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A COLD SENSITIVE RNA POLYMERASE

FROM PSEUDOMONAS AERUGINOSA

by

Rodney Jerome Sobieski

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology)
in The University of Michigan
1972

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	vii
LIST OF FIGURES	ix
ABBREVIATIONS	xii
INTRODUCTION AND HISTORY	1
Conditional Lethal Mutants	1
Research Interest	2
Research Objectives	3
Cold Sensitive Conditional Lethal Mutants	3
Bacteria	3
Viruses	5
Cold Sensitivity and Phage Dependence on Host Controlled Functions	7
Host Controlled Modification	8
Extragenic Suppressors	9
Temporal Sequence of Phage Development	10
Host Membrane and Phage DNA Synthesis	10
Host Proteins and Phage m-RNA Transcription	10
RNA Polymerase	14
Isolation and Characterization	14
Function of Subunits	15
Other Transcriptional Control Factors	18
Pseudomonas RNAP	19
Experimental System	20
Phage Isolation and Characterization	20

Page

Conditional Temperature Sensitivity of CB3 Production 20

MATERIALS AND METHODS 23

Reagents 23

Analytical Methods 25

Media 25

Organisms and Bacteriophages 26

Cultivation of Microorganisms 26

Genetics 27

Spontaneous Mutants 27

Mutagenized Cells 27

Monitoring Bacteriophage Infections 28

Radioactive Labelling of Macromolecules 28

Protein 28

DNA 29

RNA 32

Sucrose Velocity Gradients 34

CB3 Attachment Lysates 35

Sedimentation Coefficients 36

Isolation of Nucleic Acids 36

Buffers 36

Isolated CB3 DNA 38

PAT 2 DNA 40

RNA 41

DNA-RNA Hybridization Experiments 41

Filters 41

Templates 42

Hybridization 42

Page

DNA Retention Study 43

Controls 43

DNA Dependent RNA Polymerase 43

Buffers 43

Enzyme Isolation 45

Enzyme Assays 49

Enzymatic Impurities 51

SDS Polyacrylamide Gels 52

EXPERIMENTAL RESULTS 54

Biochemical Characterization of the CB3 Infection 54

Net DNA Synthesis 55

Net RNA Synthesis 55

Net Protein Synthesis 55

Rate of RNA Synthesis 62

DNA Sedimentation Analysis of CB3 Infected PAT 2 67

Early Latent Period Lysates 82

Membrane Binding Experiments 85

RNA-DNA Hybridization 101

Direct Hybridization 101

Competition Hybridization 106

RNA Polymerase 113

Travers' Enzyme Extract 113

PAT 2 113

CB3 Infected PAT 2 119

Burgess Extracted RNAP 119

E. coli D10 127

PAT 2 127

Page

PAO 1C 137

SDS Polyacrylamide Gels 139

Modified Burgess Enzyme Extraction 142

Rifampicin 155

Rifampicin Resistant Mutants 157

Rif 4 RNAP 157

Other Permissive PAT 2 Mutants 163

Conclusion 167

DISCUSSION 168

SUMMARY 185

LITERATURE CITED 188

LIST OF TABLES

Table		Page
1	Estimated $S_{20,w}$ Values of Purified CB3 DNA in Fast Neutral Sucrose Gradients	77
2	Studies on the Nature of CB3 DNA Membrane Attachment	89
3	RNA-DNA Hybridization of 20 C and 37 C Infected PAT 2	105
4	Summary of Competition Hybridization Experiments Between Phage Infected PAT 2 at 37 C and 20 C	112
5	*In Vitro* RNA Synthesis with Traver's Extracted PAT 2 RNA Polymerase	116
6	*In Vitro* RNA Synthesis with RNAP from Infected and Non-infected PAT 2	118
7	Summary of Burgess RNAP Purification from *E. coli*	120
8	Summary of Burgess RNAP Purification from PAT 2	121
9	Assay of *E. coli* D10 RNAP at 37 C	128
10	Assay of PAT 2 RNAP at 37 C and 20 C	129
11	Sigma Inhibition of Core Activity at 37 C and 20 C	131
12	Influence of the Assay Template on PAT 2 Core RNAP Activity	133
13	Assay of PAO 1C RNAP Showing the Sigma Inhibition of Core at 37 C and 20 C	138
14	PAT 2 Holoenzyme Assayed in Mammalian Buffer at 37 C and 20 C	143
15	PAO 1C Holoenzyme Assayed in Mammalian Buffer at 37 C and 20 C	144
16	Modified Burgess Extracted PAO 1C RNAP Assayed in Mammalian Buffer at 37 C and 20 C	146
17	Sigma Stimulation of Modified Burgess PAT 2 RNAP at 37 C and 20 C	149
18	Influence of Modified Burgess PAT 2 Sigma on Burgess PAT 2 Holoenzyme	151
19	Summation of Studies Using Heterologous PC Fractions in Burgess and Mammalian Assay Buffers	152

Table		Page
20	Burgess Assay of E. coli Core and Heterologous Sigma Factors	153
21	Mammalian Assay of E. coli Core and Modified Burgess PAT 2 Sigma	154
22	Influence of E. coli Sigma on Modified Burgess PAT 2 Core in a Mammalian Assay	156
23	Characterization of Rif 4	158
24	Rif 4 Burgess "Core" RNAP Assayed in Burgess Buffer at 37 C and 20 C	160
25	Rif 4 Burgess "Core" RNAP Assayed in Mammalian Buffer at 37 C and 20 C	161
26	Enzyme Activities of Burgess Extracted RNAP at 37 and 20 C with CB3 DNA in Burgess and Mammalian Assay Buffers	162
27	Effects of Rifampicin on PAT 2 and Rif 4 RNAP at 37 C and 20 C	164

LIST OF FIGURES

Figure		Page
1.	Net DNA Synthesis at Permissive and Non-permissive Temperatures	57
2.	Net RNA Synthesis at Permissive and Non-permissive Temperatures	59
3.	Net Protein Synthesis in PAT 2 at Permissive and Non-permissive Temperatures	61
4.	20 C Plate Counts of Infected and Uninfected PAT 2 at 20 C	64
5.	Rates of RNA Synthesis in Infected Cells at 37 C and 20 C	66
6.	Rates of RNA Synthesis in Uninfected Cells at 20 C	69
7.	Rates of RNA Synthesis at 37 C and 20 C in PAT 2 Infected with Ultraviolet Inactivated CB3	71
8a	PAT 2 Incorporation of Radioactivity at 37 C into DNA Unchased at the Time of Infection	73
8b	PAT 2 Incorporation of Radioactivity at 37 C into DNA Chased at the Time of Infection	73
9a	Sucrose Sedimentation of 37 C PAT 2 Lysates Unchased During Infection	75
9b	Sucrose Sedimentation of 37 C PAT 2 Lysates Chased During Infection	75
10a	PAT 2 Incorporation of Radioactivity at 20 C into DNA Unchased at the Time of Infection	79
10b	PAT 2 Incorporation of Radioactivity at 20 C into DNA Chased at the Time of Infection	79
11a	Sucrose Sedimentation of 20 C PAT 2 Lysates Unchased During Infection	81
11b	Sucrose Sedimentation of 20 C PAT 2 Lysates Chased During Infection	81
12.	Alkaline Sucrose Sedimentation of C^{14} Labelled DNA from 37 C and 20 C Infected PAT 2 Cell Lysates	84
13.	Membrane Binding of C^{14} Labelled Phage DNA Following Infection of PAT 2 at 37 C and 20 C	87

Figure		Page
14.	The Sodium Azide Inhibition of C^{14} Labelled Phage DNA Membrane Attachment in PAT 2 at 37 C and 20 C	91
15.	Fast Neutral Sucrose Sedimentation of Sodium Azide Treated PAT 2 Cells Infected with C^{14} Labelled CB3 at 37 C and 20 C	94
16.	Fast Alkaline Sucrose Sedimentation of PAT 2 Infected with C^{14} Labelled CB3 DNA at 37 C and 20 C	96
17.	Fast Alkaline Sucrose Sedimentation of C^{14} Labelled CB3 DNA Plus Uninfected PAT 2 Cell Lysate	98
18.	Fast Alkaline Sucrose Sedimentation of Sodium Azide Treated PAT 2 Cells Infected with C^{14} Labelled CB3 DNA at 37 C and 20 C	100
19.	Hybridization Saturation Curves between PAT 2 DNA-RNA and CB3 DNA - CB3 Infected PAT 2 RNA	104
20.	CB3 DNA-RNA Competition Hybridization Between RNA from 20 C Early Infected PAO 1C and 37 C Early Infected PAO 1C or PAT 2	108
21.	CB3 DNA-RNA Competition Hybridization Between 20 C Early RNA from Infected PAT 2 or PAO 1C and 37 C Early or Late RNA from Infected PAT 2	111
22.	DNA-Early RNA Hybridization Competition Between 20 C Infected PAT 2 and 37 C Infected PAT 2 or 37 C Infected, Chloramphenicol Treated PAT 2	115
23a	DEAE Cellulose Chromatography Profile of PAT 2 RNA Polymerase	124
23b	Phosphocellulose Chormatography Profile of PAT 2 RNA Polymerase	124
24a	Low Salt Glycerol Gradient Profile of PAT 2 RNA Polymerase	126
24b	High Salt Glycerol Gradient Profile of PAT 2 RNA Polymerase	126
25.	Dilution Effects of Sigma on the Activity of PAT 2 RNA Polymerase	136
26.	SDS Polyacrylamide Gel Electrophoresis of *E. coli* D10, PAO 1C and PAT 2 Burgess Phosphocellulose Enzymes	141
27.	SDS Polyacrylamide Gel Electrophoresis of PAO 1C and PAT 2 Modified Burgess Phosphocellulose Enzymes	148

Figure

Page

28. SDS Polyacrylamide Gel Electrophoresis of Rif 4 Burgess Phosphocellulose Enzyme 166

ABBREVIATIONS

cAMP	adenosine-3', 5'-cyclic phosphate
ATP	adenosine 5' triphosphate
CM	chloramphenicol
cpm	counts per minute
CTP	cytidine 5' triphosphate
DE	delayed early
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
DTT	dithiothereitol
FPA	DL parafluorophenylalanine
GTP	guanosine 5' triphosphate
HCM	host controlled modification
IE	immediate early
ilva	isoleucine valine
m-RNA	messenger ribonucleic acid
NTG	N-Methyl-N'nitro-N-nitrosoguanidine
PC	phosphocellulose
PCFM	phosphocellulose flow through material
RNA	ribonucleic acid
RNAP	ribonucleic acid polymerase
RNase	ribonuclease
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TGE(B)(A)	tryptone glucose yeast extract (agar)
UTP	uridine 5' triphosphate

INTRODUCTION AND HISTORY

Studies involving cellular regulatory control mechanisms have greatly helped our understanding of the organization of the cell. This understanding in turn allows cellular organization to be viewed at a higher level of complexity. The ability to manipulate these control mechanisms could provide a means of further pinpointing the interrelationships within the cell so that ultimately a pyramid of events leading to growth, reproduction and death could be seen as the effect of the cell "controllers".

Serious disadvantages, however, face the scientist when he seeks the mechanisms involved in the control of cell or viral macromolecular synthesis at the level of DNA replication, RNA transcription or translation. When one searches for mutations in the control of these viral processes, a control or regulatory defect in any one of them may be lethal to a living system. Consequently, techniques involving the conditional expression of a potentially lethal mutation would be a mechanism be which these "controllers" could be evaluated.

Conditional Lethal Mutants

Mutants of bacteria and viruses are available in which the expression of a potentially lethal function is conditional. This expression of a conditionally lethal mutation may be influenced by temperature.

The ability of temperature to express a potentially lethal mutation allows for a more critical evaluation of cell "controllers". The first class of temperature-dependent mutations are those in which the mutation is expressed when the temperature of the system is elevated. These temperature-sensitive mutations have been used to study vital functions

in bacteria (103, 12), yeast (62), bacterial viruses (42), plant viruses (165), animal viruses (17, 31) and even eukaryotic cells including Paramecia (67) and mammalian kidney cells (102). Some conditional lethal cell functions which have been studied are processes involving DNA synthesis (110, 111, 46), RNA synthesis (82, 70) and protein synthesis (37). Temperature-sensitive mutations arise by one of two mechanisms. The first class is due to an intrinsic heat lability of a particular cell protein at the elevated temperature (90) while the other class is due to a transient heat lability during the synthesis of a protein at an elevated temperature (42).

A class of mutants exists where the conditional lethal mutation is expressed not at an elevated temperature but only when the temperature of the system is lowered. This class of conditional mutants is referred to as cold-sensitive conditional lethal mutants. The observation that cold-sensitive mutants exist was reported by Maas in 1961(89). Unlike temperature-sensitive conditional mutations, cold-sensitive conditional mutations in general have not been well characterized except in a few cases (see below). However, those findings have shown that diverse mechanisms can be responsible for the inability of a cell or virus to reproduce at low temperatures where the wild type genome is able to normally express itself.

Research Interest

Bacteriophage systems provide model systems for the study of control functions by use of conditional lethal mutations due to their relative simplicity compared to the complex interactions in uninfected prokaryotic and eukaryotic cells. I am interested in a cold-sensitive

system originally reported by Olsen, Metcalf and Brandt (106). This system involves a strain of Pseudomonas aeruginosa, PAT 2, and one of its bacteriophages, CB3, in which the growth of the virus is conditional and hence, nonpermissive depending on the temperature at which the infection occurs. The virus is able to reproduce at 37 C but not a 20 C. This nonpermissiveness at 20 C is host strain dependent since there are hosts which are permissive for CB3 at 37 C and at 20 C. The conditionally restricting host grows normally at 20 C and is able to reproduce other phages at this temperature. (106) My work reported here is concerned with host dependent mechanisms involved in the conditional cold temperature-sensitive reproduction of CB3 at 20 C.

Research Objectives

Two objectives existed at the beginning of this work: 1) The biochemical characterization of the CB3 phage infection in the fully permissive and conditionally non-permissive host at 37 C and 20 C. This objective was undertaken in order to gain information as to the probable host function responsible for the phenomenon of cold sensitivity.
2) The isolation and characterization of the host dependent function responsible for the cold sensitivity of CB3.

Cold Sensitive Conditional Lethal Mutants

Bacteria. Studies of cold sensitive bacterial mutants show that many diverse mechanisms may be responsible for the inability of a cell to carry out required functions at low temperatures. Some cold-sensitive bacterial mutations are known to involve the synthesis of an enzyme at low temperature. In Maas"s original report (89) on cold-sensitive

mutations, he stated that a mutant was unable to synthesize an amino acid biosynthetic enzyme at low temperatures. Therefore, his mutant was auxotrophic at 25 C but prototrophic at 37 C. A similar mechanism was responsible for a cold-sensitive mutant of Pseudomonas putida reported by Codon and Ingraham (30).

Other cold-sensitive bacterial mutations are concerned with cellular proteins but for reasons unlike those found in the above examples. O'Donovan and Ingraham in 1965 (105) detailed a cold-sensitive conditionally lethal mutation in Escherichia coli. This mutation was due to increased feedback inhibition by histidine at 20 C. Phosphoribosyl ATP pyrophosphorylase was a thousand times more sensitive to feedback inhibition by histidine at 20 C than at 37 C. This allosteric enzyme had a mutation in its primary structure which resulted in a normal feedback inhibiiton at elevated temperatures but increased feedback inhibition at lower temperatures.

Low temperature can also affect enzymes in other ways. For example, Hoffman and Ingraham (64) reported a mutant which had a requirement for tryptophan only at low temperature. They found that at non-permissive temperatures an enzyme in the biosynthesis of this amino acid was irreversibly inactivated.

A rather involved mechanisms was found by Squires and Ingraham (140) in a cold-sensitive mutant of E. coli. The organism lacked galactose 1-phosphate uridyl transferase, although its UDP galactose 4-epimerase was normal. However, the cell's galacto-kinase was temperature sensitive at 37 C. Thus, this organism grew on lactose and could phosphorylate galactose at 20 C, but lacking the transferase, it was poisoned by the accumulation of galactose 1-phosphate. At 37 C, galactose-

kinase was nonfunctional so the mutant was unable to phosphorylate galactose, therefore, it was not poisoned and was able to grow at 37 C on the glucose moiety of lactose.

At still another level of cellular organization, several workers have reported the involvement of cell ribosomes in cold sensitive conditional lethal mutations. These mutants have shown defects in ribosomal subunit assembly (59, 148), r-RNA synthesis (149) or in the condensation of the ribosomal subunit with m-RNA to form an initiation complex for protein synthesis (47).

Viruses. Cold sensitive viruses have been found. However, they do not involve a host dependent control function as in the Pseudomonas-CB3 phage system. Of the reports on cold sensitive viruses, many only give the circumstances surrounding the discovery of each particular mutant. Those same reports lack information concerning either the cold sensitive site, its genetic behavior or its mechanisms.

Those uncharacterized cold sensitive viruses are members of the coliphage family and include T1 (27). ØX174 (41), T6 (118) and T4D (133, 117). Superficially, these cold sensitive mutations could be classed as affecting either an early phage function (ØX174), a late function (T1), DNA modification (T6) or as aftecting unknown functions (T4D). Concerning the cold senstitive mutants with DNA modifications alterations, T6 phage was unable to grow at 37 C but grew normally at 42 C. Revel (118) did not determine if the lack of glucosylation on phage DNA was involved with this "cold" sensitive mutation.

This first characterized cold sensitive virus is also an enteric phage. The growth of P1 appeared superficially to involve a cold sensitive phage mutation. Bertani and Nice (9) reported in 1954 that if

Shigella dysenteriae is infected with P1 at 20 C, no virus progeny are produced. On the other hand, infections at 37 C are productive. The apparent inability of P1 to be lytic at 20 C was found due to the phage undergoing lysogenization at this temperature. Thus, lysogeny control mechanisms were temperature dependent and responsible for the apparent cold sensitivity of phage production.

Studies on the RNA phage, f2, by Silverman and Valentine (135) in 1969 showed that a cold sensitive mutation was involved with the RNA injection step of that virus's growth cycle. The virus was able to attach to phage sensitive cells, but the normal removal of its coat protein allowing RNA entry into the cell did not occur. Ribonuclease sensitivity occurs at this phase in the development of RNA viruses. These mutants of f2 were ribonuclease resistant.

In 1971, cold-sensitive mutants of phage lambda were reported by Cox and Strack (32). Genetic characterization of some thirty mutants revealed that some mutations did not map in the same cistrons. There were mutants which fell in cistron A coding for head protein, G for tail protein, J for tail structural protein and one mutant in gene N, the antiterminator cistron (57).

Mammalian viruses are also known (132) which have mutations expressed only at incubation temperatures nearer the lower limit of their growth temperature range. A strain of fowl plaque virus, one of the members of the myxoviruses, was found unable to grow below 34 C. The virus was found to code for an RNA dependent RNA polymerase which had a higher activation energy below 34 C than was required above 34 C. Below 34 C the enzyme needed 16 Kcal of energy more per mole enzyme than was needed above 34 C.

Cold Sensitivity and Phage Dependence on Host Controlled Functions

Those few systems will be detailed in which there is a cold-sensitive host function indispensible for virus development. In general, these systems result in a non-permissive environment for the virus at low temperatures.

The influence of temperature on host dependent phage resistance in a strain of Heamophilus influenza was reported by Stuy in 1968 (142). It was found that cells grown at 30 C did not adsorb phage at either 30 or 37 C. However, cells grown at 37 C adsorbed virus at either temperature. The cold sensitivity of phage development in cells grown at 30 C was due to the inability of this host to synthesize receptors for the virus at the lower growth temperature..

In the same year Holland (65) reported the occurrence of E. coli K12 mutants which showed conditional refractivity to colicin E2. The mechanism responsible for refractivity of cells at the lower temperature and sensitivity of cells to colicin at the higher temperatures of incubation was not due to the lack of adsorption by the colicin. In 1970 Samson and Holland (131) found that the conditional inability of the colicin to manifest itself at 30 C may be due to the differences seen in the host membrane proteins between cells grown at 30 C and those at 40 C. These differences, then, may reflect the lack of transport of the colicin across the cell membrane which occurs in this system.

As described earlier, this thesis deals with a host dependent control on phage development which is cold-sensitive. This system involves a strain of Pseudomonas in which the growth of a virus is conditional depending on the temperature at which the infection occurs. The system will be described in more detail below, however it should be noted

that, it is one of the few systems in which a host controlled function in phage development is cold-sensitive.

A series of papers of Linial and Malamy (81) and Morrison and Malamy (99) report on some unusual characteristics of E. coli. Cells carrying the F'lac episome do not allow phage ØII to grow at either 37 or 25 C. This restriction is due to an episomal product since isogeneic females of this strain are fully permissive for ØII. Further, they can isolate conditional cold-sensitive mutants in this episomal function that allow the virus to grow at 37 C but still restrict it at 25 C. They attribute the restriction of ØII at 37 and 25 C to be due to a ØII translation inhibitor normally synthesized by the plasmid. The plasmid inhibitor in their "cold-sensitive" mutant is temperature-sensitive at 37 C, thus, the infection is permissive at 37 C and non-permissive at 25 C.

Other obligatory host functions are also involved with phage development; however, their mechanisms are quite different form those host functions implicated by studies of temperature-sensitive or cold-sensitive conditional lethal mutations. Several of these host mechanisms are discussed below which do govern, in one way or another, the expression of viral or plasmid genomes.

Host Controlled Modification

Bacteriophage adapt to new hosts either by spontaneous mutation (87) or by the result of a chemical modification of their DNA after its synthesis (88). This chemical modification is termed host controlled modification (HCM) and is a non-hereditable, phenotypic character bestowed on the virus by the host. (1). HCM involves two processes:

The first is modification and the second is restriction. Modification of DNA (1) results either in the methylation of DNA as in the case of fd or lambda phages or in glucosylation of DNA as in the T-even phages. The absence of modification in the DNA of an infecting virus results in its complete degradation (96). Restriction of unmodified DNA can occur with bacterial chromosomal material involved in conjugation or with the transfer of episomes. Thus, restriction or modification by a bacterium of heterologous DNA is a host function which can control genetic expression. For more details on HCM see any of the recent reviews on this subject (2, 3, 119).

Extragenic Suppressors

As first suggested by Yanofsky in 1960 (166), and since studied in some detail, missense and nonsense mutations cause respectively either the incorporation of the incorrect amino acid in the growing peptide chain or early termination of chain synthesis. Operationally, a host can control phage development by the presence or absence of an extragenic suppressor. Theoretically this suppressor can be in any of the molecules involved either with transcription or translation. Attention has focused on translational suppressors, specifically, the roles of t-RNA and the ribosome. Transfer RNA nonsense suppressors repress chain termination (23, 44) while ribosome alterations involving either the RAM locus (125) or Sm resistance (154) influence the precision of fit of the codon to the anticodon and, thus, incorporate and acceptable amino acid with a missense mutation. These extragenic suppressors can control phage development by counteracting the lethal mutation in the viral genome. For a further discussion of suppressors see the reviews by Gorini and Beckwith (55), Garen (51) or Gorini (54).

Temporal Sequence of Phage Development

Host Memberane and Phage DNA Synthesis. Studies on the replication of bacterial DNA in 1963 by Jacob, Brenner and Cuzin (68) suggested that a cell component, the cytoplasmic membrane, was involved in the process. Subsequently, Smith and Hanawalt (136) demonstrated that pulse labelled cell DNA is attached to a fast sedimenting cell component of E. coli. Other workers then found 25 to 50% of the total DNA polymerase activity is associated with the membrane of Bacillus subtilis (50). Sueoka and Quinn showed the initiation point, termination point and growing point of the B. subtilis chromosome are all attached to a fast sedimenting cell componenet (143) presumed to be from chemical analysis the cell membrane. Knippers and Sinsheimer found that incoming ØX174 DNA becomes associated with a rapidly sedimenting host cell component (76) later found also with lambda DNA to be cell membrane material (129). It is now recognized that the host cell membrane can serve as an attachment and replicating site for viral DNA. With lambda and ØX 174, upwards of 60 to 80 sites are available per cell (128, 127). Preliminary evidence with lambda places the phage recognition site for membrane attachment in the right half of the genetic map (127). Membrane attachment sites are host dependent entities; lack of this function or the prior occupation of the site would result in a non-permissive situation for phage replication (127). Nothing is known concerning the mechanisms involved with attachment of viral DNA to cell membrane material nor have the temporal relationships between the injection of DNA, the initiation of the first m-RNA transcript and DNA attachment been determined.

Host Proteins and Phage m-RNA Transcription. Seymor Cohen in 1967 (29) summarized the phage studies completed to that time as being primarily

concerned with reproducing viral genomes and not with the beginning or final steps of the infection. As early as 1957 (69) Jacob, Fuerst and Wollman established a genetic basis for the temporal sequence of phage development. Biochemical evidence then began to accumulate that the regulation of phage development occurred at the level of gene transcription (75). It was found that patterns of synthesis of m-RNA change with time and that the genome was read in a non-random manner. Early in vitro work (13, 94) showed that transcription was a specifically regulated process since m-RNA synthesis occurred from the 5' PO_4 end of the molecule to the 3'OH end, and the 5' end of the chain retained its triphosphate.

Well documented proof on the sequential appearance of m-RNA in T4 infected E. coli (56, 130, 97) showed that a serial order occurs in the transcription of the viral genome. The techniques of DNA-RNA hybridization and hybridization competition established that species of phage specific m-RNA were synthesized prior to and after the initiation of phage DNA synthesis (60). These species were termed early and late m-RNA. Hybridization competition experiments further divided the early species of T4 m-RNA into subsets (10, 130). The subset of early m-RNA termed immediate early (IE) or pre-early is that RNA synthesized up to 1.5 minutes into the phage infection. It is identical to the RNA synthesized in infected cells which have been treated with chloramphenicol (CM) (56). The second subset of early RNA whose synthesis occurs from 2 to 5 minutes of the infection is termed delayed early (DE) or quasi-late (130). Studies with CM have shown that if protein synthesis is blocked no DE m-RNA is produced (56, 45). Therefore, the DE m-RNA species are a direct effect of the proteins of the IE transcripts.

Studies on the temporal sequence of T4 synthesis immediately after infection brought forth new aspects of the regulatory controls of transcription. In vitro, using purified E. coli DNA dependent RNA polymerase (RNAP) with T4 DNA, the synthesis of phage specific m-RNA occurs (7, 98, 21, 15). This m-RNA corresponds by hybridization competition to the in vivo phage RNA normally fully expressed at 1.5 minutes post infection or that synthesized in the presence of CM. Delayed early m-RNA which is normally synthesized from 2 to 5 minutes and whose appearance is inhibited by CM, was synthesized in response to a phage coded protein that changes the initiation specificity of host RNAP (33). The phage dependent component is called T4 sigma factor. Thus, the cell's RNAP with new initiation specificity, due to sigma T4 replacing host sigma on RNAP, is more efficient in transcribing phage DNA than it is in transcribing host DNA (155, 156). Thus, for this reason, DE m-RNA synthesis begins. This is also the reason no DE m-RNA is synthesized in phage infected cells treated with CM, since protein synthesis is required to produce the T4 coded sigma factor.

When prolonged incubation is used for the in vitro synthesis of phage specific m-RNA some RNA is transcribed which competes with the DE RNA synthesized in vivo (14). This species should not be made in vitro since it is blocked by CM in vivo and requires a phage coded protein for its synthesis (130). This discrepancy was subsequently resolved when it was found that the probable cause of the DE m-RNA synthesis was due to the unmodified host RNAP reading through the normal termination site of the IE species. This in vitro produced molecule was considerably longer than the in vivo molecule because the DE species was synthesized adjacent to the IE. These observations resulted in the discovery that

uninfected cells of E. coli have an RNA termination factor, called rho (123). This new transcription control factor in conjunction with uninfected host RNAP and T4 DNA results only in the production of IE variety of T4 specific m-RNA.

The involvement of host RNAP in forming the first phage transcript has been shown with other phages to be similar to the T4-E. coli system detailed above. However, with these host transcribed immediate early messages, viruses can either modify the existing cell RNAP or synthesize a completely new enzyme. Phage T4 of E. coli and SPO1 of B. subtilis modify existing RNAP (157). On the other hand, T3 (144) and T7 (146, 147) synthesize new enzymes.

The transcription of a B. subtilis bacteriophage demonstrates a different role for the host in allowing the develoment of a virus. If phage ØE infects vegetative cells of B. subtilis, the infection is successful, however, if this phage infects cells committed to undergo sporulation, no viral progeny are produced. The virus does replicate when the spore containing the injected genome of the virus germinates to produce the vegetative cell. It was found in vitro that the RNAP from sporulating cells failed to transcribe ØE DNA while vegetative cell RNAP does transcribe the template (83). Further work showed that the RNAP from sporulating cells has a structure which differs from the vegetative enzyme (138, 139). Thus, the state of the cell governs the expression of a virus at the level of the cell's ability to transcribe the viral DNA.

RNA Polymerase

Isolation and Characterization. At the time studies were beginning on the sequential regulation of m-RNA in T4 and other viruses, the enzyme, RNAP, was isolated in 1959 from rat liver by Weiss (163). Shortly thereafter it was isolated from E. coli by Chamberlin and Berg (25) and by Furth, Hurwitz and Anders (48). Burgess in 1969 (18) reported a simplified method for the large scale purification of the enzyme from E. coli and at that time (19) characterized it in detail by the use of sodium dodecyl sulphate (SDS) acrylamide and urea acrylamide gels. RNAP was found to have a subunit composition $\alpha_2\beta\beta'\sigma$ where alpha, beta, beta-prime and sigma in E. coli have molecular weights of 41,000, 155,000, 165,000 and 86,000 respectively. In addition to these main subunits, two other subunits, tau and omega, are found associated with the enzyme. Omega has a molecular weight of 12,000 while the molecular weight of tau has not been reported. Tau, however, does migrate in SDS acrylamide gels between beta and sigma (158, 35, 93, 95) in E. coli and in B. subtilis RNAP (84). It is not known if these two proteins are true components of the enzyme or tightly binding contaminants.

The Burgess purification procedure uses chromatography on phosphocellulose (18, 164) to dissociate the sigma subunit from the holoenzyme ($\alpha_2\beta\beta'\sigma$) in order to yield sigma and core polymerase ($\alpha_2\beta\beta'$). Both core and the holoenzyme transcribe calf thymus DNA and poly dAT but differ in the ability to transcribe native DNAs; only holoenzyme transcribes native DNA in an efficient manner. RNAP has now been isolated from several bacteria including Azotobacter vinlandii (77), Micrococcus lysodekticus (141), B. subtilis (4, 40), Ps. putida (71), Ps. aeruginosa (164) and Holobacterium citrirubrum (85).

Function of Subunits: Beta-Prime. As detailed in a review by Chamberlin (24), a variety of methods have been used to determine the function of some of the subunits of RNAP. The first known step in the process of reactions leading to the synthesis of a transcript is the primary binding of the enzyme to the template. This primary binding is rapid, completely reversible (74 , 120) and is non-specific. Lack of specificity in the initial reaction is evident from electron microscopy of isolated polymerase-DNA complexes where the enzyme molecules are seen to be tightly packed along the template (114). Both core polymerase lacking sigma and holoenzyme bind to DNA in this manner (6). Of the subunits of RNAP, beta-prime has the highest isoelectric point between pH 8 and 9 (167). This high basicity may be connected to its DNA binding ability (167).

A simple model proposed by Chamberlin for the binding reaction first involves the binding of RNAP at random sites all along the template which the enzyme encounters by diffusion. This process leads ultimately to an encounter between the enzyme and a specific binding site on the DNA which is termed a promoter site. He defined promoter sites as a site which has specific binding and other initiation properties that have some function in vivo. The affinity of the enzyme for the template in this promoter region is enhanced over that of the primary non-specific binding. It is believed that binding at a promoter site causes the enzyme to locally unwind the template in this area (63) to form an enzyme promoter complex. It is at this point that the initiation process of transcription begins.

Chamberlin defined initiation (24) as "involving the oriented binding of two nucleoside triphosphates to the enzyme followed by the elimination of inorganic pyrophosphate to form a dinucleoside tetraphosphate." The enzyme then proceeds to synthesize a molecule of RNA

by the orderly addition of nucleoside monophosphates to the 3'OH position of the nascent polynucleotide (94) presumably involving other subunits of the enzyme.

Function of Subunits: Beta. The role of the beta subunit of RNAP in the transcription process was determined through work with the drug rifampicin. Under normal conditions both in vivo and in vitro, binding, promoter complex formation, initiation and chain elongation are not separable processes. However, several drugs are available which have been shown to interfere with one stage and not the other of these transcription processes. Initiation can be studied since enzymes and templates which have not undergone initiation are sensitive to the drug rifampicin and thereby further initiations are prevented. In the absence of the drug, once initiation occurs, chain elongation can continue and when the transcript and enzyme are released from the template, the enzyme then becomes sensitive to the drug if it was added during elongation. (167) This antibiotic from Streptomyces mediterranei (134) is known to inhibit initiation in vivo and in vitro (77). Both core polymerase ($\alpha_2\beta\beta'$) and holoenzyme ($\alpha_2\beta\beta'\sigma$) are equally inhibited by rifampicin. Of the purified subunits of RNAP, only beta is able to bind rifampicin irreversibly (167). A rifampicin resistant mutant of E. coli was shown to possess a beta polypeptide (115) which had a slightly different amino acid composition than the drug sensitive subunit. These observations place the role of the beta subunit as one involved in the initiation of transcription. It is not known if beta has any other functions.

Function of Subunits: Sigma. The sigma subunit has been shown to be involved in the specificity of transcription and, thus, to be a positive control factor of RNAP (144). For example, host RNAP holoenzyme with

phage T4 initiates only immediate early transcripts and transcribes the templates assymetrically (7, 146, 144) until the viral-induced sigma causes a new specificity to be acquired by the enzyme. (70). In contrast, core polymerase lacking sigma has no strand selectivity and fails to synthesize detectable levels of specifically initiated strands of RNA (21, 155). Core activity appears due only to the ability of the incomplete enzyme to initiate synthesis at sites on the template which have single strand nicks (21). Thus, sigma is responsible for initiation of transcription at the "correct" (promoter) site on the template. An interaction of the beta subunit with sigma occurs and is necessary for the functioning of sigma. Beta does function at a stage in initiation as determined from studies with rifampicin on core and holoenzyme, yet beta is the subunit of RNAP which binds sigma in vitro (167). Sigma by itself does not bind to DNA: thus, it seems able to control the initiation of specific transcripts only when bound to core through its interaction with beta. Recently, Pettijohn (113) found that the in vivo RNAP which is active in transcription lacks sigma. Likewise in vitro, sigma is found to be released after initiation occurs (126) when the nascent chain reaches some minimal length. It is believed the action of sigma may be a local unwinding of the duplex DNA (63) thereby producing specificity in some form of promoter recognition to allow core to initiate. Its release after initiation allows its reuse with other core enzymes, thus, resulting in a sigma cycle in the intact cell (158).

Function of Subunits: Alpha, Tau, Omega. Currently no information is known regarding the function of the subunit alpha found in RNAP. Tau and omega, as previously discussed, are subunits associated with purified RNAP, and are released when RNAP is treated with SDS and electrophoresed in acrylamide gels. Their functions, if any, are unknown. It

is conceivable that any of these subunits could also be involved in the binding or initiation sequences of transcription, or they could function either in chain elongation or termination. It is known that the rates of RNA chain growth *in vitro* and the role of each method in the *in vivo* termination of nascent chains is unknown. The protein component called rho (123) was discussed regarding the *in vitro* synthesis of immediate early T4 RNA. DNA sequence induced termination (150) and salt induced termination (122) are also known to occur at least *in vitro*. Whether alpha, tau and omega are involved in these processes is also unknown.

Other Transcriptional Control Factors

There are other protein factors in addition to sigma and rho in uninfected *E. coli* which are able to influence the transcription process. These additional factors function either in specifying initiation or increasing the number of transcripts from a single cistron.

M Factor. Factor M isolated from ribosome pellets stimulates transcription of lambda DNA in the presence of host RNAP and in the absence of ribosomes (35). Factor M is not sigma, since sigma saturated enzyme can be stimulated over twenty-fold by the addition of M.

ψ r. Travers (159) reported the isolation of factor, ψ r, which in the presence of purified *E. coli* DNA possessed no RNAP activity and could not stimulate core enzyme. However, with core and sigma factor, ψ r stimulates RNA synthesis. In hybridization competition experiments 30% of the RNA produced in the presence of ψr *in vitro* was competed by r-RNA whereas in the absence of ψr no detectable ribosomal RNA is transcribed. At this time the absolute role of ψr in transcription has not been established since the above experiments have yet to be reproduced

by other workers.

CAP. Another protein from E. coli called CAP or CR stimulates transcription of the lac operon both in vivo and in vitro (109, 160). CAP binds cyclic 3'-5' AMP and is able to increase the number of chain initiations of lac m-RNA (112). AMP-CR complexes function in a positive manner thereby allowing the interaction of RNAP with promoter loci of inducible operons (36, 161).

Pseudomonas RNAP

Studies reported to this point on RNAP and factors which control transcription have involved E. coli and its phages. RNAP has been isolated from other bacterial species as listed earlier. This current work involves a Pseudomonas-phage system, thus, information regarding the RNAP of Pseudomonads will be discussed briefly.

Pseudomonas putida RNAP. Johnson, Boezi and DeBacker (71) isolated RNAP from P. putida by technology similar to that of Burgess (18). However, the modifications they employed used primarily a low ionic strength environment to extract the cell and may be responsible for the unusual enzyme elution profiles from phosphocellulose (PC) chromatography. Unlike E. coli where PC yields core polymerase only, they found two enzymes, PCI and PCII. PCI was found to be holoenzyme by its SDS gel pattern and PCII was core enzyme. However, these two enzymes did not behave in other respects any differently than the enzymes from the Burgess extraction of E. coli.

Pseudomonas aeruginosa RNAP. Whiteley and Hemphill (164), using the technique of Brugess, studied the enzymes from E. coli, P. aeruginosa and B. subtilis. They found the purification patterns and enzyme prop-

erties of E. coli and Pseudomonas to be very similar. Furthermore, E. coli and Pseudomonas core enzymes were able to use heterologous sigma factors for RNA synthesis. B. subtilis proved to be the enzyme with widely differing properties, and it was not able to be stimulated by heterologous sigma preparation.

Experimental System

Phage Isolation and Characterization. The virus, CB3, was first isolated from sewage and characterized by Olsen, Todd and Metcalf (107). It is differentiated from other Pseudomonas phages by being resistant to both heat and an acid pH. CB3 is not inactivated by a temperature of 60 C for one hour nor by a pH of 2.0 for one hour at 25 C. Its DNA has a guanine plus cytosine content of 60.4 mole percent. Morphologically, CB3 has a contractile tail with tail fibers. Tail pins have not been observed on the base plates. Cross striations are present on uncontracted tails while contracted tails show longitudinal striations. The head diameter of CB3 is 52 mμ.

Conditional Cold Temperature Sensitivity of CB3 Production. The biology of the conditionally lethal cold-sensitive phenomenon was described (106) in 1968. It was observed during the study of phage typing of Pseudomonas that host susceptibility to a group of phages (CB3 included) varied with the temperature of incubation. Specifically, phage growth was non-permissive at 20 C and permissive at 37 C. There was segregation for phage restriction among recombinants from genetic crosses between male restricting and female non-restricting hosts. Out of the phages in the CB series which underwent this conditional restriction, CB3 was chosen for further study because it had an effeciency of plating of 1 at 37 C on both the conditionally restricting host, PAT 2, and for the fully permissive host,

PAO 1C. Studies on the infection of CB3 at 37 C showed the virus to have a latent period of 45 minutes in both hosts while in the fully permissive PAO 1C at 20 C the latent period is 145 minutes. Burst sizes at 37 C for PAO 1C and PAT 2 were 75 and 50 respectively. At 20 C the fully permissive host had a burst size of 45 to 50. The conditionally non-permissive host survives the non-permissive infection of CB3 at 20 C, and there is no measureable virus burst as detected by a one step growth curve experiment.

The restriction of CB3 was found to correlate with a temperature of 32 C. As the incubation temperature of the infection was raised from 20 to 32 C, progressively higher virus titers are produced, indicating a gradual loss of restriction. These titers were considerably lower than those seen at 37 C since the most significant increase in phage yields occurs at temperatures above 32 C.

In another series of experiments to determine the fate of CB3 under restricting conditions, it was found that of the conditionally restricting cells infected at 20 C, 13 to 14% of them are yielder cells. These cells do produce CB3 but the burst sizes, as determined by single cell burst experiments, are on the average 3 to 5% of the normal number of 50.

Temperature shift experiments were performed to determine the nature of the restricting event at 20 C. When 20 C infected PAT 2 cells were shifted up to 37 C at various times after the addition of CB3, it was found that after 30 mintes at 20 C there was a 50% loss in surviving infective centers.

The reverse experiment in which cells were infected at 37 C then shifted to 20 C various times thereafter was also performed. It was

discovered that the cold-sensitive event could be bypassed by only a 5 minute temperature pulse at 37 C. Five minutes after infecting PAT 2 at 37 C, a shift rapidly to 20 C allowed PAT 2 to produce CB3 at levels comparable to the fully permissive host under similar manipulations. Since the cold-sensitive event could be bypassed by only a 5 minute pulse at 37 C, this showed that the host dependent function in the restriction of CB3 was an event involved early in the CB3 latent period.

A temperature dependent host controlled modification or restriction was eliminated as a mechanism for the phenomenon. Lysogeny at low temperature by CB3 was also eliminated as a mechanism.

Nothing was known regarding the biochemical events involved in any virus infection of a Pseudomonas. With this fact and the background of the CB3-Pseudomonas sytem this present study was undertaken to:

a) biochemically characterize the CB3 infection in both hosts in order to i) characterize the physiological aspects of a Pseudomonas infection, ii) to gain information as to the probable cold-sensitive host dependent function in virus development at 20 C and

b) to isolate this host dependent function and learn something about its nature.

MATERIALS AND METHODS

Reagents

The following listings are those chemicals used in methods to be described below. The sources of these various reagents will now be given.

All inorganic chemicals were either Baker Analyzed Reagent grade or Mallinckrodt Analyzed Reagent grade.

Tryptone, yeast extract and Bacto-agar were obtained from Difco Laboratories, Detroit, Michigan.

Glucose (anhydrous), sucrose, angio conray, toluene, phenol and trichloroacetic acid (TCA) were from Mallinckrodt.

Tris-(hydroxymethyl)- aminomethane (Sigma 7-9) was manufactured by Sigma Chemical Co., St. Louis, Missouri.

Acrylamide and N, N'-methylene-bisacrylamide were the highest purity available from Eastman Organic Chemicals, Rochester, New York.

N,N,N' and N'-Tetramethylethylenediamine and glycine (ammonia free) were purchased from Matheson Coleman and Bell, Cincinnati, Ohio.

Folin-Ciocolteau reagent for Lowry (128) protein determinations was obtained from E. H. Sargent and Co., Chicago, Illinois.

Deoxyribonuclease (2X crystallized), ribonuclease (5X crystalline), Uracil, DL para fluorophenylalanine, 3,5-dihydroxytoluene (orcinol), lysozyme (3X crystallized), bovine albumin powder (Fraction V), yeast ribonucleic acid, L leucine, L isoleucine, and adenosine 3'5' cyclic phosphate were purchased from the Nutritional Biochemical Company, Cleveland, Ohio.

Cleland's Reagent (dithiothreitol (DTT) A grade; pronase, B grade

and rifampicin, B grade were obtained from Calbiochem, Los Angeles, California.

N-Methyl-N'nitro-N-nitroso guanidine was obtained from Aldrich Chemical Company Inc., Milwaukee, Wisconsin.

Buffalo Black NBR was procured from Allied Chemical, Morristown, New Jersey.

Ammonium sulphate, enzyme grade and Coomassie brilliant blue R 250 were manufactured by Schwarz-Mann, Orangeburg, New Jersey as was the uracil 2-C^{14} at a specific activity of 55 mc/mmole and the L leucine-4,5, H^3 at 15 C/mmole.

Chloramphenicol was the product of Parke, Davis and Company, Detroit, Michigan.

Siliclad was obtained from Clay Adams Inc., New York, New York.

Adenosine-C^{14} (U) 5' triphosphate, tri-lithium form was manufactured by New England Nuclear of Boston, Massachusetts at a specific activity of 462 mc/mmole.

The sodium salts of cytidine 5' triphosphate, uridine 5' triphosphate and guanosine 5' triphosphate were the products of P and L Biochemical Inc., Milwaukee, Wisconsin.

Whatmann Cellulose-Phosphate, PC11, 7.4 meq/g and Whatmann DEAE-Cellulose, DE52, 1.0 meq/g dry weight were obtained through Reeve Angel, New York, New York.

2,5-diphenyloxazole (PPO) and 1,4-bis-(2(5-phenyl oxazolyl))-benzene (POPOP) were obtained in scintillation grade from Packard Instrument Co., Downers Grove, Illinois.

Analytical Methods

Protein. Protein concentrations were chemically determined by the method of Lowry (86) using bovine serum albumin as a standard. Optical determinations at 280 nm of the purified protein RNA polymerase (RNAP) were calculated from its extinction coefficient of $E_{280}^{1\%}$ =6.5 (164). Protein elution profiles from chromatography columns were monitored at 280 nm with the aid of a LKB Uvicord II (LKB Producter, Brooma Sweden) coupled to an E. H. Sargent model SR recorder.

RNA. Determinations of RNA content were performed either by the orcinol (101) method using yeast RNA as standard or by the extinction coefficient at 260 nm of $E_{260}^{1\%}$ = 250 (56).

DNA. DNA was determined by its extinction coefficient at 260 nm of $E_{260}^{1\%}$ = 200 (11).

Bacteria. Cell absorbancies (O.D.) were determined using a B & L Spectronic 20 at 425 nm. An absorbancy of 1.0 equals 4 X 10^8 cells/ml.

Media

The medium for the routine broth cultivation of bacteria and bacteriophage was composed of 0.5% tryptone, 0.25% yeast extract and 0.1% glucose (TGEB) in volumes of 20 to 100 ml. When cells or viruses were cultivated in volumes larger than 100 ml, KNO_3 was added to 0.4%. A solid medium (TGEA) included TGEB plus Bacto-agar (1.5%) and $CaCl_2$ (0.015%). All liquid media for viral infections included 0.015% $CaCl_2$. Soft agar overlays for virus enumeration consisted of TGEA with one half the amount of Bacto-agar. The minimal medium for all genetic experiments was that of Vogel and Bonner (162) supplemented with 1.8% glucose (VBG) and 0.005 mg/ml of any required nutrient. Some

experiments used cells which were starved in the starvation buffer of Denhardt and Sinsheimer (39). Recovery medium after mutagenesis consisted of 0.5% tryptone, 1.0% glucose, 1.0% yeast extract, 1.5% agar and 1.0% casamino acids, pH 7.0.

Organisms and Bacteriophage

All the bacterial strains and bacteriophage used in this work were obtained from Dr. Ronald H. Olsen, Department of Microbiology, The University of Michigan. These included Pseudomonas phages CB3 and PX4 as well as Pseudomonas aeruginosa strains PAO 1C and PAT 2 and Escherichia coli D 10. Both these strains of Pseudomonas are wild type (106). PAO 1 C is resistant to chloramphenicol while PAT 2 is auxotrophic for isoleucine and valine.

Cultivation of Microorganisms

The routine cultivation of bacteria was accomplished by first making a lightly turbid suspension of cells from a fresh (1 or 2 day old) TGEA slant. The size and dilution of the turbid suspension was such that after 14 to 16 hours at each temperature the aerated cultures had an absorbancy at 425 nm of 0.3 to 1.0.

High titer lysates of CB3 were produced according to the technique as described by Olsen, Metcalf and Todd (107).

Large volumes of cells were grown in a New Brunswick Scientific Co., Fermacell, model F250. 50 to 120 liters of broth was prepared and sterilized in the Fermacell the day of use. The inoculum for the Fermcell consisted of 8 to 10 liters of actively growing cells at an O.D. of 0.8 to 1.0. This inoculum was grown overnight at 37 C in 2-liter Erlenmyer or Fernbach flasks with 1200 ml TGEB per flask. After inoc-

ulating the fermentor the system was maintained at 37 C. Aeration in the Fermacell was accomplished by use of the ring sparger and the turbine type impeller operating at 125-150 r.p.m. After 4 to 5 hours (at a cell density of approximatedly 1.0 to 1.25) the culture was cooled to 14 to 16 C by running cold tap water through the fermentor jacket. The cells were harvested with a continuous flow Sharples centrifuge (open type, Pennsalt Chemical Corp., Warmister, Pennsylvania) at a flow rate of 0.8 to 0.9 l/min. After centrifugation the cell paste was immediately frozen. Typical yields from a 120 liter volume was 200 g wet weight cell paste. Sterility was monitored prior to inoculation of the Fermacell by incubation at 37 C of a TGEA plate streaked for isolated colonies. At the time of harvest, a gram stain and a TGEA plate streaked for isolated colonies confirmed the presence of a pure culture.

Genetics

Spontaneous Mutants. PAT 2 cells which occurred spontaneously and were resistant to the amino acid analogue DL parafluorophenylalanine (FPA) were selected on VBG containing isoleucine, valine (ilva) and FPA at 0.25 mg/ml. Overnight TGEB grown cells were washed in VBG ilva broth and resuspended at an O.D. of 1.0 Then dilutions of the suspension were plated on the above selective medium. Colonies were picked and tested against CB3 after one week of incubation at 37 C.

Mutagenized Cells. Cells mutagenized with N-methyl-N' nitro-N-nitrosoguanidine (NTG) were prepared by first subculturing in TGEB at 37 C an overnight culture of 37 C TGEB grown cells of PAT 2. After 2 to 4 doublings, the cells were washed once at ambient temperature in 0.9% sterile NaCL(saline). The final cell pellet was suspended in 0.1 M

citrate (pH 5.4) to a cell density of 2 x 10^8/ml. Then 1.0 ml of the cells were added to a tube containing an equal volume of NTG (1 mg/ml). The cells were incubated for 30 min at 30 C in a water bath, centrifuged and suspended in 1.0 ml recovery medium. This suspension was incubated without shaking for 2 hr at 37 C prior to selecting mutants on VBG-ilva plates containing 0.025 mg/ml rifampicin. Plates were incubated for 3 to 5 days at 37 C prior to picking colonies. Colonies were streaked for isolation three times on VBG ilva containing rifampicin (0.02 mg/ml) prior to their characterization.

Monitoring Bacteriophage Infections

In all experiments routine checks were performed to insure the successful virus infection of bacteria. This was accomplished in several ways depending on the experiment. Either infectious centers were titrated or the percentages of unadsorbed phage in infected cell supernatants were determined. At times, 37 C plate counts of pre- and post-infected cells were used to determine the success of the infection. Occasionally, several methods were used in combination to determine both the percentage of virus which were adsorbed and the number of cells surviving the infection. These procedures showed that more than 99% of the added CB3 could attach to PAT 2 or PAO 1C and that greater than 95% of the cells were infected when the multiplicity of infection (m.o.i.) was three. When the m.o.i. was greater than three, more than 99% of the cells were infected.

Radioactive Labelling of Macromolecules

Protein. Cell proteins were labelled by first resuspending cells grown overnight at either 37 or 20 C to an absorbancy of 1.0 in TGEB containing

0.005 mg/ml C^{12} leucine. Adaptation to exogenous leucine occurred for 15 min for cells grown at and used at 37 C. Cells grown and used at 20 C were adapted for 30 min at this temperature. After this incubation period, more C^{12} leucine was added so that final concentration was 0.010 mg/ml. At this time, cells which were to remain uninfected were added to an equal volume of TGEB containing 1.25×10^{-3} mC of L-leucine-4,5 H^3 at a specific activity of 15.0 C/mmole. Cells to be infected were added to an equal volume of TGEB and label containing CB3 at an m.o.i. of 4 to 5. Sampling occurred by removing 0.1 ml of the reaction mixture and pipetting it onto a 1 cm^2 piece of Whatmann 3MM filter paper which was suspended on a pin. The straight pin was held rigid by being pushed up through a piece of cardboard. The filter paper was immediately placed face down into a beaker of cold 5% TCA (10 ml/filter paper). Subsequent to collecting all of the filter papers, they were boiled for 20 min then washed twice for 10 min in 5% TCA (5 ml/filter paper) prior to a 5 min wash in a 1:1 solution of absolute ethanol:ether. After air drying, the filter papers were placed in glass scintillation vials containing 15 ml toluene-Liquifluor and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer. Liquifluor was prepared by adding 100 g PPO and 1.25 g POPOP to one liter of toluene. Cocktails contained 42 ml Liquifluor/liter toluene.

Continuous Labelling of DNA. Bacterial nucleic acids were continuously labelled using C^{14} uracil then radioactivity in DNA was determined after alkaline hydrolysis of RNA. PAT 2 or PAO 1C were grown overnight in TGEB at 37 or 20 C containing 0.020 mg/ml C^{12} uracil. Cells were then resuspended and grown in uracil-free TGEB for 30 min at both 37 and 20 C. To obtain linear incorporation of the label into DNA after this starvation period, PAT 2 was re-exposed to C^{12} uracil at a concentration of 0.005 mg/ml while PAO 1C was re-exposed to 0.0025 mg/ml C^{12} uracil.

Re-adaptation to exogenous uracil occurred for 15 minutes for cells grown and used at both 37 and 20 C. After this time, cells were added to an equal volume of TGEB C^{12}/C^{14} uracil or TGEB C^{12}/C^{14} uracil containing CB3 at an m.o.i. of 4 to 5. Radioactive C^{14} uracil was 0.25 mc/ml of a solution with a specific activity of 55 mc/mmole. Also in the TGEB was enough C^{12} uracil to maintain a final uracil concentration equal to that to which each strain was re-exposed to following the initial starvation. Samples of 0.1 ml were removed from the reaction vessel, placed in a test tube, then 0.05 ml of KOH (0.75 N) was added to hydrolyze the RNA. Hydrolysis occurred overnight at an incubation temperature of 37 C. The sample was transferred to a one inch square Whatmann 3MM filter paper with the aid of a 5 1/2 inch Pasteur pipette. The filter paper was dried in an oven at 60 C then had its acid soluble radioactivity extracted by a 30 min exposure to cold 10% TCA (10 ml/filter paper). During this initial extraction the beaker containing the filter paper was swirled every 5 to 10 min. Following this initial extraction, two cold 5% TCA extractions of 15 min each were done prior to the 1:1 ethanol;ether extraction and counting as described under the procedure for labelling cell proteins.

Labelling DNA for Velocity Gradients: Early Latent Period Lysate. Cells were grown and starved according to the procedure used for the continuous uptake of label into DNA. After starvation in uracil free TGEB, C^{12} uracil was added to a final concentration of 0.001 mg/ml for ten min for cells grown at both 37 and 20 C. After this period, 0.25 ml of the re-adapted cells was added to 0.05 ml of C^{14} uracil (0.020 mg/ml) and 0.20 ml of TGEB or TGEB with CB3 at an m.o.i. of 6 to 7. Samples were taken from 37 C infected cells after 15 min and from 20 C infected cells after 60 min. The lysing procedure consisted of adding 0.34 ml of a lysing

mixture to 0.5 ml of infected cells and immediately placing the vial in a 65 C water bath. The 0.34 ml of lysing mixture was 0.125 M Tris-HCl, pH 7.9; 0.125 M EDTA, pH 8.0; 7.5% SDS and 0.1875 M NaCl. After 10 min at 65 C with gently rolling of the suspension at five minute intervals, 0.34 ml of 3.8 M NaCl was added. The same gentle rolling regime was followed for ten additional minutes at this same temperature. After which time 3.4 ml of distilled water was added, and the lysed cell mixture was stored at ambient temperature until centrifuged.

Labelling DNA for Velocity Gradients: Pre-labelled Cells Chased During Phage Infection. Cells were processed and lysed as above except that the concentration of C^{14} uracil after starvation was 0.005 mg/ml. After labelling for 30 min at 37 C or 80 min at 20 C, cells were infected the same way as those used for the production of the early latent period lysates. At infection, however, C^{12} uracil was added so the final concentration in the flask was 0.5 mg/ml. Samples were removed prior to chasing and subsequent to it at each temperature to follow the labelling pattern. Lysates at 37 and 20 C were made immediately before CB3 infection and at times equivalent to one half and the end of the phage latent periods at both temperatures.

Labelling DNA for Velocity Gradients: Pre-labelled Cells Labelled During CB3 Infection. Cells were processed as above except that at the time of virus addition, labelling was continued by the further addition of C^{14} uracil so that the final concentration was maintained at 0.005 mg/ml. Samples for the analysis of labelling patterns and lysates were taken as above.

CB3 Labelled DNA. Labelled CB3 DNA was produced in broth cultures. PAO 1C cells grown overnight in uracil (0.02 mg/ml) were resuspended in TGEB containing 0.005 mg/ml C^{14} uracil and infected at an m.o.i. of 0.01.

Following a 5 min adsorption period at 37 C, the culture was aerated for 5 hr, then it was made 1% in $CHCl_3$. The labelled lysate was processed as any high titer lysate (107). Prior to the final centrifugation and resuspension, it was treated with 0.05 mg/ml RNAase for 1 hr at room temperature and then it was made 5×10^{-3} M in $MgSO_4$ before DNAase was added to 0.005 mg/ml for 1 hr at ambient temperature.

Labelling of RNA. Labelling of this nucleic acid involved the use of overnight cultures of cells grown at either 37 or 20 C in 0.020 mg/ml C^{12} uracil. Cells were resuspended and starved of uracil for one half hour at both temperatures. Readaptation to exogenous C^{12} uracil occurred for 15 min at both 37 and 20 C. For this procedure uracil at a concentration of 0.01 mg/ml was used for continuous labelling experiments and for cells which were to be pulsed with C^{14} uracil to determine the rate of RNA synthesis. Subsequent to readaptation, cells used for continuous uptake were diluted into C^{14} uracil TGEB or C^{14} uracil TGEB and CB3 as described under the procedure for the continuous labelling of cell DNA. Counts in RNA were determined by substracting DNA counts from the total acid precipitable radioactivity in each sample. Total acid precipitable counts were obtained by first pipetting 0.1 ml of sample on a filter paper and immediately placing it face down in 10% cold TCA. The filter papers were then TCA extracted as before, washed with ethanol-ether, and counted as described under the section ofr the continuous labelling of cell DNA. Cells used to determine the rates of RNA synthesis were diluted into broth containing C^{12} uracil or C^{12} uracil and

phage having enough C^{12} uracil to have the final C^{14} and C^{12} uracil concentration of 0.01 mg/ml. This combination was aerated for 5 min then quickly chilled in an ice bath. Then 0.1 ml was removed for the determination of total precipitable radioactivity while the remaining 0.1 ml was processed for the radioactivity in DNA. (See DNA, Continuous Labelling).

RNA labelled for hybridization experiments used similar treatment of cells prior to readaptation to C^{12} uracil. After starvation, cells were exposed to 0.003 mg/ml C^{12} uracil for one half hr then diluted into TGEB or TGEB and CB3 with only C^{14} uracil present at 1.25 uc/ml. Labelling occurred for 5 min at 37 C and for 16 min at 20 C prior to beginning the extraction of this nucleic acid (see below).

Sucrose Velocity Gradients

Neutral and alkaline 5 to 20% linear sucrose gradients were prepared with the aid of a Beckman density gradient former. Neutral gradients were 0.02 M Tris-HCl, pH 7.9; 0.01 M EDTA, pH 7.9 and 0.3 M NaCl. Alkaline gradients were 0.02 M Tris-HCl, pH 12.1; 0.01 M EDTA; 0.3 N NaOH and 0.7 M NaCl. Each gradient of final volume 5.0 ml was formed above a high density shelf of 0.3 ml of a solution which was 80% Angio Conray-20% sucrose (148). Alkaline gradients utilized a pretreatment of lysates prior to centrifugation which consisted of removing 0.1 ml of a lysate warmed to 45 C and adding it to 0.05 ml of 0.9 N NaOH for 2 hr at 37 C. Then 0.1 ml of this mixture was gently layered on top the gradient with a tuberculin syringe. This syringe has as a needle a piece of glass tubing having an I.D. of 2.5 mm and a length of 65 mm. Neutral gradients used no lysate pretreatment except for warming the lysate to 45 C. Gradients were centrifuged at 25 C in either a Beckman L2 65B or an L3 50 using a SW 50.1 rotor with polyallomer tubes. "Fast" centrifugation conditions utilized a running time of 90 min at 30,000 r.p.m. "Slow" conditions used 25,000 r.p.m. for 40 min. Neutral and alkaline gradients were collected using a Beckman fractionation system. Neutral gradient tubes were punctured from the bottom, and approximately 0.15 ml fractions were allowed to drip into test tubes for alkali hydrolysis of RNA. Hydrolysis and extraction of soluble radioactive counts occurred as described earlier. Alkaline gradients were collected in the same manner, but each fraction was collected on a filter paper and processed for total acid precipitable counts.

CB3 DNA Attachment Lysates

Experiments to determine the attachment of CB3 DNA to cell cytoplasmic membrane material were done using overnight grown cells of PAT 2. These cells were grown at the temperature at which the assay was to be performed. They were resuspended to absorbancy 1.0 in TGEB plus uracil C^{12} (0.75 mg/ml) then immediately 3.0 ml of cells was mixed with 3.0 ml TGEB containing CB3 labelled in its DNA. The m.o.i. was 1 to 2. Attachment and penetration were allowed to occur for 5 min at 37 C and for 16 min at 20 C before the mixtures were centrifuged at room temperature for 5 min at 17,300 x g to remove unattached virus and concentrate the cells. The infected cell pellets were resuspended in 0.3 ml TGEB uracil (0.75 mg/ml) then immediately lysed by the addition of 0.2 ml lysing mixture and processed as a cell lysate (See "Labelling DNA for Velocity Gradients: Early Latent Period Lysate").

Some attachment experiments used UV-treated phage which had been inactivated to produce a log decrease of 3.8. Some cells used in attachment were also treated with U.V. to result in 10% survival as determined by plate counts on TGEA at 37 C. Other cells were starved in S.B. for one half hour at 37 C prior to infection in either 0.01 M sodium azide or 0.1 mg/ml chloramphenicol. Some lysates of infected cells were treated with a fresh pronase solution (0.2 mg/ml) for 4 hr at 37 C before layering on sucrose gradients. Centrifugation conditions were as described under the section "Sucrose Velocity Gradients."

Sedimentation Coefficients

Estimated values for all sedimentation coefficients from sucrose gradients were calculated using the formula of Burgi and Hershey (22). No corrections in estimated S 20,w values were done for any differences between my centrifugation condition and those of Burgi and Hershey.

$$S\ 20,w = \frac{6.45 \times 10^{10} d}{\omega^2 t}$$

where d = distance (cm) travelled from meniscus and $\omega^2 t$ = r.p.m.^{2} hr.

Isolation of Nucleic Acids

Buffers. The buffers described here were used both in the isolation of nucleic acids and in DNA-RNA hybridization experiments (See below). The pH was determined using a Beckman Expandomatic pH Meter. Buffers were stored in the cold to prevent contamination.

1) Saline Citrate (SCC)

0.15 M NaCl
0.02 M Na-Citrate
pH 7.0

2) EDTA, NaCl, SLS (ENS)

10% SLS
0.15 M NaCl
0.10 M EDTA, pH 7.9

3) Tris, KCl, $MgCl_2$ (TKM)

0.001 M Tris-HCl, pH 7.4
0.001 M KCl
0.001 M $MgCl_2$

CB3 DNA

All DNA isolations used redistilled phenol. DNA isolations were performed with a piece of apparatus called a rotator which revolved the

extraction tubes end over end. The extraction tubes were fastened at the circumference of a metal disc having a radius of 19 cm and rotation occurred at a rate of 50 r.p.m. The following flow diagram describes the isolation of phage DNA:

3 to 5 ml CB3 HTL
greater than 10^{11}/ml

↓ centrifuge 105,000 x g
4 C, 2 hr

Pellet

↓ resuspend in 3 to 5 ml
1X SSC, overnight, 4 C

CB3 suspension (screw cap tube)

↓ 4 ml phenol saturated with SSC
30 min on rotator

centrifuge, 20-22 C, 800 r.p.m.,
10 min,

remove lower phenol phase

DNA/SSC

↓ 4 ml phenol sat. with SSC
10 min on rotator

centrifuge, 20-22 C, 800 r.p.m.,
10 min

remove lower phenol phase

DNA/SSC

↓ repeat 2nd phenol extraction

DNA/SSC

↓ equal volume ether saturated SSC
and hand mix 1 min

centrifuge, 20-22 C, 800 r.p.m.,
10 sec

discard ether phase

repeat ether extraction 6X

Ether Extracted
CB3 DNA/SSC

decant to 50-ml beaker

allow ether to evaporate at ambient temperature

3 volumes cold 95% ethanol, gently layered

strand DNA by gently stirring with glass rod

vigorously stir until homogenous ethanol-SSC phase

Stranded DNA on glass Rod

wash 3X in absolute ethanol by immersion

place in tube with 3.6 ml 0.1X SSC to dissolve overnight at 4 C

add 0.4 ml 20X SSC

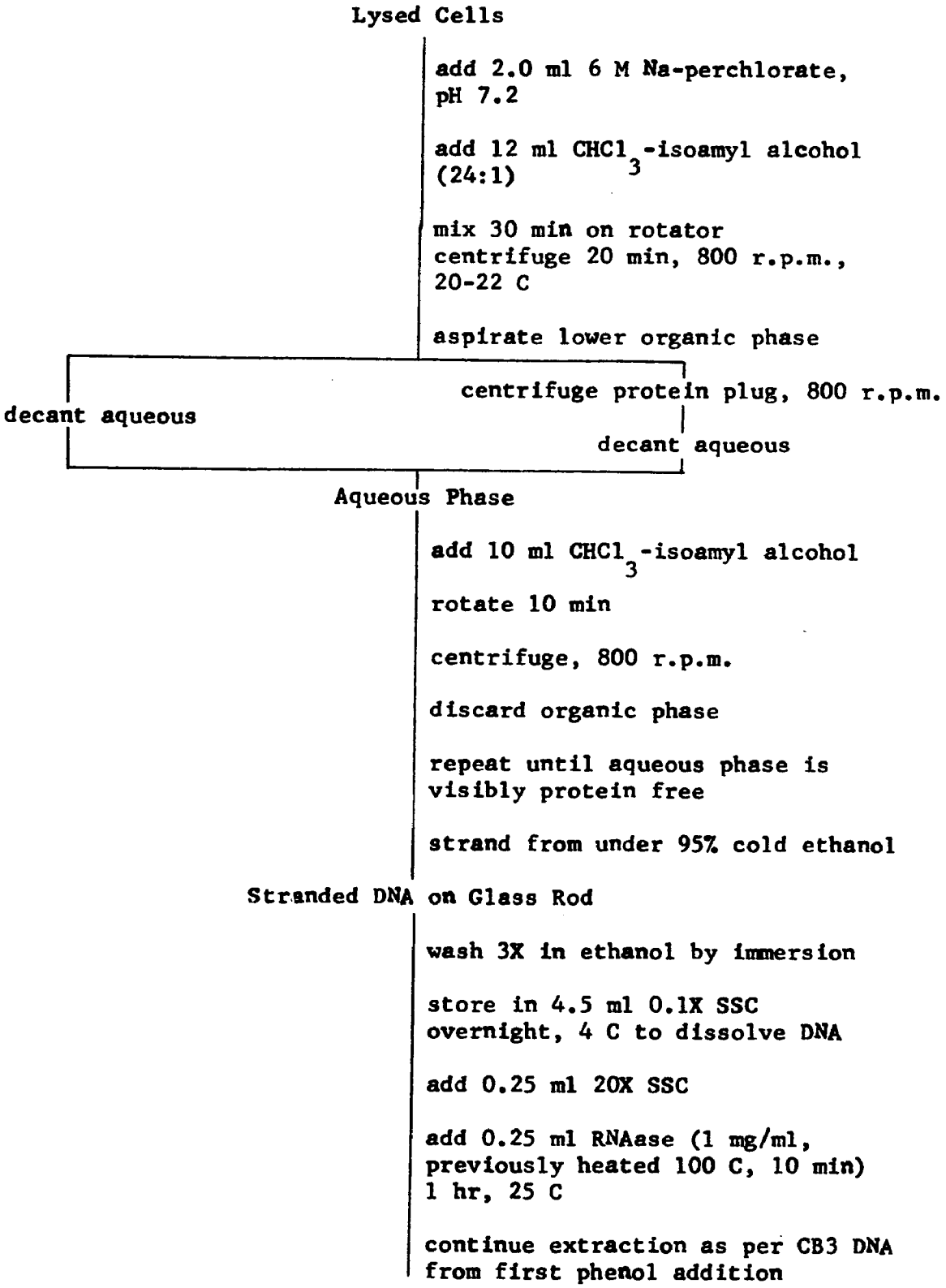
Isolated CB3 DNA

DNA prepared in this manner has a 260/280 nm absorbancy ratio of between 1.81 to 2.01 and was stored at 4 C at a concentration of greater than 0.2 mg/ml. Yield for 5 x 10^{12} viruses was 1.0 to 1.3 mg.

PAT 2 DNA. PAT 2 cells were grown overnight with aeration in 1200 ml volumes in Fernbach flasks. At O.D. 0.5, 250 ml of culture was centrifuged and washed in 33 ml of 0.15 M NaCl followed by resuspension in 5.0 ml of 0.3 M NaCl. The cells were frozen for at least 18 hr at -20 C in 19 x 450 mm screw cap tubes. The following flow diagram describes the extraction of one of these frozen cell suspensions

5.0 ml Frozen Culture

add 5.0 ml ENS, 65 C, 10 min gentle mixing


Lysed Cells
add 2.0 ml 6 M Na-perchlorate, pH 7.2
add 12 ml $CHCl_3$-isoamyl alcohol (24:1)
mix 30 min on rotator
centrifuge 20 min, 800 r.p.m., 20-22 C
aspirate lower organic phase
centrifuge protein plug, 800 r.p.m.
decant aqueous
decant aqueous
Aqueous Phase
add 10 ml $CHCl_3$-isoamyl alcohol
rotate 10 min
centrifuge, 800 r.p.m.
discard organic phase
repeat until aqueous phase is visibly protein free
strand from under 95% cold ethanol
Stranded DNA on Glass Rod
wash 3X in ethanol by immersion
store in 4.5 ml 0.1X SSC overnight, 4 C to dissolve DNA
add 0.25 ml 20X SSC
add 0.25 ml RNAase (1 mg/ml, previously heated 100 C, 10 min) 1 hr, 25 C
continue extraction as per CB3 DNA from first phenol addition

PAT 2 DNA

Host DNA prepared in this manner yielded approximately 2 mg of DNA from 2 liters of cells. These preparations had a 260/280 nm ratio of between 1.91 to 2.08. DNA was stored at concentrations greater than 0.2 mg/ml.

RNA for hybridization experiments. The extraction of RNA for hybridization experiments is a modification of the technique of Taylor, Hradecna and Szybalski (151).

5.0 ml of Infected or Uninfected Cells

terminate labelling by quick cooling in ice bath

0.5 ml 10^{-1} M Na azide

10 min, 10,000 x g, 4 C

resuspend in 5.0 ml TKM

Cells/TKM

centrifuge 10 min, 10,000 x g, 4 C

4.0 ml TKM

Cells/TKM

add to tube with 1.0 ml TKM, 1 mg lysozyme, 0.2 mg DNAase and 1 mg BSA

dry ice-ethanol bath to freeze cells

melt slowly (10 min) with vortexing every 3 to 4 min

Frozen Thawed Cells

0.5 ml 20% SLS

invert tube after 2.5 and 5 min

Lysed Cells

add equal volume phenol saturated with SSC

60 C, reciprocating shaker (300 cycles/min), 15 min

centrifuge and discard organic phenol phase

repeat phenol extractions 2X

extract 6X with 1.5 to 2.0 volumes ether saturated by TKM

dialyze each 10 ml in 2 changes (1500 ml) 2X SSC, 4 C, 18 hr

RNA

RNA prepared in this manner had a 260/280 nm ratio of 1.86 to 1.94. Radioactive preparations had less than 0.096% DNA as determined by alkali hydrolysis.

Unlabelled RNA from infected or uninfected cells was isolated by these identical procedures. At times 0.50 to 1.0 liter volumes of infected unlabelled cells were concentrated by centrifugation and resuspended to 5.0 ml prior to extraction of RNA. Some cells prior to infection and extraction of RNA were treated with 0.1 mg/ml chloramphenicol for 5 min.

DNA-RNA Hybridization

Methodology for the preparation of filter templates, hybridization and washing of filters is basically that of Gillespie and Spielgemann (52).

Filters. Membrane filters for DNA-RNA hybridization and hybridization competition experiments were obtained from Schliecker and Schuell Inc. Keene, New Hampshire (Bac-T-Flex, B6, 24 mm). Filters were prepared

the day used by first soaking them in 6X SSC (5ml/filter)for one hour at room temperature. This solution was then poured off and the filters washed in 1X SSC (10 ml/filter) for one half hour at room temperature. Finally, they were soaked in 6X SSC at 4 C until used.

Templates. The desired amount of DNA to be fixed on each filter was placed in a siliclad screw cap tube. The final volume was 0.5 ml. The tubes were plunged into a boiling water bath for 15 min then plunged into an ice bath. To each tube 2.0 ml of cold 7.2X SSC was quickly added and the contents were gently mixed by inversion and maintained at 4 C. Template filters were prepared in the cold using a Millipore filter assembly. The contents of each tube of denatured DNA were poured into the filter assembly containing the pre-washed, moist filter. The rate of filtration was such that 5 to 7 min were required for the 2.5 ml volume. Following this filtration, 2.0 ml of 6X SSC was added to the assembly as a wash. The filters were then removed and placed face up to air dry. Filters were dried overnight at room temperature in a Precision Scientific Model 10 Vacuum Oven. The bleeder valve of the oven was opened enough to produce a reading on the vacuum gauge of between 26 and 28. The following morning the oven was heated to 80 C for 2 1/2 hr, and the bleeder valve adjusted as necessary to maintain this same reading. This step is critical for DNA retention.

Hybridization. Filters with fixed DNA were incubated in separate siliclad scintillation vials for 22 to 23 hr at 60 C in the presence of RNA. In this vial was the test RNA and enough phenol saturated 2X SSC to give a final volume of 1.0 ml. The RNA was either C^{14} labelled for the direct DNA-RNA hybridization experiments or a mixture of C^{14} and varied amounts of C^{12} labelled RNA for hybridization competition experiments.

Following incubation each filter was washed in a millipore filter assembly with 2X SSC at 10 ml/side. The washed filters were placed in clean siliclad scintillation vials and incubated for one hour at 25 C in 2 ml 2X SSC containing 0.02 mg/ml boiled (10 min) RNAase. Subsequent to this treatment, each side of the filter was washed with 10 ml of 1X SSC, and following air drying, the filters were placed in separate vials with scintillation fluid and counted.

DNA Retention Study. The retention of denatured DNA on membrane filters was checked by the use of the procedure of Brown and Weber (16). Oven-cured filters were dissolved in 0.5 ml $CHCl_3$, and the DNA hydrolyzed with 1 N HCl for 15 min in a boiling water bath. Final volume was 3.0 ml. The extinction coefficient for DNA in 1 N HCl is $E_{260}^{1\%} = 278$ (16).

Controls. All hybridization and hybridization competition experiments included several controls. These controls were handled similar to the experimental filters through pre-washing, curing, hybridization, washing and counting. The controls included two DNA-free filters, two filters with DNA for each type of DNA used which were not reacted with the test RNA, and two DNA-free filters which had only test RNA added to them. In all cases these controls produced counts no greater than the two filter free cocktails.

DNA Dependent RNA Polymerase

Buffers. Buffers described here were used in the isolation of RNAP. Double distilled water was used for all solutions. Stock solutions of the following were diluted to prepare each buffer prior to use: 1 M Tris-HCl, pH 7.9; 1 M Tris-HCl pH 7.5; 1 M Tris-acetate, pH 8.0; 1 M Mg-acetate; 1 M ammonium acetate; 0.1 M $MnCl_2$; 0.1 M EDTA, pH 7.9;

1 M $MgCl_2$; 0.1 M K_2HPO_4, pH 7.9; 1 M KCl and 0.1 M dithiothreitol (DTT).

1) Standard Buffer

10 mM Tris-HCl, pH 7.9
5.0 mM $MgCl_2$
0.1 mM DTT
0.1 mM EDTA
0.5 M KCl
5.0 % glycerol

2) Buffer A

10.0 mM Tris-HCl, pH7.9
10.0 mM $MgCl_2$
0.1 mM EDTA
0.1 mM DTT
5.0 % glycerol

3) Buffer C

$MgCl_2$-free Buffer A

4) Buffer G

50.0 mM Tris, pH 7.5
200.0 mM KCL
10.0 mM $MgCl_2$
0.1 mM EDTA
0.1 mM DTT
5.0 % glycerol

5) Storage Buffer (Burgess, (90))

10.0 mM Tris-HCl, pH 7.9
10.0 mM $MgCl_2$
0.1 M KCl
0.1 mM EDTA
0.1 mM DTT
50.0 % glycerol

6) Storage Buffer (Johnson (93))

50.0 mM Tris-acetate, pH 8.0
10.0 mM Mg-acetate
0.1 mM EDTA
0.1 mM DTT
50.0 % glycerol

Saturated ammonium sulphate was prepared at 25 C, and the acidity controlled by the addition of concentrated NH_4OH so that a 1:20 dilution produced the desired pH (7.0 or 8.15).

Enzyme Isolation. Preliminary experiments with RNAP used a modification of an extraction procedure developed by Travers (155) for T4 infected cells of E. coli. With E. coli this procedure gives a relatively pure core enzyme preparation in the 40% ammonium sulphate fraction while sigma is found in the 55% fraction. All manipulations are performed at 4 C. Sonic disruption of cells occurred in a Raytheon 10 kc Sonic Oscillator (Raytheon Manufacturing Co., Waltham, Massachusetts) operated at maximum output. Efficiency of sonication was monitored by microscopic determination.

40 g Frozen Cell Paste

| 40 ml Standard Buffer
| sonicate 5 to 7 min

Cell Lysate

| 14,000 x g, 15 min

Crude Extract

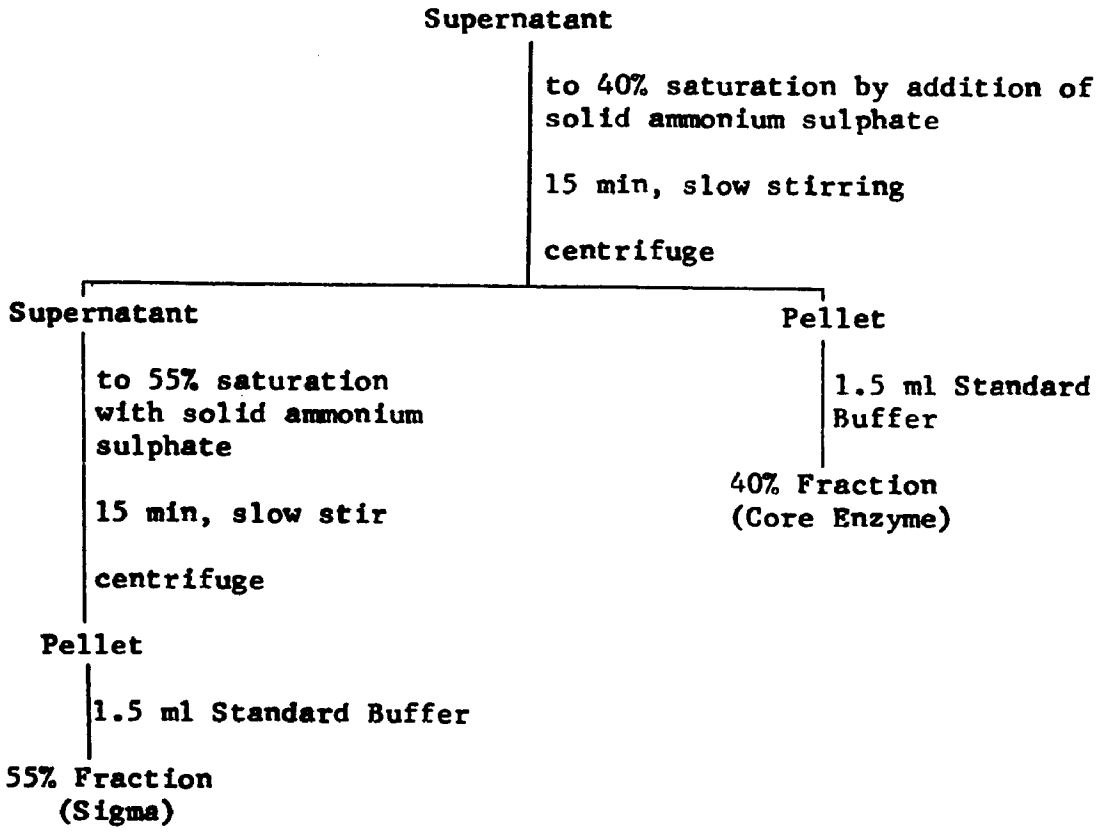
| 105,000 x g, 2 hr

Ribosomal Pellets

| resuspend in 10 ml Standard Buffer plus 0.5 M KCl
| 105,000 x g, 2 hr

Ribosomal Wash

| make 25% saturated in ammonium sulphate by addition of saturated solution, pH 8.15
| static, 15 min
| centrifuge, 8,000 x g, 10 min



A second extraction procedure utilized the technique described by Burgess (18). Chromatography columns were prepared and equilibrated according to his description and were recycled by using 1.0 M KCl in Buffer A for DEAE and 1.0 M KCl in Buffer C for PC. All manipulations were done at 4 C. Glycerol gradients were prepared as described in the section entitled "Sucrose Velocity Gradients," and centrifuged in a Spinco SW 27 rotor. The collection of material from glycerol gradients and chromatography columns used a LKB model 7000 Ultrorac Fraction Collector coupled to the previously described Uvicord II with chart recorder. A hydrostatic column (425 cm) maintained positive pressure for chromatography. The flow diagram below depicts the manipulations of cells to obtain purified RNAP.

100 to 200 g Frozen Cell Paste

↓ add volume Buffer G equal to cell wet weight

↓ sonicate 5 to 7 min

Cell Lysate = Fraction 1

↓ 1 ml DNAase (1 mg/ml) in Buffer G per 100 ml lysate

↓ one half hr, gentle stirring

↓ 105,000 x g, 2 hr

Supernatant = Fraction 2

↓ add equal volume Buffer G

↓ 23.1 g solid ammonium sulphate/ 100 ml

↓ pH monitored and controlled to keep pH above 7.0 with 0.5 N NaOH

↓ 30 min with stirring
centrifuge to remove precipitate

Supernatant

↓ 10.75 g solid ammonium sulphate/ 100 ml

↓ pH monitored

↓ 30 min with stirring

↓ centrifuge

Precipitate

↓ 130 ml 42% ammonium sulphate saturated Buffer A/100 g cells

↓ 45 min with stirring

↓ centrifuge

Precipitate

↓ 250 ml Buffer A/100 g cells

Fraction 3

applied at 1.0 ml/min to freshly equilibrated DEAE-Buffer A (100 ml column volume/100 to 200 g cells)

column washed 50 ml Buffer A

400 ml Buffer A + 0.13 M KCl

300 ml Buffer C + 0.23 M KCl

assay RNAP in effluent

pool fractions and dilute with Buffer C at 53 ml/100 RNAP

RNAP Fractions = Fraction 4a

precipitate RNAP fraction with concentrated ammonium sulphate

dissolve in Buffer A to give 20 to 40 mg/ml protein

dialyze against 100 volumes Buffer A two times, 2 hr each

1.5 to 2.0 ml layered on 10 to 30% glycerol gradients in Buffer A

Non-Centrifuged RNAP

23 hr, SW 27, 24,000 r.p.m.

assay for RNAP

precipitate protein and dialyse against Buffer A + 1.0 M KCl overnight

layer on 10 to 30% glycerol gradient in Buffer A + 1.0 M KCl

22 hr, SW 27, 22,000 r.p.m.

assay for RNAP

precipitate protein with saturated ammonium sulfate and dialyse against Buffer C

RNAP Fractions = Fractions 4b

apply to 50 ml PC column recently equilibrated with Buffer C, flow rate = 0.5 column volumes/hour

column washed with 50 ml Buffer C + 0.1 M KCl

100 ml Buffer C + 0.25 M KCl

100 ml Buffer C + 0.35 M KCl

assay RNAP in effluent

Core RNAP = Fraction 5; Sigma = PC flow through material

RNAP was stored by precipitating the enzyme with 1 1/2 volumes of saturated ammonium sulphate (pH 7.0) and redissolving in 0.5 to 1.0 ml Burgess storage buffer after centrifugation. Storage of the enzyme occurred in this buffer at -20 C. Likewise when desired various column eluates were also precipitated and stored in this manner.

Modification of the Burgess procedure involved lysing cells in KCl-free Buffer G, eluting from DEAE with NaCl and finally a stepwise elution from PC using a procedure described by Whitely and Hemphill (164). Stepwise elution involves desalting fraction 4a (non-centrifuged RNAP) by dialysis against Buffer C then eluting the protein from the column by 0.05 M increments of KCl-Buffer C. This enzyme was stored in Johnson storage buffer after it was concentrated by precipitation.

Enzyme Assays. RNAP activities were determined using the assay conditions described by Burgess (18). All reaction tubes were 11 x 72 mm and siliclad treated. The assay mixtures in 0.15 ml were 40 mM Tris-HCl, pH 7.9; 10 mM $MgCl_2$ 0.1 mM EDTA; 0.1 mM DTT; 0.15 M KCl; 0.15 mM UTP, GTP and CTP. Assay of RNAP purified by the Traver's procedure included 1 mM K_2HPO_4. The DNA concentration in either assay systems was 0.035 mg/tube. The order of addition of reagents to the cold assay tubes was to add the buffer; labelled and unlabelled triphosphates plus diluent, if

any, as a mixture; then the protein and finally the DNA. Assays were incubated for 15 min at 37 C or for 52 min at 20 C after which the tubes were chilled in an ice bath and the content processed on filter paper squares for total acid precipitable counts. All TCA contained 0.01 M Na-pyrophosphate (18). ATP C^{14} has a specific activity of 462 mc/mmole and 0.0125μc was used per assay. The C^{14} ATP was diluted upon arrival in a buffer 1:4 which was 40 mM Tris-HCl, pH 7.9; 10 mM $MgCl_2$; 150 mM KCl and 0.1 mM EDTA. This diluted nucleotide was then added in 0.1 ml aliquots to cold two-dram vials and immediately shell frozen in a dry-ice acetone bath and stored at -60 C.

The frozen ATP was thawed and diluted 20-fold prior to use. Sufficient C^{12} ATP was added to each assay to bring the total ATP concentration to 0.037 mM. These RNAP assays did not require bovine serum albumin probably due to the use of siliclad coated assay tubes. Rifampicin was used at a final concentration of 0.0125 mg/ml assay. When cyclic AMP was used, this compound was added to assays with the buffer mixture or to sigma fractions according to the technique of Martelo et. al. (95).

These authors incubated sigma and cyclic AMP for five minutes at 37 C prior to addition to the assay tube. The concentration of cyclic AMP during incubation with sigma was 0.005 mg/ml. Enzymatic assays using Traver's purified RNAP used between 0.0020-0.0037 mg of the 40% saturated ammonium sulphate fraction (core)/assay and between 0.0036-0.0041 mg of the 55% saturated fraction (sigma)/assay. Enzymatic assays of Burgess purified RNAP (PC binding protein) used between 0.0025-0.0035 mg protein/assay. Between 0.0150-0.0175 mg of the flow through material from PC was used/assay to test that fraction's ability to stimulate the activity of PC binding protein. All enzyme assays were performed at least three times.

Assays were linear with time and protein concentration. Reproducibility of the enzyme assays varied with the method of enzyme isolation. Enzyme fractions isolated by the Travers method were more variable in their in vitro transcription than those produced using Burgess purified RNAP. Results of Travers RNAP assays are reported showing either the results between different experiments or the radioactivity incorporated in a typical experiment. Burgess or modified Burgess RNAP assays are reported in terms of the radioactivity incorporated by a particular fraction. Assays of RNAP use the fully permissive temperature, 37 C, as representing 100% enzyme activity. Radioactivity incorporated at 20 C was compared to the 37 C incorporation to report the influence of temperature upon the efficiency of transcription. The variability between experiments for the enzyme efficiencies at 20 C was $\pm$ 0.20 of the reported efficiency. The variability within an experiment for the radioactivity incorporated between replicate assays did not exceed 3%. Controls for enzyme assays included: processing a blank filter paper with the experimental filter papers, a reaction tube lacking the template for each protein tested, and a reaction tube with template but no RNAP for each DNA species. Any radioactivity incorporated by these controls was subtracted from the appropriate experimental filter paper.

Enzymatic Impurities. RNAase in enzyme preparations was determined by incubating 0.015 mg (0.02 ml) of the protein fraction to be tested with 0.02 ml of C^{14} purified RNA. This RNA had 710 c.p.m. and 0.014 mg/ml RNA. Samples of various proteins were incubated with RNA for one hour at 37 C and processed for total radioactivity.

DNAase was determined by incubating the same volume and amount of proteins with 0.05 ml of C^{14} labelled and purified CB3 DNA. This DNA

preparation had 1378 c.p.m. Incubation and processing was identical to that for the detection of RNAase activity.

SDS Polyacrylamide Gels

SDS polyacrylamide gels were run according to the general methods of Laemmli (78). The 90 x 6 mm (I.D.) separating gel in a 15 cm tube was 5% acrylamide, 0.08% N,N' methylene bisacrylamide, 0.375 M Tris, pH 8.8 and 0.1% SDS. The gels were polymerized chemically by the addition of 0.01% of N, N, N' and N'-tetramethylenthylenediamine and by 0.01% fresh ammonium persulphate. The Laemmli procedure used one fourth the percentages used here for polymerization. The electrophoresis apparatus is that described by Kulpa and Olsen (in press). The electrode buffer (pH 8.3) contained 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS. The protein 0.010 mg to 0.020 mg (0.005 to 0.01 ml) was placed in a sample buffer so that in the final volume of 0.1 ml the mixture was 0.0625 M Tris (pH 6.8), 2% glycerol, 0.1% DTT and 0.001% bromophenol blue as a marker dye. The protein sample was then completely dissociated by immersing the tubes in a boiling water bath for 1 1/2 min. The samples were applied under the electrode buffer to the top of the gels by the use of a tuberculin syringe which had as a needle a 150 x 1 mm (I.D.) piece of soft plastic tubing. Electrophoresis occurred with a current of 3 ma/gel until the marker dye reached the bottom of the tube (about seven hours). The proteins were fixed in the gels with fresh 50% TCA overnight, stained for 1 hr at 37 C with a 0.1% Coomassie brilliant blue R 250 made up in fresh 50% TCA. The gels were diffusion-destained at room temperature by repeated washing over a 36 hr period in 7% acetic acid. Destained gels were stored in

7% acetic acid. Gels were also destained electrophoretically and found to be unsatisfactory. Likewise, staining gels for 1 hr in 1% Buffalo black NBR/7% acetic acid followed by overnight elution in 60 ml 7% acetic acid with subsequent electrophoretic or diffusion destaining was also unsatisfactory.

EXPERIMENTAL RESULTS

The experimental section has been subdivided for convenience into three major units. The first objective of this work, the biochemical characterization of the CB3 infection, evolved naturally into the second major unit on RNA-DNA hybridization. Likewise the organization of the last section dealing with DNA dependent RNA polymerase, evolved from the information of section two.

I. Biochemical Characterization of the CB3 Infection.

The biochemical characterization of the CB3 infection of PAO 1C and PAT 2 encompasses experiments dealing with various aspects of the infection. These include studies evaluating the ability of the infection to alter macromolecular synthesis of the cell's DNA, RNA and protein. Also included in the first subdivision are experiments characterizing the DNA of infected cells by sucrose gradients and a gradient analysis on the role of viral DNA attachment to cell cytoplasmic membrance material.

These data establish that the infected, fully permissive host, PAO 1C, shows similar changes in its macromolecular synthesis at both 37 C and 20 C. At 37 C the conditionally permissive PAT 2 has alterations in its synthetic abilities comparable to the fully permissive host at either temperature; however, non-permissive infections of PAT 2 do not show these changes. Sucrose gradients establish that viral DNA is not synthesized at 20 C in PAT 2 but the viral genome does attach to cell cytoplasmic material at both 37 C and 20 C. Thus, the data of this first section establish that the cold sensitive event in the development of CB3 at 20 C is subsequent to viral DNA attachment but prior

to viral DNA synthesis.

A. Net DNA Synthesis

PAT 2 cells infected with CB3 show a slight depression in net incorporation of radioactive precursors into DNA at 37 C when compared to uninfected cells (fig 1). However at a non-permissive temperature this depression of incorporation in infected cells is more pronounced. PAO 1C has an identical pattern for net DNA synthesis at both permissive and non-permissive temperatures. These incorporation curves for DNA establish that PAO 1C infected with CB3 responds with a similar pattern at both 37 and 20 C while PAT 2 infected cells produce a dissimilar pattern between permissive and non-permissive conditions.

B. Net RNA Synthesis

Both strains infected at 37 C show a similar pattern of label incorporation into RNA (fig 2). The levels of incorporation in infected cells are depressed from that seen in control cells, and this incorporation abruptly ends after 45 min. This corresponds to the end of the latent period for CB3 in both PAO 1C and PAT 2. Infected PAT 2 at 20 C has a net incorporation curve unlike the permissive infection at 20 C in that there is no plateauing of RNA counts at the end of the theoretical latent period. In PAO 1C the latent period at 20 C is 145 min.

C. Net Protein Synthesis

Protein synthesis as measured by the uptake of tritiated leucine shows that PAT 2 infected at both 37 and 20 C does not greatly differ from the pattern in uninfected cells (fig 3). Net synthesis in infected cells is slightly depressed at both temperatures. At the permissive temperature, incorporation ceases at the end of the viral latent

Figure 1. Net DNA synthesis at permissive and non-permissive temperatures.

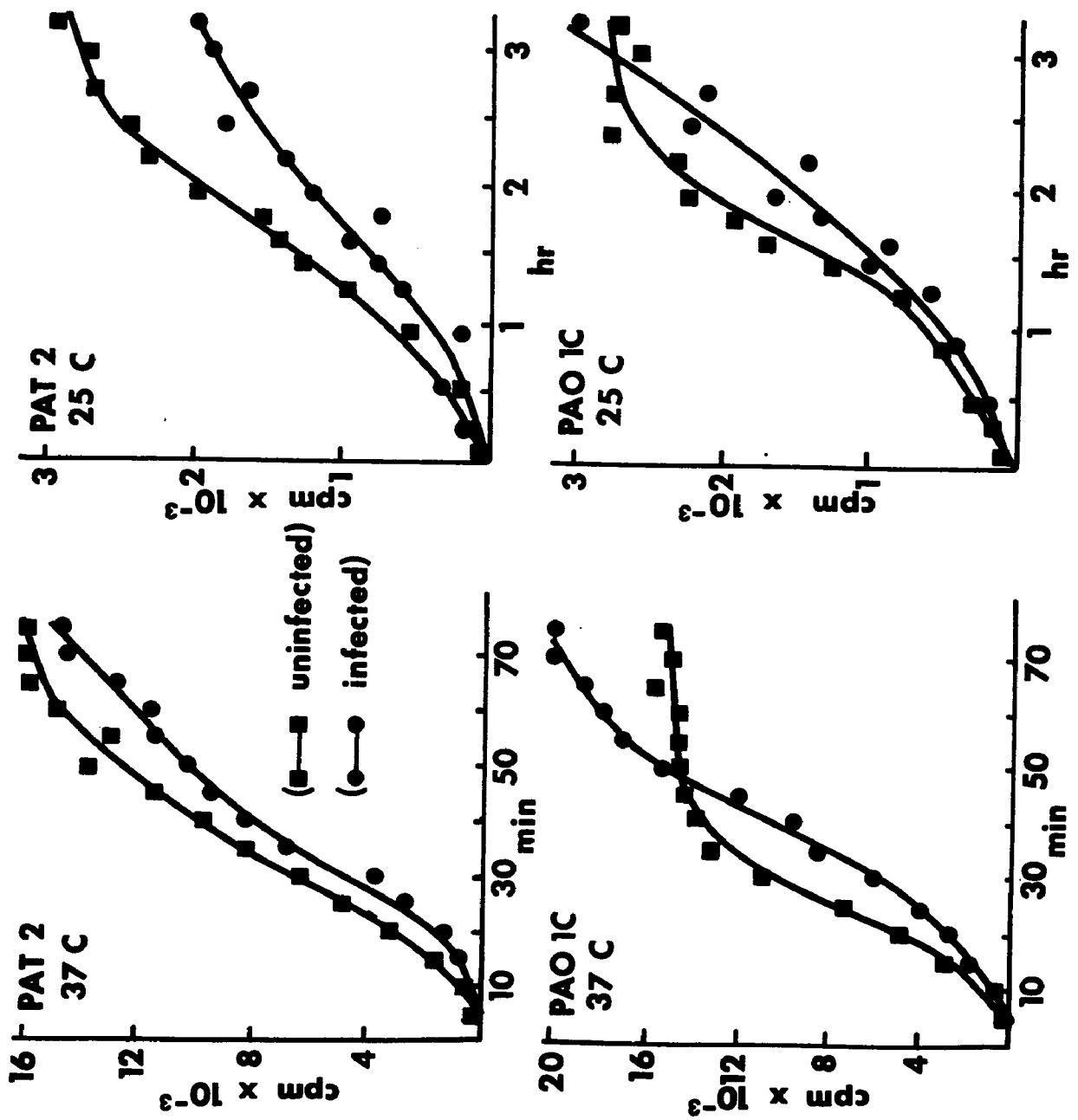

PAT 2
37 C
PAT 2
25 C
PAO 1C
37 C
PAO 1C
25 C
cpm x 10⁻³
min
hr
(■ uninfected)
(● infected)

Figure 2. Net RNA synthesis at permissive and non-permissive temperatures.

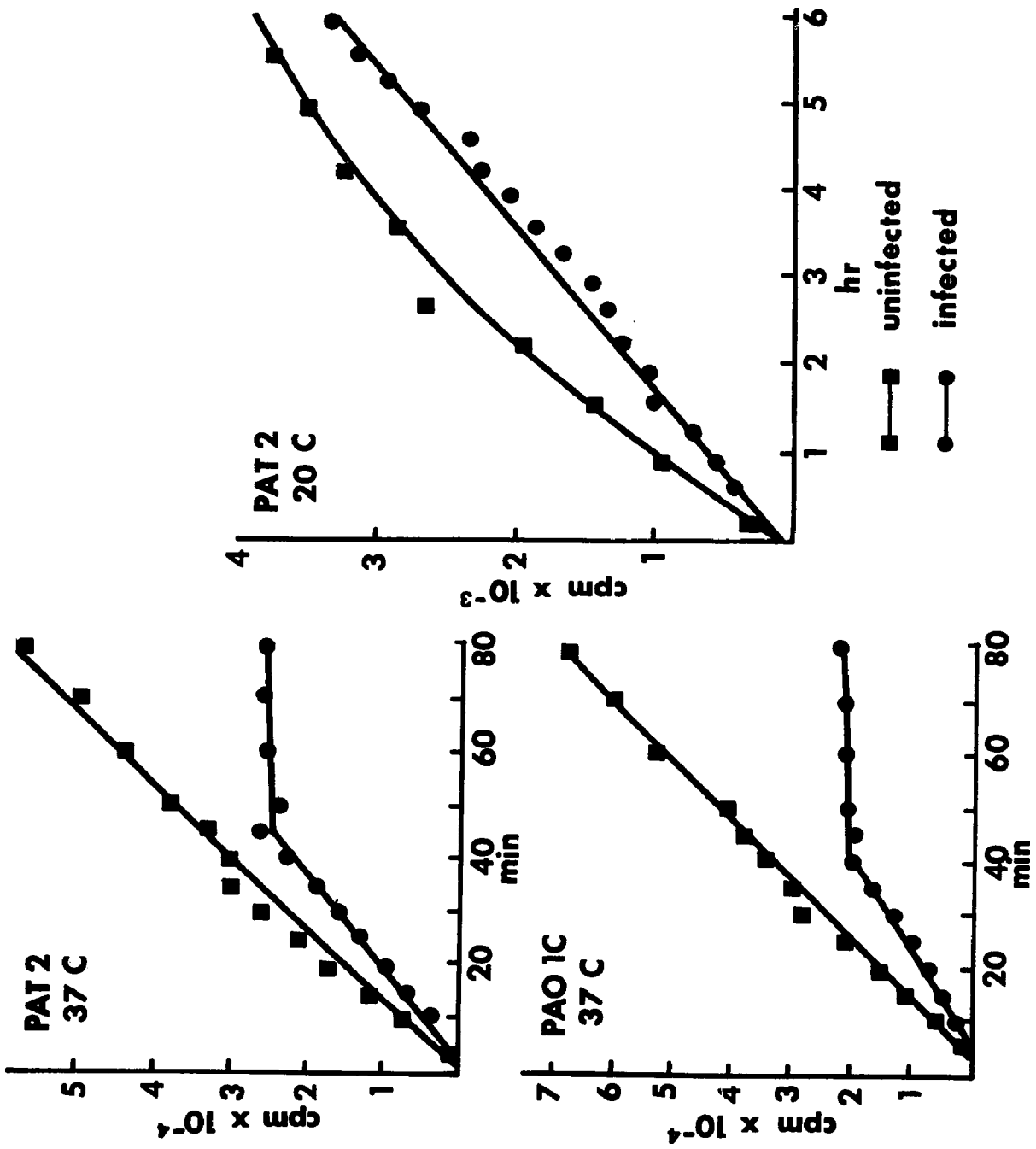

PAT 2
20 C
cpm x 10⁻³
hr
uninfected
infected
PAT 2
37 C
cpm x 10⁻⁴
min
PAO 1C
37 C
cpm x 10⁻⁴
min

Figure 3. Net protein synthesis in PAT 2 at permissive and non-permissive temperatures.

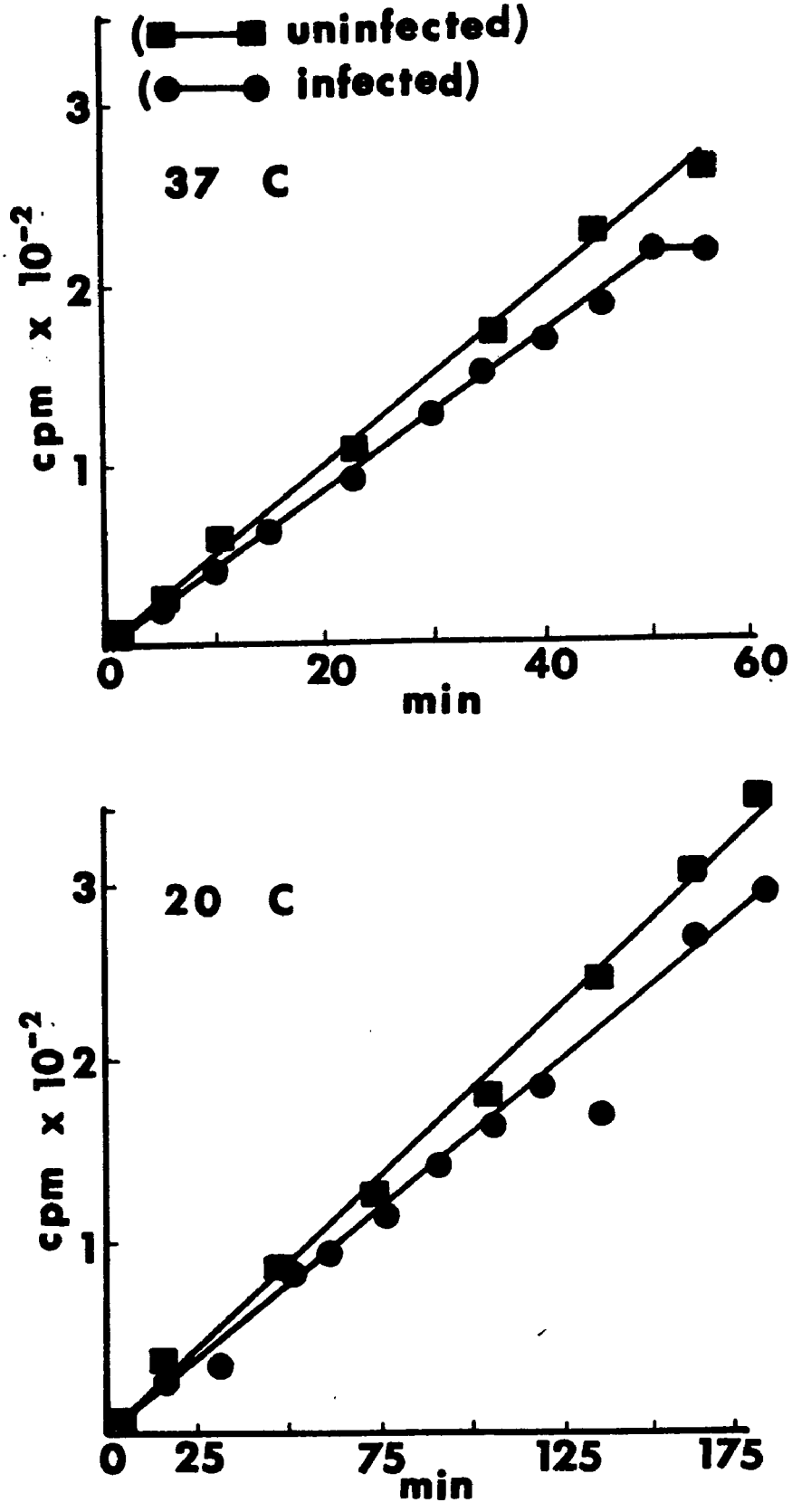

(■—■ uninfected)
(●—● infected)
37 C
cpm x 10^{-2}
3
2
1
0
20
40
60
min
20 C
cpm x 10^{-2}
3
2
1
0
25
75
125
175
min

Figure 3

period; no such change is seen at 20 C.

Monitoring net macromolecular synthesis in CB3 infected cells brings to light several observations. First, the infection by CB3 of either PAT 2 or PAO 1C does not markedly alter net synthesis of DNA, RNA or protein when compared to uninfected cells. Secondly, this data establishes that both hosts react biochemically at permissive and non-permissive temperatures as they do biologically in respect to their ability to undergo infection by CB3. PAO 1C produced a similar DNA synthesis pattern at both temperatures while PAT 2 produced dissimilar patterns. RNA synthesis in infected cells is similar in both hosts at 37 C while PAT 2 at 20 C has a pattern indicating in a biochemical sense its survival to infection at this temperature. This biochemical survival is consistent with 20 C plate counts of infected PAT 2 cells (fig 4) and with information reported in previous work (106).

D. Rate of RNA Synthesis

Experiments to determine the effects of CB3 on the rates of RNA synthesis were performed. These experiments also show that there is a relationship between the biological permissiveness of infection with the observed biochemical alterations in infected cells. Figure 5 depicts that both PAT 2 and PAO 1C show identical effects on RNA synthetic rates when they are infected at 37 C. The rates decrease from that of pre-infected levels and reach a low point between 15 to 20 min into the latent period. The rates then increase until the end of the latent period at which time incorporation rapidly diminishes. At non-permissive temperatures PAO 1C produces a pattern similar to that seen in both hosts at 37 C. The rates at 20 C in PAO 1C diminish from the pre-infected levels for the first 1/3 of the latent period then increase

Figure 4. 20 C plate counts of infected and uninfected PAT 2 at 20 C.

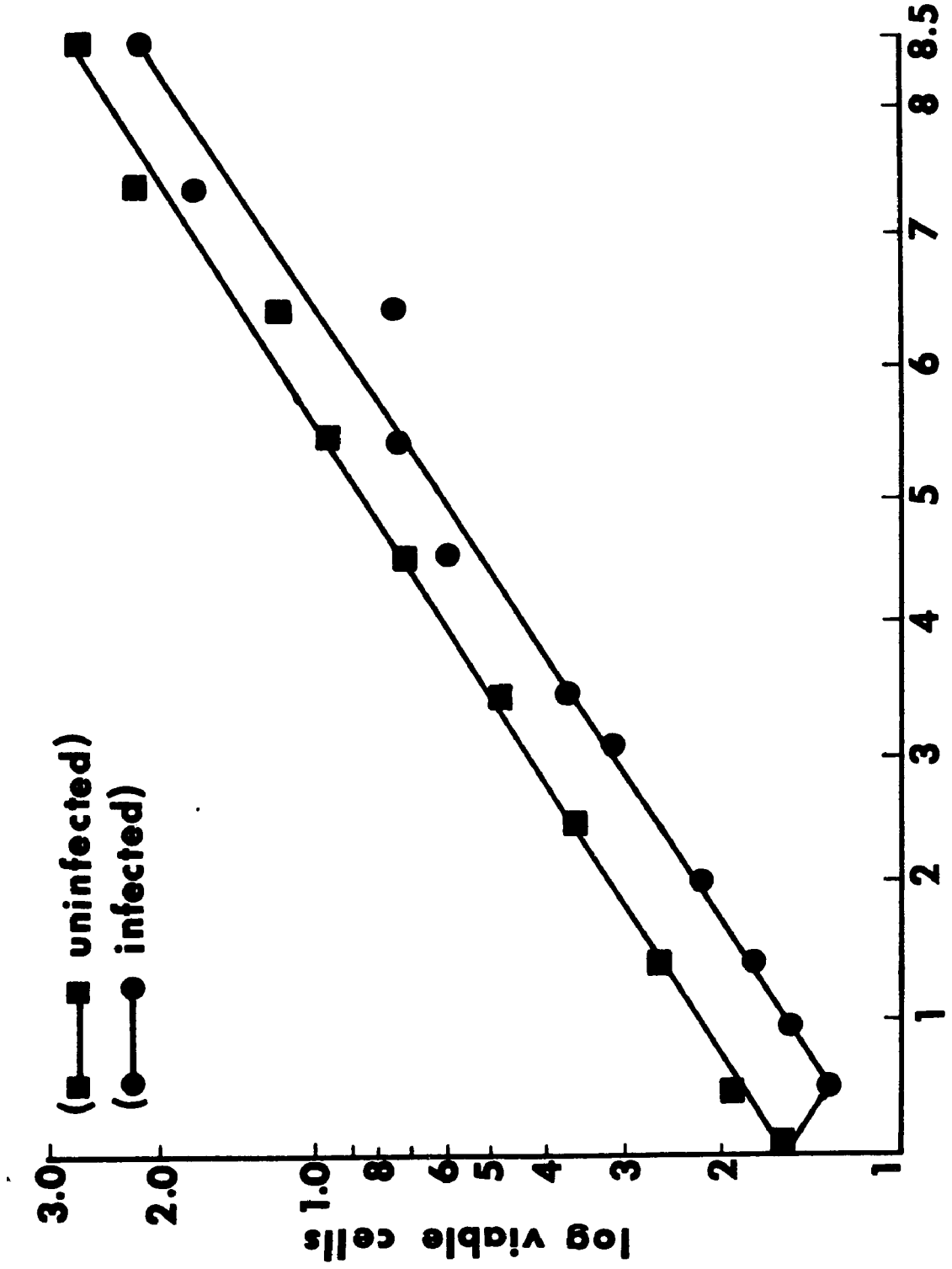
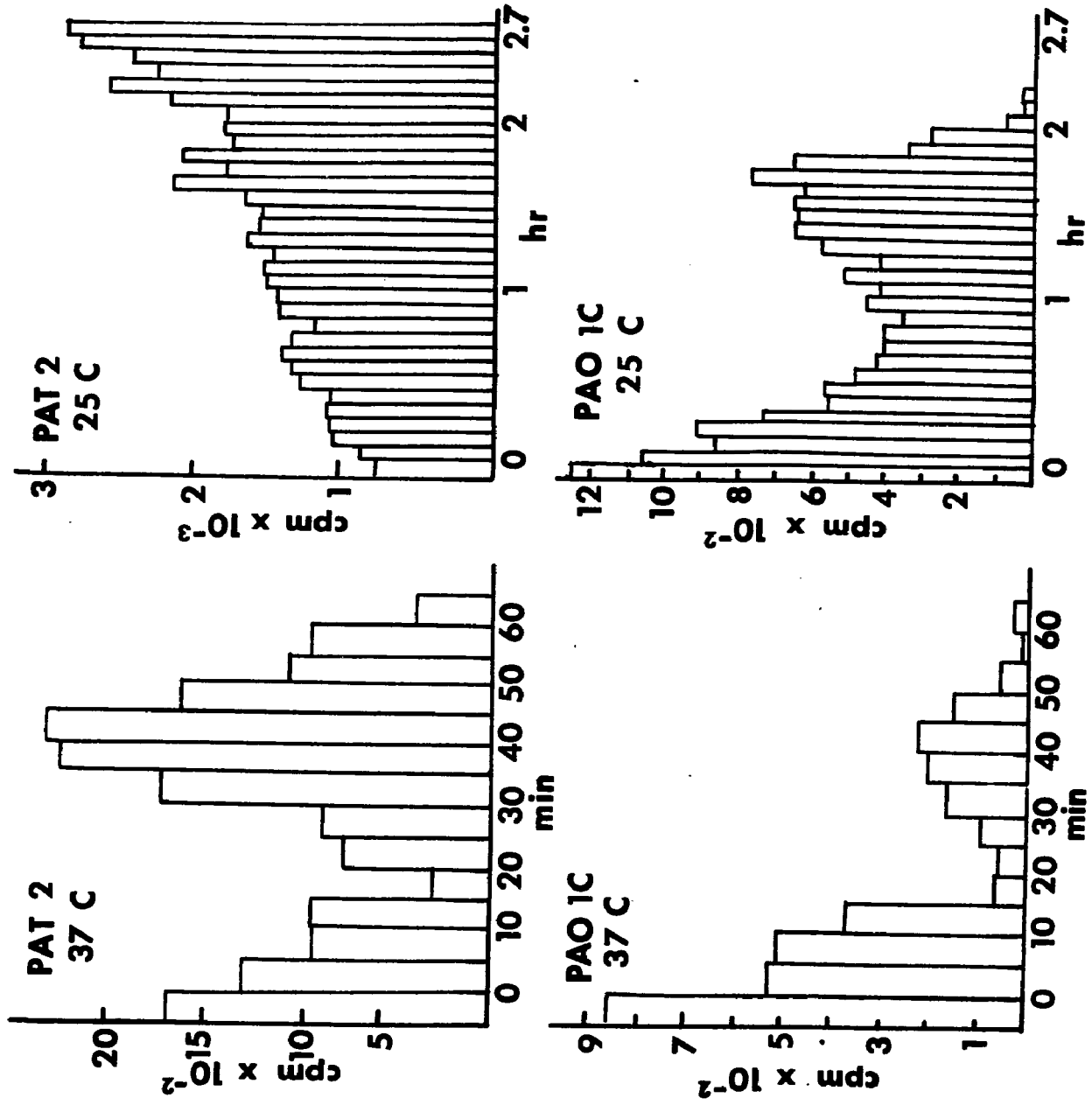

(■ uninfected)
(● infected)
log viable cells
3.0
2.0
1.0
8
6
5
4
3
2
1
1
2
3
4
5
6
7
8
8.5
hr

Figure 4

Figure 5. Rates of RNA synthesis in infected cells at 37 C and 20 C.


PAT 2
25 C
cpm x 10⁻³
hr
PAO 1C
25 C
cpm x 10⁻²
hr
PAT 2
37 C
cpm x 10⁻²
min
PAO 1C
37 C
cpm x 10⁻²
min

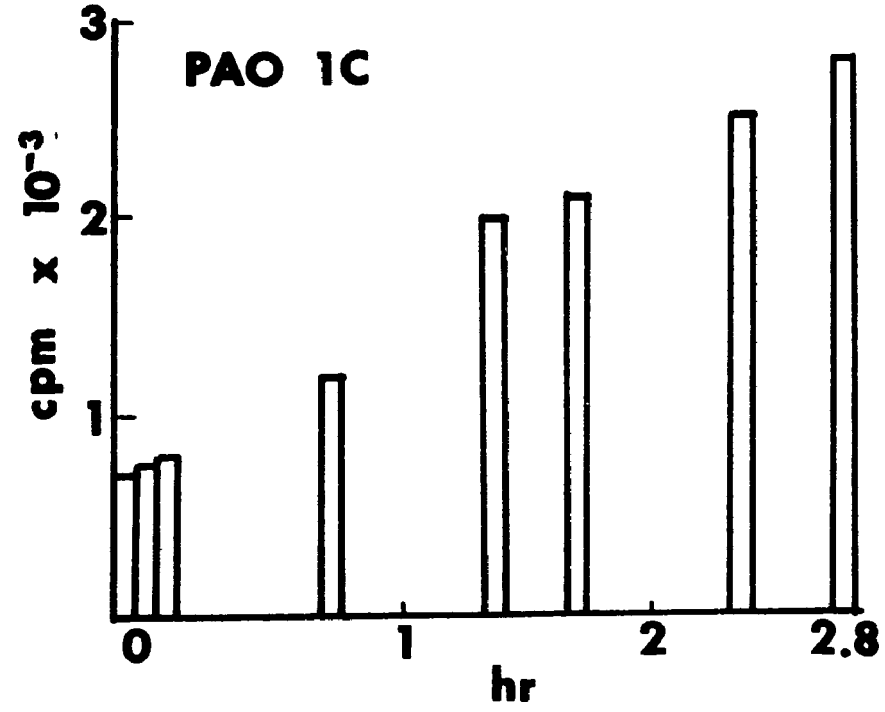
until the end of the latent period. The rates in PAT 2 at 25 C (fig 5) differ from the rates seen in PAO 1C at this temperature and differ from the patterns of both infected hosts at 37 C. In fact, the rates in PAT 2 at 20 C, which continually increase in magnitude from the time of phage addition, are similar to the pulse data from uninfected cells. Figure 6 shows that in uninfected PAT 2 and PAO 1C at 25 C the rates increase in magnitude throughout the experiment. Likewise, when PAT 2 is infected with ultraviolet inactivated CB3 at either 37 or 20 C (fig 7), the rates also increase in magnitude throughout the experiment. Thus, the rates seen in uninfected cells are similar to the rates seen in infected PAT 2 at 20 C or in PAT 2 at 20 or 37 C when it is infected with ultraviolet inactivated virus.

These experiments to determine the effects of CB3 on the rate of RNA synthesis in P. aeruginosa have shown that a correlation exists between the RNA pulse labelling pattern and the in vivo permissiveness of the infection. It appears then that the viral function(s) responsible for the alteration of RNA synthesis are absent or defective at 20 C. In addition, these studies point out that the changes seen in infected cells under permissive conditions are dependent on a biologically active phage genome.

E. DNA Sedimentation Analysis of CB 3 Infected PAT 2

Cells which were labelled prior to and during the latent period at 37 C showed net DNA incorporation curves as those in Fig 8A. These lysates centrifuged in fast neutral sucrose gradients (90 min, 30,000 r.p.m.) gave the radioactive profiles shown in Fig 9A. Lysates of cells made prior to infection showed that all the labelled DNA sediments to the high density shelf of angio-conray at the bottom of the centrifuge

Figure 6. Rates of RNA synthesis in uninfected cells at 20 C.



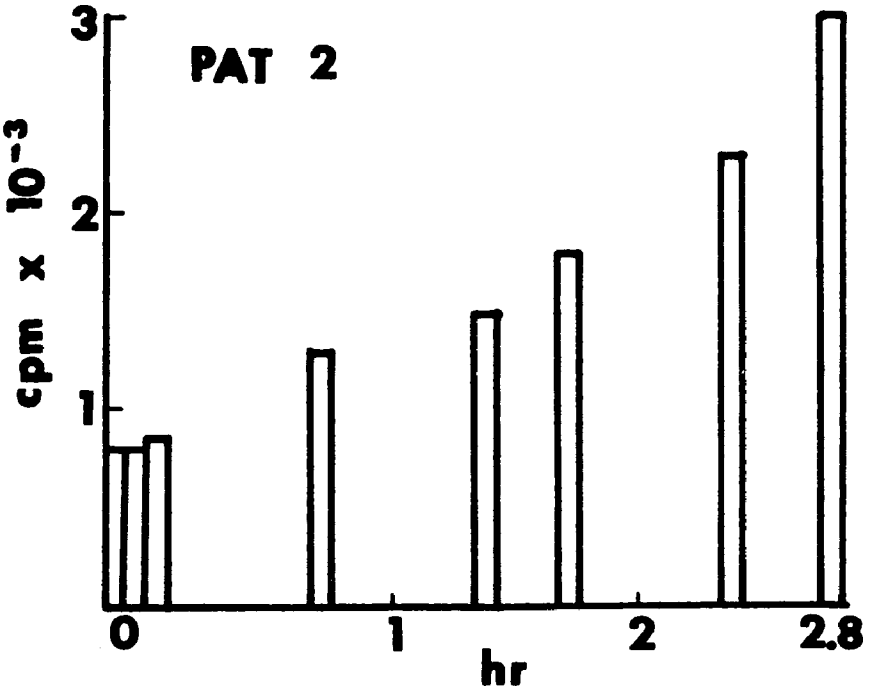
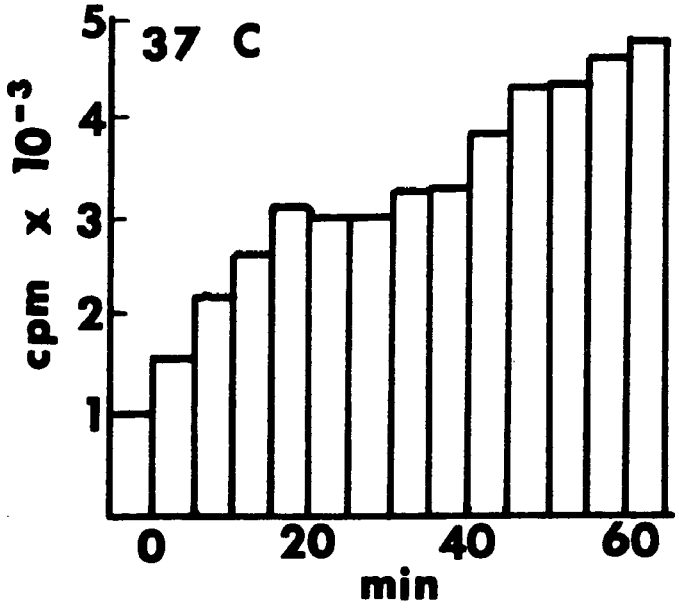


Figure 6

Figure 7. Rates of RNA synthesis at 37 C and 20 C in PAT 2 infected with ultraviolet inactivated CB3.



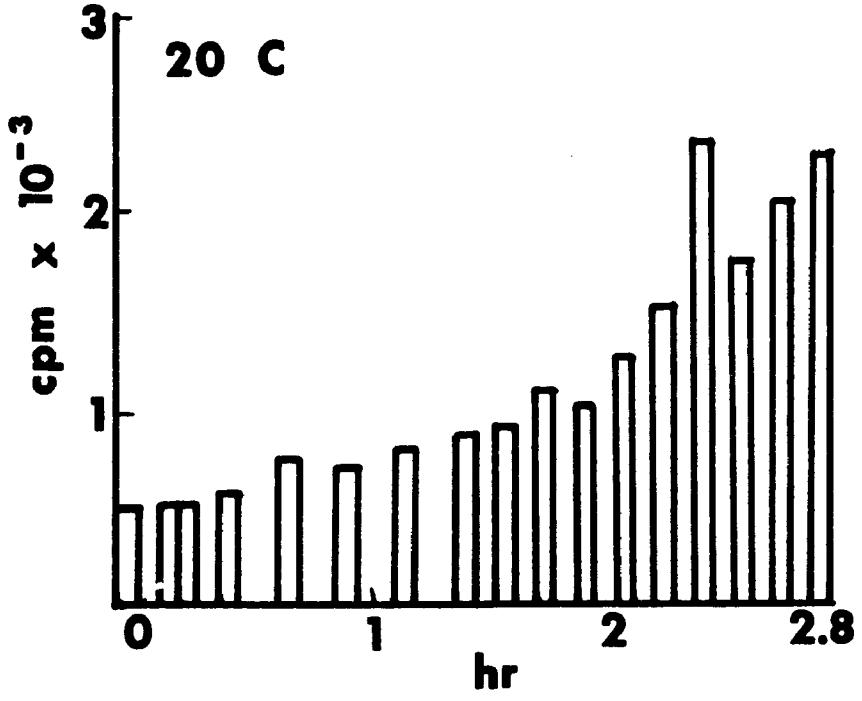


Figure 7

Figure 8a. PAT 2 incorporation of radioactivity at 37 C into DNA unchased at the time of infection.

Figure 8b. PAT 2 incorporation of radioactivity at 37 C into DNA chased at the time of infection.

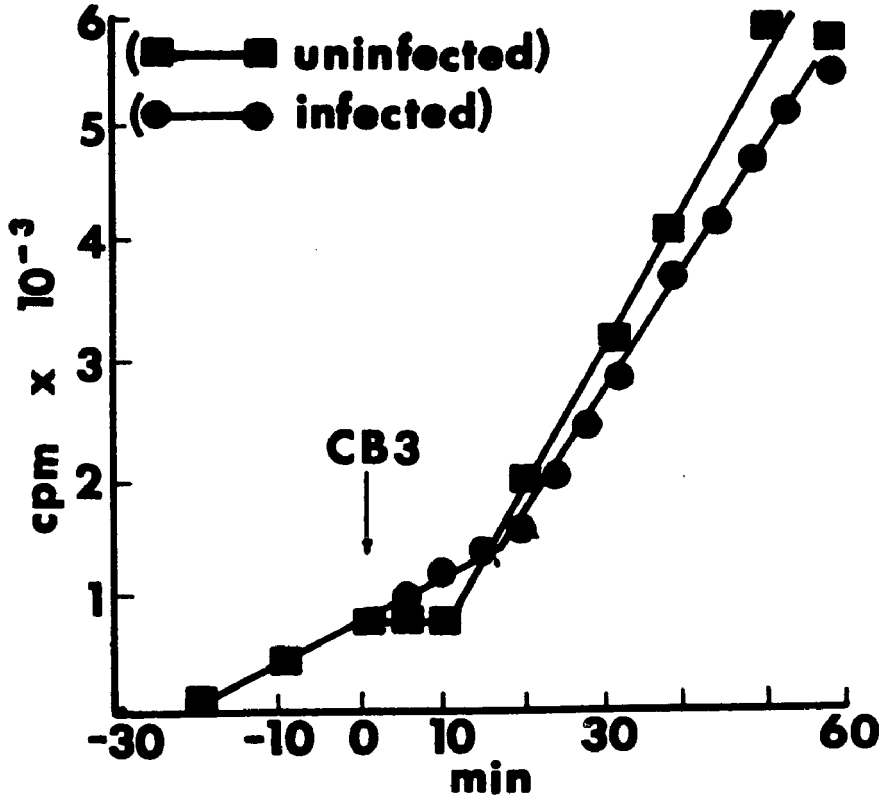


Figure 8a

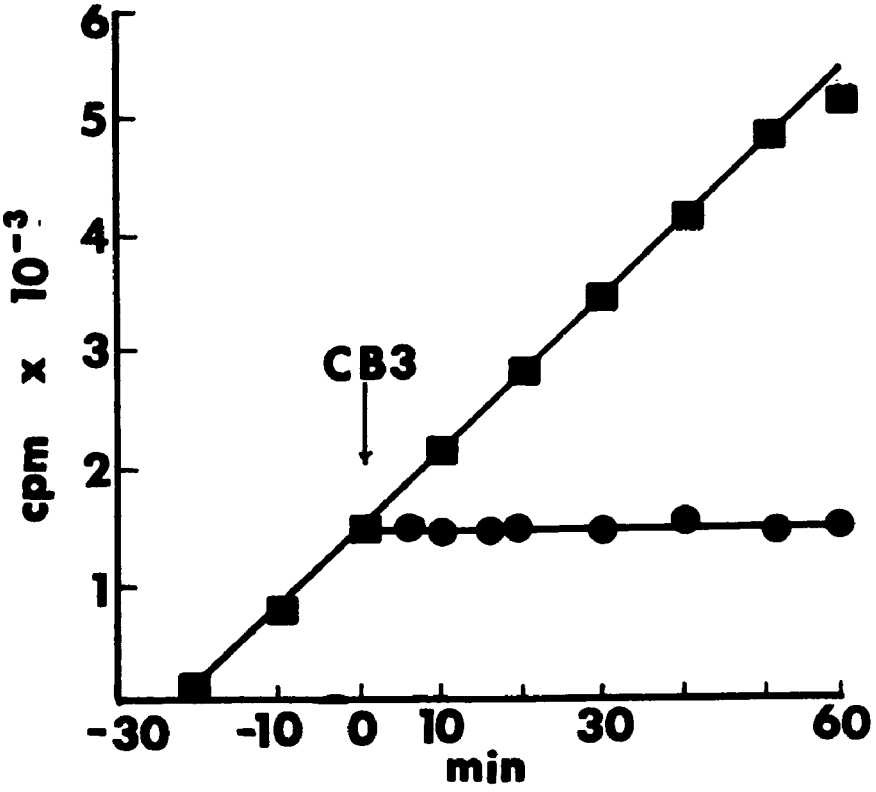


Figure 8b

Figure 9a. Sucrose sedimentation of 37 C PAT 2 lysates unchased during infection.

Figure 9b. Sucrose sedimentation of 37 C PAT 2 lysates chased during infection.

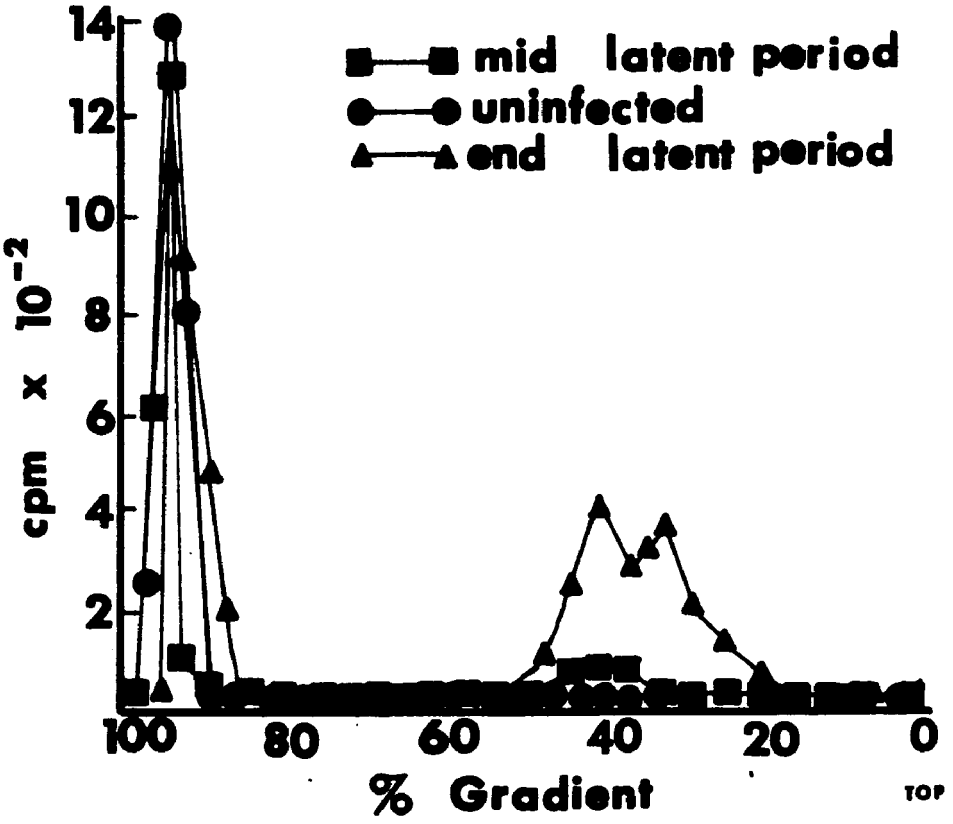


Figure 9a

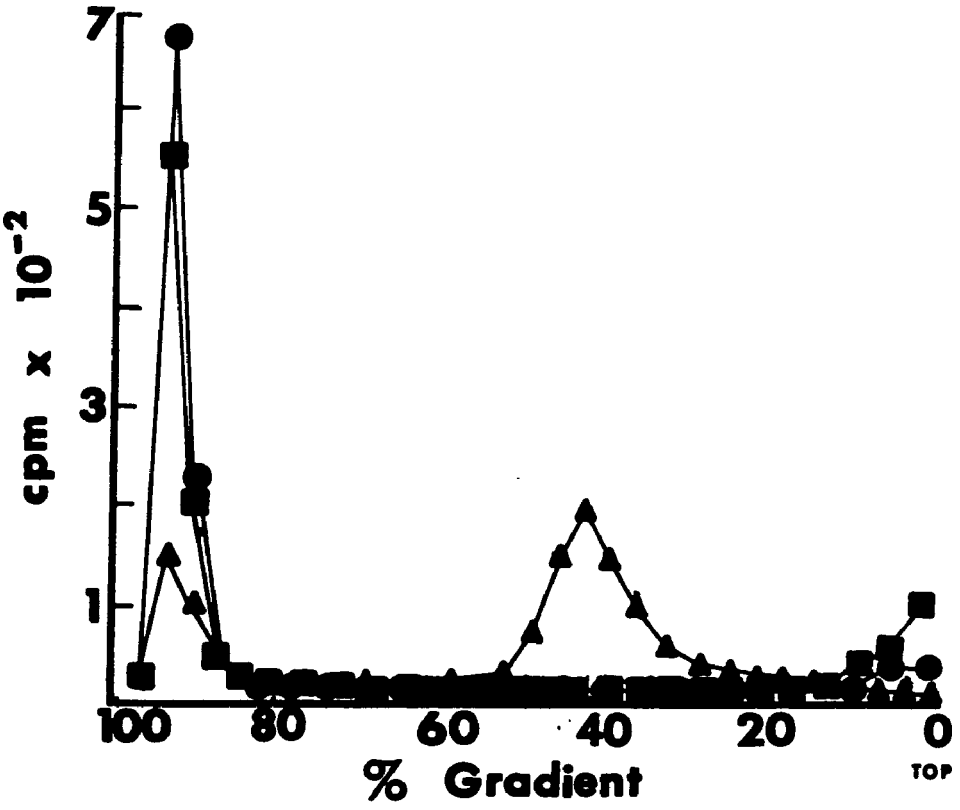


Figure 9b

tube. This represents a distance travelled through the gradient of 100%. A mid-latent period lysate from this same infection has a peak which sediments only 40% of the distance through the gradient which is equivalent to an estimated $S_{20,w}$ value of 78 S. The lysates taken at the end of this infection show that the 78 S peak present midway through the latent period has increased, and a new slower sedimenting peak appears having an estimated sedimentation value of 63.2 S. The identification of these peaks occurred when C^{14} DNA isolated from CB3 was centrifuged under identical conditions. This gradient formed a profile in which the peak of radioactivity has an $S_{20,w}$ equal to 63.2 (Table 1). The identity of the faster sedimenting 78 S peak was shown from experiments using pre-labelled cells which were chased at the time of phage addition. Figure 8B shows the efficacy of the chase procedure at 37 C since no further radioactivity was incorporated after chasing. In Figure 9B the gradient profiles show that prior to infection, chased cells produce a peak of DNA sedimenting to the bottom of the gradient. Following infection, a peak appears in the mid-latent period sample which continues to increase in size until the end of the infection. This peak sedimented to a position in the gradient representing a molecule with an estimated $S_{20,w}$ of 78. These gradients therefore establish that permissively infected PAT 2 has its DNA degraded during the course of viral development to a molecule with a size of 78 S. Likewise at 37 C the appearance of phage DNA is detected during the last half of the latent period as a molecule with an estimated $S_{20,w}$ of 62 to 63 S.

Experiments at 20 C with PAT 2 infected cells produce the net DNA synthesis and chase incorporation curves shown in Figure 10a and 10b. Fast neutral gradients (90 min, 30,000 r.p.m.) of lysates from contin-

TABLE 1

ESTIMATED $S_{20,w}$ VALUES OF PURIFIED CB3 DNA IN FAST NEUTRAL SUCROSE GRADIENTS

Sample	$S_{20,w}$
C^{14} purified CB3 DNA	63.2
C^{14} purified CB3 DNA and uninfected PAT 2 cell lysate	62.0

Figure 10a. PAT 2 incorporation of radioactivity at 20 C into DNA unchased at the time of infection.

Figure 10b. PAT 2 incorporation of radioactivity at 20 C into DNA chased at the time of infection.

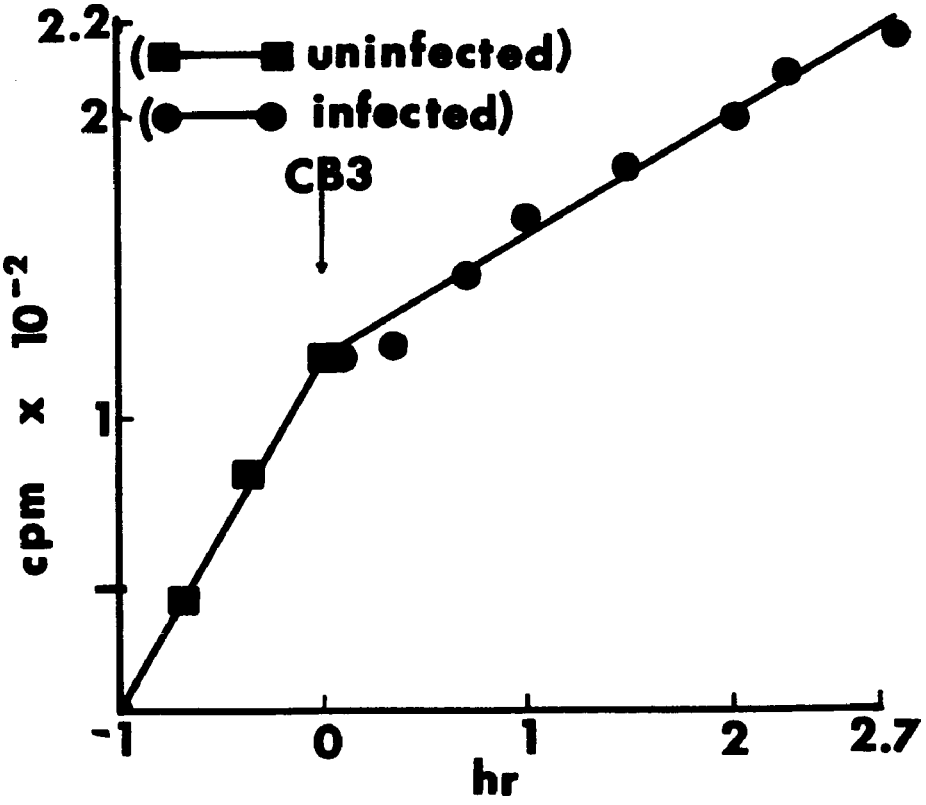


Figure 10a

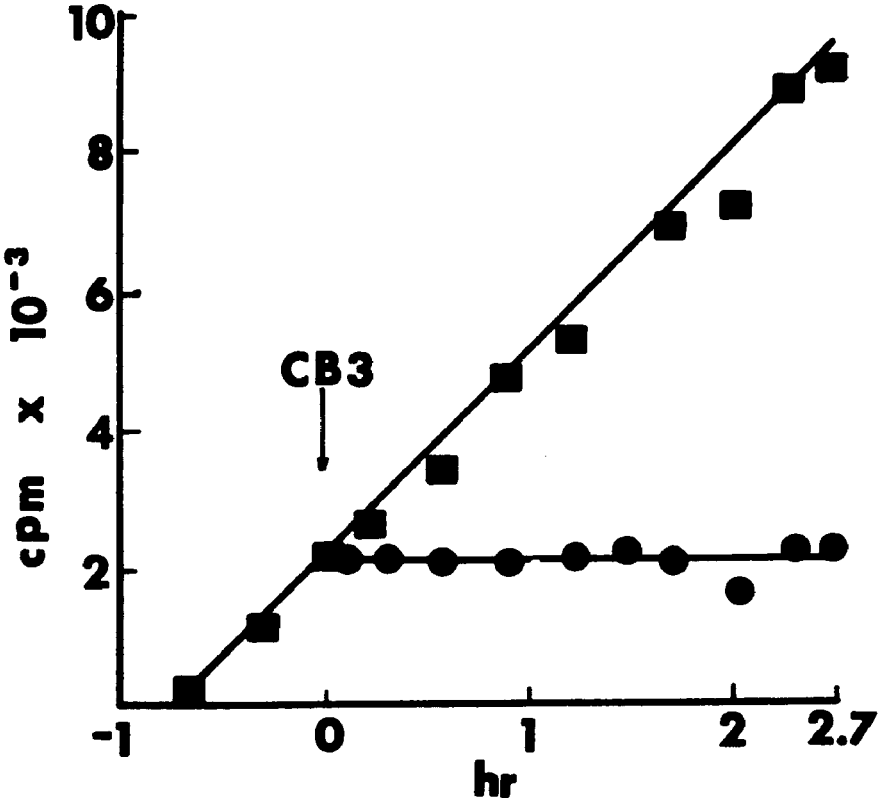


Figure 10b

Figure 11a. Sucrose sedimentation of 20 C PAT 2 lysates unchased during infection.

Figure 11b. Sucrose sedimentation of 20 C PAT 2 lysates chased during infection.

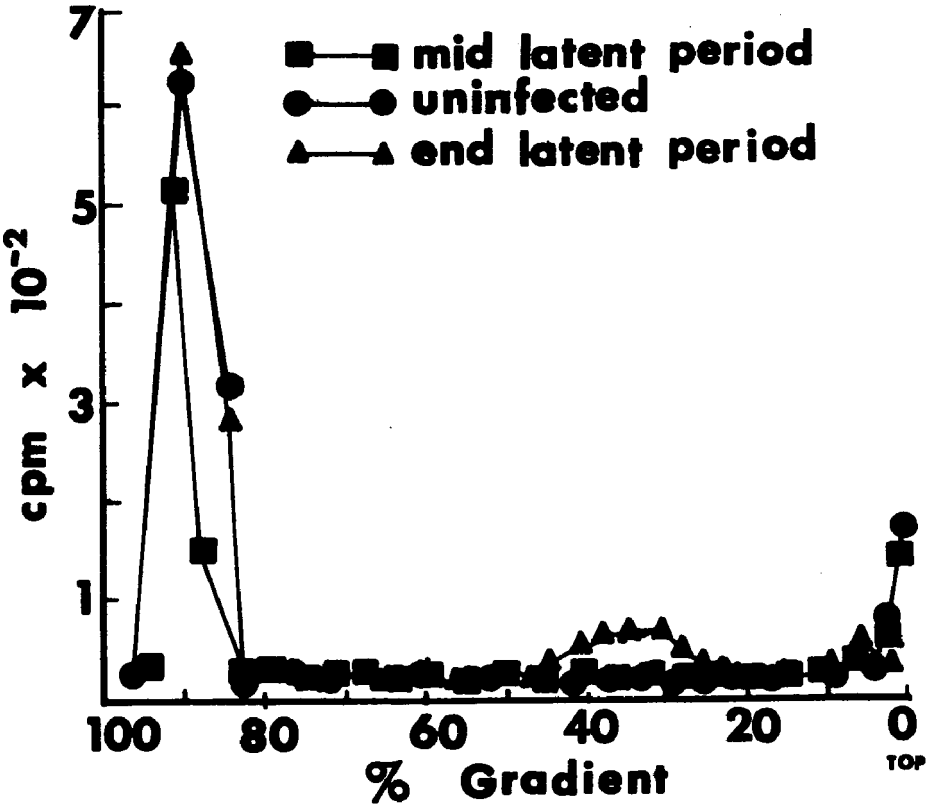


Figure 11a

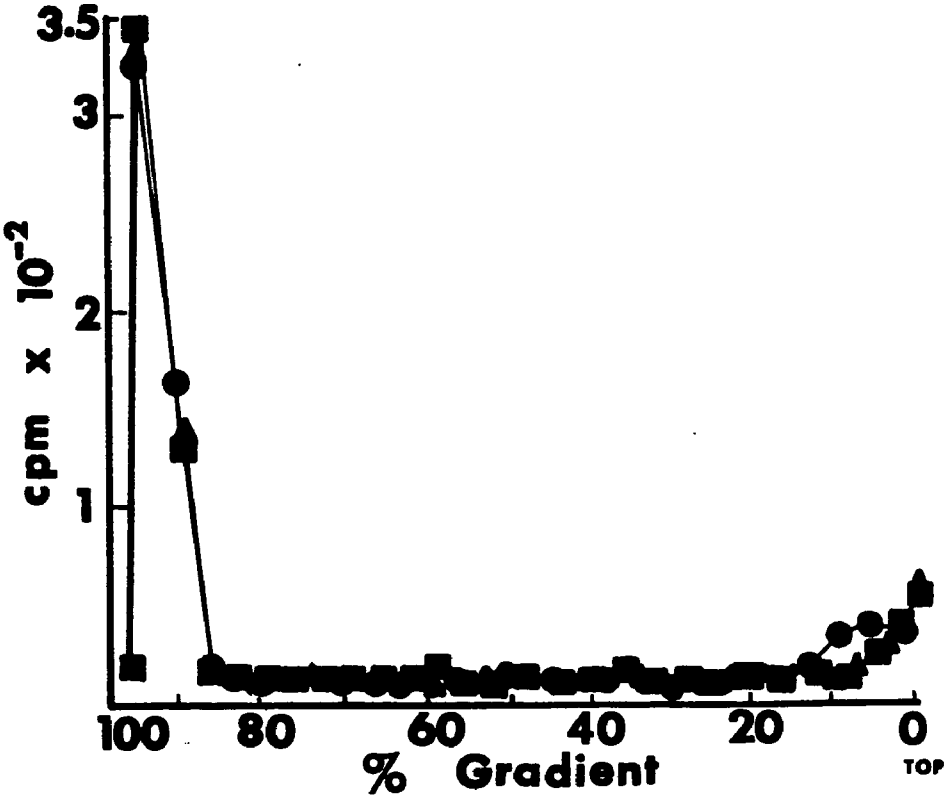


Figure 11b

uously labelled, 20 C, infected cells (fig 11a) only show minor peaks of radioactivity representing either 78 S host degradation product or mature 63 S phage DNA. Pre-labelled chased and infected cells (fig 11b) show that no host degradation product appears in any of the lysates made during the theoretical latent period at 20 C in PAT 2. These gradients of non-permissively infected PAT 2 show that those changes seen in the permissive situation do not occur. Thus, these observations support previous data that PAT 2 does not respond to CB3 infections at non-permissive temperatures. In a more specific sense, cold sensitivity of CB3 appears to prevent phage DNA synthesis based on the samples taken late in the 20 C infection.

F. Early Latent Period Lysates

PAT 2 cells were labelled with radioactive uracil from the start of the infection by CB3. Samples for these lysates were taken 15 min after phage addition at 37 C and after 60 min at 20 C. Both these samples, when centrifuged under fast neutral conditions (90 min, 30,000 r.p.m.), produced peaks of radioactivity which sediment to the bottom of the gradient. These same lysates in fast alkaline sucrose gradients show two different profiles (fig 12). Lysates of 37 C infected cells have a peak of radioactivity sedimenting through 20% of the gradient while 20 C lysates have a peak sedimenting through 35 to 50% of the gradient. The profile of the 20 C sample is identical to profiles of labelled uninfected cells in fast alkaline sucrose (not shown). The position of the 37 C peak corresponds to the position in fast alkaline gradients of CB3 C^{14} DNA which was added to uninfected cell lysates prior to centrifugation (see fig 17). Thus, these early latent period lysates demonstrate that there is a differentiation of macromolecular synthesis

Figure 12. Alkaline sucrose sedimentation of C^{14} labelled DNA from 37 C and 20 C infected PAT 2 cells lysates.

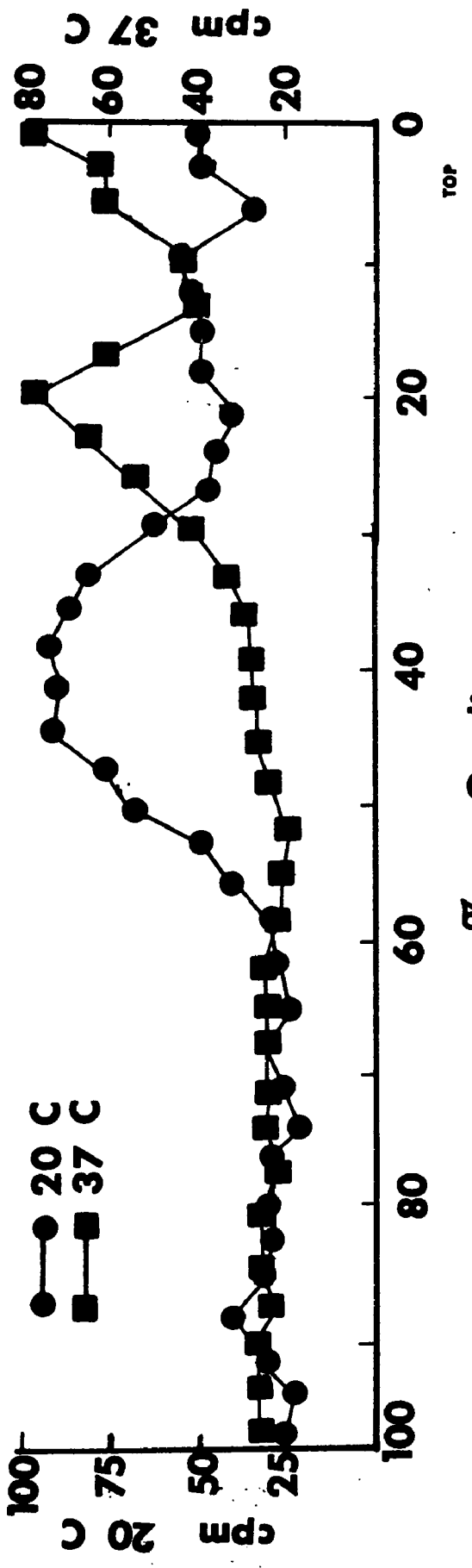

cpm 37 C
80
60
40
20
0
top
20
40
60
80
100
% Gradient
20 C
37 C
cpm 20 C
100
75
50
25

Figure 12

under permissive and non-permissive conditions. PAT 2 DNA synthesis occurs in infections at 20 C while at 37 C host DNA is not detected, but phage synthesis is evident. On the other hand, this implies that the cold sensitive defect at low temperatures is prior to the onset of viral DNA synthesis since these parameters failed to detect CB3 DNA.

G. Membrane Binding Experiments

The cold sensitivity of CB3 at 20 C can be overcome by a 5 min temperature pulse at 37 C from the beginning of the infection (106). Studies were performed with viable CB3 radioactively labelled in its DNA to determine if the attachment of viral DNA to membrane material was involved in this temperature dependent phenomenon. These experiments utilized three different C^{14} CB3 preparations which after nuclease treatment and purification by differential centrifugation had respectively 2.86×10^{-7}, 2.9×10^{-6} and 2.3×10^{-6} c.p.m./viable virion. The studies showed that the CB3 genome does attach to cell cytoplasmic membrane material at both temperatures. Figure 13 shows the gradient profiles of cells infected at 37 and 20 C and processed to determine if viral DNA binds to host cell material. It demonstrates that after 5 min at 37 C or 15 min at 20 C, lysates centrifuged under slow neutral conditions (40 min, 25,000 r.p.m.) have peaks of radioactivity represented by estimated $S_{20,w}$ values of 600 or more. The S value must be at least this large in order for the material to reach the bottom of the tube in the time and speeds used for slow gradients. Identical profiles are obtained in slow neutral gradients with 37 C lysates made after 2 or 10 min following phage addition, or with 20 C lysates made 32 min into the latent period. Thus, it appears that phage DNA does attach to membrane material at both temperatures and is not the cause of the cold sensitive restriction of CB3. Necessary controls further establish the validity

Figure 13. Membrane binding of C^{14} labelled phage DNA following infection of PAT 2 at 37 C and 20 C.

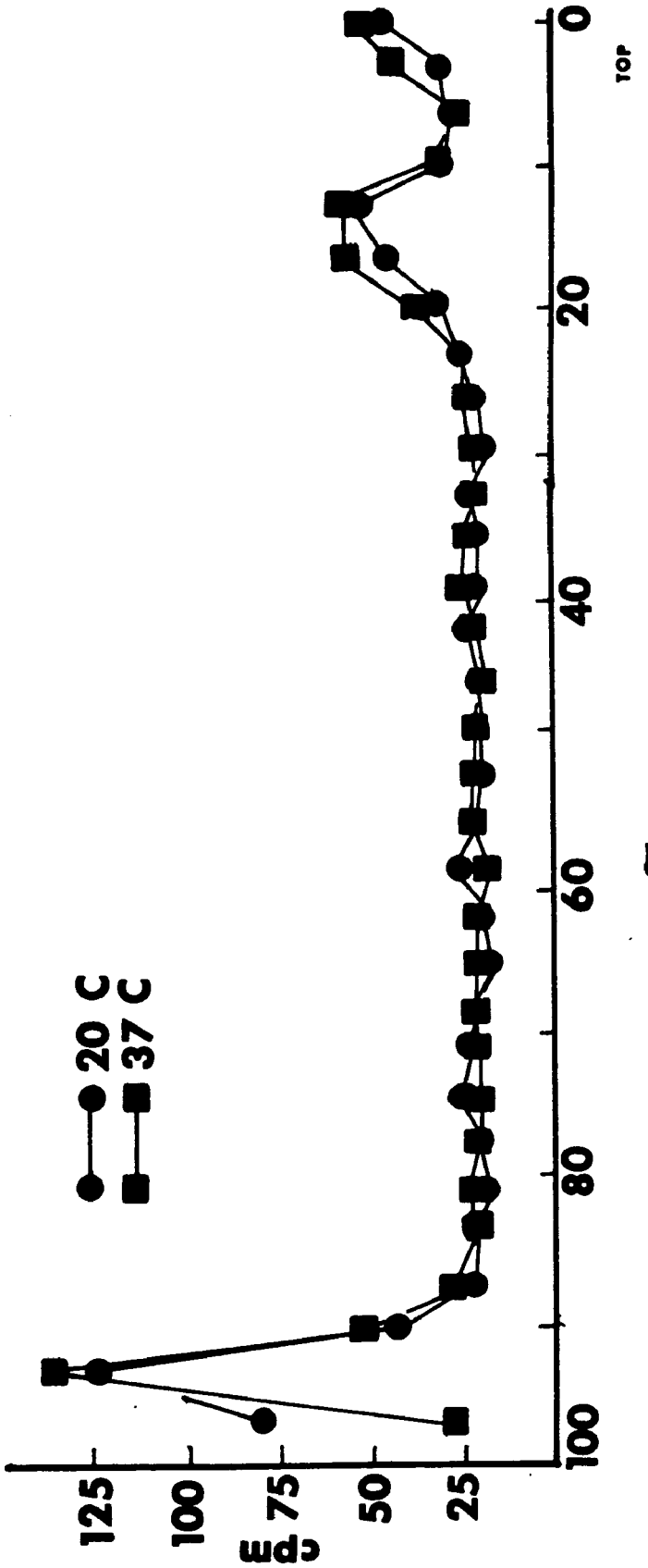


Figure 13

of this observation. Table 1 lists the positions in fast neutral gradients of purified phage DNA, and the positions of this DNA when added to a lysate of uninfected PAT 2 growing at 37 C. Under these fast neutral conditions the peaks of radioactivity in these controls are not at the bottom of the gradient but only at positions represented by S values of 62 and 63.2. These S values correspond to S values for phage DNA seen in lysate samples taken at the end of the viral latent period (see fig 9a). Therefore, these control experiments establish the validity of the earlier observation that phage DNA does become membrane attached in vivo at both temperatures.

Infected cells were treated in various ways to gain information on the nature of the attachment process. These treatments included: the ultraviolet irradiation of PAT 2 prior to infection, chloramphenicol pretreatment prior to infection and the starvation of cells by depletion of intracellular nutrients using starvation buffer. These treatments of PAT 2 still allow viral attachment (Table 2). Ultraviolet inactivation of CB3 also does not prevent the attachment process at either 37 or 20 C (Table 2) since the peaks of radioactivity are found at the bottom of the gradients. Interestingly, the changes in the rates of RNA synthesis were shown not to occur in infected cells using ultraviolet inactivated phage (see fig 7), yet attachment of the genome to membrane material is not prevented by this treatment. This finding implies that the changes seen in the rates of RNA synthesis under permissive conditions are dependent on some phage function which occurs subsequent to attachment.

Another treatment used to gain information on the nature of the attachment process was exposure to the metabolic inhibitor, sodium azide. Pretreating PAT 2 for 5 min in 0.01 M sodium azide prior to

TABLE 2

STUDIES ON THE NATURE OF CB3 DNA MEMBRANE ATTACHMENT

Conditions	Temperature of the Experiment	$S_{20,w}$ Value
U.V. treated PAT 2	37 C	600+
CM treated PAT 2	37 C	600+
Starved PAT 2	37 C	600+
U.V. inactivated CB3	37 C	600+
U.V. inactivated CB3	20 C	600+

Figure 14. The sodium azide inhibition of C^{14} labelled phage DNA membrane attachment in PAT 2 at 37 C and 20 C.

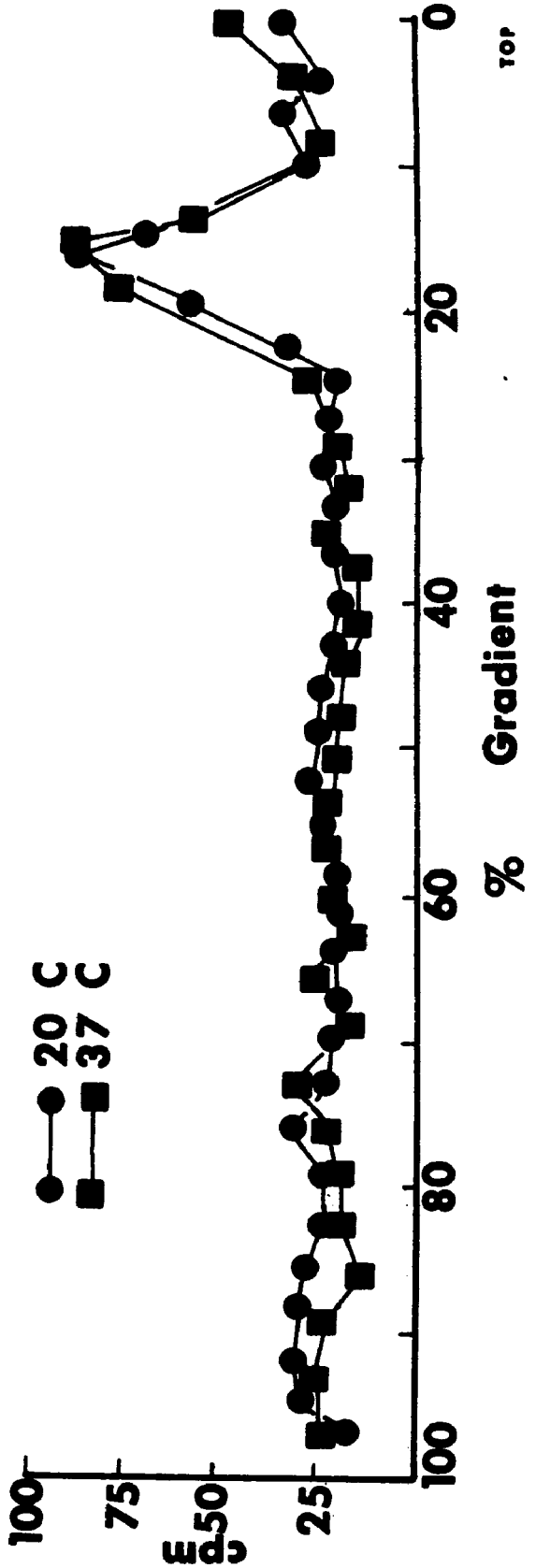


Figure 14

infection did prevent attachment of the viral genome. Gradients of lysates made after 10 min at 37 C and after 32 min at 20 C show that radioactive DNA now sediments in slow neutral gradients (40 min, 25,000 r.p.m.) near the top of the centrifuge tube representing a distance travelled of 15% (fig 14). Other lysates of azide-treated cells centtrifuged under fast neutral condition (90 min, 30,000 r.p.m.) produced radioactive peaks at an $S_{20,w}$ value of 62 (fig 15). This is identical to the S value of mature phage DNA found in infected cells at the end of the latent period (see fig 9a) and to the S value for mature phage DNA isolated from intact virus particles (Table 1).

Fast alkaline gradients were also used to study the nature of attached viral DNA in regard to its sedimentation properties compared to free viral DNA. Gradients of cells infected at 37 C for 2 and 10 min or at 20 C for 16 or 32 min showed the same profiles. Figure 16 reproduces the gradient profile of 37 C-10 min and 20 C-32 min lysates centrifuged in fast alkaline gradients. Both these lysates travelled 23% into the gradient. C^{14} labelled DNA from CB3 when added to uninfected cell lysates prior to centrifugation in fast alkaline sucrose also produced a profile having the radioactive material at a position of 22 to 23% into the gradient (fig 17). Interestingly, fast alkaline gradients of azide-treated cells infected with virus for 10 min at 37 C or for 32 min at 20 C produced similar peaks in an identical position (fig 18). Thus these gradients establish that the physical form of each strand of CB3 DNA is identical prior to and subsequent to attachment. In addition, even mature virus DNA showed the same properties.

These alkaline gradients also show that alkali pretreatment of cell lysates in conjunction with the alkaline gradients removes the

Figure 15. Fast neutral sucrose sedimentation of sodium azide treated PAT 2 cells infected with C^{14} labelled CB3 DNA at 37 C and 20 C.

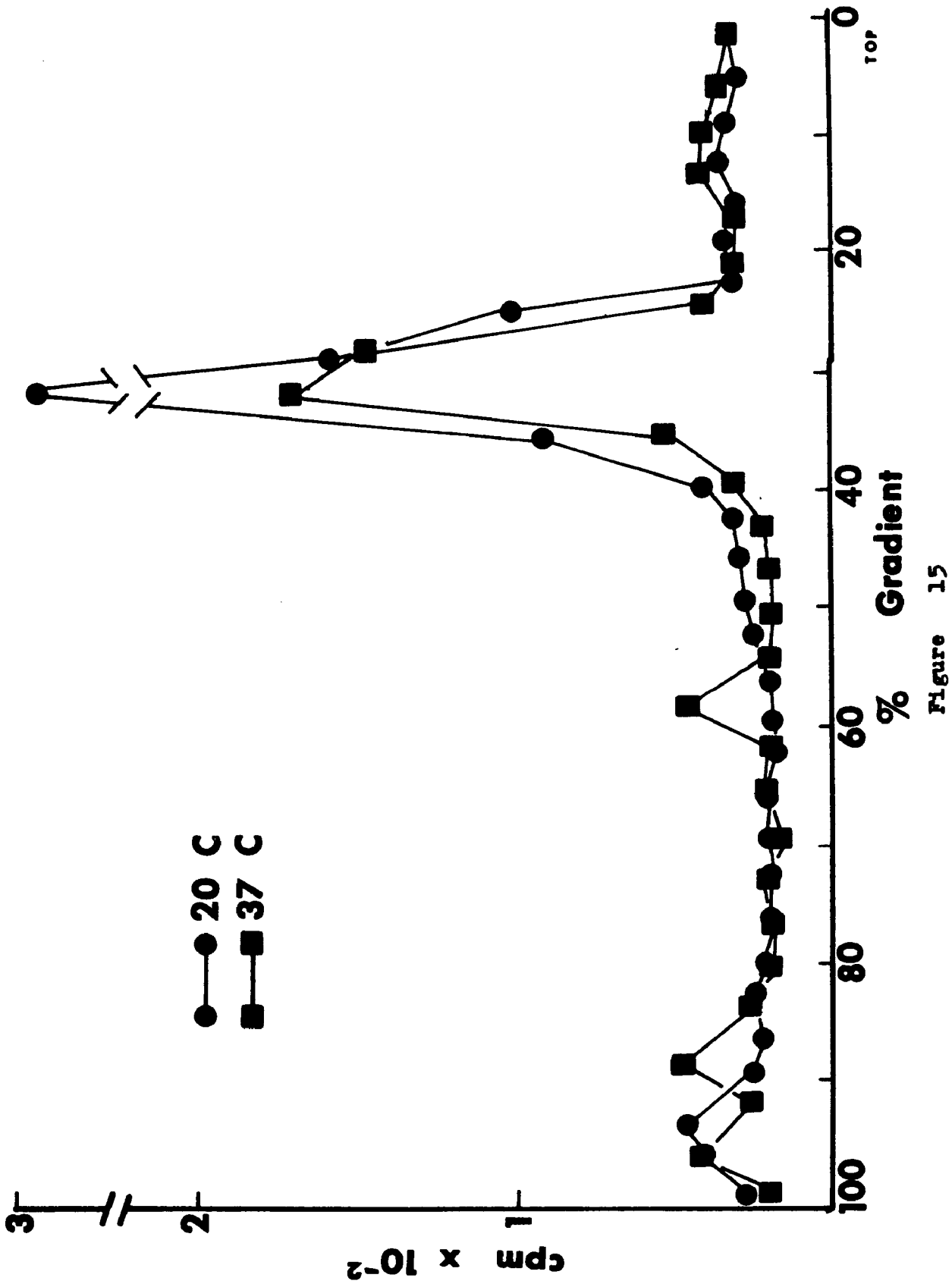


Figure 15

Figure 16. Fast alkaline sucrose sedimentation of PAT 2 infected with C^{14} labelled CB3 DNA at 37 C and 20 C.

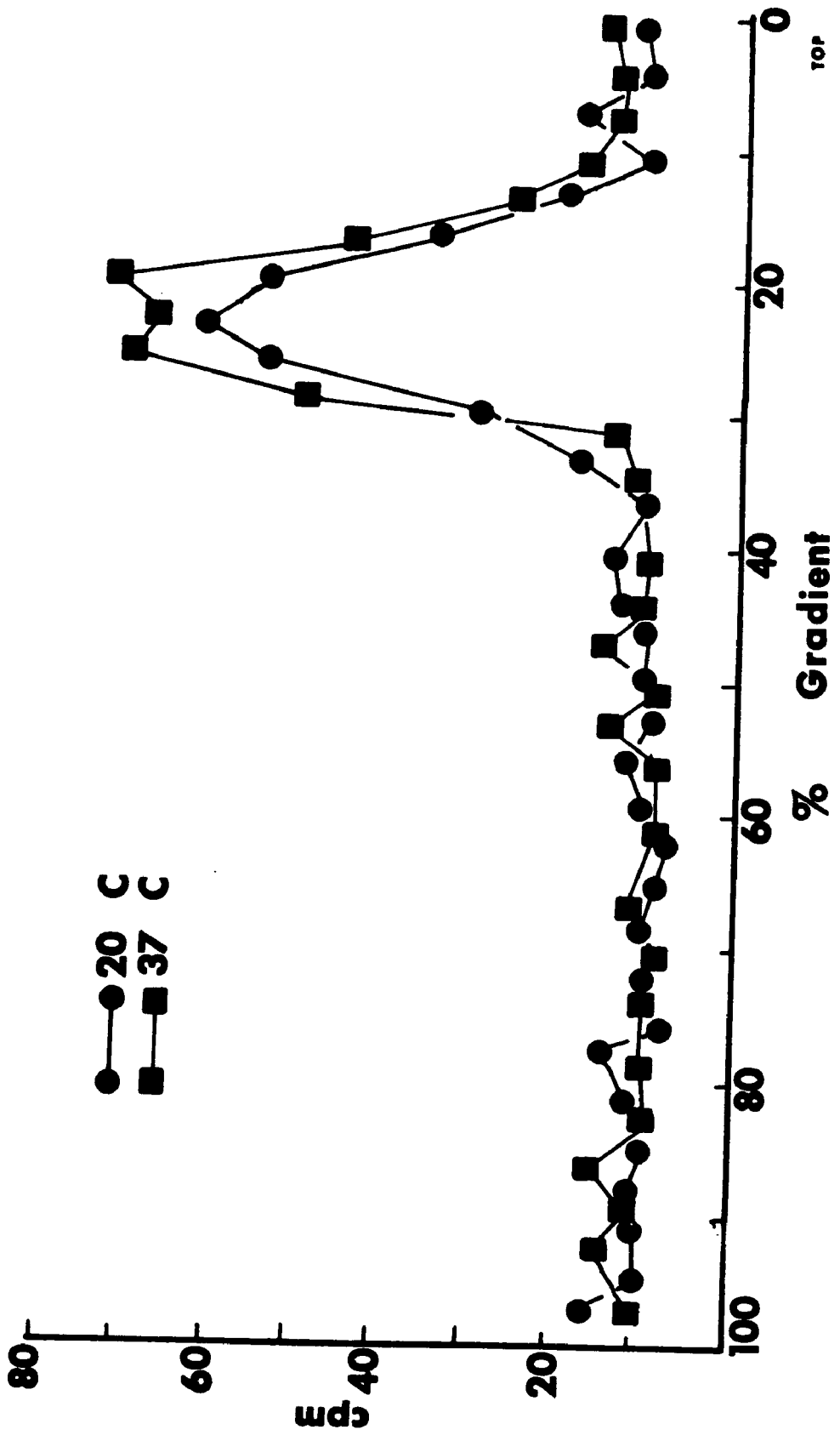


Figure 16

Figure 17. Fast alkaline sucrose sedimentation of C^{14} labelled CB3 DNA plus uninfected PAT 2 cell lysate.

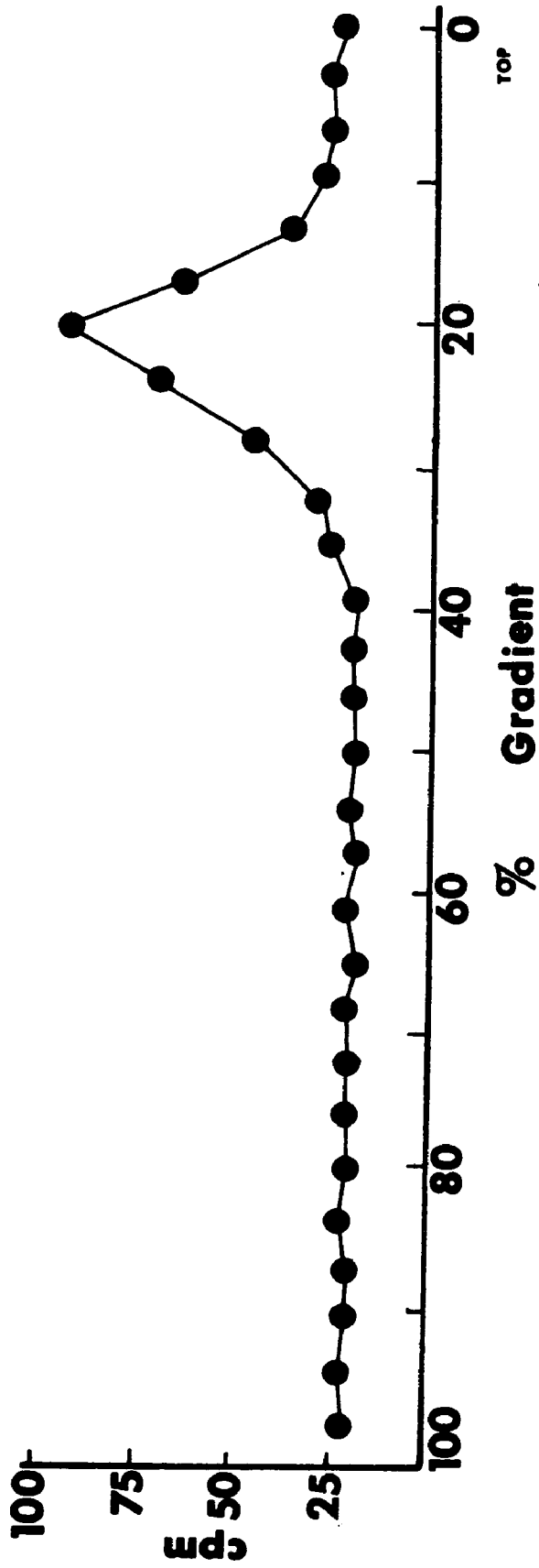


Figure 17

Figure 18. Fast alkaline sucrose sedimentation of sodium azide treated PAT 2 cells infected with C^{14} labelled CB3 DNA at 37 C and 20 C.

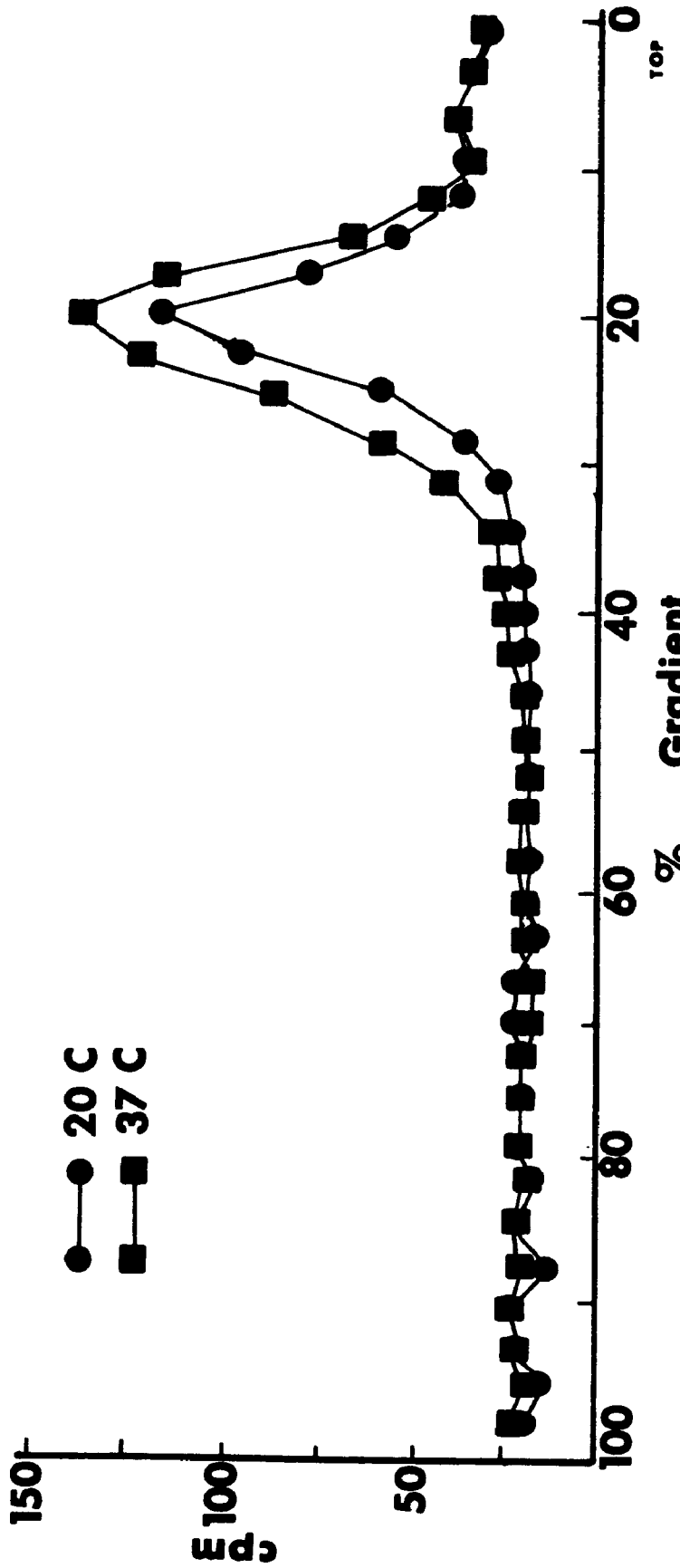


Figure 18

membrane from phage DNA which is responsible for its fast sedimentation properties in slow neutral sucrose. Pronase treatment also removed the membrane material from phage DNA to allow it to sediment with an S value of 62 in fast neutral gradients. These findings are analogous to those found with E. coli (129).

Membrane attachment studies have eliminated the absence of this host virus interaction as the cold sensitive defect in the cold sensitivity of CB3 in PAT 2. Further, these studies have pointed up several observations on the nature of phage DNA attachment which could only be prevented by sodium azide. Membrane binding of viral DNA was found to be insensitive to prior starvation of the cell, ultraviolet irradiation of either the host or the virus and the process was not prevented by an inhibitor of protein synthesis. Finally, the physical form of each strand of CB3 DNA is not altered as a result of attachment when compared to mature viral DNA.

II. RNA-DNA Hybridization

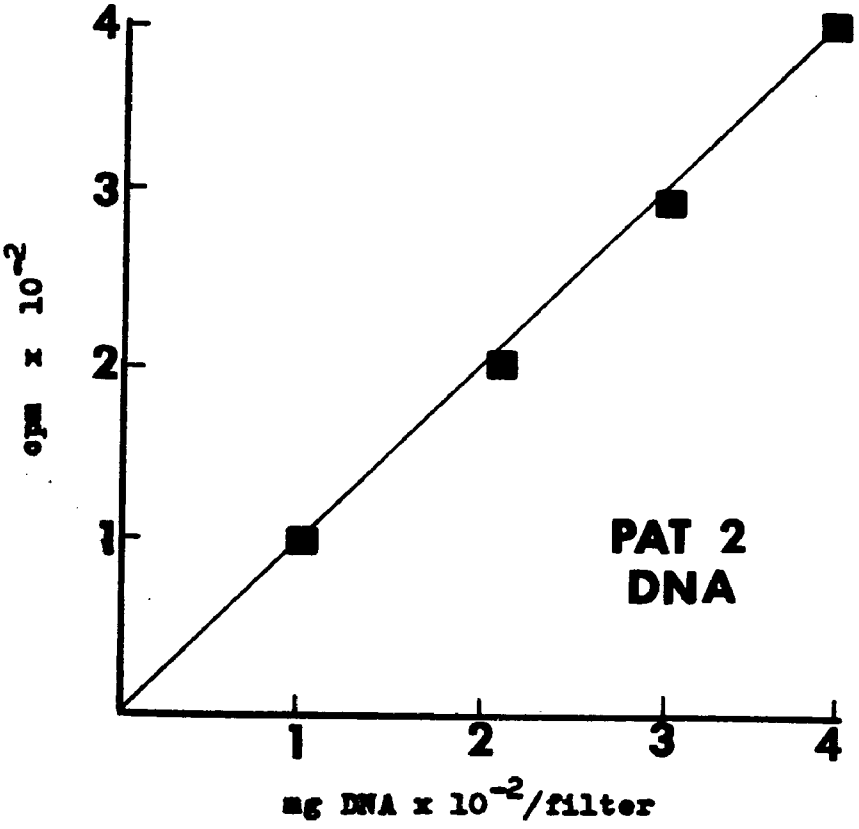
A. Direct Hybridization

Prior experiments characterizing the biochemistry of the CB3 infection showed that at 20 C in PAT 2 phage functions subsequent to DNA membrane binding appeared to be absent (influence of CB3 on RNA synthesis; appearance of viral DNA). For these reasons a RNA-DNA hybridization approach was undertaken to determine if the cold sensitive defect at low temperature was the result of the inability of CB3 specific RNA synthesis to occur. It is known in other systems that viral specific messages are produced within the first five minutes of a permissive infection.

Saturation curves for CB3 and PAT 2 DNA in which filters with

increasing amounts of DNA are reacted with a constant amount of either phage infected or uninfected cell RNA preparations are shown in Fig 19. It can be seen that with the concentrations of PAT 2 DNA used (range 0.01 to 0.04 mg), progressively more counts of the uninfected RNA preparations become hybridized. With CB3 DNA, however, as little as 5×10^{-5} mg DNA is able to completely hybridize with the phage specific early RNA from a permissive infection. Routinely, 2×10^{-2} mg CB3 DNA was immobilized per filter while a level of 2.5×10^{-2} mg PAT 2 DNA was used so that the percentage of counts to hybridize to both DNA's in a permissive RNA preparation is approximately equal (see below). The results of one direct DNA-RNA hybridization experiment are shown in Table 3; RNA used was isolated from permissive and non-permissive infections. Section A of Table 3 shows that the control, uninfected PAT 2, possesses no RNA capable of hybridizing to CB3 DNA but has RNA hybridizing to homologous host DNA, in this case 4.05%. This is an important control; since there is no RNA in uninfected cells hybridizing to phage DNA, it means that all RNA present in infected cells which does hybridize to phage DNA is viral specific. Section B of this table used RNA isolated from permissively or non-permissively infected cells and shows that the RNA labelled for the first 5 min of the latent period, and hence called "early", has material which hybridizes to both host and CB3 DNA. Specifically 4.36% of the 37 C, early, infected cell RNA hybridized to PAT 2 while 4.58% hybridized to CB3 DNA. The results of hybridization with RNA isolated from other 37 C infected cells but labelled between 25 to 30 min of the latent period, and hence called "late" RNA, are also shown in Section B. In that preparation more RNA hybridizes to phage DNA than to host DNA (18.1% vs. 2.41%) when compared to the percentages seen in early RNA preparations (4.58% vs. 4.36%). Also shown in Section

Figure 19. Hybridization saturation curves between PAT 2 DNA - uninfected PAT 2 RNA and CB3 DNA - CB3 infected PAT 2 RNA.


4
3
2
1
cpm x 10^{-2}
PAT 2
DNA
1
2
3
4
ng DNA x 10^{-2}/filter

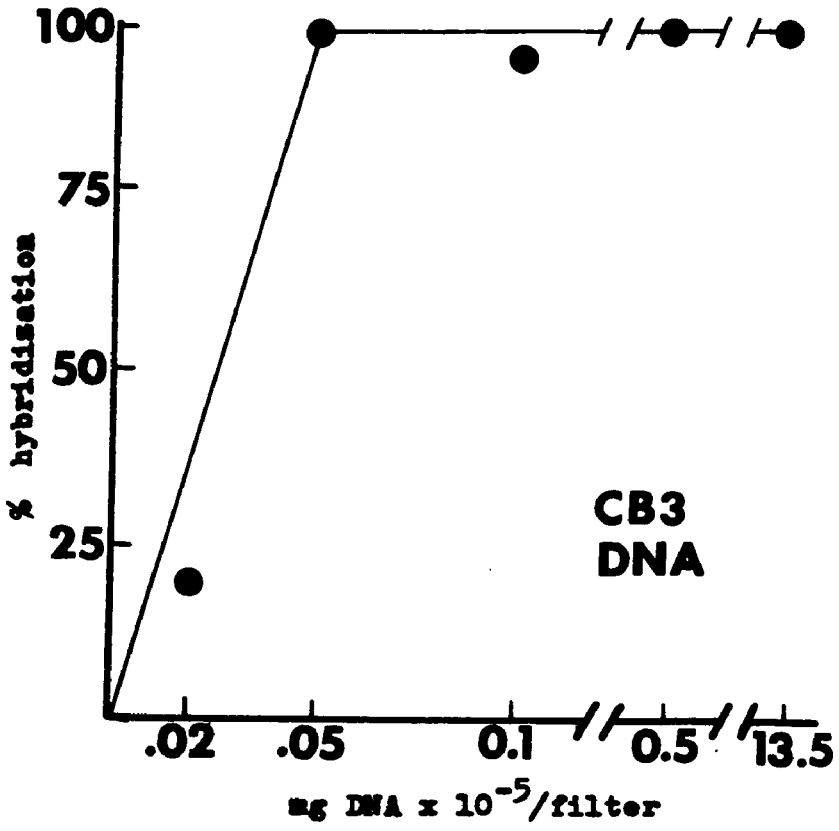

100
75
50
25
% hybridisation
CB3
DNA
.02
.05
0.1
0.5
13.5
ng DNA x 10^{-5}/filter

Figure 19

TABLE 3

RNA-DNA HYBRIDIZATION OF 20 C AND 37 C INFECTED PAT 2

DNA on filter	Temperature/Time of RNA Sample	CPM Added	Percent CPM Hybridized
A. Non-infected			
PAT 2	(Control Cells)	1,635	4.05
CB3	(Control Cells)	1,635	0.0
B. Infected Cells			
PAT 2	37 C, early*	1,089	4.36
CB3	37 C, early	1,089	4.58
PAT 2	37 C, late**	507	2.41
CB3	37 C, late	507	18.10
PAT 2	20 C, early	6,447	2.33
CB3	20 C, early	6,447	0.33

* Early RNA was isolated from cells infected and labelled for zero to 5 min at 37 C or zero to 16 min at 20 C.

** Late RNA was isolated from cells infected and labelled for 25 to 30 min.

B are hybridization results of early RNA isolated from 20 C infected cells labelled for 16 min of the latent period. Sixteen min is the fraction of the 20 C latent period for PAO 1C comparable to 5 min of the 37 C latent period. In this 20 C infected RNA preparation, 2.33% of the radioactive counts hybridize to PAT 2 DNA while only 0.33% hybridize to CB3 DNA. Two other DNA-RNA hybridization experiments using RNA preparations from cells infected with CB3 produced similar hybridization percentages following 37 C infections. Results with the 20 C infected preparations with CB3 DNA yielded percentages of 0.14% and 0.13%. These DNA-RNA hybridization experiments establish that in a non-permissive infection, CB3 infected PAT 2 produces very little phage specific m-RNA while host RNA synthesis does occur. On the other hand, permissively infected cells synthesize both phage and host m-RNA during the same early fraction of the viral latent period. These experiments have also shown that later during the permissive infection (25 to 30 min) more of the isolated RNA hybridizes to phage DNA than to host DNA.

B. Competition Hybridization

Competition hybridization experiments were used to study the nature of the phage specific RNA synthesized in PAT 2 at 20 C which was detected in the previous section as that fraction of 20 C infected cell RNA which does hybridize to phage DNA (0.33%). Since uninfected cells were found to lack any RNA hybridizable to CB3 DNA (see Table 3) all remaining experiments used only filters with viral DNA. A control experiment which used early, unlabelled, 20 C, infected PAO 1C RNA to compete against early, C^{14} labelled, 37 C RNA from PAO 1C and from PAT 2 is shown in Figure 20. PAO 1C RNA (20 C) competes identically against both PAO 1C and PAT 2 37 C infected host preparations. This establishes the similarity of the m-RNA from both infected hosts at 37 C to the RNA synthesized

Figure 20. CB3 DNA-RNA competition hybridization between RNA from 20 C early infected PAO 1C and 37 C early infected PAO 1C or PAT 2.

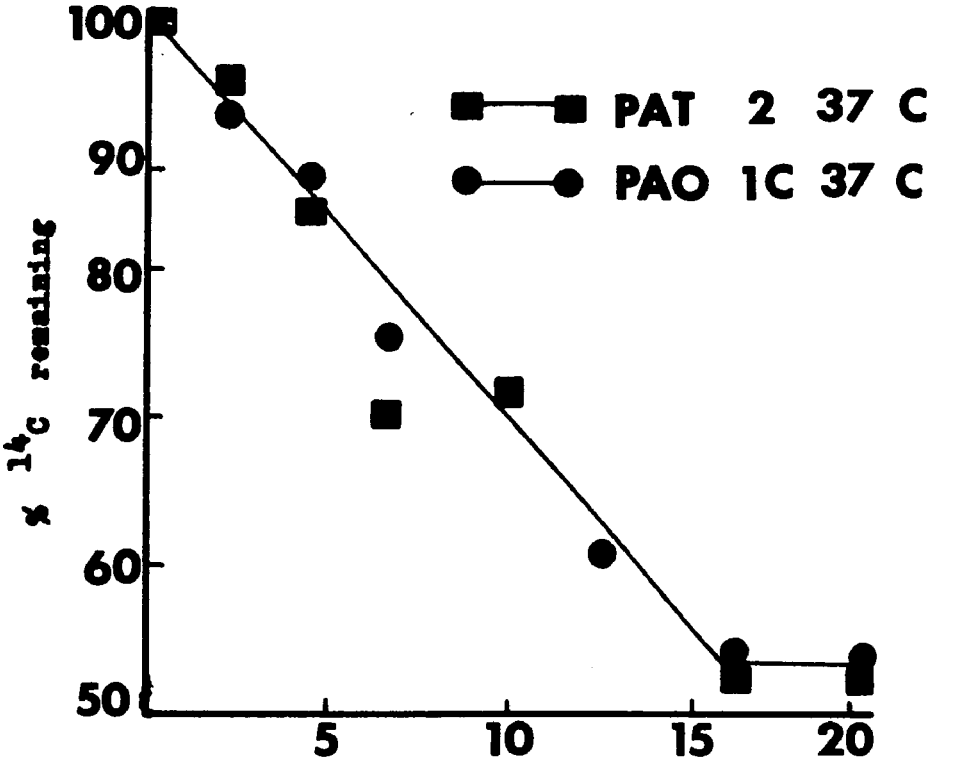
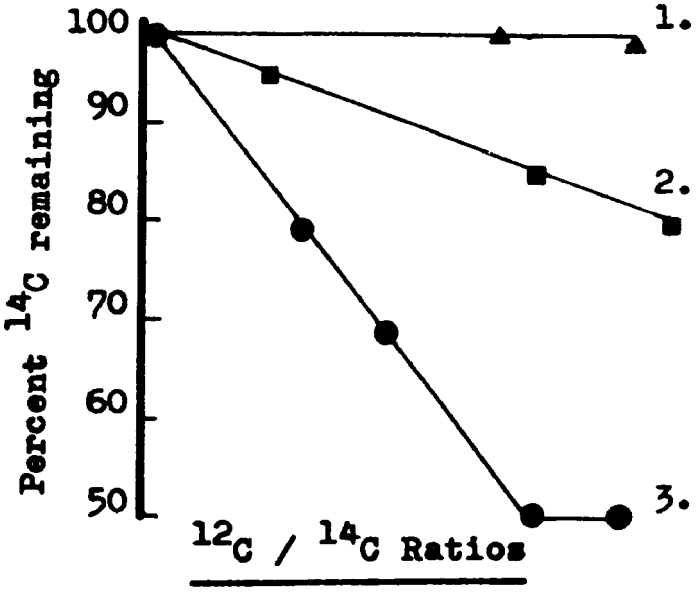

100
90
80
70
60
50
% ^{14}C remaining
5
10
15
20
PAT 2 37 C
PAO 1C 37 C
Ratio ^{12}C, PAO 1C 20C RNA/ ^{14}C, PAO 1C 37 C and
^{14}C, PAT 2 37 C RNA

Figure 20

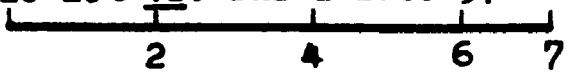
in the fully permissive PAO 1C at 20 C. Figure 21 shows the results of the critical experiments. Curve 1 shows that 37 C late phage RNA is not competed aganst by 20 C early PAO 1C RNA. This establishes that CB3 infections at permissive temperatures produce at least two classes of phage specific RNA, "early" and "late". Curve 2 shows the competition slope of an infected PAO 1C 20 C early RNA preparation competing against infected 37 C PAT 2 early RNA. This curve is similar to the same experimental combination shown in the previous figure and is included here only as a reference. Curve 3 in Figure 21 shows the results of competing infected, PAT 2 20 C early RNA against infected PAT 2 37 C early RNA. The use of three different absiccas in this figure emphasizes the finding that much more infected, PAT 2, 20 C early RNA is needed to produce a similar degree of competition to that seen in permissive phage infected 20 C, PAO 1C early RNA preparations. The amount of 20 C, infected, unlabelled PAT 2 RNA required (line 3) is 6.6 times more than the amount necessary from PAO 1C (line 2) to give a similar percentage of competition. This value of 6.6 is called a competition ratio. A 6.6 fold increase in the amount of PAT 2 RNA over PAO 1C is also equivalent to saying that only 15.1% of the PAT 2 cells are producing phage specific RNA at 20 C. Table 4 lists the results of five different hybridization competition experiments which show that the average competition ratio is 6.82 and results in an average yielder value of 14.5%. This value is very near the figure obtained by single cell burst experiments (106) which found that 13 to 14% of infected PAT 2 cells are phage yielders at 20 C. Therefore, from this data it appears that non-yielders do not synthesize phage m-RNA at 20 C.

20 C, early RNA prepared from PAO 1C (fig 20) or from PAT 2 (fig 21) was able to compete against only 50% of the early phage specific RNA

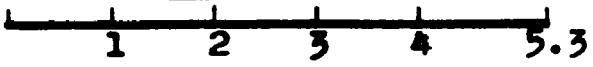
Figure 21. CB3 DNA-RNA competition hybridization between 20 C early RNA from infected PAT 2 or PAO 1C and 37 C early or late RNA from infected PAT 2.



1. PA01C 20 C vs. PAT 2 late 37 C



2. PA01C 20 C vs. PAT 2 early 37 C



3. PAT 2 20 C vs. PAT 2 early 37 C

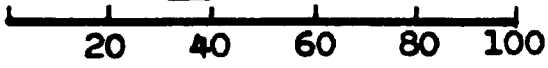


Figure 21

TABLE 4

SUMMARY OF COMPETITION HYBRIDIZATION EXPERIMENTS BETWEEN PHAGE INFECTED PAT 2 AT 37 C AND 20 C

Experiment no.	Competition Ratio	Percentage
1	6.16	16.2
2	6.60	15.1
3	8.96	11.3
4	7.34	13.6
5	5.85	17.1
Average	6.82	14.5

produced at 37 C in either host. This fact will be considered in the light of subsequent data and incorporated into a model presented in the last section of this dissertation.

The drug, chloramphenicol (CM), was used to determine if the phage specific m-RNA synthesized in infected cells at 20 C was dependent on protein synthesis. Figure 22 shows that unlabelled, infected, 20 C, early PAT 2 RNA competes as effectively against 37 C CM treated or untreated cells infected with CB3. This inhibition of protein synthesis indicates that the RNA synthesized under early conditions in PAT 2 yielder cells is probably analogous in nature to the T4 pre-early or immediate early species of m-RNA synthesized under the direction of host RNAP, Therefore, these same phage specific, host enzyme transcribed messages are probably absent in non-yielder cells of PAT 2 at 20 C since these cells had no viral specific m-RNA after infection. From these data the theory was proposed that restriction of phage CB3 was due to some unusual property of host RNAP, thus, experiments were performed to determine if PAT 2 RNAP was involved in cold sensitive restriction of CB3.

III. RNA Polymerase

A. Travers Enzyme Extraction

1. PAT 2

An extract from 40 g of cells of PAT 2 (according to the techniques of Travers) DNA dependent RNA polymerase activity appeared in a fraction precipitating at 40% ammonium sulphate saturation having 2.54 g of protein while the 55% fraction had 3.85 g of protein. In using T4 infected *E. coli*, Travers found that core enzyme was concentrated in the 40% ammonium sulphate fraction while sigma activity occurred in the 55% fraction. Assays for the PAT 2 enzyme activity showed that acid precipitable radio-

Figure 22. DNA-early RNA hybridization competition between 20 C infected PAT 2 and 37 C infected PAT 2 or 37 C infected, chloramphenicol treated PAT 2.

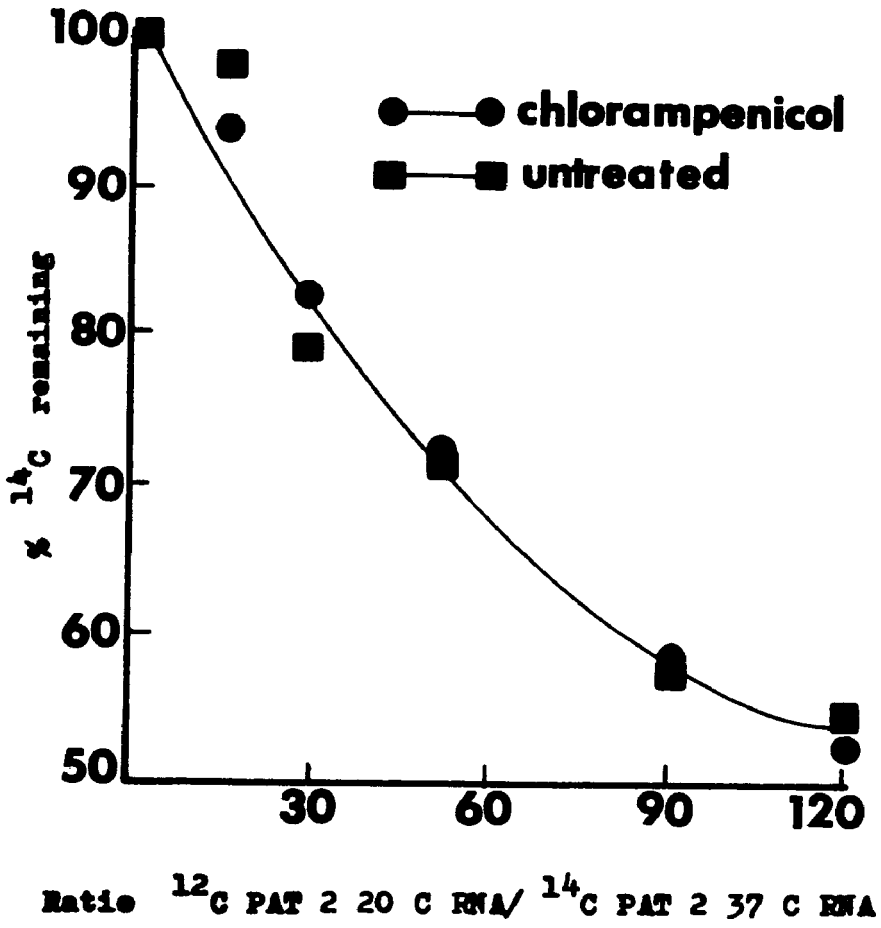

100
90
80
70
60
50
% ^{14}C remaining
chlorampenicol
untreated
30
60
90
120
Ratio ^{12}C PAT 2 20 C RNA/ ^{14}C PAT 2 37 C RNA

Figure 22

TABLE 5

IN VITRO RNA SYNTHESIS WITH TRAVER'S EXTRACTED PAT 2 RNAP

DNA Template	RNAP Components	Relative Incorporation at Each Temperature			
		37 C		20 C	
		Exp. no.		Exp. no.	
		I	IV	II	IV
Calf Thymus	core + sigma	100 (270)*	100 (465)	100 (218)	100(471)
	core	49	49	100	66
	sigma	85	83	91	32
		I	III	III	
PAT 2	core + sigma	100 (154)	100 (207)	100 (214)	
	core	2.4	4.9	0	
	sigma	132	101	82	
		II	III	III	IV
CB 3	core + sigma	100 (123)	100 (280)	100 (275)	100 (213)
	core	8.5	36	0	16
	sigma	134	126	24	79

* indicates cpm incorporated

active counts were sensitive to alkali hydrolysis or RNAase. These PAT 2 enzyme fractions assayed in the absence of DNA had incorporation levels approaching 15% of the DNA containing assays and thus were equivalent to those levels observed by Travers. Table 5 compares the levels of activity for core, sigma and the combination of core and sigma preparations for three different DNA templates at both 37 and 20 C. Incorporation of 100% was designated for the combination of core and sigma at 37 C since with other systems (E. coli) this combination produces maximum incorporation. With respect to RNA synthesis using calf DNA in Table 5, no significant temperature effect is seen in the enzyme fraction or combinations tested.

The fraction called sigma which should not possess enzymatic activity according to Travers' work with E. coli must be contaminated by core since it incorporates a significant number of acid precipitable counts. This observation of core contamination in sigma applies to PAT 2 and CB3 when they are the templates. A hint of cold temperature sensitivity is evident when PAT 2 "sigma" which has RNAP activity is assayed with CB3 DNA. Enzyme activity in "sigma" at 20 C is lower than it is at 37 C - experiment III performed at both 37 and 20 C with CB3 DNA showed that at low temperatures the enzyme had only 19% of its 37 C activity (126 c.p.m. vs. 24 c.p.m.). Although the purification of RNAP according to Travers produced a contaminated "sigma" fraction, these pilot studies did furnish the impetus to undertake a more refined isolation of the enzyme. Experiments were also performed on these Travers preparations to determine the influence, if any, of fractions which are normally discarded during the purification (see flow diagram, Materials and Methods). These various discards, including fresh sonicates of PAT 2, neither inhibited nor stimulated the activity of "core", "sigma" or "core and "sigma" in

TABLE 6

IN VITRO RNA SYNTHESIS WITH RNAP FROM INFECTED AND NON-INFECTED PAT 2

DNA Template	RNAP Components	Enzyme Source: Non-infected PAT 2	Enzyme Source: Infected PAT 2	Enzyme Source: Non-infected PAT 2	Enzyme Source: Infected PAT 2
		cpm at 37C		cpm at 20 C	
Calf Thymus	core + sigma	379	490	361	1546
	core	231	430	204	1157
	sigma	320	770	343	1892
PAT 2	core + sigma	179	238	181	345
	core	119	207	89	281
	sigma	184	270	193	388
CB3	core + sigma	167	191	194	286
	core	161	163	145	198
	sigma	225	215	96	310

combination with any of the three templates DNA's.

2. CB3 Infected PAT 2

RNAP was also isolated by the Travers technique from PAT 2 infected for 15 min at 37 C. These results (Table 6) also show the "core" contamination of "sigma" and the observation that "core-sigma" combinations were less efficient than "sigma fraction" by itself. The cold sensitivity of PAT 2 enzyme (sigma) isolated for this experiment is not as dramatic with CB3 DNA seen in the previous table since the enzyme was only 42% as active at 20 C as it was at 37 C (225 c.p.m. vs. 96 c.p.m.). However, there is a significant change in enzyme activity (sigma) with the phage infected cell preparations when compared to uninfected host enzyme. With calf DNA at 20 C, CB3 infected cell enzyme (sigma) is four times more efficient than host enzyme (sigma) at this temperature (343 c.p.m. vs. 1892 c.p.m.). Also at 37 C, phage infected cell enzyme (sigma) is more active than host enzyme (sigma) toward calf DNA. No other significant changes are seen between these enzymes on CB3 or PAT 2 DNA's. Thus, CB3 infection does change the apparent activity of the RNAP compared to the uninfected host enzyme when both "sigma fractions" are assayed on calf DNA. This may suggest that permissive CB3 infections either modify the existing host RNAP or synthesize a new enzyme molecule.

B. Burgess Extracted RNAP

The isolation of RNAP from cells by the Burgess procedure was performed a total of ten times. Three purifications were done using a modification of the Burgess technique. Of the remaining extractions four were carried out with PAT 2, and one each on PAO 1C, E. coli D10 and a rifampicin resistant mutant of PAT 2. The summary of the Burgess extraction of RNAP from E. coli is given in Table 7 while the results of a typical Pseudomonas purification appears in Table 8. Activity elution

TABLE 7

SUMMARY OF BURGESS RNAP PURIFICATION FROM *E. COLI*

Fraction	Total Protein mg	Total Activity units*	Activity units/mg	Yield %
1-Lysate	9,560	23,600	2.4	100
2-Supernatant	4,950	22,000	4.4	93
3-$(NH_4)_2SO_4$ pool	340	19,120	56.0	81
4-DEAE pool	59	17,200	293.0	73
5-PC pool	7	11,560	1,850.0	49

*one activity unit of enzyme incorporates 1 mμ mole of AMP in 10 min at 37 C.

TABLE 8

SUMMARY OF BURGESS RNAP PURIFICATION FROM PAT 2

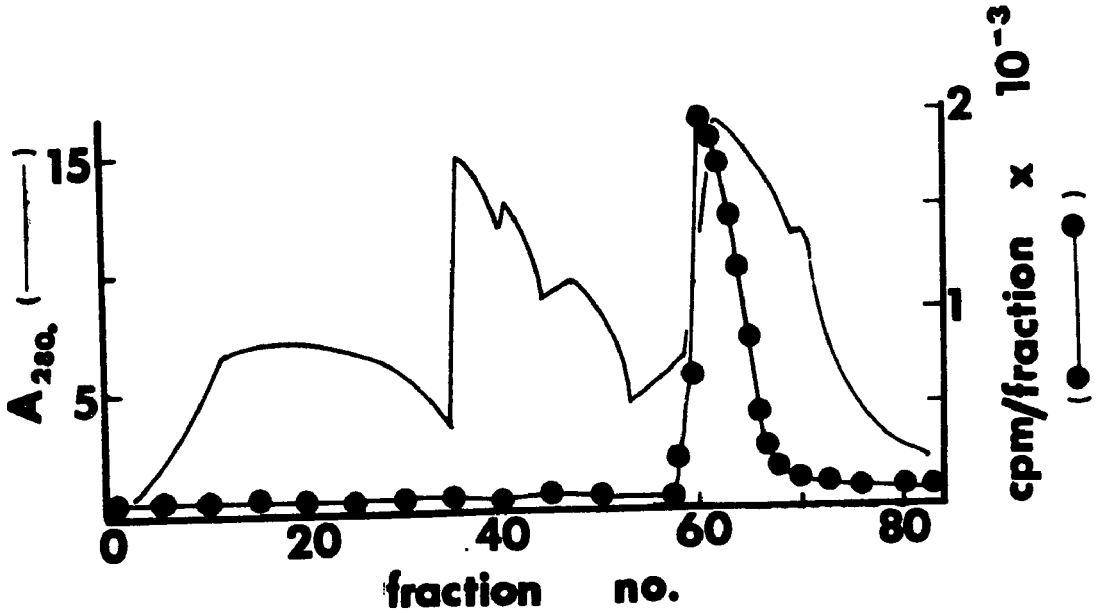
Fraction	Total Protein mg	Total Activity units*	Activity units/mg	Yield %
1-Lysate	22,960	40,800	1.7	100
2-Supernatant	12,720	39,600	3.2	97
3-$(NH_4)_2SO_4$	800	36,300	4.5	89
4-DEAE pool	122	37,600	319.0	92
5-PC pool	15	39,600	2570.0	97

*one activity unit of enzyme incorporates 1mμ mole of AMP in 10 min at 37 C.

profiles from chromatography columns were essentially similar for all extractions including the modified Burgess procedure. Figure 23a shows the protein elution profile of the third PAT 2 Burgess preparation from DEAE along with the enzyme activity profiles. Enzymatic activity always appeared in fraction 59 or 60 and included up to nine fractions which were pooled to produce fraction 4a of the enzyme extraction (see flow diagram, Materials and Methods). Protein elution profiles and RNAP assays for a typical phosphocellulose column (PC) are shown in Fig. 23b. In two of the PAT 2 Burgess extractions a protein was eluted between the column flow through material and core enzyme; however, in both cases this protein showed no RNAP activity either by itself or in combination with other protein fractions from PC. In the stepwise elution from PC, as described by Whitely and Hemphill (164), a small protein peak was eluted immediately after the flow through material. This was also inactive when assayed. These DEAE and PC elution and assay profiles are similar to those reported by Burgess (18). Figs. 24a and 24b show the profiles of Burgess PAT 2 enzyme centrifuged first in low salt-Buffer A, 10 to 30% glycerol (ionic strength $\Gamma/2 = 0.04$) then in high salt-Buffer A, 10 to 30% glycerol ($\Gamma/2 = 1.0$). In low salt gradients, PAT 2 RNAP sedimented to the lower 1/2 of the gradient while the pooled enzyme from these gradients, when centrifuged in high salt gradients, remained in the upper portion of the tube. These were the expected results since it is known that RNAP from both *E. coli* (18) and *P. putida* (71) respond in this manner to changes in the ionic strength of glycerol gradients. In an ionic environment of less than 0.12, RNAP holoenzyme undergoes aggregation to form a hexamer (8) that has an S value of 21. This allows the enzyme to migrate in a low salt glycerol

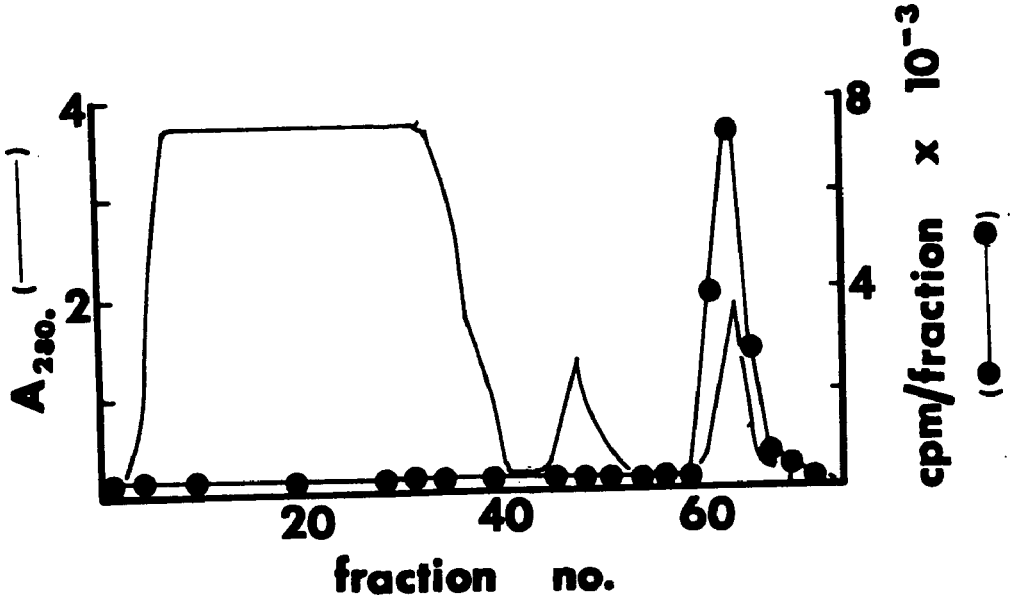
Figure 23a. DEAE cellulose chromatography profile of PAT 2 RNA polymerase.

Figure 23b. Phosphocellulose chromatography profile of PAT 2 RNA polymerase.



Tubes 1 to 30 contain the flow through material, tubes 32 to 50 contain the buffer A-0.13M KCl eluate, tubes 54 to 70 contain the buffer C-0.23M KCl eluate.

Figure 23a



Tubes 1 to 21 contain the flow through material, tubes 22 to 28 contain the buffer C-0.1M KCl eluate, tubes 29 to 58 contain the buffer C-0.23M KCl eluate, tubes 59 to 70 contain the buffer C-0.35M KCl eluate.

Figure 23b

Figure 24a. Low salt glycerol gradient profile of PAT 2 RNA polymerase.

Figure 24b. High salt glycerol gradient profile of PAT 2 RNA polymerase.

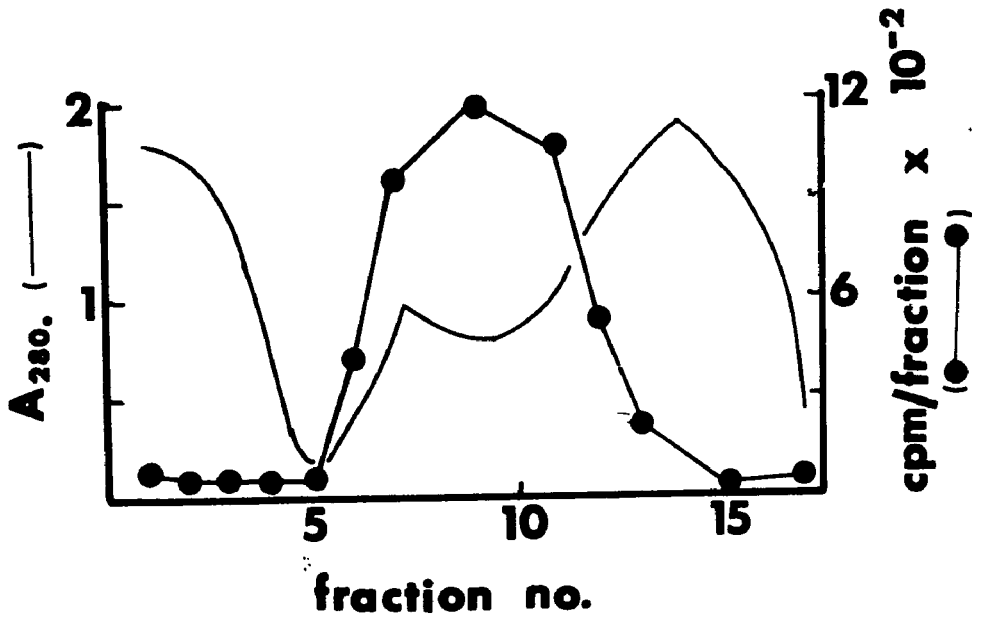


Figure 24a

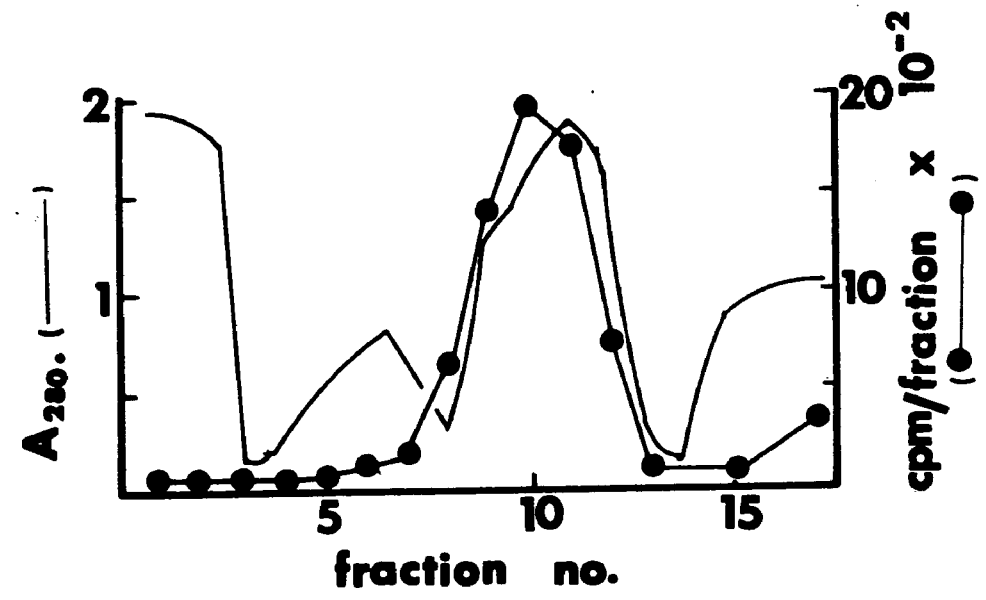


Figure 24b

gradient well ahead of the bulk of the co-purifying protein which sediments at 6 to 14S (71). At ionic strengths greater than 0.26, RNAP sediments as a monomer of 13S (9) thus allowing it to be separated from the protein which still sediments at 22S. The PAT 2 enzyme centrifuged through these two ionic gradients was then processed on PC as usual according to the Burgess procedure. The Burgess protocol for the isolation of DNA dependent RNAP produced the following results:

1. E. Coli D10

The purification of RNAP from E. coli produced the PC fraction which had the enzymatic activity at 37 C shown in Table 9. This table shows that core and sigma (PC flow through material) by themselves are not very active on these templates but when they are used in combination on either PAT 2 or CB3, there is an expected synergistic stimulation of enzyme activity. Stimulation equals the summation of counts of core and counts of sigma divided into the incorporation of the core-sigma combination. This stimulation by sigma or core amounts to 2.4 for PAT 2 DNA and 3.7 for CB3. Thus, E. coli purifies as reported by others using the Burgess technique in that RNAP holoenzyme upon being chromatographed on PC yields a core enzyme fraction and a fraction called sigma which stimulates core activity. The stimulation seen in my preparations is comparable to the levels reported in the literature - for example, Whitely and Hemphill (164) had stimulation ratios of as low as 1.5 and as high as 7.1 for their enzyme preparations from E. coli, B. subtilis and P. aeruginosa.

2. PAT 2

Surprisingly, the purification of RNAP from PAT 2 did not produce PC fractions behaving as those from E. coli D10. Table 10 lists the

TABLE 9

ASSAY OF *E. COLI* D10 RNAP AT 37 C

PC Fraction	cpm/DNA	
	PAT 2	CB3
core	390	260
sigma	124	86
core + sigma	1,092	1,061
Stimulation	2.4	3.7

TABLE 10

ASSAY OF PAT 2 RNAP AT 37 C AND 20 C

Temperature	PC Fraction	cpm/DNA		
		Calf	PAT 2	CB3
37 C	core	6,560	706	4,830
	sigma	141	122	128
	core + sigma	191	104	299
20 C	core	6,979	681	387
	sigma	111	97	121
	core + sigma	572	174	167

activity of the PAT 2 PC fractions on different DNA templates at both 37 and 20 C. First of all, core enzyme possesses much more specific activity than E. coli core, while on the other hand sigma had a level of incorporation similar to E. coli sigma. Further, when sigma is combined with PAT 2 active core, there is no synergistic stimulation but an inhibition of activity. In fact, the pronounced inhibition of core by sigma was as high as 97% (Table 11). With all three templates the greatest inhibition is seen at 37 C while the least amount of "inactivation" occurs at 20 C. However, if for the moment one disregards this unusual outcome of the assay using core and sigma in combination and only compares core activity at 37 C and 20 C some interesting observations come to light. With calf thymus and PAT 2 DNA approximately equal numbers of acid precipitable counts are synthesized after 15 min at 37 C or after 52 min at 20 C. With CB3 DNA however, the enzyme is only 8% as efficient at 20 C as it is at 37 C (387 c.p.m. vs. 4830 c.p.m.). So, the unusual behaviour of the reconstituted holoenzyme (core and sigma) when compared to E. coli not withstanding, the PC core from PAT 2 does show an inability to use CB3 as a template at 20 C. In view of the fact that 14.5% of infected cells produce phage RNA (Table 4) and synthesize viral progeny in vivo (106), this residual 8% activity implies a similar degree of in vitro permissiveness with the isolated host enzyme.

Many experiments were designed to study sigma's ability to inactivate or "kill" core enzyme activity. Some studies tested the possibility that some manipulation of the extraction procedure affected sigma. These studies included testing various extract discards with purified PAT 2 core, sigma and core-sigma combinations in an in vitro RNAP assay. Along with using freshly lysed cells, aliquots of the following were also tested: 33% ammonium sulphate pellet, 42% ammonium

TABLE 11

SIGMA INHIBITION OF CORE ACTIVITY AT 37 C AND 20 C

Temperature	Percent Inhibition/DNA		
	Calf	PAT 2	CB3
37 C	97.1*	85.4	92.8
20 C	91.8	74.5	57.0

* Percentages were calculated from data in Table 10.

sulphate supernatant, 50% ammonium sulphate supernatant and alternating tubes from DEAE flow through and elution fractions. Also tested was the ability of Travers' core contaminated sigma to influence PC PAT 2 core. In addition, a fresh cell lysate was tested which was incubated at 37 C for 7 min prior to adding it to the various PC protein fractions. These experiments were all unsuccessful in detecting any change in the sigma inactivation of core enzyme synthesis.

Studies were carried out to determine if the sigma "kill" of core was related somehow to the enzyme assay. These experiments were designed to check the influence of the order of addition of the assay ingredients, the effect of the prior pre-incubation of core and sigma at 37 C, and the influence of the nature of the DNA template. None of these experiments showed any change in the sigma "kill" phenomenon. The studies with DNA templates, however, found that freshly extracted or non-ethanol precipitated DNA produced the same results as the DNA stocks which were stored for these assays. Heat denatured DNA produced the results tabulated in Table 12 with PAT 2 core enzyme at 37 C. This data with PAT 2 core is similar to that reported by Johnson et. al (164) for P. putida RNAP holoenzyme. They found a similar percentage of decrease in activity on calf DNA and on the DNA of a Pseudomonas putida phage. However, their core preparation from P. putida showed no change in synthetic ability with their viral DNA. Thus, PAT 2 "core" enzyme appears to respond to native and denatured DNA unlike the core enzyme from P. putida but rather, like RNAP holoenzyme of P. putida.

The possibility that sigma "kill" was related to other factors was also checked. Martello and co-workers (95) showed that protein kinase

TABLE 12

INFLUENCE OF THE ASSAY TEMPLATE ON
PAT 2 CORE RNAP ACTIVITY

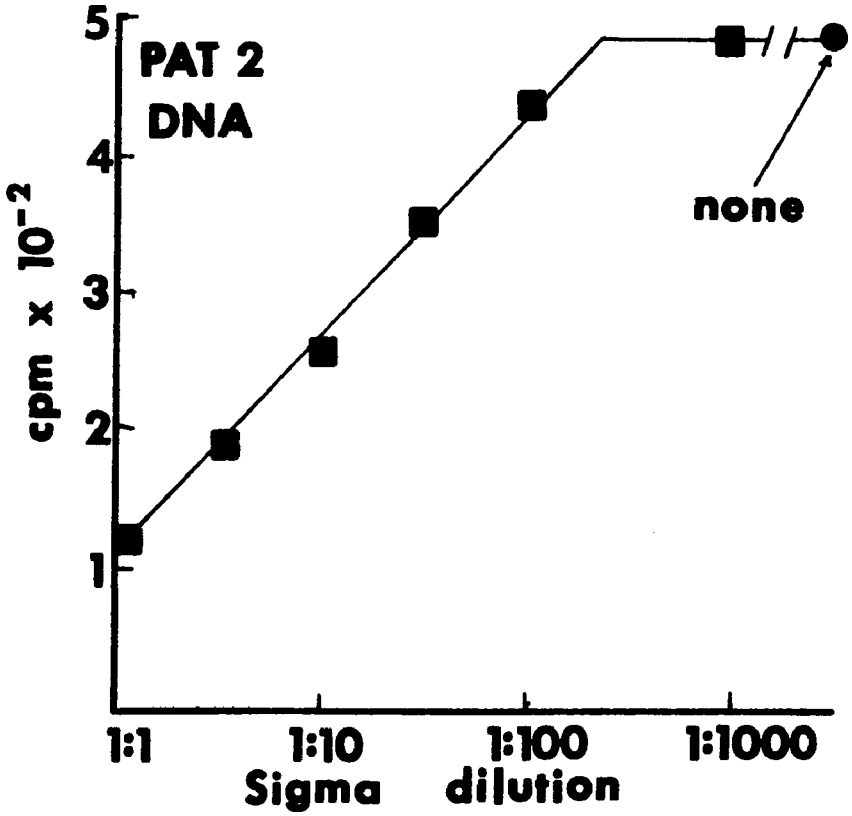
DNA	Native	Denatured
Calf	7,140	810
PAT 2	469	341
CB3	3,993	410

from rabbit skeletal muscle could in the presence of 3'-5' cyclic AMP and E. coli sigma cause sigma to be phosphorylated. The stimulation as a result of using phosphorylated sigma was as high as 30 fold over that stimulation seen with unphosphorylated sigma. They also reported that even pre-incubating sigma with 10^{-4} M 3'-5' AMP at 37 C for 5 min also was able to stimulate core over and above that seen with unincubated sigma. This preincubation procedure with PAT 2 sigma did not increase, eliminate or alter in any way the synthesis seen with core-sigma combinations or with core and sigma by themselves.

Chellals and co-workers (26) found that E. coli RNAP exists in two forms which are interconvertible. One form is less active than the other. They further discovered that the active enzyme can be formed from the inactive one simply by the presence of 10 mM Mg++ in the assay. Accordingly, the effect of added Mg++ to the assays for PAT 2 RNAP was evaluated. No effect was seen with PAT 2 PC fractions or combinations of them when either 10 or 50 mM Mg++ was used with calf thymus or CB3 DNA at 37 C. Other experiments showed that the sigma "kill" of core can be diluted out (Fig 25) of the PC flow through material.

Studies to detect DNAase and RNAase in PC core and PC sigma showed that these fractions were free of these nucleases. In addition, RNAP purified on glycerol gradients prior to PC chromatography still produced a sigma fraction having the ability to markedly depress core activity with all templates at both temperatures. Thus, if an inhibitor was copurifying with RNAP, it would have to undergo changes in sedimentation velocity as does RNAP in low and high salt glycerol gradients. Of all the various parameters examined none of them resolves the sigma "killing" phenomenon. Several things are evident however: first, PAT 2 PC core has very high specific activity when compared to

Figure 25. Dilution effects of sigma on the activity of PAT 2 RNA polymerase.


PAT 2
DNA
cpm x 10^{-2}
5
4
3
2
1
none
1:1
1:10
1:100
1:1000
Sigma dilution

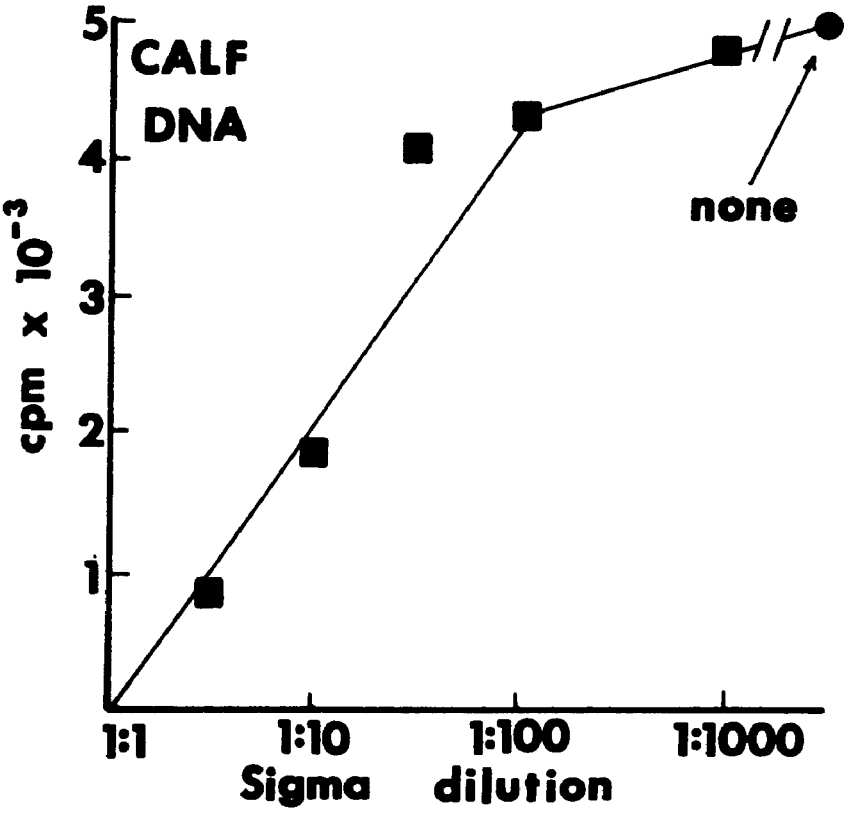

CALF
DNA
cpm x 10^{-3}
5
4
3
2
1
none
1:1
1:10
1:100
1:1000
Sigma dilution

Figure 25

E. coli; secondly, PAT 2 core behaves with denatured DNA's as does P. putida holoenzyme. This suggests that PAT 2 core may be contaminated with sigma, thus, explaining both of the above observations. However, the factor in PCFM responsible for inactivation is unknown, and further, PC chromatograpy itself seems responsible for this phenomenon since the pooled enzyme fractions from DEAE possess as much total activity as do the PC core fractions. In this same context is the observation that the PAT 2 Travers' enzyme fractions (core and sigma) were also not synergistic when combined. Travers' PAT 2 "core" and Travers' PAT 2 "sigma" always incorporated fewer counts than the summation of either fractions independently (see Tables 5 and 6). This suggests that the sigma inactivation of core may be bacterial strain related phenomenon since E. coli RNAP purified and behaved as expected with respect to the sigma stimulation of its core activity.

3. PAO 1C

Isolation of the enzyme from PAO 1C resulted in the PC fractions having the properties tabulated in Table 13. Again, the sigma inhibition of core is seen with PAO 1C RNAP, and as with PAT 2, the greatest inhibition occurs in assays incubated at 37 C. The ability of the fully permissive host's enzyme to synthesize RNA with calf or PAT 2 DNA is not impaired at a phage non-permissive temperature. However, with CB3 DNA the enzyme appears to be slightly cold sensitive. At 20 C, core has only 49.6% of its 37 C activity. PAO 1C core at 20 C is more active than PAT 2 core at 20 C by a factor of 6.2. Interestingly, this factor of 6.2 is similar to the ratio of biological phage yielders of PAT 2 vs. PAO 1C at 20 C. Recall that 13 to 14% of PAT 2 cells are yielders which means that PAO 1C is more efficient at 20 C in phage

TABLE 13

ASSAY OF PAO 1C RNAP SHOWING THE SIGMA INHIBITION OF CORE AT 37 C AND 20 C

Temperature	PC Fraction	cpm/DNA		
		Calf	PAT 2	CB3
37 C	core	3,567	402	1,860
	sigma	328	341	227
	core + sigma	403	354	315
	Percent Inhibition	90.3	52.5	85
20 C	core	3,581	435	921
	sigma	401	457	336
	core + sigma	587	448	381
	Percent Inhibition	85.2	49.5	67

production by a factor of 7.1. Further, RNA-DNA competition hybridization experiments produced a competition ratio of 6.82 which is another way of stating that PAO 1C is more efficient in CB3 RNA synthesis *in vivo* by a factor of 6.82. Thus entirely different experiments, both *in vivo* (single cell burst experiments and RNA-DNA hybridization competition) and *in vitro* (RNAP assays) establish a similar value for the ratio of restrictive host permissiveness to permissive host synthesis of phage or its gene products.

4. SDS Polyacrylamide Gels

In view of the unusual behavior of core enzyme from both PAO 1C and PAT 2, analysis of their subunit composition was done with the aid of SDS polyacrylamide gels. Figure 26 shows the subunits of *E. coli* D10 PC core. Four bands are visible which are labelled beta-beta', tau , alpha and omega. The subunits always associated with *E. coli* core are beta-beta' and alpha. In Burgess' characterization of the enzyme (19) on SDS, he states that a subunit called omega was often found in preparations of core. In addition, he mentions the occurrence of tau in some preparations of the enzyme. Since his characterization, other workers have reported finding omega in core. Recently, many authors report finding tau in both *E. coli* (35, 93, 53, 158) and *B. subtilis* (84) enzymes. Thus my SDS gel analysis of *E. coli* core corresponds to preparations by other workers in its subunit composition. SDS analysis of Burgess purified PAT 2 and PAO 1C core shows, in addition to the bands found in *E. coli*, the occurrence of a band migrating between tau and alpha (Fig 26). This band migrates in the same position as sigma (155). Thus, PAT 2 and PAO 1C RNAP on chromatography on PC yield a "core" fraction composed of both core enzyme and sigma protein. The

Figure 26. SDS polyacrylamide gel electrophoresis of E. coli D10, PAO 1C and PAT 2 Burgess phosphocellulose enzymes.

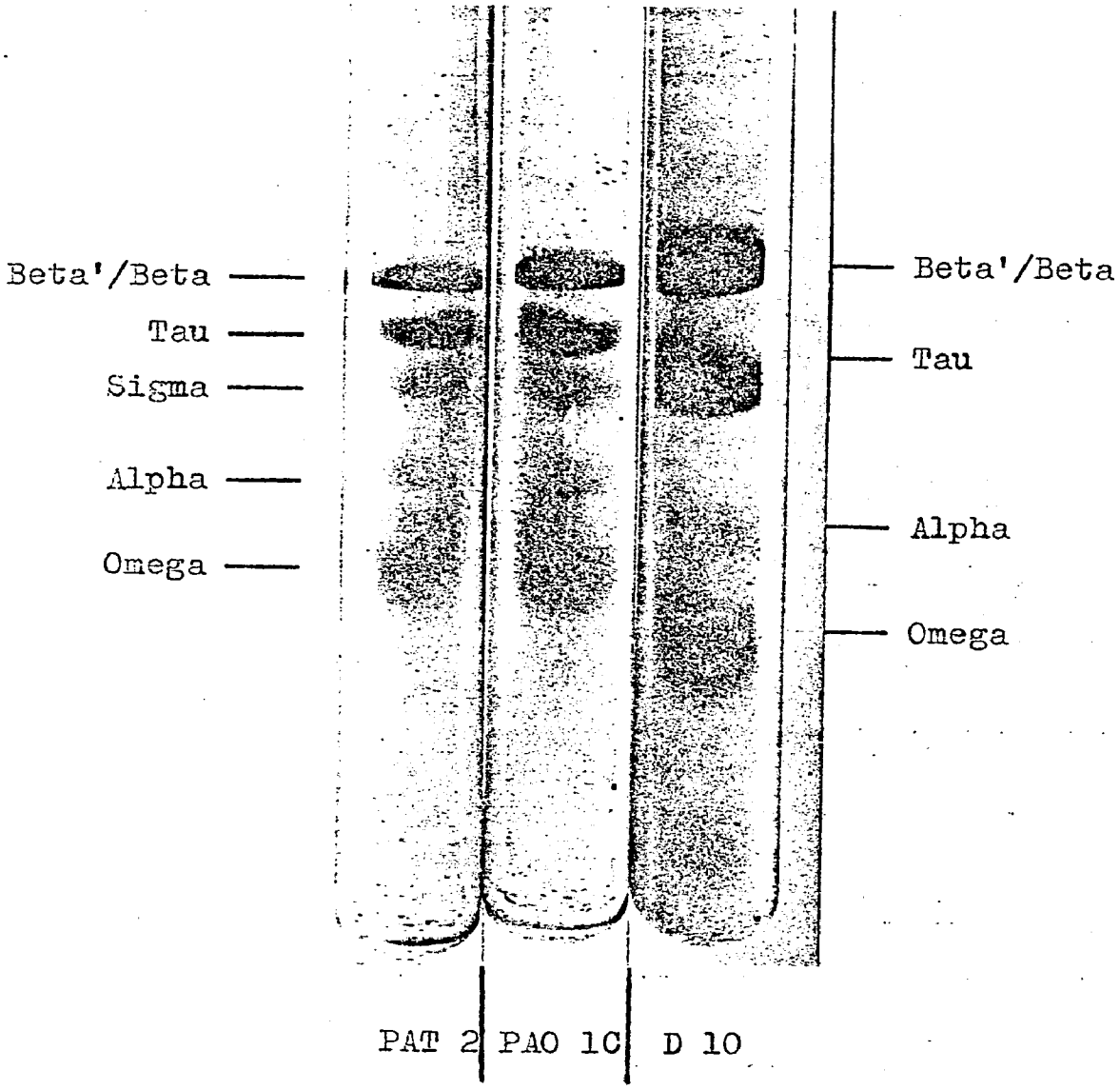

Beta'/Beta
Tau
Sigma
Alpha
Omega
Beta'/Beta
Tau
Alpha
Omega
PAT 2
PAO 1C
D 10

Figure 26

finding of sigma in Pseudomonas core explains the behavior of PC "core" in respect to its high activity in relation to E. coli core (Tables 9 and 10) plus its reaction with denatured templates (Table 12). PAT 2 "core" acted like P. putida holoenzyme in respect to its activity with natured and denatured phage DNA.

C. Modified Burgess Enzyme Extraction

During still other studies on the nature of the loss of PC "core" activity in the presence of PCFM (see Table 11), an enzyme assay was used which is similar to the assay conditions used in studies of eukaryotic RNAP. This mammalian RNAP assay is also similar to the assay used by Johnson et al. (164) with P. putida. This assay uses the same concentrations of DNA, enzyme and triphosphates as the Burgess assay but is 20 mM tris acetate (pH 8.0), 4 mM Mg-acetate, 1mM $MnCl_2$ and 60 mM ammonium acetate. When PAT 2 PC purified RNAP was assayed in the above mixture, the specific activity of the enzyme increased 3 to 125 fold (Table 14) over the activity of this same enzyme in Burgess buffer. In addition, the cold sensitivity of the enzyme at 20 C disappeared with CB3 DNA. In fact, at 20 C, the enzyme was 60% more active with Calf DNA and 78% more active with phage DNA than it was at 37 C. Assays of PAO 1C PC purified RNAP produced results similar to the PAT 2 enzyme (Table 15), but the stimulation of activity was not as high.

One difference between the two assay buffers is the ionic strength. The Burgess assay $\Gamma/2$ is 0.235 while the mammalian assay $\Gamma/2$ is 0.035. In their work with P. putida, Johnson et al. used a Burgess protocol but extracted their enzyme under conditions of very low ionic strength by eliminating KCl from the various buffers. In view of the dramatic alteration in enzyme activity using this mammalian assay which is similar to that used by Johnson et al. a modified Burgess extraction tailored

TABLE 14

PAT 2 HOLOENZYME ASSAYED IN MAMMALIAN BUFFER AT 37 C AND 20 C

Temperature	cpm/DNA		
	Calf	PAT 2	CB3
37 C	20,700 (6,560)*	5,500 (706)	38,000 (4,830)
20 C	33,600 (6,979)	4,630 (611)	48,500 (387)

* PAT 2 "core" cpm in Burgess buffer from table 10. The data for Table 10 and this Table were obtained by assaying the PAT 2 enzyme simultaneously in each assay buffer.

TABLE 15

PAO 1C HOLOENZYME ASSAYED IN MAMMALIAN BUFFER AT 37 C AND 20 C

Temperature	cpm/DNA		
	Calf	PAT 2	CB3
37 C	3,024 (3,567)*	4,573 (402)	4,390 (1,860)
20 C	10,132 (3,581)	6,433 (435)	5,852 (921)

* PAO 1C "core" cpm in Burgess buffer from table 13. The data for Table 13 and this Table were obtained by assaying the PAO 1C enzyme simultaneously in each assay buffer.

to my system was attempted on PAO 1C and PAT 2 (see Materials and Methods). When PAO 1C RNAP was extracted in KCl free buffers and eluted from DEAE and PC with NaCl, the procedure resulted in PC fractions with the activities listed in Table 16. These assays were carried out in mammalian buffer. The enzyme from PAO 1C behaves as did Burgess extracted enzyme assayed in a mammalian buffering system. This comparison included the severe depression of enzyme activity by PCFM. Figure 27 shows that the subunit composition of this core enzyme is identical to Burgess extracted RNAP in that it also has sigma protein.

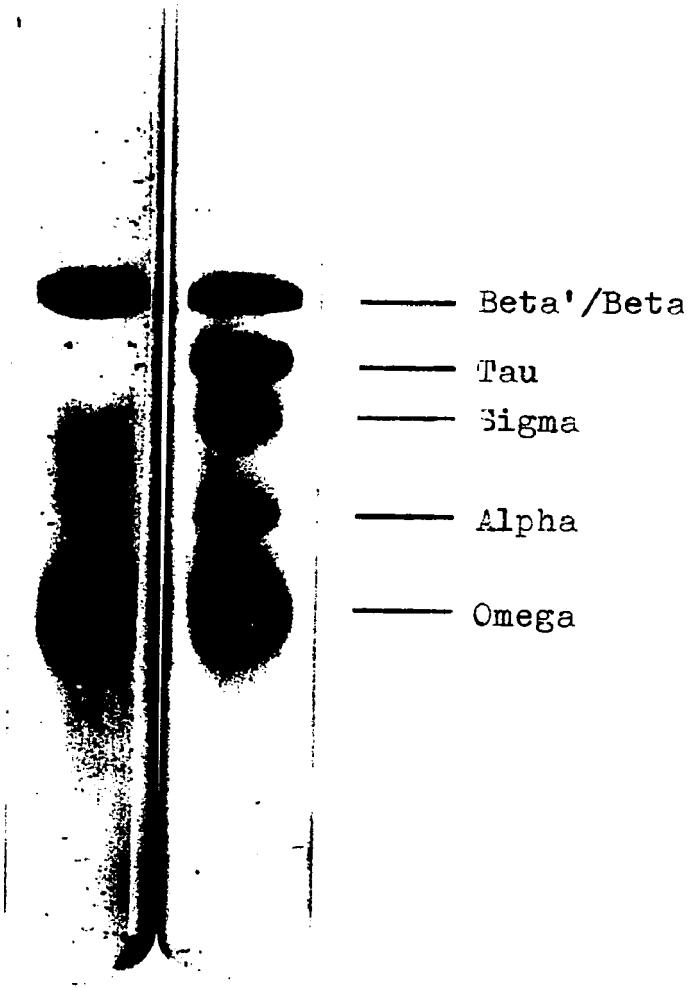
Isolation of PAT 2 RNAP by the modified Burgess technique and assay in the mammalian buffer system produced enzyme fractions from PC that behaved like those of E. coli D 10. Table 17 shows core enzyme has little activity by itself as did E. coli core. Sigma (PCFM) had higher activity than did E. coli even after having been chromatographed on PC three times in the hope of eliminating this high background. However, when core and sigma are both included in an assay, there is a marked synergism occurring with all three templates. The magnitude of the stimulation at 37 C is similar to that seen in the E. coli PC fractions. Moreover, this synergism does manifest a cold sensitivity with CB3 DNA at 20 C. Although the reconstituted holoenzyme (core plus sigma) is not as efficient at 20 C as at 37 C with either Calf or PAT 2, the greatest loss of synergism occurs with CB3 DNA. The SDS subunit analysis of PAT 2 core using my modification of the Burgess purification is surprising. Figure 27 shows this core does lack the sigma subunit which is present in the modified Burgess PAO 1C core preparation. Since the modified Burgess extraction produced with PAO 1C an enzyme which differed from PAT 2 in its synthetic ability and subunit composition, it implies a basic difference in the enzymes from these two hosts.

TABLE 16

MODIFIED BURGESS EXTRACTED PAO 1C RNAP ASSAYED IN MAMMALIAN BUFFER AT 37 C AND 20 C

Temperature	PC Fraction	cpm/DNA		
		Calf	PAT 2	CB3
37 C	core	5,080	7,023	15,810
	core + sigma	104	90	76
20 C	core	7,500	6,740	21,420
	core + sigma	121	1,111	83

Figure 27. SDS polyacrylamide gel electrophoresis of PAO 1C and PAT 2 modified Burgess phosphocellulose enzymes.



PAT 2 PAO 1C

Figure 27

TABLE 17

SIGMA STIMULATION OF MODIFIED BURGESS PAT 2 RNAP AT 37 C AND 20 C

Temperature	PC Fraction	cpm/DNA		
		Calf	PAT 2	CB3
37 C	core	144	109	124
	sigma	937	670	1,077
	core + sigma	2,857	3,014	3,948
	sigma stimulation	2.64	3.75	3.28
20 C	core	337	227	411
	sigma	1,025	1,652	1,328
	core + sigma	1,700	2,620	1,745
	sigma stimulation	1.25	1.40	0.97

Combination Studies with Heterologous PC Fractions

The inhibition or "killing" of core by PCFM was eliminated in the case of PAT 2, by using a modification of the Burgess isolation procedure. However, the nature of this inhibition with the PC holoenzymes from PAT 2 and PAO 1C extracted by the unmodified Burgess procedure was not understood. For this reason studies were initiated combining heterologous PC fractions in order to gain insight into this behavior of Burgess extracted RNAP. For example, using Burgess PC PAT 2 holoenzyme with modified Burgess PAT 2 sigma, it was found that even this sigma preparation known to work with homologously extracted PAT 2 core still caused an inhibition of Burgess PC holoenzyme (Table 18) in a mammalian assay. Table 19 shows the other combinations of PC fractions tested and in all of those experiments PCFM inhibited Pseudomonas RNAP holoenzyme from PC.

E. coli core when tested in a Burgess assay with Burgess PCFM from PAT 2 or PAO 1C showed that it could not be stimulated by these heterologous "sigma" preparations (Table 20). Even in a mammalian assay, modified Burgess PAT 2 sigma is not synergistic with E. coli core (Table 21) in spite of the fact that this sigma is stimulatory with its homologous core. This data is in direct contradiction to the work of Whitely and Hemphill (164) who found that their Pseudomonas sigma was able to function with E. coli core.

When modified Burgess PAT 2 core and E. coli sigma are assayed under conditions in which Pseudomonas core responds to its homologous sigma, there is a synergistic effect (Table 22). This result agrees with the work of Whitely and Hemphill (164). They found that Pseudomonas core enzyme can be stimulated by E. coli sigma.

TABLE 18

INFLUENCE OF MODIFIED BURGESS PAT 2 SIGMA
ON BURGESS PAT 2 HOLOENZYME

Enzyme Source	cpm/DNA/37 C	
	PAT 2	CB3
Burgess PAT 2 Holoenzyme	1,969	12,768
Modified Burgess PAT 2 Sigma	670	1,077
Holoenzyme + Sigma	2,306	2,241
Percent Inhibition	12	85

TABLE 19

SUMMATION OF STUDIES USING HETEROLOGOUS PC FRACTIONS IN BURGESS AND MAMMALIAN ASSAY BUFFERS

Assay Buffer	Fraction Tested	
	Core or Holoenzyme	PCFM
Burgess	Burgess PAT 2	Modified Burgess PAT 2
	Burgess PAO 1C	Burgess PAT 2
	Burgess PAO 1C	Modified Burgess PAT 2
	Modified Burgess PAO 1C	Burgess PAT 2
	Modified Burgess PAO 1C	Modified Burgess PAT 2
Mammalian	Burgess PAO 1C	Burgess PAT 2
	Burgess PAO 1C	Modified Burgess PAT 2
	Modified Burgess PAO 1C	Burgess PAT 2
	Modified Burgess PAO 1C	Modified Burgess PAT 2

TABLE 20

BURGESS ASSAY OF *E. COLI* CORE AND HETEROLOGOUS SIGMA FACTORS

Source of PCFM*	cpm/DNA/37 C	
	PAT 2	CB3
None	390	261
Burgess PAT 2	178	126
Modified Burgess PAT 2	219	178
Burgess PAO 1C	171	150

* Phosphocellulose Flow Through Material.

TABLE 21

MAMMALIAN ASSAY OF *E. COLI* CORE AND MODIFIED BURGESS PAT 2 SIGMA

Enzyme Source	cpm/CB3 DNA/37 C
core (*E. coli*)	849
sigma (Modified Burgess PAT 2)	748
core + sigma	845

These studies with heterologous PC fractions show that the PCFM from these strains of Pseudomonas inhibit heterologous core preparations regardless of their bacterial source or their method of preparation. The only Pseudomonas PC fraction responding to a heterologous PC fraction is PAT 2 modified Burgess core with E. coli sigma. Even the reverse combination of PAT 2 modified Burgess sigma and E. coli core was found not to be synergistic.

Rifampicin

As detected in glycerol gradients, PAT 2 RNAP is affected by changes in the ionic strength as are other bacterial RNAP preparations. The Burgess and mammalian assays differ in their ionic strength to such a degree that in the mammalian assay the enzyme would be in an aggregated state while in the Burgess assay it would be a monomer (8). These states of the enzyme may explain the increased activity and loss of cold sensitivity on CB3 DNA in a mammalian assay by PAT 2 PC holoenzyme (Table 14). However, changes in the ionic strength of the magnitude used in these assays are also known to influence the formation of the initiation complex between DNA and RNAP. Specifically, high salt dissociates the initiation complex between RNAP and DNA (137). Bautz and Bautz also showed (5) that the formation of the initiation complex can be altered by an ionic strength of 0.1 or higher. The differences seen in enzymatic activity in the two assays may then reflect some influence on the formation of the initiation complex. The beta subunit of RNAP has been shown to be involved with the initiation process of RNA transcription (see History, "Function of Subunits"). Thus, experiments were designed to test the effect of a mutation in PAT 2 RNAP specifically involved with initiation by selecting rifampicin resistant mutants and

TABLE 22

INFLUENCE OF *E. COLI* SIGMA ON MODIFIED BURGESS PAT 2 CORE IN A MAMMALIAN ASSAY

Enzyme Source	cpm/CB3 DNA/37 C
core (Modified Burgess PAT 2)	124
sigma (*E. coli*)	598
core + sigma	1,399
Sigma Stimulation	2.1

determining their ability to be permissive for CB3 at both 37 and 20 C.

Rifampicin Resistant Mutants

Of 212 rifampicin resistant (Rif^{res}) mutants of PAT 2 from two nitrosoguanidine mutagenesis experiments, 26 were found which were permissive (by cross streaking) for CB3 at 20 C. A third mutagenesis produced 100 colonies of which 18 were permissive for CB3. Controls establishing the parentage of the permissive PAT 2 mutants used both nutritional markers and phage sensitivity patterns. PAT 2 wild type has an ilva requirment while PAO 1C is a prototroph. Phage PX4 only forms plaques on PAT 2 below 32 C. Phage LP infects only PAO 1C and does not adsorb to PAT 2. One of the Rif^{res} mutants was studied in detail (Table 23). This isolate, Rif 4, had the pigmentation of PAT 2 but its generation time at 37 C was 25 to 27 min longer than that of PAT 2 (65 to 67 min vs. 40 min). This alteration of growth rate in Rif 4 was the only change detected between it and wild type PAT 2 (Table 23). At 37 C and 20 C, PAO 1C has an efficiency of plating CB3 of 1.0. Rif 4 has an efficiency of plating of 0.91.

Rif 4 RNAP

The Burgess isolation of RNAP from Rif 4 produced fractions from PC showing the same depressions of activity as for its parent, PAT 2, when PCFM was added to "core". The incorporations by Rif 4 PC binding protein "core" in a Burgess assay at 37 and 20 C are listed in Table 24. It is apparent that Rif 4 is much more active with all templates at 20 C than it is at 37 C. The identical Burgess procedure for the extraction and assays of RNAP from PAT 2, PAO 1C and Rif 4 shows that in vitro the PC products behave toward CB3 DNA as do intact cells to the in vivo CB3

TABLE 23

CHARACTERIZATION OF RIF 4

Test	Bacterial Strain		
	PAO 1C	Rif 4	PAT 2
ilva required	-	+	+
CB3 sensitive at 37 C	+	+	+
CB3 sensitive at 20 C	+	+	-
PX4 sensitive at 37 C	-	-	-
PX4 sensitive at 20 C	-	+	+
LP sensitive at 37 C	+	-	-

infection. Thus, the genetic modification of the restriction of CB3 further confirms that the inability of PAT 2 to reproduce CB3 at 20 C is due to the host's RNAP.

When Rif 4 is assayed in a Burgess buffer (Table 24), it has synthetic activities similar to that of both wild type enzymes assayed in a mammalian buffer (Table 15 and 16) in that its activity at 20 C is 2.3 fold higher than its activity at 37 C. In a mammalian assay (Table 25), Rif 4 behaves as did the fully permissive, wild type, PAO 1C PC holoenzyme assayed in a Burgess buffer system (Table 13). Where the fully permissive PAO 1C PC was only 49.6% as active at 20 C, Rif 4 at 20 C in a mammalian buffer has 56% of its 37 C activity (Table 25).

These experiments conducted to determine the influence of a mutation in the initiation subunit of RNAP on the restriction of CB3 have brought forth several observations which link cold sensitivity to a defect in initiation. First, an alteration in the beta subunit which is known to be involved in transcription initiation alleviates the restriction of CB3. Secondly, the <u>in vitro</u> correlation between enzyme responsiveness to CB3 DNA at 20 C correlates with the cell's ability to undergo infection by the phage at this temperature. Third, the restricting enzyme under the proper assay conditions can have its cold sensitivity overcome; under these same conditions the Rif 4 enzyme acts more like the fully permissive wild type enzyme. Table 26 summarizes those changes of enzyme activity in each RNAP when assayed in the two different assay systems with CB3 DNA. Thus, this implies that cold promoted restriction of CB3 by PAT 2 resides in some aspect of the ability of PAT 2 RNAP and CB3 to form an initiation complex for the synthesis of pre-early phage m-RNA.

TABLE 24

RIF 4 BURGESS "CORE"

RNAP ASSAYED IN BURGESS BUFFER AT 37 C AND 20 C

Temperature	cpm/DNA		
	Calf	PAT 2	CB3
37 C	2,325	609	811
20 C	4,147	1,329	1,897

TABLE 25

RIF 4 BURGESS "CORE"

RNAP ASSAYED IN MAMMALIAN BUFFER AT 37 C AND 20 C

Temperature	cpm/CB3 DNA	Percentage
37 C	12,141	100
20 C	6,841	56

TABLE 26

ENZYME ACTIVITIES OF BURGESS EXTRACTED RNAP AT 37 C AND 20 C WITH CB3 DNA IN BURGESS AND MAMMALIAN ASSAY BUFFERS

Assay Buffer	Temperature	Percent Activity of Each Enzyme		
		PAT 2	PAO 1C	Rif 4
Burgess	37 C	100	100	100
	20 C	8	49.6	233
	Data Source	Table 10	Table 13	Table 24
Mammalian	37 C	100	100	100
	20 C	128	134	56
	Data Source	Table 14	Table 15	Table 25

Rif 4 core enzyme was tested for its in vitro resistance to rifampicin. While the enzyme from PAT 2 was severely inhibited (Table 27), Rif 4 was resistant to the drug.

SDS acrylamide gels of Rif 4 "core" (Fig 28) show the presence of sigma in this PC enzyme. This correlates with the earlier finding that "core" preparations which contain sigma undergo the depression of activ- by PCFM.

Other Permissive PAT 2 Mutants

Other genetic manipulations of PAT 2 which are seemingly unrelated to RNAP allow for the selection of fully permissive mutants. Rolfe and Holloway (124) reported that mutants of P. aeruginosa spontaneously resistant to dl-fluorophenylalanine (fpa) were fully permissive for viruses which are under host controlled modification functions (HCM). They found that one in 10^3 to 10^4 cells are spontaneously resistant to fpa and that 50% of those cells isolated are fully permissive for previously restricted viruses. They showed fpa resistance involves at least three loci, two of which map near streptomycin (str). In fact, cells which are fpa^{res} and phage sensitive when they are made str^{res} will become fpa^{sens} but are still not able to restrict HCM phages. With PAT 2, one in 10^3 to 10^4 cells are resistant to fpa, and of those colonies isolated, 71 out of 158 or 45% were fully permissive for CB3. One of those colonies, Fpa 1, was further studied and shown not to be a contaminant as judged by the same criteria that established the purity of Rif 4. Like Rolfe and Holloway's observation, spontaneous resistance to str by Fpa 1 suppressed fpa resistance, but Fpa 1 str^{res} is still permissive for CB3. No other experiments were performed with Fpa 1 or Fpa 1 str^{res} to determine if their RNAP was somehow altered. A discussion of this mutant is given in the following section of this work.

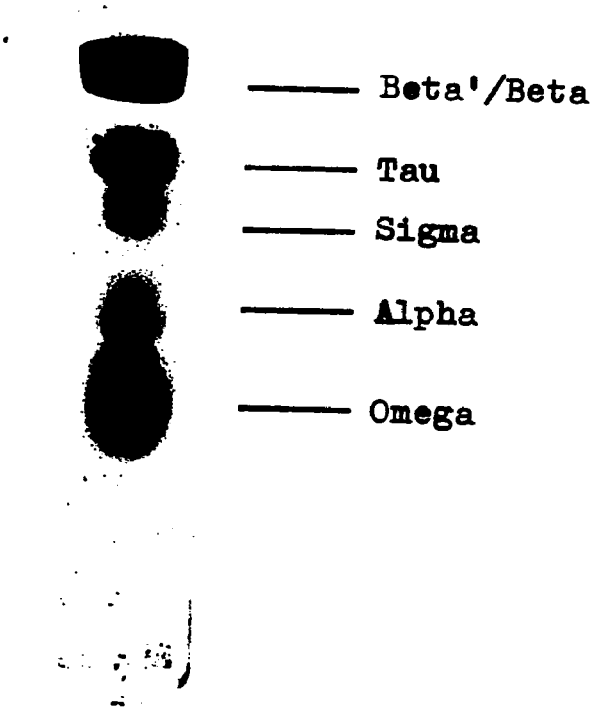
TABLE 27

EFFECTS OF RIFAMPICIN ON PAT 2 AND RIF 4 RNAP AT 37 C AND 20 C

Assay Condition*	cpm/RNAP/Temperature			
	PAT 2		Rif 4	
	37 C	20 C	37 C	20 C
Control	606	48	811	1,197
Rifampicin	56	0	746	1,642
Percent Inhibition	92	100	9.4	none

* Burgess buffer, CB3 DNA.

Figure 28. SDS polyacrylamide gel electrophoresis of Rif 4 Burgess phosphocellulose enzyme.



Rif 4

Figure 28

Conclusion.

In conclusion the objectives set forth at the beginning of this study have been met. Aspects of the infection by CB3 in P. aeruginosa relating to the inhibition of phage synthesis at 20 C have been characterized. That work showed that phage CB3 caused no detectable changes in PAT 2 at non-permissive temperatures. In fact, it appears that the expression of phage functions is completely absent. Those studies also implicated a defect in the host RNAP as the cause of CB3 cold sensitivity since no phage specific RNA is synthesized in non-yielder cells. Genetic manipulations of PAT 2 established a correlation between in vitro and in vivo permissiveness for CB3 when alterations are induced in RNAP subunits apparently involved with the initiation of transcription. Thus, the host dependent function in the restriction of CB3 may involve the gene coding for the beta subunit of PAT 2's RNAP.

DISCUSSION

When bacteria are infected with phage, various viral genes are expressed sequentially; so called early genes are transcribed immediately after infection, but late genes are transcribed only after some lag (56, 130, 97). RNAP prepared from uninfected E. coli can transcribe some of the early genes of its bacteriophages and thus host functions control the initial events in phage development. However, prior to viral gene transcription, several other host determined functions can also influence the success of a particular viral infection. Some of these other host functions include the synthesis of a viral receptor site, the presence of membrane replication sites for phage DNA, the possession of an extragenic suppressor for a phage missense or nonsense mutation and host determined modification and/or restriction enzymes for foreign DNA. Several lines of evidence are therefore necessary to adequately demonstrate the involvement of a particular host determinant in the absolute control of virus development. With the temperature permissiveness of the CB3 infection of P. aeruginosa PAT 2, the evidence does establish a link between permissiveness and host RNAP, and, in fact, the evidence goes further to implicate the beta polypeptide of the enzyme as the possible cold sensitive site.

My initial objectives were concerned with the biochemical characterization of the CB3 infection of strain PAT 2 and PAO 1C. In those studies, the first experiments followed the incorporation of labelled precursors into the macromolecules of infected and uninfected cells. With CB3 infected cells those studies established that the incorporation of radioactive precursors into DNA, RNA and protein did show a correspon-

dence between a partcular biosynthetic event and the *in vivo* infection with both the conditionally and fully permissive host.

In other experiments directed toward the characterization of biochemical events in the CB3 infection, it was found that permissive infections of PAT 2 or PAO 1C do influence the rates of RNA synthesis. Unlike coliphage T7 (145) which causes a continual decrease in the rates of RNA synthesis from the start of the infection, permissive CB3 infections show a decrease in the rates for only the first one third of the latent period. After this initial decrease the rates increase until the end of the latent period. This increase in rate may be the result of a phage RNAP molecule. Pilot experiments with RNAP isolated from infected cells did show that this enzyme differed from uninfected cell RNAP in respect to calf DNA template activity. Thus, the decrease in rates would be terminated when the phage coded or phage modified molecule was produced. The infection of *E. coli* by T4 not only causes host RNAP to acquire new specificity through the action of a phage coded sigma factor, but still later in the virus infection the core molecule is extensively modified (157). The changes result in an enzyme which has a decreased affinity for mature coliphage DNA. The species of m-RNA synthesized subsequent to the changes would differ from the species synthesized prior to the changes. CB3 RNAP may act in a similar manner and be responsible for the increase in the rates of RNA synthesis seen during the last one third of the latent period.

Rates of RNA synthesis are not affected by CB3 when it infects PAT 2 at non-permissive temperatures. In fact, PAT 2 cells behave as do uninfected cells at either temperature in that the rates of RNA synthesis continue to increase with time. This implies that the viral functions

responsible for altering the rates of RNA synthesis at 37 C are absent or inactive at 20 C. In this context even ultraviolet irradiated CB3 was unable to affect the rates of RNA synthesis in either host at 37 or 20 C. However, this same treatment of lambda does not prevent this phage from inhibiting all macromolecular synthesis in E. coli (28). Thus, the CB3 function(s) responsible for altering RNA synthesis is unlike those seen in phage lambda. However, ultraviolet treatment of CB3 did not prevent the viral genome from becoming membrane bound or attached. Therefore, mere binding of phage DNA to host membrane is not the phage function responsible for the changes in RNA synthesis.

DNA sedimentation analysis of CB3 infected cells detected viral DNA synthesis shortly after the start and at the end of the latent period at 37 C. At a non-permissive temperature CB3 DNA is not detected in samples taken at corresponding times of the latent period. However at 20 C, PAT 2 DNA was synthesized while at 37 C host DNA synthesis was not detected. These biochemical studies then have shown that not only is CB3 unable to control the rates of RNA synthesis at low temperatures, but also viral DNA is not synthesized. In view of the continued synthesis of host DNA at 20 C, these studies imply that the CB3 functions controlling these processes are not functioning or are absent at this temperature.

Sedimentation analysis of infected cells also showed that a normal event of the permissive infection was the degradation of host DNA. This degradation product was first detected midway through the CB3 latent period (25 min) as a molecule with an estimated $S_{20,w}$ of 78. This molecule increased in amount until the end of the latent period.

The mechanism responsible for the occurrence of the PAT 2 host

degradation product and its uniform size may be the result of packaging viral DNA into phage capsids. This is based on observations from other systems in which viral DNA is in a large replicating complex, called a concatomere, which has more than 100 equivalents of phage DNA linked end to end (66). This structure must be "cut" into genetically identical phage genomes in order for packaging to occur. Several methods have been proposed for obtaining identical phage equivalents from concatomeres (152). The first method is called the headfull hypothesis wherein phage DNA is cut on the basis of equal lengths. The other method used DNA sequence specific scissions to produce mature phage equivalents. Many double stranded DNA viruses use concatomeres (66) in their replication (T5, lambda, P22C, T7). Therefore, the 78S host degradation product may reflect the outcome of the interaction between CB3 concatomere processing enzymes and PAT 2 DNA.

Other information from the sedimentation analysis of the CB3 infection offered a clue to the mode of the early replication of CB3. Tomazawa and Ogawa (153) using lambda found that, following injection, DNA undergoes several cycles of replication which result in sister copies of the infecting genomes. Later in that infection, "polymer" DNA structures are found. CB3 DNA is probably undergoing similar duplication of the penetrating DNA, thus accounting for the alkaline sucrose profiles seen during the early part of the permissive latent period. Those profiles showed that phage DNA synthesized early in the infection had a size and structure which was identical to mature CB3 DNA. Further support for this mode of early DNA replication is seen in studies on the membrane binding of viral DNA. Those studies found that attached viral DNA or DNA prevented from becoming membrane bound

were also identical to mature phage DNA when analyzed in alkaline sucrose gradients.

Although the above information concerning the viral replication was ancillary from the biochemical characterization of the CB3 infection, the primary goal of the study was to use a biochemical approach to determine the host dependent controller responsible for the non-permissive nature of the CB3 infection at 20 C. Since CB3 DNA synthesis was not detected at 20 C, experiments were designed to test for the membrane binding of incoming phage DNA to host cytoplasmic membrane material. It is known that the attachment of viral DNA to membrane occurs immediately after DNA penetration in the temporal development of many bacteriophages (76, 129). Thus, the absence of this event would have explained the apparent lack of CB3 functions at 20 C. This was found not to be the case.

Ancillary information from these studies indicated that membrane binding of CB3 DNA is an event independent of protein synthesis. Pretreatment of cells prior to infection by a variety of methods failed to prevent CB3 DNA from attaching to host membrane material.

These attachment studies also point up several observations on the nature of the viral DNA- host membrane complex. The binding of phage DNA to cell cytoplasmic membrane may on the one hand be of a covalent nature since alkali removed membrane from DNA, and, on the other hand, to involve a protein since the proteolytic enzyme, pronase, also releases phage DNA. These observations are in accord to those found by Knippers and Sinsheimer (76). In addition, the inhibition of attachment by sodium azide points out that this process may involve ATP or an ATP-dependent enzyme. Azide, cyanide and CO are all known to

specifically inhibit the generation of ATP through their action on the cytochrome A redox system (91). In E. coli, DNA replication depends on continual ATP generation since both cyanide and CO inhibit replication (38) even in the presence of all four desoxyribonucleotides. In the presence of these inhibitors, DNA polymerase and ligases function normally, but DNA synthesis is blocked (49). Sodium azide (49) prevents DNA synthesis in Bacillus subtilis, and in E. coli it additionally inhibits nucleases. That the continual generation of ATP is a necessary function for viral attachment may also point to the involvement of some DNA replication enzymes for the formation of the CB3 DNA-host-membrane complex.

Hybridization experiments provided the first clue that the inability of CB3 to reproduce in PAT 2 at low temperature may be related to some defect in the synthesis of phage dependent RNA. Competition hybridization experiments showed that at 20 C, phage specific RNA was produced only in the same fraction of infected cells which were detected as biological yielders at 20 C in single cell burst experiments. Thus, it may be that CB3 RNA was not transcribed in non-yielder PAT 2 cells at 20 C. Competition experiments also indicated that protein synthesis was not required for the appearance of the viral specific RNA detected in PAT 2 yielder cells at 20 C. Thus, the species of phage specific RNA absent in 20 C infected PAT 2 seem identical to those species termed immediate early or pre-early in T4 infected E. coli (7, 98, 21, 15). These immediate early species in T4 are synthesized exclusively by E. coli RNAP and also do not require protein synthesis for their occurrence. Therefore, the RNAP from PAT 2 was investigated to determine its involvement in the conditional temperature sensitive growth of CB3 at temperature below 32 C.

Isolation of RNAP from E. coli D10 by the technique of Burgess produced, as others have reported, two distinct PC protein fractions. The protein that binds to PC is core enzyme. The sigma protein of RNAP is found in the PC flow through material (PCFM). Sigma does not bind to PC since it is acidic and is released from holoenzyme when it binds to PC. The mechanism for sigma release on PC is unknown. However, RNAP is unusual in its ability to bind to both DEAE and PC at the same pH. RNAP is acidic with a net negative charge at pH 8, and, therefore, it is not expected to bind to the anionic PC. But, RNAP binds to DNA and other polyanionic materials. In view of the fact that in vivo sigma is released shortly after binding and initiation of holoenzyme to DNA (126), PC chromatography may be causing similar changes in RNAP to allow in vitro release of sigma.

When RNAP was extracted and purified from PAT 2 according to the Burgess procedure, several unusual properties of the enzyme were found. The Pseudomonas PC binding protein was not "core" but RNAP holoenzyme. The binding of holoenzyme to PC in itself is not a new finding with pseudomonads. P. putida RNAP when chromatographed on PC yields two PC binding proteins (21). One of the proteins is core enzyme, and the other was found to be holoenzyme. With P. aeruginosa, however, Whitely and Hemphill found their strain to behave in the Burgess isolation procedure as did E. coli (164). Thus, my finding is not in accord with their finding.

A second unusual property of PAT 2 RNAP extracted and purified according to the Burgess procedure was the ability of the enzyme to have virtually all of its activity depressed by the addition of PCFM. Such a phenomenon has not been reported for any RNAP. The implication of this

unusual property of RNAP will be considered.

This multi-functioning enzyme, RNAP, must carry out its tasks (template finding, promoter site recognition, transcript initiation, elongation and chain termination) in an efficient manner. Consequently, the positive control elements, such as sigma, and CAP protein are all able to recognize the enzyme and govern its efficiency by stimulating transcription in one way or another. The question I pose is the following: does Burgess PCFM contain an activated negative control element released from its inactive role on the holoenzyme by PC chromatography? Because the DEAE enzyme pools possessed RNAP activity even after undergoing precipitation by ammonium sulphate, dialysis, and purification on glycerol gradients; and the unusual behavior of the enzyme occurs only subsequent to chromatography on PC, it indicates that some factor from RNAP which is apparently inactive prior to PC chromatography is released by this procedure in an active form. This factor appeared to be present even in the modified Burgess PCFM, since this PCFM was not stimulatory with E. coli core and with the Burgess Pseudomonas PC holoenzyme. This PCFM also depressed its activity. Therefore, the Burgess PCFM-Burgess PC holoenzyme provides an opportunity to study a possible new element of RNAP which is negative in its control on the enzyme.

Burgess PCFM can be used as a starting material to isolate and characterize the negative control element. Cationic exchange columns would allow a separation of the elements in the flow through material from PC. The factor can be assayed in the eluates by its ability to inhibit the activity of PC holoenzyme. Further purification could be accomplished, if necessary, either by molecular sieving, acrylamide gel electrophoresis or density gradients to allow a characterization of the

"killer" factor in terms of its molecular weight or chemical composition. At this point a study of the mechanisms of action or interaction of the factor with PC holoenzyme could be undertaken. Techniques are currently available to allow at least a superficial examination of where the factor interferes with enzyme action. For example, the binding of enzyme to template or its prevention by the factor could be detected in a glycerol gradient. Likewise synthetic polynucleotides could be used to determine if elongation is influenced by the action of the factor. However, since the enzymes from PAT 2 are naturally defective in initiation at least with viral DNA, this may be the transcription step to investigate first. Supporting this is the observation that E. coli PCFM is not inhibitory to heterologous or homologous core enzymes or to PC holoenzymes from either PAT 2 or PAO 1C. This indicates that the factor is not present in a "normal" enzyme extracted and purified by the techniques used. PAO 1C PCFM was also capable of depressing PC holoenzyme, and it will be shown below that even this enzyme is abnormal for reasons other than its inability to be fractionated on PC into core and sigma.

However, equally important to the process of transcription that the factor inhibits are the cellular mechanisms which govern its expression. It is easiest to assume that the factor normally is an integral part of the enzyme molecule and PC chromatography removes it, but in the process "killer" factor is also activated. In any event, cellular mechanisms must be available to activate and inactivate the factor on the enzyme as a response to changes in cellular growth or metabolism.

A role for this factor may be in the control of the synthesis of transfer and ribosomal RNA (stable RNA). The rel locus in E. coli is an example in which this negative controller of RNAP would be used by

the cell to great advantage. When E. coli rel$^+$ cells are starved for a required amino acid the cells regulate the synthesis of stable RNA in a stringent manner (43). Stringency of the rel$^+$ locus causes an abrupt halt to the accumulation of stable RNA during amino acid starvation. Thus, there is a precedent for the cellular utilization of such a RNAP control mechanism involving a negative control factor. The chemical and enzymatic characterization of the factor may provide clues to the cellular control of this element of RNAP and its role in the cell.

In spite of the unusual purification of RNAP, the conditionally permissive host's PC holoenzyme shows a cold sensitivity toward CB3 DNA in an in vitro enzyme assay. The activities of PC holoenzyme from PAT 2 and PAO 1C also establish that the permissiveness of PAO 1C and the non-permissiveness of PAT 2 at 20 C detected in vitro correspond to the same permissiveness and non-permissiveness of these hosts toward CB3 production in vivo. Even the modified Burgess extraction of PAT 2 RNAP which resulted in core and sigma fractions when recombined in vitro were also found to be cold sensitive at 20 C when CB3 DNA was the template. The similarity of the ratios of PC RNAP activities in vitro between PAT 2 and PAO 1C at 20 C to the ratios of in vivo phage production and to the ratios of in vivo phage specific RNA synthesis establish an equivalency supporting this host enzyme as responsible for the cold sensitive host function in CB3's development at low temperature.

The low ionic strength mammalian assay used in studies on PCFM inhibition of PC holoenzyme provided the impetus to extract the enzyme by use of modified Burgess procedure. Those extractions established another difference between the fully permissive PAO 1C and the conditionally permissive PAT 2. This modified procedure resulted in holo-

enzyme eluting from PC with PAO 1C, but using PAT 2, PC chromatography resulted in the only separation of core from sigma in these strains of Pseudomonas.

One unanswered observation which may relate to the initiation of the enzyme is the apparent leakiness of the non-permissive host at 20 C. Both in vivo and in vitro PAT 2 possesses about 15% of the permissiveness of PAO 1C in terms of its residual phage production, hybridizable phage specific RNA or enzymatic activity with CB3 DNA. These findings are resolved by recalling the experiments of Olsen et al. which dealt with the influence of temperature on the infection of CB3. They found that restriction of CB3 was correlated to a temperature of 32 C. As the incubation temperature of the infection was increased from 20 to 32 C, progressively higher virus titers were produced, indicating a gradual increase in host permissiveness. However, these titers are considerably lower than those seen above 32 C since the most significant increase in phage production occurred when the temperature was higher than 32 C.

The marked stimulation of phage titers in PAT 2 above 32 C may be involved with some unusual findings seen in the DNA-RNA hybridization competition experiments. Those experiments showed that phage specific RNA synthesized at 20 C from either host was only able to compete against 50% of the phage specific RNA synthesized in 37 C infected PAT 2 or PAO 1C. The marked increase in permissiveness of PAT 2 above 32 C may be due to the influence of temperature on the initiation of the enzyme (see discussion below for a possible mechanism). Not only would an increase in temperature allow initiation of those cistrons needed for phage production, but also a temperature above 32 C would allow initiation for cistron unnecessary for phage production. In other words, temperatures above 32 C cause the initiation of cistron other than

those defined as immediate early m-RNA. Since PAO 1C in hybridization experiments was also seen to compete with only 50% of the 37 C RNA, the temperature influence on unnecessary cistron initiations above 32 C would have to apply to the fully permissive host's RNAP. Surprisingly, the PC holoenzyme from PAO 1C was found to possess only 49.6% of its 37 C activity when assayed at low temperature. Thus, these observations implicate an influence by temperature on both the permissive and non-permissive host enzymes.

How can a temperature above and below 32 C produce such a dramatic effect in the polymerase from these two hosts? Temperature is known to influence proteins. In fact, any change brought about by temperature in the configuration of a protein can be defined as denaturation (72). Consequently, various levels of denaturation are distinguished according to whether the secondary, tertiary or quaternary structure of the protein is involved. Reversible denaturation or conformational changes in a protein affecting its activity can occur in the quaternary structure of that protein. It has been proposed and indirectly tested to date that RNAP undergoes changes in its configuration in order for it to carry out each of the steps of RNA synthesis (20). Indirect proof of these changes come from studies on the sensitivity of the enzyme to salt, proteases and inhibitors (rifampicin). Polymerase responds differently to these agents after it binds to template or when it is in the process of synthesizing a transcript. For example, the enzyme becomes more resistant to the action of trypsin and NH_4Cl after binding to DNA, and this resistance increases upon the addition of the four nucleoside triphosphates which allow RNA synthesis to occur (20). Results of this type have been interpreted to be due to

changes in the conformation of the enzyme. As discussed earlier, low ionic strength favors initiation, and this can be detected by the formation of rifampicin resistant enzyme-DNA complexes (167, 137, 5); however, subsequent to the release of the transcript the enzyme is sensitive to this inhibitor. The current studies with CB3 have also indicated that low ionic strengths alter enzyme activity. Recent work (100) strongly suggests that a conformational change must also occur in the enzyme prior to initiation and that this change occurs as a result of sigma factor binding to core. This is probably involved in sigma's ability to recognize the correct promoter sites on DNA.

An increase in the temperature from 20 to 32 C influences PAT 2 and RNAP in a manner that alters its conformation (denatures quaternary structure) so that these configurational changes allow a greater number of molecules to initiate, and hence progressively higher virus production is seen. PAO 1C is fully permissive because its renaturation below 32 C does not prevent its initiation with CB3 DNA. However, both hosts must undergo significant conformational alterations above 32 C. These changes would account for the marked increase in virus production by PAT 2 above this temperature. They would also account for the observation in hybridization experiments and enzyme activity assays showing the excess activity or transcript production at 37 C.

A number of substances that are denaturing agents or structural modifiers have an effectiveness correlating to their capability to form bifunctional hydrogen bonds. Water is known to be such a structural modifier even at room temperature. For instance, poly N^5-(3 hydroxypropyl)-L-glutamine possesses an alpha helical configuration in organic solvents while in the presence of water its structure is largely dis-

organized (73). Several laboratories have reported that water itself undergoes subtle structural changes at specific temperatures (58, 79). Of further interest to this discussion is that at these temperatures significant affects can be shown on biological systems. These changes in the properties of water occur within a rather narrow range (± 2 C) near 15, 30, 45, and 60 C. It has been shown that a sulfide reducing bacterium (108), Streptococcus faecalis (34) and that Neurospora crassa (58) all grow less favorably at temperatures near 15, 30 and 45 C. More optimal growth occurred at a temperature midway between these minima. Thus a temperature of 30 to 32 C has been shown to influence biological systems and is also a temperature where water undergoes a slight change in its state. It is not unreasonable to assume that the dramatic 32 C temperature effect on PAT 2 and probably on PAO 1C is due to the changes in the properties of water allowing a more significant change to occur in the conformation of RNAP. Physical methods to date have not been successful in directly proving any conformational changes in RNAP (20). One study using optical rotatory dispersion and circular dichroism showed that the alpha helical content of core enzyme does not change when it binds to DNA (104). In view of the marked influence of temperature on PAT 2 RNAP, this system seems more ideally suited to detecting conformational changes required by core and sigma for transcription. Thus, it would offer the first definitive demonstration that such a change does indeed occur and would thereby open new approaches to the study of this enzyme in its interaction with DNA.

One experimental finding that does not appear logical in view of the observation on the influence of temperature on RNAP and initiation is the suppression of the cold sensitivity in PAT 2 cells spon-

taneously resistant to FPA. Hardy and Binkly (61) reported in studies on E. coli B that FPA not only inhibited the initiation of DNA synthesis but also inhibited RNA synthesis. This inhibition of RNA by FPA was shown to be due first to the immediate influence of the analogue on RNAP activity, and secondly, to FPA's later affect on the synthesis of new polymerase molecules. The mechanisms of this action was unknown, but other enzymes of the cell (isocitric dehydrogenase) were not affected by FPA in this manner. Thus, there is a precedent for the involvement of FPA in RNAP so that the suppression of cold sensitivity in PAT 2 by this analogue is not inconsistent with other findings in this study.

A note of caution must be introduced in the interpretations of these observations implicating the beta subunit's responsibility for the cold sensitivity of CB3 development. The many functions of RNAP involve the interactions of five or possibly six different polypeptides which together determine the role of this enzyme within the cell. The beta subunit is also known to be the polypeptide to which sigma binds in E. coli (167). Thus, pleitropic effects on RNAP through the action of the beta subunit must also be evaluated in respect to their function in the cold sensitivity of the enzyme with CB3 DNA. It is not inconceivable that sigma's recognition of the correct promoter sites could be influenced by temperature through some conformation change of the beta subunit and thus account for the observations in this system. As an example, rifampicin can cause pleitropic effects as shown by the work of Jacobsen and Gillespie (70) with E. coli. They found that rifampicin resistance suppressed a mutant which was "warm temperature sensitive" in its RNA synthesis. Rifampicin resistance in their system caused an alteration in the enzyme in that it allowed RNA synthesis at

elevated temperatures. However, independent revertants of the temperature sensitive mutant did not have enzymes resistant to the drug. Thus, a pleitropic effect is involved in their system.

Recently a cold sensitive rifampicin resistant RNAP was produced in E. coli. Reid (116) used tritium suicide in his protocol and was able to isolate survivors resistant to the antibiotic. Thus, in his system, rifampicin resistance appeared associated with cold sensitivity of the enzyme while in my system cold sensitivity is suppressed by rifampicin resistance. Since the antibiotic in these two systems reversed cold sensitivity in one case and in the other case is associated with cold sensitivity, this points up the justification for a more cautious approach in viewing the role of the drug exclusively with the beta subunit of the enzyme.

Further justification for this cautious outlook is the fact that CB3 is only one phage out of an immunologically related group of CB phages (106). Some of these other viruses were found unable to plate on either host at 20 C, and still others were able to plate at this temperature on both strains. Thus, phage DNA may also be a determining factor that allows these host enzymes to be cold sensitive. These phages could accomplish this by slight variation in their template promoter regions for immediate early m-RNA and thus effect the sigma recognition of this sites, therefore, the absolute role of the beta subunit of PAT 2 needs more vigorous experimental study to conclusively rule out the possible influence of sigma or some other polypeptide of the enzyme.

The CB series of phages may be used as a system to study the interaction of RNAP with DNA promoter sites and the process of transcript initiation. One would have to first establish the involvement of

the RNAP of PAT 2 and PAO 1C with the cold sensitivity of these phages. The isolated enzymes from PAT 2, PAO 1C and Rif 4 may be sufficient to do this. Mechanisms are available to isolate the subunits of the enzyme from E. coli (167) and to reassociate these subunits into an active molecule (80). Therefore, using heterologous subunits, one could construct hybrid polymerase molecules, then using the CB DNA's as template, one could screen the hybrid enzymes and determine how their activities were altered. Differences in activity between hybrid subunits (loss or gain of CB3 transcription) would allow pinpointing the subunit involved and allow an amino acid analysis of the subunit. From here differences in amino acid composition to determine altered enzyme activity would provide clues into the mechanisms of the function of the enzyme. This procedure would also determine the absolute role of the beta subunit in the cold sensitivity of CB3.

In conclusion, this work has used many different experiments to establish the link between the inability of CB3 to reproduce at low temperatures and the conditional non-permissive host's DNA dependent RNAP. The host dependent function in the inability of CB3 to reproduce at 20 C in PAT 2, is from the current evidence, the beta subunit of the enzyme. Further, the specific fault in the subunit resides in its apparent inability to initiate RNA transcripts from CB3 DNA. The influence of temperature on permissiveness also points up some properties of the fully permissive host's enzyme which tend to support findings from enzyme purifications, hybridization and enzymatic activities that implicate that both hosts possess unusual RNAP molecules. However, they do differ enough in their response to temperature so that the dicotomy of CB3 permissiveness is seen below 32 C.

SUMMARY

The host dependent mechanisms involved with the conditional temperature sensitive reproduction of CB3 at 20 C was sought by means of a biochemical analysis of the infection.

The biochemical analysis of the infection showed that phage CB3 causes no detectable changes in PAT 2 at non-permissive temperatures. Net macromolecular synthesis of DNA, RNA and protein and the rates of RNA synthesis were found unaltered as a result of CB3 infection of PAT 2 at 20 C. In infected PAT 2 at 37 C or in infected PAO 1C at both temperatures, alterations in macromolecular synthesis are seen.

Sedimentation analysis of cells infected at 37 C detected the cessation of host DNA synthesis and its degradation. In those same gradients new viral DNA is seen to be synthesized at a permissive temperature. Neutral and alkaline sucrose sedimentation analysis of PAT 2 cells infected at 20 C showed that host DNA synthesis is not inhibited and no viral DNA is synthesized.

Sucrose velocity gradients also established that incoming phage DNA binds to PAT 2 host "cytoplasmic material" at both 37 C and 20 C. Therefore, the general lack of phage expression at 20 C is not due to a temperature-dependent absence of this event.

Nucleic acid hybridization was used to determine the production of phage RNA. At 20 C, CB3 specific m-RNA is not synthesized in non-yielder PAT 2 cells. Chloramphenicol treatment of PAT 2 indicated that the species of m-RNA absent at 20 C in non-yielder cells is probably analogous to the so-called pre-early or immediate early species in other bacterial virus systems. Thus, the biochemical analysis of the CB3

infection indicated that some unusual property of PAT 2 DNA dependent RNA polymerase may be involved with the cold temperature sensitive restriction of CB3.

E. coli purification procedures for RNA polymerase were unable to resolve the enzymes from PAT 2 and PAO 1C into core and sigma fractions during phosphocellulose (PC) chromatography. Unlike Escherichia coli D10 the PC binding enzyme for these pseudomonads is holoenzyme (core and sigma). The PC flow through material of PAT 2 (which with E. coli D10 contained sigma factor) was found to contain an apparently new factor of RNAP. This factor is released from holoenzyme by PC chromatography and in the process appears to become activated. Readdition of the in vitro separated factor to holoenzyme and a variety of DNA templates completely depresses transcription.

In spite of the unusual purification of RNAP from PAT 2 and PAO 1C, the PC holoenzyme of PAT 2 was cold sensitive in its ability to transcribe CB3 DNA. The ratios of in vitro enzyme activities for PAT 2 and PAO 1C at 20 C correspond to the in vivo phage permissiveness and to the phage specific m-RNA synthesis detected in vivo for these two hosts.

Cold sensitivity of PAT 2 RNAP apparently resides in its inability to initiate transcription. Low ionic strength assay buffers known to increase the initiation ability of other bacterial RNAP enzymes eliminate the cold sensitivity of PAT 2. Resistance to the drug rifampicin is known to involve the beta subunit of other RNAP enzymes, and this subunit is also known to be involved with the initiation of E. coli transcription. A mutant strain of PAT 2 which is resistant to rifampicin is permissive for CB3 at 20 C. The isolation and assay of RNAP

from one rifampicin resistant mutant showed that its enzyme is not cold sensitive with CB3 DNA.

Therefore, the host dependent function in PAT 2 responsible for the inability of CB3 to reproduce at 20 C is the host enzyme RNAP.

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