

Summer 1976

BIOCHEMICAL AND GENETIC ANALYSIS
OF THE FORMATION OF MEMBRANE-
COMPLEXED DNA FOLLOWING
INFECTION OF BACILLUS SUBTILIS BY
BACTERIOPHAGE

PHYLLIS SANDRA KOHLER
University of New Hampshire, Durham

Follow this and additional works at: <https://scholars.unh.edu/dissertation>

Recommended Citation

KOHLER, PHYLLIS SANDRA, "BIOCHEMICAL AND GENETIC ANALYSIS OF THE FORMATION OF MEMBRANE-COMPLEXED DNA FOLLOWING INFECTION OF BACILLUS SUBTILIS BY BACTERIOPHAGE" (1976). *Doctoral Dissertations*. 2354.
<https://scholars.unh.edu/dissertation/2354>

This Dissertation is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact nicole.hentz@unh.edu.

INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.
2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.
3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.
4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.
5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms

300 North Zeeb Road
Ann Arbor, Michigan 48106

76-23,127

KOHLER, Phyllis Sandra, 1949-
BIOCHEMICAL AND GENETIC ANALYSIS OF
THE FORMATION OF MEMBRANE-COMPLEXED
DNA FOLLOWING INFECTION OF
BACILLUS SUBTILIS BY BACTERIOPHAGE.

University of New Hampshire, Ph.D., 1976
Chemistry, biological

Xerox University Microfilms, Ann Arbor, Michigan 48106

BIOCHEMICAL AND GENETIC ANALYSIS OF THE FORMATION
OF MEMBRANE-COMPLEXED DNA FOLLOWING
INFECTION OF BACILLUS SUBTILIS BY BACTERIOPHAGE

by

PHYLLIS SANDRA KOHLER

B.A. Boston University, 1971

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

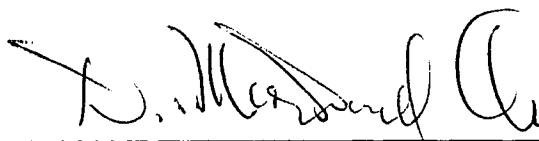
Doctor of Philosophy

Graduate School

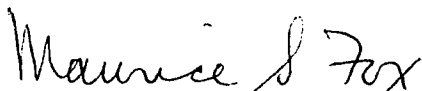
Department of Biochemistry

June, 1976

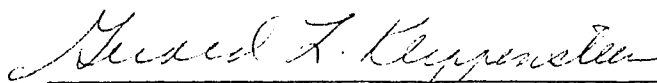
This thesis has been examined and approved.



Thesis director, D. MacDonald Green, Prof. of Biochem.



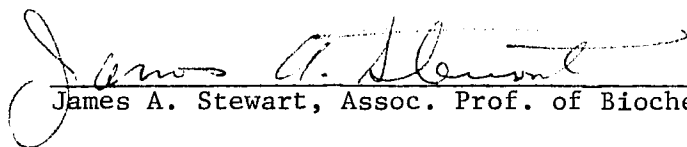
Maurice S. Fox, Prof. of Biology
Massachusetts Institute of Technology



Gerald L. Klippenstein, Assoc. Prof. of Biochem.



Samuel C. Smith, Prof. of Biochem.



James A. Stewart, Assoc. Prof. of Biochem.

To my family, whose support, understanding, patience and love were remarkable. Especially to my Mother, whose words "you'll only end up in pots and pans anyway" made me want to prove that I could do it and to my Father, who always made me feel that I would. And to David.

Acknowledgements

I wish to thank Dr. D. MacDonald Green for his guidance, support and wisdom. I also wish to thank Drs. James Stewart and Gerald Klippenstein for their most helpful advice and assistance. A most special note of thanks to Dr. Jack King, who helped me to learn how to think like a researcher and who always seemed to be there to smooth out the rough spots. Also a note of thanks to Dr. John Reeve for his time, knowledge and unique sense of humor in the joint research projects we undertook. This work was supported by Public Health Service Grant number AI 11318-02 from the National Institutes of Health.

TABLE OF CONTENTS

LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT	x
Introduction	1
Materials and Methods	15
Bacterial Strains, Growth Conditions and Bacteriophage Preparations	15
Cell Lysis	19
Renografin Density Gradients	20
Sedimentation	20
Protease Sensitivity	21
Sarkosyl Sensitivity	21
Phospholipase C Sensitivity	22
DNase/Phosphodiesterase Sensitivity.....	22
Stabilization by Phenyl Methyl Sulfonyl Flouride	22
Inhibition of RNA Synthesis	23
RNase Sensitivity	24
Metabolic Inhibitor Studies	24
Electron Microscopy	25
Marker Rescue Experiments	25
Minicell Experiments	27
SPP1 Experiments	28
Bacterial Strains, Growth Conditions and Bacteriophage Preparations	29
SPP1 DNA-Membrane Complex Analysis	29

Protease, Sarkosyl, Phospholipase C and DNase Sensitivities	29
Chloramphenicol, Rifampicin and HPUra Studies	29
RESULTS	30
Characterization of the Membrane Fraction	30
Time Course of DNA-Membrane Binding	42
Effect of Metabolic Inhibitors on Membrane Complex Dynamics	42
Stabilization With Phenyl Methyl Sulfonyl Flouride (PMSF)	54
Sensitivity to RNase	54
Minicell Experiments	59
DNA-Membrane Binding in Minicells	63
Time Course of DNA-Membrane Binding in Minicells	69
Metabolic Inhibitor Studies (Minicells)	72
Marker Rescue Studies	75
Is There A Pilot Protein? - Double-Label Infection Experiments	79
SPP1 Experiments	85
SPP1 DNA-Membrane Complex Analysis	86
Time Course of SPP1 DNA-Membrane Interactions	86
Metabolic Inhibitor Studies: SPP1	92
Location of Replicating DNA	92
DISCUSSION	101
REFERENCES	110

LIST OF TABLES

1. Summary of Characteristics of Known DNA-Membrane Associations	13
2. Some Biological Properties of Bacteriophages SP82G and SPP1	14
3. Effect of RNase on the Percent Total Label in the DNA-Membrane Complex	62
4a. Genetic Analysis of Bound and Unbound DNA by the Marker Rescue Technique	77
4b. Percent of the Total Marker Activity Found in the Bound DNA Fraction	78
5. Effect of 6-(p-Hydroxyphenylazo)-Uracil on SPP1 DNA-Membrane Binding	95

LIST OF FIGURES

1.	CsCl gradient purification of $^3\text{H}/^{32}\text{P}$ double-label SP82G bacteriophage	18
2.	Distribution of infecting SP82G DNA from sheared lysates on Renografin gradients	32
3.	Distribution of infecting SP82G DNA after treatment with DNase/Phosphodiesterase	34
4.	Distribution of infecting SP82G DNA after Protease treatment	37
5.	Distribution of infecting SP82G DNA after Sarkosyl treatment	39
6.	Distribution of infecting SP82G DNA after phospholipase C treatment	41
7.	Electron micrograph A of rapidly-sedimenting SP82G DNA	44
8.	Electron micrograph B of rapidly-sedimenting SP82G DNA	46
9.	Change in the amount of infecting SP82G DNA found in the membrane complex with time	48
10.	Effect of rifampicin and actinomycin D on ^3H -uridine incorporation	51
11.	Effect of metabolic inhibitors on the time course of SP82G DNA-membrane binding	53
12.	Effect of NaCN on the time course of SP82G DNA-membrane binding	56
13.	Stabilization of membrane-complexed DNA by PMSF	58
14.	RNase sensitivity of membrane-complexed SP82G DNA	61
15.	Distribution of infecting SP82G DNA from sheared minicell lysates on Renografin gradients	66
16.	Effect of gradient shape on the distribution of infecting SP82G from sheared minicell lysates	68

17.	Distribution of lysed and unlysed SP82G-infected minicells on Renografin gradients	71
18.	Time course of SP82G DNA-membrane binding in minicells and minicell-producer <u>B. subtilis</u>	74
19.	Distribution of infecting ³ H/ ³² P double-label SP82G from sheared lysates on Renografin gradients	82
20.	Effect of Sarkosyl on the sedimentation behavior of ³ H/ ³² P double-label SP82G DNA	84
21.	Distribution of infecting SPP1 DNA from sheared lysates on Renografin gradients	88
22.	Distribution of infecting SPP1 DNA after treatment with various chemical agents	90
23.	Effect of chloramphenicol and rifampicin on the distribution of infecting SPP1 DNA	94
24.	Distribution of newly-incorporated ¹⁴ C-thymine in SPP1-infected <u>Bacillus subtilis</u> lysates on Renografin gradients	98
25.	Distribution of labeled SPP1 and host DNAs in SPP1-infected <u>Bacillus subtilis</u> lysates on Renografin gradients	100

ABSTRACT

BIOCHEMICAL AND GENETIC ANALYSIS OF THE FORMATION
OF MEMBRANE-COMPLEXED DNA FOLLOWING
INFECTION OF BACILLUS SUBTILIS BY BACTERIOPHAGE

by

PHYLLIS SANDRA KOHLER

The attachment of viral DNA to a host cell component in SP82G-infected Bacillus subtilis was investigated by means of the Renografin density gradient technique. The host cell component has been shown to be membrane by virtue of its sensitivity to Protease, Sarkosyl and phospholipase C, and by examination of electron micrographs of the isolated complex.

The amount of infecting DNA in the membrane complex varies during the growth cycle of the phage. Three distinct phases were observed: an increase in the amount of viral DNA bound to the membrane, followed by a release of that DNA from membrane association except for a small, constant amount of viral DNA which, in the third phase, remains membrane-associated. The sensitivity of this time course to chloramphenicol, rifampicin, actinomycin D, nalidixic acid and NaCN has been studied. The complex is formed in the presence of chloramphenicol or nalidixic acid, but not in the presence of rifampicin, actinomycin D or NaCN. This suggests that RNA synthesis is necessary for the forma-

tion of the DNA-membrane complex, but that protein synthesis is not. The lack of nalidixic acid sensitivity and the time of initiation of SP82G DNA synthesis suggest that DNA synthesis is also not essential in this process. During the formation of the membrane complex, at least 40% of the complexed material becomes RNase sensitive (from 3 to 6 minutes after infection). This RNase sensitivity is transitory and, prior to the formation of maximum DNA-membrane complex, all of the complexed material becomes resistant to RNase. This suggests that the physical presence of an RNA molecule is necessary to maintain the integrity of the DNA-membrane complex during its formation; the RNA-DNA linkage must later be replaced by some other type of linkage from 7-9 minutes after infection.

The detachment of SP82G DNA from the membrane complex is inhibited by chloramphenicol and nalidixic acid. Infection of DNA synthesis-deficient Bacillus subtilis minicells by SP82G bacteriophage indicates that the phage DNA binds to the minicell membranes, but is not released. DNA-membrane binding in minicells showed chloramphenicol and rifampicin sensitivities similar to the DNA-membrane binding in normal B. subtilis SB-1 cells.

Genetic analysis of the infecting DNA associated with the membrane shows a trend which suggests that the right (last-entering) end of the molecule is the first to complex to the membrane. The unbound portions of the DNA subsequently become bound, probably in the order of right to left.

Infection experiments with SP82G bacteriophage labeled both in the protein and the DNA suggest that there is a phage protein(s) which enters the host with the viral DNA and co-sediments with the DNA-membrane

complex but not with the unbound DNA. Following treatment of the infected lysate with Sarkosyl, the protein label sediments at the top of the Renografin gradient.

SPP1 Experiments

Analysis of SPP1 bacteriophage DNA after phage infection of Bacillus subtilis indicates that SPP1 DNA also becomes associated with a host cell component. The host cell component is membrane by virtue of its sensitivity to Protease, Sarkosyl and phospholipase C. The binding process is insensitive to chloramphenicol and therefore does not require de novo protein synthesis, but is sensitive to rifampicin and hence does require RNA synthesis. 6-(p-Hydroxyphenylazo)-uracil (HPUra), which completely inhibits SPP1 DNA synthesis, has no effect on DNA-membrane binding.

INTRODUCTION

The efficient organization of metabolic functions is of fundamental importance to the viability of an organism. The bacterial cell membrane plays numerous roles in a variety of cellular activities and appears to be a "central organizer" in the procaryotic cell.

In 1963, Jacob, Brenner and Cuzin proposed the "replicon model" to explain the process of bacterial DNA replication and segregation. In this model, the genetic material is attached to the cell membrane. In effect the membrane acts as a primitive mitotic apparatus and growth of the membrane after DNA replication facilitates the separation of daughter genomes, thereby insuring accurate inheritance. In support of this idea, DNA-membrane interactions specifically involved in bacterial DNA replication have been identified and studied in recent years in, for example, Bacillus subtilis (Ganesan, 1968; Ganesan and Lederberg, 1965; Sueoka and Quinn, 1968; Ivarie and Pene, 1970; Ivarie and Pene, 1973), Bacillus megaterium (Tremblay et al., 1969) and Escherichia coli (Smith and Hanawalt, 1967; Earhart et al., 1968; Dworsky and Schaecter, 1973; Niveleau, 1974).

A possible role for the bacterial cell membrane in the replication of bacteriophage DNA during phage infection was first proposed in 1968 when Gilbert and Dressler presented the "rolling circle" model for phage DNA replication. This model proposes that the 5' end of one of the template bacteriophage DNA strands becomes anchored to the host cell membrane. Transfer of this exposed 5' end to a membrane

site would be necessary to prevent the repair of a nick, essential to the replication of the DNA, by DNA ligase enzyme. In addition, Ganesan (1968) demonstrated the association of the B. subtilis replicative enzymes with the cell membrane. This suggested a further reason for suspecting a direct involvement of specific membrane sites in bacteriophage DNA replication, assuming these bacterial enzymes proved to be involved in the replication of some bacteriophage DNA, or if specific phage-coded DNA polymerases associated with the membrane in a similar manner. The idea that the host cell membrane might play a role in the replication of the viral genome encouraged further research into phage DNA-membrane associations. However, in spite of many subsequent studies, the subject is still at an early stage of development.

In all systems examined so far, phage DNA attaches to the host membrane at some point(s) during the growth cycle of the phage. As will be mentioned later in this Introduction, not all of this attachment is at sites active in bacteriophage DNA replication. With rare exception the early steps involved in the formation of the DNA-membrane complex have not been examined in detail. Many important questions have been studied rather incompletely, or not at all. For example, the exact nature of the bonds involved in maintaining the integrity of the DNA-membrane complex remains unknown and hence there are no rigorous criteria to define a DNA-membrane complex. For most phage, it is not known whether a specific region of the viral genome and/or host membrane are involved. Is membrane attachment obligatory for the DNA replication of certain phages, but not others?

Nevertheless, a variety of observations with different phages

suggest substantial variability in the requirements for the initial binding process. The variety of modes of formation of phage DNA-membrane complexes by different phages warrants their examination on a case by case basis. The remainder of this Introduction will present information on DNA-membrane associations which has been derived from the more well-studied phage systems. A summary of this material can be found in Table 1. In conclusion, the scope of this thesis work will be discussed.

BACTERIOPHAGE T₄

Participation of host membrane in the replication of coliphage T₄ has been studied extensively. The early experiments of Kozinski and Lin (1965) and Frankel (1966) suggested that soon after T₄ infection, the linear viral DNA duplex attaches to a host cell component, where it replicates. Earhart (1973) has shown that synthesis of T₄ DNA begins at about six to seven minutes after infection, and that prior to the onset of DNA replication, over 80% of the parental viral DNA can be found in association with membrane. The formation of the DNA-membrane complex is sensitive to NaCN and rifampicin and thus requires ATP and RNA synthesis (Earhart, 1973); there have been conflicting reports as to whether the complex itself is sensitive to RNase (Earhart, 1973; Miller, 1972). The binding of T₄ DNA to host membrane is not dependent upon de novo viral protein or DNA synthesis (Earhart, 1973). The RNA synthesis requirement is suggestive of the reported role of the synthe-

sis of a primer RNA in the initiation of DNA replication. This model of initiation of de novo DNA synthesis was proposed originally by Brutlag, Schekman and Kornberg (1971). In support of this model for the initiation of T₄ DNA synthesis, in 1972 Buckley et al. isolated an RNA-DNA copolymer from T₄-infected E. coli. The RNA and DNA were found to be covalently linked and in the same strand. In hybridization experiments, they found that the copolymer DNA specifically annealed to the left strand of T₄ DNA and that the DNA portion of the copolymer is single stranded. Direct involvement of the host in the formation of this copolymer has been suggested.

The finding that there is a stimulation of membrane-associated DNA polymerase activity after T₄ infection (Frankel et al., 1968) lends support to the idea that the end purpose of the DNA-membrane association is the replication of T₄ DNA. In some very elegant genetic experiments (Mosig, 1970; Marsh et al., 1971) the origin of replication on T₄ DNA was found to lie in the region of gene 43 (coding for the T₄ DNA polymerase) and, further, this origin is preferentially associated with the cell membrane. Finally, in pulse label experiments (Siegel and Schaecter, 1973) newly synthesized (i.e., progeny) DNA was found exclusively in association with the membrane.

From 12 to 14 minutes after T₄ infection, there occurs a release of approximately 50% of the parental viral DNA and 85% of the progeny viral DNA (Earhart, 1973; Siegel and Schaecter, 1973) from membrane association. It has been determined (Earhart, 1970; Earhart, 1973) that membrane detachment is a late phage function and that late protein synthesis is required; the gene products involved in T₄ head formation

and DNA packaging are also necessary (Siegel and Schaecter, 1973). It has been suggested that T_4 DNA is replicated and then packaged in a partially complete capsid while associated with a particular membrane site (Siegel and Schaecter, 1973).

BACTERIOPHAGES T_7 AND \emptyset II

The coliphage T_7 contains a linear DNA duplex of molecular weight 24.3×10^6 daltons (Sober, 1968). At early times after T_7 infection, the parental viral DNA can be found in two rapidly-sedimenting complexes (Center, 1972). It has been suggested that Intermediate I, sedimenting at approximately 1500 S, represents a complex of T_7 DNA and host membrane (Center), however proof for this proposal is far from conclusive. The function and nature of Intermediate II (sedimenting 2-3 times faster than mature T_7 DNA [32 S]) is not known. The formation of Intermediate I in T_7 , like the membrane-association of T_4 phage, does not require de novo protein synthesis (Center, 1972). RNase has no effect on the integrity of the membrane complex (Center, 1972). At approximately 11 minutes after T_7 infection, pulse-labeled DNA is released from the membrane complex (Center, 1972); the significance of the dissociation is not known.

\emptyset II bacteriophage is a female-specific coliphage which is reported to be closely related to phage T_7 (Siegel and Schaecter, 1973). By 3 minutes after \emptyset II infection at 37°C, approximately 70% of the infecting phage DNA can be found in a complex with the host cell

membrane (Linial and Malamy, 1970). Between 5 and 8 minutes after infection, this DNA leaves the complex so that eventually only 10% of it remains membrane-associated. It seems likely that either phage DNA synthesis and/or protein synthesis is required for the detachment phenomenon since ϕ II infection of a male host, resulting in a cessation of all protein and DNA synthesis by 4 to 6 minutes after infection, leads to the normal, initial DNA-membrane binding, but no detachment occurs (Linial and Malamy, 1970).

BACTERIOPHAGE M13

The bacteriophage M13 is a rod-shaped filamentous coliphage. Its genetic material (molecular weight 2×10^6 daltons) is single stranded (ss). The pattern of DNA replication for this phage is similar to that displayed by the more well-known ϕ X174; that is to say, parental ss ----> parental replicative form (RF) ----> progeny RF ----> viral ss.

There is not an extensive amount of information known relative to M13 DNA-membrane binding but, briefly, the following pattern is emerging. By 5 minutes after M13 infection, 85-90% of the intracellular parental phage DNA molecules are found in a reversible association with the host membrane (Kluge et al., 1971; Forsheit and Ray, 1971). De novo protein synthesis is not an essential event in the binding process (Brutlag et al., 1971). An intriguing aspect in the binding of infecting M13 DNA to membrane is that a minor coat protein (the gene

3 product) enters the host in association with the viral genome and seems to play a role in the complex formation (Jazwinski et al., 1973). Search for proteins like the M13 gene 3 product in other systems has not generally been fruitful (Siegel and Schaecter, 1973). M13 progeny RF DNA replication does occur on the membrane (Forsheit and Ray, 1971), but the question as to whether ss viral DNA synthesis is membrane-associated is still in dispute (Siegel and Schaecter, 1973; Forsheit and Ray, 1971). The parental viral DNA strand is released from the membrane complex but the rate and degree of detachment is highly host strain-dependent. It has been suggested that this dependency reflects differences in the interactions of parental M13 DNA with the host membrane (Kluge et al., 1971).

BACTERIOPHAGE ϕ X174

During infection of a susceptible E. coli host, bacteriophage ϕ X174 injects its single-stranded DNA molecule into the cell, where host enzymes synthesize a complementary strand. This double-stranded replicative molecule (RF I) associates with a site on the membrane wherein it acquires a nick in one strand (RF II) and replicates semi-conservatively. The parental viral strand remains membrane-associated while the progeny RF molecules are released into the cell cytoplasm where they will serve as templates for the synthesis of progeny single-stranded viral DNA molecules. De novo bacteriophage protein synthesis is not required for membrane attachment of RF I, but it has

been suggested (Jazwinski et al., 1973) that a protein similar to the M13 gene 3 product may play a role in the initial binding. A unique aspect of the ϕ X174 system is that there is no detachment of infecting parental DNA from the membrane (Knippers and Sinsheimer, 1968).

A major effort has been made to elucidate the nature of the bonds involved in the ϕ X174 DNA-membrane complex. The approach has been the treatment of the complex with a variety of potentially disruptive agents. The following destroy the complex: ionic detergents (Knippers and Sinsheimer, 1968; Loos et al., 1971), phenol (Knippers and Sinsheimer, 1968; Loos et al., 1971; Salivar and Sinsheimer, 1969), and alkali (Knippers and Sinsheimer, 1968; Loos et al., 1971; Salivar and Sinsheimer, 1969). These agents do not disrupt the complex: high salt (Knippers and Sinsheimer, 1968; Loos et al., 1971; Salivar and Sinsheimer, 1969), nonionic detergents (Loos et al., 1971; Salivar and Sinsheimer, 1969) and microbial lipase (Loos et al., 1971). There are conflicting results with pronase sensitivity (Siegel and Schaecter, 1973) and this, in conjunction with the lack of lipase sensitivity, is hard to reconcile with the fact that membrane is primarily composed of protein and lipid.

BACTERIOPHAGE LAMBDA

Conflicting results concerning the role of the host membrane in lambda DNA replication seem to be a rule of thumb. Indeed, the only piece of data that appears to be universally accepted is that during

virulent lambda infection of E. coli, the viral DNA becomes associated with the host membrane. Most of the discrepancy seems to be methodology-related. However, the following picture can be constructed. When lambda DNA is injected into the host, the viral genome will associate with the membrane only when there are no active lambda repressor molecules present so that the rightward operon is derepressed (Hallick et al., 1969; Kolber and Sly, 1971). DNA replication is not required for the formation of the DNA-membrane complexes (Hallick et al., 1969) but transcription (Shuster, 1974) as well as the product of lambda gene N is required (Hallick et al., 1969; Kolber and Sly, 1971; Hallick and Echols, 1973). The N gene product, which is an early function, stimulates transcription and is required for the synthesis of early and late proteins. The level at which the N product acts on DNA-membrane binding is as yet unknown. There does seem to be synthesis of viral progeny DNA on the membrane and detachment of parental DNA from the membrane occurs (Siegel and Schaecter, 1973). Nothing further is known, however, about the fate of lambda DNA with respect to the membrane during vegetative replication of the phage.

BACTERIOPHAGE Ø29

With the exception of the small bacteriophage Ø29, DNA-membrane association has not been studied for the virulent phages of Bacillus subtilis. Ø29 is a small, thymine-containing B. subtilis phage whose DNA duplex has a molecular weight of 11×10^6 daltons. The DNA-membrane

binding pattern found in this phage system is quite different from that generally found in the coliphage systems examined. After injection of $\phi 29$ DNA into the host, no membrane association of the viral genome occurs until approximately ten minutes after infection whereupon $\phi 29$ DNA synthesis begins and one can find the parental DNA in a membrane complex (Ivarie and Pene, 1973). The amount of bound $\phi 29$ DNA is proportional to the degree of viral DNA synthesis taking place. Maximum membrane binding occurs around 30 minutes after $\phi 29$ -infection; lysis is at 35 minutes after infection (Sober, 1968). De novo phage protein synthesis, RNA synthesis and DNA synthesis are all prerequisites for the binding event (Ivarie and Pene, 1973). Marker rescue experiments revealing that the left-most and right-most $\phi 29$ markers are not rescuable in membrane-bound $\phi 29$ DNA, suggest that the $\phi 29$ DNA may circularize on the membrane (M. Inciarte, personal communication). Pulse-chase experiments indicate that newly synthesized $\phi 29$ DNA is preferentially membrane-associated (Ivarie and Pene, 1973). Detachment of membrane-bound DNA does occur. Experiments with a thermosensitive mutant of phage $\phi 29$, TS35, indicated that membrane binding of the mutant phage DNA does not occur at the restrictive temperature (Ivarie and Pene, 1973). It has been suggested that the TS35 gene product is involved in the binding of the viral genome to the host membrane (Ivarie and Pene, 1973). However, in view of the fact that the TS35 mutation is phenotypically DNA synthesis-negative, it is not clear whether the lack of DNA-membrane binding is a result of the lack of a particular gene product or the total lack of de novo DNA synthesis. Nevertheless, it is conceivable that the TS35 gene product will be found to function in a manner similar to the lambda N gene product.

BACTERIOPHAGE P22

Information on the role of the host cell membrane in the replication of the temperate Salmonella typhimurium phage, P22, is obscured by the fact that identification of the host cell component involved as being unequivocally cell membrane has never been made. In 1968, it was suggested that Intermediate I, the "replication complex" into which P22 DNA first enters after lytic infection, was a DNA-membrane complex (Botstein and Levine, 1968). However, the actual presence of membrane, or even membrane-like material, in this complex was never proven. In fact, the host component in Intermediate I may be the cell envelope and not membrane at all (Siegel and Schaecter, 1973).

Intermediate I is only formed in the absence of P22 repressor protein (Levine et al., 1970). The above two phenomena mirror the early events in lambda DNA-membrane complex formation. Intermediate I formation is, in part, a phage function under the control of the immunity system (Botstein and Levine, 1968). Binding does not require DNA synthesis but does require RNA synthesis (Botstein and Levine, 1968). Intermediate I is enriched for newly synthesized P22 DNA (Levine et al., 1970) and parental and newly synthesized DNA begin to disappear from the complex at approximately 20 minutes after infection, or about the time that late phage functions are initiated (Botstein and Levine, 1968). Experiments with a thermosensitive mutant of P22, ts 12.1, which fails to initiate late phage functions, indicated that there was no detachment of P22 DNA from Intermediate I, suggesting that detachment is a late phage function (Botstein and Levine, 1968).

In conclusion of this review of viral DNA-membrane interactions, Table 1 organizes all major information on the common phenomena studied in these various phage systems. Table 1 also presents information on the membrane binding of SP82G DNA and SPP1 DNA. Table 2 lists some distinguishing biological properties of these two bacteriophages.

It is the primary objective of this thesis to investigate the events involved in the formation of the DNA-membrane complex during SP82G infection of Bacillus subtilis. Requirements for, and a model of that binding will be the subject of this thesis. Information on DNA-membrane binding after infection with the smaller B. subtilis phage SPP1 was the result of two joint research ventures with Dr. John N. Reeve (Max Planck Institut fur Molekulare Genetik, West Berlin, Germany). That data will be presented in this thesis as an addition to the main research.

Table 1. SUMMARY OF CHARACTERISTICS OF KNOWN DNA-MEMBRANE ASSOCIATIONS

Phenomenon	Bacteriophage								
	T4	T7,ØII	M13	ØX174	lambda	P22	Ø29	SP82G	SPP1
Attachment of parental DNA	Y	Y	Y	Y	Y ^a	Y ^a	Y	Y	Y
Requirements for binding:									
<u>de novo</u> protein synthesis	N	N	N	N	Y	Y	Y	N	N
<u>de novo</u> RNA synthesis	Y	-	-	-	Y	Y	Y	Y	Y
phage DNA synthesis	N	N	N	N	N	N	Y	N	N
Sensitivity of complex to RNase	Y	N	-	-	-	-	-	Y	-
Detachment of parental DNA (fraction detached)	Y (1/2)	Y	Y (1/4)	N	Y	Y (all)	-	Y (3/4)	Y
Requirements for detachment of DNA from membrane:									
phage DNA synthesis	Y	P	Y	-	-	-	-	Y	-
phage protein synthesis	Y	Y	Y	N	Y	Y	-	Y	-
Synthesis of viral progeny DNA on membrane	Y	Y	Y	N	Y	Y	Y	-	-
Area of the genome initially bound to membrane	0	-	-	-	-	-	P both ends	right end	-

abbreviations: Y= yes N= no P= probably 0= origin of replication - = unknown

a= only in absence of repressor

Table 2. SOME BIOLOGICAL PROPERTIES OF
BACTERIOPHAGES SP82G AND SPP1

	Bacteriophage	
	SP82G	SPP1
Size (Å)		
Head	1000 x 1000	450 x 450
Tail	200 x 1650	65 x 1400
Type of infection	Virulent	Virulent
Latent period (37 C)	40 min.	40 min.
Burst size (37 C)	100-200	100-300
Molecular weight of DNA (x 10 ⁶ daltons)	115	25
Base composition of DNA (% GC)	43	43
Unusual bases in DNA	HMU replaces T	No

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions and Bacteriophage Preparations

Techniques for the isolation, growth and assay of bacteriophage SP82G are identical to those of Green (1964). Bacillus subtilis strain SB-1 (his_1^- , try_2^-) was the host cell for all experiments. The media for bacterial growth was Nomura salts (NM) (Nomura et al., 1962) supplemented with 0.5% glucose, 0.2% casein hydrolysate, 2×10^{-3} M $MgCl_2$, 0.1% yeast extract, 0.05 mg/ml D,L-tryptophan, 2 mg/ml arginine and 0.2 mg/ml L-histidine.

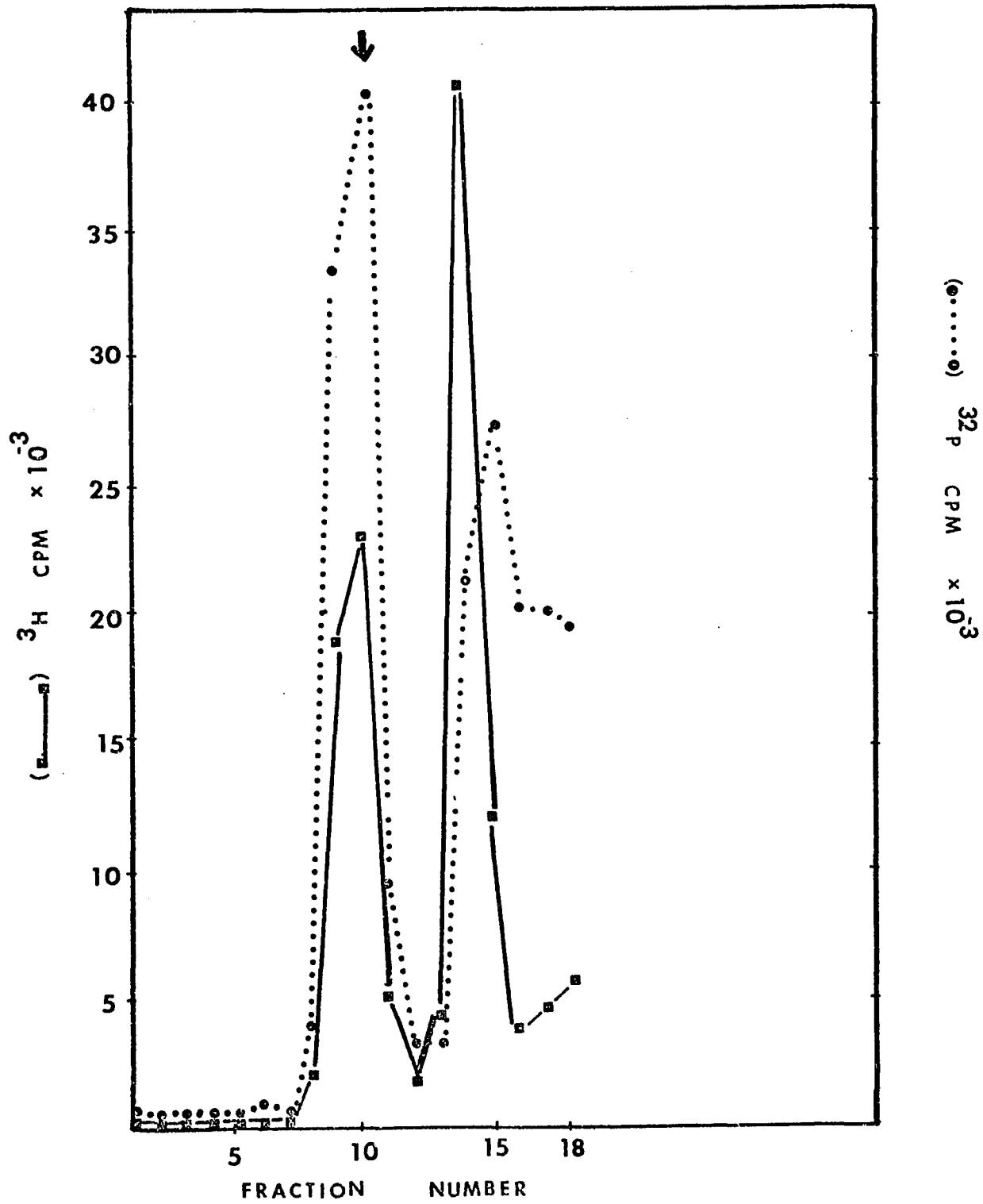
For preparation of 3H -adenine labeled SP82G bacteriophage, Bacillus subtilis strain G-1 (pur^-) isolated from gamma-irradiated spores of B. subtilis strain 168, was the host cell used. Bacteria were grown to approximately 10^8 cells/ml in NM supplemented with 0.2% casein hydrolysate, 2×10^{-3} M $MgCl_2$ and 5 ug/ml adenine. 3H -adenine (New England Nuclear, Boston, Mass.; 200 mCi/mg) was added to a concentration of 1 mCi/liter along with SP82G bacteriophage at a multiplicity of infection (moi) of 0.1. Phage lysates were concentrated by the method of Yamamoto et al. (1970). The crude phage solutions, in approximately 4-5 ml NM salts which was 2×10^{-3} M in $MgCl_2$ and 10^{-3} M in EDTA, were layered onto pre-formed CsCl gradients consisting of 1.0 ml layers of CsCl with densities of 1.65, 1.60, 1.55, 1.50, 1.46, 1.42 and 1.38 gm/ml and centrifuged at $37,000 \times g$ for 30 minutes in an SS-34 rotor in a Sorvall RC2B refrigerated centrifuge. The purified phage were dialyzed against 1X NM salts, 2×10^{-3} M $MgCl_2$ and 10^{-3} M EDTA and stored at $4^\circ C$.

For preparation of $^3\text{H}/^{32}\text{P}$ double label SP82G bacteriophage, Bacillus subtilis SB-1 cells were grown at 37°C to a cell concentration of approximately 5×10^7 cells/ml in media (NM) without the phosphate salts and supplemented with 0.5% glucose, 2×10^{-3} M MgCl_2 , 0.05 mg/ml D,L-tryptophan, 0.2 mg/ml L-histidine and 0.2% casein hydrolysate. $\text{H}_3^{32}\text{PO}_4$ (25 mCi/ml in 0.02 M HCl; New England Nuclear, Boston, Mass.) was added to the media to a final concentration of 1.5 mCi/100 ml. After a 10 minute incubation with shaking, SP82G bacteriophage were added to an moi of approximately 1. Following a further 10 minute incubation, a mixture of $^3\text{H}(\text{G})$ -labeled L-amino acids (New England Nuclear, Boston, Mass.) was added to a concentration of 2 mCi/100 ml, and incubation with shaking at 37°C was continued until lysis was complete. Concentration and purification of the $^3\text{H}/^{32}\text{P}$ -labeled phage stock was identical to that for the ^3H -adenine labeled SP82G, except that the CsCl step gradients (4.0 ml) were made up in 1/2" x 2" cellulose nitrate tubes (Beckman Inc., Palo Alto, Cal.). One ml of concentrated phage stock was layered on each gradient, and sedimentation was in an SW65 rotor in the Beckman L2-65B ultracentrifuge (Beckman Inc., Palo Alto, Cal.) at 23°C and 30,000 RPM for 30 minutes. Tubes were punctured from the bottom and fractions collected. Aliquots of each fraction were added to 10 ml of an Omnifluor (New England Nuclear, Boston, Mass.) solution (5.6 g/liter in a toluene-Triton X-100 [2:1] base) in scintillation vials and counted in a Nuclear Chicago Mark II Liquid Scintillation Counter. The appropriate fractions were pooled (Fig. 1) and the purified phage dialyzed overnight against 2 liters

Fig. 1. CsCl gradient purification of $^3\text{H}/^{32}\text{P}$ double-label SP82G bacteriophage

Experimental procedure described in Materials and Methods.

^{32}P (●.....●) and ^3H (■——■) radioactivity are shown for each fraction. Direction of sedimentation is from right to left. Arrow (↓) denotes area containing intact infective SP82G bacteriophage.



of 1X NM salts plus MgCl_2 (2×10^{-3} M) and EDTA (10^{-3} M). Phage preparations were stored at 4°C for no longer than two weeks.

For all infection experiments, overnight cultures of B. subtilis diluted 1:10 into fresh NM media were grown to a concentration of 6×10^7 cells/ml at 37°C . The cells were concentrated ten-fold by centrifugation and infected with SP82G, labeled in the DNA with ^3H -adenine, at an approximate moi of 5. After a 2 minute adsorption period, the preparations were diluted to the original cell concentration with fresh warm NM and incubated for the designated amount of time. At the appropriate time, 5.0 ml samples were withdrawn, and the infection process halted by rapid cooling in a dry ice/acetone bath; thereafter, all samples were kept at 4°C .

Cell Lysis

The lysis procedure was essentially that of Ivarie and Pene (1970). All glassware was pretreated with a silicone solution (Siliclad; Clay Adams, Parsippany, N.J.) to prevent adsorption. 5.0 ml samples of infected bacteria were centrifuged at $12,000 \times g$ for 10 minutes at 4°C in a Sorvall RC2B centrifuge to collect all cells. The cells were washed 2 times with lysis buffer (0.01 M sodium citrate, 0.12 M potassium phosphate, pH 7.4) and resuspended in lysis buffer at a four-fold increase in concentration. Actinomycin D (Merck, Sharpe and Dohme, Rahway, N.J.) was added to a concentration of 4 ug/ml followed by the addition of lysozyme (Miles-Seravac (PTY), Berks, Eng.) to a final concentration of 0.5 mg/ml. Samples were incubated at 37°C for 10 minutes; lysis was halted by means of rapid cooling on ice. All lysates were then made 0.1 M in NaCl, placed in 16 mm diameter tubes, and sheared on a Vortex mixer

at setting number six for 20 seconds in order to mildly shear the DNA, thereby maximizing sedimentation differences between the bound and unbound DNA in the Renografin gradient.

Renografin Density Gradients

The technique used to differentiate the bound from the unbound DNA is basically that developed by Ivarie and Pene (1970). This involves the use of linear Renografin gradients in which high molecular weight complexes, such as membrane-bound DNA, come to equilibrium density before smaller components such as unbound DNA.

Renografin-76 (76% solution of methyl glucamine N,N'- diacetyl - 3,5 diamino - 2, 4, 6 - triiodobenzoate containing 0.32% sodium citrate, 0.04% disodium ethylene-diaminetetraacetate dihydrate, 0.1% methyl p-hydroxy-benzoate and 0.3% p-hydroxypropylbenzoate, pH 7.0-7.6; E.R. Squibb and Sons, N.Y., N.Y.) was diluted to 38% with phosphate buffer (0.02 M sodium citrate, 0.24 M potassium phosphate, 0.2 M sodium chloride, pH 7.4). Linear 0 to 38% ($\rho = 1.00 \text{ g/cm}^2$ to $\rho = 1.2 \text{ g/cm}^2$) Renografin gradients (17.5 ml) were made at 4°C in cellulose nitrate tubes (5/8" x 4"; Beckman Instruments, Palo Alto, Cal.).

Sedimentation

Samples (0.55 ml) of chilled lysates prepared as described above were layered on the 0 to 38% linear Renografin gradients and centrifuged in a Spinco SW27.1 rotor in an L265B ultracentrifuge (Beckman Instruments, Palo Alto, Cal.) at 27,000 RPM, 2°C for 5 hours. Tubes were punctured from the bottom and 20 drop fractions were collected

directly into scintillation vials. 10 ml of the Omnifluor solution previously described was added along with 1.0 ml of water, and the samples were counted for radioactivity in a Nuclear Chicago Mark II Liquid Scintillation Counter.

Protease Sensitivity

A stock solution of protease (Streptomyces griseus type III, 5.28 mg/ml; Sigma Chemical Co., St. Louis, MO.) was auto-digested for 30 minutes at 37°C; 0.05 ml of this solution was then added to a sheared lysate sample that was removed at 9 minutes after infection (final protease concentration 200 ug/ml). This was incubated at 37°C for 20 minutes. A duplicate 9 minute sample was incubated for the same amount of time without protease (incubation control) and another identical sample was kept at 4°C for the 20 minute incubation period (enzyme control). Incubation was terminated by rapid chilling. Samples (0.55 ml) were then centrifuged on Renografin gradients as described above.

Sarkosyl Sensitivity

Sarkosyl (sodium lauroyl sarcosine; Schwarz/Mann, Orangeburg, N.J.; 0.05 ml of a 26.4% solution) was added to a 9 minute sheared lysate sample prepared as previously described (final Sarkosyl concentration 1%). This sample was incubated at 37°C for 20 minutes; a duplicate sample without Sarkosyl (Sarkosyl control) was kept at 4°C during the incubation period. An incubation control as described above was also run. Incubation was terminated by rapid chilling and samples (0.55 ml) were centrifuged on Renografin gradients and fractions were collected as described above.

Phospholipase C Sensitivity

Phospholipase C (phosphatidylcholine:choline phosphohydrolase; I.U.B. 3.1.4.3, Worthington Biochemicals, Freehold, N.J.) was made up to a 20 mg/ml concentration in 0.1 M Tris-chloride, 0.05 M CaCl₂ buffer, pH 7.3. Enzyme solution, 0.1 ml, was then added to a 9 minute sheared lysate sample (final enzyme concentration 1.5 mg/ml) and incubated for 20 minutes at 37°C. An incubation control (duplicate sample but without the enzyme) was incubated simultaneously, and an identical sample without enzyme was kept at 4°C during the incubation period (enzyme control). Incubation was terminated by chilling, followed by centrifugation and fractionation as previously described.

DNase/Phosphodiesterase Sensitivity

Deoxyribonuclease I (beef pancreas deoxyribonucleate oligonucleotidohydrolase; I.U.B. 3.1.4.5, Sigma Chemical Co., St. Louis, MO.) and snake venom phosphodiesterase I (orthophosphoric diester phosphohydrolase, Crotalus Atorx venom, type VII, I.U.B. 3.1.4.1; Sigma Chemical Co., St. Louis, MO.) were added (final concentrations 50 ug/ml and 2.5 ug/ml respectively) to a 7 minute sheared lysate sample and incubated for 2 minutes at 37°C. An incubation control and an enzyme control, as described above, were also included. Incubation was terminated and centrifugation carried out in the usual manner.

Stabilization by Phenyl Methyl Sulfonyl Flouride (PMSF)

Preparation of infected lysates was the same as described earlier.

Samples were removed at 7 minutes after infection. Duplicate lysate samples were incubated for varying amounts of time at 37°C. For each time point, one of the duplicate samples contained phenyl methyl sulfonyl flouride (PMSF) (Sigma Chemical Co., St. Louis, MO.) at a final concentration of 320 ug/ml, and the other sample was a control. After incubation at 37°C, the samples were rapidly chilled and then sedimented on Renografin gradients.

Inhibition of RNA Synthesis

B. subtilis SB-1 cells were grown in NM salts supplemented as previously described to a cell concentration of approximately 6×10^7 cells/ml. Either rifampicin (Mann Research Laboratories, N.Y., N.Y.; final concentration 100 ug/ml) or actinomycin D (Merck, Sharp and Dohme; 50 ug/ml) was added. At various times after addition of the inhibitor, 0.5 ml samples were withdrawn and added to test tubes containing 0.025 uCi of ^3H -uridine (Uridine- ^3H (G), 1 mCi/ml in sterile water; New England Nuclear, Boston, Mass.) in each. Samples were incubated at 37°C for 3 minutes at which time duplicate 0.2 ml aliquots for each time point were added to 3 ml of cold 5% trichloroacetic acid (TCA). Precipitation at 4°C was allowed to proceed for 30 minutes. Precipitates were collected on membrane filters (B-6, Schleicher and Schuell) and washed with 20 ml of cold ethanol (95%). The filters were placed in liquid scintillation vials and dried at room temperature overnight. 10 ml of an Omnifluor-toluene solution (4 g/liter) were added and the radioactivity determined in a Nuclear Chicago liquid scintillation counter.

RNase Sensitivity

Bacteria were infected with SP82G as previously described. At various times after infection, triplicate samples were removed, and sheared lysates were prepared in the usual manner. For each time point after infection, the triplicate lysate samples were treated in the following way. One sample (control) was kept at 4 °C for 2 minutes. To the second sample (incubation control), PMSF was added (final concentration 320 ug/ml) followed by incubation at 37 °C for 2 minutes. To the third sample, RNase A (bovine pancreas RNase A- electrophoretically pure, I.U.B. 2.7.7.16; Worthington Biochemicals, Freehold, N.J.; final concentration 500 ug/ml) and PMSF (final concentration 320 ug/ml) were added, followed by incubation at 37 °C for 2 minutes. After incubation, all lysate samples were quickly chilled and sedimented on Renografin gradients in the usual manner.

Metabolic Inhibitor Studies

(Rifampicin, Chloramphenicol, Nalidixic Acid, NaCN, Actinomycin D)

Bacteria were grown in NM media as described. The inhibitor was added to the growth media and preincubation of the bacteria with the inhibitor for a specific period followed. The final concentrations and preincubation times for each inhibitor tested were as follows: rifampicin (Mann Research Laboratories, N.Y., N.Y.), 100 ug/ml, 25 min; chloramphenicol (Sigma Chemical Co., St. Louis, MO.), 500 ug/ml, 10 min; nalidixic acid (Mann Research Laboratories, N.Y., N.Y.), 75 ug/ml, 10 min; actinomycin D (Merck, Sharp and Dohme, Rahway, N.J.), 100 ug/ml and 50 ug/ml, 10 min; and 0.01 M NaCN (Fischer Scientific, Fairlawn, N.J.),

10 minutes.

Following the designated incubation period, the bacteria were concentrated by centrifugation and infected as previously described; all subsequent work with these samples was done in media with the indicated concentration of inhibitor. At the designated time points, 5 ml samples were withdrawn, washed, lysed and fractionated as was described earlier.

Electron Microscopy

Samples of B. subtilis SB-1 cells taken at 9 minutes after ^3H -SP82G infection were washed, lysed and fractionated on Renografin gradients. An aliquot of each fraction was placed in a scintillation vial along with 10 ml of the Omnifluor-Triton X-100-toluene solution routinely used for scintillation counting, and the radioactivity determined in a Nuclear Chicago Liquid Scintillation Counter. The membrane-bound DNA fractions were pooled and diluted 1:5 into SSC (0.15 M NaCl, 0.015 M sodium citrate). Specimen drops were applied to 200 mesh copper grids with carbon-coated Formvar films and allowed to stand for 1 minute. The excess liquid was then drawn off with a square of roughly-cut blotter paper. Grids were rotary-shadowed with a platinum/palladium mixture and placed in a desiccator overnight. The rotary-shadowing procedure deposited approximately 25-50 Å of metal on each surface. Micrographs were taken with a Phillips EM-200 electron microscope at 40 kV.

Marker Rescue Experiments

Genetic analysis of the phage DNA found in the bound state com-

pared to that in the unbound state was carried out by the marker rescue technique as previously described (Green, 1966). A typical marker rescue experiment would proceed as follows. Competent SB-1 cells (grown in LS-complete media for 90 minutes at 37°C) were pre-infected with the indicated ts mutant SP82G bacteriophage for 2 minutes at 33°C. The cells were diluted 1:10 into fresh LSC and incubated with shaking for 6 minutes. Cells were then exposed to isolated "bound" or "free" phage DNA for 18 minutes at 33°C with shaking. Aliquots of each sample were plated in duplicate at a selective temperature (45°C), at which only wild-type recombinants can give rise to plaques, and the plates scored after incubation overnight at 45°C.

Three ts mutants of SP82G phage, H177-G55 (a close double mutation in the left end of the genome), H167-A4 (a close double mutation in the middle of the genome) and E14-H24 (a close double mutation in the right end of the genome) (Green and Laman, 1972) were used in this study.

"Bound" and "free" SP82G DNA used for these experiments was prepared in the following manner. B. subtilis SB-1 cells were grown and infected with ³H-SP82G (adenine) wild-type phage. At either 4, 7 or 9 minutes after infection, samples were withdrawn and prepared for analysis of membrane-bound DNA in the usual manner. However, shearing at Vortex position 6 was reduced to 10 seconds rather than 20 seconds. The bound and free DNA peak fractions were pooled separately. To the bound sample, Protease was added to a final concentration of 1 ug/ml followed by incubation at 37°C for 10 minutes to release this DNA from the membrane. Both the bound and free samples were then dialyzed overnight against SSC. Part of each sample was then counted for radioactivity. The free DNA was invar-

iably more concentrated (i.e., more CPM) than the bound DNA and therefore was diluted with SSC so that concentrations of DNA in both samples were approximately the same. DNA final concentrations in the range of 0.024-0.04 ug/ml was used in the marker rescue experiments. Concentrations in this range are linear in respect to infective center production (Green, 1966).

Minicell Experiments

All minicell experiments were done in cooperation with Dr. John N. Reeve (Max Planck Institut fur Molekulare Genetik, W. Berlin, Germany) as a joint undertaking.

Techniques for the isolation of minicells by sucrose gradient technology are identical to those of Reeve (1975). The parental cells used in this study as a source of minicells were Bacillus subtilis CU 403 thy⁻ met⁻ div IVB1 (gift from J.N.R.; Reeve et al., 1973).

Minimal media for minicell-producer growth and minicell isolation consists of (in grams per liter): (NH₄)₂SO₄, 2.0; K₂HPO₄, 14.0; KH₂PO₄, 6.0; sodium citrate·2H₂O, 1.0; MgSO₄·7H₂O, 0.2; glucose, 2.0 and was supplemented by the addition of 20 ug/ml of both thymine and methionine. The media for phage infection was NM supplemented in the previously described manner with the addition of 20 ug/ml each of thymine and methionine.

All minicell-phage infection experiments were done at 37°C. Minicells were infected at an input multiplicity of approximately 5, using our standard ³H-labeled SP82G bacteriophage. The infection, sample withdrawal, washing, lysis and Renografin sedimentation regimens

were identical to those described earlier for analysis of membrane-bound DNA using B. subtilis SB-1 as host. The control experiments using the minicell-producer strain B. subtilis CU403 as the host for SP82G infection and analysis of membrane-bound DNA were also done following the usual protocol.

SPPI EXPERIMENTS

All SPPI experiments were done jointly with Dr. John N. Reeve (Max Planck Institut fur Molekulare Genetik, W. Berlin, Germany) as a cooperative project.

Bacterial Strains, Growth Conditions and Bacteriophage Preparations

³H-thymine-labeled SPPI bacteriophage was a gift from Drs. Tom A. Trautner and John N. Reeve. The host bacteria used was Bacillus subtilis strain SB-1 as described previously for SP82G infection experiments. Bacillus subtilis strain VUB 210 try⁻ thy⁻ nal^r nov^r (gift from J.N.R.) was used in the ³H/¹⁴C double label de novo DNA synthesis experiment. The media for growth and infection of the host was MIII salts consisting of (in grams per liter): (NH₄)₂SO₄, 1.32; K₂HPO₄, 1.4; KH₂PO₄, 0.56; sodium citrate·2H₂O, 2.9; and supplemented with (per 200 ml): D,L-tryptophan, 12.0 ml of a 1 mg/ml stock solution; MgSO₄, 0.9 ml of a 20% stock; MnSO₄, 0.15 ml of a 0.1 M stock; yeast extract, 0.8 ml of a 25% stock; FeCl₃, 0.005 ml of a 0.05 M stock; glucose, 3.5 ml of a 20% stock; histidine, 1.0 ml of a 50 mg/ml stock, and casein hydrolysate, 0.375 ml of a 10% solution.

All infection experiments were performed according to the

protocol described earlier in this thesis for SP82G infection. The input multiplicity of infection for SPP1 was approximately 2.

SPP1 DNA-Membrane Complex Analysis

Preparation, lysis and centrifugation of membrane-bound DNA from SPP1-infected *B. subtilis* SB-1 cells on Renografin gradients was identical to the previously described procedure for SP82G-infected cells. In this case, however, lysates (0.55 ml) were fractionated on 13.0 ml linear 0 to 38% Renografin gradients in a Spinco SW41 rotor. Centrifugation was in a Beckman L2-65B ultracentrifuge at approximately 29,000 RPM, 2°C for 4 hours.

Protease, Sarkosyl, Phospholipase C and DNase Sensitivities

Enzyme sources, activation, concentrations, incubation times and temperatures were identical to those described previously in this thesis for work with membrane-bound SP82G DNA. All SPP1 lysate samples were withdrawn at 6 minutes after infection at 37°C.

Chloramphenicol, Rifampicin and HPUra Studies

The protocol for determination of the effect of chloramphenicol and rifampicin on the DNA-membrane binding process was identical to that previously described under Metabolic Inhibitor Studies for SP82G-infection experiments. For 6-(p-hydroxyphenylazo)-uracil (HPUra) sensitivity, the experimental design was also identical, and HPUra (gift from Dr. Asad Ahmad, Max Planck Institut fur Molekulare Genetik) was added 10 minutes before infection at a final concentration of 150 ug/ml.

RESULTS

Characterization of the Membrane Fraction

Ivarie and Pene (1970) showed that DNA from sheared lysates of Bacillus subtilis can be resolved into two components on linear density gradients of Renografin. The rapidly-sedimenting fraction of DNA, which reaches equilibrium quickly, was shown to be DNA in association with membrane. The slower-sedimenting material, determined to be free DNA, is smaller and does not reach iso-density by the time the DNA-membrane complex does. This technique was later shown to be effective in the resolution of membrane-bound and free Ø29 bacteriophage DNA during phage infection (Ivarie and Pene, 1973).

The Ivarie-Pene Renografin technique applied to lysates of cells infected by SP82G for 9 minutes yields the sedimentation pattern shown in Fig. 2. Two distinct peaks are evident, one sedimenting at approximately 75% of the distance down the gradient (rapidly-sedimenting) and one at approximately 28% (slower-sedimenting). At 9 minutes after infection, approximately 38% of the input viral ³H-adenine label is located in the rapidly-sedimenting peak.

To confirm that the radioactivity in the peaks detected on the Renografin gradients had remained in the viral DNA, the effect of a DNase I/ snake venom phosphodiesterase enzyme mixture on the sedimentation behavior of SP82G-infected lysates was examined (see Materials and Methods). As can be seen in Fig. 3, the material in both peaks was reduced to a smaller size by the DNase/phosphodiesterase thus indicating that the radioactivity is contained in DNA.

Fig. 2. Distribution of infecting SP82G DNA from sheared lysates
on Renografin gradients

Logarithmically growing cells (5.7×10^7 /ml) were infected with SP82G bacteriophage labeled in the DNA with ^3H -adenine (moi= 5). After a 9 minute incubation period at 37°C , including a 2 minute adsorption period, 5 ml samples were withdrawn, washed and concentrated. Sheared lysates were prepared as described in Materials and Methods. Samples were centrifuged on linear 0 to 38% Renografin gradients (100% of recovered radioactivity= 5431 counts/minute).

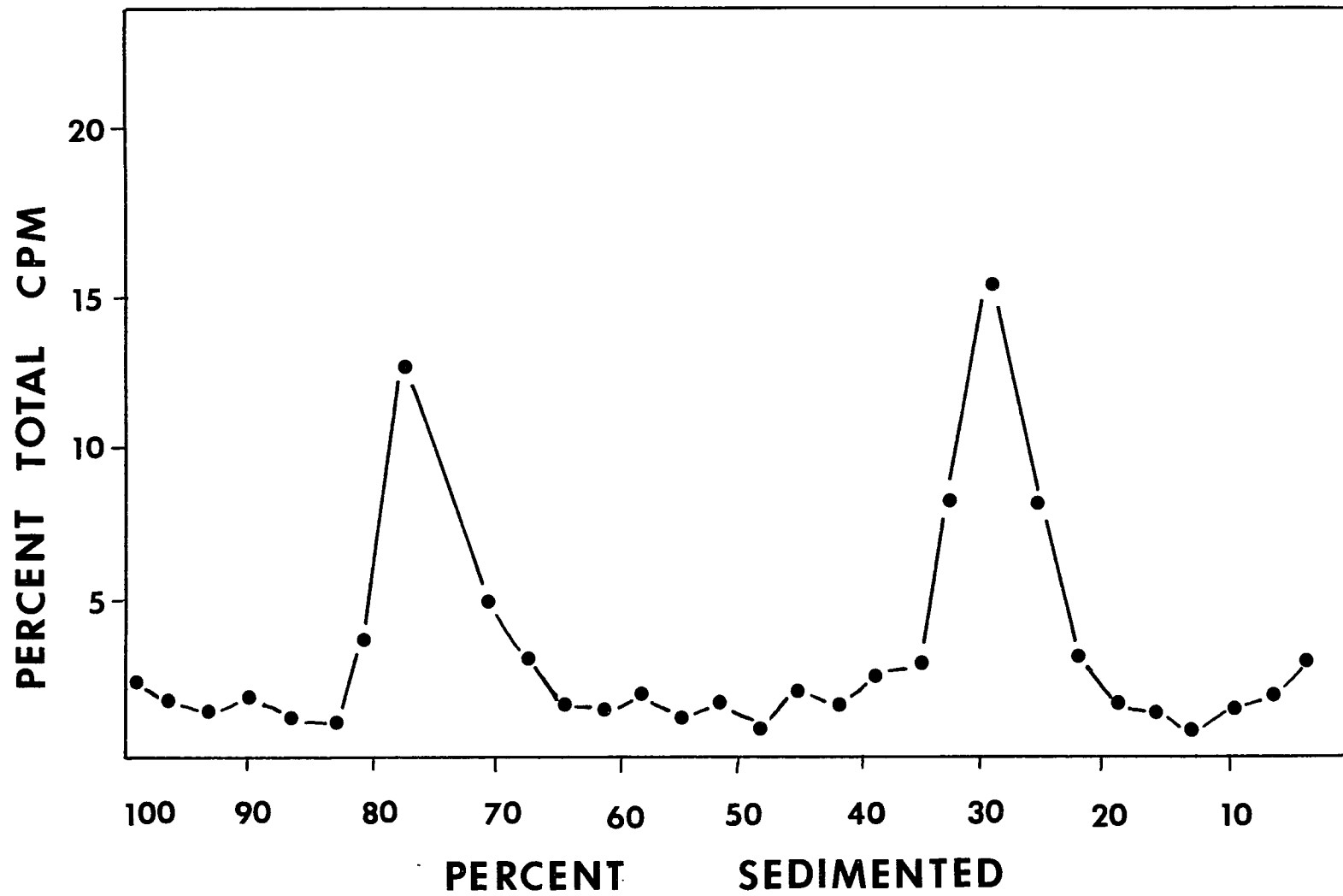
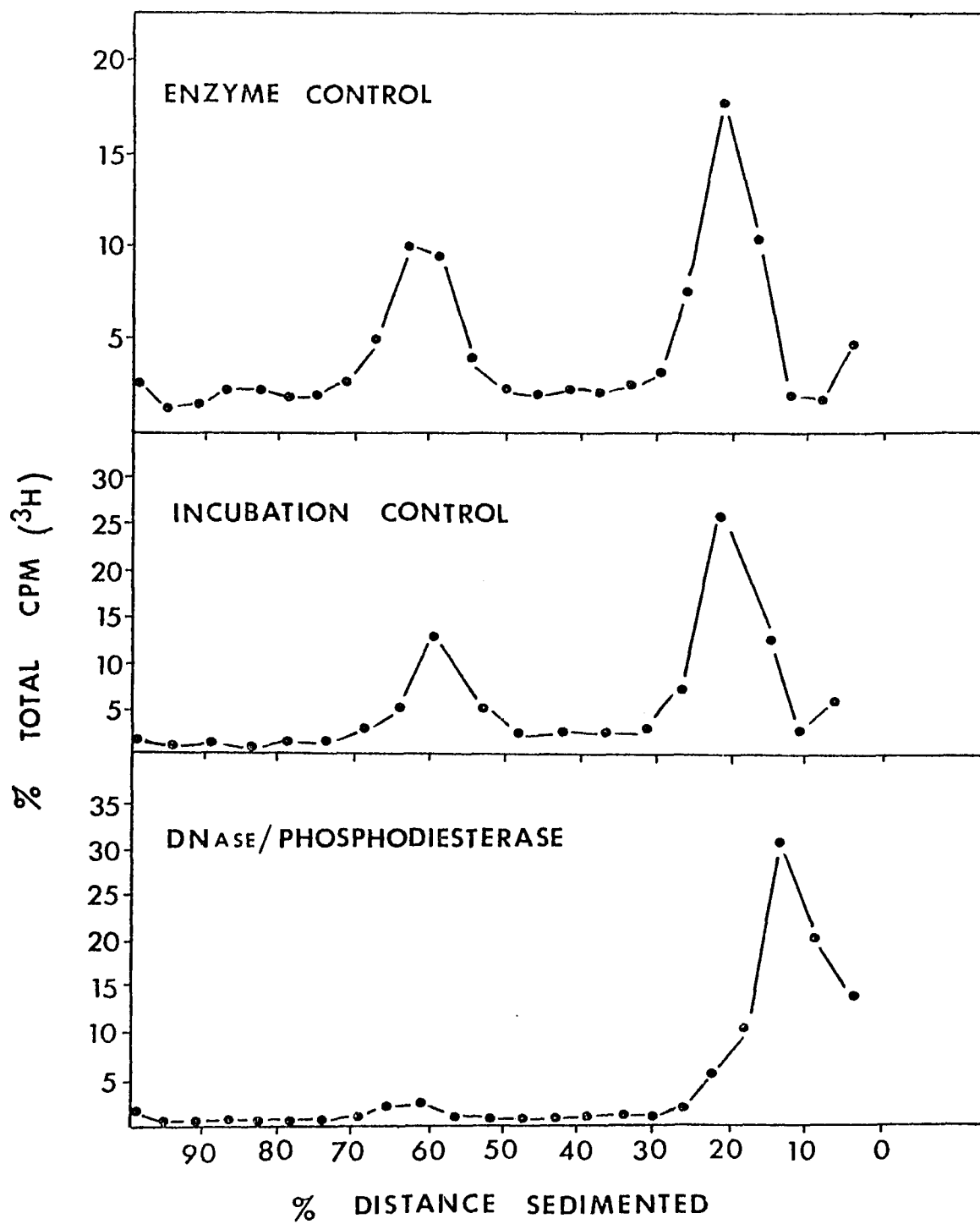


Fig. 3. Distribution of infecting SP82G DNA after treatment with
DNase/phosphodiesterase

Logarithmically growing cells were infected with SP82G bacteriophage (^3H -adenine) at an moi= 5. Three 5 ml samples were withdrawn at 7 minutes after infection and sheared lysates were prepared as described in Materials and Methods. To one sample, a DNase I/snake venom phosphodiesterase enzyme mixture was added (final concentrations 50 ug/ml and 2.5 ug/ml respectively) followed by incubation at 37°C for 2 minutes (DNase/phosphodiesterase; 100% of recovered radioactivity= 6251 counts/minute). To the other two samples, no enzyme was added. One of these was kept at 4°C (Enzyme control; 100% of recovered radioactivity= 6785 counts/minute) and the other was incubated at 37°C for 2 minutes (Incubation control; 100% of recovered radioactivity= 7762 counts/minute). Samples were centrifuged as in Fig. 2.



To determine if the rapidly-sedimenting DNA was a DNA-membrane complex, the sensitivity of that fraction to three agents which are capable of destroying the integrity of the host cell membrane was examined. The following agents were tested following the protocols described in Materials and Methods:

1) Protease, a mixture of proteolytic enzymes possessing both tryptic and chymotryptic activities,

2) Sarkosyl, an ionic detergent and therefore a lipid solubilizer, and

3) phospholipase C, which will hydrolyze phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin. Two controls were run for each experiment: the incubation control which was included due to the extreme lability of the DNA-membrane complex at 37°C, and an enzyme control. The results of these experiments are shown in Figs. 4-6. Incubation with Protease, Sarkosyl or phospholipase C causes the rapidly-sedimenting DNA to shift and sediment with the slower-sedimenting DNA. The slower-sedimenting DNA does not appreciably change in sedimentation behavior by treatment with any of these reagents or upon incubation in their absence at 37°C (incubation controls). The response to the enzymes and Sarkosyl suggests that it is a membrane component which differentiates the rapidly-sedimenting from the slower-sedimenting DNA. While this characterization is not definitive, it is consistent with the hypothesis that the associated cellular component is membrane. Henceforth, the rapidly-sedimenting DNA will be referred to as a DNA-membrane complex.

As another means of characterizing the physical nature of the DNA-membrane complex, the material in the rapidly-sedimenting DNA frac-

Fig. 4. Distribution of infecting SP82G DNA after Protease treatment

Logarithmically growing cells were infected with SP82G bacteriophage (^3H -adenine) at a multiplicity of infection of 5. Three 5 ml samples were withdrawn at 9 minutes after infection and sheared lysates were prepared as described in Materials and Methods. To one sample, Protease was added to a final concentration of 100 ug/ml followed by incubation at 37°C for 20 minutes (Protease; 100% of recovered radioactivity= 6153 counts/minute). To the other two samples, no enzyme was added. One of these was kept at 4°C (Enzyme control; 100% of recovered radioactivity= 7706 counts/minute) and the other was incubated at 37°C for 20 minutes (Incubation control; 100% of recovered radioactivity= 6594 counts/minute). Samples were centrifuged as in Fig. 2.

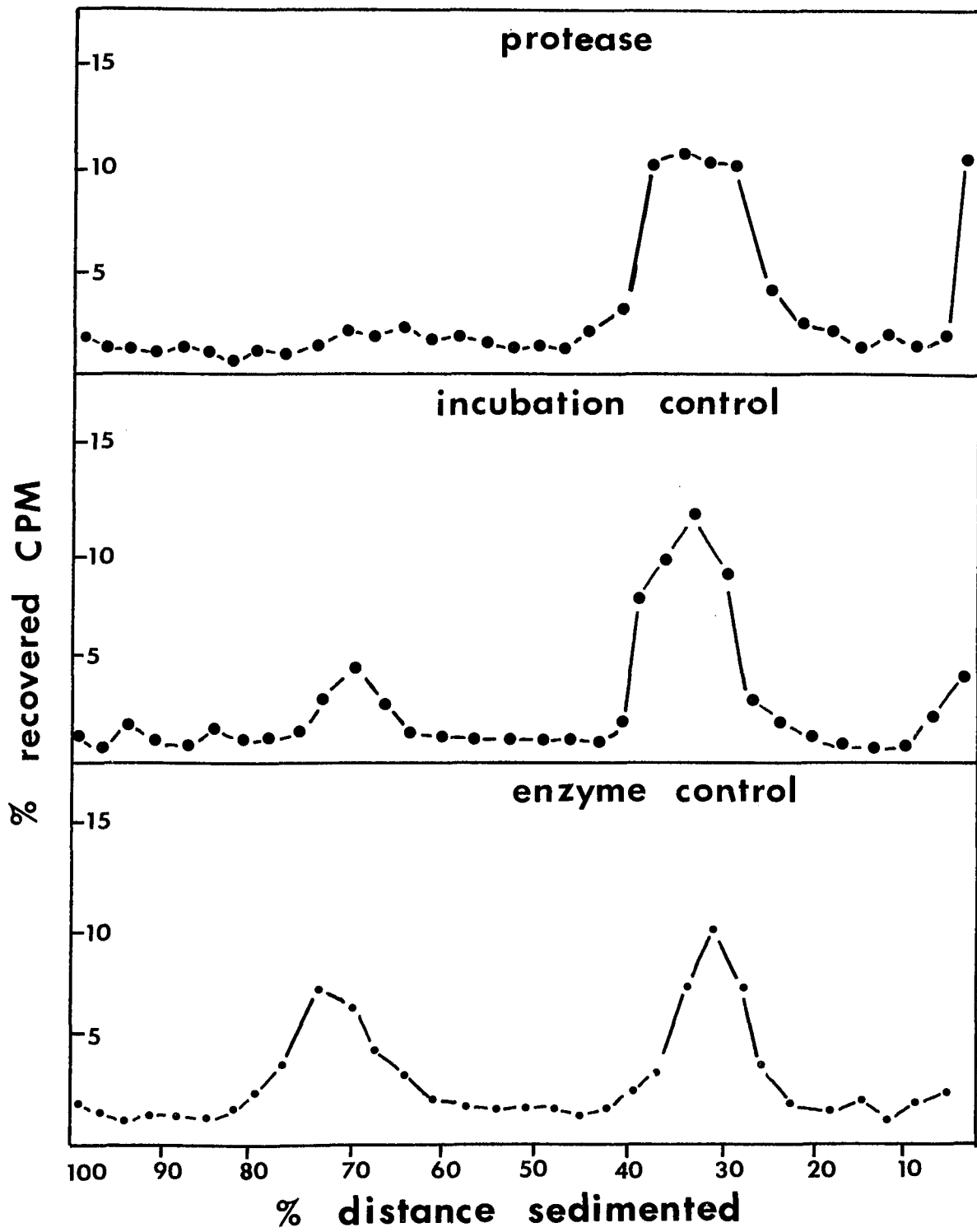


Fig. 5. Distribution of infecting SP82G DNA after Sarkosyl treatment

Logarithmically growing cells were infected with SP82G (^3H -adenine) at an moi= 5. Three 5 ml samples were withdrawn at 9 minutes after infection and sheared lysates were prepared as described in Materials and Methods. To one sample, Sarkosyl was added to a final concentration of 1% and an incubation of 20 minutes was allowed to proceed at 37°C (Sarkosyl; 100% of recovered radioactivity= 9291 counts/minute). To the other two samples, no detergent was added; one of these was incubated at 37°C for 20 minutes (Incubation control; 100% of recovered radioactivity= 6410 counts/minute) and the other was kept at 4°C for the same time period (Sarkosyl control; 100% of recovered radioactivity= 6791 counts/minute). Samples were centrifuged as in Fig. 2.

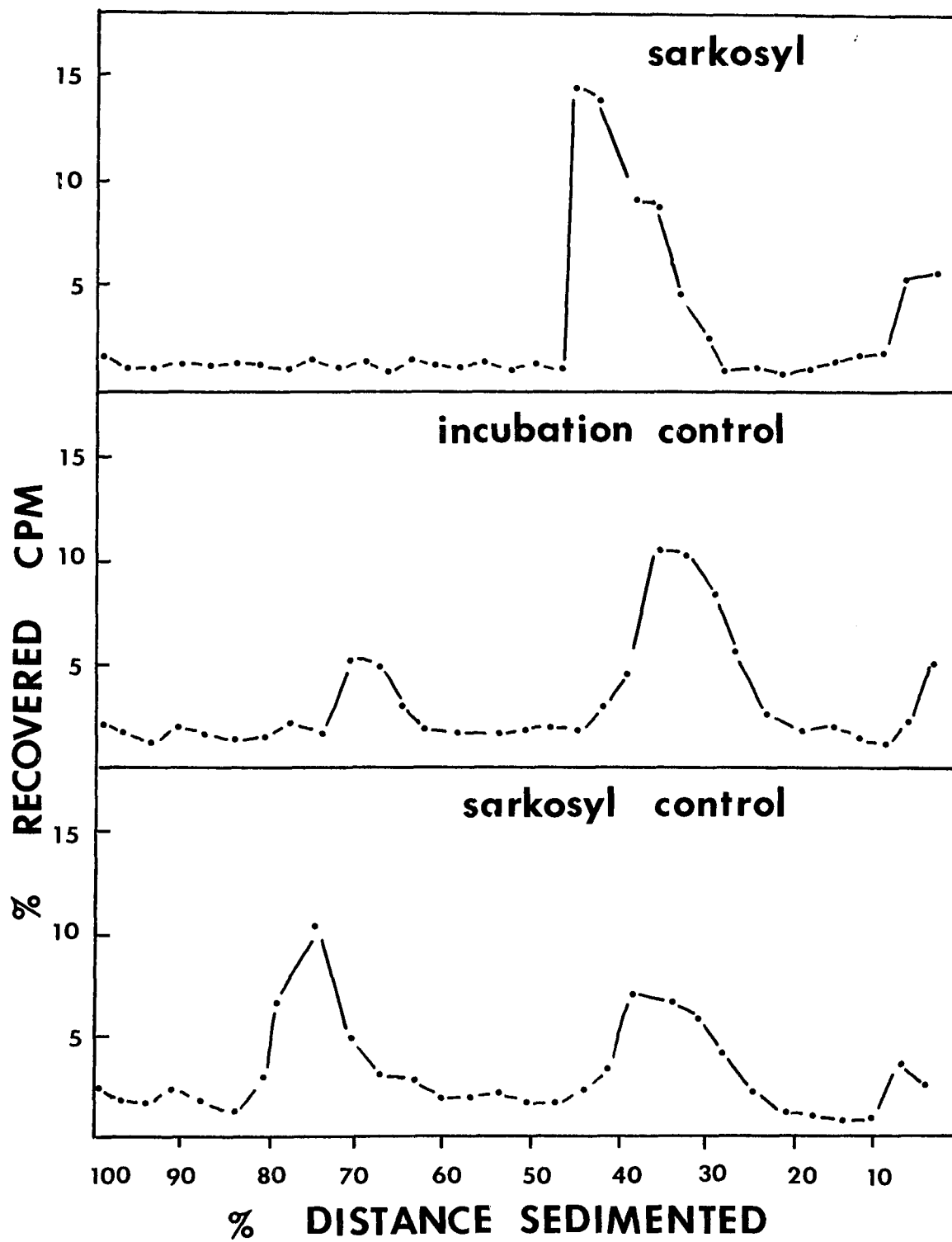
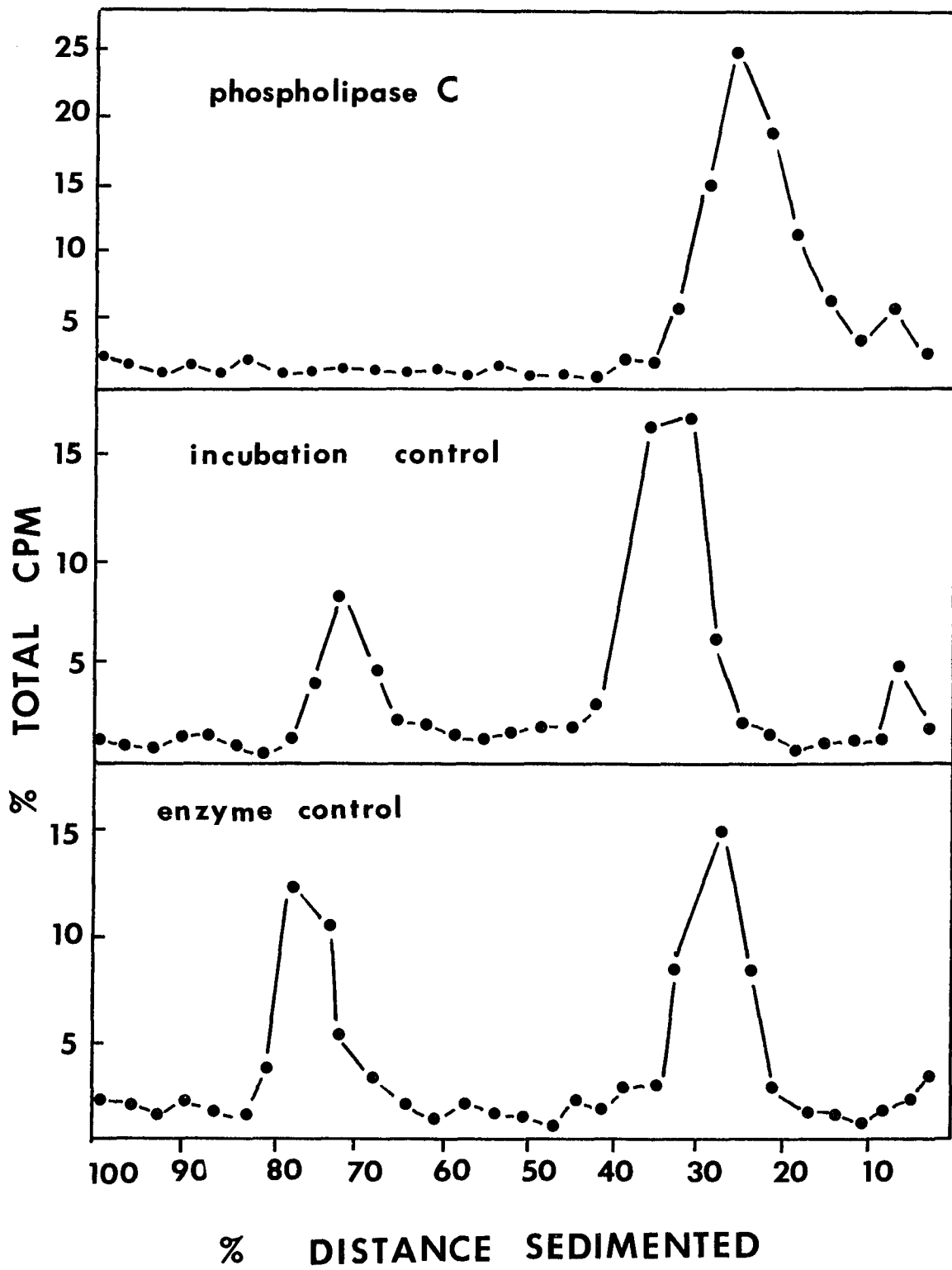


Fig. 6. Distribution of infecting SP82G DNA after phospholipase C treatment

Logarithmically growing cells were infected with SP82G bacteriophage (^3H -adenine) at an input multiplicity of 5. Three 5 ml samples were withdrawn at 9 minutes after infection and sheared lysates were prepared. To one of these samples phospholipase C (final concentration 1.33 mg/ml) was added and incubation for 20 minutes at 37° C was allowed to proceed (phospholipase C; 100% of recovered radioactivity= 6547 counts/minute). To the other two samples, no enzyme was added. One of these was incubated for 20 minutes at 37°C (Incubation control; 100% of recovered radioactivity= 4015 counts/minute) and the other was kept at 4°C for the incubation period (Enzyme control; 100% of recovered radioactivity= 5762 counts/minute). Samples were centrifuged as in Fig. 2.



tion was collected and examined under the electron microscope as detailed in Materials and Methods. The micrographs in Figs. 7 and 8 reveal a DNA molecule (average width 21-23 Å) with an associated cellular component.

Time Course of DNA-Membrane Binding

Demonstration that the rapidly-sedimenting SP82G DNA is a DNA-membrane complex raises the question of whether the relative amount of DNA sedimenting as membrane-bound DNA changes during the growth cycle of the phage. Samples were withdrawn at intervals from 2 to 30 minutes after infection with ³H-adenine-labeled SP82G. Lysates were prepared and analyzed for the amount of membrane-bound phage DNA. The results (Fig. 9) indicate that the 30 minute time period can be divided into three phases. In the first phase there is an increase in the amount of rapidly-sedimenting DNA; a maximum of approximately 38% is reached at 9 minutes after infection at 37°C. Phase II is distinguished by a decrease in the amount of DNA in the rapidly-sedimenting peak. This reaches a minimum of about 10-12% at 19 minutes after infection. During phase III (19 to 30 minutes after infection), the amount of bacteriophage DNA in the rapidly-sedimenting peak remains constant at approximately 10-12% (baseline amount).

Effect of Metabolic Inhibitors on Membrane Complex Dynamics

The effects of a variety of metabolic inhibitors were studied in order to gain information on the mechanism of formation of the SP82G DNA-membrane complex. The effects of two transcriptional inhib-

Fig. 7. Electron micrograph A of rapidly-sedimenting SP82G DNA
Experimental procedure described in Materials and Methods.
Micrographs taken on a Phillips EM-200 electron microscope at 40 kV.
Magnification: 24,057 X.

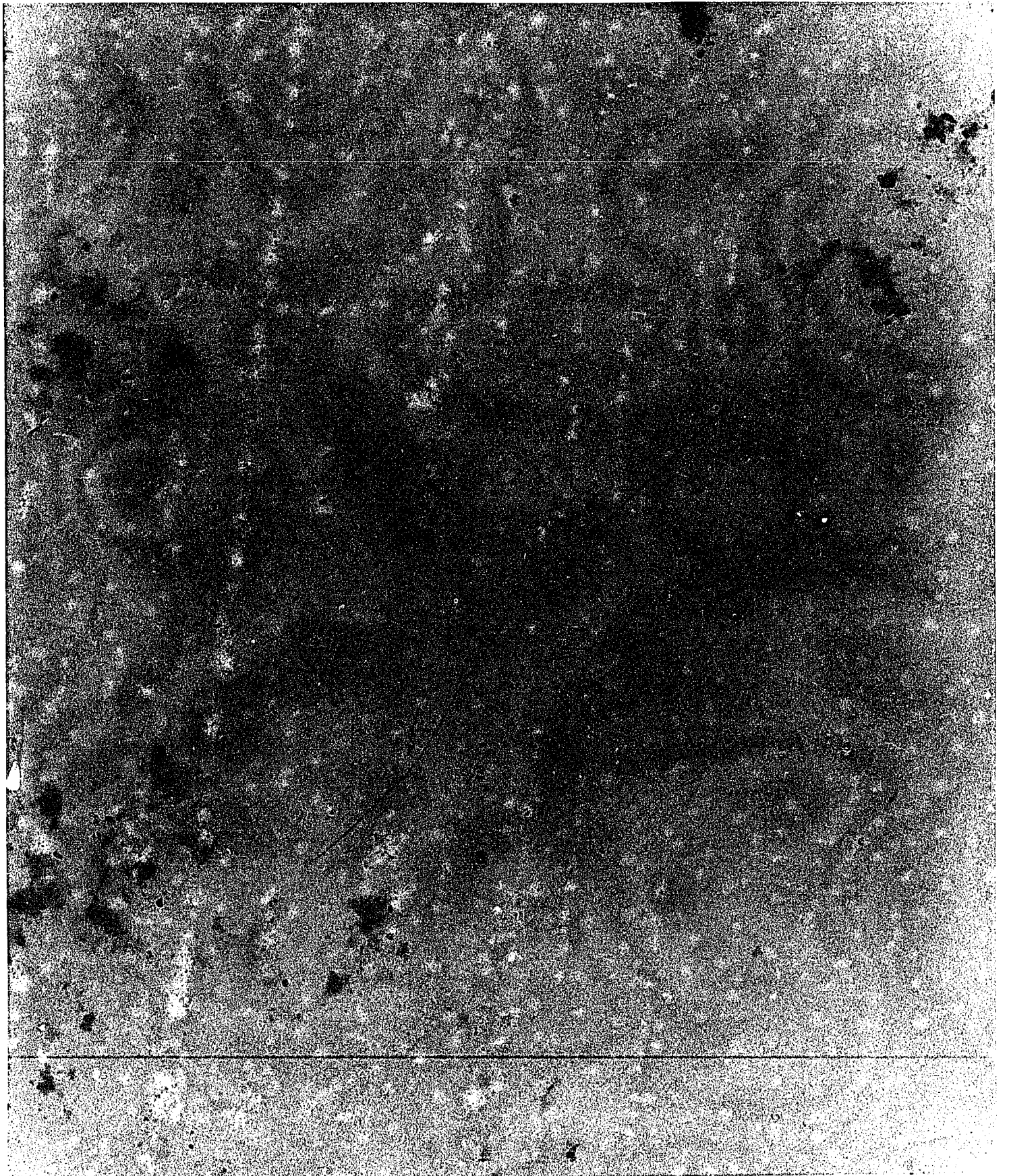


Fig. 8. Electron micrograph B of rapidly-sedimenting SP82G DNA

Experimental procedure described in Materials and Methods.

Micrographs taken with a Phillips EM-200 electron microscope at 40 kV.

Magnification: 142,435 X.

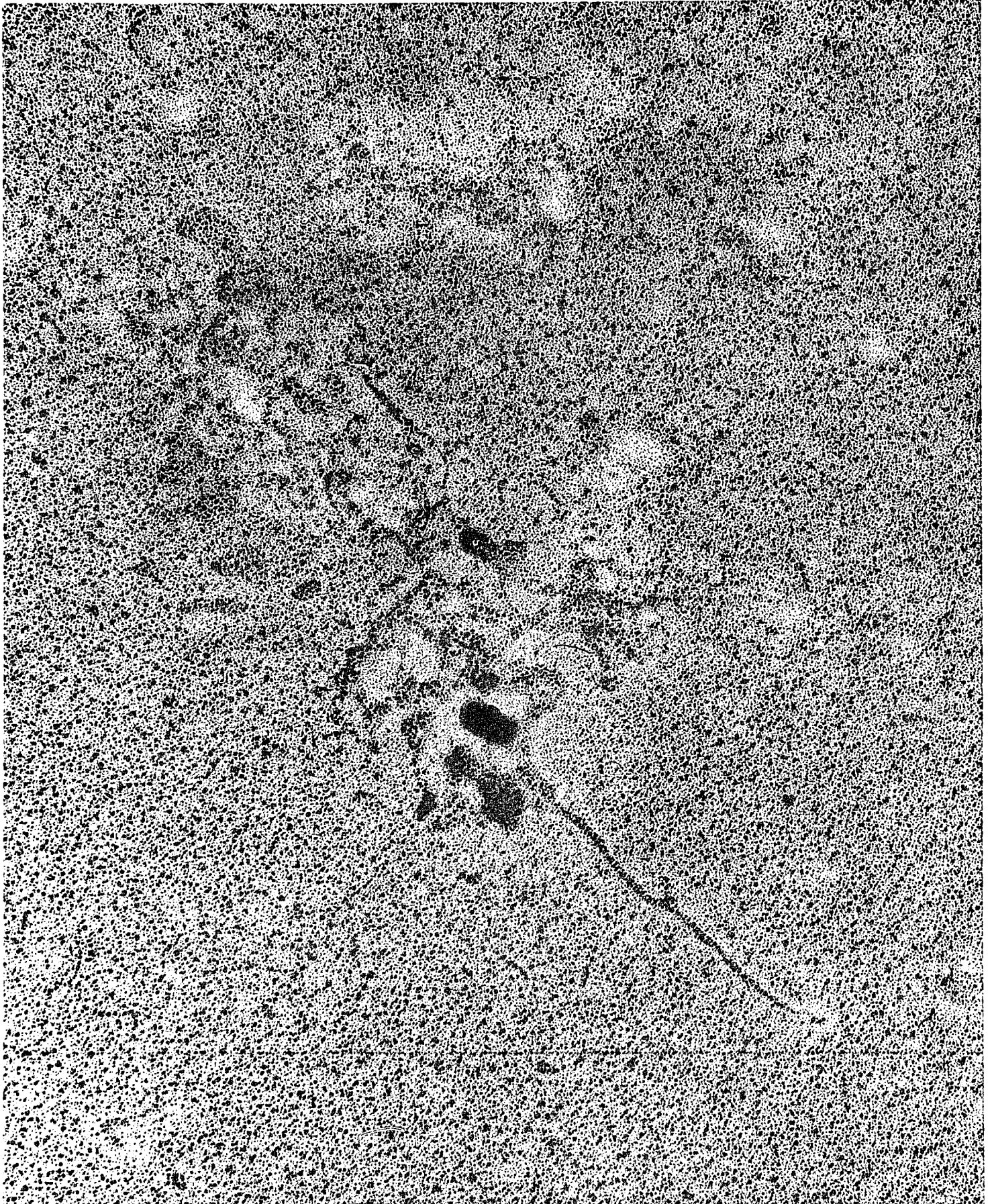
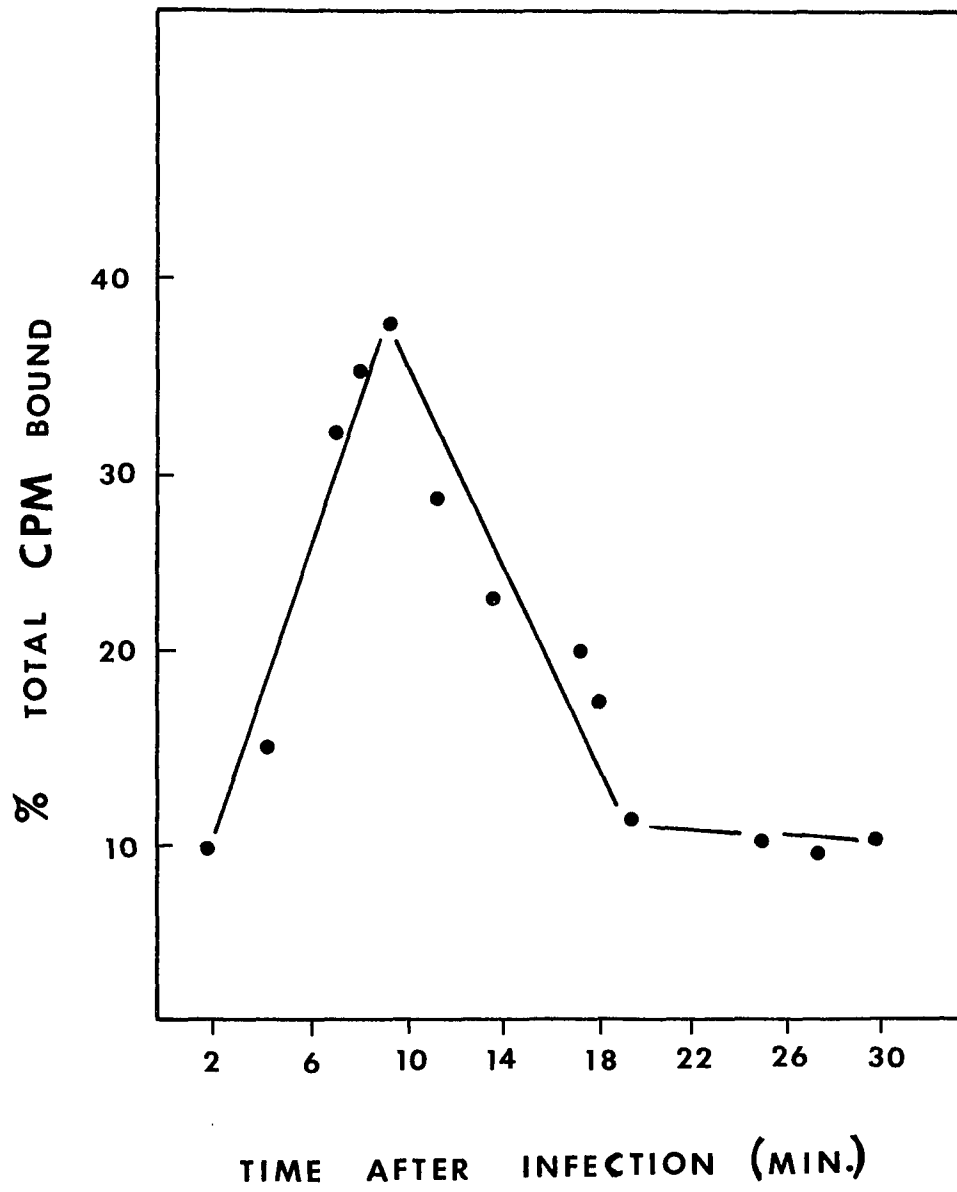


Fig. 9. Change in the amount of infecting SP82G DNA found in the membrane complex with time

Logarithmically growing cells (5.7×10^7 /ml) were concentrated ten-fold and infected with SP82G bacteriophage (^3H -adenine) at a multiplicity of infection of 5. Following a 2 minute adsorption period, subsequent dilution and incubation, 5 ml samples were withdrawn at the designated times and lysates were prepared, sheared and centrifuged as described in Materials and Methods. The percent of the total label occurring in the rapidly-sedimenting DNA peak was calculated and plotted as a function of time after infection.



itors (rifampicin and actinomycin D), an inhibitor of protein synthesis (chloramphenicol), a DNA synthesis inhibitor (nalidixic acid) and a respiratory inhibitor (NaCN) were examined.

An experiment was done to confirm that the intended concentrations of rifampicin and actinomycin D were sufficient to inhibit RNA synthesis. Rifampicin at 100 ug/ml or actinomycin D at 50 ug/ml are sufficient to effectively inhibit at least 95% of the incorporation of ^3H -uridine into TCA-precipitable material (RNA) in Bacillus subtilis SB-1 (Fig. 10).

When B. subtilis SB-1 cells are pre-treated with rifampicin, the amount of SP82G DNA which binds to the membrane is drastically reduced. As can be seen in Fig. 11, in the presence of rifampicin only the baseline 10-12% of phage DNA associates with the membrane. Identical results were obtained using cells pre-treated with actinomycin D (data not shown).

If samples of B. subtilis SB-1 are pre-treated with chloramphenicol at a concentration of 500 ug/ml (sufficient to yield a 95% inhibition of protein synthesis [King, 1974]), the time course of DNA-membrane binding shown in Fig. 11 is obtained. It is clear that the inhibition of protein synthesis has no effect on the ability of the SP82G DNA molecule to bind to the host cell membrane. In contrast in the presence of chloramphenicol the bound DNA is not released from membrane association as it is in the untreated control.

Consistent with the apparent requirement for RNA synthesis in the establishment of the membrane complex, pre-treatment of cells with 0.01 M NaCN permitted the formation of only the baseline level of DNA-

Fig. 10. Effect of rifampicin and actinomycin D on
³H-uridine incorporation

Experimental procedure detailed in Materials and Methods. The amount of ³H-uridine incorporated into acid precipitable RNA is expressed as a percent of the value obtained for cells withdrawn and treated immediately after addition of inhibitor (0-time control). Percentages are shown as a function of time of incubation with inhibitor. 100% of ³H-uridine incorporation= 632 counts/ minute for rifampicin-treated cells and 570 counts/ minute for actinomycin D-treated cells.

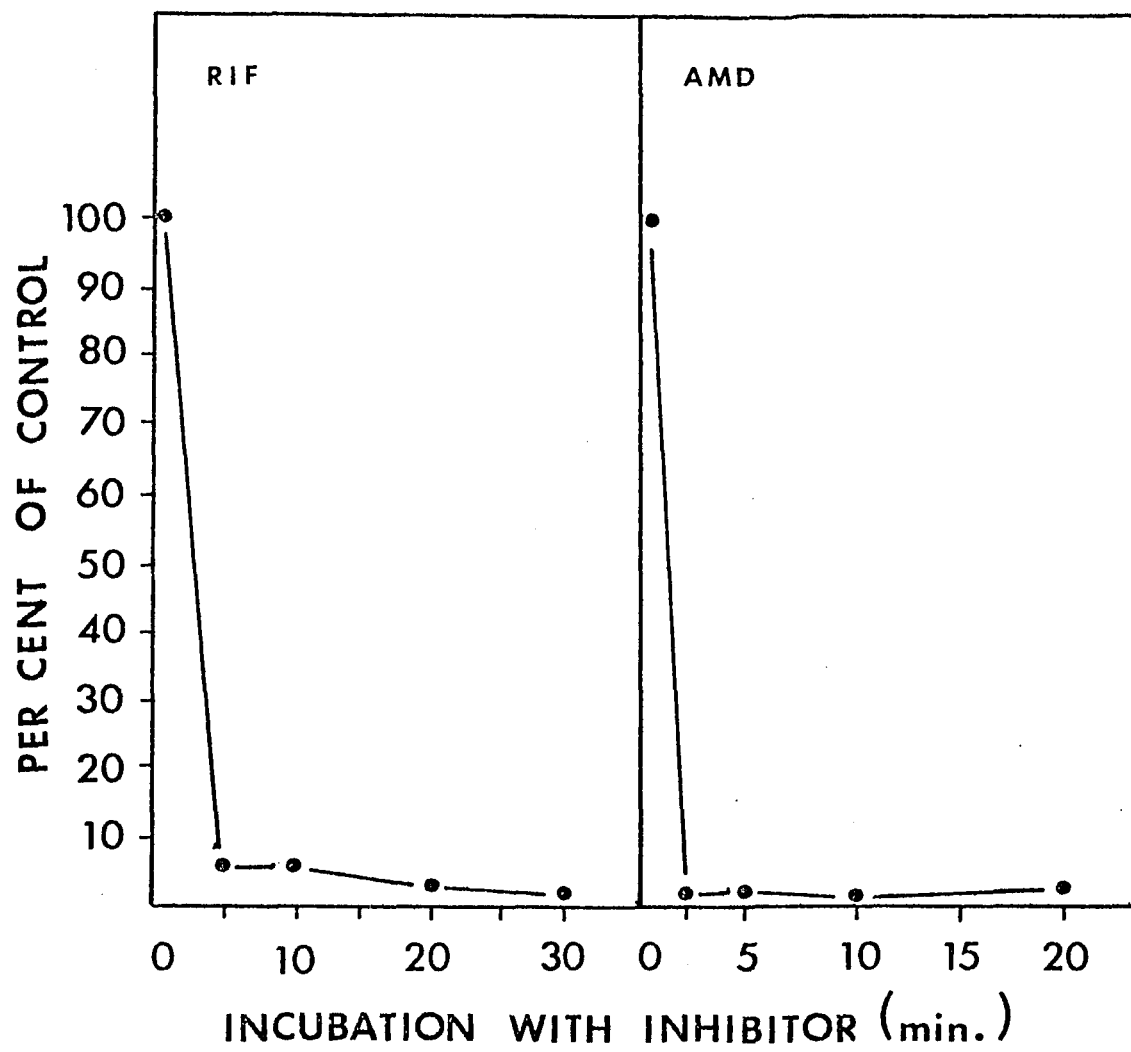
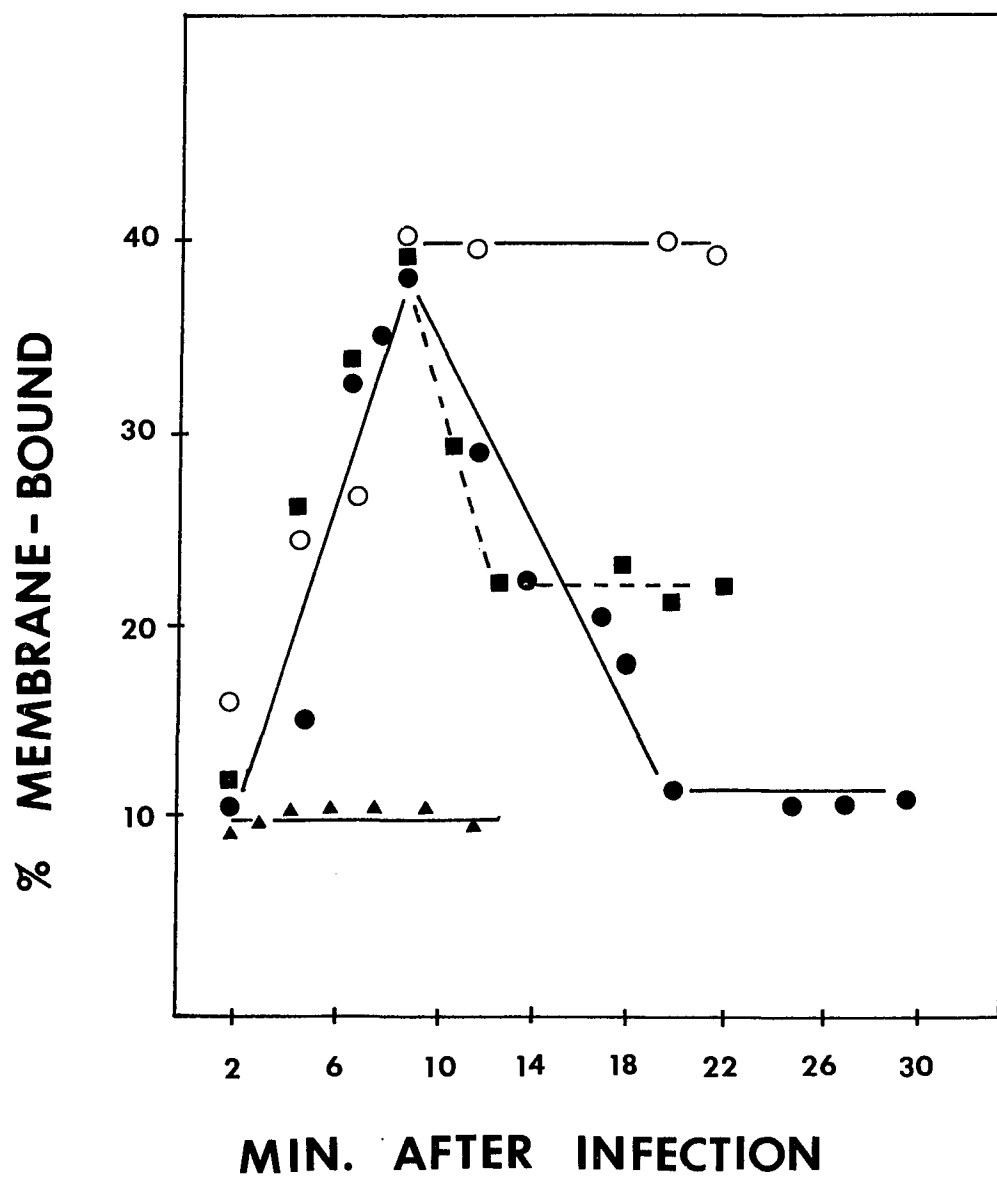


Fig. 11. Effect of metabolic inhibitors on the time course
of SP82G DNA-membrane binding

Experimental procedure identical to that described in Fig. 9
and Materials and Methods. The percent of the total label occurring
in the rapidly-sedimenting DNA peak was calculated and plotted as a
function of time after infection. (●) untreated; (▲) rifampicin-
treated; (○) chloramphenicol-treated; and (■) nalidixic acid-treated.



membrane binding to occur (Fig. 12).

Nalidixic acid (75 ug/ml) is only a weak inhibitor of bacteriophage DNA synthesis in this and related systems (Baird et al., 1972; Gage and Fujita, 1969) and therefore results using this inhibitor must be viewed with this fact in mind. Nevertheless, pre-treatment of the host cells with nalidixic acid has no detectable effect on the formation of the membrane complex (Fig. 11), whereas only about one half of the normal release occurs. This suggests that active DNA synthesis may be needed for the release of the bound DNA from membrane association.

Stabilization With Phenyl Methyl Sulfonyl Flouride (PMSF)

The results with rifampicin and actinomycin D suggest that the DNA-membrane complex requires RNA synthesis for its formation. However, before attempting to verify this hypothesis, techniques had to be developed to decrease the lability (see controls Figs. 4-6) of the complex when incubated at 37°C. On the assumption that this lability could result in part from proteolytic activity, the effect of the seryl protease inhibitor phenyl methyl sulfonyl flouride (PMSF) on the complex integrity during incubation at 37°C was examined. Typical results are presented in Fig. 13. Virtually complete stabilization for incubation periods up to 3 minutes can be obtained by adding PMSF (final concentration 320 ug/ml) to the lysate before incubation.

Sensitivity to RNase

Having eliminated the 37°C lability as an experimental variable, the question of the RNase sensitivity could be investigated.

Fig. 12. Effect of NaCN on the time course of SP82G DNA-
membrane binding

Experimental procedure described in Fig. 9 and Materials and Methods. The percent of total label occurring in the rapidly-sedimenting DNA peak after pre-treatment of the host cells with NaCN (0.01 M) was calculated and plotted as a function of time after infection.

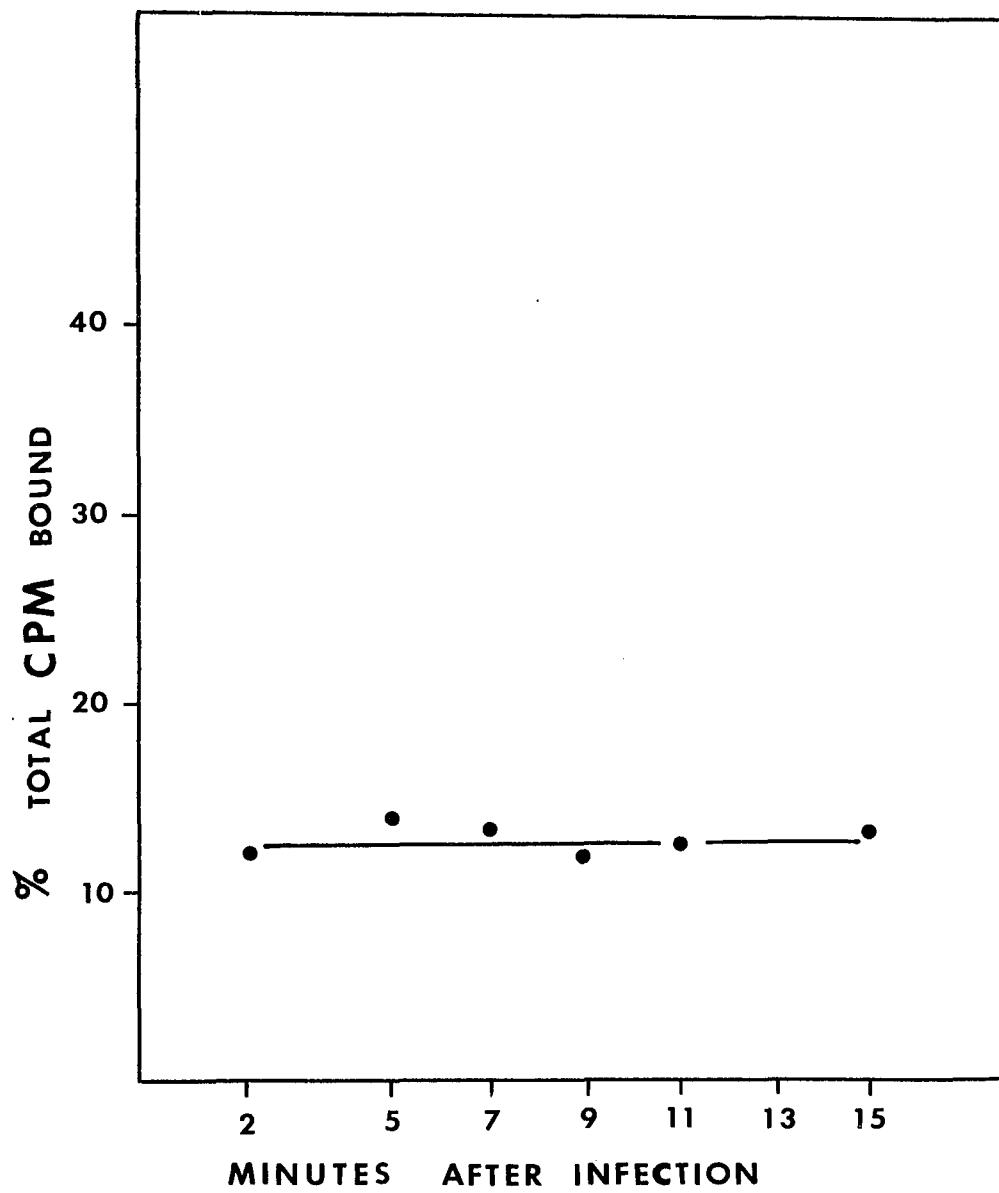
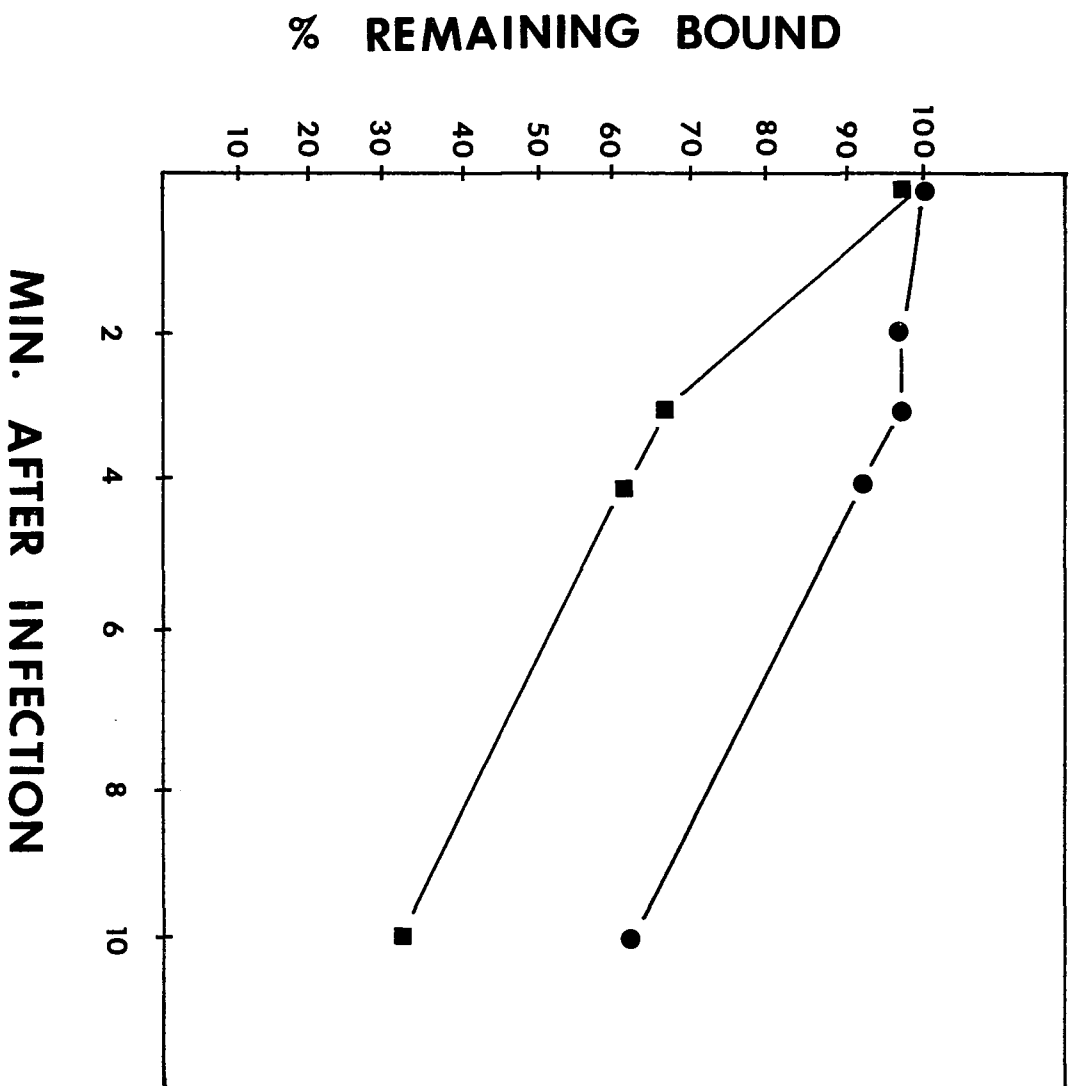


Fig. 13. Stabilization of membrane-complexed DNA by PMSF

Cells infected with ^3H -adenine labeled SP82G bacteriophage were collected and lysed at 7 minutes after infection as described in Materials and Methods. To one portion of the lysate, PMSF was added at a concentration of 320 $\mu\text{g}/\text{ml}$ (●), to the other (■) no addition. Both samples were incubated at 37°C for the indicated times, rapidly chilled and the amount of membrane-bound DNA determined as previously described. The fraction of total DNA bound at 0 time was 33%.



An analysis of the sensitivity of the DNA-membrane complex to Ribonuclease A (RNase) at various times after infection was undertaken. The results of this study are shown in Fig. 14 and Table 3. The data indicates that initially the complex is insensitive to RNase. From 3 to 6 minutes after infection (37°C) the complex is sensitive to RNase. The sensitivity is maximum at the 4th minute when approximately 40% of the membrane-bound DNA can be released by RNase. Ultimately the RNase sensitivity disappears. It is important to note that this temporary RNase sensitivity occurs in Phase I of the binding time course (see Fig. 9) at the time when the amount of membrane-bound DNA increases from approximately 12 to 25%. This suggests that Phase I may consist of at least 2 types of membrane association. The first is dependent upon the presence of an RNA species; this is followed by a further increase in DNA-membrane binding in which the linkage is different. The conclusions that can be drawn from this experiment are that:

- 1) the involvement of RNA in the complex is critical to the integrity of the complex,
- 2) at the time of maximum DNA-membrane binding (9 minutes after infection), there is no RNase sensitivity and,
- 3) the shift from the RNase-sensitive state to the RNase-insensitive state at 7-9 minutes after infection shows that the initially formed RNA structure is superseded by some other linkage.

Minicell Experiments

The results of the nalidixic acid study (see Fig. 11 and

Fig. 14. RNase sensitivity of membrane-complexed SP82G DNA

Lysates prepared at the indicated times after infection were incubated with RNase A and PMSF for 2 minutes at 37°C as described in Materials and Methods. Following treatment, the amount of membrane-bound phage DNA was determined in the usual manner. The percentages observed in this and control experiments are presented in Table 3. The percent RNase releasable is calculated as percent bound in incubation control - percent bound in RNase-treated \div percent bound in incubation control.

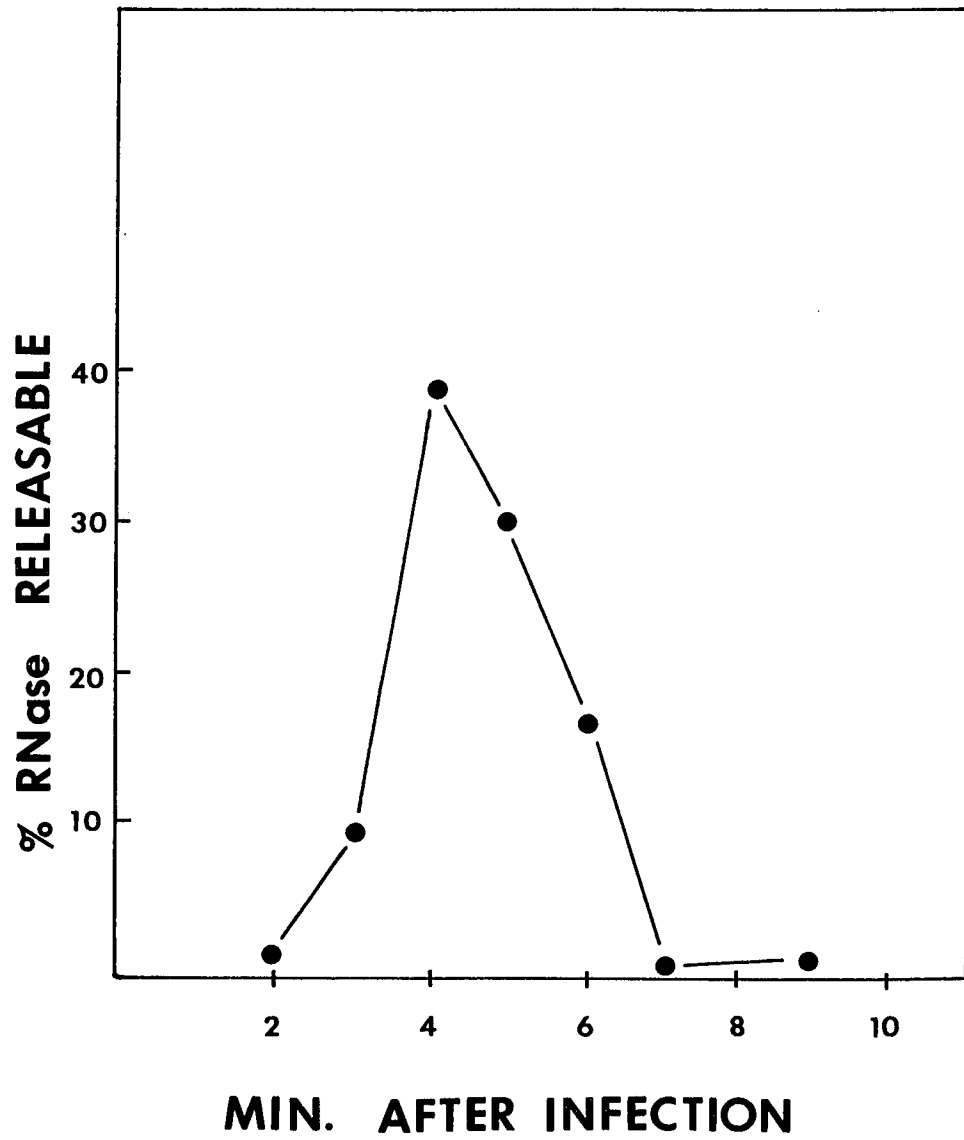


Table 3. EFFECT OF RNase ON THE PERCENT TOTAL LABEL IN THE DNA-MEMBRANE COMPLEX

	Time After Infection (min.)						
	2	3	4	5	6	7	9
Control	20	24	25.7	24	32	37.8	35.2
Incubation Control	18.4	23.3	25.7	21.1	30.8	38.9	38.1
RNase-Treated	18.7	21.2	15.7	14.7	25.4	39.1	38.6

page 54) indicate that active DNA synthesis is not a prerequisite for membrane complex formation (Phase I) but might be important in the detachment process (Phase II). Unfortunately, however, the weak inhibition of DNA synthesis by nalidixic acid in this system (Baird et al., 1972; Gage and Fujita, 1969) prevents a firm conclusion concerning the extent to which the detachment is dependent on DNA synthesis. To examine the role of DNA synthesis in DNA-membrane interactions, a system exhibiting a DNA synthesis-negative phenotype had to be devised. Use of an appropriate ts-mutant phage (DNA-0), while possible, would not be ideal since the mandatory temperature shift to 45°C would alter the kinetics of the DNA-membrane interactions and perhaps unfavorably affect the stability of the complex. Therefore, in an effort to effectively inhibit DNA synthesis, minicells of Bacillus subtilis were used as the host for SP82G infection and DNA-membrane complex analysis. Minicells are produced during abnormal, asymmetric cell division; they lack DNA and are deficient in DNA synthesis, but are capable of limited protein synthesis and RNA synthesis when a DNA template is introduced (Reeve and Cornett, 1975; Reeve, in press). For purposes of this study, they would seem an ideal DNA synthesis-negative host.

DNA-Membrane Binding in Minicells

Experiments were undertaken to determine whether infecting SP82G DNA bound to the cell membrane in minicells as it did in the normal B. subtilis host. The results (Fig. 15) indicate that bacteriophage SP82G injects its genetic material into minicells and that the phage DNA does bind to the minicell membrane as determined by the

the presence of rapidly-sedimenting DNA. However, the Renografin gradient profile for infected minicells is distinctly different from the sedimentation pattern obtained for infected B. subtilis SB-1 cells (Fig. 2). The infected minicell lysates have two peaks in the region of membrane-bound phage DNA. The infected SB-1 lysates always indicate a single symmetrical membrane-bound DNA peak.

In order to separate the rapidly-sedimenting peak into its two components, the Renografin gradient was changed. An SP82G-infected minicell lysate (6 minutes after infection) was made as described in Materials and Methods and centrifuged on a linear 15-30% Renografin gradient. The results (Fig. 16) indicate that the double peak contains two distinct components.

One possible explanation for the presence of two rapidly-sedimenting species is that a percentage of the infected minicells were resistant to the lysis procedure, and that these unlysed minicells sedimented to a position near that of the membrane-bound DNA. In support of this idea, Reeve (personal communication) has noted that minicells are very resistant to normal lysis procedures and usually require harsh techniques to achieve complete lysis. Examination of the minicell lysates in these experiments by phase contrast microscopy revealed that only about 70% of the minicells were lysed. To test this hypothesis, the following experiment was designed. One sample of isolated minicells was infected with SP82G; at 6 minutes after infection, an aliquot was withdrawn, lysed, and analyzed for membrane-bound DNA by the usual protocol. At the same time an aliquot was withdrawn but

Fig. 15. Distribution of infecting SP82G DNA from sheared
minicell lysates on Renografin gradients

Minicells were isolated, infected with ^3H -adenine-labeled
SP82G and analyzed for membrane-bound DNA as described in Materials
and Methods. The multiplicity of infection (moi) was approximately 5.
Direction of sedimentation is from right to left.

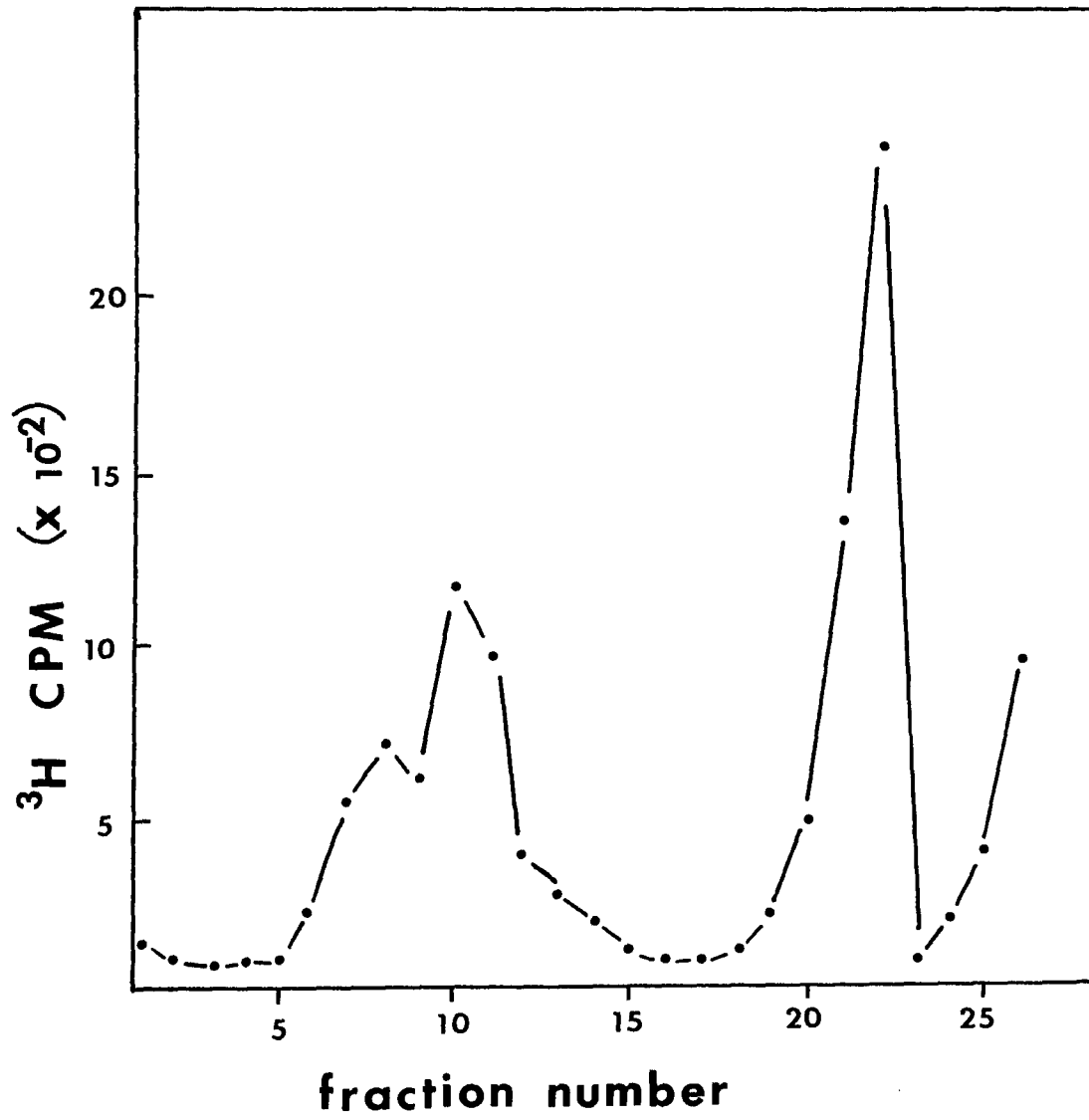
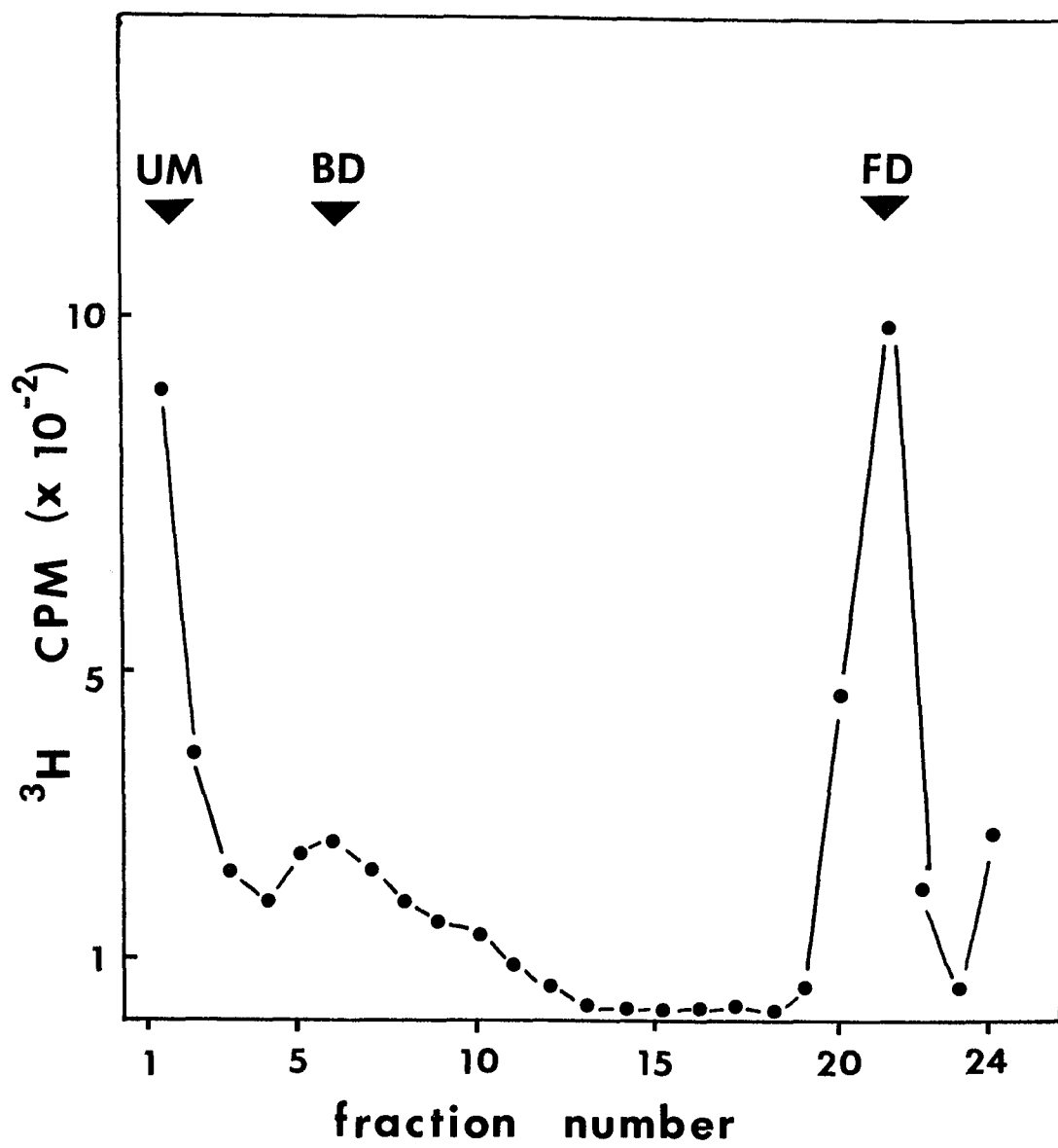


Fig. 16. Effect of gradient shape on the distribution of infecting SP82G DNA from sheared minicell lysates

Experimental procedure described in text. The sheared minicell lysate was sedimented on a 15-30% linear Renografin gradient at 27,000 RPM for 5 hours at 2°C in an SW27.1 rotor. Direction of sedimentation is from right to left. UM denotes unlysed minicells; BD denotes membrane-bound phage DNA; and FD denotes free phage DNA.



was not lysed prior to centrifugation. The results are shown in Fig. 17. The unlysed minicells sediment just below, and partially overlapping, the membrane-bound phage DNA. Thus the above hypothesis was confirmed. Attempts to obtain more complete lysis of the minicells were unsuccessful. Therefore, all of the rapidly-sedimenting radioactively-labeled material cannot be attributed to membrane-bound DNA. Nevertheless, the percentage of unlysed minicells was found to remain fairly constant (within 20-30%) from experiment to experiment. Therefore, although the calculated percentage of membrane-bound DNA is over-estimated by the fraction of unlysed minicells, the binding trends observed in subsequent time course studies are valid.

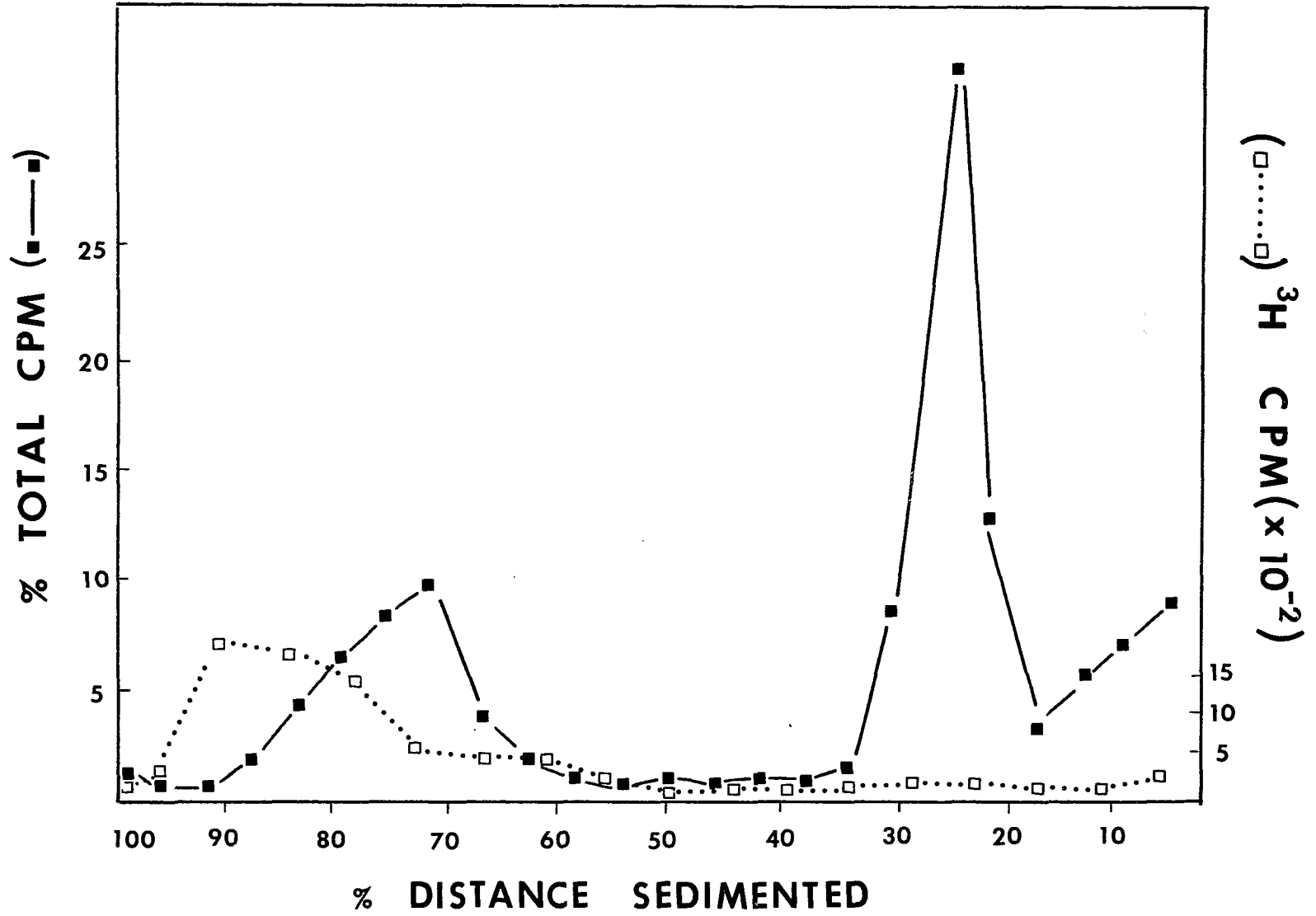
Time Course of DNA-Membrane Binding in Minicells

The kinetics of DNA-membrane association in the minicell system as compared to normal host cell infection was next investigated. Before this could be done it was essential to determine a control time course of DNA-membrane binding using the parental minicell-producer strain, B. subtilis CU 403, as the host cell. Although CU 403 and SB-1 are related strains derived from Bacillus subtilis 168, the kinetics of SP82G DNA-membrane binding in both strains need not be identical. B. subtilis strain CU 403 is thus the appropriate control.

It is clear that the general time course of DNA-membrane binding in CU 403 is similar to that exhibited by B. subtilis SB-1 (Fig. 18). For both systems the overall pattern consists of an increase followed by a decrease in DNA-membrane binding. However, the timing is different in the two systems. CU 403 shows a binding maximum of

Fig. 17. Distribution of lysed and unlysed SP82G-infected minicells on Renografin gradients

Experimental procedure described in text. The sedimentation profiles for a lysed (■—■) SP82G-infected minicell sample and an unlysed, but otherwise identical, SP82G-infected minicell sample (□...□) are shown.



approximately 50% at 15 minutes after infection whereas SB-1 showed a binding maximum of approximately 38% at 9 minutes after infection.

The results of the time course studies using minicells as the host for SP82G infection are also shown in Fig. 18. It is obvious that in this system phage DNA-membrane binding does occur, but that detachment does not. It is not understood why the maximum level of DNA-membrane binding is reached by 3 minutes after infection, rather than increasing with time. Nevertheless, these results are consistent with the hypothesis that active DNA synthesis is a prerequisite for the release of SP82G DNA from membrane association.

Metabolic Inhibitor Studies (Minicells)

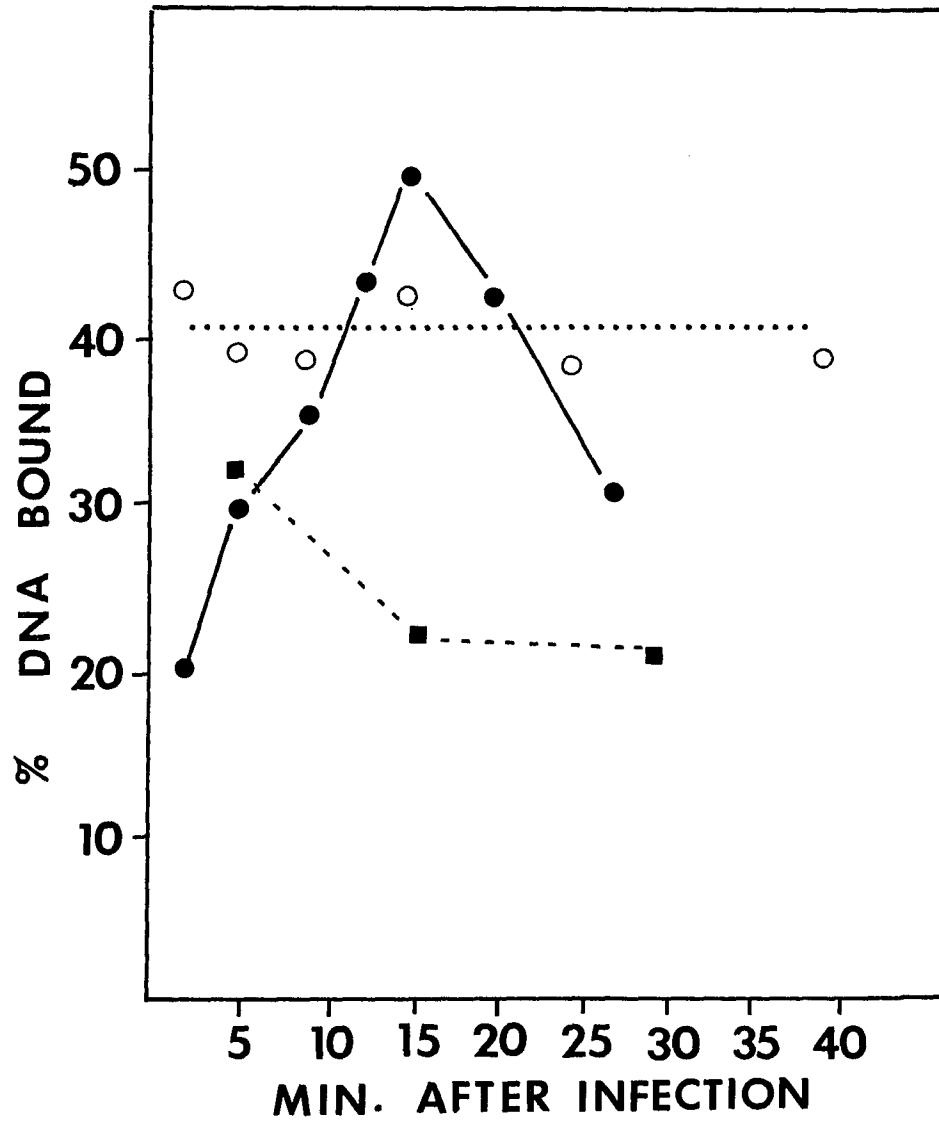
The effects of chloramphenicol and rifampicin were examined in the minicells. Similar sensitivities to these inhibitors using normal cells and minicells would suggest that the membrane binding processes in both cell types had similar transcriptional and translational requirements. To this end, minicells pre-treated with these metabolic inhibitors were infected and analyzed for membrane-bound SP82G DNA. As shown in Fig. 18, pre-treatment with rifampicin reduces the amount of membrane-bound DNA to the CU 403 baseline level (c.a. 20%). This result is similar to that obtained with rifampicin in the SP82G/SB-1 system where the amount of membrane-associated DNA is also reduced to the baseline level (Phase III) of 10-12%.

Pre-treatment of the minicells with chloramphenicol has no detectable effect (data not shown) on the time course of DNA-membrane binding; a constant level (c.a. 45%) of the recovered radioactivity

Fig. 18. Time course of SP82G DNA-membrane binding in minicells
and minicell-producer B. subtilis

Logarithmically growing B. subtilis CU 403 cells (6×10^7 /ml) were infected with labeled SP82G bacteriophage (^3H -adenine) at an moi=5 and analyzed for the amount of membrane-bound phage DNA at various times after infection as described in Materials and Methods. The percent of the total label occurring in the membrane-bound DNA peak was calculated and plotted as a function of time after infection (●—●).

Minicells were isolated, treated with inhibitor or not as described in Materials and Methods, and then were infected with SP82G bacteriophage (^3H -adenine) at an approximate moi of 5. At various times after infection, samples were removed and analyzed for the amount of membrane-bound phage DNA. The percent of the total label occurring as membrane-bound DNA was calculated and plotted as a function of time after infection for control (○····○) and rifampicin-treated (■---■) minicells.



sedimented as membrane-bound DNA. Chloramphenicol also had no effect on the membrane-binding phenomenon in the SB-1 host system, but it did inhibit detachment; there is no detachment event in the minicell system. The results of the minicell experiments indicate that:

- 1) minicell membranes are able to bind SP82G DNA,
- 2) the DNA is not released from membrane association,
- 3) the DNA binding process in minicells is sensitive to rifampicin as it is in the control system and,
- 4) the binding process in minicells is insensitive to chloramphenicol as it is in control B. subtilis cells. These results suggest that:

- 1) the type of DNA-membrane binding is the same in both systems and,
- 2) the lack of DNA synthesis in minicells may be responsible for the absence of the membrane release process.

Marker Rescue Studies

One virtue of the Ivarie-Pene technique is that the shearing step facilitates identification of those portions of the genome most closely associated with the membrane. The marker rescue technique (Materials and Methods) can be used to determine if specific areas of the DNA form a membrane complex. In an effort to answer this question and to determine if in fact there is a specific sequence of binding for different portions of the DNA molecule, marker rescue experiments were undertaken. Three closely-linked pairs of markers representative of the left (H177-G55), middle (H167-A4) and right (E14-H24) ends of the genome, were analyzed for association with the Bacillus subtilis membrane at different

times after infection. Three times, up to and including the time of maximum complex formation, were examined. The results of these experiments are presented in Table 4a. Two experiments using independent DNA preparations were performed for each of the three time points. At the earliest time (4 minutes after infection) there is an enrichment for right end markers in isolated bound DNA relative to unbound DNA. The left and middle markers are found with a lower frequency. This relationship is most clearly seen in Table 4b, which presents the percent of the total marker activity found in the bound DNA for each marker pair. At 7 minutes after infection, both right and middle markers are enriched in the bound DNA relative to the unbound DNA. The left end marker is found with a lower frequency. By 9 minutes after infection (maximum complex formation) all three markers are enriched in the membrane-bound fraction of DNA with equal frequency. This data suggests that SP82G DNA binds to the membrane in a polar fashion beginning from the right end.

Comparison of the multiple assays (see Table 4a) of the individual experiments for DNA isolated at a given time reveals that there is variability in marker frequency for individual marker pairs between experiments. Among the possible sources of variability are the following.

- 1) Slight variations in the time of sample withdrawal could occur. If the SP82G DNA does indeed bind to the membrane in a polar fashion, it could be expected that individual markers would bind at distinct times. The time of isolation of the DNA would, therefore, be critical. Examination of Table 4b indicates that at 7 minutes after infection, the frequency of the middle marker (H167-A4) in the bound DNA fraction represents 68% of the total marker activity in one experiment and 40% in the other. If 7 minutes after infection is the time at which the middle mar-

Table 4a. GENETIC ANALYSIS OF BOUND AND UNBOUND DNA BY THE
MARKER RESCUE TECHNIQUE*

Experiment #	Bound			Free		
	H177/G55	H167/A4	E14/H24	H177/G55	H167/A4	E14/H24
4 minute DNA						
Exp. 1 ^a	1890	1350	1620	3090	1640	1150
Exp. 2 ^b	590	720	1030	590	875	813
7 minute DNA						
Exp. 1 ^a	1220	2760	1600	2220	1320	1220
Exp. 2 ^b	650	1250	1330	2660	1870	1150
9 minute DNA						
Exp. 1 ^a	5220	3700	1960	1300	590	740
Exp. 2 ^c	22000	11100	10500	5940	3510	2760

* all numbers are given as PFU/ml cells

a= experiment is the average of 2 independent assays of a single DNA preparation. Each assay was plated in duplicate.

b= experiment results from a single assay (plated in duplicate) of a new preparation of DNA.

c= experiment is the average of 3 independent assays of a single DNA preparation. Each assay was plated in duplicate.

Table 4b. PERCENT OF THE TOTAL MARKER ACTIVITY
FOUND IN THE BOUND DNA FRACTION *

Experiment	H177/G55 (left end)	H167/A4 (middle)	E14/H24 (right end)
4 minute DNA			
Exp. 1	38	45	58
Exp. 2	50	45	56
7 minute DNA			
Exp. 1	36	68	57
Exp. 2	20	40	54
9 minute DNA			
Exp. 1	80	86	73
Exp. 2	79	76	79

* numbers are calculated as the rescuable markers in the bound DNA fraction \div the rescuable markers in the bound DNA fraction + the rescuable markers in the comparable unbound DNA fraction (Table 4a) x 100.

ker is being bound to the membrane, a slight variation in the time of DNA isolation between the two DNA preparations could result in the middle marker being enriched in one case and not in the other.

2) Variation in the degree of shearing might affect the activity of the DNA in marker rescue experiments.

3) Slight variations in the multiplicity of rescuing phage could result in variability between experiments.

4) Fluctuations in cell competency and daily plating conditions may also cause experimental variability.

Is There A Pilot Protein? - Double Label Infection Experiments

As mentioned in the Introduction there is a minor coat protein in coliphage M13 (the product of gene 3) which enters the host in association with the viral genome and plays a role in the DNA-membrane complex formation (Jazwinski et al., 1973). It has been suggested that a similar "pilot protein" plays a rôle in the ϕ X174 DNA-membrane binding system (Jazwinski et al., 1973).

In an effort to determine if there is such a pilot protein involved in the SP82G DNA-membrane binding process, the following experiment was done. SP82G bacteriophage that carried ^3H label in the protein and ^{32}P label in the DNA (see Materials and Methods) were used to infect B. subtilis SB-1 cells. Lysates were prepared and analyzed on Renografin gradients in the usual manner. The results are shown in Fig. 19. It is clear that there is a protein (i.e., ^3H -label) co-sedimenting with membrane-bound DNA but not with the free DNA fraction. Since the bacteriophage were pre-labeled, and there is no in vivo labeling taking place, these

results suggest that there is a bacteriophage protein which enters the host with the viral genome and attaches with the DNA to the membrane. The sedimentation pattern in Fig. 19 is highly reproducible.

In order to examine the relative affinity of the protein(s) for the membrane and the DNA, a double-label lysate was treated with Sarkosyl (final concentration 1%) to see where the ^3H label sediments after disruption of the membrane. The results shown in Fig. 20 demonstrate that:

- 1) the membrane-bound DNA is released and sediments with the free DNA as in Fig. 5 and,

- 2) the ^3H label co-sedimenting with the membrane-bound DNA is released and stays at the top of the Renografin gradient.

These results suggest that the protein(s) is more strongly associated with the membrane than with the viral DNA.

Fig. 19. Distribution of infecting $^3\text{H}/^{32}\text{P}$ double-label SP82G

DNA from sheared lysates on Renografin gradients

$^3\text{H}/^{32}\text{P}$ double-label SP82G bacteriophage were isolated as described in Materials and Methods. Logarithmically growing B. subtilis SB-1 cells were infected with $^3\text{H}/^{32}\text{P}$ phage (moi= 2) and analyzed for membrane-bound DNA at 7 minutes after infection as described in text and Materials and Methods. ^{32}P (■—■) and ^3H (●.....●) radioactivities were determined for each fraction.

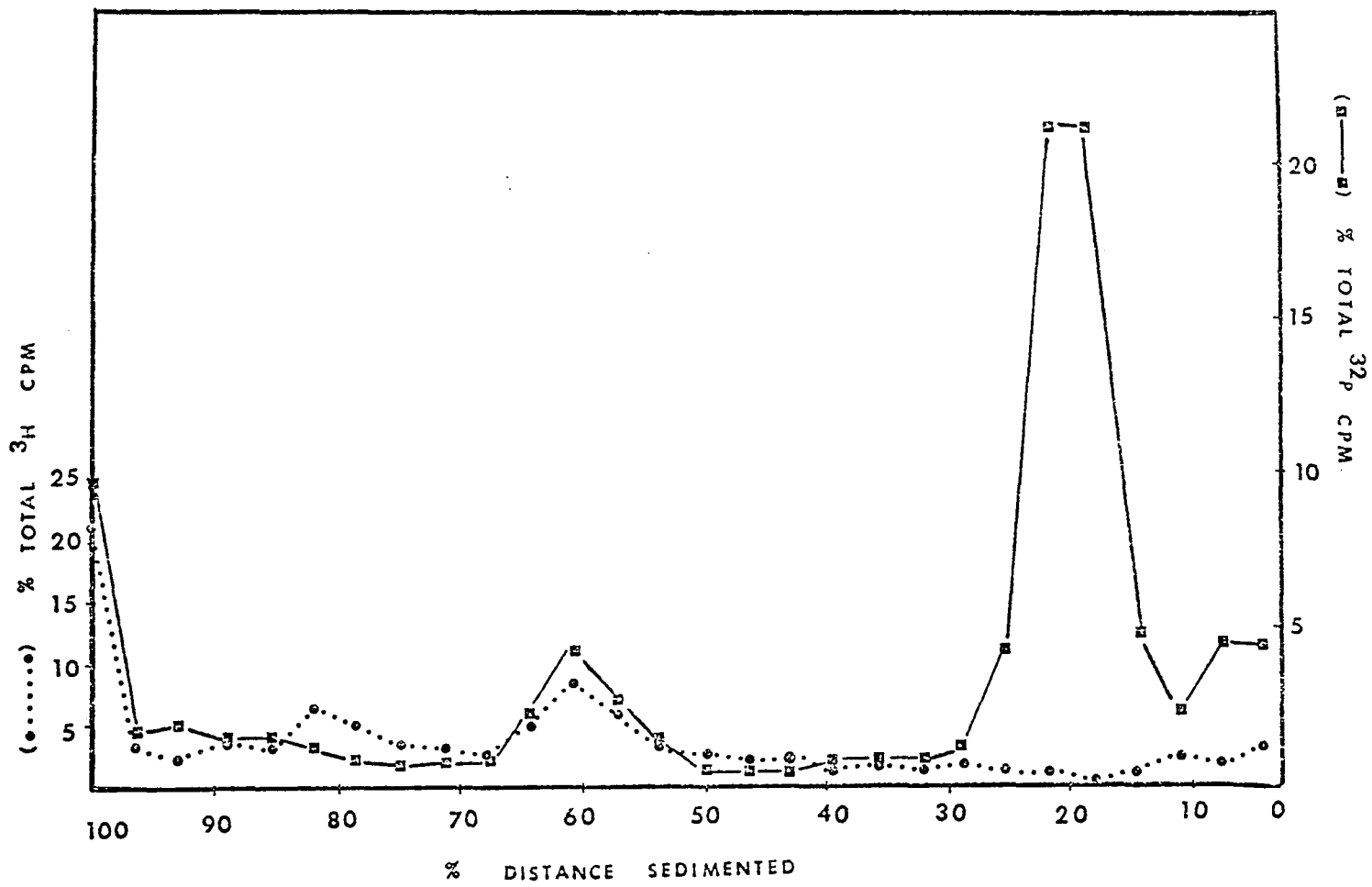
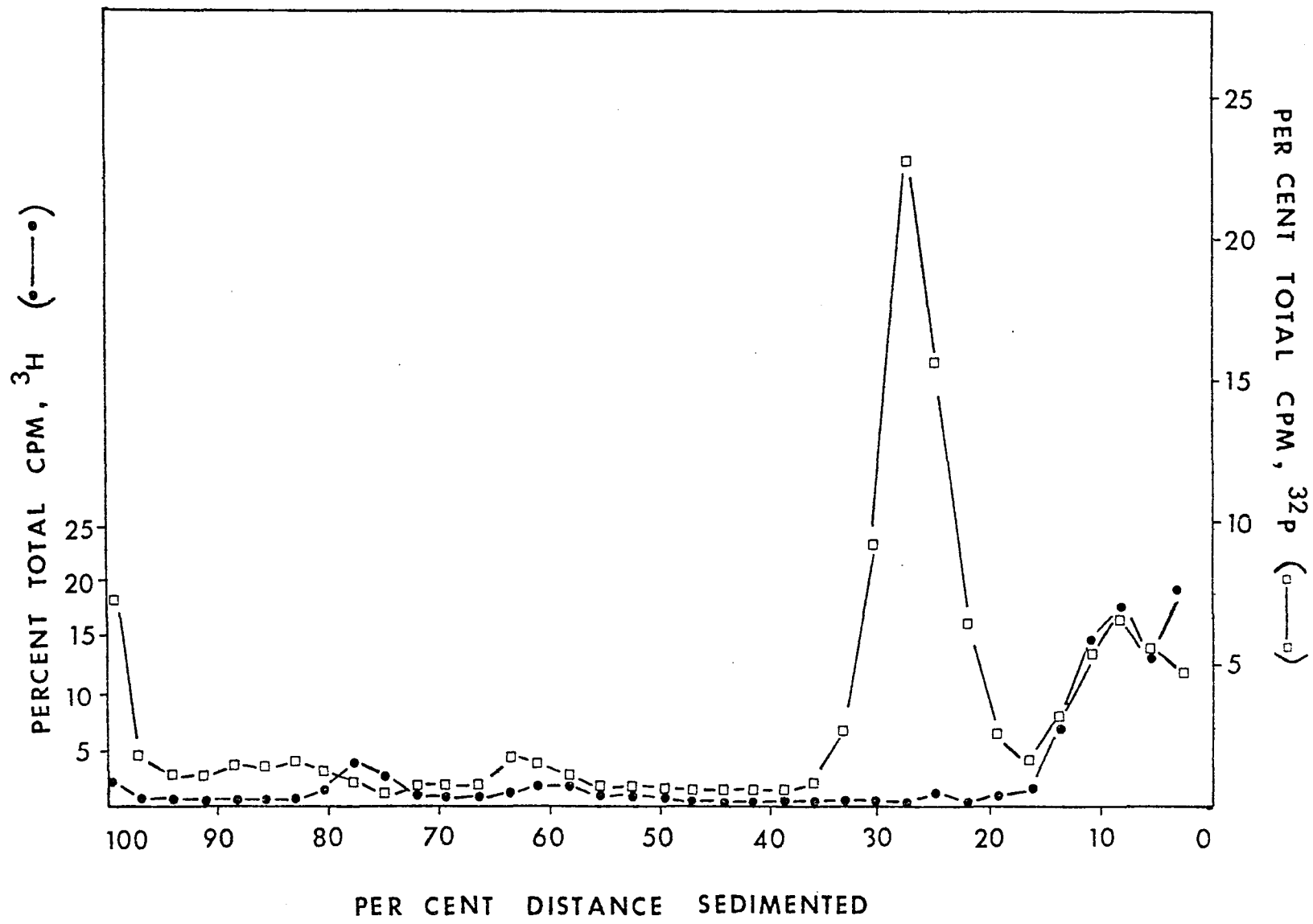


Fig. 20. Effect of Sarkosyl on the sedimentation behavior of
 $^3\text{H}/^{32}\text{P}$ double-label SP82G DNA

A sheared lysate of $^3\text{H}/^{32}\text{P}$ SP82G-infected cells was made as described in Fig. 19. The lysate was made 1% in Sarkosyl and incubated at 37°C for 10 minutes. Centrifugation in Renografin for analysis of membrane-bound DNA is described in text and Materials and Methods.

^3H (•——•) and ^{32}P (□——□) radioactivities are shown.



SPP1 Experiments

The small Bacillus subtilis bacteriophage SPP1 is unique among other known B. subtilis bacteriophages in that the replication of its DNA can be inhibited by the drug 6-(p-hydroxyphenylazo)-uracil (HPUra) (Esche, 1975; N.C. Brown, personal communication). Brown has shown that HPUra inhibits DNA synthesis in B. subtilis by interference with DNA polymerase III (Brown, 1970; Brown, 1971; Brown et al., 1972; Cozzarelli et al., 1973). This suggests that SPP1 uses the host DNA polymerase III for its own DNA replication; this hypothesis has recently been found to be correct (N.C. Brown, personal communication). Therefore, it would seem that the use of an SPP1/ B. subtilis system, where phage DNA synthesis can be effectively inhibited, would be a direct way to ascertain the role of DNA synthesis in DNA-membrane interactions.

Experiments were undertaken to determine if SPP1 DNA did in fact associate with the host membrane during phage infection. The Renografin gradient profile of one such experiment is shown in Fig. 21. The following points should be noted:

- 1) As in the SP82G/SB-1 system, there is a rapidly-sedimenting peak at approximately 78% of the gradient distance.

- 2) There is a slower-sedimenting peak at approximately 24%, whereas the gradient position of slowly-sedimenting SP82G DNA was 28%.

- 3) The slower-sedimenting SPP1 DNA peak is free, whole SPP1 DNA by virtue of its position relative to purified, radioactively-labeled marker SPP1 DNA (Fig. 21).

- 4) Simultaneous sedimentation of sheared and unsheared SPP1 DNA indicates that SPP1 DNA is not fragmented by the Vortex shearing. The lack

of shear sensitivity is understandable since the molecular weight of SPP1 DNA of 25×10^6 daltons is approximately one fourth the size of SP82G DNA (115×10^6 daltons).

5) There is a small, very rapidly-sedimenting peak at approximately 90% of the gradient distance. This peak was most often not present, and was shown to be intact SPP1 bacteriophage by virtue of its position relative to the sedimentation position in Renografin of radioactively-labeled, purified SPP1 phage.

6) The vast majority of the recovered ^3H -thymine-labeled DNA is found at the top of the gradient. This low molecular weight material is most likely degradation product, but the cause of its appearance is not known at this time.

SPP1 DNA-Membrane Complex Analysis

The SPP1 DNA-membrane complex was examined for sensitivity to Protease, Sarkosyl, phospholipase C and DNase/phosphodiesterase. The results are presented in Fig. 22. It is clear from these experiments that the rapidly-sedimenting material is a complex of SPP1 DNA and the host cell membrane.

Time Course of SPP1 DNA-Membrane Interactions

The results obtained in a time course experiment indicate that at very early times after infection with SPP1 (4 minutes) up to 75% of the recovered intact viral DNA can be found in a membrane complex. With increasing time after infection, both bound and free phage DNA are susceptible to extensive degradation; by 12 minutes after infection up to 80% of the recovered SPP1 CPM can be found at the top of the Renografin

Fig. 21. Distribution of infecting SPP1 DNA from sheared lysates on Renografin gradients

Experimental procedure described in Materials and Methods. Gradient profile is traced from a computer plot of the radioactivity determined in each of the 37 fractions. Direction of sedimentation is from right to left. Radioactivity from ^3H -SPP1 (thymine)-infected *B. subtilis* lysates (—) and ^{14}C - SPP1 (thymine) marker DNA (---) added to the infected cells before lysis are shown. Arrow (↓) denotes position of whole, infective marker SPP1 phage. Arrow (↓) denotes position of ^{14}C - SPP1 marker DNA.

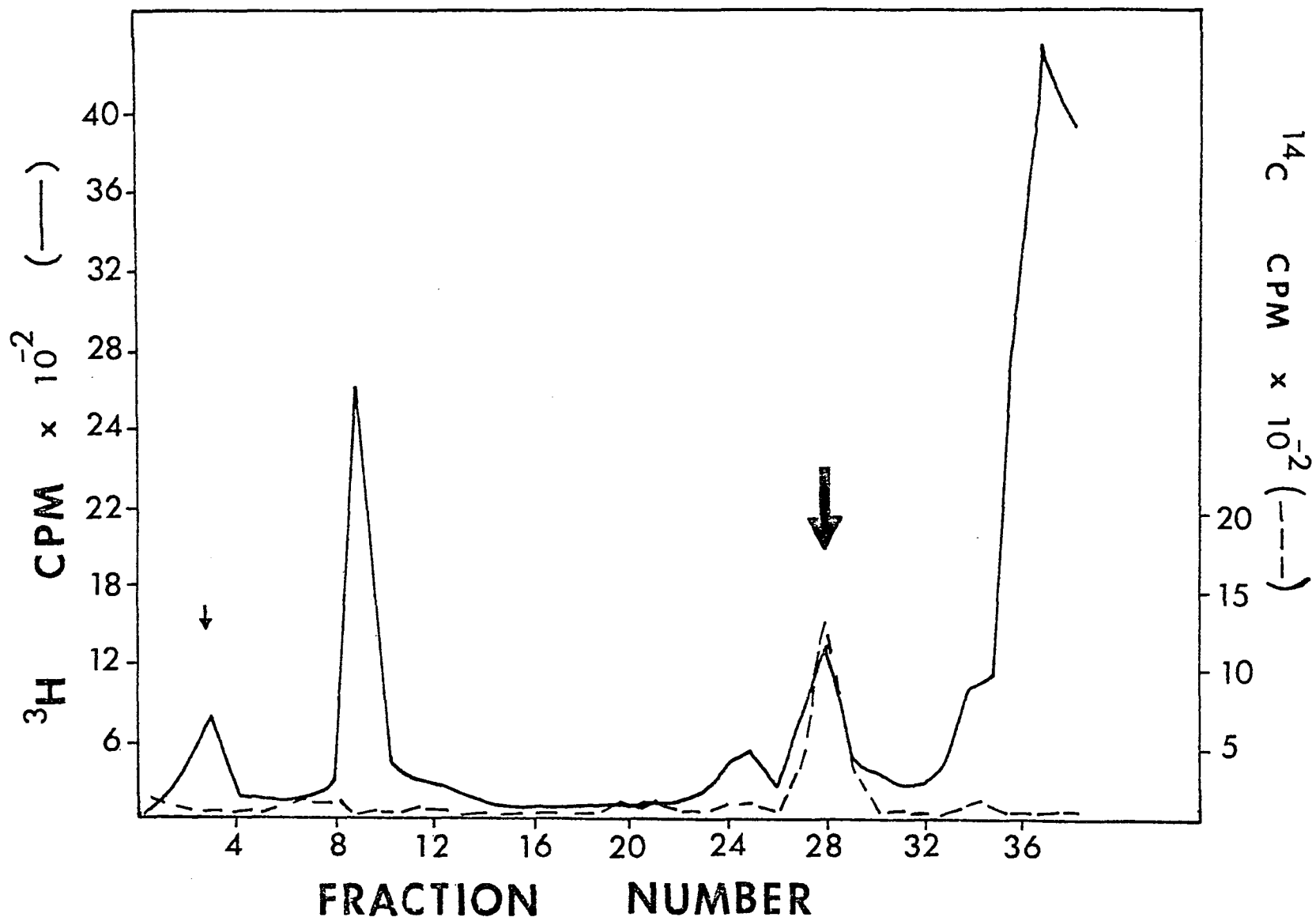


Fig. 22. Distribution of infecting SPP1 DNA after treatment
with various chemical agents

Experimental procedure described in Materials and Methods.

A: Sedimentation profile of an untreated (control) ^3H -SPP1-infected B. subtilis lysate. 100% of recovered radioactivity= 16,535 counts/minute.

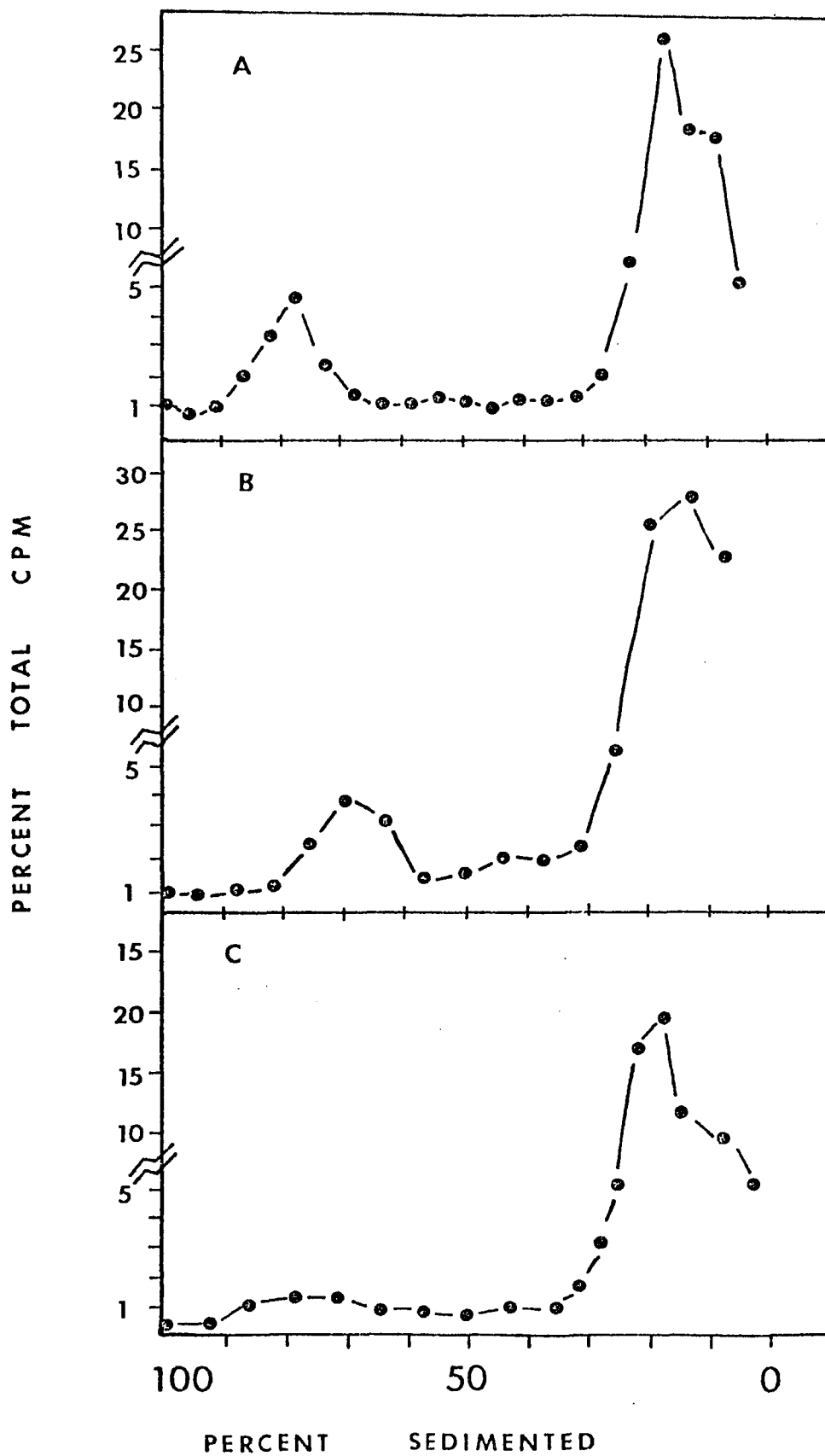
B: Sedimentation profile of an untreated (incubation control) ^3H -SPP1-infected lysate incubated at 37°C for 10 minutes. 100% of recovered radioactivity= 17,093 counts/minute.

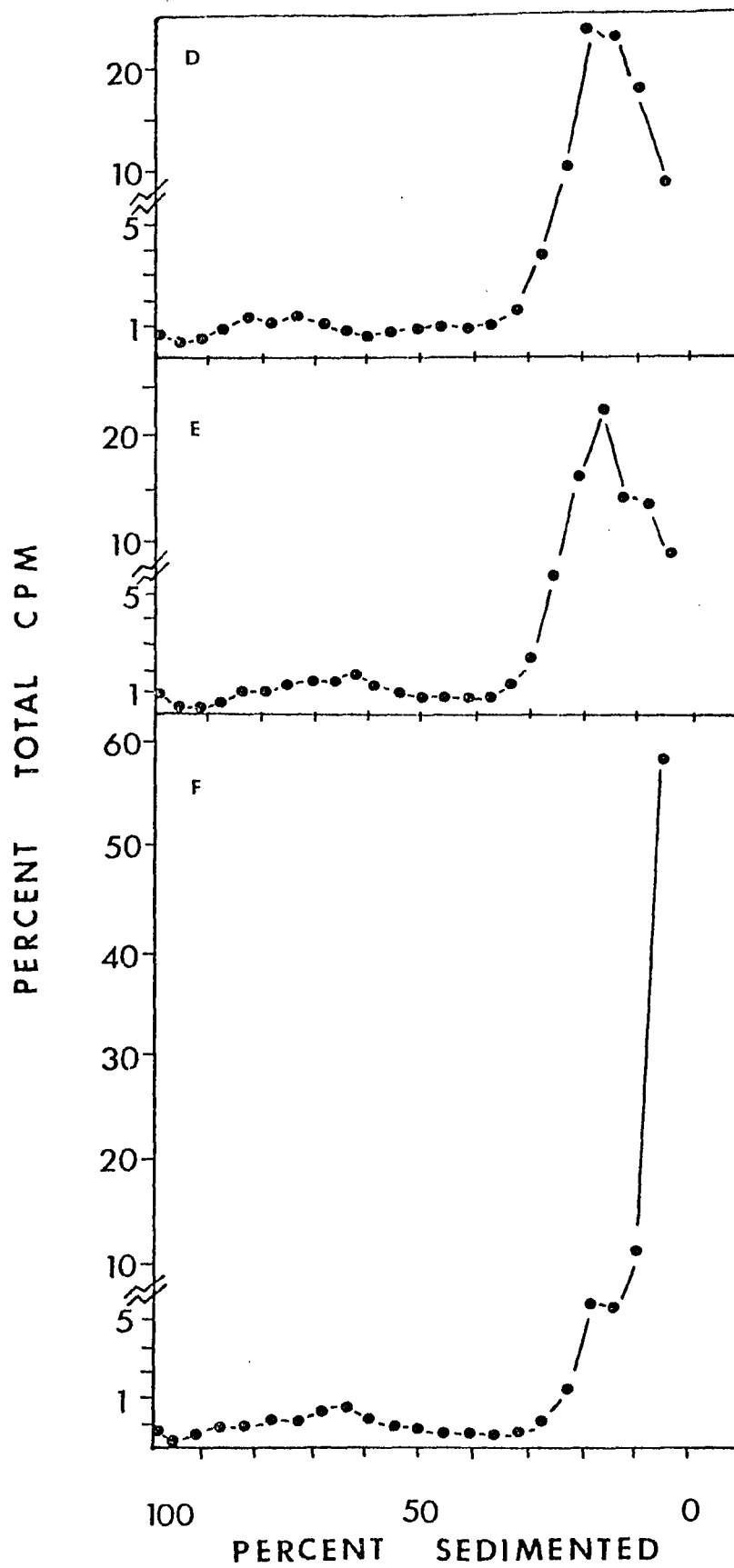
C: Sedimentation profile of a Sarkosyl-treated ^3H -SPP1-infected lysate. 100% of recovered radioactivity= 16,377 counts/minute.

D: Sedimentation profile of a phospholipase C-treated ^3H -SPP1-infected lysate. 100% of recovered radioactivity= 17,686 counts/minute.

E: Sedimentation profile of a Protease-treated ^3H -SPP1-infected lysate. 100% of recovered radioactivity= 16,987 counts/minute.

F: Sedimentation profile of a DNase/phosphodiesterase-treated lysate. 100% of recovered radioactivity= 18,099 counts/minute.





gradient. The bound DNA is less susceptible to degradation than the unbound DNA. Limited observations show that the rate of disappearance of the membrane-bound DNA peak is slower (approximately one half) than the rate at which the free DNA peak disappears.

Metabolic Inhibitor Studies: SPP1

The ability of SPP1 DNA to bind to the host cell membrane during the inhibition of protein synthesis and the inhibition of RNA synthesis was investigated. Following the protocols used in the SP82G investigations, the sensitivity of the SPP1 binding process to chloramphenicol and rifampicin was analyzed. The data, presented in Fig. 23, suggests that, like SP82G, SPP1 DNA-membrane binding does not require protein synthesis but does require RNA synthesis.

To investigate the role of DNA synthesis in the binding event, B. subtilis SB-1 cells were pre-treated with HPUra at a final concentration of 150 ug/ml, which is sufficient to cause a 95% inhibition of host and SPP1 DNA replication (Dr. Asad Ahmann, personal communication). They were then infected with SPP1 bacteriophage and analyzed for membrane-bound DNA (see Materials and Methods). The results are presented in Table 5 and suggest that the inhibition of DNA synthesis has no effect on SPP1 DNA-membrane binding. This result was not unexpected due to the very early presence of large amounts of SPP1 DNA in membrane association.

Location of Replicating DNA

Enrichment for replicating DNA molecules in the membrane-

Fig. 23. Effect of chloramphenicol and rifampicin on the distribution of infecting SPP1 DNA

Experimental procedure described in Materials and Methods.

A: Sedimentation profile of an untreated (control) ^3H -SPP1-infected B. subtilis lysate. 100% of recovered radioactivity= 19,449 counts/minute.

B: Sedimentation profile of chloramphenicol-treated cells infected with ^3H -SPP1 phage and lysed in the usual manner. 100% of recovered radioactivity= 22,793 counts/minute.

C: Sedimentation profile of rifampicin-treated cells infected with ^3H -SPP1 and lysed in the usual manner. 100% of recovered radioactivity= 21,397 counts/minute.

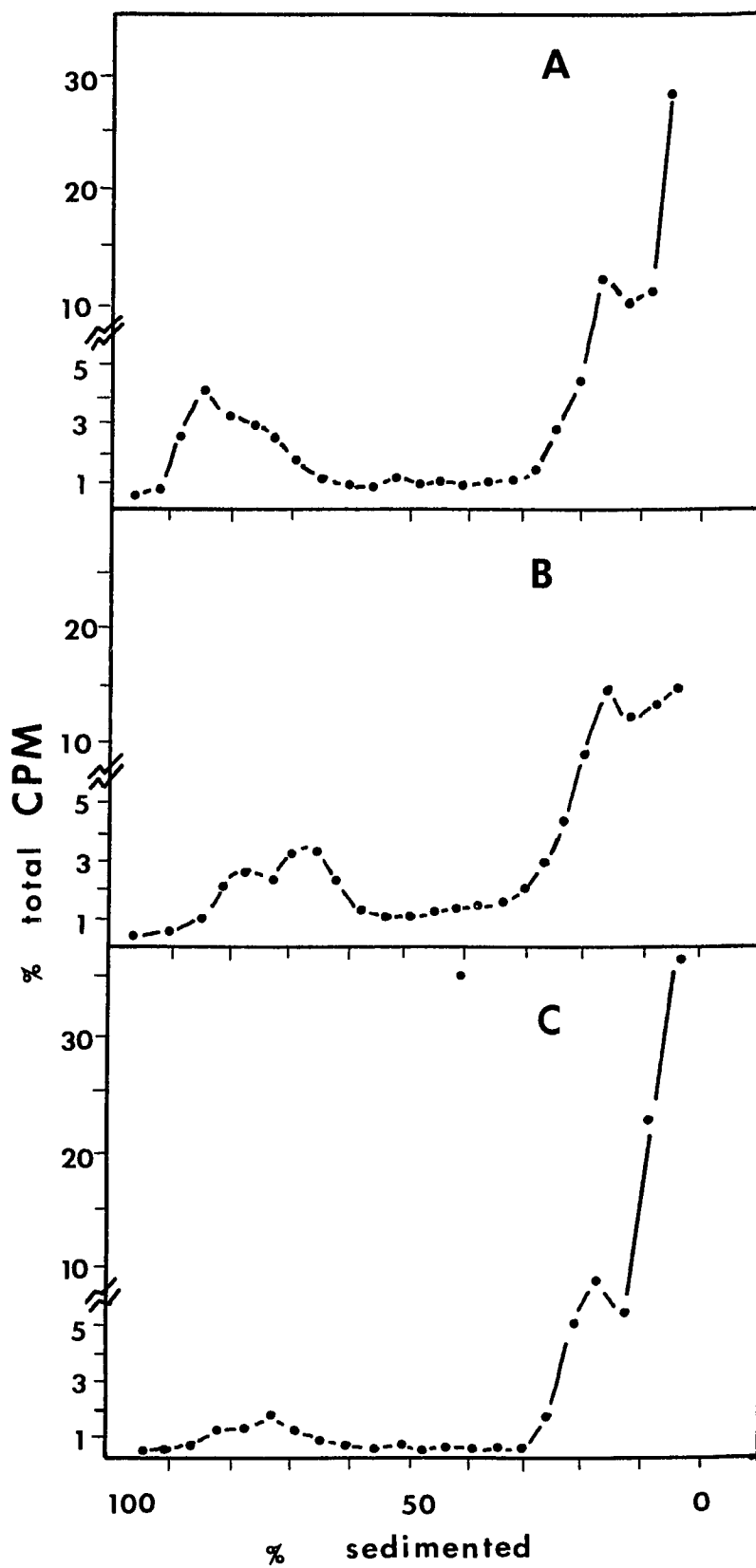


Table 5. EFFECT OF HPURA ON SPP1 DNA-MEMBRANE BINDING

Time after Infection (minutes)	Control %	HPUra-Treated %
2	30	38
4	32	35
6	40	35

*Numbers represent the percent recovered CPM in the bound DNA peak divided by the percent recovered CPM in the bound + the unbound DNA peaks.

bound DNA fraction has been reported for several bacterial and bacteriophage systems (see Introduction). In an effort to determine where replicating DNA molecules are preferentially located in SPP1-infected and uninfected cells, the following experiments were performed. A thymine-requiring Bacillus subtilis host was grown in the presence of non-radioactive thymine for 2 hours. The cells were then pelleted, washed and resuspended in a small volume of the same media. Cells were infected with ^3H -thymine-labeled SPP1 and following a 2 minute adsorption period, the infected cells were diluted back to the original cell concentration with media containing 2 ug/ml of ^{14}C -thymine. At various times after infection, samples were withdrawn and analyzed for membrane-bound DNA in the usual manner. The results are shown in Fig. 24.

It is clear that the replicating DNA molecules are exclusively located in the membrane-associated fraction of DNA. However, this DNA synthesis cannot unequivocally be attributed to either bacteriophage or host DNA replication. The reason for this is two-fold.

- 1) SPP1 bacteriophage does not shut down host DNA synthesis (Esche, 1975).

- 2) The results of the experiment shown in Fig. 25 indicate that both phage and host DNA are associated with the host cell membrane at the times assayed.

Therefore, it can be concluded from these experiments that in vivo DNA synthesis is occurring on the host membrane in SPP1-infected Bacillus subtilis.

Fig. 24. Distribution of newly-incorporated ^{14}C -thymine in SPP1-infected Bacillus subtilis lysates on Renografin gradients

Experimental procedure described in text. Sedimentation profiles (traced from computer plots) are shown for lysates made at 2, 6 and 10 minutes after ^3H -SPP1 infection of B. subtilis UVB210 cells. Direction of sedimentation is from right to left. Solid line (————) represents ^3H radioactivity derived from the pre-labeled ^3H -thymine SPP1 bacteriophage. Broken line (-----) represents ^{14}C -thymine added to the cells simultaneously with the SPP1 phage.

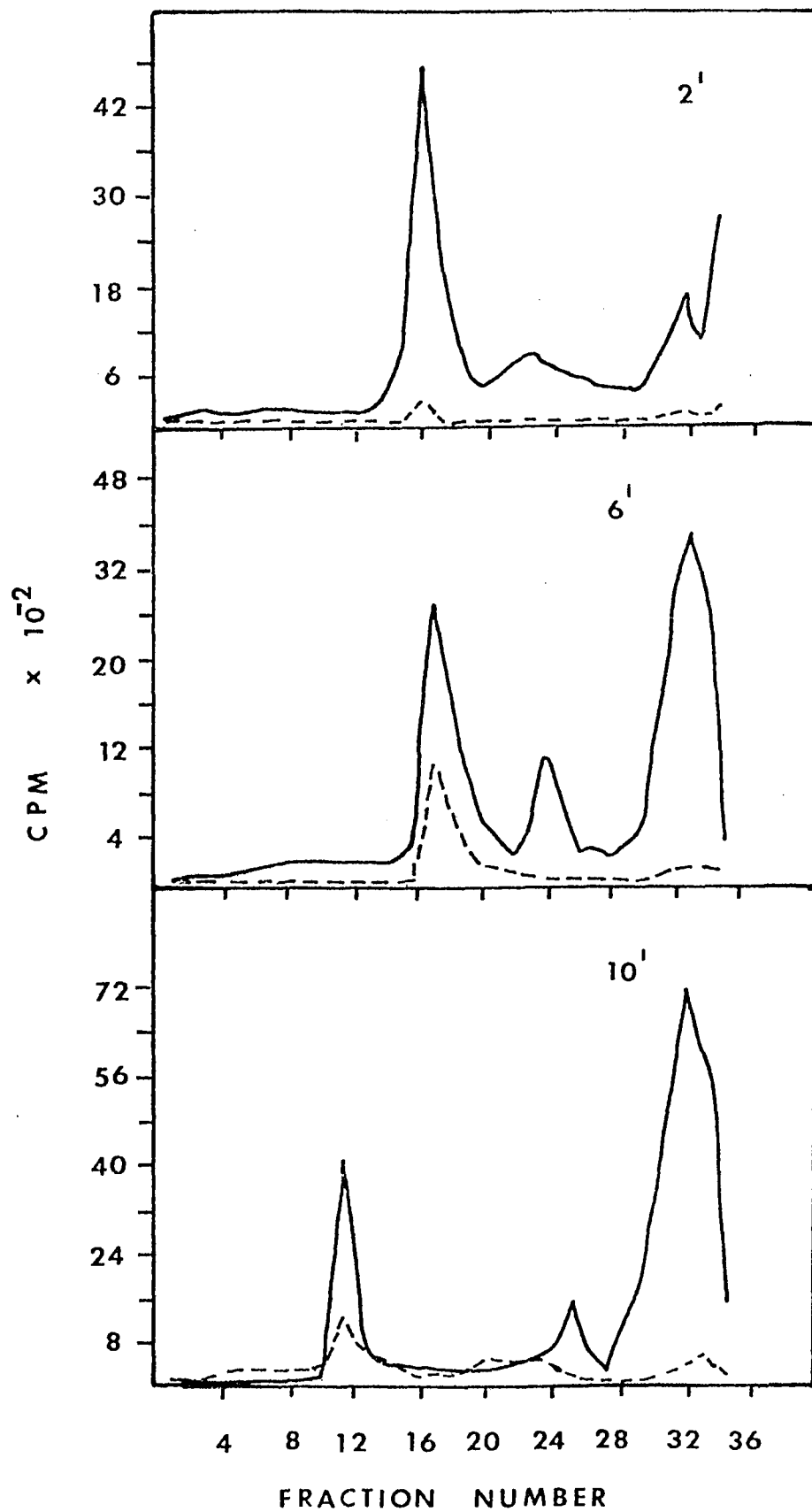
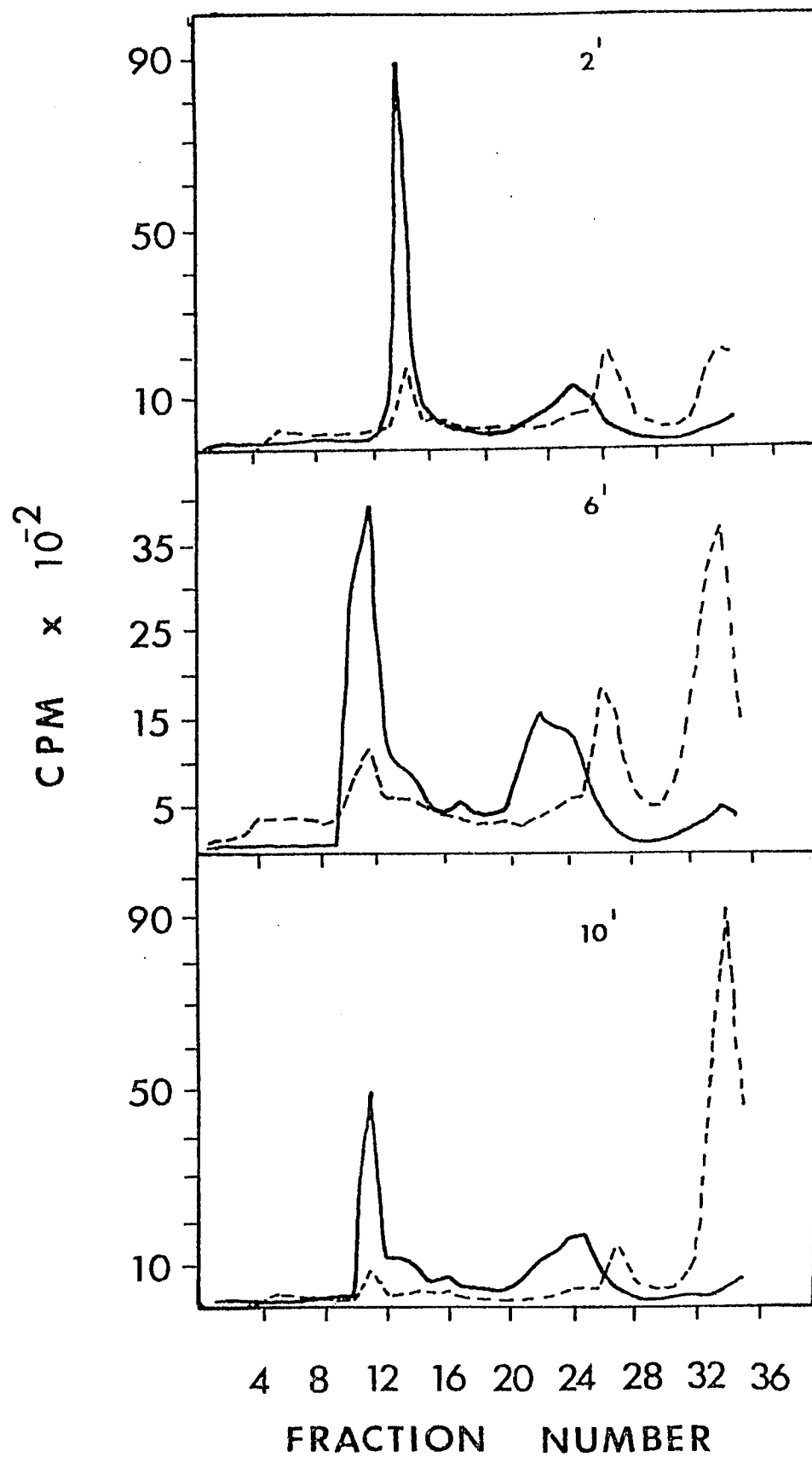


Fig. 25. Distribution of labeled SPP1 and host DNAs in SPP1-infected Bacillus subtilis lysates on Renografin gradients

B. subtilis VUB210 cells (thy^-) were grown for 2 hours in media containing 2 ug/ml ^{14}C -thymine plus 20 ug/ml non-radioactive thymine to a cell concentration of approximately $6 \times 10^7/\text{ml}$. The cells were pelleted, washed and resuspended in media containing only non-radioactive thymine (20 ug/ml). Bacteria were then infected with ^3H -SPP1 (thymine) bacteriophage ($\text{moi}=2$) and analyzed for membrane-bound DNA at 2, 6 and 10 minutes after infection in the usual manner. Gradient profiles are traced from the computer plots. Solid line (————) represents the ^{14}C -thymine in which the cells were grown and therefore which was incorporated into the host DNA. Broken line (-----) represents ^3H radioactivity derived from the ^3H -thymine-labeled SPP1 phage. Direction of sedimentation is from right to left.



DISCUSSION

The examination of lysates of SP82G-infected Bacillus subtilis cells by sedimentation on 0 to 38% Renografin gradients indicates that the rapidly-sedimenting SP82G DNA component exhibits enzyme and detergent sensitivities typical of membrane-complexed DNA. The time course of SP82G DNA-membrane binding has been shown to include an initial phase where the amount of complexed DNA increases, and a secondary phase where the complexed DNA is released from membrane association. This pattern of viral DNA binding followed by detachment is not uncommon (for summary see Introduction, Table 1); ØX174 appears to be the only bacteriophage known so far that does not release even a fraction of its membrane-complexed DNA into the cell cytoplasm.

The insensitivity of the formation of the DNA-membrane complex (Phase I) to chloramphenicol indicates that active protein synthesis is not required for its formation. However, inhibition of membrane complex formation to baseline levels by rifampicin and actinomycin D suggests that de novo RNA synthesis is an essential requirement for the formation of the complex. At early times during the formation of the complex (3-6 minutes after infection), at least 40% of the complex is sensitive to RNase. Therefore, the required RNA synthesis forms a structure essential for the integrity of the complex. The RNA findings, in conjunction with the lack of a protein synthesis requirement, suggest that the structural role for RNA in the membrane complex may represent an RNA primer for covalent extension by a DNA polymerase. This model for the initiation of DNA synthesis was origin-

ally proposed by Brutlag, Schekman and Kornberg (1971) and has been implicated in the initiation of bacteriophage T₄ DNA synthesis, where an RNA-DNA copolymer has been isolated (Buckley et al., 1972).

Genetic analysis of the membrane-associated DNA suggests that the right end of the molecule is the first end to become complexed to the membrane. The timing of the early formation of the complex, e.g. that portion which is RNase sensitive, must be viewed in terms of what is known about the timing and direction of entry of the SP82G DNA molecule. McAllister (1970) has presented evidence in the timing for entry of rescuable genetic markers of SP82G. When extrapolated to 37°C, this data suggests that the time for entry of the earliest marker is about 3 minutes, and the entire genome requires a further 1.2 minutes for entry. The direction of entry of the viral DNA was determined in the above study and confirmed in a subsequent analysis of the inhibition of genome transfer by ³²P suicide (McAllister and Green, 1973). The genome is injected in a linear, polar fashion with the right end of the molecule always entering last. The time at which maximum RNase-sensitive binding takes place (4 minutes after infection) coincides with the time of entry of the right end of the molecule. This, taken together with the higher frequency of right end genetic markers in the complex at 4 minutes after infection, supports the conclusion that an early step in the formation of the complex requires RNA synthesis to physically link the right end of the molecule to the membrane. It is a reasonable assumption that linkage at this time is a consequence of transcription of the right end, but direct evidence to bear on this possibility was not obtained in this work. It could be argued that the differences in marker frequencies which are seen are the result of intracellular

nuclease or denaturation activities. It is, nevertheless, clear that these phenomena are distributed unequally between the bound and free DNA fractions. Thus, even if these alternative explanations are offered for the differences observed it is necessary to implicate a specificity in membrane association to explain the protective effect.

Examination of Table 4b reveals that the specific activity of the 9 minute DNA is higher than that of the 4 and 7 minute DNAs. The reason for this is unknown at the present time. It is possible that some type of SP82G DNA replication has been initiated at this time. An increase in certain gene copies might then be reflected in an increased activity of the DNA in marker rescue experiments.

Specific regions of infecting bacteriophage DNA have previously been reported to be membrane-associated. Marsh *et al.* (1971) have shown that T₄ genes 40 and 42 are membrane-bound. The initiation of T₄ DNA replication was shown to proceed from this region and among the first genes replicated are several involved with the replication of T₄ DNA. Hellend and Nygaard (1975) have shown that T₇ DNA is bound to the membrane and that DNA complementary to early transcripts is bound first. In contrast to these observations, the initial SP82G binding involves association of a region of the genome in which all genes so far identified in SP82G are late-acting (Green and Laman, 1972). The direction of replication of SP82G or related phages is unknown.

During this period when SP82G DNA being bound to membrane is increasing, there are periods (2-3 minutes and 7-9 minutes) in which the complex is insensitive to RNase. At the earliest time, the amount of membrane complex formed is similar to the baseline levels observed from 20 minutes on (i.e., Phase III). This baseline level might represent a

nonfunctional, nonspecific association although direct evidence for or against this was not obtained in this study. The genetic study suggests that all markers tested are present in the complex at 4 minutes after infection, albeit right-end markers are more frequent than left-end markers. If this baseline value were a non-specific association, and did not represent DNA in the normal association-dissociation pathway, then the amount of complex which becomes maximally bound in an RNase-sensitive state would represent all of the remaining complexed DNA.

The shift from the RNase-sensitive state to the RNase-insensitive state at 7-9 minutes after infection, shows that the initially-formed RNA structure is superseded by some other linkage. The genetic evidence suggests that the alternate form of binding is gradually distributed over different regions of the molecule, perhaps starting in a polar fashion from the right end. This shift is reminiscent of the extension-replacement of the RNA initiating transcript (RNA primer) by DNA that was previously mentioned.

The present studies with double-label bacteriophage suggest that a viral protein(s) is injected into the host along with the viral DNA and is found in the DNA-membrane complex. It is possible that this protein(s) is a so-called "pilot protein" which promotes binding of the infecting DNA to a particular membrane site. Thus, a DNA-protein-membrane linkage would be the earliest linkage formed (i.e., 2-3 minutes after infection). One would predict that this earliest attachment would be chloramphenicol and RNase insensitive. The fact that at 2-3 minutes after infection, a low level of viral DNA is found bound to the membrane (baseline level) is not inconsistent with the pilot protein hypothesis. Therefore, it remains a possibility that this early bound DNA truly rep-

resents a functional attachment.

The data presented indicate that nalidixic acid has no effect on membrane complex formation. However, in the system studied, nalidixic acid is an incomplete inhibitor of SP82G DNA synthesis and, thus, it can be assumed that limited DNA synthesis is taking place. In one report (Pisetskyy et al., 1972) it was suggested that the action of nalidixic acid is directed towards the conversion of 30-40 S fragments to higher molecular weight DNA. Therefore, limited DNA synthesis in the replacement of an RNA initiator by DNA may be all that is required for the Phase I binding increase. In the Bacillus subtilis minicell studies, minicells were found to form membrane-complexed DNA following SP82G infection despite the fact that minicells are deficient in DNA synthesis. These cells are capable of RNA synthesis, which has been shown to be a prerequisite for the initial attachment of SP82G DNA to membrane. Conceivably, the later association (i.e., the later RNase-resistant period) which may involve the RNA replacement, is mediated by either an atypical replicative DNA synthesis (i.e., repair synthesis) or some novel biochemical process.

A detachment of the viral DNA from the host cell membrane takes place in Phase II of the binding time course. This release is a separate and distinct event apart from the binding process, since the chloramphenicol studies indicate that if protein synthesis is inhibited, the normal binding process takes place, but release from membrane association is prevented. Detachment of T₄ DNA from membrane association was investigated by Earhart in 1970; he found that chloramphenicol and tetracycline prevent the normal release of the T₄ genome from the E. coli cell membrane.

Earhart suggested that this detachment is a late phage function (Earhart, 1970; Earhart, 1973); later it was shown that the viral genes involved in T₄ head formation and DNA packaging are required for the release phenomenon to occur (Siegel and Schaecter, 1973).

Several explanations are possible to account for the detachment of SP82G DNA from the host cell membrane:

- 1) the infection process may alter the membrane structure,
- 2) a specific phage-directed release function might be present to effect the DNA detachment or,
- 3) the detachment may represent a "peeling-away" from the membrane once DNA synthesis on a particular segment of DNA is completed.

The present studies indicate that even the incomplete inhibition of SP82G DNA synthesis by nalidixic acid does have a detectable effect on membrane detachment; approximately half of the normal release occurs. The DNA synthesis-deficient minicells do not release any of the bound SP82G DNA from the membrane-associated state. The time at which the DNA release commences (9-10 minutes after infection at 37°C) closely corresponds with the time of initiation of DNA synthesis in this and related phage (Kahan, 1971). Thus, the above three findings strongly suggest that active DNA synthesis is required for membrane release to occur, although the exact mechanism of release cannot be determined from this work.

In summary, these observations lead to the following conclusions.

- 1) During the infection of Bacillus subtilis by bacteriophage SP82G, the infecting viral genome binds to a host cell component which

most likely is membrane.

2) The DNA-membrane association shows a specific time course.

3) The time course consists of three phases: an increase in the amount of viral DNA bound to the membrane, followed by a release of the DNA from membrane association except for a small, constant amount of DNA which remains membrane-associated.

4) The DNA-membrane binding process is dependent upon RNA synthesis and an energy source. The binding is independent of protein and replicative DNA synthesis.

5) The release from membrane association requires viral protein synthesis and probably viral DNA synthesis.

6) The integrity of the DNA-membrane complex is sensitive to RNase from 3 to 6 minutes after infection. Both before and after this period, the complex is insensitive to RNase.

7) Genetic analysis of the infecting DNA associated with the membrane suggests that the right (last-entering) end of the molecule is the first to bind to the membrane. The unbound portions of the DNA subsequently become bound, probably in the order of right to left.

8) ^3H -labeled protein from the infecting virus enters the host, becomes membrane-associated and co-sediments with the membrane-bound DNA.

BACTERIOPHAGE SPP1

Experiments presented indicate that infecting SPP1 DNA also binds to the host cell membrane. However, these were brief experiments designed only to obtain an overview of the SPP1 DNA binding. The smaller

size of SPP1 DNA (25×10^6 daltons) made it nonsusceptible to the shear forces generated by the Vortex shear step. Therefore, rather than looking at those portions of the genome most closely associated with the membrane, it can only be discerned from these experiments whether whole SPP1 DNA molecules are membrane-associated or not membrane-associated.

It is clear from the present work that SPP1 DNA binds to the host membrane at early times after infection. However, an interesting observation from the SPP1 analyses is that shortly thereafter, the viral DNA is subject to extensive nucleolytic degradation. Membrane association seems to afford some protection to bound DNA that is not enjoyed by the free DNA but, nevertheless, the degradation is so extreme that it is difficult to imagine how this virus reproduces itself at all. Perhaps only a small proportion of the infecting phage lead to a successful infection.

The degradation is not likely to be an artifact of the lysis procedure since pre-labeled marker SPP1 DNA added to the samples of infected bacteria before going through the lysis regime is not affected, and sediments in the position of whole, intact SPP1 DNA. It also seems unlikely that this is all in vivo degradation since the sedimentation of infected lysates on sucrose gradients to detect membrane-bound DNA (Reeve, personal communication) does not reveal a similar large amount of small molecular weight material at the top of the gradients. The extensive degradation of SPP1 DNA after phage infection has been observed before (Dr. T.A. Trautner, personal communication) but as yet there is no reasonable explanation for it. Also, it must be kept in mind that SPP1 infection has been shown not to turn off host DNA or protein synthesis

(Esche, 1975).

The original intent in developing an SPP1 DNA-membrane binding system was to examine the inhibitory effect of HPUra on DNA synthesis in that system. The experiments presented suggest that inhibition of DNA synthesis by HPUra does not affect the membrane binding process. Unfortunately, however, a firm conclusion cannot be made since it is not presently known when SPP1 DNA replication is initiated.

REFERENCES

1. Baird, J., Bourguignon, G., and Sternglanz, R. Effect of Nalidixic Acid on the Growth of Deoxyribonucleic Acid Bacteriophages. *J. Virology* 9, 17 (1972).
2. Botstein, D. and Levine, M. Intermediates in the Synthesis of Phage P22 DNA. *CSH Symp. Quant. Biol.* 33, 659 (1968).
3. Brown, N. 6-(p-Hydroxyphenylazo)-Uracil: A Selective Inhibitor of Host DNA Replication in Phage-Infected *Bacillus subtilis*. *Proc. Nat. Acad. Sci. (Wash.)* 67, 1454 (1970).
4. Brown, N. Inhibition of Bacterial DNA Replication by 6-(p-Hydroxyphenylazo)-Uracil: Differential Effect on Repair and Semiconservative Synthesis in *Bacillus Subtilis*. *J. Mol. Biol.* 59, 1 (1971).
5. Brown, N., Wisseman, C., and Matsushita, T. Inhibition of Bacterial DNA Replication by 6-(p-Hydroxyphenylazo)-Uracil. *Nature New Biol. (Lond.)* 237, 72 (1972).
6. Brutlag, D., Schekman, R., and Kornberg, A. A Possible Role for RNA Polymerase in the Initiation of M13 DNA Synthesis. *Proc. Nat. Acad. Sci. (USA)* 68, 2826 (1971).
7. Buckley, P., Kosturko, L., and Kozinski, A. *In Vivo* Production of an RNA-DNA Copolymer after Infection of *Escherichia coli* by Bacteriophage T₄. *Proc. Nat. Acad. Sci. (USA)* 69, 3165 (1972).
8. Center, M. Replicative Intermediates of Bacteriophage T₇ Deoxyribonucleic Acid. *J. Virology* 10, 115 (1972).

9. Cozzarelli, N. and Low, R. Mutational Alteration of Bacillus subtilis DNA Polymerase III to Hydroxy-Phenylazo-Pyrimidine Resistance: Polymerase III is Necessary for DNA Replication. *Biochem. Biophys. Res. Comm.* 51, 151 (1973).
10. Dworsky, P. and Schaecter, M. Effect of Rifampin on the Structure and Membrane Attachment of the Nucleoid of Escherichia coli. *J. Bact.* 116, 1364 (1973).
11. Earhart, C., Tremblay, G., Daniels, M., and Schaecter, M. DNA Replication Studied by a New Method for Isolation of Cell Membrane-DNA Complexes. *CSH Symp. Quant. Biol.* 33, 707 (1968).
12. Earhart, C. The Association of Host and Phage DNA with the Membrane of Escherichia coli. *Virology* 42, 429 (1970).
13. Earhart, C., Sauri, C., Fletcher, G., and Wulff, J. Effect of Inhibition of Macromolecule Synthesis on the Association of Bacteriophage T₄ DNA with Membrane. *J. Virology* 11, 527 (1973).
14. Esche, H. Gene Expression of Bacteriophage SPP1, II. Regulatory Aspects. *Mol. Gen. Genetics* 142, 57 (1975).
15. Forsheit, A. and Ray, D. Replication of Bacteriophage M13, VI. Attachment of M13 DNA to a Fast Sedimenting Host Cell Component. *Virology* 43, 647 (1971).
16. Frankel, F., Majumdar, C., Weintraub, S., and Frankel, D. DNA Polymerase and the Cell Membrane after T₄ Infection. *CSH Symp. Quant. Biol.* 33, 495 (1968).
17. Frankel, F. The Absence of Mature Phage DNA Molecules from the Replicating Pool of T-Even-Infected Escherichia coli. *J. Mol. Biol.* 18, 109 (1966).

18. Gage, L. and Fujita, D. Effect of Nalidixic Acid on Deoxyribonucleic Acid Synthesis in Bacteriophage SP01-Infected Bacillus subtilis. J. Bact. 98, 96 (1969).
19. Ganesan, A. and Lederberg, J. A Cell Membrane Bound Fraction of Bacterial DNA. Biochem. Biophys. Res. Comm. 18, 824 (1965).
20. Ganesan, A. Studies on In Vitro Replication of Bacillus subtilis DNA. CSH Symp. Quant. Biol. 33, 45 (1968).
21. Gilbert, W. and Dressler, D. DNA Replication: The Rolling Circle Model. CSH Symp. Quant. Biol. 33, 473 (1968).
22. Green, D. Infectivity of DNA Isolated from B. subtilis Bacteriophage SP82. J. Mol. Biol. 10, 438 (1964).
23. Green, D. Intracellular Inactivation of Infective SP82 Bacteriophage DNA. J. Mol. Biol. 22, 15 (1966).
24. Green, D. Physical and Genetic Characterization of Sheared Infective SP82 Bacteriophage DNA. J. Mol. Biol. 22, 1 (1966).
25. Green, D. and Laman, D. Organization of Gene Function in Bacillus subtilis Bacteriophage SP82G. J. Virology 9, 1033 (1972).
26. Hallick, L., Boyce, R., and Echols, H. Membrane Association by Bacteriophage λ DNA: Possible Direct Role of Regulator Gene N. Nature 233, 1239 (1969).
27. Hallick, L. and Echols, H. Genetic and Biochemical Properties of an Association Complex Between Host Components and λ DNA. Virology 52, 105 (1973).
28. Hellend, D. and Nygaard, A. Selected Genes of T7 DNA Associated with the Escherichia coli Membrane Following Phage Infection. Febs. Letters 50, 105 (1975).

29. Ivarie, R. and Pene, J. Association of the Bacillus subtilis Chromosome with the Cell Membrane; Resolution of Free and Bound Deoxyribonucleic Acid on Renografin Gradients. *J. Bact.* 104, 839 (1970).
30. Ivarie, R. and Pene, J. Association of Many Regions of the Bacillus subtilis Chromosome with the Cell Membrane. *J. Bact.* 114, 571 (1973).
31. Ivarie, R. and Pene, J. DNA Replication in Bacteriophage ϕ 29: The Requirement of a Viral-Specific Product for Association of ϕ 29 DNA with the Cell Membrane of Bacillus amyloliquefaciens. *Virology* 52, 351 (1973).
32. Jacob, F., Brenner, S., and Cuzin, F. The Role of Membrane in Bacterial Replication. *CSH Symp. Quant. Biol.* 28, 329 (1963).
33. Jazwinski, S., Marco, R., and Kornberg, A. A Coat Protein of the Bacteriophage M13 Virion Participates in Membrane-Oriented Synthesis of DNA. *Proc. Nat. Acad. Sci. (USA)* 7, 205 (1973).
34. Kahan, E. Early and Late Gene Function in Bacteriophage SP82. *Virology* 46, 634 (1971).
35. King, J. The Molecular Basis of Intracellular Inactivation in Bacillus subtilis: An Endonuclease Inhibited During Infection by Bacteriophage SP82G. Ph.D. Thesis, University of New Hampshire (1974).
36. Kluge, F., Staudenbauer, W., and Hofschneider, P. Replication of Bacteriophage M13- Detachment of the Parental DNA From the Host Membrane and Transfer to Progeny Phages. *Eur. J. Biochem.* 22, 350 (1971).

37. Knippers, R. and Sinsheimer, R. Process of Infection with Bacteriophage ϕ X174, XX. Attachment of the Parental DNA of Bacteriophage ϕ X174 to a Fast-Sedimenting Cell Component. *J. Mol. Biol.* 34, 17 (1968).
38. Kolber, A. and Sly, W. Association of Lambda Bacteriophage DNA with a Rapidly-Sedimenting *Escherichia coli* component. *Virology* 46, 638 (1971).
39. Kozinski, A. and Lin, H. Early Intracellular Events in the Replication of T₄ Phage DNA, I. Complex Formation of Replicative DNA. *Proc. Nat. Acad. Sci. (USA)* 54, 273 (1965).
40. Levine, M., Chakravorty, M., and Bronson, M. Control of the Replication Complex of Bacteriophage P22. *J. Virology* 6, 400 (1970).
41. Marsh, R., Breshkin, A., and Mosig, G. Origin and Direction of Bacteriophage T₄ DNA Replication, II. A Gradient of Marker Frequencies in Partially Replicated T₄ DNA as Assayed by Transformation. *J. Mol. Biol.* 60, 213 (1971).
42. Linial, M. and Malamy, M. Studies with Bacteriophage ϕ II. Events Following Infection of Male and Female Derivatives of *Escherichia coli* K-12. *J. Virology* 5, 72 (1970).
43. Loos, L., Tessler, P., and Salivar, W. Host Cell Membrane-Associated Phage ϕ X174 DNA Replication, I. Isolation and Partial Characterization of ϕ X DNA-Containing Complexes Released from The Membrane. *Virology* 45, 339 (1971).

44. McAllister, W. Bacteriophage Infection: Which End of the SP82G Genome Goes in First? *J. Virology* 5, 194 (1970).
45. McAllister, W. and Green, D. Effects of the Decay of Incorporated Radioactive Phosphorous on the Transfer of the Bacteriophage SP82G Genome. *J. Virology* 12, 300 (1973).
46. Miller, R. Association of Replicative T₄ Deoxyribonucleic Acid and Bacterial Membranes. *J. Virology* 10, 920 (1972).
47. Niveleau, A. Electron Microscope Studies of Deoxyribonucleic Acid Synthesis in *Escherichia coli*: Localization of (³H) Thymidine in Deoxyribonucleic Acid-Membrane Complexes. *J. Bact.* 120, 433 (1974).
48. Nomura, M., Matsubara, K., Okamoto, K., and Fujimara, R. Inhibition of Host Nucleic Acid and Protein Synthesis by Bacteriophage T₄: Its Relation to the Physical and Functional Integrity of the Host Chromosome. *J. Mol. Biol.* 5, 535 (1962).
49. Pisetskys, D., Berkower, I., Wickner, R., and Hurwitz, J. Role of ATP in DNA Synthesis in *Escherichia coli*. *J. Mol. Biol.* 71, 557 (1972).
50. Reeve, J., Mendelson, N., Coyne, S., Hallock, L., and Cole, R. Minicells of *Bacillus subtilis*. *J. Bact.* 114, 860 (1973).
51. Reeve, J. and Cornett, J. Bacteriophage SP01-Induced Macromolecular Synthesis in Minicells of *Bacillus subtilis*. *J. Virology* 15, 1308 (1975).
52. Reeve, J. Macromolecular Synthesis in Bacteriophage-Infected Minicells of *Bacillus subtilis*. In Press.
53. Salivar, W. and Sinsheimer, R. Intracellular Location and Number of Replicating Parental DNA Molecules of Bacteriophage Lambda and

- ØX174. J. Mol. Biol. 41, 39 (1969).
54. Shuster, R. The Effect of Chloramphenicol and Rifampicin on the "Nicking" and "Membrane Attachment" of Bacteriophage λ DNA. Virology 62, 472 (1974).
55. Siegel, P. and Schaecter, M. The Role of the Host Cell Membrane in the Replication and Morphogenesis of Bacteriophage. Ann. Rev. Micro. 27, 261 (1973).
56. Siegel, P. and Schaecter, M. Bacteriophage T₄ Head Maturation: Release of Progeny DNA from the Host Cell Membrane. J. Virology 11, 359 (1973).
57. Smith, D. and Hanawalt, P. Properties of the Growing Point Region in the Bacterial Chromosome. Biochimica and Biophysica Acta 149, 519 (1967).
58. Sober, H. (editor) Handbook of Biochemistry. The Chemical Rubber Co., Cleveland Ohio (1968).
59. Sueoka, N. and Quinn, W. Membrane Attachment of the Chromosome Replication Origin in Bacillus subtilis. CSH Symp. Quant. Biol. 33, 695 (1968).
60. Tremblay, G., Daniels, M., and Schaecter, M. Isolation of a Cell Membrane-DNA-Nascent RNA Complex from Bacteria. J. Mol. Biol. 40, 65 (1969).
61. Yamamoto, K., Alberts, B., Benzinger, R., Lawhorne, L., and Treiber, G. Rapid Bacteriophage Sedimentation in the Presence of Polyethylene Glycol and its Application to Large Scale Virus Purification. Virology 40, 734 (1970).