at the same rate whether induction is arrested by phage infection or by inducer withdrawal. But in F^- cells, the rate of decay after T-even phage infection is twice as fast as after inducer removal (Kaempfer & Magasanik, 1967a).

Similar results were obtained by Rouviere *et al.* (1968). The decrease in translation yield after T4 infection was not due to a loss in the capacity for initiating message reading. Addition of purified

T4 ghosts also led to a dramatic fall in β-galactosidase synthesizing capacity; the rate of this decrease was dependent on multiplicity, unlike the inactivation of β-galactosidase messenger by phage infection. In infection with T4 phage, the remaining synthetic ability was not due to uninfected cells, but the residual β-galactosidase synthetic capacity after ghost adsorption was proportional to the number of survivors.

The inhibition of host mRNA synthesis does not depend on the expression of T4 genes, since the inhibition occurs promptly on infection, does not require protein synthesis or breakdown of host DNA (Nomura *et al.*, 1962; Terzi, 1967) and is unaffected by UV irradiation of phage (Kaempfer & Magasanik, 1967a). But the enhancement of mRNA destruction appears to require viral gene expression since it is abolished by UV irradiation.

The synthesis of β-galactosidase after R23 infection was studied in an attempt to elucidate the mechanism of its inhibition of bacterial protein synthesis and to determine the requirement for viral genetic information for this effect.

Results

a) Effect of RNA Phage on β-Galactosidase Synthesis

A study of the effect of R23 infection on β-galactosidase synthesis was somewhat complicated by the fact that rapid infection of an entire bacterial population could not be obtained. Maximal phage adsorption required 4 min and 10% of the bacterial population survived the initial infection. Despite this limitation, the effect of R23 on β-galactosidase synthesis could be determined. The important

overall effect was a reduction of β-galactosidase synthesis. The synthesis of this enzyme was not completely inhibited after R23 infection (Fig. C1). The enzyme activity in an infected culture was greater than that which could be accounted for by the population of uninfected bacteria, although the latter (which varied from one culture to the other) affected the final level of enzyme activity observed.

Detailed examination of the course of enzyme induction in R23 infected cells showed 3 characteristic stages. An immediate phase, occurring during the first 10-15 min of infection, was characterized by levels exceeding those in uninfected cells (Fig. C2). (This phase will be considered in more detail in section D). It was followed by a gradual diminution in enzyme synthesis in infected cells from 10 to 25 min (the "early" inhibitory stage) (Fig. C2), and then by a rapid and more pronounced inhibition (the "late" inhibitory period) (Fig. C3). This period of maximal inhibition after the first 20 min of infection corresponds to the time of maximal phage coat protein synthesis (Watanabe *et al.*, 1968).

Maximal inhibition by R23 was achieved at a multiplicity of 10 phage/cell (Fig. C4). This multiplicity is associated with the largest number of infected cells and the smallest number of bacterial survivors, as already shown. Since there is a small population of uninfected bacteria, the level of enzyme synthesized was a function of the effective multiplicity of infection and the number of surviving bacteria.

The effect of the other RNA phages (described in section A) was compared to that of R23 using a multiplicity of 20 phage/cell.

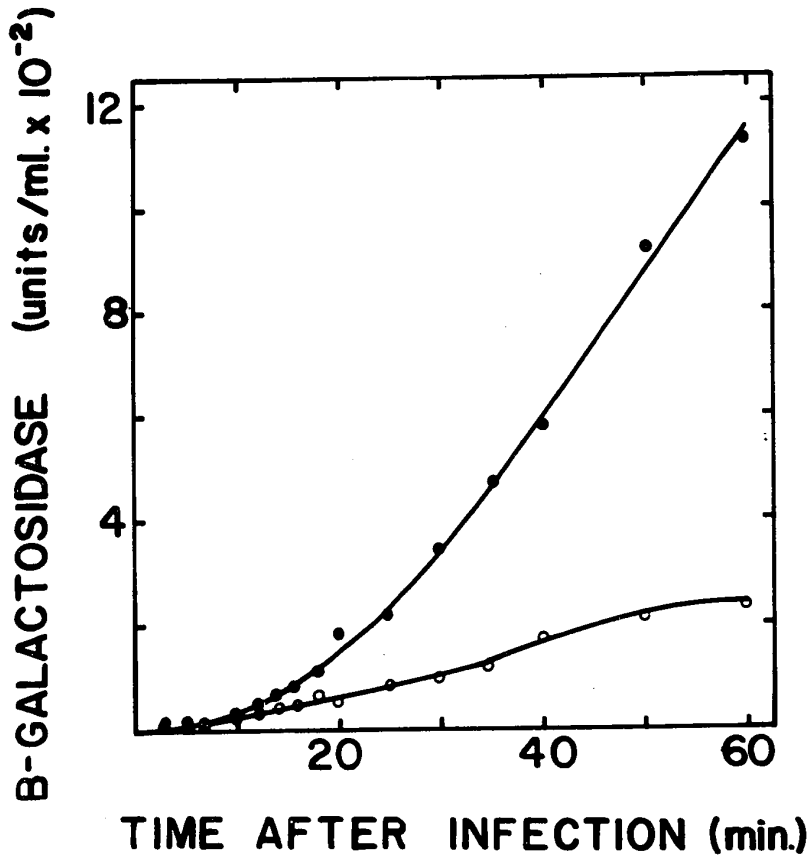

Fig. C1. Effect of R23 Infection on β-Galactosidase Induction.
β-Galactosidase was induced and assayed in *E. coli* K38 as described in Materials and Methods. R23 was added at a MOI of 10 phage/cell.
●: control. o: infected.

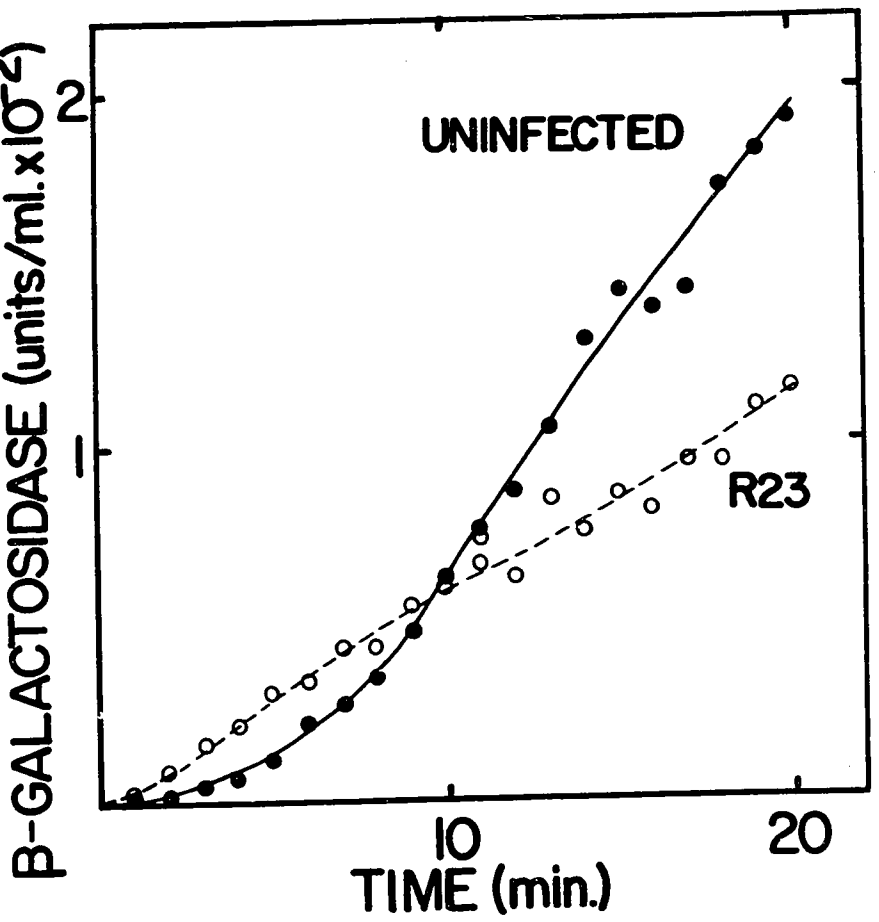

Fig. C2. Immediate Effect of R23 Infection on β-Galactosidase Synthesis. *E. coli* K38 was infected at a MOI of 20 phage/cell. β-Galactosidase was induced and assayed as described in Materials and Methods.

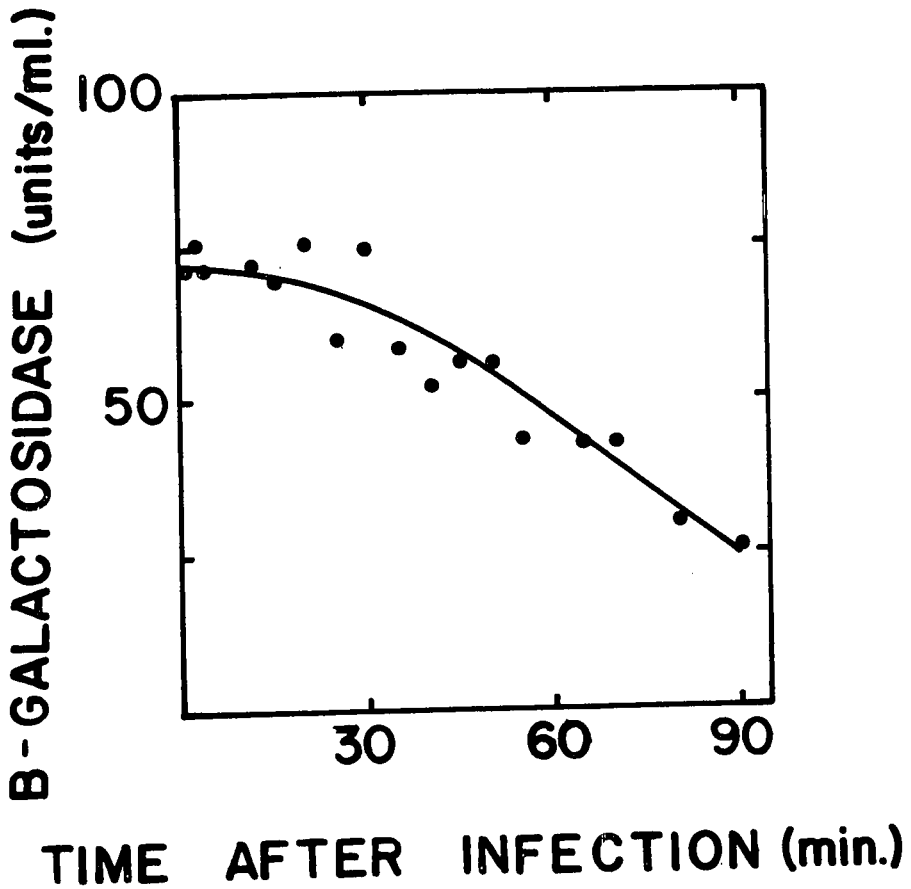

Fig. C3. Rate of β-Galactosidase Synthesis in R23-Infected Bacteria. *E. coli* K38 was infected with R23 at a MOI of 20 phage/cell. At various times after infection IPTG was added and 15 min later the cultures were analyzed for β-galactosidase activity.

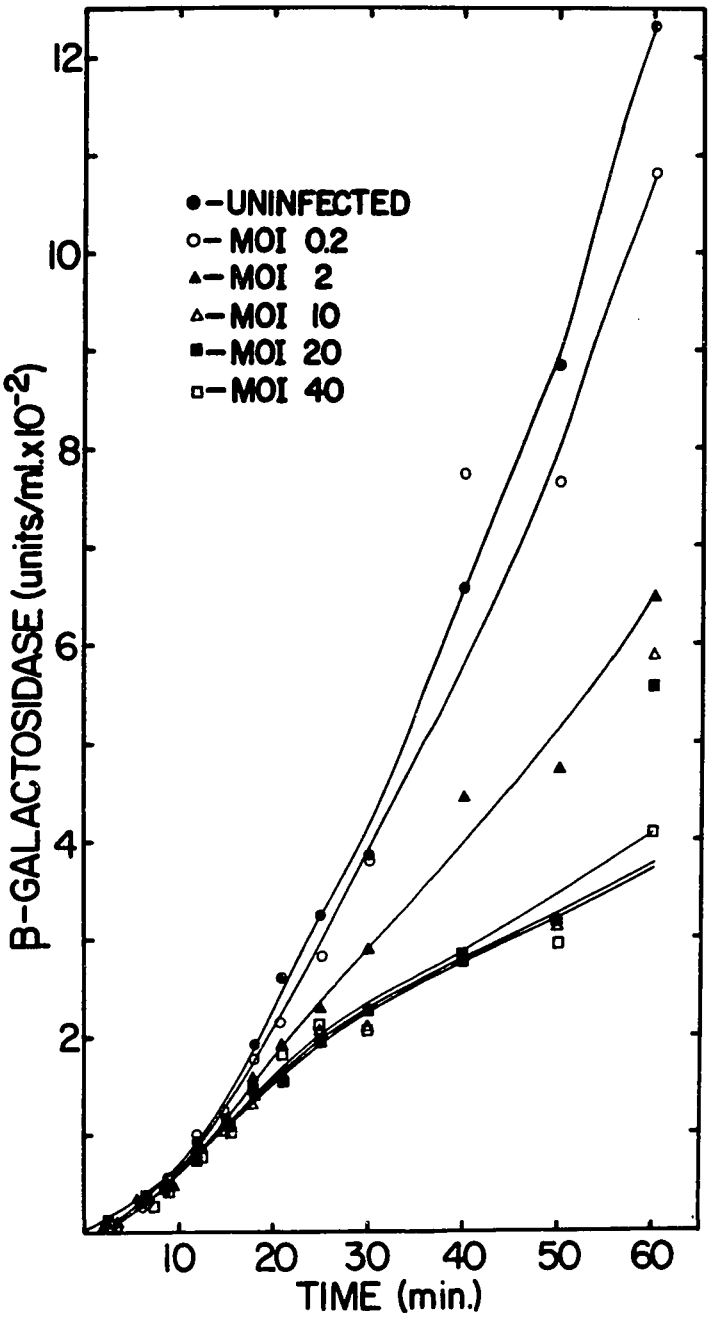

Fig. C4. Effect of Multiplicity of Infection on β-Galactosidase Synthesis. *E. coli* K38 was infected with R23 at a MOI of 0.2 to 40 phage/cell. β-Galactosidase was induced and assayed as described in Materials and Methods.

The inhibition of β-galactosidase synthesis produced by Qβ was minimal compared to R23, while f2, R34 and R40 had intermediate effects (Fig. C5). The inhibition by these latter phages did not differ significantly from the effect of R23 during the first 25-30 min but was noticeably less during the late phase.

b) Requirements for Effect of R23 on β-Galactosidase Synthesis

1. Effect of UV-irradiation of R23. To determine whether inhibition of β-galactosidase following R23 infection required genetic function(s) of the phage, the effect of UV-irradiated R23 was investigated. UV-irradiated phage inhibited enzyme synthesis during the first 20-30 min of infection like unirradiated R23, but not after this time (Fig. C6). It thus appeared that the inhibition of β-galactosidase production by R23 could be considered to consist of a component which did not require expression of the viral genome and a second component which occurred only in the presence of a functional phage genome. For convenience, these will be referred to as the "early" and "late" phases of inhibition.

2. Gene Responsible for "Late" Inhibition. To determine the gene(s) required for late inhibition of bacterial protein synthesis, the effect of R23 amber mutants was examined. None of the maturation protein mutants demonstrated a defect in the ability to inhibit β-galactosidase synthesis either early or late. On the other hand, a mutant defective in the viral RNA synthetase cistron was unable to inhibit β-galactosidase synthesis late in infection. Like the UV-irradiated phage, this mutant was able to transiently inhibit enzyme synthesis (Fig. C7), but the inhibition was reversed after

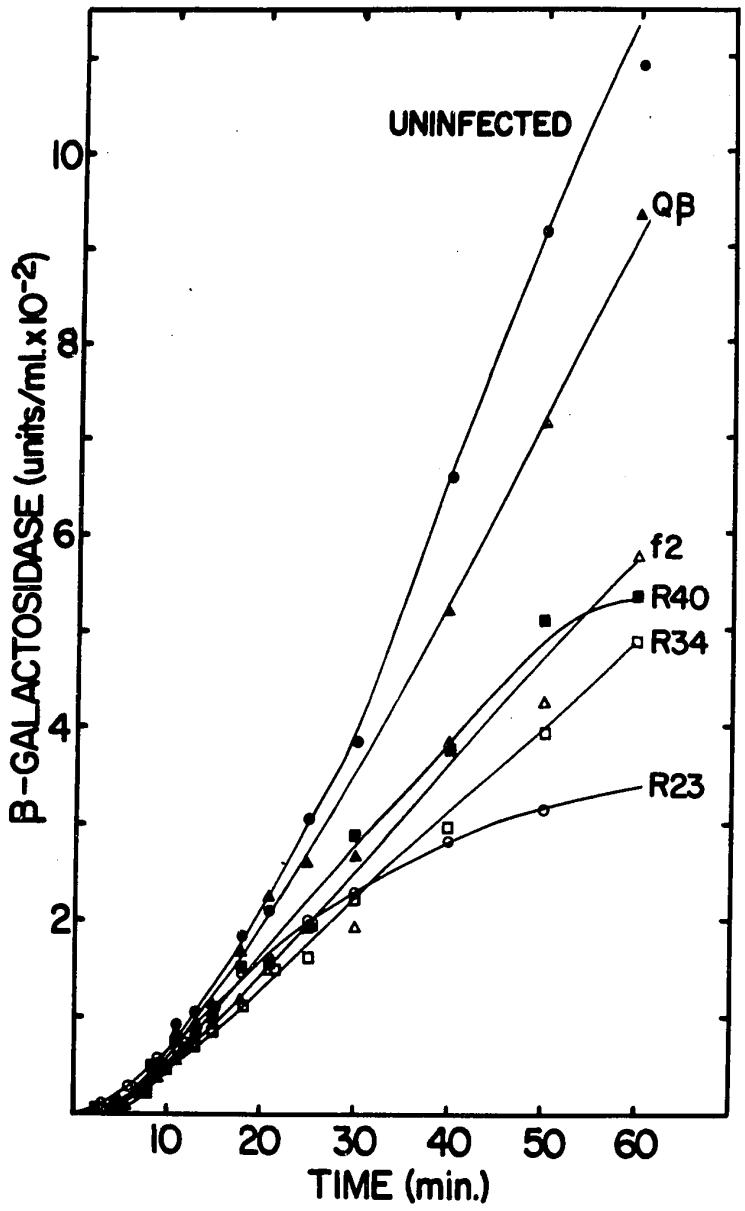

Fig. C5. Effect of Different RNA Phages on β-Galactosidase Synthesis. *E. coli* K38 was infected with each phage at a MOI of 20 phage/cell. β-Galactosidase was induced and assayed as described in Materials and Methods.

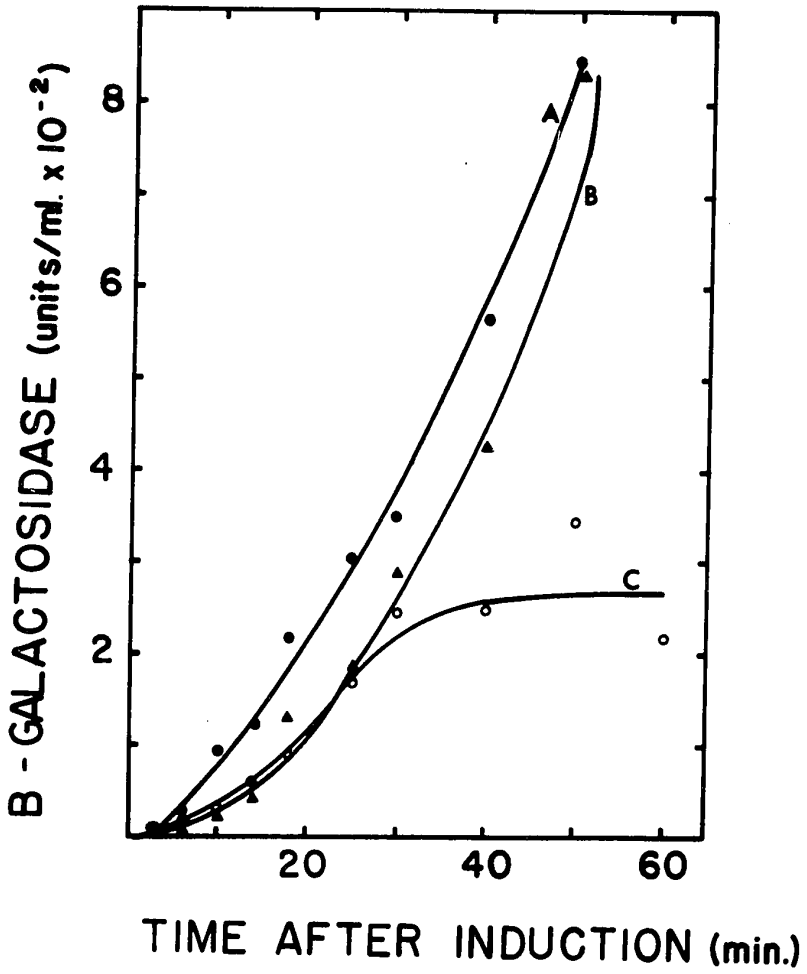

Fig. C6. Effect of UV-Irradiated R23 on β-Galactosidase Synthesis. *E. coli* K38 was infected with R23 at a MOI of 10 phage/cell (C) or with the same number of UV-irradiated phage particles (B). β-Galactosidase was induced in these cultures and an uninfected culture (A) and assayed as described in Materials and Methods.

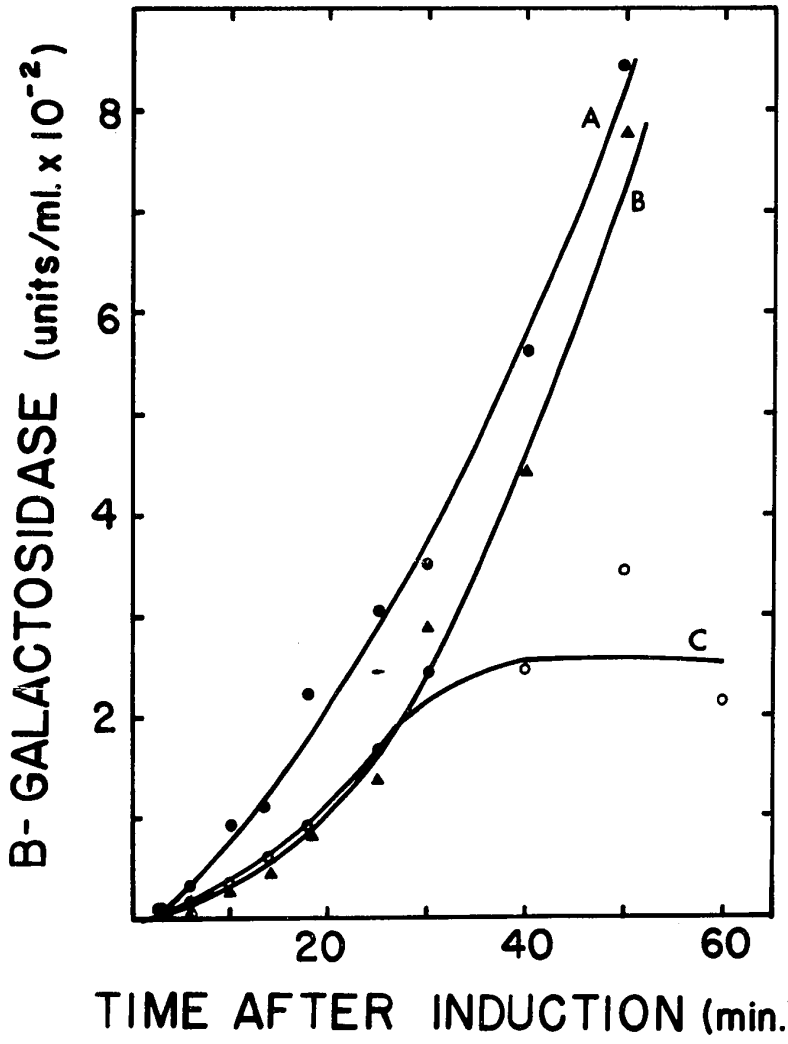

Fig. C7. Effect of an RNA Synthetase Mutant of R23 on β-Galactosidase Synthesis. The experiment was performed as in Fig. C6 except that curve B represents a culture infected with an RNA synthetase mutant at a multiplicity of 10 phage/cell.

25-30 min of infection. It appeared, therefore, that expression of the RNA synthetase cistron was necessary for maintenance of the inhibition of bacterial protein synthesis late in infection.

3. Requirements for "Early" R23 Effect. The requirements for the early phage effect on β-galactosidase synthesis were investigated. The inability of defective phage particles (produced by propagating maturation protein mutants in a non-suppressor host) to inhibit enzyme synthesis like wild type R23 (Fig. C8) indicated that early inhibition required interaction of the maturation protein with the F pilus.

Penetration of viral RNA was necessary for prolonged inhibition but not for immediate stimulation or transient inhibition of β-galactosidase synthesis. Infection in the presence of RNase, which incompletely prevents injection of RNA, caused early but not late inhibition (Fig. C9), a pattern similar to that seen with infection by UV-irradiated phage or enzyme mutants. This point was confirmed by studying enzyme induction in the presence of EDTA, which prevents penetration, but does not affect injection of viral RNA. EDTA, at a concentration of 5 mM, inhibited enzyme synthesis in uninfected cells by 60% and reduced the infective centre titre to 9.1% of control values, but did not prevent the immediate increase in enzyme synthesis in infected cultures which lasted 15 min (Fig. C10). This initial stimulation was followed by depression of enzyme synthesis; it was unlikely that this inhibition could be accounted for solely by the small population (0.1%) of successfully infected cells. It could be equated with the early phage effect since late inhibition, which is dependent on intracellular expression of the phage genome, would not

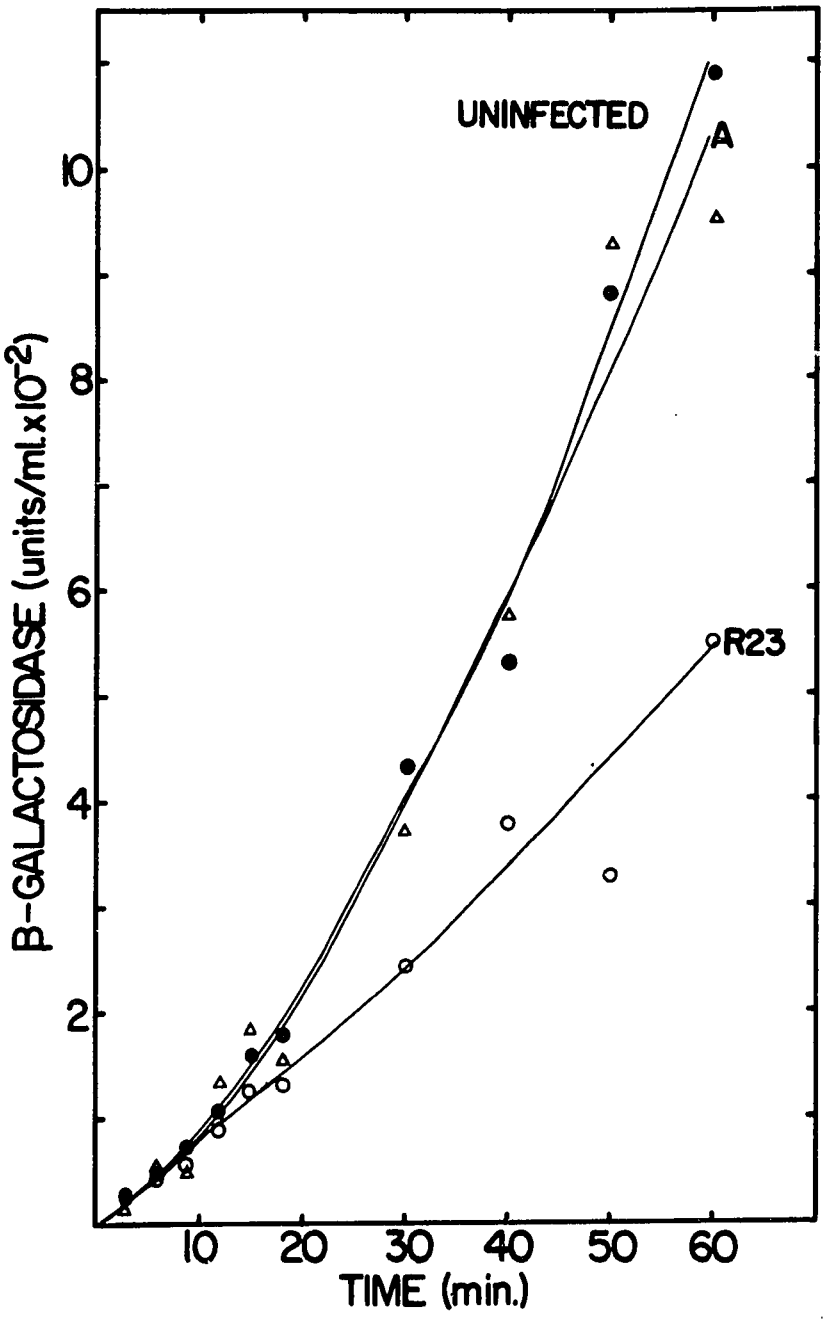

Fig. C8. Effect of Defective Particles on β-Galactosidase Synthesis. *E. coli* K38 was infected with R23 or defective particles produced by propagating maturation protein mutants in the Su^- host (curve A) at a MOI of 10 phage/cell. β-Galactosidase was induced and assayed as described in Materials and Methods.

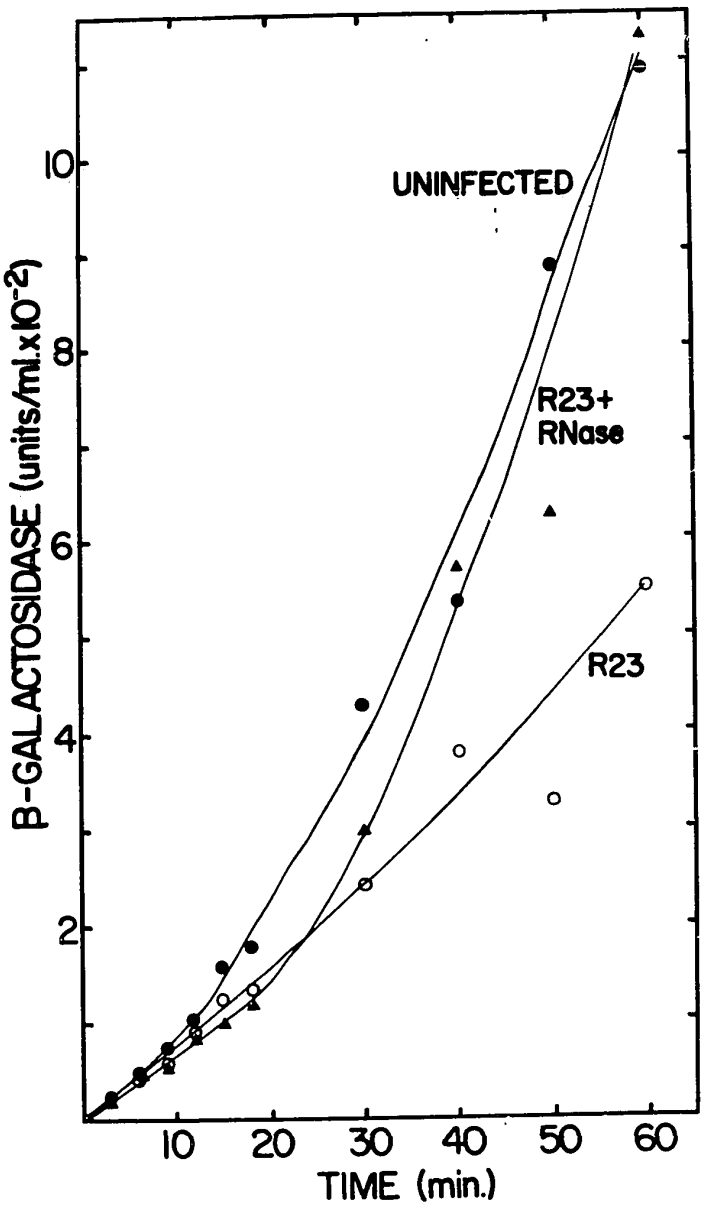

Fig. C9. Effect of RNase on R23-Induced Inhibition of β-Galactosidase Synthesis. *E. coli* K38 was infected at a MOI of 10 phage/cell. RNase was added at the time of infection to one culture of infected cells to a concentration of 100 μg/ml. β-Galactosidase was induced and assayed as described in Materials and Methods.

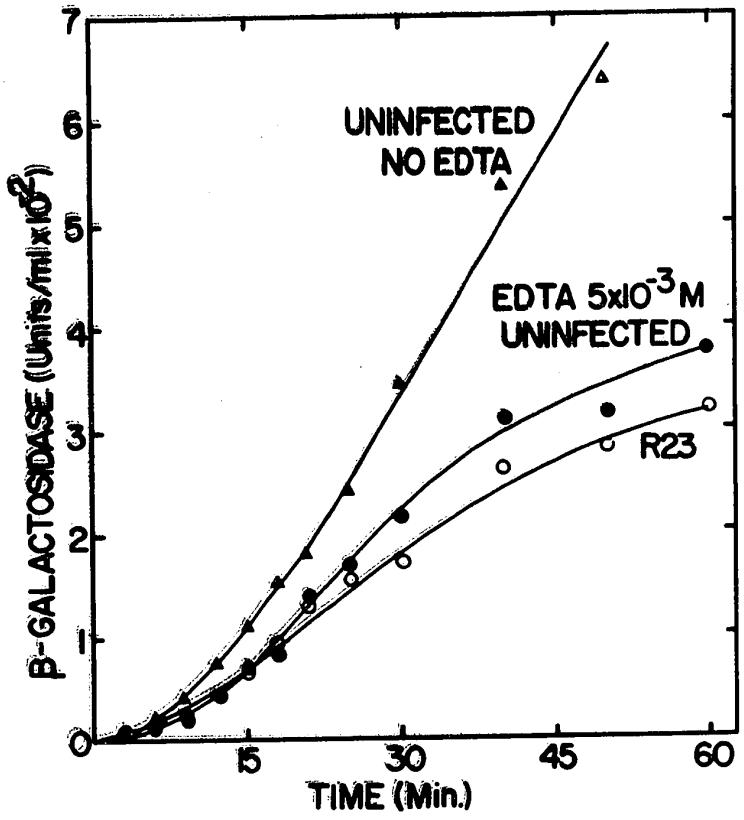

Fig. C10. Effect of EDTA on R23-Induced Inhibition of β-Galactosidase Synthesis. *E. coli* K38 was grown in medium A lacking $CaCl_2$ to a density of 2 X 10^8 cells/ml. The culture was divided into 3 parts and EDTA added to 2 of the cultures to a concentration of 5 mM. R23 was added at a MOI of 20 phage/cell. β-Galactosidase was then induced and assayed as described in Materials and Methods.

be expected to occur in the absence of penetration of viral RNA. In the presence of 5 mM EDTA, the β-galactosidase synthesized in infected cells at 60 min was 85% of that synthesized in uninfected cells, while in the absence of EDTA, the corresponding value was 35%, i.e., enzyme synthesis was inhibited by 15% in the presence of EDTA and by 65% in its absence. Thus about 23% of the total enzyme inhibition was related to the early phase and 77% to the late phase.

c) Mechanism of "Early" Inhibition by R23

The synthesis of β-galactosidase continued for a longer period after R23 infection than after inducer removal (Fig. C11), suggesting that R23 differed from T2 in its ability to inhibit mRNA synthesis (Kaempfer & Magasanik, 1967a). Removal of inducer and resuspension in inducer-free medium allowed induced cells to express their enzyme-forming capacity. In uninfected and R23-infected cultures, enzyme synthesis ceased 7-10 min after inducer removal (Fig. C12). The final enzyme level, however, was lower in infected cells than in uninfected cells. Thus, although R23 infection was unable to completely prevent the induction of β-galactosidase, it limited the capacity for enzyme synthesis. This effect was even more marked when inducer was added for a period of 3 min at 30 min after infection. However, when R23 was added immediately after a 3 min exposure to IPTG and removal of inducer, the appearance and final level of enzyme activity were identical to that for inducer removal in uninfected cells. Thus, the final level of enzyme activity was reduced when R23 was present during the period of synthesis of enzyme-specific mRNA, but infection by R23 did not

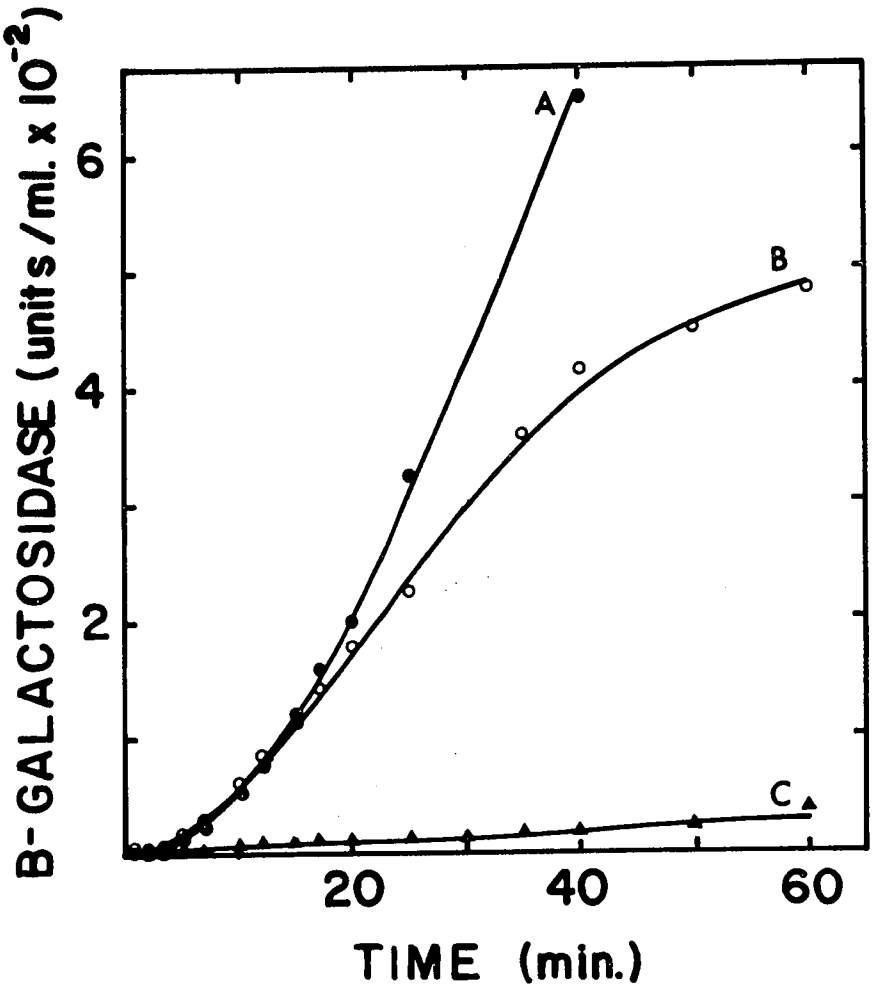

Fig. C11. Effect of Inducer Removal and R23 Infection on β-Galactosidase Synthesis. To cultures of *E. coli* K38, IPTG was added at 0 min. A: control (●). B: infected with R23 at a multiplicity of 20 phage/cell at 0 min (○). C: inducer removed at 3 min by filtration (▲) as described in Materials and Methods.

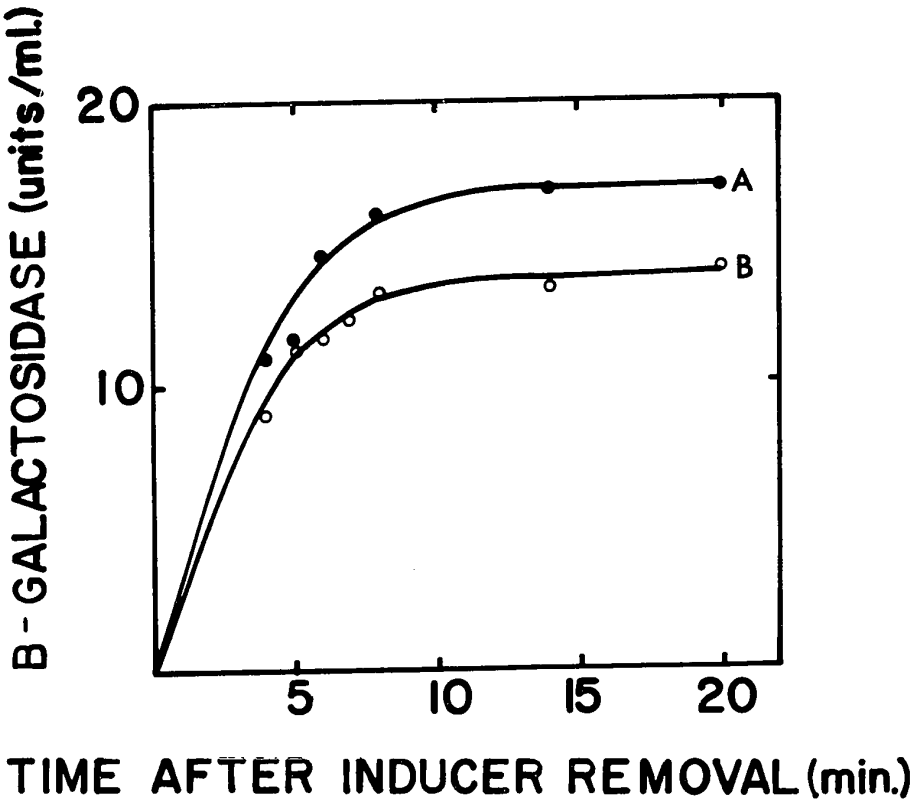

Fig. C12. β-Galactosidase Synthesis Following Inducer Removal in Infected and Uninfected Cultures. *E. coli* K38 was infected with R23 at a MOI of 10 phage/cell. Inducer was removed from uninfected (A) and infected (B) cultures 3 min after induction by filtration. Induction and assay of β-galactosidase and inducer removal were performed as described in Materials and Methods.

affect the enzyme-forming capacity once the mRNA had been formed.

The effect of R23 infection on the rate of decay of enzyme-forming capacity after inducer removal was examined in cells infected for 3 min. The capacity for enzyme synthesis decayed at an exponential rate with a $t_{\frac{1}{2}}$ of 1.6 min in uninfected cells (Fig. C13). The rate of decay was unaltered by the presence of R23 during the period of induction, though the final level of enzyme synthesized was diminished. Similarly, the rate of decay was unaltered when cultures infected with R23 for 30 min were exposed to inducer for 3 min. Finally, addition of R23 after the period of enzyme induction did not affect the rate of decay of enzyme-forming capacity. Thus, it appeared that R23 did not at any time affect the stability of enzyme-specific mRNA.

Discussion

Infection by R23 reduced both the rate of incorporation of radioactive amino acids (section A) and the synthesis of β-galactosidase in *E. coli*. Although the inhibition of β-galactosidase was noted shortly after infection, the maximal effect occurred after the first 20 min. This was compatible with the large amount of phage coat protein synthesized late in infection. The inhibition of β-galactosidase by R23 differed from that observed with T-even phage infection. Infection of *E. coli* by T-even phage at the time of inducer addition completely prevented the synthesis of β-galactosidase (Benzer, 1953; Kaempfer & Magasanik, 1967a). R23, in contrast, limited the enzyme-forming capacity but was unable to completely inhibit it.

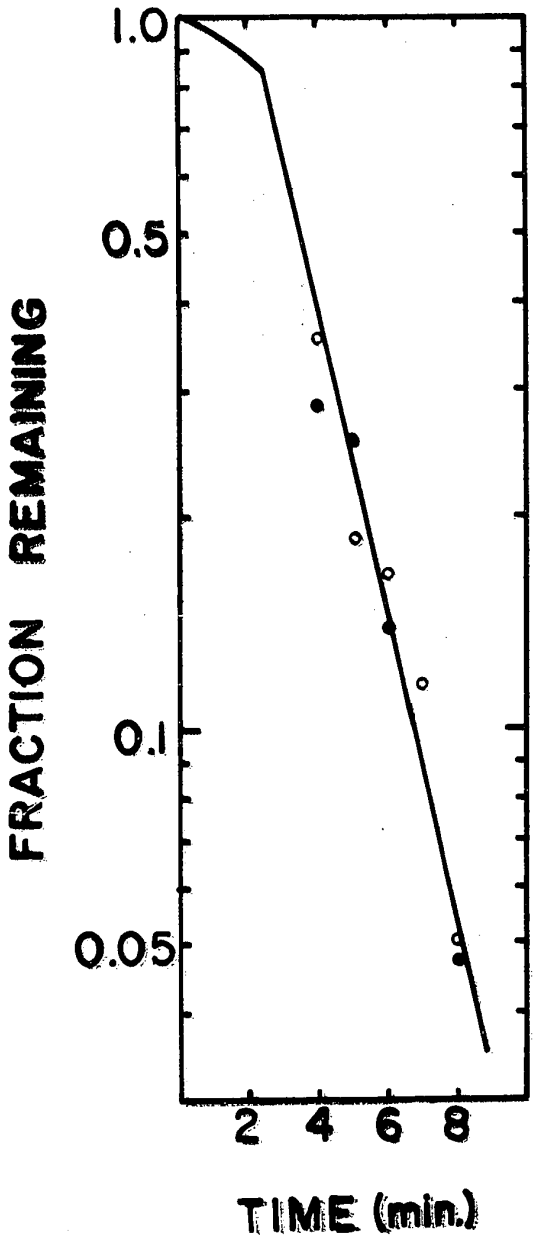

Fig. C13. Decay of β-Galactosidase Synthesis as a Function of Time Following Inducer Removal in Uninfected and R23-Infected Cultures. Uninfected (●) and infected (o) cells of *E. coli* K38 were induced for 3 min and transferred to inducer-free medium (from Fig. C12). The points represent $(E_{max} - E_t)/ E_{max}$ at any given time t; E_{max} is the final level of enzyme activity reached after inducer removal.

The diminution in β-galactosidase synthesizing capacity could result from 1) an effect of R23 on the rate of overall protein synthesis, 2) interference with synthesis of enzyme-specific mRNA 3) suppression of expression of this message, 4) alteration of stability of enzyme-specific mRNA, or a combination of these 4 factors. It seemed unlikely that the diminution in enzyme synthesis was due to an effect of R23 on the rate of overall protein synthesis since the latter did not appear to be greatly different in infected cultures during the first 20 min compared to uninfected cultures (Fig. A5). Furthermore, R23 affected the final level of enzyme activity when present during the period of synthesis of enzyme-specific mRNA, but not once the enzyme-specific mRNA had been formed. Thus, the inhibition by R23 could not be attributed to a suppression of translation of enzyme-specific mRNA. Finally, unlike T-even phage infection, (Kaempfer & Magasanik, 1967a), R23 infection did not at any time affect stability of the mRNA. Thus, it appeared that R23 reduced the β-galactosidase synthesizing capacity by interfering with the synthesis of enzyme-specific mRNA.

Inhibition of enzyme synthesis by R23 appeared to have 2 distinct phases. Inhibition during the first 20-25 min of infection did not require viral penetration but was dependent on phage adsorption; it was absent in infection with defective particles lacking the maturation protein. The role of RNA injection was not unequivocally established. It seemed likely that injection was not a requirement for early inhibition since the latter was observed in the presence

of RNase, but RNase does not completely prevent injection of viral RNA. The early phase did not require viral genome expression since it was produced by UV-irradiated phage and viral RNA synthetase mutants.

A second function appeared to determine the inhibition of β-galactosidase after the first 20-25 min of infection. This late inhibition was absent in infection by UV-irradiated R23 and R23 enzyme mutants, but present during infection by phage defective in the maturation protein. Expression of the viral RNA synthetase cistron appeared to be required for maintenance of inhibition since, in its absence, bacterial protein synthesis resumed after a transient arrest.

The difference in effect of the 5 phages R23, R34, R40, f2 and Qβ was predominantly in their ability to produce late inhibition. There was a correlation between the inhibition of β-galactosidase and the amount of viral RNA produced by these phages (Watanabe *et al.*, 1968).

From quantitative analysis of enzyme synthesis in the presence and absence of EDTA, it was estimated that 75-80% of the total inhibition could be attributed to the late inhibition and 20-25% to the early effect. These figures agreed with the greater inhibition of β-galactosidase after the first 25 min of infection and the major synthesis of phage products at this time.

D. Effect of R23 on Inducer Transport and Lac Operon Expression

Introduction

Transport of β-galactosides into *E. coli* is mediated by the galactoside permease and may occur by facilitated diffusion, a process independent of metabolic energy, or active transport. The lac permease or M protein (Scarborough, Rumley & Kennedy, 1968), located in the spheroplast membrane, specifically binds certain galactosides and transports them through the membrane. According to Kennedy's scheme, in the presence of metabolic energy, the M protein is converted to a carrier (M_i) with greatly reduced affinity for the substrate which thus accumulates within the cell. The M_i carrier moves back to the exterior surface and is converted to the active form M. The conversion of M to M_i and M_i to M is promoted by K^+ or NH_4^+ ions and by Mg^{++} and ATP, respectively.

Changes in β-galactosidase synthesizing capacity after phage infection could conceivably result from a primary effect of phage on accumulation or transport of the inducer. The effect of R23 on the uptake of ^{14}C-IPTG was therefore examined with concurrent studies of β-galactosidase induction to determine the relationship of changes in β-galactosidase levels to inducer uptake and permease activity, as well as to establish the effect of R23 on induction of the lac permease. The fluctuations in enzyme and inducer levels were analyzed in terms of 3 phases or components and the requirements for all stages were examined.

Results

The pattern of inducer uptake was determined concurrently with the curve of β-galactosidase activity (Fig. D1). Corresponding to the immediate stage of enzyme stimulation, infected cells consistently exhibited a more rapid uptake of ^{14}C-IPTG than uninfected cells. During the early phase of inhibition of enzyme synthesis the intracellular concentration remained elevated, but the rate of inducer accumulation decreased so that at 40 min the concentration of inducer was identical in uninfected and infected cells. Thereafter, the concentration of ^{14}C-IPTG was less in infected than in uninfected cells. A similar pattern was observed whether the concentration of inducer was 0.5 or 0.04 mM (Fig. D2). In each case, entry of inducer in infected cells preceded and exceeded inducer uptake in uninfected cells. However, the level of enzyme activity was depressed in infected cells compared to uninfected cells from about 10 min after infection.

The influx of inducer paralleled the synthesis of β-galactosidase under other conditions of infection. The immediate stimulation of inducer uptake occurred on infection with an RNA synthetase mutant of R23 and in wild type phage infected in the presence of RNase (Fig. D3). In both cases, the initial increase was followed by transient inhibition and then resumption of inducer accumulation between 20 and 40 min at an accelerating rate. This pattern was similar to that of β-galactosidase synthesis in cultures infected with an RNA synthetase mutant, UV-irradiated phage or R23 in the presence of RNase (Section C). In contrast,

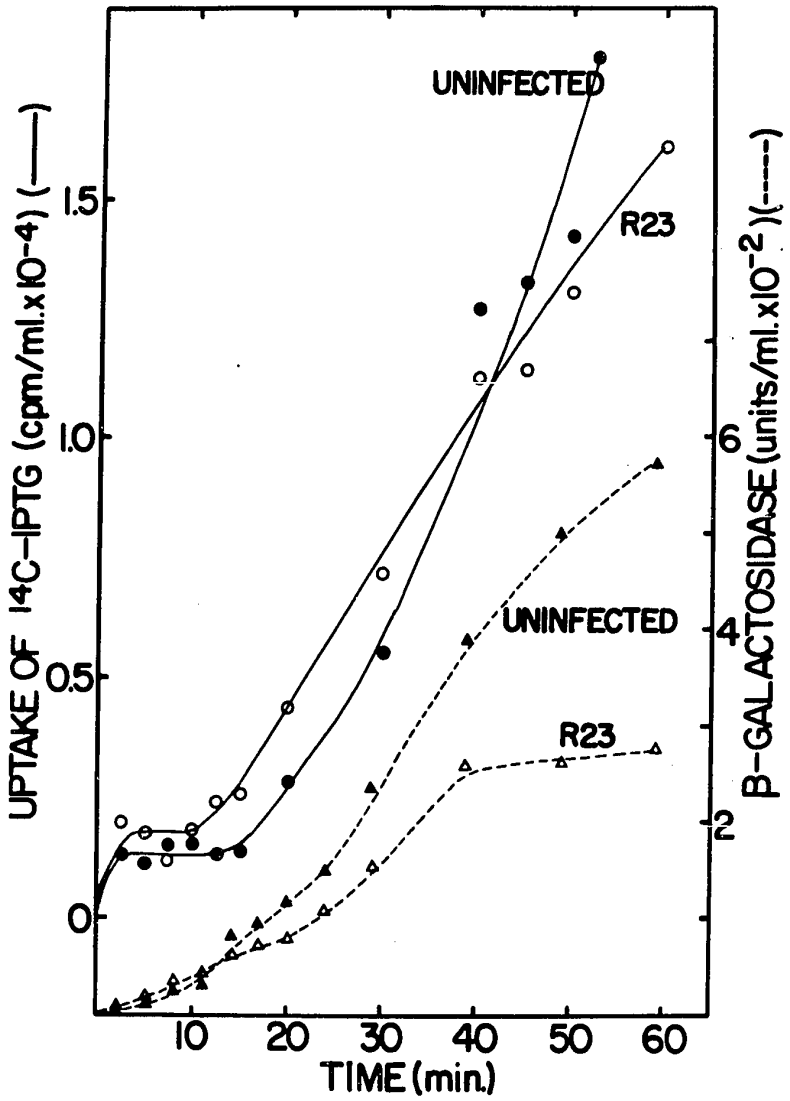

Fig. D1. Effect of R23 Infection on β-Galactosidase Synthesis and ^{14}C-IPTG Uptake. The experiment was performed as described in Materials and Methods. R23 was added at a MOI of 20 phage/cell.

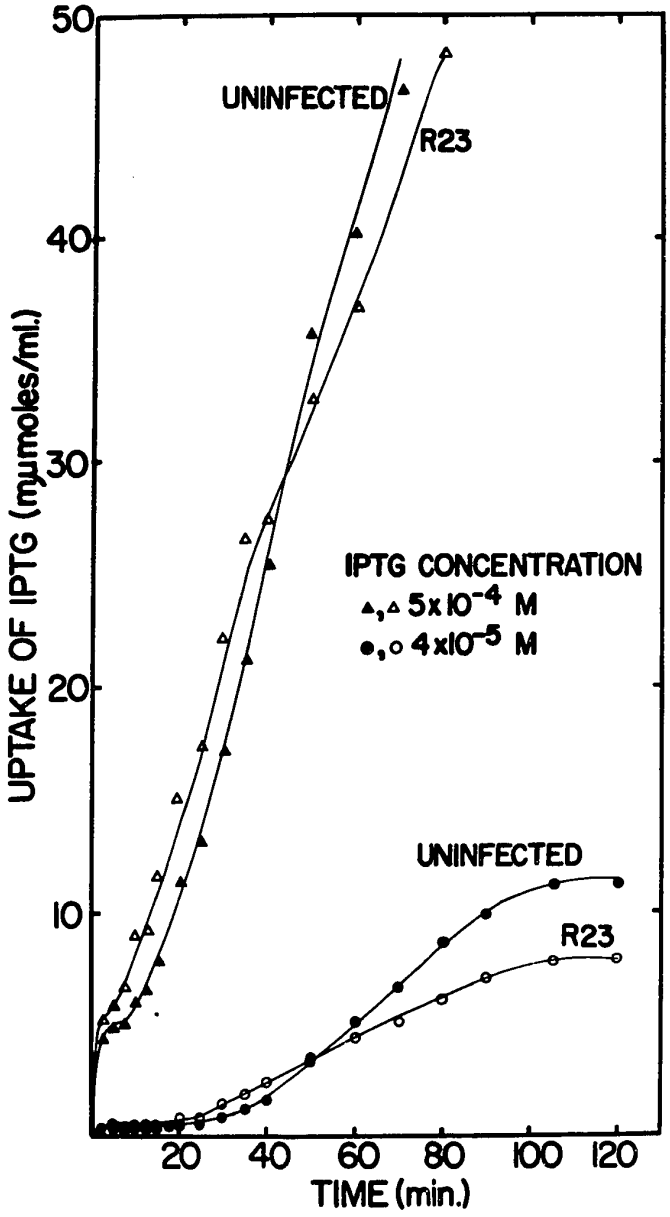

Fig. D2. R23 Infection, ^{14}C-IPTG Uptake and the Effect of Inducer Concentration. The experiment was performed as in Fig. D1 except that two concentrations of IPTG were used.

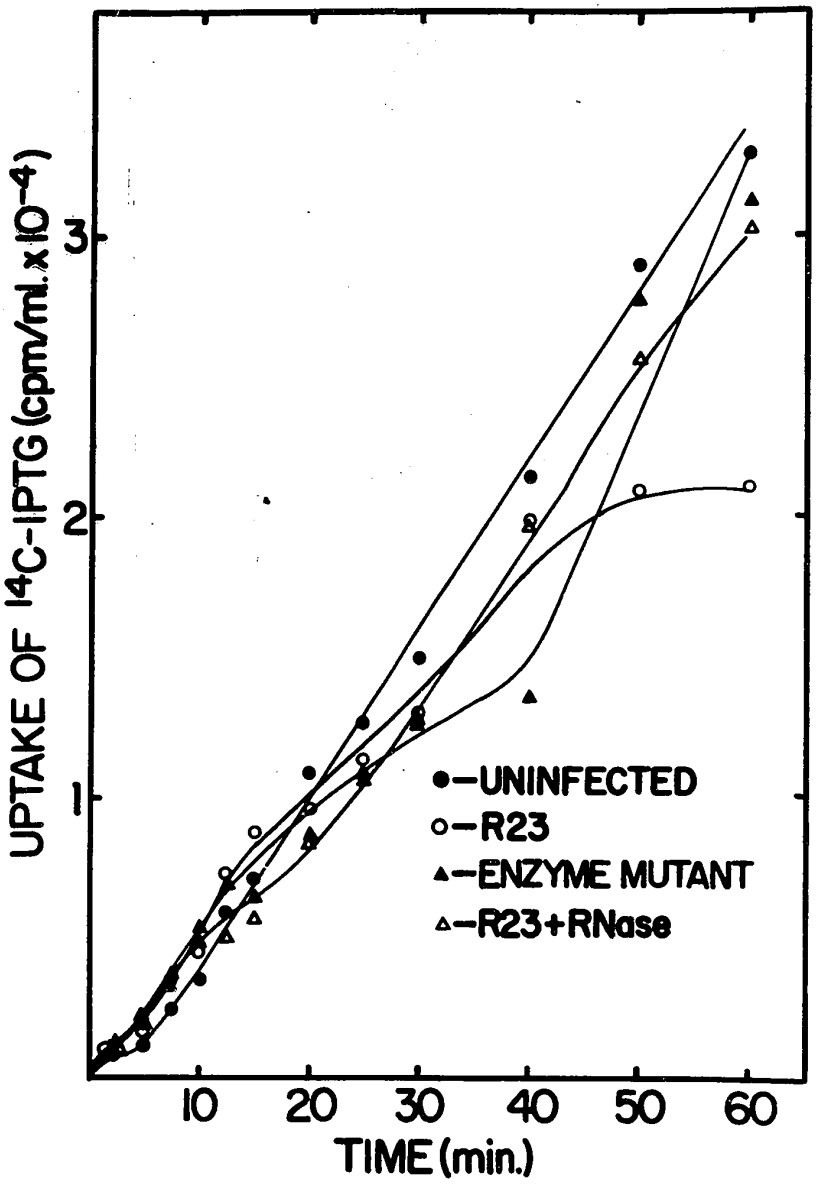

Fig. D3. Uptake of ^{14}C-IPTG in *E. Coli* Infected with an RNA Synthetase Mutant or R23 in the Presence of RNase. The experiment was performed as in Fig. D1 using a multiplicity of 10. One of the cultures infected with R23 contained RNase at a concentration of 100 μg/ml. Another culture was infected with an RNA synthetase mutant.

in the culture infected normally with wild type R23, the inhibition of inducer uptake persisted and became more pronounced after 40 min (Fig. D3).

The phage effect on uptake of ^{14}C-IPTG, like that on synthesis of β-galactosidase, was dependent on the multiplicity, increasing as the multiplicity was increased from 1 to 25 (Fig. D4). The phages R34, R40, f2, Qβ and R23 differed in their effects on ^{14}C-IPTG uptake (Fig. D5) in a manner commensurate with their effects on β-galactoside synthesis.

The effect of R23 on cells already containing significant galactoside permease activity was examined by infecting cells 10 min after addition of inducer (Fig. D6). There was an initial rapid increase in inducer accumulation for 10 min as before, but the period of increased inducer concentration evident between 10 and 40 min in cultures infected and induced simultaneously was abolished, and the inducer concentration was less than that in uninfected cells from 10 min after infection. The level of inducer accumulated at 60 min was the same whether induction preceded or accompanied addition of phage to the culture.

The effect of R23 on inducer uptake was also examined under conditions of catabolite repression by glucose. R23 was able to stimulate inducer accumulation in spite of glucose repression during the first 40 min (Fig. D7) and to subsequently inhibit it. Similarly, β-galactosidase activity was repressed by glucose in both uninfected and R23-infected cells, but the 3 stages of stimulation and inhibition were clearly visible in infected cells even in the presence

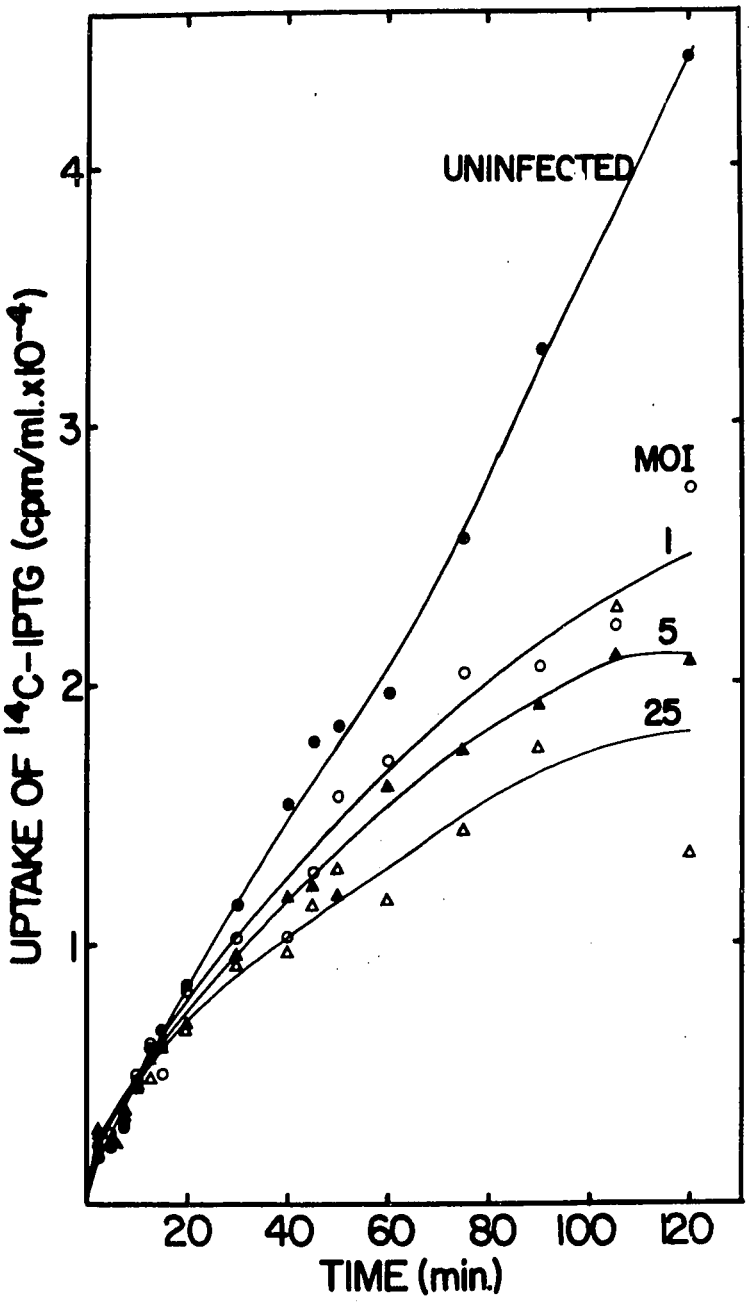

Fig. D4. Effect of Multiplicity of Infection on Uptake of ^{14}C-IPTG. The experiment was performed as in Fig. D1 except that R23 was added at a multiplicity of 1, 5 or 25 phage/cell.

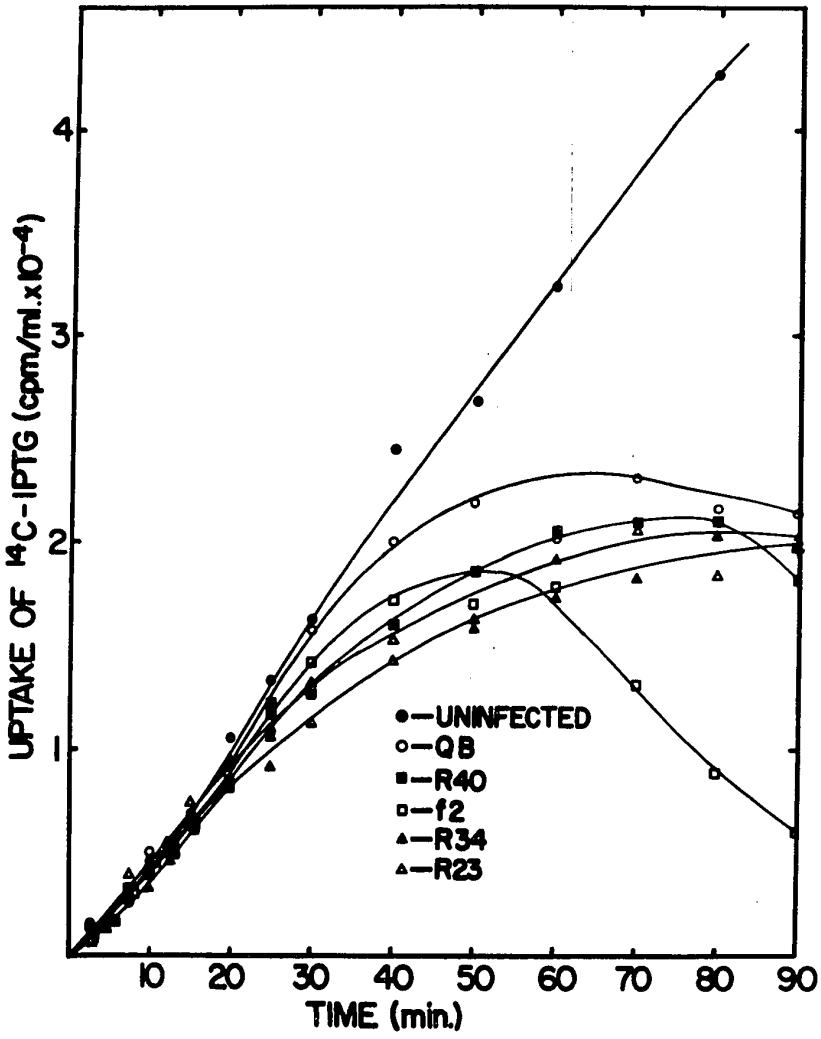

Fig. D5. Effect of RNA Phages on Uptake of ^{14}C-IPTG. The experiment was performed as described in Materials and Methods. Cultures were infected with R23, R34, R40, f2 or Qβ at a MOI of 20 phage/cell.

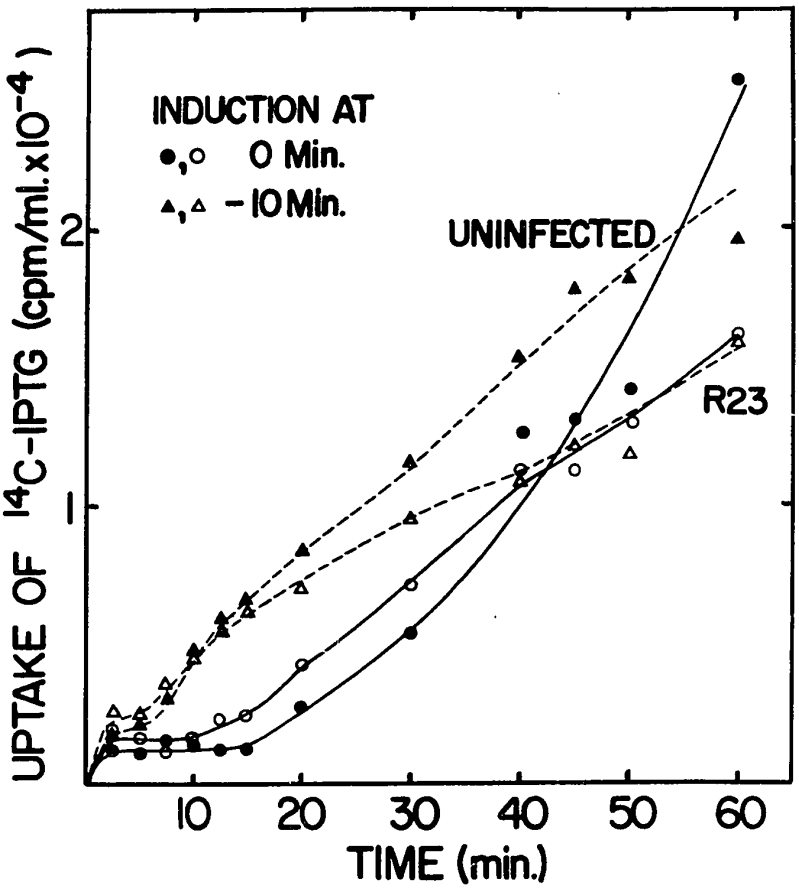

Fig. D6. Effect of R23 Infection on ^{14}C-IPTG with and without Pre-Induction. The experiment was performed as in Fig. D1 except that in one set of cultures non-radioactive IPTG was added 10 min prior to infection and ^{14}C-IPTG addition. In the other set of cultures, non-radioactive IPTG and ^{14}C-IPTG were added at the time of infection.

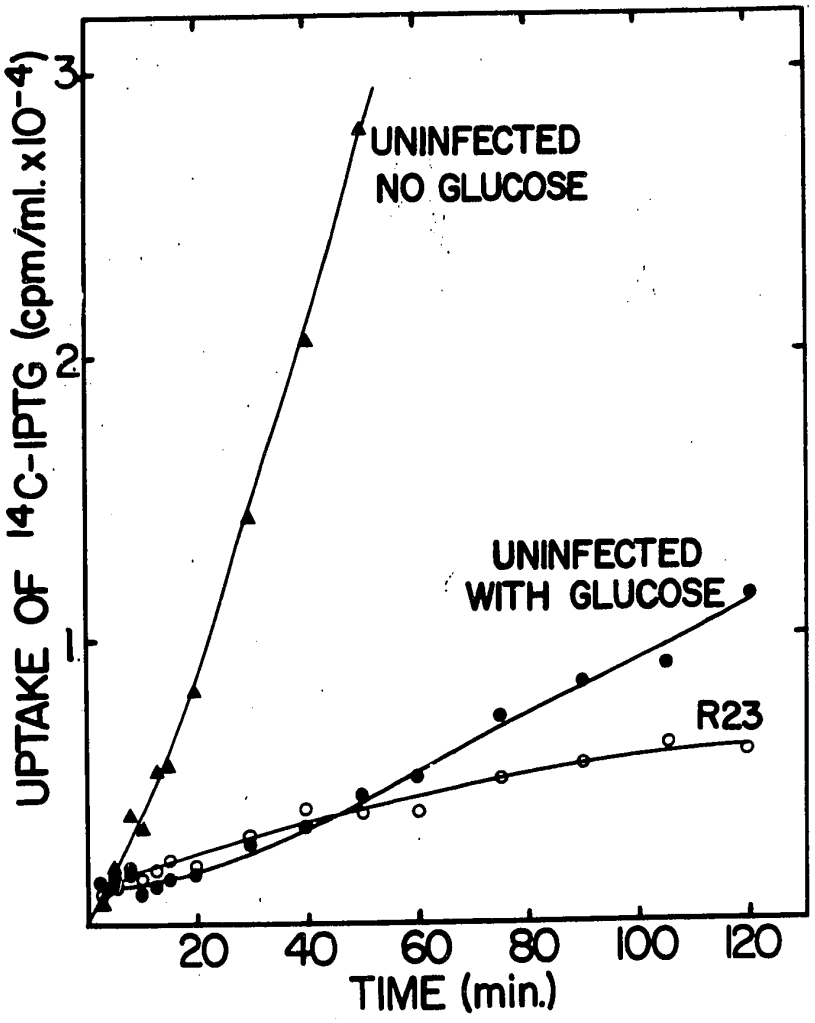

Fig. D7. Uptake of ^{14}C-IPTG and the Effect of Glucose. The experiment was performed as in Fig. D1 except that glucose (28 mM) was added to one set of uninfected and infected cultures at the time of infection.

of glucose.

Discussion

The pattern of uptake of ^{14}C-IPTG has been shown to parallel that of β-galactosidase synthesis in *E. coli* cells infected with R23. The degree of inhibition of inducer uptake and enzyme synthesis depended on the multiplicity of infection and on the bacteriophage, the greatest effect being demonstrated by R23 at an input of at least 10 phage/cell. The effect of R23 on both β-galactosidase synthesis and uptake of inducer can be conveniently analyzed and interpreted in terms of the 3 components of the curves which have been described. The following discussion represents a hypothetical explanation of the results.

1) Immediate stimulation (during the first 10 min of infection). Inducer entry was enhanced by an increase in membrane permeability and possibly by a stimulatory effect on the carrier-mediated transport. The earlier and more rapid entry of inducer allowed faster and greater synthesis of permease and β-galactosidase, i.e., expression of the lac operon, in infected cells compared to uninfected cells. The stimulatory phase occurred under conditions of repression of enzyme synthesis by glucose which has been reported to inhibit mRNA synthesis at the level of the lac promoter (Pastan & Perlman, 1968). The stimulatory phase also occurred in cells containing significant permease activity. This could be compatible with the proposal that the initial and primary effect was an increase in membrane permeability which allowed rapid inducer entry and led only secondarily to increased transcription of the lac operon.

The immediate stimulation may result from interaction of the phage with the cell membrane via its hydrophobic maturation protein. Whether R23 only produces a general increase in cell permeability, or promotes the activity of the galactoside permease or M protein as well remains to be determined. This initial enhancement of inducer uptake may be one reason why inhibition of β-galactosidase synthesis by R23 is not as immediate and complete as that characteristic of T-even phage infection (Kaempfer & Magasanik, 1967a).

2) Early inhibition (the period from 10 to 30 min after infection). During this stage the concentration of inducer remained elevated in infected cells due to the immediate stimulation of inducer uptake and increased permease activity, but the rates of inducer uptake and enzyme synthesis began to decelerate. It is thought that this deceleration was due to disappearance of the stimulatory membrane effect, appearance of an inhibitory membrane effect and commencement of the late inhibitory effect. The level of enzyme synthesis in infected cells was less than that in uninfected cells after 10 min despite the presence of a higher concentration of inducer in infected cells. Thus, inhibition of β-galactosidase synthesis by R23 was not due to defective inducer entry (and resultant low permease activity), rather, permease and β-galactosidase were coordinately inhibited. It is possible that contact of phage with the cell membrane disturbed transcription of DNA (which is thought to be attached to the membrane), and produced an early inhibition of β-galactosidase and permease synthesis.

In cells induced for 10 min prior to infection the period of

increased inducer concentration, seen between 10 and 40 min after infection in cultures not pre-induced, was not visible. During the 10 min preinduction period, both cultures accumulated sufficient inducer and permease activity that the increase in inducer level and expression of the lac operon following phage addition became insignificant or undetectable. The late inhibition by phage (component 3) thus was the predominant effect visible and appeared to occur earlier than in a culture to which phage and inducer were added simultaneously.

3) Late inhibition (after about 30 min). R23 infection produced a pronounced depression of synthesis of both permease and β-galactosidase, i.e., inhibited expression of the lac operon, beginning 25-30 min after infection. Thus, both the intracellular concentration of ^{14}C-IPTG and the synthesis of β-galactosidase were depressed (Fig. D1). This effect required viral adsorption, RNA penetration and replication; it was absent in infection with enzyme mutants or in the presence of RNase (or EDTA).

These studies indicated that R23 infection produced coordinate or concurrent inhibition of permease and β-galactosidase (as measured by ^{14}C-IPTG uptake and enzymatic assay of β-galactosidase). This suggested that its action might be mediated at the level of a controlling site of the lac operon, such as the lac promoter, operator or regulatory gene. This possibility was considered in further studies.

E. Effect of Cyclic 3',5'-AMP on Inhibition of β-Galactosidase Synthesis by R23 and T2.

Introduction

The synthesis of β-galactosidase is transiently repressed when glucose is added to cultures of *E. coli* growing on another carbon source such as glycerol and permanently repressed when cells are grown for many generations in a glucose medium (Monod,1947; Tyler & Magasanik, 1967). Glucose represses the synthesis of β-galactosidase mRNA (Nakada & Magasanik, 1964).

Cyclic 3',5'-AMP, a nucleotide present in *E. coli* (Makman & Sutherland, 1965), stimulates β-galactosidase synthesis in *E. coli* cells made permeable by treatment with Tris-EDTA by stimulating synthesis of enzyme-specific mRNA (Perlman & Pastan, 1968a,b). It overcomes transient (but not permanent) repression of β-galactosidase synthesis by glucose both in cells treated with Tris-EDTA and in untreated cells. Transient glucose repression of β-galactosidase is believed to be mediated by change in concentration of cyclic 3',5'-AMP (Pastan & Perlman, 1968). Glucose lowers cyclic 3',5'-AMP concentration in bacterial cells (Makman & Sutherland, 1965). The site at which cyclic 3',5'-AMP acts to stimulate β-galactosidase synthesis and abolish transient glucose repression is believed to be the lac promotor, where RNA polymerase attaches and transcription begins (Ippen *et al.*, 1968). Thus, it does not stimulate β-galactosidase synthesis in certain promotor mutants which make much less β-galactosidase than the wild type strain

(Pastan & Perlman, 1968; Silverstone *et al.*, 1969).

The inhibition of β-galactosidase synthesis by R23 and T-even phage infection (Kaempfer & Magasanik, 1967a,b) is partly due to an arrest of synthesis of enzyme-specific mRNA, but the locus and exact mechanism of action have not been defined. Studies previously described (Section C & D) indicated that permease and β-galactosidase were coordinately inhibited after R23 infection and that this inhibition was not a result of primary inhibition of inducer transport. These results suggested that the action of R23 might involve a controlling site of the lac operon, such as the promotor.

It, therefore, appeared possible that studies of the effect of cyclic 3',5'-AMP on β-galactosidase synthesis in phage-infected cultures might contribute to the delineation of the site and mode of inhibition by R23. It was of interest to determine whether cyclic 3',5'-AMP could prevent or reduce the inhibition of β-galactosidase synthesis produced by phage infection, or whether the phage could still exert its usual drastic effect in the presence of cyclic 3',5'-AMP. The effect of this nucleotide on T2-induced inhibition of β-galactosidase synthesis was also examined in order to compare the mechanism of action of T2 and R23.

Results

The effect of cyclic 3',5'-AMP on the synthesis of β-galactosidase was examined in *E. coli* made permeable by treatment with Tris-EDTA (Fig. E1). Infected and uninfected Tris-EDTA treated

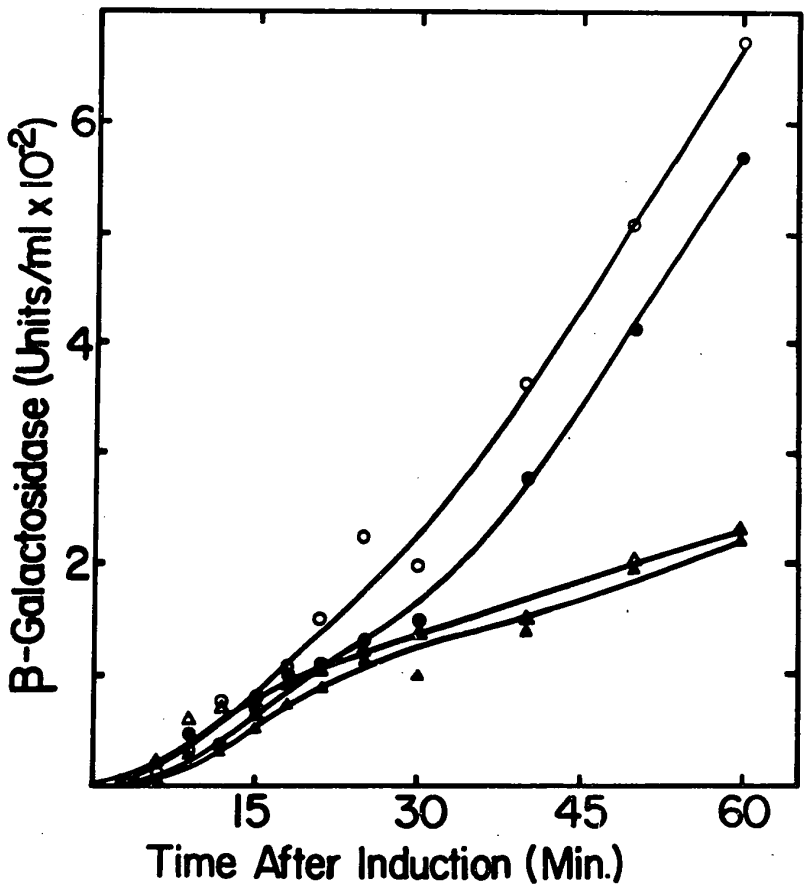

Fig. E1. Effect of Cyclic 3',5'-AMP on β-Galactosidase Synthesis in Uninfected and R23-Infected *E. Coli*. *E. coli* K38 was treated with Tris and EDTA. R23, at a MOI of 20 phage/cell, and cyclic 3',5'-AMP at a concentration of 1 mM, were added 3 min prior to induction. Induction and assay of β-galactosidase and Tris-EDTA treatment of bacteria were performed as described in Materials and Methods. Uninfected: with (○) and without (●) cyclic 3',5'-AMP. Infected: with (△) and without (▲) cyclic 3',5'-AMP.

cells were incubated with IPTG in the presence and absence of cyclic 3',5'-AMP. In the culture infected with R23, IPTG was added 3 min after phage (represented as time 0). R23 infection resulted in an inhibition of enzyme synthesis similar to that seen in cells untreated with Tris-EDTA. Cyclic 3',5'-AMP stimulated the synthesis of β-galactosidase in both uninfected and infected cultures, in agreement with the results reported by Perlman and Pastan (1968a,b) for uninfected bacteria. Cyclic 3',5'-AMP, however, was unable to reverse the inhibition by R23.

This was also true in glucose-repressed cultures. Addition of glucose to the cultures (which had been grown in a minimal glycerol medium for several generations) repressed β-galactosidase synthesis in both uninfected and R23-infected cells. Cyclic 3',5'-AMP restored enzyme synthesis to unrepressed levels (Fig. E2), but was unable to reverse the inhibitory effect of R23 infection.

Perlman and Pastan (1968b) reported that the stimulatory effect of cyclic 3',5'-AMP on glucose-repressed cells occurred whether the cells were made permeable by Tris-EDTA or were normally growing untreated cells. In contrast, stimulation by cyclic 3',5'-AMP in cells not repressed by glucose required that the cells be made permeable by Tris-EDTA. In R23-infected cells, cyclic 3',5'-AMP, in concentrations up to 5 mM, was unable to stimulate the synthesis of β-galactosidase in cells not made permeable by Tris-EDTA (Fig. E3).

Infection of *E. coli* by T2 results in an immediate cessation of β-galactosidase synthesis. If phage infection follows a short

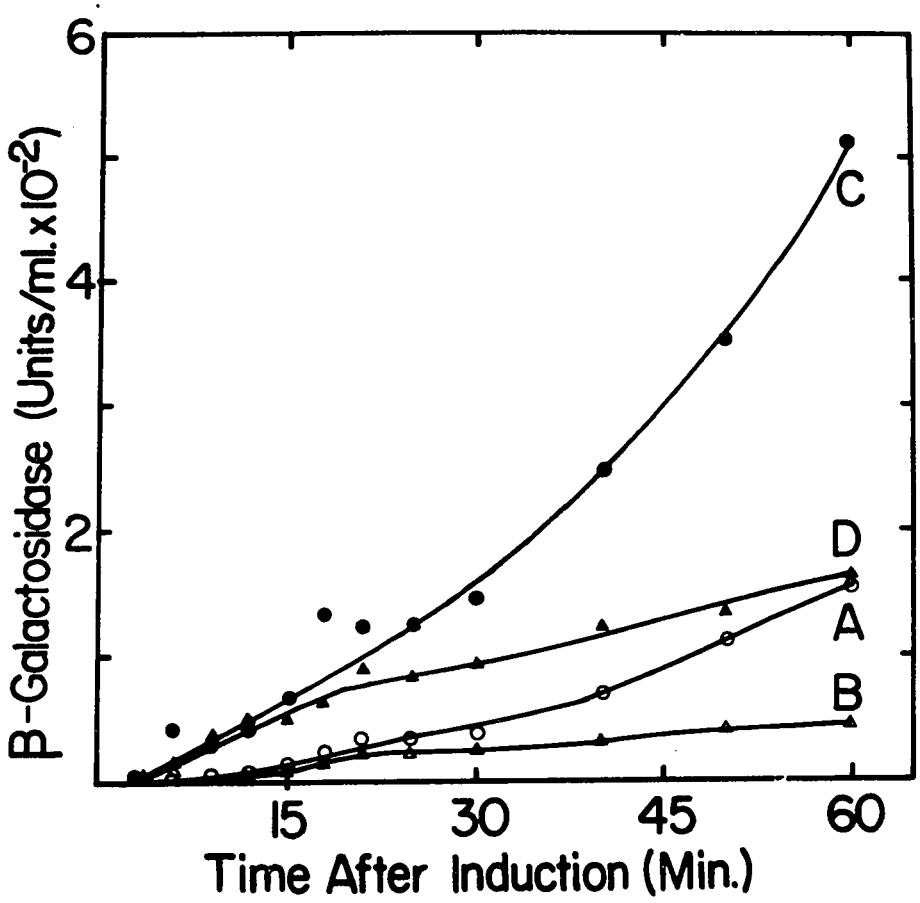

Fig. E2. Effect of Glucose and Cyclic 3',5'-AMP on β-Galactosidase Synthesis in R23-Infected and Uninfected Cultures. The experiment was performed as in Fig. E1 except that all cultures contained 0.02 M glucose. A: uninfected. B: infected. C: uninfected, cyclic 3',5'-AMP. D: infected, cyclic 3',5'-AMP.

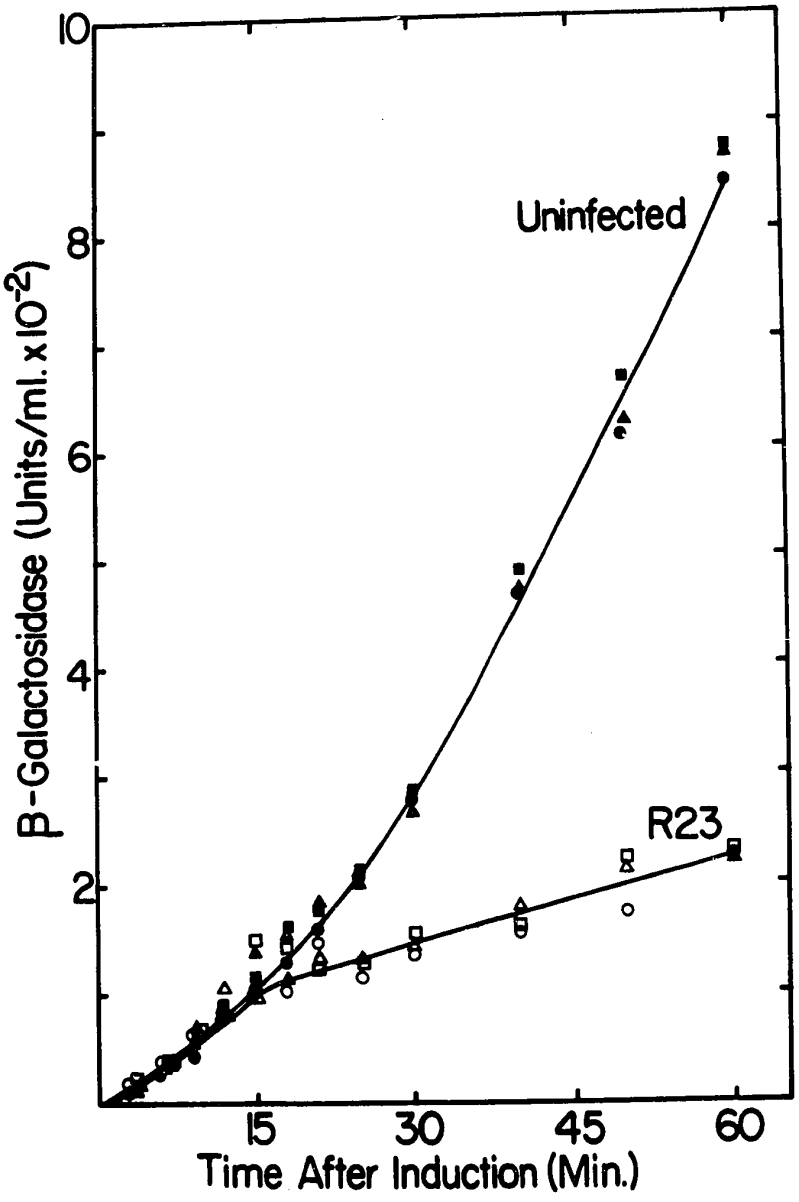

Fig. E3. Effect of Cyclic 3',5'-AMP on β-Galactosidase Synthesis in Untreated Uninfected and R23-Infected Cells. Half a culture of *E. Coli* was infected with R23 at a multiplicity of 20 phage/cell. Uninfected and infected cultures were each divided into 3 portions, to 2 of which were added cyclic 3',5'-AMP to final concentrations of 0.001 M and 0.005 M. IPTG was added 3 min later. Control, without added cyclic 3',5'-AMP: uninfected (●), infected (○). Cyclic 3',5'-AMP, 1 mM: uninfected (▲), infected (△). Cyclic 3',5'-AMP, 5 mM: uninfected (■), infected (□).

period of exposure to inducer, however, the enzyme-forming capacity can be expressed (Pardee & Prestidge, 1961). On addition of T2 3 min after inducer (Fig. E4), a small amount of enzyme was synthesized. Cyclic 3',5'-AMP increased the synthesis of β-galactosidase in both uninfected and T2-infected cells, but was unable to reverse the inhibitory effect of phage infection. The stimulatory effect of cyclic 3',5'-AMP was also more pronounced in the presence of glucose, but again a complete reversal to non-infected levels did not occur (Fig. E5).

Discussion

Makman and Sutherland (1965) demonstrated the presence of cyclic 3',5'-AMP in *E. coli* and found an abrupt rise in cyclic 3',5'-AMP content coincident with the limitation of carbon and energy sources. In the presence of glucose, cyclic 3',5'-AMP rapidly disappeared from the cells and appeared in the medium. It was suggested that this nucleotide might be important in the synthesis of inducible enzymes, and that control of enzyme activity might be at the cell surface.

Part of the T-even phage effect and the early stage of inhibition of β-galactosidase synthesis by R23 may be mediated by contact of the phage with the cell surface and/or some step which occurs soon thereafter since they occur in the absence of viral genome expression. Since the exact mechanism of inhibition of host mRNA synthesis by these phages is unknown, however, the possibility that it involves a specific genetic locus such as the operator or promotor deserves consideration. Thus (in accordance with current concepts of the regulation of the lac operon), phage could inhibit initiation of

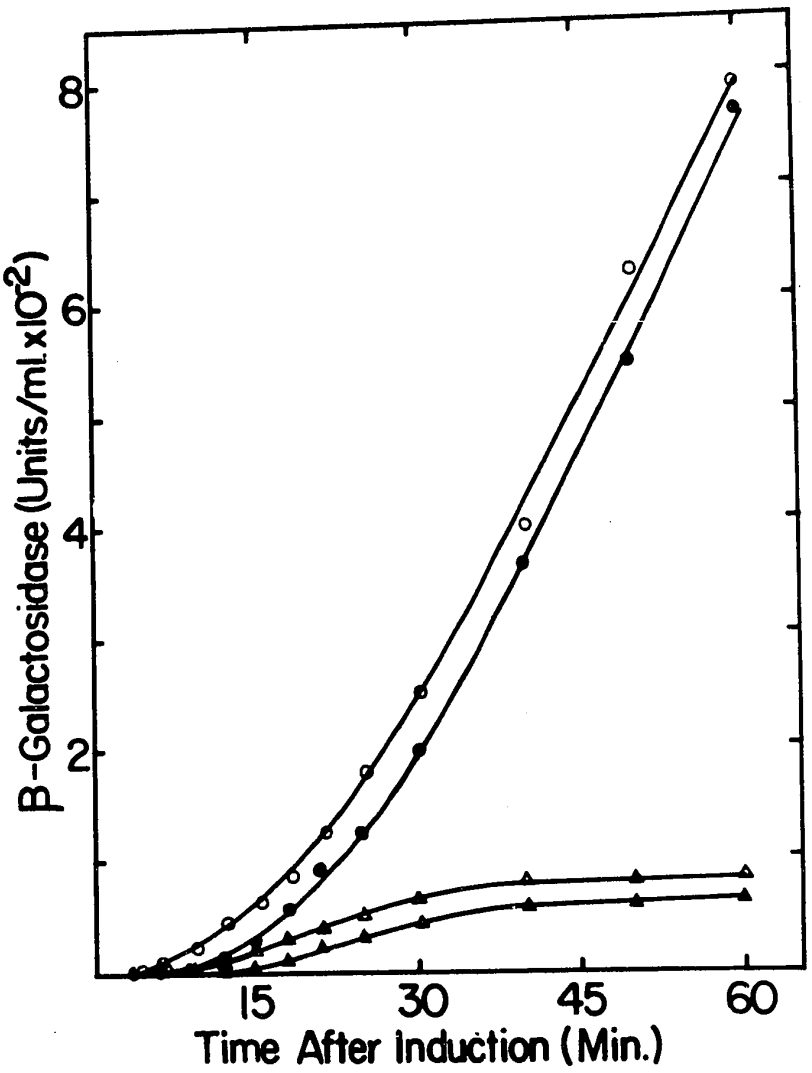

Fig. E4. Effect of Cyclic 3',5'-AMP on β-Galactosidase Synthesis in Uninfected and T2-Infected *E. Coli*. The symbols and experimental procedure are the same as in Fig. E1 except that bacteria were infected with T2 (at a multiplicity of 2.5 phage/cell), rather than R23.

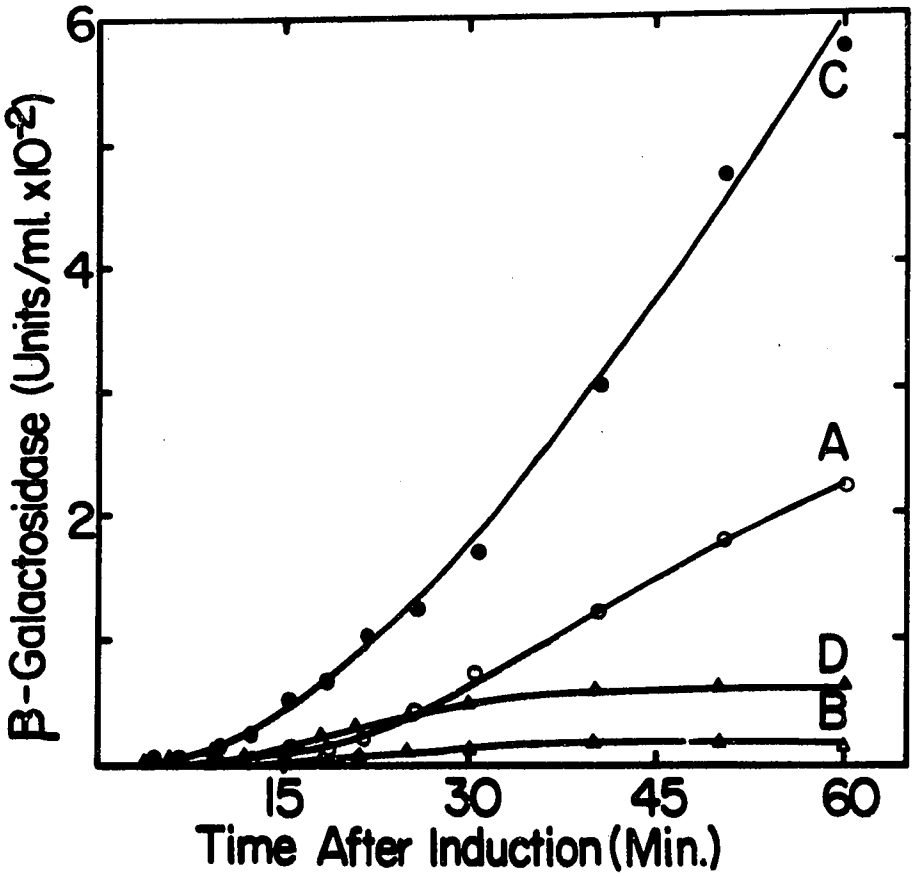

Fig. E5. Effect of Glucose and Cyclic 3',5'-AMP on β-Galactosidase Synthesis in T2-Infected and Uninfected Cultures. The symbols and experimental procedure are the same as in Fig. E2 except that T2 (2.5 phage/cell) was added instead of R23.

transcription by 1) preventing normal binding of the DNA-dependent RNA polymerase to the promoter site by affecting the enzyme or the promoter, 2) promoting the binding of repressor to the operator which blocks the progress of the polymerase, or 3) inhibiting binding of inducer to repressor.

It appeared that indirect support for the promoter as the target of phage action might be provided by studies with cyclic 3',5'-AMP in view of its postulated action. Thus if phage infection inhibited synthesis of β-galactosidase mRNA by preventing initiation of transcription at the lac promoter, cyclic 3',5'-AMP might reverse or reduce the inhibition by stimulating initiation of mRNA synthesis.

The results indicate, however, that alteration in cyclic 3',5'-AMP concentration or interference of its action is unlikely to be the mechanism by which phage infection causes inhibition of β-galactosidase synthesis. In cells made permeable by Tris-EDTA, cyclic 3',5'-AMP stimulated the synthesis of β-galactosidase in uninfected, R23-infected and T2-infected cells in the presence and absence of glucose but the inhibitory phage effect was not reversed. It was unable to affect the synthesis of β-galactosidase in R23-infected cells untreated with Tris-EDTA, in contrast to the stimulatory effect in untreated cells repressed by glucose. R23 and T2 inhibited β-galactosidase synthesis in the presence of cyclic 3',5'-AMP to the same extent as in its absence, and produced an inhibitory effect in cells already repressed by glucose.

From this study alone, firm conclusions regarding the mode of inhibition of β-galactosidase synthesis by R23 and T2 cannot be drawn.

The results suggest, however, that the phage effect is not directed at the lac promoter site (at least in a similar location and/or manner as cyclic 3',5'-AMP is postulated to act), and that phage infection, nucleotide and glucose effects are entirely unrelated. Phage infection did not prevent the stimulatory effect of the nucleotide on β-galactosidase synthesis. The outstanding significant finding was that the inhibitory effect of R23 (and T2) infection persisted in spite of stimulation of β-galactosidase synthesis by cyclic 3',5'-AMP.

F. Effect of R23 on Membrane Permeability and Potassium Fluxes

Introduction

The studies on β-galactosidase synthesis and ^{14}C-IPTG uptake suggested that the initial effects of infection by R23 could be related to the contact of the phage particle with the cell membrane and its immediate consequences. Thus, the immediate stimulation and early inhibition of enzyme synthesis and inducer uptake were independent of viral RNA penetration and replication. The increased influx of ^{14}C-IPTG indicated increased membrane permeability. To determine whether this effect extended to other transport systems and affected both uphill and downhill transport, the effect of R23 on influx and efflux of $^{42}K^{+}$ was investigated.

The bacterial membrane has been implicated as the site of DNA replication, protein synthesis, electron transport and oxidative phosphorylation, transport processes and other phenomena. Since Jacob, Brenner and Cuzin (1963) proposed that bacterial DNA is connected to the cell surface, certain agents which inhibit bacterial growth and macromolecular synthesis have been postulated to do so by producing changes in surface properties.

Changes in phospholipid synthesis or metabolism and membrane proteins have been reported in *E. coli* infected with T4 (Buller & Astrachan, 1968; Frankel *et al.*, 1968; Furrows & Pizer, 1968; Peterson & Buller, 1969) and colicins (Nomura & Maeda, 1965). Increased membrane permeability has been demonstrated on infection

of *E. coli* by T-even phage (Puck & Lee, 1954, 1955; Silver, Levine & Spielman, 1968), T phage ghosts (Herriott & Barlow, 1957; Lehman & Herriott, 1958), rII mutants of T4 (Ames & Ames, 1965; Garen, 1961; Buller & Astrachan, 1968; Brock, 1965) and colicins (Nomura & Maeda, 1965). T-even phages (Silver *et al.*, 1968), colicins (Nomura & Maeda, 1965), antibiotics such as phenethyl alcohol (Silver & Wendt, 1967), gramicidin and valinomycin (Harold & Baarda, 1967), and steroidal diamines (Silver & Levine, 1968) increased potassium efflux in bacteria without increasing (or altering) influx and were therefore considered to induce permeability changes without directly altering transport.

Potassium is an important cation present in high concentrations in growing *E. coli*; its depletion in bacterial mutants defective in K^+ transport and concentration resulted in depression of protein synthesis (Ennis & Lubin, 1965). Schultz and Solomon (1961) showed that the intracellular K^+ concentration greatly exceeded and was independent of extracellular K^+ in *E. coli* during the early log phase of growth.

With the ultimate aim of elucidating the mechanism of inhibition of host macromolecular synthesis by R23, an investigation of the nature and consequences of its interaction with the membrane was initiated by focusing on its effects on K^+ transport. The requirements for these effects were examined. The most striking finding was that R23, in contrast to T-even phage and other agents listed

above, produced an increase in the rates of both influx and efflux of $^{42}K^+$ in *E. coli*.

Results

a) Effect of R23 on Potassium Efflux. Infection of *E. coli* by R23 resulted in a rapid loss of intracellular potassium as measured by efflux of $^{42}K^+$ (Fig. F1). The accelerated loss was detected almost immediately after addition of phage; it was maximal for the first 10-15 min and became negligible thereafter. The average $^{42}K^+$ remaining within the cells at 10 min was 55% and 25% of the radioactivity present at time zero in uninfected and infected cells, respectively. A similar pattern was observed with R34, R40, f2 and Qβ (Fig. F2). The K^+ leakage increased with increasing MOI to a maximum at about 12.5 phage/cell (Fig. F1). The lesser effect of lower multiplicities may be partly due to the larger number of uninfected cells, since the maximum number of infected cells occurs at a multiplicity of 10 or over. At multiplicities over 25, there appeared to be a slight diminution in the loss of K^+.

b) Requirements for Phage-Induced Potassium Efflux. The rapidity with which K^+ efflux was induced by phage infection suggested that intracellular expression of a phage function was not required. UV-irradiated phage (Fig. F3) and amber mutants defective in the viral RNA synthetase and maturation protein cistrons (Figs. F4,5) induced a K^+ leakage comparable to that produced by wild type R23. The RNA synthetase mutant produced a slightly greater effect than the

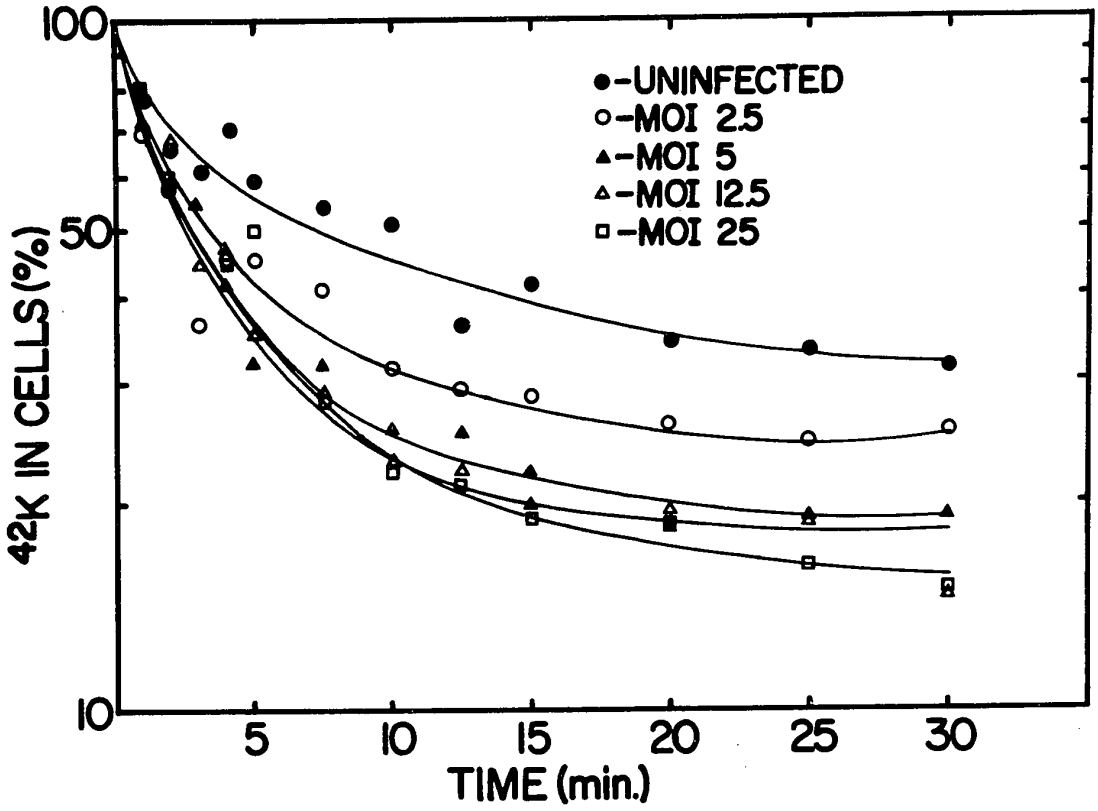

Fig. F1. Potassium Efflux: Effect of R23. The experiment was performed as described in Materials and Methods except that R23 was added at time zero at multiplicities of 2.5 to 25 phage/cell. The radioactivity present within the cell at the time of infection is represented as 100%.

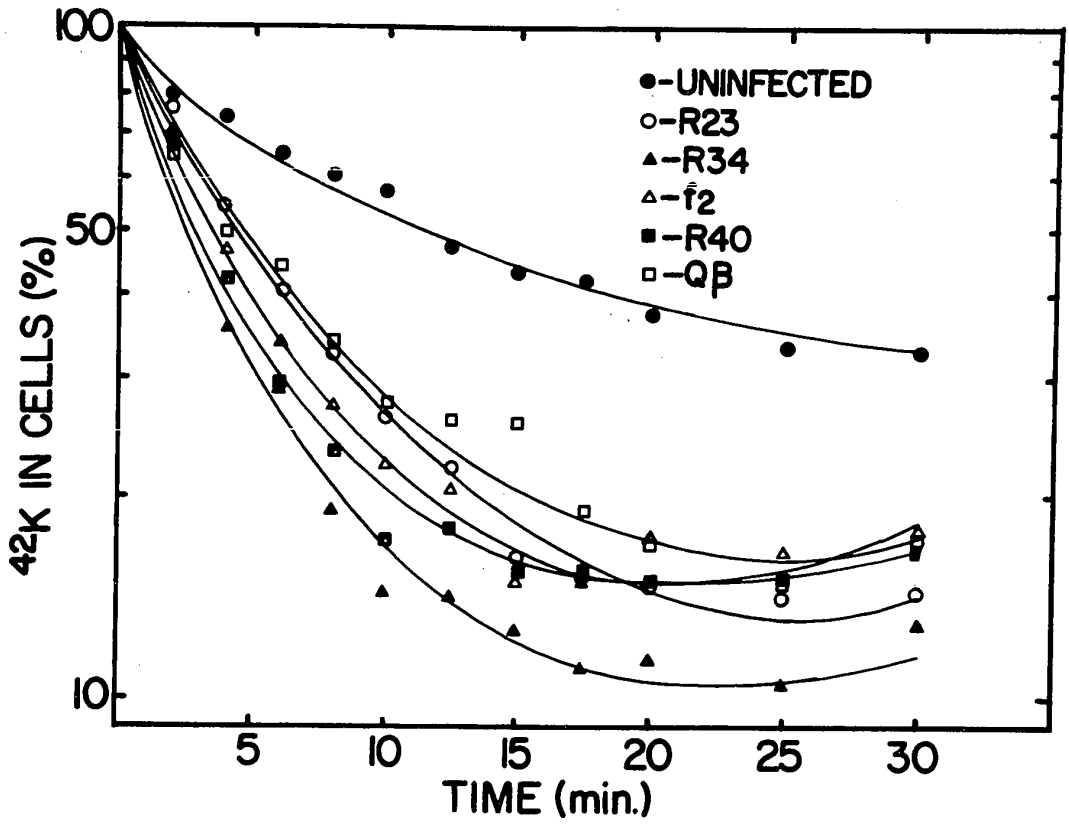

Fig. F2. Effect of RNA Phages on Potassium Efflux. The experiment was performed as described in Materials and Methods.

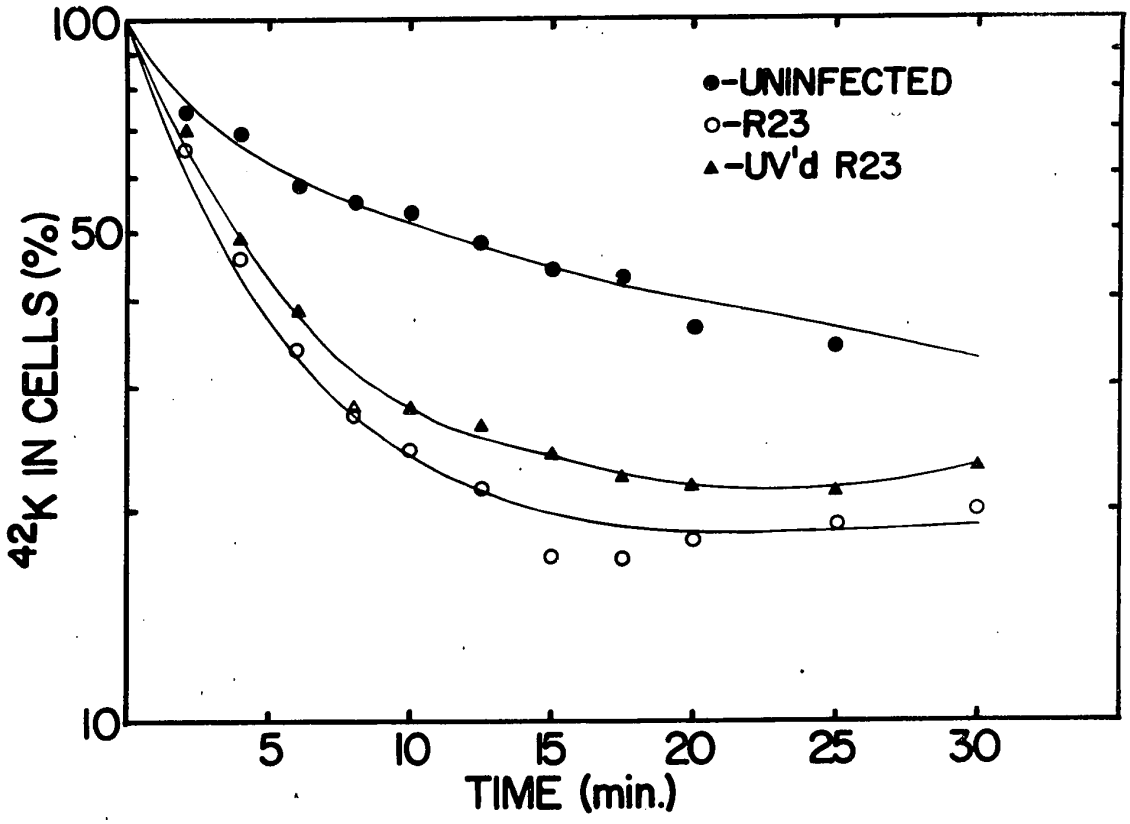

Fig. F3. Potassium Efflux: Effect of UV-Irradiated R23. The experiment was performed as described in Materials and Methods. Phages were added at a multiplicity of 20 particles/cell.

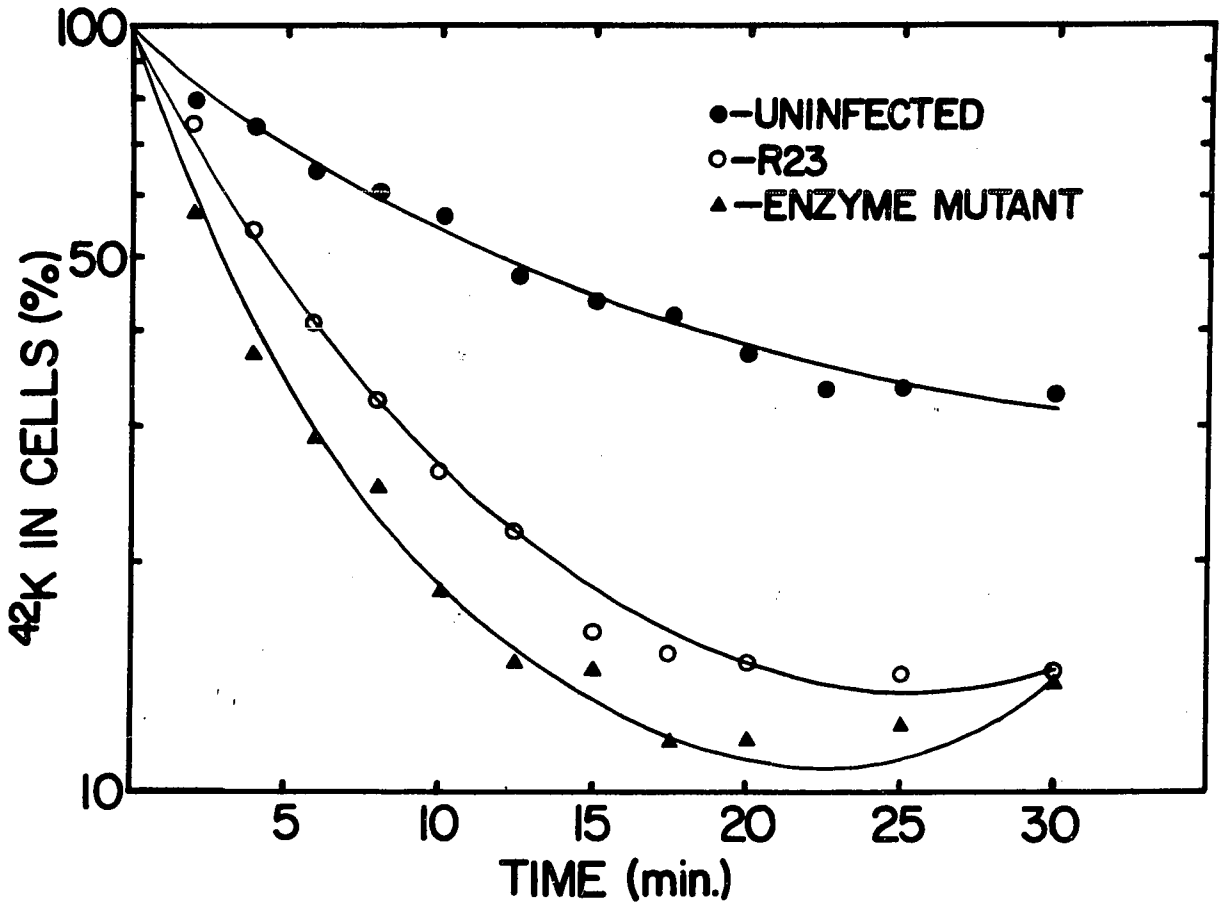

Fig. F4. Potassium Efflux: Effect of an RNA Synthetase Mutant. The experiment was performed as described in Materials and Methods except that phages were added at a multiplicity of 10 phage/cell.

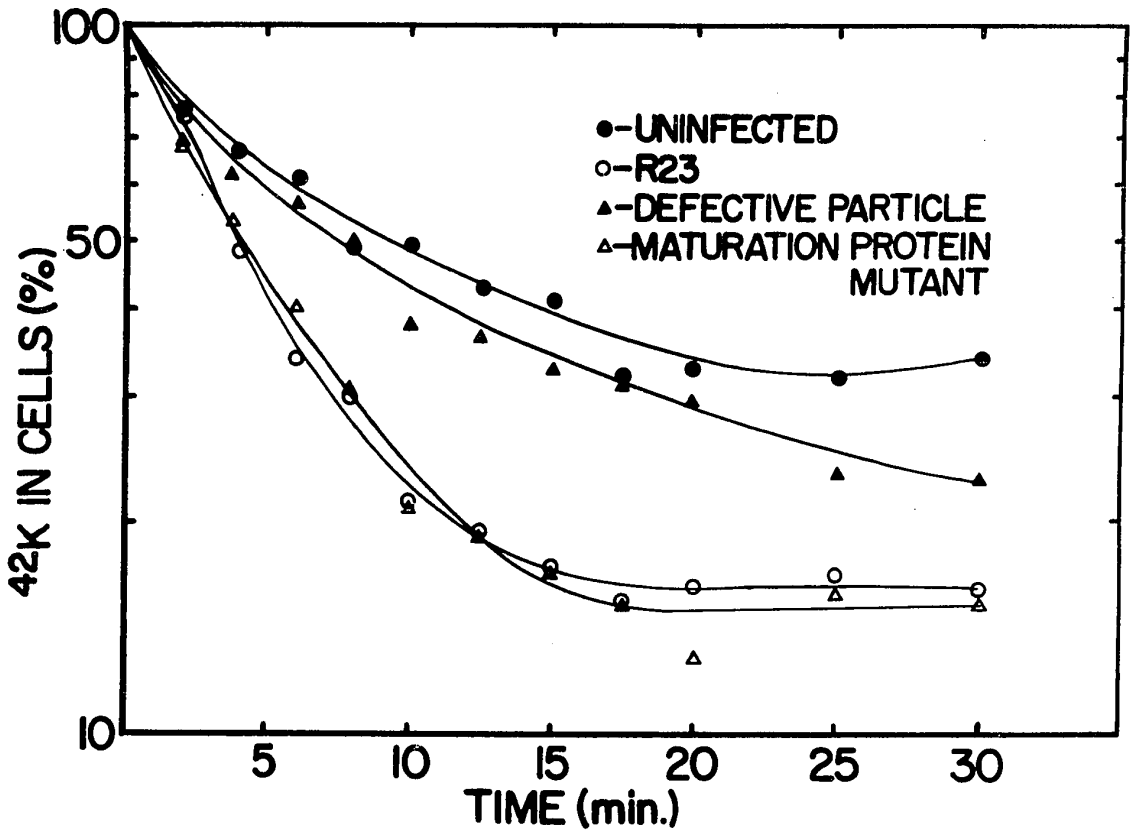

Fig. F5. Potassium Efflux: Effect of Defective Particles and Maturation Protein Mutants. The experiment was performed as described in Materials and Methods except that phages were added at a multiplicity of 10 phage/cell. Mutants and defective particles were obtained by growing the maturation protein mutant in *E. coli* K37 (Su^+) and K38 (Su^-), respectively.

wild type phage. Chloramphenicol, in a concentration sufficient to depress the phage yield to 0.01% of the control, did not prevent the phage-induced K^+ loss (Fig. F6), nor did rifampicin (Fig. F7). Rifampicin, an inhibitor of DNA-dependent RNA polymerase (Hartmann *et al.*, 1967),is known to depress RNA phage yields by blocking some early step after penetration if added before 5 min after infection (Fromageot & Zinder, 1968). Therefore, host or viral RNA or protein synthesis was not required for phage-induced K^+ efflux.

Increased K^+ leakage was not observed on infection with defective particles lacking the maturation protein (Fig. F5), or when adsorption was prevented by R23 antiserum (Fig. F8). Purified coat protein subunits were incapable of reproducing the effect of the complete phage (Fig. F8). Penetration, and probably injection, of phage RNA were not necessary since R23 enhanced efflux in the presence of EDTA (Fig. F9), although the phage yield was depressed to 0.025% of the control value, and in the presence of RNase (Fig. F10). RNase, however, does not completely prevent RNA injection.

c) Effect of Temperature. The phage effect on K^+ efflux was reduced at temperatures below 33° C and essentially absent at 4° C (Fig. F11), 22° C and 24° C (Table F1). At 29° C, the increment due to R23 was reduced to 56% of that observed at 37° C. The leakage from uninfected cells, on the other hand, was reduced at 4° C but was comparable to the efflux at 37° C at all the other temperatures tested. Thus, the reduction in the phage effect at low temperatures (22-33° C) was attributable to the inability of RNA to successfully infect *E. coli* at these temperatures (Zinder, 1965).

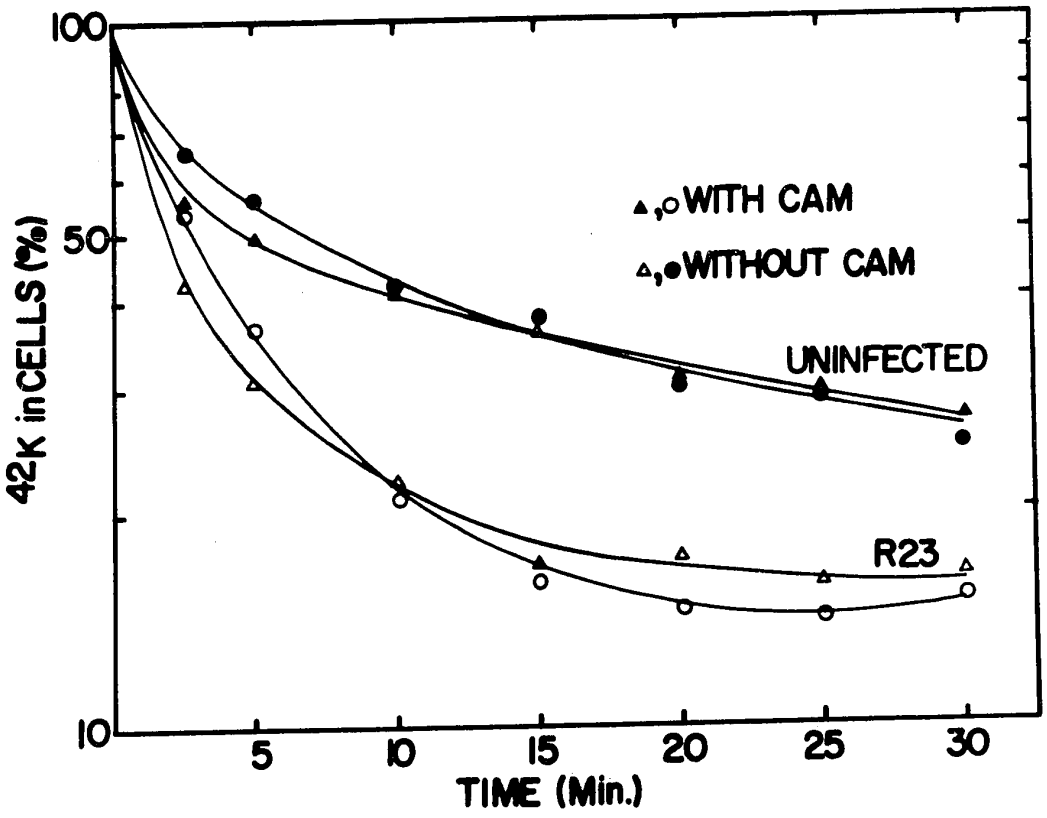

Fig. F6. Potassium Efflux: Effect of Chloramphenicol. The experiment was performed as described in Materials and Methods. Chloramphenicol was added to a concentration of 1 mg/ml at the time of infection; it depressed the phage yield to 0.01% of the normal yield.

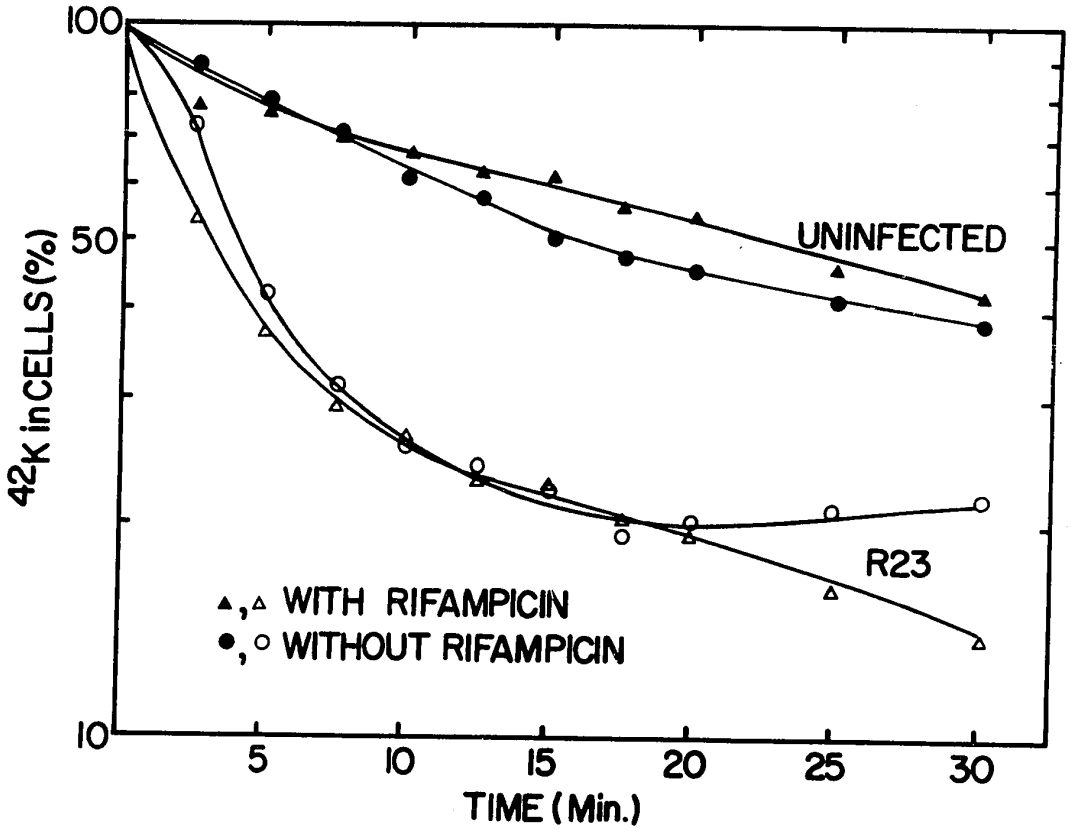

Fig. F7. Potassium Efflux: Effect of Rifampicin. The experiment was performed as described in Materials and Methods. Rifampicin was added to a concentration of 150 μg/ml at the time of infection; the phage yield was 2.6% of untreated cultures.

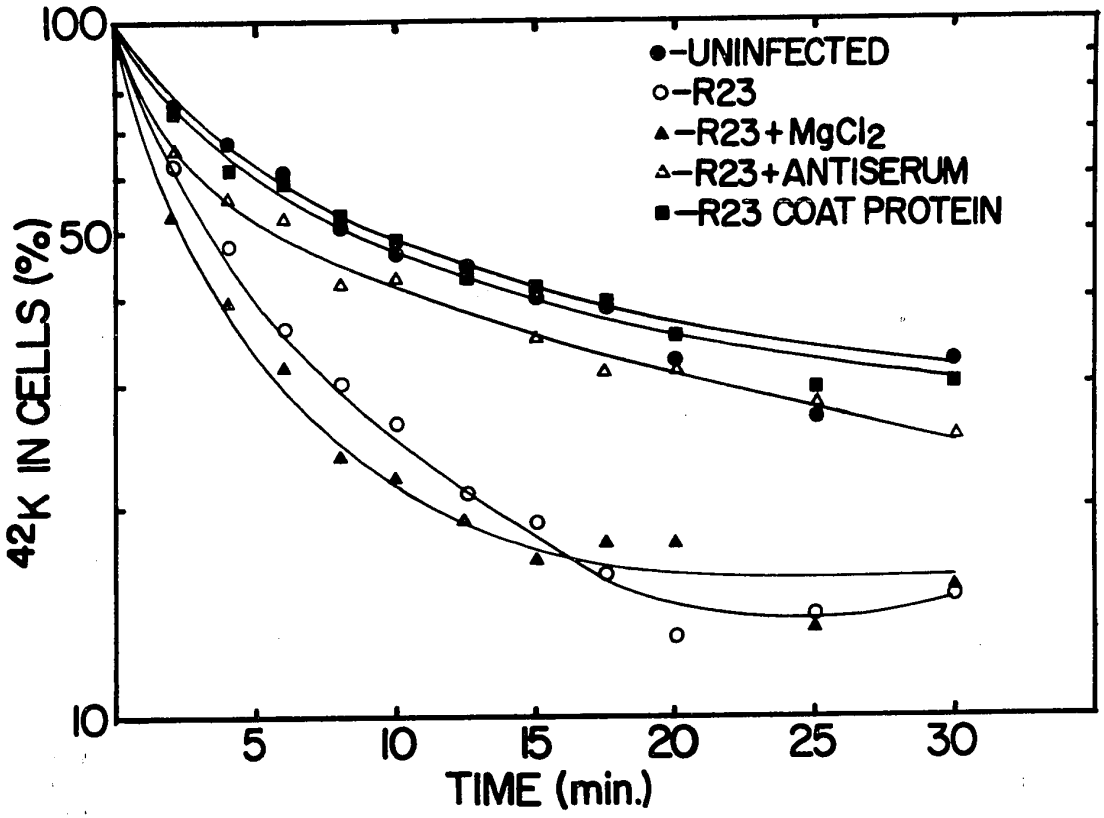

Fig. F8. Potassium Efflux: Effect of $MgCl_2$, R23 Coat Protein and Antiserum. The experiment was performed as described in Materials and Methods. $MgCl_2$ was added to 0.1 M and purified coat protein subunits in a concentration equivalent to a multiplicity of 10 phage/cell. R23 antiserum was used in excess to produce maximum inactivation of phage infectivity.

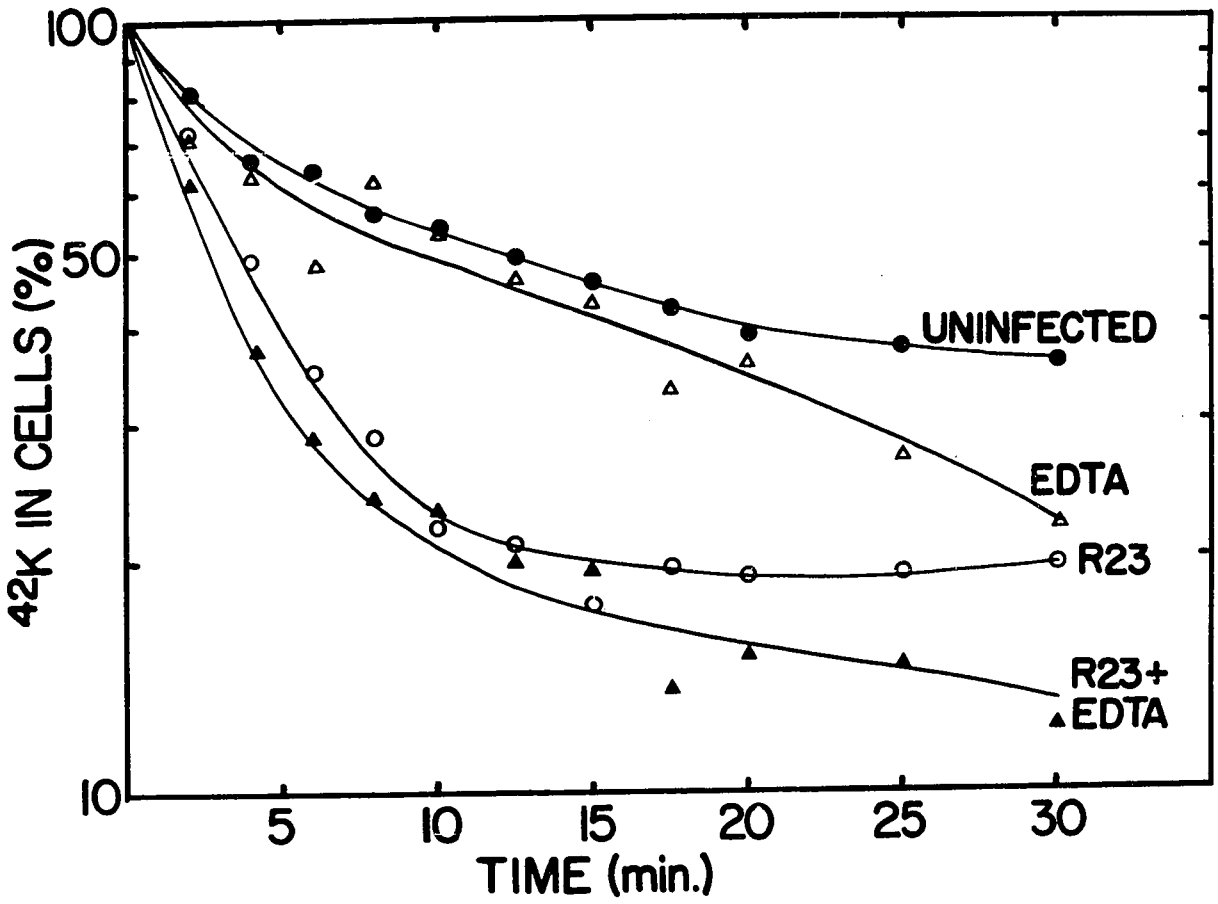

Fig. F9. Potassium Efflux: Effect of EDTA. The experiment was performed as described in Materials and Methods. EDTA was added to a final concentration of 10 mM at the time of infection.

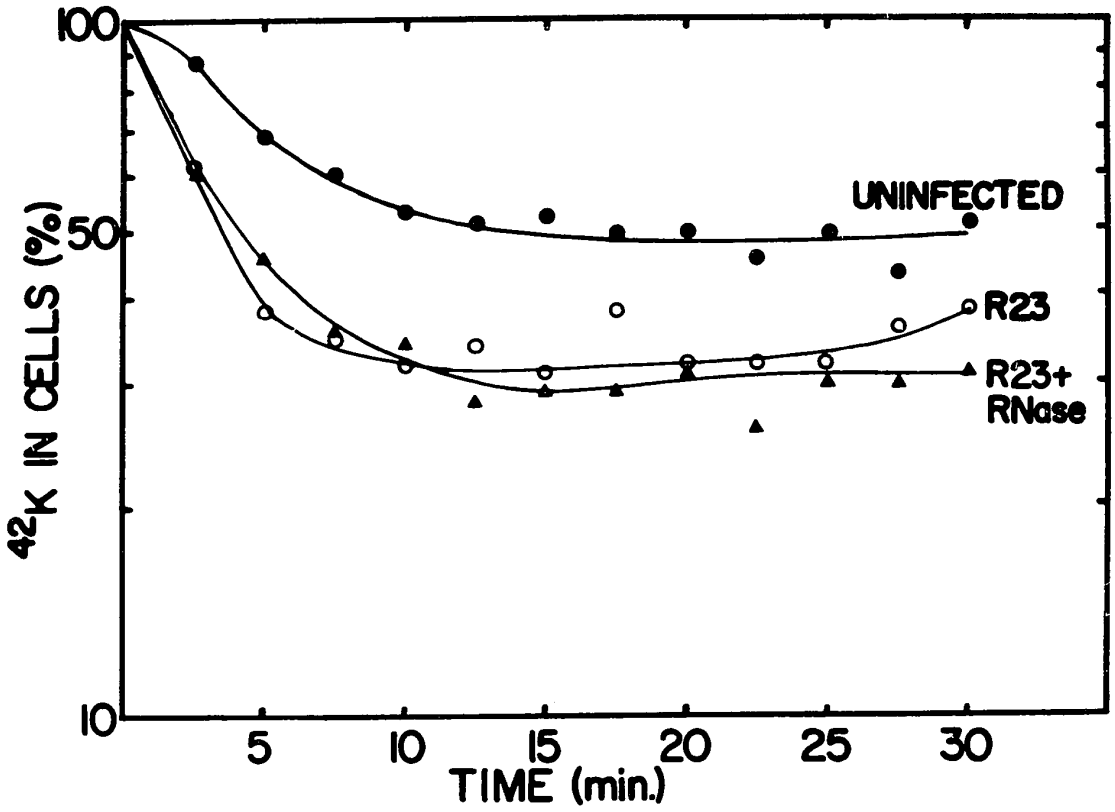

Fig. F10. Potassium Efflux: Effect of Ribonuclease. The experiment was performed as described in Materials and Methods. RNase was added to a concentration of 100 µg/ml.

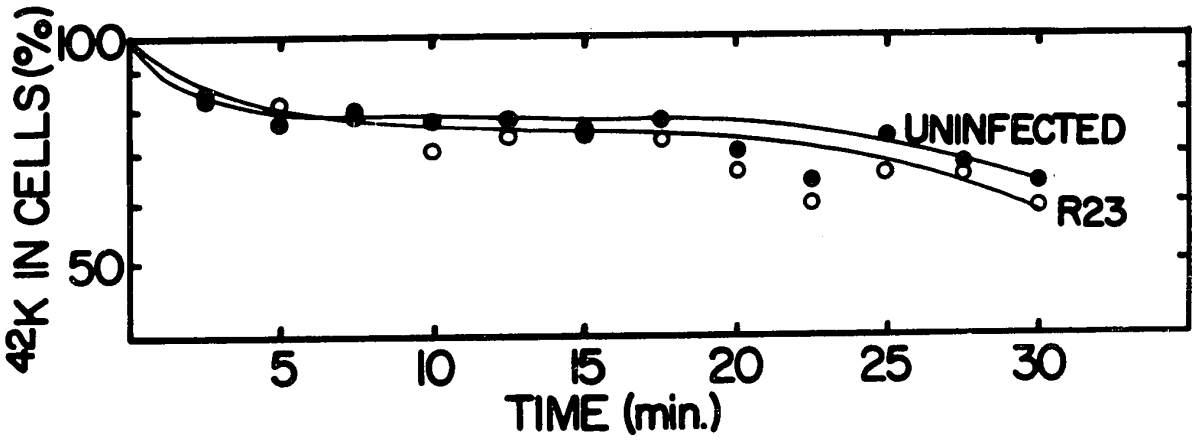

Fig. F11. Potassium Efflux at 4° C. The experiment was performed as in Fig. F1 except that the culture was maintained at 4° C after infection with R23.

TABLE F1

EFFECT OF TEMPERATURE ON R23-INDUCED POTASSIUM LOSS

Experiment	Temperature °C	$^{42}K^+$ Remaining in Cells: Uninfected %	$^{42}K^+$ Remaining in Cells: Infected %	Loss Induced by R23 %	% of Loss at 37° C %
1	4	79.8	78.4	1.4	5.6
	37	60.4	35.6	24.8	
2	22	55.0	54.9	0.1	0.3
	37	54.2	26.9	7.3	
3	24	61.7	59.8	1.9	6.8
	37	61.1	33.2	27.9	
4	29	57.5	39.3	18.2	56.0
	33	58.4	26.5	31.9	98.1
	37	60.3	27.8	32.5	

The experiments were performed as described in Materials and Methods except that the cultures were incubated at different temperatures. The $^{42}K^+$ remaining within the cells 9 min after infection was determined in uninfected and infected cultures and the difference between these values represented as the loss induced by R23 infection.

At temperatures such as 4° C, K^+ migration was arrested in uninfected cultures as well.

d) Effect of Ions. R23 infection increased ^{42}K efflux from cells suspended in media completely lacking potassium (Fig. F12) or sodium. Efflux of ^{42}K was enhanced by the presence of KH_2PO_4 (Fig. F12), KCl (Fig. F13) or NaCl (Fig. F14) in the medium in both uninfected and R23-infected cultures; this enhancement increased with increase in K^+ concentration (Fig. F13). In each case, however, a further and similar increment in efflux was produced by R23 infection over uninfected control levels (Figs. F12-14). Therefore, the R23 effect was independent of concentration of K^+ or Na^+ in the medium. Also, high concentrations of Mg^{++} (Fig. F8) and NH_4^{++} ions did not eliminate the phage effect.

e) The "Sealing Reaction". The accelerated K^+ efflux ceased after the first 10-15 min of infection, and in studies continued for 60 min, a slight increase in intracellular radioactivity was noted after the first 30 min, indicating reaccumulation of $^{42}K^+$. This arrest in K^+ loss was comparable to the "sealing reaction" described in T phage infection of *E. coli* (Puck & Lee, 1954, 1955; Silver *et al.*, 1968). Superinfection with R23 at 5 and 10 min did not release significant additional K^+ (Fig. F15), nor did superinfection at 15, 20 and 30 min (data not shown). "Sealing" occurred in cells infected with amber mutants, UV-irradiated phage (Figs. F3-5) and R23 in the presence of chloramphenicol (Fig. F6); it therefore did not appear to be related to the process or products of viral replication.

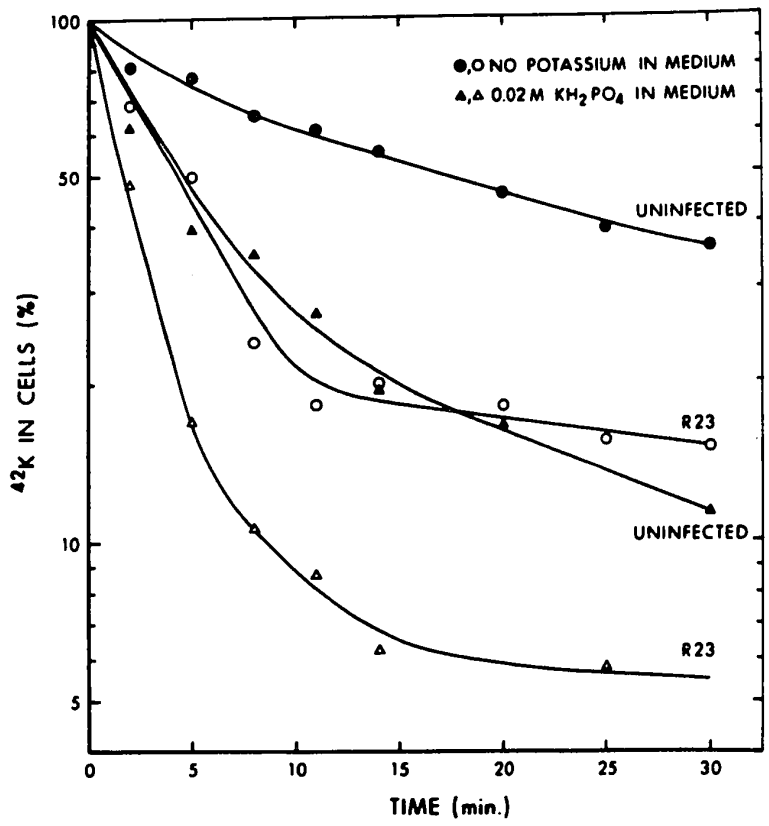

Fig. F12. Potassium Efflux: Effect of K^+-Deficient Medium. The experiment was performed in medium A (20 mM KH_2PO_4) or medium A lacking K^+ (KH_2PO_4 substituted by NaH_2PO_4).

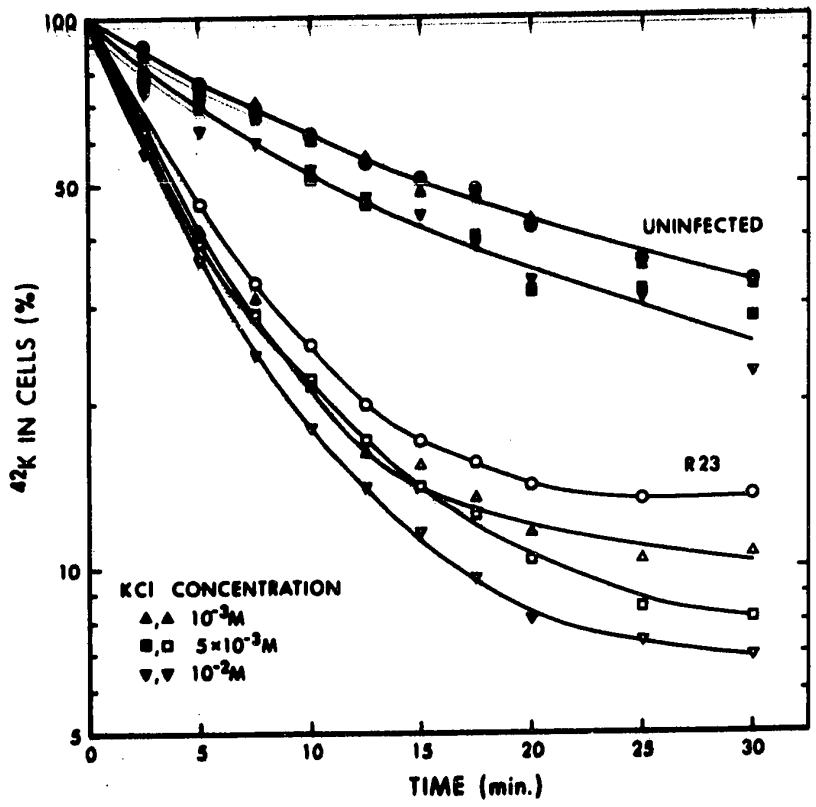

Fig. F13. Potassium Efflux: Effect of KCl in Medium. The experiment was performed as described in Materials and Methods, except that bacteria were suspended in Tryptone medium containing different concentrations of KCl or no added KCl.

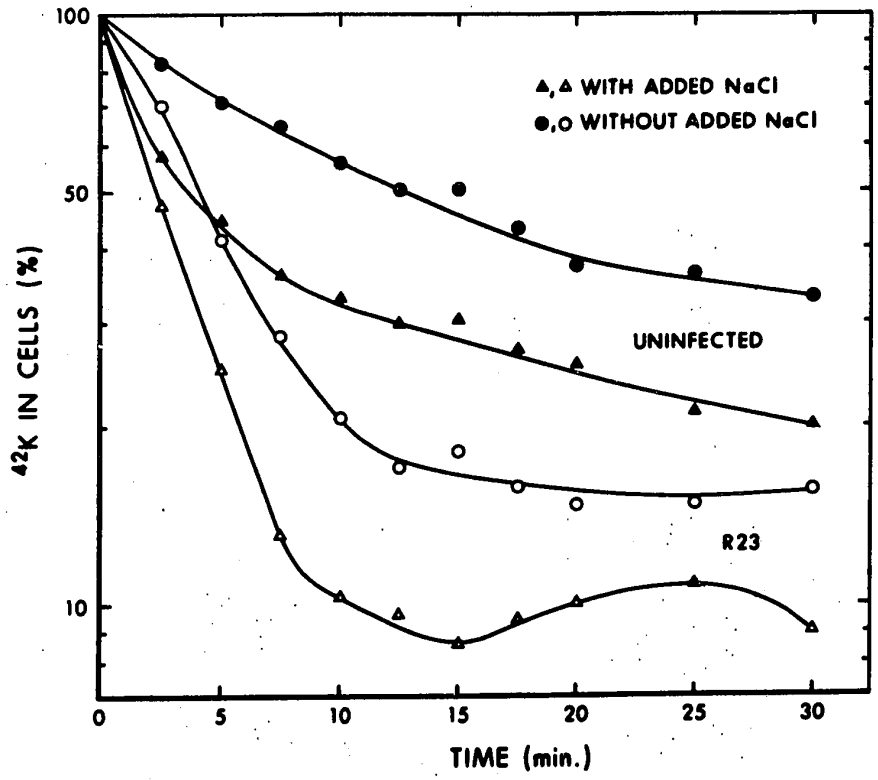

Fig. F14. Potassium Efflux: Effect of Added NaCl. The experiment was performed as described in Materials and Methods, except that NaCl was added to one pair of cultures to a final concentration of 0.44 M.

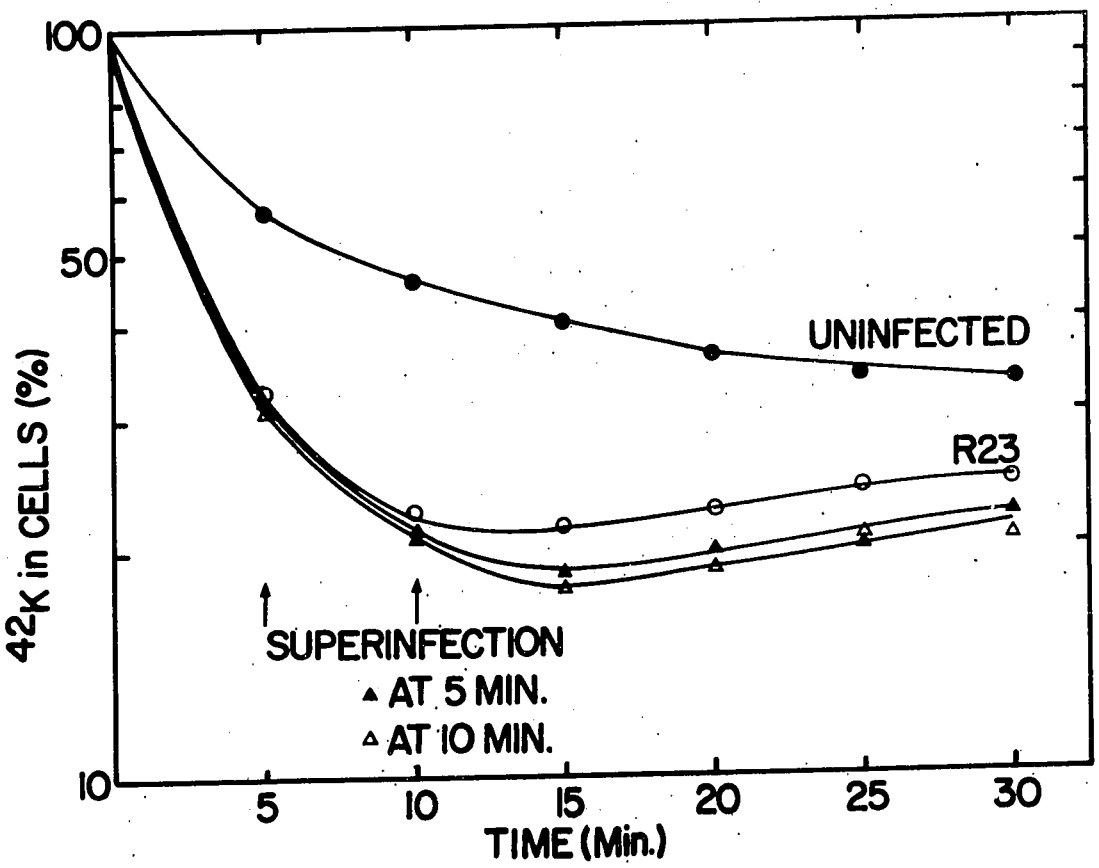

Fig. F15. Potassium Efflux: Effect of Superinfection. The experiment was performed as described for Fig. F1. Five or 10 min after the initial infection, R23 was again added at a multiplicity of 20 phage/cell.

f) Effect of R23 on Potassium Influx. Infection by R23 also resulted in an increased rate of $^{42}K^+$ influx during the first 5-10 min (Fig. F16). Thereafter, there was a gradual decline so that by 25-30 min the radioactivity accumulated was identical in uninfected and infected cells. This effect was also noted with f2, Qβ, R34 and R40 (Fig. F17). The phage effect on influx increased with increase in MOI up to 12.5 to 25 phage/cell (Fig. F16) and appeared to be diminished at higher multiplicities.

As with efflux, the increased influx was observed during infection by UV-irradiated phage or viral RNA synthetase mutants and in the presence of chloramphenicol and EDTA, and thus did not require viral RNA replication or penetration. In the presence of EDTA, the increased uptake was not quite as marked as in its absence (Fig. F18), but EDTA diminished the rate of influx after the first 5 min in uninfected cells as well.

Finally, quantitative analysis of the results indicated a close correspondence between the $^{42}K^+$ lost and gained in the first 10 min after R23 infection (Table F2), although extracellular potassium concentrations in influx and efflux experiments were not identical and the techniques not absolutely comparable. Thus, there was probably no marked change in net cell K^+ content.

Discussion

a) Effect of R23 on K^+ Flux. Potassium flux was investigated in K^+-rich cells suspended in tryptone medium poor in K^+ (0.6 mM) (Silver *et al.*, 1968), i.e., under conditions favouring the concentration gradient normally present during the early logarithmic

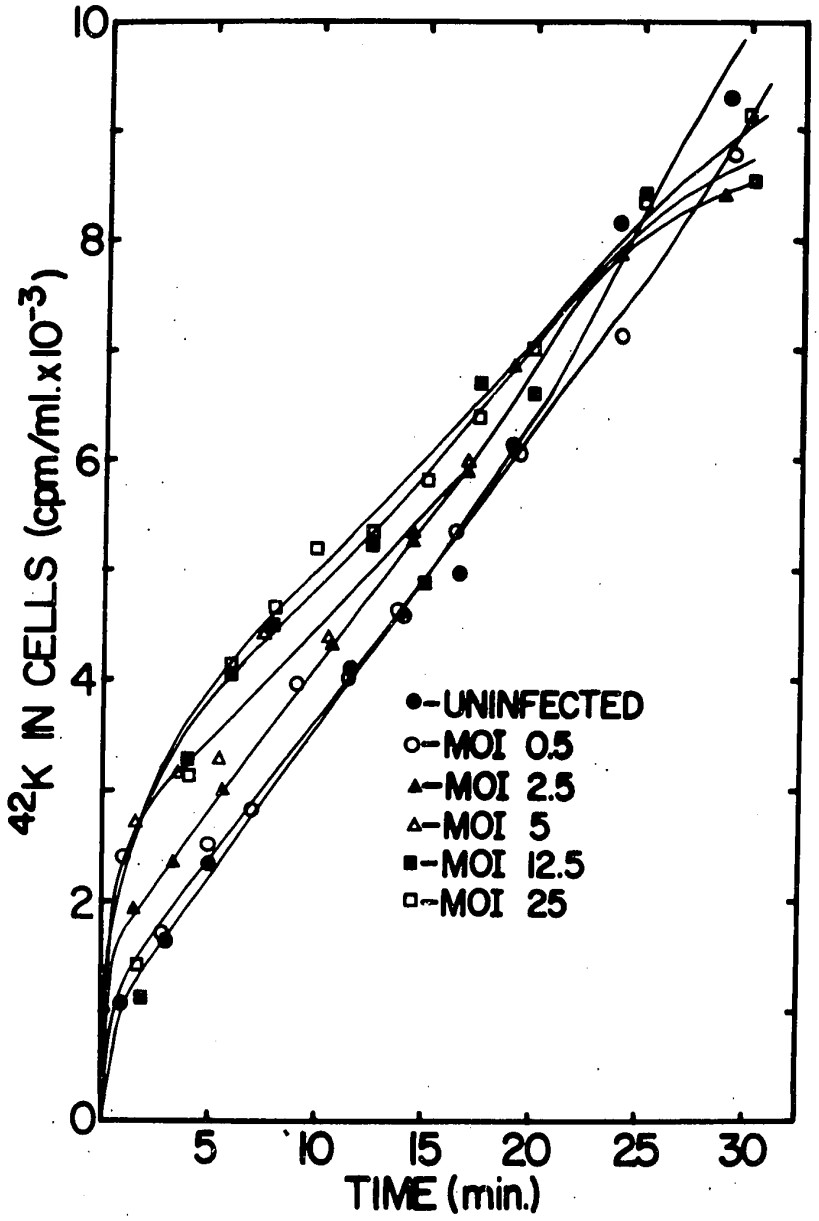

Fig. F16. Potassium Influx: Effect of R23 at Different MOI.

The experiment was performed as described in Materials and Methods. R23 was added at multiplicities of 0.5 - 25 phage/cell, at the time of addition of $^{42}K^{+}$.

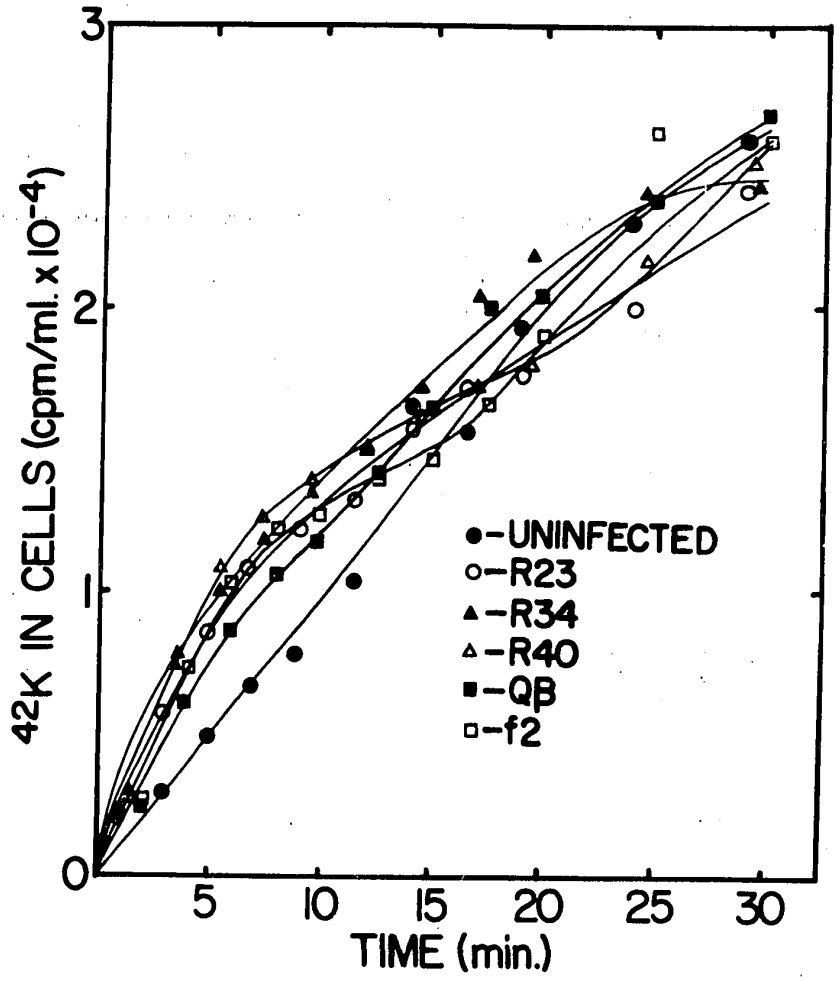

Fig. F17. Effect of RNA Phages on Potassium Influx. The experiment was performed as described in Materials and Methods.

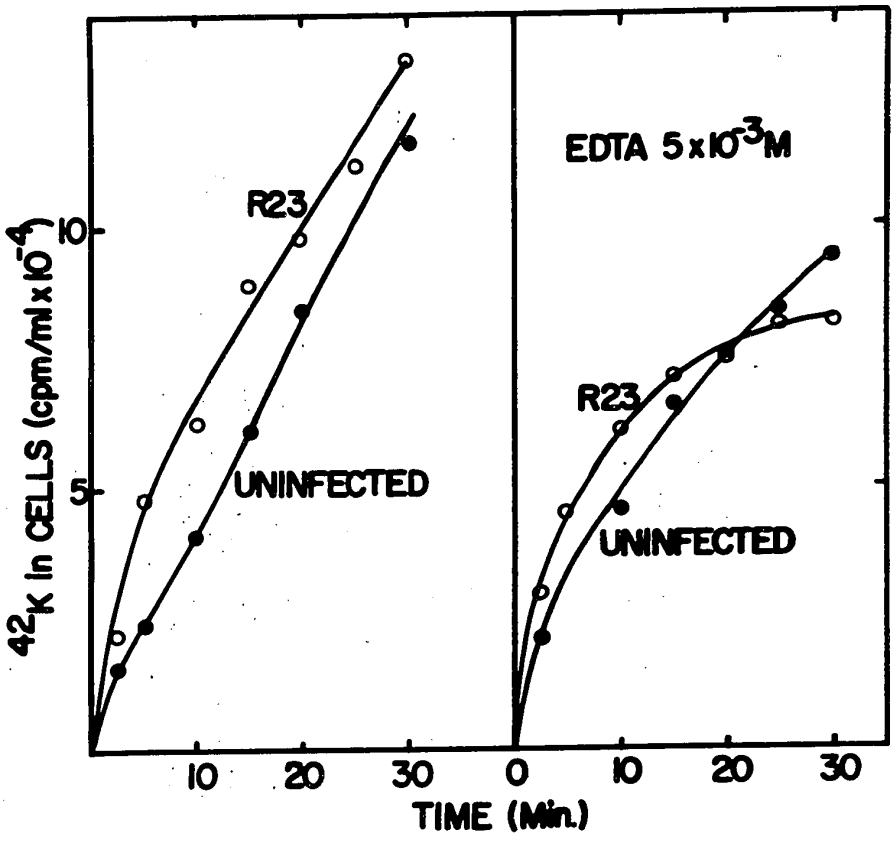

Fig. F18. Potassium Influx: Effect of EDTA. The experiment was performed as described in Materials and Methods. EDTA was added to a final concentration of 5 mM. The phage yield in EDTA-treated cultures was 0.02% of untreated cultures.

TABLE F2

POTASSIUM INFLUX AND EFFLUX INDUCED BY R23 INFECTION

Experiment	Efflux Increment cpm/ml at 10 min	Influx Increment cpm/ml at 10 min
1	4600	6200
2	1450	1480

The experiments were performed as described in Materials and Methods. The increment in potassium efflux and influx 10 min after R23 infection was analyzed.

phase of the growth of *E. coli* (Schultz & Solomon, 1961). R23 induced a transient increase in both efflux and influx of $^{42}K^{+}$ which increased with increase in MOI up to 12.5 and was not dependent on protein synthesis or intracellular functioning of the phage nucleic acid. These effects, and their arrest, were demonstrated during infection with UV-irradiated R23, amber mutants, and R23 in the presence of chloramphenicol and rifampicin. Penetration and injection of phage RNA did not appear to be necessary since EDTA and RNase did not prevent the phage effect on K^{+} flux. (However, RNase does not completely prevent injection). Adsorption was not the sole requirement since the phage effect diminished with reduction in incubation temperature. Though adsorption occurs at low temperatures, including 4° C, successful phage invasion or "irreversible adsorption" does not occur. Therefore, it appeared that a further step in phage infection beyond adsorption, and perhaps involving injection, was involved.

The effect of R23 on K^{+} flux in *E. coli* shows many similarities and some important differences compared to that of T-even phage (Silver *et al.*, 1968). In contrast to R23, T-even phage did not affect K^{+} influx, and leakage was prevented by a high Mg^{++} concentration. Since no leakage occurred at 4° C (at which temperature adsorption proceeds but injection of phage DNA is blocked), it was considered that the leakage might be related to the injection stage. It was suggested that leakage could be associated with enzymatic digestion of the cell wall at the site of adsorption or could accompany injection of the T phage tail needle through the cell wall.

b) Mechanism of R23 Effect. R23 infection stimulated movement of K^+ in both directions, both along and against the initial concentration gradient (due to a high cellular K^+ concentration). It is difficult to distinguish between a primary action of R23 on influx and an initial effect on efflux, or to postulate an independent action on both fluxes, since K^+ influx and efflux are probably coupled. It is likely that phage infection produced an increase in membrane permeability which led to enhanced K^+ efflux or diffusion in the direction of the concentration gradient. The increased influx may be attributed to the increased phage-induced passive release of K^+ allowing the carrier-utilizing uptake of K^+ to proceed more rapidly.

The RNA phage does not possess a tail needle like T phage; an enzyme able to digest the cell wall has not been demonstrated. The early leakage likely represents increased membrane permeability without actual cell lysis. Some event in phage-host interaction consequent on adsorption is involved in the enhancement of K^+ flux by R23. One speculation is that contact of the phage (maturation protein) with the F pili triggers a conformational change in the membrane which leads to increased permeability and enhanced activity of the K^+ carrier. Or the interaction may somehow widen or create a pore in the membrane. Perhaps later removal of the maturation protein or its effects from the perturbed membrane allows an arrest in K^+ leakage by a conformational shift or by an enzymatic repair which requires energy.

It is unlikely that the increased K^+ efflux after R23 infection is mechanistically involved in its characteristic inhibition of host

protein synthesis, but it may be a minor contributory factor if it leads to a significantly decreased cellular content of K at any time. The conjecture that structural and functional alteration of the bacterial membrane following R23 infection is responsible for part of its effect on host macromolecular synthesis is plausible, however. This initial study has illustrated that R23 is able to affect the membrane to the extent that transport and provision of a permeability barrier are significantly impaired or altered. The determination of the exact molecular events involved in this effect will require extensive investigation.

G. Effect of R23 and Metabolic Inhibitors on Potassium Fluxes

Introduction

Transport through the cell membrane may occur by passive (or simple) diffusion, facilitated diffusion (with a carrier) or active transport (utilizing energy). Inhibitors or activators of transport may act on the metabolic systems involved in supplying energy for transport or in maintaining the membrane, or on the membrane or transport system directly. Some inhibitors, such as cyanide, undoubtedly act mainly by disturbing metabolism, whereas SH reagents are more apt to act on the membrane, since the membrane proteins contain SH groups important in maintaining the integrity of the membrane (Webb, 1966).

Studies on the mechanisms of K^+ transport in normal and mutant *E. coli* have been reported (Lubin & Kessel, 1960; Schultz & Solomon, 1961; Schultz, Epstein & Goldstein, 1963; Schultz, Epstein & Solomon, 1963; Epstein & Schultz, 1965, 1966; Damadian, 1968; Lubochinsky, Meury & Stolkowski, 1964). Schultz *et al.* favoured the view that intracellular cation binding was not responsible for the K^+ distribution , that at least 50% of K^+ efflux was a carrier-mediated process tightly coupled to K^+ influx and that mechanisms for Na^+ and K^+ transport were independent. One model for exchange of cations mediated by a carrier protein assumes that interaction of the carrier with ATP induces a conformational change in the protein that rotates the external binding sites inward. This lowers their relative affinity for the external ion which dissociates from it and allows binding of an extracellular cation in exchange. A Na^+-K^+-ATPase has been implicated

in active transport of K^+ and Na^+ in animal cells (Katz & Epstein, 1968); ATPases associated with bacterial membranes may also be involved in active K^+ transport (Harold *et al.*, 1969; Hayashi & Uchida, 1968).

The use of radioactively-labeled ions can provide information on influx and efflux rates independently of total or overall changes. Since a given flux may be the resultant of simple diffusion, exchange diffusion and active transport, R23 could have stimulated $^{42}K^+$ influx and efflux (Section F) by increasing passive or facilitated diffusion or active transport or by acting on the energy-supplying system. It was thought that the use of specific metabolic inhibitors would help delineate the component affected by R23. The effects of a respiratory inhibitor (NaCN), an uncoupler of oxidative phosphorylation (DNP) and a SH reagent (NEM) on $^{42}K^+$ flux in uninfected and R23-infected *E. coli* were investigated to ascertain the conditions allowing or abolishing the stimulatory phage effect and to compare the effects of inhibitors to that of R23.

Results

a) Effect of NaCN, DNP, NaN_3. The K^+ efflux during the first 10-15 min of infection appeared to be inhibited in the presence of NaCN when compared to the loss in untreated infected cells (Fig. G1). NaCN, however, diminished the efflux in uninfected cells as well. Taking this into consideration, R23 induced an equal loss from cyanide-treated and untreated cells during the first 10 min, although the amount remaining in the cells at 10 min was much higher in the treated cells (Table G1). After 15 min of infection, however, the K^+ loss in cyanide-treated cells was much enhanced and the final level of $^{42}K^+$ was lower than in unpoisoned

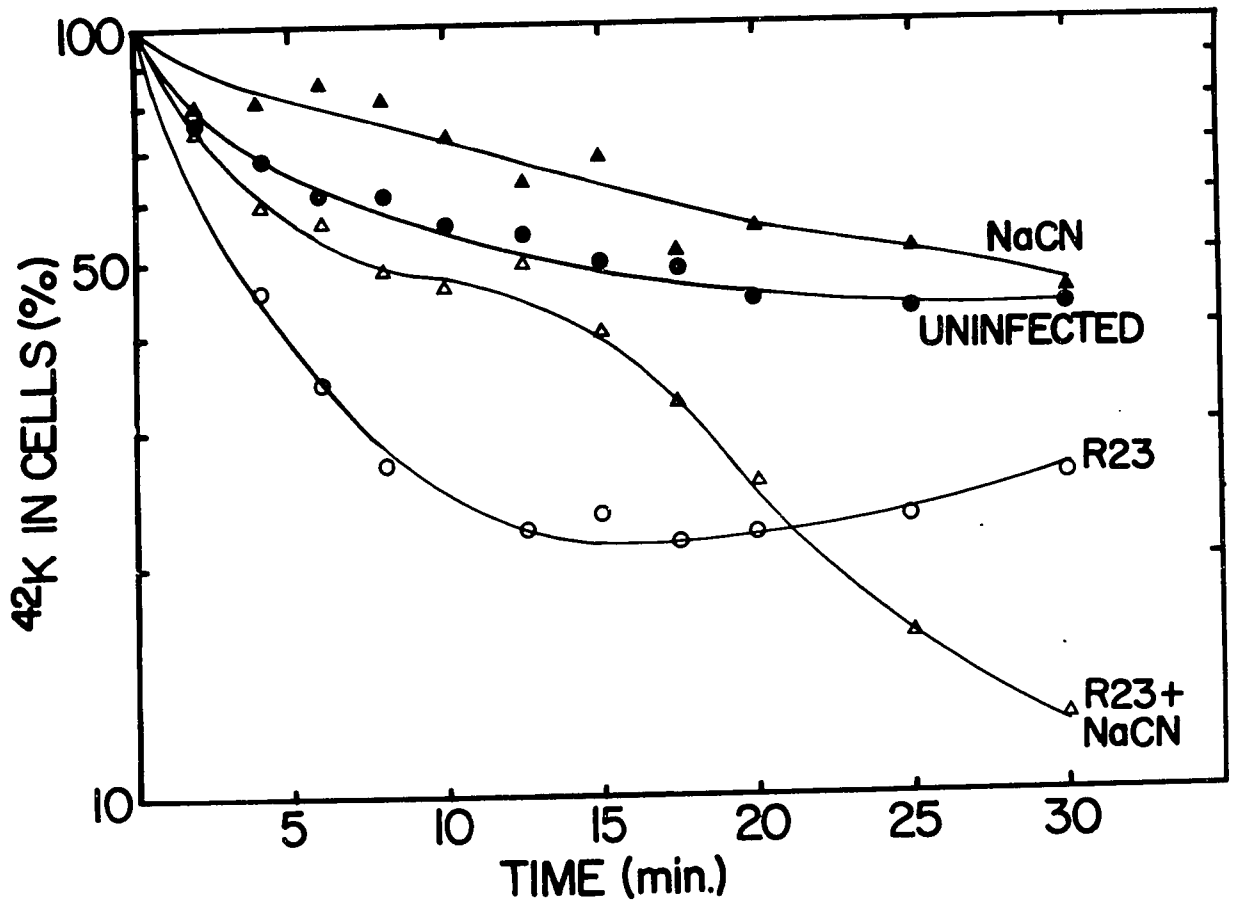

Fig. G1. Potassium Efflux: Effect of Sodium Cyanide. The experiment was performed as described in Materials and Methods. NaCN was added at the time of infection to a final concentration of 1 mM. The phage yield in NaCN-treated cultures was 4% of untreated cultures.

TABLE G1

POTASSIUM EFFLUX: EFFECT OF NaCN AND ATP

Addition to Culture	Experiments Analyzed	^{42}K Remaining in Cells at 10 min		Loss Induced By R23 Infection
	No.	Uninfected %	Infected %	%
Control	30	55 ±7	25 ±4	30
ATP 5 mM	1	64	29	35
NaCN 1 mM	14	88 ±8	56 ±10	32
NaCN 1 mM and ATP 5 mM	3	81 ±7	27 ±4	54

The experiments were performed as described in the legends to Figs. F1, G1, 6. The amount of ^{42}K in the cell at 10 min was analyzed.

cells (Fig. G1). Sodium cyanide also produced a marked depression of $^{42}K^+$ accumulation but R23 infection still enhanced K^+ entry to the same extent as in unpoisoned cells (Fig. G2, Table G2).

Sodium azide and DNP produced a reduction in the rate of efflux in R23-infected cells (and to a lesser degree in uninfected cells), especially after the first 2 min of infection (Figs. G3,4). The leakage continued linearly, without the late accentuation produced by NaCN, until the intracellular $^{42}K^+$ levels at 30 min in uninfected and infected cultures equalled or exceeded the levels achieved in control cultures. DNP also inhibited $^{42}K^+$ influx and the stimulatory effect of R23 (Fig. G5).

b) Effect of ATP. Addition of ATP alone to uninfected or infected cells did not alter K^+ efflux (Fig. G6) or the amount of $^{42}K^+$ remaining in the cells (Table G1). Addition of ATP to infected cultures in the presence of NaCN, however, accentuated the K^+ loss during the first 10 min, mimicking the pattern in infected cells untreated with ATP or cyanide (Fig. G6). Fifty-four % of the cellular $^{42}K^+$ was lost in 10 min in contrast to about 30% in infected, untreated cells or infected cells treated with cyanide or ATP (Table G1). ATP did not affect the late accentuation induced by cyanide; the intracellular concentration of $^{42}K^+$ was similar at 30 min in cultures infected in the presence or absence of ATP (Fig. G6). Addition of ATP plus NaCN did not affect K^+ loss from uninfected cells. The effect of ATP on cyanide-treated, infected cells could not be reproduced by 5'-AMP, cyclic 3',5'-AMP, 5'-CMP, CDP, CTP, 5'-UMP, UTP, 2',3'-GMP or GTP, but was duplicated by dATP. The addition of ADP in equimolar concentrations did not diminish the effect of ATP.

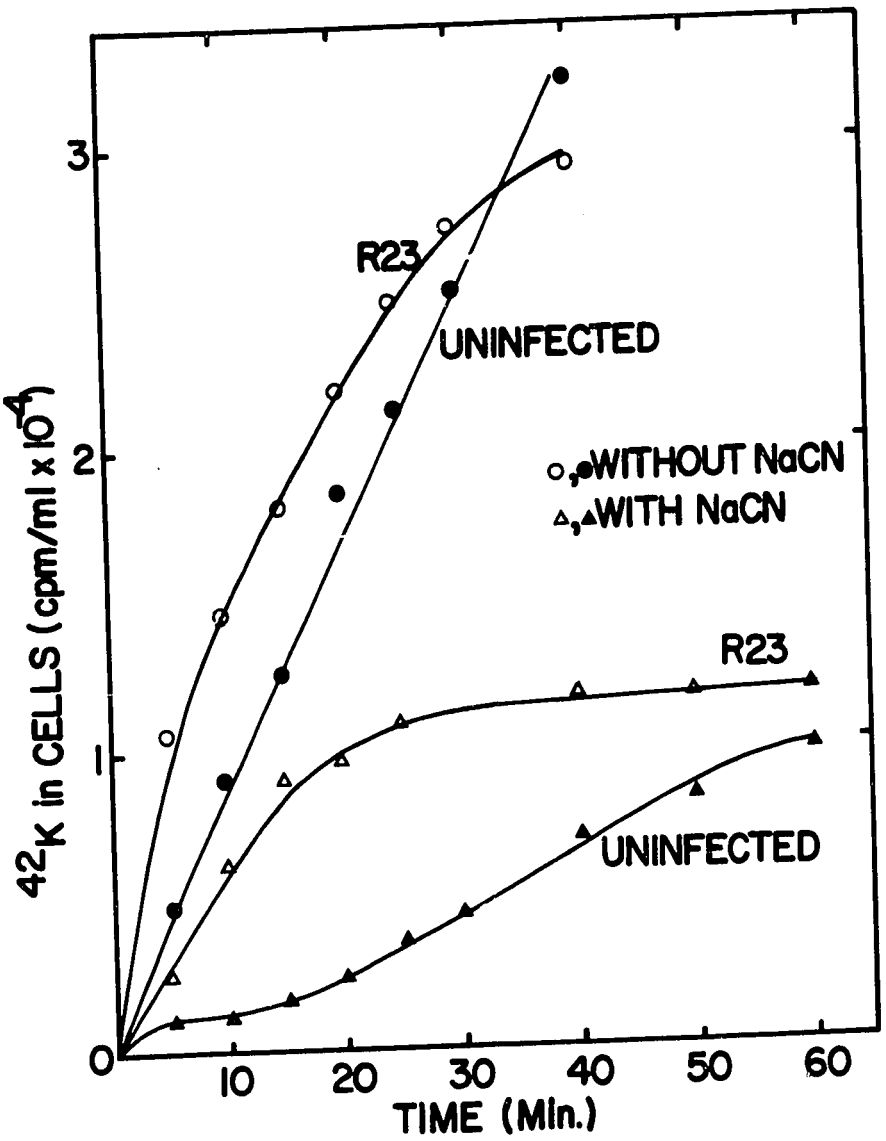

Fig. G2. Potassium Influx: Effect of Sodium Cyanide. The experiment was performed as described in Materials and Methods. NaCN was added at the time of infection to a final concentration of 1 mM. The phage yield in the NaCN-treated cultures was 10% of that in the untreated cultures.

TABLE G2

POTASSIUM LOSS AND INTAKE INDUCED BY R23 INFECTION: EFFECT OF NaCN

Experiment	Potassium Loss after R23 Infection cpm/ml at 10 min	Potassium Intake Stimulated by R23 Infection cpm/ml at 10 min
1. Control	4600	6200
NaCN 1 mM	5430	5600
NaCN 1 mM + ATP 5 mM	-	6320
2. Control	1450	1480
NaCN 1 mM	920	1310

The experiments were performed as described in the legends to Figs. F1, G1, 2, 6, 7. The increment in potassium efflux and influx 10 min after R23 infection was analyzed.

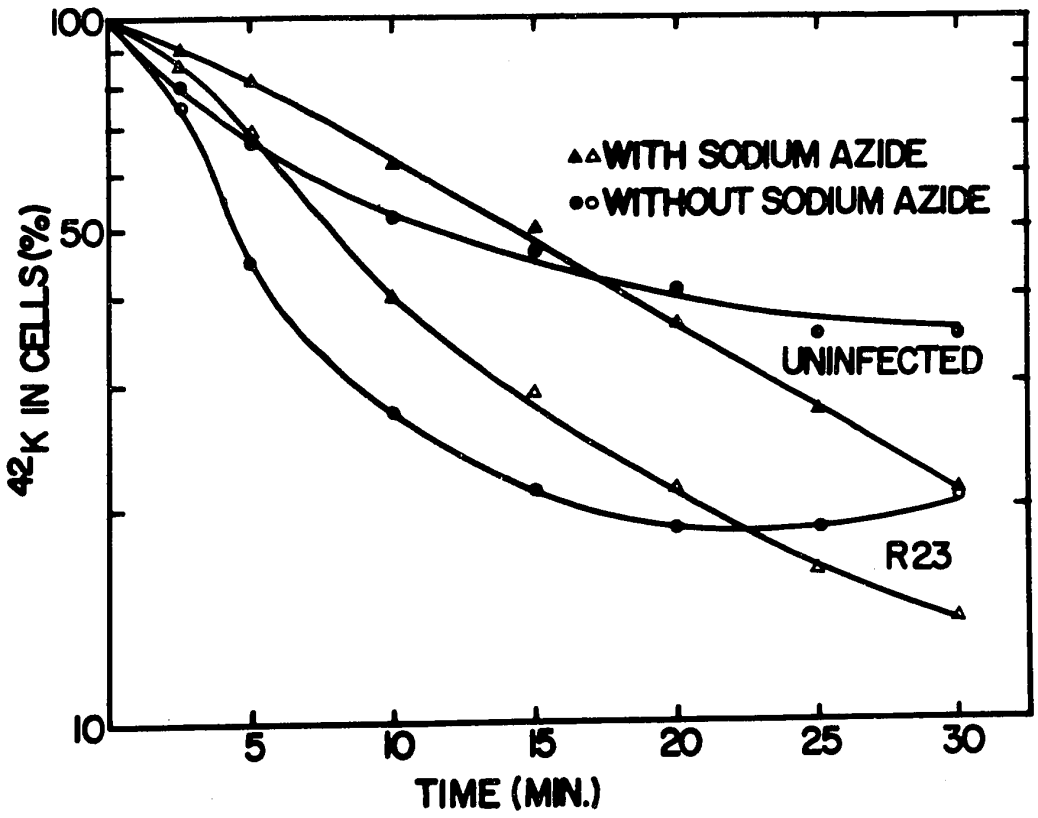

Fig. G3. Potassium Efflux: Effect of Sodium Azide. The experiment was performed as described in Materials and Methods. NaN_3 was added at a concentration of 10 mM.

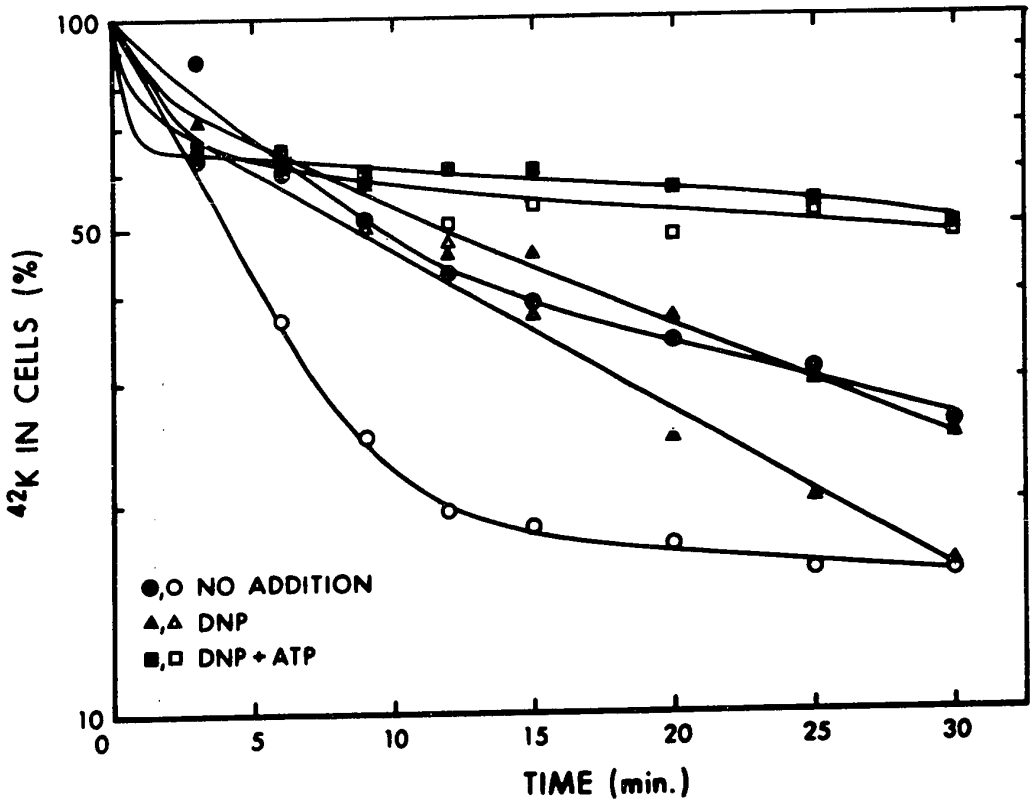

Fig. G4. Potassium Efflux: Effect of DNP and ATP plus DNP. The experiment was performed as described in Materials and Methods. DNP and ATP were added to concentrations of 1 mM and 5 mM, respectively. Open symbols: R23. Closed symbols: uninfected.

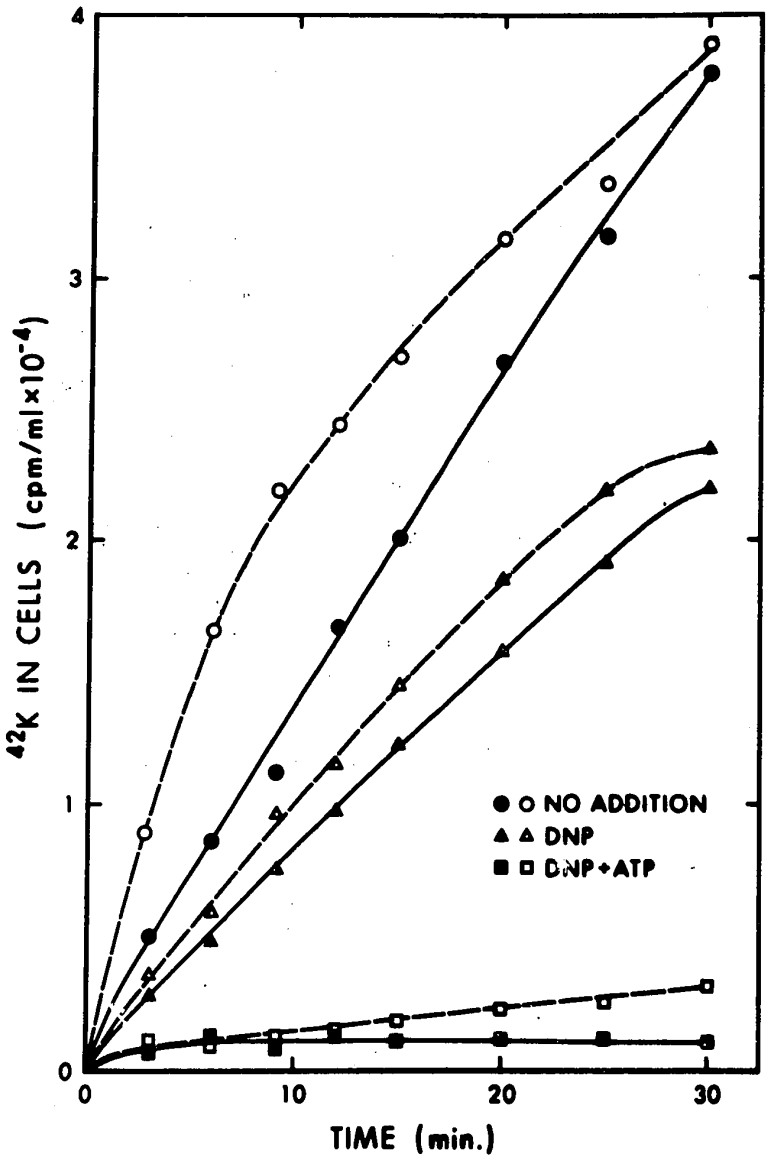

Fig. G5. Potassium Influx: Effect of DNP and DNP plus ATP. The experiment was performed as described in Materials and Methods. DNP and ATP were added to concentrations of 1 mM and 5 mM, respectively. Open symbols: R23. Closed symbols: uninfected.

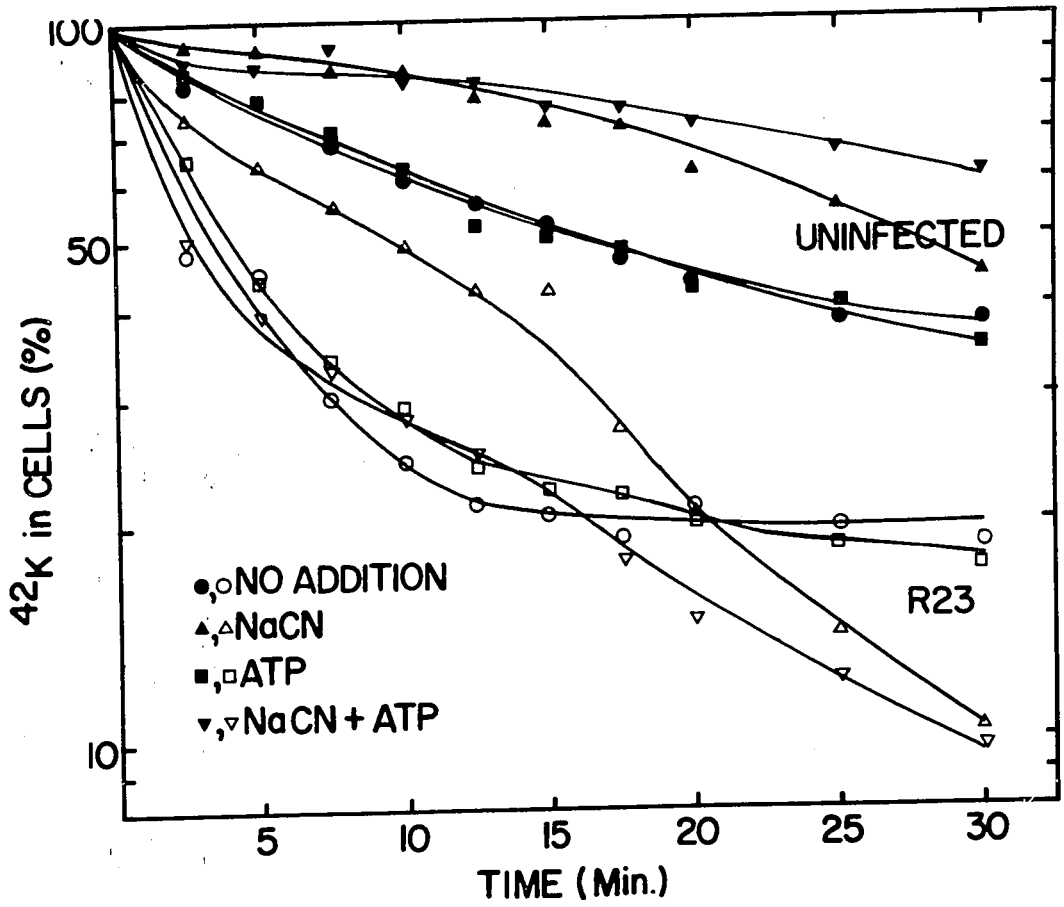

Fig. G6. Potassium Efflux: Effect of Sodium Cyanide and ATP. The experiment was performed as described in Materials and Methods. NaCN and ATP were added to final concentrations of 1 and 5 mM, respectively. The phage yields in cultures containing ATP, NaCN, and NaCN plus ATP were 91, 22 and 13%, respectively, of untreated cultures.

The addition of ATP to infected (or uninfected) cyanide-treated cells did not affect $^{42}K^+$ accumulation significantly, in contrast to its effect on efflux (Fig. G7, Table G2). Addition of ATP alone, however, consistently diminished $^{42}K^+$ accumulation after the first 10 min (Fig.G8).

On addition of ATP simultaneously with DNP, there was virtual arrest of leakage after the first 2 min; the intracellular $^{42}K^+$ levels achieved were 55-60% of the initial values in both uninfected and infected cultures (Fig. G4). Thus, efflux in uninfected and infected cultures and the R23 effect were inhibited to a more drastic degree by DNP and ATP together than by DNP alone. ATP addition to DNP-treated cells also drastically inhibited $^{42}K^+$ influx and the stimulatory effect of R23 (Fig. G5).

Although the injection and penetration of viral RNA requires energy (Brinton & Beer, 1967), the effect of ATP on K^+ efflux in cyanide-treated, infected cells was not due to an enhancement of penetration. Addition of ATP or NaCN inhibited penetration during the first 10 min of infection (Fig. G9) and the addition of both together had an even greater inhibitory effect. Penetration achieved normal levels by 30 min in cyanide-treated cells, but the inhibitory effect of ATP was not reversed with time.

c) Effect of NEM. N-ethylmaleimide (NEM) increased $^{42}K^+$ efflux in uninfected and infected cultures (Fig. G10). The enhancement of the initial rate of efflux depended on the concentration of NEM and decreased in the order $10^{-4}>10^{-5}>10^{-3}$ and 10^{-2}. NEM at 10^{-6} M had no effect on efflux. The losses induced by 10^{-2} and 10^{-3} M NEM were similar and consistently less than that observed at 10^{-4} M NEM, and the intracellular

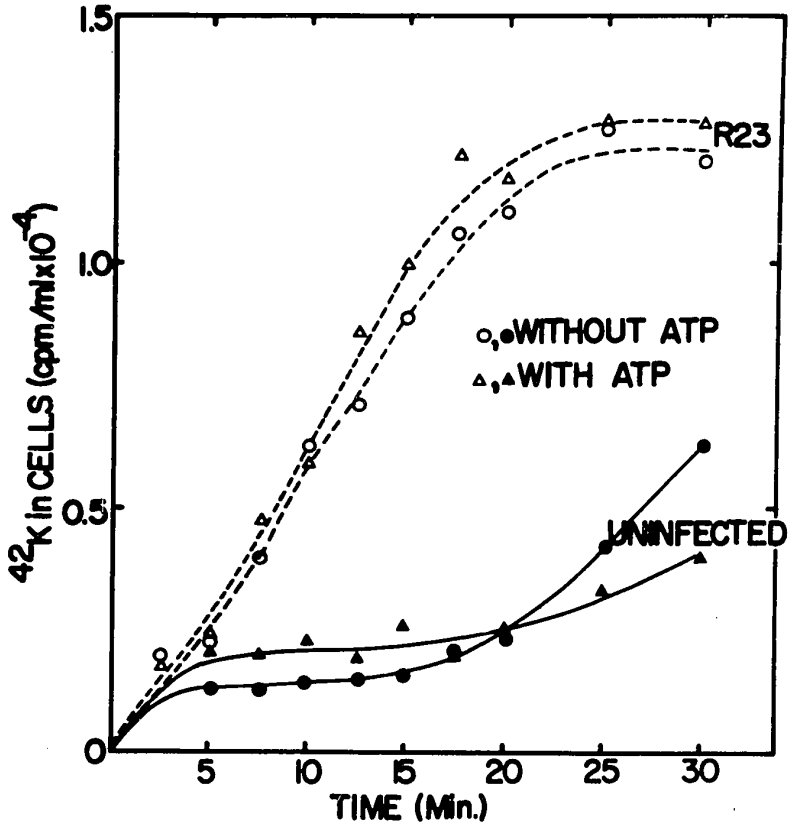

Fig. G7. Potassium Influx: Effect of Sodium Cyanide and ATP.

The experiment was performed as described in Materials and Methods. NaCN and ATP were added to concentrations of 1 and 5 mM, respectively.

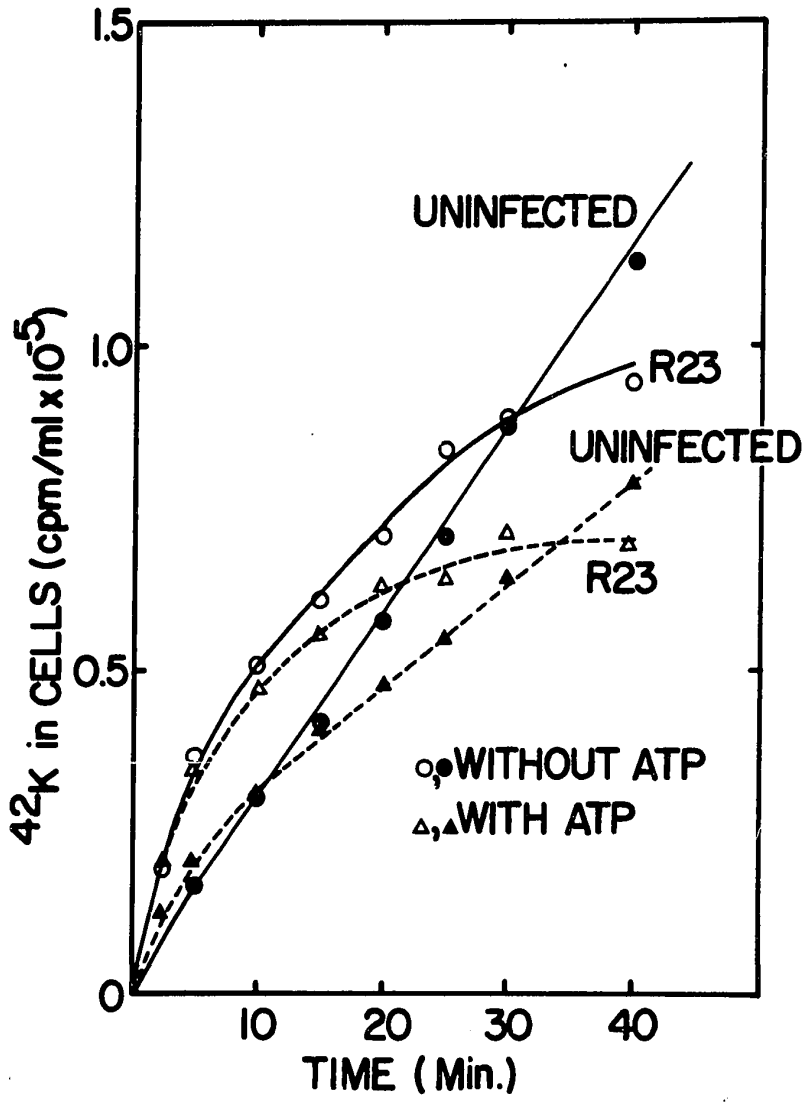

Fig. G8. Potassium Influx: Effect of ATP. The experiment was performed as described in Materials and Methods. ATP was added to a concentration of 5 mM.

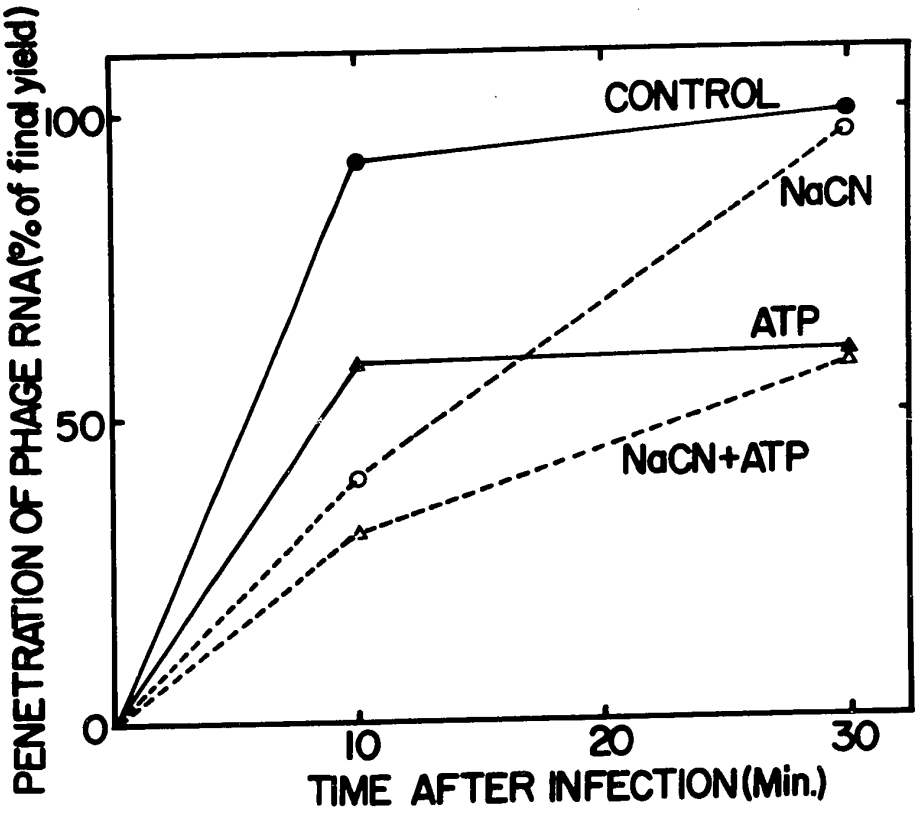

Fig. G9. Penetration of Phage RNA: Effect of Sodium Cyanide and ATP. The experiment was performed as described in Materials and Methods. NaCN (1 mM), ATP (5 mM), or both, were added 1 min before infection with R23. The final level of radioactivity associated with vigorously washed cells 30 min after infection is represented here as 100%.

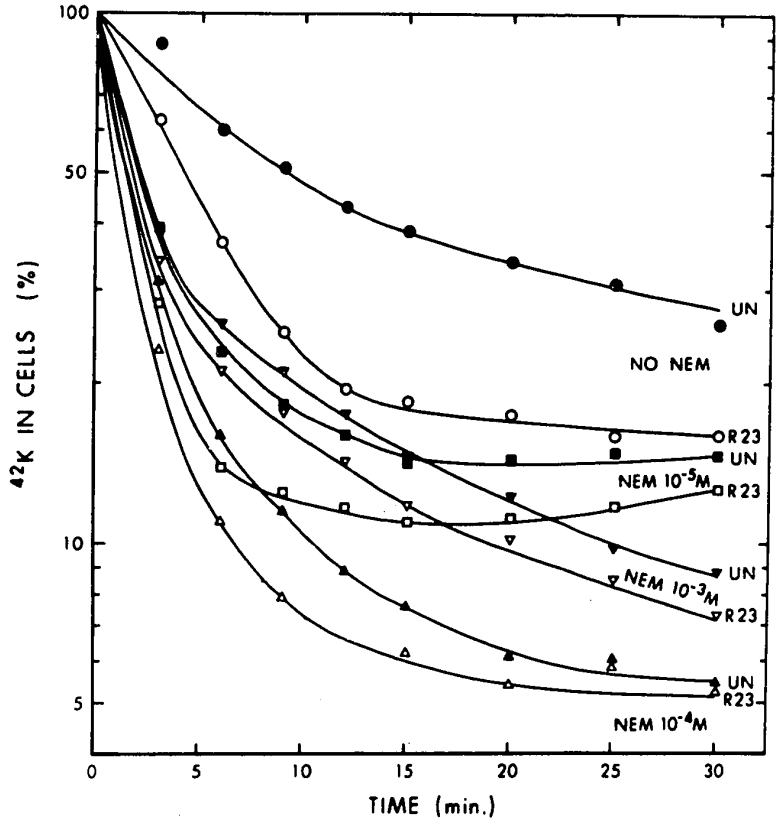

Fig. G10. Potassium Efflux: Effect of NEM. The experiment was performed as described in Materials and Methods. N-ethylmaleimide was added at the concentrations indicated.

levels of $^{42}K^+$ decreased progressively with time (up to 30 min). In the case of 10^{-4} and 10^{-5} M NEM, the pattern of efflux after the first 10 to 20 min resembled the R23 effect (although the loss was slightly greater in the case of NEM). The final level of intracellular $^{42}K^+$, therefore, was reduced by different concentrations of NEM in the order: $10^{-5}<$ 10^{-2} and $10^{-3}<10^{-4}$ M. Thus, a concentration of 10^{-4} M NEM produced the maximum rate of efflux and the maximum depression of intracellular $^{42}K^+$. R23 was able to induce K^+ loss in the presence of NEM but the capacity to induce K^+ loss was markedly reduced with increasing concentrations of NEM.

The effect of NEM on influx was also dependent on concentration (Fig. G11). 10^{-6} M NEM had no effect while 10^{-5} M NEM increased K^+ influx in uninfected and infected cells to a similar extent. 10^{-4} M NEM produced a transient stimulation of K^+ influx for 5 min followed by a rapid decline; the pattern produced by 10^{-3} M NEM was similar but the stimulatory phase was minimal or nonexistent. K^+ influx was also markedly reduced by 10^{-2} M NEM. The influx in R23-infected cells in the presence of 10^{-3} and 10^{-4} M NEM was less than that in uninfected cells and equal to the latter at 10^{-5} M NEM.

The stimulatory effect of 10^{-5} M NEM on $^{42}K^+$ influx disappeared when NEM was removed after a 15 min exposure by washing, centrifugation, and resuspension in NEM-free medium. On subsequent infection, R23 induced its usual stimulation of K^+ accumulation. On the other hand, the effect of 10^{-3} and 10^{-4} M NEM was not reversed by NEM removal after 15 min. Addition of 10 mM 2-mercaptoethanol simultaneously with NEM completely prevented the effects of NEM on K^+ influx and efflux.

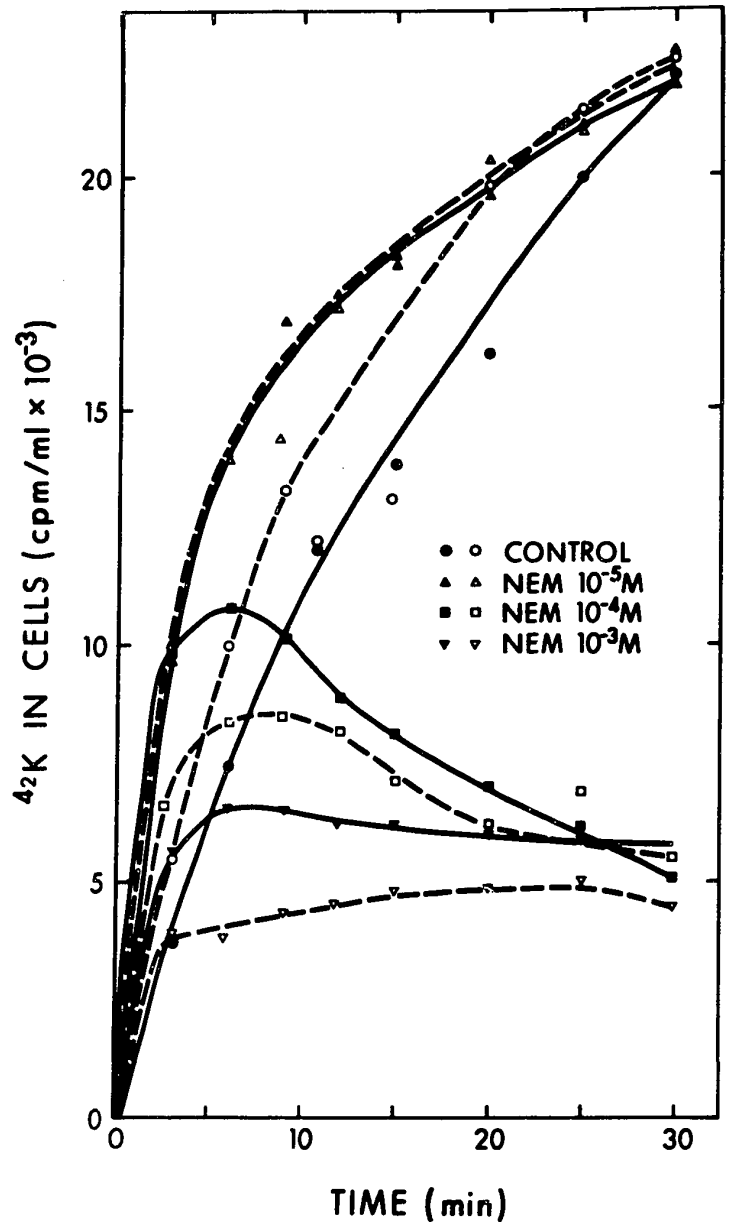

Fig. G11. Potassium Influx: Effect of NEM. The experiment was performed as described in Materials and Methods. Open symbols: R23. Closed symbols: uninfected.

Sodium cyanide reduced the loss of $^{42}K^+$ from uninfected and infected cells (Fig. G1). NaCN, 1 mM, completely prevented the marked efflux of K^+ seen with 10^{-5} M NEM in uninfected and infected cells (Fig. G12) but the same concentration of NaCN diminished only slightly the loss of K^+ induced by 10^{-2} M NEM. ATP in combination with NaCN and 10^{-5} M NEM had an effect similar to ATP and NaCN together (Fig. G12).

Discussion

When *E. coli* cells preloaded with $^{42}K^+$ were resuspended in isotope-free, low K^+ medium, there was a gradual loss of $^{42}K^+$ from the cells amounting to 45% (at 10 min) of the K^+ present at the time of resuspension. About 75% of this loss appeared to be dependent upon an energy supply since NaCN diminished the loss to 12%. Uncouplers of oxidative phosphorylation, on the other hand, diminished K^+ loss only to a slight degree.

Infection by R23 increased potassium efflux so that 75% of the K^+ originally present was lost by 10 min. Therefore, approximately 30% of the K^+ loss was due to the effect of R23 infection. The R23-induced loss was independent of an energy supply since 30% of the K^+ was still lost after infection in the presence of NaCN. The addition of ATP to the external medium enhanced the loss of K^+ from infected cells; that portion of K^+ loss which had been blocked by NaCN was restored so that in R23-infected cells about 75% of the K^+ was again extruded. The ability of ATP to enhance K^+ loss was observed only in infected cells; this suggested that infection altered the conformation of the bacterial membrane in such a way that it became permeable or responsive to added ATP.

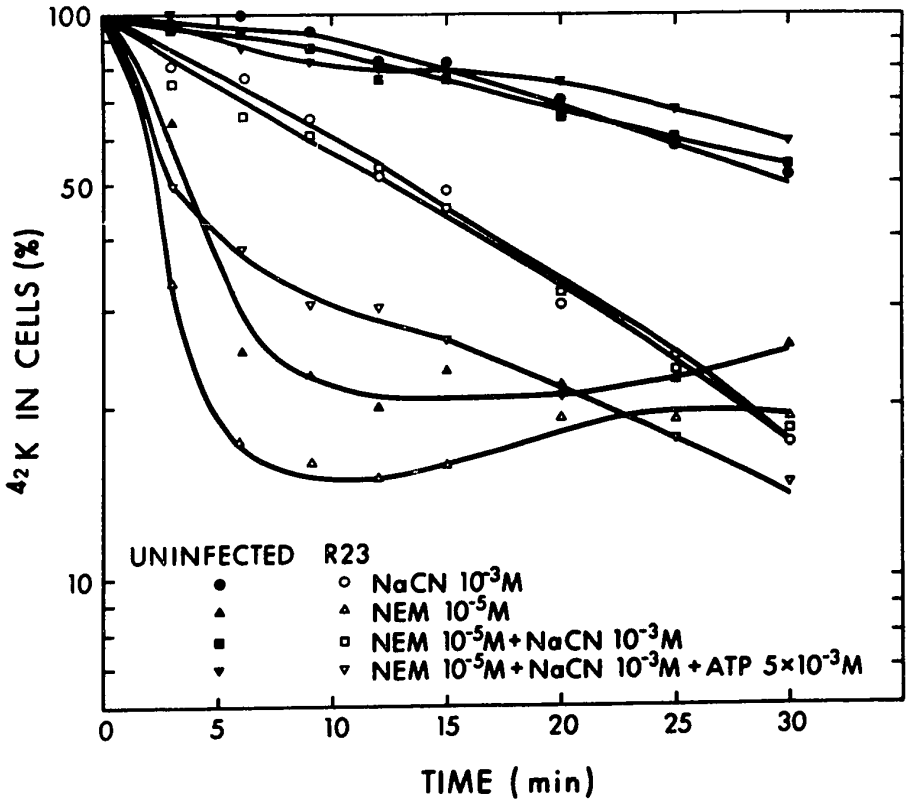

Fig. G12. Potassium Efflux: Effect of NEM (10^{-5} M), NaCN and ATP. The experiment was performed as described in Materials and Methods.

Although the effect of R23 on K^+ loss was unaffected by NaCN, uncouplers of oxidative phosphorylation diminished the initial rate of K^+ loss in infected cells. By 30 min, however, the loss was equal to that observed in untreated control cultures. The mechanism for this effect is not yet clear but it may be that oxidative phosphorylation is required for initiating the early stages of phage infection.

The requirement for transport protein(s) for R23-induced K^+ loss was examined in cells treated with NEM. The effect of R23 was diminished in the presence of NEM. At 10^{-5} M NEM, the rate of R23-induced efflux (during the first 5-10 min) was 24-45% of untreated controls, and at 10^{-4} and 10^{-3} M NEM, 12-30% of the untreated control value. These studies indicated that only about 10-30% of K^+ loss induced by R23 occurred independently of a carrier protein, i.e., by passive diffusion, whereas 70-90% was due to facilitated diffusion or carrier-mediated transport.

R23 infection also induced an increase in K^+ uptake against a concentration gradient. Such an uptake would be expected to involve active transport. R23 was, however, able to stimulate uptake in the presence of a metabolic inhibitor such as NaCN. Therefore, it appears that R23 stimulated influx by increasing exchange diffusion. According to Schultz and coworkers at least 50% of K^+ efflux is a carrier-mediated process tightly coupled to K^+ influx. It would appear that R23 infection activates this process in some way. On the other hand, there is some evidence which indicates that the effect of R23 on efflux of K^+ may be independent of the effect on influx. Although ATP enhanced

the efflux of K^+ in the presence of NaCN in infected cells, there was no significant effect of ATP on K^+ influx. Furthermore, although NEM in concentrations of 10^{-4} M or greater blocked R23-induced influx, R23-induced efflux of K^+ persisted.

Effect of NEM. The results obtained with NEM have been interpreted as follows. At 10^{-5} M, NEM produced a stimulatory effect on K^+ efflux and influx which was strikingly similar to that observed with R23. The protection against the effect of NEM by mercaptoethanol and the reversal by its removal indicated that 10^{-5} M NEM probably produced its effect by modifying the external thiol groups of the membrane. It affected proteins primarily involved in maintaining the permeability barrier, allowing intracellular K^+ to escape by passive diffusion, facilitated diffusion and energy-dependent efflux. Addition of NaCN to 10^{-5} M NEM reduced K^+ loss by preventing the energy-dependent extrusion. At this concentration, the influx mechanism was intact and influx was also increased. The increased K^+ influx may have been a reflection of the activation of exchange diffusion or a compensatory mechanism for maintaining the intracellular ionic concentration at a constant level. At 10^{-4} M NEM, on the other hand, active transport for K^+ uptake was damaged. Maximum efflux occurred at this concentration. As NEM concentration increased (10^{-2} and 10^{-3} M), deeper SH groups on other proteins such as carrier proteins involved in facilitated diffusion and energy-coupled K^+ efflux were damaged. Under these conditions, the exit of K^+ was by passive diffusion only and thus occurred at a slower rate than in the case of lower concentrations of NEM. Inhibition of energy sources would not be expected to alter the K^+ loss; thus NaCN

did not affect the K^+ efflux, in contrast to its effect with 10^{-5} M NEM.

From these studies we have concluded that R23 infection, like treatment with 10^{-5} M NEM, leads to conformational changes in the membrane or membrane components, producing a change in the permeability barrier to K^+. Since K^+ is an intracellular cation in *E. coli* which is present in higher concentrations than in the external medium, alteration in the permeability barrier leads to the efflux of K^+. In the case of R23 infection, the efflux occurs mainly by carrier-mediated facilitated diffusion and partly by passive diffusion. This efflux is not dependent upon an energy source. In addition to its effect on K^+ efflux, R23 induces an increased influx of K^+ probably by exchange diffusion or coupled influx-efflux. An alternative explanation is that influx is increased by homeostatic mechanisms involved in maintaining K^+ balance within the cell. In support of R23-induced alteration of membrane function or structure is the fact that K^+ efflux in R23-infected cells becomes sensitive to ATP added to the external medium.

CHAPTER IV

GENERAL DISCUSSION

A major problem pursued by both bacterial and animal virologists is the elucidation of the mechanism(s) by which viruses control host biosynthetic processes and redirect the metabolic machinery of the host towards synthesis of viral products. The isolation of the RNA bacteriophage R23, which completely dominates its host, has provided a convenient model for probing this problem of cellular control. Its profound effect on host macromolecular synthesis is especially striking in view of its uncomplicated architecture and genome. The organized pattern of transcription and translation occurring within a bacterial cell manufacturing innumerable macromolecules is disrupted on injection of a RNA molecule coding for only 3 known proteins.

Viruses, with their limited genetic information, must carry out their life cycle as intracellular parasites using the synthetic machinery of the host. Synthesis of viral rather than host products requires an actively metabolizing cell (Cohen, 1963) to provide basic synthetic units and an intact framework or cell wall. Virus-host interaction entails a competition for polymer precursors (amino acids, purine and pyrimidine bases, etc.) as well as for ribosomes, transfer RNAs, etc. The outcome of each interaction depends on the specific virus and bacterium. The effect of T4 differs in *E. coli* and *S. dysenteriae* (Terzi, 1967). The viability of *E. coli* after infection with DNA and RNA phages depends on the viral strain. Rapid inhibition of host macromolecular synthesis and killing of the cell characterize the most virulent viruses, such as the T-even phages. Other viruses allow the cell to multiply and may require host participation for their own propagation. The DNA-dependent RNA polymerase appears to be

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required for T-phage replication throughout the infectious cycle (Geiduschek & Sklar, 1969) and Qβ replicase requires a bacterial component for activity (Eikhom & Spiegelman, 1967; Eikhom et al, 1968).

E. coli metabolism is altered by DNA and RNA phages to different extents and in different ways. After ϕX174 infection, host DNA synthesis ceases 12-14 min after infection, but RNA and protein synthesis is affected only slightly (Lindquist & Sinsheimer, 1967). In the case of T5, the first-step-transfer DNA, which represents the first 8% of the total DNA molecule, controls degradation of host DNA (Lanni, 1968). The inhibition of host DNA, RNA and protein synthesis by λ (Howes, 1965; Terzi & Levinthal, 1967) is more profound after infection than following induction of the prophage (Howes, 1965; Siminovitch & Jacob, 1952; Waites & Fry, 1964). Howes (1965) reported that λ at high MOI interrupts protein synthesis prior to interference with RNA synthesis, but Terzi & Levinthal (1967) suggests that λ interferes at the level of host RNA synthesis. The depression of RNA and protein synthesis is dependent on the MOI. RNA synthesis is depressed in cells pretreated with chloramphenicol and affects all molecular species of RNA equally. Since host-restricted λ and ghosts of λ do not interfere with host metabolism, the effects are considered to be due to phage DNA or a gene product rather than to a protein component of the phage itself.

When *E. coli* is infected with T4, there is prompt and almost complete cessation of synthesis of host DNA, rRNA, tRNA, mRNA and protein synthesis; induction of bacterial enzymes is blocked and phage enzymes are induced. Host DNA is destroyed by DNase but the disruption of the nuclear apparatus is not responsible for inhibition of host macromolecular

synthesis since 1) *E. coli* DNA retains its integrity for 5 min following infection, by which time the major changes of host metabolism have occurred (Nomura *et al.*, 1962), 2) mutants which cannot degrade host DNA can inhibit host RNA (Wiberg, 1966) and protein (Hosoda & Levinthal, 1968) synthesis, and 3) when infection occurs in the presence of chloramphenicol or streptomycin, host DNA is not destroyed but host DNA and mRNA are inhibited. The extent of mRNA inhibition depends on the MOI (Nomura *et al.*, 1966). Synthesis of rRNA and tRNA continues in the absence of protein synthesis (Nomura, Okamoto & Asano, 1962), suggesting that phage DNA directs the synthesis of some protein which inhibits synthesis of host rRNA and sRNA.

There may be 2 mechanisms for T4 inhibition of host nucleic acid synthesis: 1) dependent on protein synthesis by phage and inhibiting nucleic acid synthesis completely and 2) insensitive to chloramphenicol, dependent on MOI, and producing nonspecific inhibition of all cell activity (Nomura *et al.*, 1966). The first mechanism requires a protein synthesized under the direction of the injected phage genome and may correspond to the enhancing effect of T-even phage of β-galactosidase mRNA destruction which is absent in UV-irradiated phage (Kaempfer & Magasanik, 1967a). The second mechanism may occur through the action of the coat protein and may correspond to the inhibition of β-galactosidase mRNA synthesis which does not depend on viral gene expression (Kaempfer & Magasanik, 1967a). Thus phage ghosts of T2 inhibit synthesis of host nucleic acid and protein (French & Siminovitch, 1955). In *Shigella*, T4 does not produce a complete arrest of host synthesis; DNA synthesis is only slightly decreased. The reduction in nucleic acid and protein synthesis is dependent on the multiplicity of infection. The mechanism dependent on protein synthesis

in *E. coli* does not function in *Shigella* (Terzi, 1967).

The inhibitory effect of RNA phage on nucleic acid and protein synthesis depends on the viral strain (Watanabe *et al.*, 1968). Inhibition of host DNA, RNA and protein synthesis has been reported for some RNA phages (Ellis & Paranchych, 1963; Nonoyama *et al.*, 1963; Bishop, 1965, 1966; Granboulan & Franklin, 1966; Hudson & Paranchych, 1967; Watanabe *et al.*, 1968).

The mechanism of inhibition of host macromolecular synthesis by R23 can be compared to that of T-even phages. An effect on decay of β-galactosidase mRNA was not detected in the case of R23. R23 may inherently lack the ability to facilitate mRNA destruction like T4. Like the T-even phages, however, the inhibition of host functions may be considered to include two components which differ mainly in their requirement for viral gene expression. There is an early effect, shown also by R23 enzyme mutants and UV-irradiated phage, during which host RNA, DNA and protein syntheses are inhibited rapidly, transiently and perhaps, simultaneously and nonspecifically. This inhibition is dependent on multiplicity of infection and phage adsorption to F pili by the maturation protein. It is independent of viral replication and insensitive to chloramphenicol, and may be somewhat obscured by an initial stimulation of membrane permeability.

The second effect of R23, not shared with RNA synthetase mutants or UV-irradiated phage, produces inhibition of RNA, DNA and protein synthesis in a more marked fashion and mainly after the first 20 min of infection. This phase is followed by cell lysis. It requires viral RNA synthetase activity as well as phage RNA penetration and an actively metabolizing host.

Increase in multiplicity increases the inhibition, but a maximum effect is achieved at about 10 phage/cell. This component is responsible for the major inhibitory effect of R23 and is thus more important for the phage than the early effect.

The early effect of R23 and the second mechanism of T-even phage may be comparable to the effects of T2 ghosts (French & Siminovitch, 1955), colicins (Nomura & Maeda, 1965), formalin-treated T phage (Nomura, 1967), abortive infection by T phage (Fields, 1969) and defective phage of *Bacillus subtilis* (Seaman, Tarmy & Marmur, 1964; Okamoto *et al.*, 1968) all of which inhibit nucleic acid and protein synthesis. T2 ghosts and colicins act from the cell surface and produce inhibition of macromolecular synthesis without gross cell damage. Formalin-treated phage and defective phage PBSX from lysogenic *B. subtilis* also inhibit nucleic acid and protein synthesis without injecting their DNA.

The early effect of R23 resembles the effect of colicins, T-even phage and T-phage ghosts in multiplicity dependence and reversibility. The number of killed bacteria is minimal. After being repressed for about half a generation, bacteria infected with enzyme mutants of R23 or UV-irradiated R23 recover and multiply at an increased, rapid rate. Recovery is demonstrated by resumption of cell division in liquid cultures and on agar plates, incorporation of radioactive precursors of nucleic acid and protein synthesis, and synthesis of β-galactosidase. Recovery is demonstrable in both Tryptone broth and synthetic media; it occurs earlier than in the case of T2 ghosts (which occurred at 80 min after infection) and it occurs without intervention, such as the addition of trypsin in the case of colicins (Nomura & Nakamura, 1962). The ready reversibility

indicates that the synthetic machinery and overall integrity of the cell must be grossly intact. This is proven also by the ability of superinfecting R23 to replicate in cultures already infected with R23 or its enzyme mutants.

Nomura (1964) has postulated that the attachment of a single killing particle of colicin to a receptor site causes a change at the receptor site which is transmitted to the killing and/or biochemical target, and causes eventual death and/or biochemical change. A similar change may occur on adsorption of phages such as R23 and T-even phage. An effect on the cell membrane has been indicated by permeability studies with T-even phage (Silver *et al.*, 1968; Puck & Lee, 1954, 1955) and R23 (Section D, F). Leakage is caused by T2 ghosts (Herriott & Barlow, 1957; Lehman & Herriott, 1958), infection of *E. coli* K12 (λ) with T4rII mutants (Ames & Ames, 1965), megacins (Ivanovics, Alfoldi & Nagy, 1959) and abortive infection by T-even or T5 phage (Fields, 1969). Contact of phage with the cell membrane may halt protein and nucleic acid synthesis and ribosome function because of their physical connection with the membrane. This may even be implicated in the change in the ability of ribosomes to bind tRNA (Hung & Overby, 1968) and in the polysome distribution (Hotham-Iglewski & Franklin, 1967; Godson & Sinsheimer, 1967) after RNA phage infection.

The early effect requires interaction between the viral maturation protein and a specific receptor site of the F pilus. Thus defective particles lacking the maturation protein produce no effect and addition of phage antiserum prevents the early effect. However, simple adsorption is not sufficient as shown by the need for incubation temperatures above

4^{o} C; the adsorption must be irreversible as a minimal requirement. This implies active involvement of the host membrane in some way. Adsorption may produce a transient conformational alteration in the membrane leading to functional changes. Recovery may represent disappearance of the stimulus for membrane alteration on ejection of the phage particle or maturation protein from the bacterial surface. Or the phenomenon may require some active bacterial repair of the membrane and other structures, or resistance in the form of an accelerated synthesis of macromolecules.

The effects of contact between RNA phage and the F pilus can be compared to the reversible inhibition of cell division and β-galactosidase synthesis in F^{-} cells during conjugation (Riley *et al.*, 1960). The surface reaction initiated on cell contact in mating may activate the F replicating system necessary for chromosome transfer (Jacob, Brenner & Cuzin, 1963). The F factor may determine the synthesis of a surface protein in F pili which specifically interacts with the RNA phage or F^{-} cell. Injection of phage RNA may also be stimulated by a surface contact reaction. It is of interest that R23 synthetase mutants inject their RNA though the RNA is not replicated; thus, replication does not appear necessary for phage RNA transport into the cell.

Whether the early phage effect is due to or accompanied by change in cell ATP levels, as in the case of colicins E1 and K (Fields & Luria, 1969), remains to be seen. The effect on membrane permeability does not appear to be as gross as that of megacins or abortive infection; it is likely a consequence rather than a cause of the effects of R23 infection. The effect and mechanism of action of ghosts may be different from that of

intact or untreated phage. Thus ghosts produce an "all or none" effect (Rouviere *et al.*, 1968), while phage produce a more subtle derangement of host function at a specific level or locus. However, the effect of R23 enzyme mutants need not be irrelevant or unphysiological since they resemble wild type phage except for their ability to replicate RNA.

It is likely that normal R23 shares the early action of UV-irradiated R23 and enzyme mutants and has in addition a mode of host suppression dependent on viral RNA penetration and replication. The viral RNA synthetase may replicate phage RNA more efficiently and rapidly than bacterial polymerases replicate bacterial nucleic acid, and indirectly inhibit host transcription (and translation) due to incorporation of commonly needed precursors into viral RNA. In turn, the abundance of viral RNA messengers may usurp ribosomes and make them unavailable to host messengers or competitively inhibit host mRNA binding to ribosomes. Infection by RNA phage may also inhibit host tRNA binding to ribosomes (Hung & Overby, 1968), perhaps by producing an allosteric change in the ribosome structure.

It is possible that, instead of viral RNA synthetase or its RNA product, the viral coat protein (or some other viral protein not yet identified) whose synthesis depends on synthesis of progeny RNA is responsible for host inhibition, perhaps by binding to host DNA as it does to viral SS and DS RNA (Sugiyama *et al.*, 1967; Engelhardt *et al.*, 1968). The viral RNA synthetase cistron may be only one of several cistrons whose expression is required for the inhibition of bacterial protein synthesis late in infection. It is also possible that the same factor, be it RNA or protein, may be responsible for both early and late

inhibition, the 2 phases then representing "parental" and "progeny" effects.

Elucidation of the mechanism of host inhibition by R23 is relevant not only for the general problem of viral propagation in a bacterial cell, but for its potential contribution to the unravelling of the basis of viral carcinogenesis as well as cellular differentiation and development. The regulatory processes involved in the metabolic changes which ensue following virus infection may serve as a model for the study of control mechanisms in the uninfected and in the cancerous cell in which this regulation may be lost. The mechanism which compels the cell to recognize an invading message and manufacture foreign proteins may underlie the basis for loss of control in cancerous cells. The process by which the RNA phage injects its RNA may be important in understanding nucleic acid transfer in conjugation, transformation, etc. and may be useful in devising ways of introducing nucleic acid experimentally into cells to produce desirable effects or of preventing undersirable entry. The effects of adsorption of phage protein to the bacterial membrane may be analogous to interactions between other molecules and other membranes, such as antigen-antibody reactions, and may provide a prototype for studying means of combating phenomena such as rejection or for elucidating the nature and basis of membrane alterations in tumor cells and their loss of contact inhibition. In short, the remarkable efficiency and simplicity of the RNA bacteriophage render it uniquely suitable and deserving of investigation from many points of view.

CHAPTER V

SUMMARY

The properties of infection with R23, a recently isolated RNA bacteriophage, have been investigated with particular emphasis on its effects on the host bacterium. R23 produced a marked inhibition of rRNA, tRNA, mRNA, DNA and protein synthesis in *E. coli*. The process of infection by R23 was examined in detail and compared to that of the RNA phages R34, R40, f2 and Qβ. There was little difference between R23, R34, R40 and f2 in rate of adsorption and eclipse of infectivity, time of appearance of the first intracellular phage, and overall efficiency of infection at multiplicities over 5 phage/cell. But the phage yield of R23, in terms of plaque-forming units and total particles, was higher than that of R34, R40 and f2. R23 also induced the greatest level of RNA synthesis in UV-irradiated bacteria and had the longest life cycle with a relatively prolonged latent period. Qβ appeared to infect cells less efficiently under the experimental conditions employed. In successfully infected cells the total particle yield appeared to be similar to that of R23 but the yield of viable Qβ phage was less.

A genetic analysis of R23 was performed by isolating amber mutants after mutagenesis with nitrous acid and fluorouracil. The defect in each mutant was analyzed and the mutants grouped into cistrons. These mutants were utilized to determine the requirement for viral genome expression in the various effects of phage infection.

R23 is distinguished by its marked inhibitory effect on host nucleic acid and protein synthesis (Watanabe *et al.*, 1968). The effect

of R23 infection on synthesis of the enzyme, β-galactosidase, was studied in an attempt to determine the mechanism of its inhibition of host macromolecular synthesis. The inhibition of β-galactosidase, which was not complete, became apparent shortly after infection and was maximal after the first 20 min of infection. R23 diminished the β-galactosidase synthesizing capacity when inducer was added after phage infection, but not when infection followed inducer removal. These findings suggested that the primary effect of R23 on enzyme-forming capacity was a limitation of synthesis of enzyme-specific mRNA. Studies with EDTA-treated R23, UV-irradiated phage and enzyme mutants of R23 indicated that early inhibition did not require the penetration and replication of the viral genome, whereas late inhibition required the intracellular expression of the viral RNA synthetase cistron.

The effect of R23 on the uptake of the inducer, ^{14}C-IPTG, was investigated to show that the inhibition of enzyme synthesis was not due to an impairment of transport of inducer into the bacterial cell. The fluctations in inducer uptake were correlated with changes in β-galactosidase levels and conveniently analyzed in terms of three phases. The sequence of changes in enzyme synthesis and inducer accumulation included 1) an immediate stimulation lasting 10-15 min, 2) a gradual diminution over the next 10-15 min, and 3) a more rapid and pronounced inhibition, compared to uninfected cells. The first two phases required phage adsorption but not penetration of viral RNA. The results suggested that stimulation of inducer uptake by contact of phage with the cell membrane resulted in earlier and greater enzyme synthesis in infected cells. This was followed by

a reduction in inducer influx and a gradual decrease in the rate of enzyme synthesis which accounted for 20-25% of the total inhibition. The late inhibition, which amounted to 75-80% of the overall inhibition, required penetration and replication of viral RNA and was not due primarily to inhibition of inducer transport. R23 infection resulted in co-ordinate repression of the β-galactosidase and permease cistrons of the lac operon.

The possibility that the target of R23 action was the lactose operon promoter was indirectly investigated by infecting in the presence of cyclic 3',5'-AMP, a nucleotide which has been shown to stimulate β-galactosidase mRNA synthesis by an action on the lac promoter. Cyclic 3',5'-AMP stimulated the synthesis of β-galactosidase in cultures of *E. coli* made permeable by treatment with Tris-EDTA, both in the absence and presence of glucose. It did not, however, affect the inhibition of β-galactosidase synthesis produced by infection with R23 or T2.

That R23 had a direct effect on membrane permeability and transport processes was also illustrated by its effect on potassium fluxes in *E. coli*. R23 infection produced a rapid transient increase in potassium efflux from cells preloaded with $^{42}K^{+}$. The phage effect on potassium flux, which was multiplicity-dependent, did not appear to require viral RNA penetration or replication but was related to phage adsorption. It was observed following infection with UV-irradiated phage and amber mutants defective in the viral RNA synthetase and maturation protein cistrons. It occurred during R23 infection in the

presence of EDTA, RNase, sodium cyanide, chloramphenicol, rifampicin or a high magnesium concentration but not at temperatures less than 29° C. R23 infection also stimulated $^{42}K^{+}$ influx. Addition of ATP to cyanide-treated infected cells enhanced efflux but not influx of $^{42}K^{+}$.

R23 thus appeared to act by producing 1) a transitory or reversible early effect, initiated by adsorption of the phage particle to the F pilus, which possibly stimulated transmission of a perturbation in conformation and function throughout the cell membrane and led to interruption of synthetic processes associated with the membrane, and 2) a major late effect dependent on intracellular expression of the viral RNA synthetase cistron which eventually led to complete inhibition of host macromolecular synthesis and lysis of the bacterium.

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