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DNA METABOLISM DURING THE INFECTION OF ANACYSTIS
NIDULANS BY THE CYANOPHAGE AS-1

City University of New York

PH.D.

1980

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DNA Metabolism During the Infection of
Anacystis nidulans by the Cyanophage AS-1

by

Lee Hwang

A dissertation submitted to the Graduate Faculty
in Biology in partial fulfillment of the require-
ments for the degree of Doctor of Philosophy

The City University of New York

1980

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1980

Lee Hwang

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

January 15, 1980

Date

Roy E McGowan
Chairman of Examining Committee
Professor R. McGowan

January 21, 1980

Date

L.G. Moriber
Executive Officer
Professor L.G. Moriber

J. Blamire
Prof. J. Blamire

Brooklyn College
Institution

J. Nishiura
Prof. J. Nishiura

Brooklyn College
Institution

S. Cosloy
Prof. S. Cosloy

City College
Institution

K. Blashka
Prof. K. Blashka

Brandeis University
Institution

H. Lyman
Prof. H. Lyman

State University of N.Y. Stony Brook
Institution

Institution

The City University of New York

ABSTRACT

The metabolism of DNA during the course of infection of Anacystis nidulans by the cyanophage AS-1 was studied, utilizing several different approaches. One approach was to follow the patterns of DNA metabolism in infected cells using a ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ dual pulse label technique and subsequently separating the newly labeled DNA in CsCl density gradients. Incorporation of ^3H -adenine into DNA of buoyant density 1.715 g/cm^3 was observed throughout the entire duration of the time course experiments. ^{14}C , from the photoreduced $^{14}\text{CO}_2$, however, was associated with a lighter peak of buoyant density (1.701 g/cm^3), particularly at the early hours (2-6 hr) post infection. Some incorporation into the normal phage DNA density (1.715 g/cm^3) occurred only at much later times (6 hr). The light peak material has been termed PIL-DNA (phage-induced-light-DNA). OD_{260} detectable, non-radioactive PIL-DNA was collected from CsCl density gradients and found to be insensitive to RNase, protease, or lipase treatment, but susceptible to DNase digestion.

Another approach was to use Poly-L-lysine Kieselguhr (PLK) chromatography to fractionate and analyze the DNA of ^3H -adenine, $\text{NaH}^{14}\text{CO}_3$ pulse labeled AS-1 infected cultures of Anacystis nidulans. The ^{14}C -DNA peak obtained by this

procedure repeatedly fractionated coincidentally with the ^3H -adenine DNA peak. No separation of the two DNA species was achieved using PLK column chromatography, thus indicating a similar % G + C content of both DNAs.

A determination of the thermal denaturation profile of host, phage and PIL-DNA was also performed on repurified DNA collected from CsCl density gradients. The thermal denaturation pattern of PIL-DNA (collected from a CsCl gradient region with a 260/280 ratio of 1.94) bears great similarity to those of the host and phage DNA, and displays a T_m of approximately 76°C . Thus, the % G + C content calculated from the buoyant density data is not in agreement with that calculated from the thermal denaturation data. This discrepancy is most readily resolved by attributing the lighter density of PIL-DNA on a CsCl gradient to a modification of that DNA. Indeed, analysis of the base and nucleotide composition by thin layer chromatography reveals the presence of an additional component in the PIL-DNA samples. Similar results were obtained from the autoradiographs of hydrolysis and radioactive PIL-DNA.

Ultraviolet irradiation and inhibitor experiments, conducted to further elucidate patterns of DNA metabolism in infected cells, have led to several conclusions. One is that photoreactivation systems are to be found in UV-irradiated Anacystis nidulans cells and can be stimulated by UV-irradiated phage AS-1 once inside the host. The

cyanophage itself cannot repair UV-damage to its own DNA. Second, hydroxyurea and nalidixic acid at a concentration of 500 ug/nl inhibit DNA synthesis in synchronized Anacystis nidulans. Protein and chlorophyll synthesis remain essentially unchanged as do turbidity increases. Third, the complete burst size of the cyanophage AS-1 is severely affected by UV-irradiation, hydroxyurea and nalidixic acid treatment, and burst sizes of 24, 8, 2.6, respectively, were obtained. UV or inhibitor treatment of infected cultures causes a premature release of phage at 12 hr instead of 16 hr post infection. Fourth, the use of UV-damaged phage AS-1 to infect A. nidulans seriously affects the formation of PIL-DNA, thus healthy phage is necessary for the induction of PIL-DNA. Finally, ultraviolet irradiation and hydroxyurea treatment of the host cell has no effect upon the formation of PIL-DNA, whereas nalidixic acid pre-treatment blocks its formation.

Thus it seems from these and other experiments reported here that PIL-DNA possibly arises by a modification of infecting phage DNA and could possibly be a protecting mechanism to prevent phage DNA catabolism within the host cell.

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Acknowledgements

I wish to express my sincerest gratitude to Professors Roy McGowan and John Blamire, whose helpful suggestions and criticisms, along with their friendship, were invaluable to me throughout the course of my study. I would also like to thank Dr. Nishiura for providing many stimulating discussions.

Special thanks are extended to Gabriel Cohen for his valuable assistance in my research, and to Faye Teichman and my aunt, Elizabeth Palacios, who were responsible for proofreading the rough draft of this manuscript. I would also like to thank my uncles and my aunts for their encouragement, and Anthea Blamire for the wonderful job she performed in typing and designing the figures for this thesis.

Lastly, I would like to thank my husband to whose patience and encouragement I am deeply indebted.

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INTRODUCTION

I. The Blue-green Algae

Cyanobacteria (blue-green algae) constitute the largest, most diverse and widely distributed group of photosynthetic prokaryotes. They can be succinctly defined as organisms that harbor, within a prokaryotic cell, a photosynthetic apparatus remarkably similar in functional, structural, and molecular aspects to that contained in the eukaryotic chloroplast. The blue-green algae include unicellular, colonial and filamentous forms, with the multi-seriate branched filament being the most advanced type of morphological structure attained. The broad classification of these forms into four subgroups: chroococcacean, pleurocapsalean, oscillatorian and heterocystous subgroups (155). The main features of blue-green algae as seen under the electron microscope are: a prokaryotic organization, an elaborate photosynthetic membrane system, a fibrillogranular nucleoplasmic region, a diversity of characteristic cytoplasmic inclusions (glycogen granules, cyanophycin granules, polyhedral bodies etc.), and a rigid multilayered cell wall and a fibrous sheath. In these cells cytoplasmic regions are in direct contact with the region containing DNA fibrils and ribosomes. Thus the cells can be considered as a single physiological unit. The protoplasm contains, on a dry weight basis, an average of 50% protein, 30% carbo-

hydrate, 5% lipid and 15% ash. Thus, the protein content is higher than that in other algal groups (33), and the carbohydrates and lipids make up a correspondingly smaller proportion of the cellular contents. Amino acid analyses of the bulked algae proteins are no different to those of other algal groups (145). Their molecular properties show an extremely wide variation in DNA base composition among the cyanobacteria, ranging from 39-51% G + C in filamentous varieties to 35-70% in unicellular forms (153). The genome size ranges from 2.4 to 4.6×10^9 daltons (155). Sexual reproduction does not occur in the cyanophycean; however, like bacteria, genetic recombination occurs by a parasexual process (74).

The blue-green algae derive their energy from photosynthesis. Most of the protoplast is occupied by the photosynthetic apparatus which is the site of the photosynthetic pigments (chlorophyll a and carotenoids), the photochemical reaction contents and the photosynthetic electron transport chain. The scheme for photosynthesis is similar to that found in eukaryotic algae and plants. Two photosystems, acting in series (45), are connected by a photosynthetic electron transport chain and are coupled with photophosphorylation to form ATP. Their "carbon" source is derived from the fixation of carbon dioxide. Carbon dioxide is added to a 5-carbon acceptor substance, ribulose diphosphate (RuDP), and photoassimilated through a reductive pentose

phosphate pathway. Blue-green algae are therefore considered photoautotrophs.

It is evident that the blue-green algae share the prokaryotic cellular organization of bacteria as seen by the absence of membrane-enclosed nucleus present in eukaryotes. Structure and chemistry of the cell wall (6,50), cell division (6), genetic recombination (74) and nitrogen fixation (158) are all examples of processes that further relate bacteria to blue-green algae. Classification of the blue-green algae alongside the bacteria in the kingdom Prokaryota is therefore a true representation of their phylogenetic position. There are, however, striking differences between the two prokaryotes. Blue-green algae appear to possess different biochemical machinery, since they lack certain enzyme synthesis control mechanisms that are characteristically possessed by the true bacteria (99,100, 101). The photosynthetic mechanism of the blue-green algae also differs distinctly from that of bacteria, both in the chemical nature of the pigments involved in the photochemical reactions and in the role of oxygen liberation from water. The blue-green algae also exhibit a greater degree of cellular differentiation, as the presence of heterocysts and akinetes indicates.

Cyanophyceae possess, in addition, a significant resemblance to an eukaryotic group, the rhodophyceae (red algae) (9,66). The most obvious similarity between these

two groups is seen in their photosynthetic apparatus, where, in both organisms, chlorophyll a serves as the principal pigment, and β -carotene and phycobiliprotein serve as accessory pigments. In fact, the chloroplast of the red algae, and all chloroplasts in general, are organelles with some of the attributes of prokaryotic cells (25,32,64). The evolutionary ancestors of chloroplasts might therefore have been photosynthetic prokaryotes that were endosymbionts of primitive eukaryotes, and which subsequently lost their genetic independence (155).

A great variety of general biological problems have been investigated in bacteria. The biochemistry of cellular metabolic processes and genetic mechanisms has been carried out as far as the molecular level. However, work with blue-green algae has so far played little part. It is important to note that this kind of investigation should also be made with blue-green algae. The greater extent of cellular differentiation and morphological elaboration exhibited by the blue-green algae makes feasible the extension of molecular biology into fields which could not be explored using only bacteria as the experimental organism. Study of blue-green algae may also shed light on the origin of life and the evolution of the Eukaryota.

II. Cyanophages

A. History of the Discovery and Classification of Cyanophages

The presence of blue-green algae in fresh water, such as the dense blooms that grow in polluted lakes, poses a threat to the water's ecosystem and hence creates a serious problem. No satisfactory means of prevention is yet available, although much attention has been paid to the factors regulating the growth of blue-green algae in various ecosystems. In 1962 Krauss (72) suggested that algal viruses may be the regulatory agent and even be responsible for the disappearance of algal blooms. Two years later Safferman and Morris (121) isolated, for the first time, a virus pathogenic to photosynthetic algae, and demonstrated the cyanophage's usefulness in the control of algal growth (123).

More recently the use of cyanophage has been suggested as a pollution indicator in waste water (144). Since cyanophages are non-pathogenic, and are generally found in waters that contain significant amounts of organic material (181), they may provide a suitable model for indicating the potential hazard from infecting viruses in waste water. The current method using fecal coliforms only serves as an indicator of bacterial pollution, not viral contamination. Chlorine disinfection, designed to eliminate bacteria, usually removed 99% of the coliform indicators but does not accomplish complete removal of virus (144). Thus, a new

biological pollution indicator that monitors both bacteria and viruses is necessary to assure that the animal viruses will also be inactivated by the waste water treatment.

Cyanophage may provide this indicator. The study of cyanophage infection is not only of particular intrinsic interest for biology but is also of economic importance.

In addition, the cyanophage presents a new system, similar to bacteriophage, for probing host metabolic pathways, including photosynthesis, as well as for studying the interactions between the host's metabolism and the regulation of viral-specific synthesis. Plant virologists have looked to the cyanophage as a model system to help resolve discrepancies arising in the study of plant viral-host interactions. Furthermore, the discovery of cyanophages introduced the possibility of transduction, whereby the viruses could be utilized in a multitude of genetic experiments.

Following isolation and characterization of the first cyanophage LPP-1 by Safferman, many other viruses of blue-green algae have been reported. According to their host ranges, serological and morphological properties, cyanophages have been classified into four groups: LPP group (filamentous hosts), N group (filamentous hosts), SM group (unicellular hosts) and AS group (unicellular hosts) (140). The LPP group is subdivided into two serologically distinct groups, LPP-1 and LPP-2 (125), and the SM group is sub-

divided into two groups, SM and S. Because only one virus is known for each of the SM, S, N and AS groups, they have been designated SM-1, S-1, N-1 and AS-1, respectively. LPP-1 cyanophage infects three different genera of non-heterocystic filamentous blue-green algae, Lyngbya, Plectonema, and Phormidium (121). Cyanophage N-1 infects the filamentous strain Nostoc muscorum, which has differentiated heterocysts among the vegetative cells (1). The SM-1 strains attack the unicellular blue-green algae, Synechococcus elongatus and Microcystis aeruginosa (124), and S-1 infects Synechococcus strain NRC₁. AS-1, another cyanophage, infects the unicellular cyanobacteria Anacystis nidulans and Synechococcus cedrorum. It appears that the cyanophage groups isolated so far have rather narrow host ranges.

B. Morphology

The morphological and physical characteristics of the cyanophages are summarized in Table I, adapted and revised from Sherman (140). The structural work performed so far on the cyanophages indicates that they have much in common with bacteriophages but not with plant viruses. The cyanophages characterized to date are morphologically related to one of the three types of bacteriophages, (A, B, C) proposed by Bradley (26).

The viral head capsid of LPP-1 is hexagonal in projection. The edge-to-edge diameter of the head is 586 ⁰Å (125)

TABLE I
Morphological and Physical Characteristics of the Cyanophages

Parameter	Filamentous host			Unicellular host		
	LPP-1	LPP-2	N1	SM1	S1	ASM-1
Morphology						
Capsid						
Shape	Icosahedral	Icosahedral	Hexagonal	Icosahedral	Hexagonal	Hexagonal
Edge-to-edge diameter (nm)	58.6 ± 2	57.3 ± 2	61.4 ± 3	67 ± 1.8	50	90 ± 2
Tail						
Shape	Short, non-contractile	Short, non-contractile	Long, contractile	Very short collar with thin appendage	Long, non-contractile	Long, contractile
Length x width (nm)	20 x 15	20 x 15	100 x 16		140 x 5(?)	240 x 20
Morphological class and bacteriophage relationship	C T7	C T7	A T-even	C T7	B λ	A T-even
Physical characteristics						
Sedimentation coefficient, S _{20,w} (S)	526-550	490	539	820	353	754
Dry mass (daltons)	53.4 x 10 ⁶	50.9 x 10 ⁶	-	-	-	-
Buoyant density in CsCl (g/ml)	1.48	1.48	1.498	1.48	1.501	1.49
Mg ²⁺ requirement	Yes (1 mM)	Yes (1 mM)	Yes	No	Yes	No
Temperature of inactivation (°C)	55	55	-	55	-	55
Stability						
pH range	5-11	5-11	-	5-11	-	4-10
Temperature range (°C)	4-40	4-40	-	4-40	-	-
Major protein species (kilo-daltons)	44.14	42.11	37.14	40.25	39,11,10	90,45,22,16,15
Growth						
Latent period (hr)	6	6	7	32	Slow	8
Lytic cycle (hr)	14	14	14	48	-	12
Burst size (pfu/cell)	200-350	200-350	100	100	-	50
Physical Chemical Characteristics of Cyanophage DNA						
Molecular weight	27 x 10 ⁶	28 x 10 ⁶	41-45 x 10 ⁶	56-62 x 10 ⁶	23-26 x 10 ⁶	57 x 10 ⁶
Sedimentation coefficient	33.4	34.2	40.9	48	30.8	45.6
Contour length (μm)	13.2	-	-20	24.3	13.3	29.5
Buoyant density in CsCl (g/ml)	1.714	1.709	1.696	1.725	1.729	1.714
G + C content (%)	53	52	37-41	66	70-74	52-55

with a short tail (200 Å long and 150 Å wide) that attaches to one of the vertices. Several investigators have stressed the similarity of LPP to T₇ (2,80) and T₃ (51) bacteriophages, both in dimensions and morphology and may be included in the group C defined by Bradley (26) - "short noncontractile tail and head with six-sided outline."

The cyanophage N-1 contains a hexagonal capsid with a diameter of 614 Å and a long, contractile tail. The structure of N-1 is very similar to the T-even bacteriophage, which constitutes the type A group designated by Bradley and which are morphologically described as "contractile tail with six-sided outline head."

Cyanophage SM-1 has an icosahedral head capsid with an edge-to-edge diameter of 670 Å. A very short collar is attached to the head, and from this projects a thin appendage, which could be a tail (82). The morphology of SM-1 is similar to bacteriophage type C. S-1 cyanophage has a hexagonal head capsid with an edge-to-edge diameter of 500 Å and a very long noncontractile tail. This structure is similar to phage λ, with the basic morphology of type B bacteriophage designated by Bradley as "long noncontractile tail with six-sided outline head."

AS-1, the cyanophage of this study, is the largest in size among the cyanophages, and possibly the most architecturally complex. The AS-1 particle is reported to have a polyhedral structure and a contractile tail. Its head has

a diameter of 900 Å from edge to edge, and its tail measures 225 and 2435 Å in width and length, respectively. It is very similar to the T-even phage in morphology (type A) (137) bacteriophage. However, the major difference is that AS-1 does not possess the tail fibers present in T₄ and its tail length is much greater.

C. Physical Characteristics

1) Nucleic Acids

All the cyanophages analyzed so far contain linear double-stranded DNAs. The physiochemical properties of the cyanophage DNAs are summarized in Table I. The presence of double-stranded DNA in LPP-1, N-1, AS-1, S-1 and SM-1 conforms with Bradley's (26) prediction that head-tail bacteriophages contain only this type of nucleic acid. The molecular weights of the DNAs range from 23×10^6 for S-1 (5) to about 60×10^6 daltons for SM-1 (82) and AS-1M (138,139). The % G + C of these nucleic acids also varies substantially from 37% for N-1 (1) to 70% for S-1 (5). With the exception of the parameters listed in Table I, cyanophage DNA has not been studied in great detail.

2) Proteins

The structural proteins have been analyzed by SDS gel electrophoresis. The total complement of these structural proteins represents about 15% of the coding capacity of

SM-1 (82,140) and about 50% for LPP-1, LPP-2 (2) and AS-1M (138). The major protein species possess similarities to the major head proteins found in bacteriophage capsids (Table I). Because of the absence of a conditional-lethal mutant in these systems, the structural parts of the virus have not generally been characterized.

3) Other Physical Properties

Other physical properties such as pH range and cation requirement have been studied. It has been determined that the alkaline pH range for optimal blue-green algal growth also serves as the optimum pH for cyanophage development. The cation requirement for different viruses is variable with LPP-1, possessing an absolute requirement for at least 1 mM Mg^{+2} , while the presence of NaCl enhances the adsorption of AS-1.

D. Growth Cycle of Cyanophages

The mode of lytic cycle of algal viruses is characteristic of phages in general. Following phage adsorption and penetration the viral DNA multiplies and the protein capsid is formed. The assembled mature phages are eventually released when the algal cells are lysed (137,148). Adsorption of cyanophages infecting either filamentous or unicellular hosts follows the kinetics of a first-order reaction.

The replication cycles of the cyanophage are much

longer than those of bacteriophage. The lytic cycle is usually complete by 14-16 hours post infection in LPP-1, N-1 and AS-1, with average burst size of 350 plaque-forming units (PFU/cell), 100 PFU and 50 PFU, respectively. In SM-1 and S-1 complete lysis requires at least 48 hours.

Detailed investigations of the ultrastructure of cyanophage infection and the sequence of events for synthesis of the cyanophage macromolecules have only been conducted for AS-1, AS-1M/Anacystis nidulans (137,138) and LPP-1/Plectonema boryanum (132,133,134) infective systems.

a) Ultrastructure of infection:

Morphological changes of the host following cyanophage infection are separated into two groups. Cyanophages that infect unicellular blue-green algae, such as AS-1 (or AS-1M), do not greatly disturb the host ultrastructure (136,138). Replication takes place entirely within the nucleoplasm and no major morphological changes are seen. However, infection by LPP-1 and other cyanophages possessing filamentous blue-green algal hosts results in an invagination of the photosynthetic lamellae and the creation of a virogenic stroma wherein the phage ultimately replicate (145,146,148).

b) DNA synthesis:

When AS-1M replicates in its unicellular hosts, the host DNA begins to break down immediately with 15% becoming solubilized by the end of the first hour post infection and 40% by 2.5-3 hours. AS-1M DNA synthesis begins at three

hours and is then continuous throughout the latent period. (138). LPP-1 infection also causes extensive degradation of the host DNA commencing one hour after infection and reaches maximum degradation at three hours ($\sim 50\%$). Most of the viral DNA synthesis occurs between six and eight hours post infection. Although incorporation of exogenous ^3H -adenine into viral DNA was detected very early after infection (2 hours), most of the replication of viral DNA closely corresponds in time to the maturation processes (132).

c) Protein synthesis:

Protein synthesis following AS-1M infection has also been investigated (138). Cessation of host protein synthesis begins almost immediately and is complete by four hours after infection. A total of seven early viral proteins are synthesized in the 0-3 hour period post infection, and by four hours their synthesis has been arrested. After phage infection the level DNase in the infected A. nidulans increases 15-20 fold (167). This phage-directed DNase appears to be an early protein which degrades the host DNA optimally by two hours after infection (140). This correlates very well with electron microscopy findings (133) which reveal a severe disruption of the host nucleoplasm at the two hour point. Another function for the early protein may be its necessity for the formation of the middle proteins. The synthesis of middle proteins is initiated at about three hours and is continuous throughout the

infective cycle. The primary function of these proteins is DNA synthesis. The synthesis of late proteins, which function as phage structural proteins, begins at 3-4 hours and continues until lysis (140). The pattern of protein synthesis following LPP-1 infection is generally the same as that of AS-1M. Three classes of viral proteins were distinguished: early, middle and late (134). Synthesis of three early proteins begins soon after infection and is terminated by the fourth hour. The appearance of three middle proteins begins at around the second hour with synthesis continuing until lysis. These middle proteins are not found in the mature phage particles. The late proteins, which include the viral structural proteins, appear at about the fourth hour and are continually formed until lysis.

A great deal of energy has been expended in the search for lysogeny in the blue-green algae and several reports on lysogeny have appeared in the last ten years (29,30,96). However, only one lysogen, in the Plectonema boryanum strain LPP-2SPI, has been characterized (96) and examined using the electron microscope (142). Recently, a temperature-sensitive mutant, SPI_{CTS1}, was developed from the LPP-2SPI strain and carefully isolated by Rimon et al. (113). Although lysogenic strains and mutants have not been utilized in studies of gene expression or mapping of genes involved in DNA synthesis and repression, the basic

framework for research has now been established.

E. Interaction between Cyanophage Development and the Metabolism of the Host

Inhibitors such as DCMU and uncouplers such as CCCP, which disrupt the normal function of photosynthesis, have been utilized to investigate the dependence on this process for phage development. Dichlorophenyl dimethylurea (DCMU), a powerful inhibitor of photosynthetic oxygen evolution (53), also blocks the reduction of NADP (161), but permits ATP production via the mechanism of cyclic phosphorylation (10). Carbonyl cyanide m-chlorophenyl hydrozone (CCCP) uncouples the processes of photosynthetic electron transport and photophosphorylation (39) in blue-green algae. The most important effect of LPP-1 infection on host metabolism is to cause a rapid and complete cessation of CO₂ photoassimilation while allowing all other photosynthetic capacities such as phosphorylation, O₂ evolution, pigments and total ATP in cell extract to remain essentially normal (48,49). The fact that CCCP completely eliminates phage growth and that phage development proceeds normally in the presence of DCMU (95, 97,135) implies that cyclic phosphorylation can supply all of the ATP needed for phage production, i.e., only photosystem I in photosynthesis is absolutely required for phage growth. Therefore, LPP-1 biosynthesis requires photosynthesis for energy production but not for the supply of carbon. No specific light requirement for either early or late phage

function exists (94).

Similar effects were found in the N-1/Nostac muscorum infective system. Inhibition of CO₂ fixation is not so rapid and commences at about five hours after infection. Although N-1 perturbations of cellular metabolism resemble the pattern of LPP-1, it is much more dependent on host photosynthesis than LPP-1 (3). N-1 growth in the presence of DCMU is reduced to 25% of the control, while the addition of CCCP severely restricts growth.

The dependence of phage development upon host photosynthesis in the AS-1/Anacystis nidulans and SM-1/S. elongatus, M. aeruginosa infective systems, however, is quite different. The results of AS-1 and SM-1 are nearly identical. CO₂ photoassimilation is not terminated and the photosynthetic parameters measured remain almost unaffected until the end of the latent period at eight hours. Removal of light at any time during the latent period lowers the burst size appreciably (136). Phage growth is likewise inhibited by both DCMU and CCCP. This indicates a complete dependence of phage growth upon host photosynthesis for the reduced carbon supply as well as for energy.

III. The AS-1/Anacystis nidulans Infective System

A. Isolation and Characterization of AS-1

The cyanophage AS-1 was first isolated and characterized by Safferman and Morris in 1972 from a composite sample taken from a waste stabilization pond at Sun City Center, Florida, that received only domestic sewage (126). The first host was detected by its lysis of Anacystis nidulans strain Iu-625. Subsequent screening of a wide variety of both coccoid and filamentous cyanobacteria resulted in the addition of Anacystis strains 1550 and 1549 and Synechococcus cedrorum strain 1191 to the host range for this virus. This virus was not active in bacteria, actinomycete nor monkey kidney tissue cells. Therefore, the host range of AS-1 was considered to be limited to unicellular blue-green algae.

AS-1 is serologically distinct from other blue-green algal viruses, since no cross neutralization was observed when its antiserum was tested against the cyanophage LPP-1, LPP-2 or SM-1.

The virus was found to be stable in distilled water. Magnesium ions are not required for stabilization of this virus, as was found for the LPP-1 virus, while NaCl increases its adsorption rate by 34%.

A wide pH range from 4 to 10 has no significant effect on viral titer. At pH 11 a decrease of approxi-

ately 75% in virus titer was noted, while at pH 3 and 12 virtually all infectivity was lost.

High temperature affects viral infectivity. No loss in titer was observed in lysates incubated at 45°C. However, at 50°C, 77% of the infectivity remained and this decreased to 12% at 55°C and to less than 0.001% at 60°C.

The processes by which AS-1 adsorbs onto the host have been clarified. Complete adsorption of the phage AS-1 occurs in approximately 60 minutes (126). Electron microscopy of sectioned cells which were infected by AS-1 revealed that the phage attaches to the cell wall by the distal part of its tail (102). Earlier studies on the adsorption of cyanophages onto their hosts led to two major conclusions, namely that the adsorption process is quantitatively similar to that of the bacteriophage to bacteria (3,50,126), and that the adsorption process occurs relatively slowly (8,50,94). More recently Samimi and Drems, studying the biochemistry of adsorption, have found a very strong correlation between the presence of certain cell wall components and the cells' susceptibility to AS-1 infection (128). They have isolated lipopolysaccharides (LPS) as well as protein material from the cell wall of typical host strains which have the same fine structure as that found in gram-negative bacteria. Furthermore, they have observed that these materials inactivate cyanophage AS-1 (128). Highly purified material can interact with AS-1 and can

cause the tail to contract and the head to be emptied of DNA. Thus, the formation of the receptor complex and the adsorption process both involve the participation of LPS and protein. Detailed studies of this mechanism have yet to be performed. It has been reported that the bacteriophages T₃, T₄, and T₇ are specifically adsorbed by LPS which is exposed at the cell surface of their hosts (75). The receptors for T₂ and T₆ phages are proteins while T₅ receptors consist of protein and LPS (172). The adsorption process is another parallel between cyanophage and bacteriophage.

Cseke et al. have recently examined in greater detail the effect of illumination on the extent and kinetics of the adsorption of cyanophage AS-1 onto the blue-green algae A. nidulans (36). They found that the initial rate of adsorption was not significantly affected by light. The final amount of phage adsorption, however, was doubled in the illuminated cultures at the Na⁺ level used to culture the algae (Na⁺ = 11.7 mM). The amount of adsorption in the dark may be raised to the level found in the illuminated culture with a ten-fold increase in Na⁺ concentration. The effect of ions on phage adsorption may be due to some charge neutralization which permits the phage to attach more firmly to the receptors.

The one-step growth curve of AS-1 reveals a general growth pattern typical of that observed with bacteriophages.

The complete lytic cycle of AS-1 requires sixteen hours with a latent period of approximately eight hours. The burst size under optimal conditions numbers about fifty plaque forming units per infected cell.

Electron micrographs indicate that AS-1 is morphologically a group A phage, with a large, hexagonal, capsid (900 Å diameter) and a long, contractile tail. Short pins are attached to the base plate on the tail. When the particles are adsorbed to the host cells the tail sheath and base plate with pins can contract along the tail core. AS-1 is the largest and the most complex cyanophage among all the cyanophages discovered so far. It is very similar to the T-even phage in morphology, but there are a few major differences (i.e., absence of tail fibers and greater tail length).

The AS-1 particle contains double-stranded DNA of a buoyant density of 1.714 g/ml which corresponds to a G + C content of 53-54%. Thermal denaturation of AS-1 DNA dissolved in 1 x SSC and 0.01 x SSC melted with T_m values of 91.2°C and 64.4°C, respectively. The ultraviolet adsorption spectrum of the DNA has a 260 nm to 233 nm ratio of 2.0 and a 260 to 280 nm ratio of 1.7 (126).

B. Isolation and Characterization of Cyanophage AS-1M

Cyanophage AS-1M was first isolated from waste stabilization ponds in central and southern Missouri by

Sherman in 1976 (139). It is serologically and morphologically identical to AS-1. Only Anacystis nidulans Iu 625, Iu 1549, Iu 1550 and Synechococcus cedrorum 1191 are susceptible to the phage. This indicates the host range is also the same as AS-1. The only difference from AS-1 is its rapid lytic cycle which has a latent period of eight hours and completes lysis by twelve hours after infection.

C. Infection of Anacystis nidulans by the Cyanophage AS-1 and Infection of Synechococcus cedrorum by AS-1M

1) Infection of Anacystis nidulans by the Cyanophage AS-1

An electron microscopic study of the AS-1/A. nidulans infective system was reported by Pearson in 1975. Viral development in A. nidulans is not found to be accompanied by any gross changes in host cell cytology until just prior to lysis. AS-1 develops in the nucleoplasm of the cell and, in direct contrast to LPP-1/Plectonema, does not cause any invagination of the photosynthetic lamellae at the beginning of infection (133). The first changes in ultrastructure were observed only after nine hours after infection. The nuclear material in these cells appeared more condensed and more prevalent. Eleven to fifteen hours after infection aggregation of electron-dense material in the nuclear area of the host cells is evident and this has been suggested to be part of the viral head structure. In the later hours of infection (sixteen to eighteen hours) particles are observed

both inside and outside the photosynthetic membranes, and viral heads are obvious as dark condensates in the host cytoplasm. Twenty hours after infection only cell ghosts are visible.

The unicellular blue-green alga A. nidulans, unlike bacteria, is relatively impermeable to nucleic acid precursors (105,112) and only allows a low-level of incorporation of organic metabolites (71,147,149). This creates difficulties in the determination of synthesis patterns for the major macromolecules in A. nidulans. Following AS-1 phage infection, however, it has been shown that an alteration of host permeability takes place which allows the incorporation of all the nucleic acid precursors, some amino acids and two sugars (24). The use of radioactive precursors to follow the changing patterns of macromolecular synthesis in the infected cells is thus rendered feasible. In addition, this increased permeability following infection generates a potentially valuable technique for the study of all metabolic mechanisms and processes.

Blashka (24) was the first to study the synthesis patterns of DNA in phage-infected cells using isotope precursors and pulse-labeling techniques.

Samples were double labeled with ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ following infection and the DNA subsequently analyzed using CsCl density gradient ultracentrifugation. It was found that ^3H -adenine is incorporated into DNA of

buoyant density 1.715 g/cm^3 at all points during a time course experiment. However, $\text{NaH}^{14}\text{CO}_3$ was found to be associated only with a peak of lighter buoyant density (1.701 g/cm^3), particularly in the early hours of the infective cycle. This lighter peak began to accumulate at 2.5 hours, reached a maximum at five hours, and then decreased thereafter up to the time of lysis. During the later hours of infection $\text{NaH}^{14}\text{CO}_3$ was found to be incorporated into DNA at the buoyant density of normal phage.

In order to ascertain whether the existence of the ^{14}C -incorporating light peak occurs in every system infected by AS-1 or related phage, and to determine whether the phenomenon is observed with all nucleic acid precursors, alternate precursors, host and phage were used. For alternate precursors ^3H -thymidine and ^3H -uracil were used to replace ^3H -adenine. Synechococcus cedrorum (1191) was used as the alternate host in place of A. nidulans, and cyanophage AS-1 (obtained from Safferman) and AS-1M (obtained from Sherman) were used as the alternate phages. Similar results to those found using AS-1/A. nidulans were obtained from all these studies, thus indicating that the formation of the ^{14}C -incorporating light peak is universal in all AS-1 infected unicellular blue-green algal systems. The ^{14}C -incorporating light peak was named PIL-DNA (phage induced light DNA) (24), but the nature and the function of this PIL-DNA was still uncertain.

2) Infection of Synechococcus cedrorum by AS-1M

Electron microscopic descriptions of ultrastructural changes after infection were reported by Sherman et al. (137). The first noticeable change of phage infection was a degradation of host nucleoplasm which becomes almost electron transparent compared to the same area in the uninfected controls which contain a rather even density with ribosomes and DNA distributed throughout the nucleoplasm. At four hours post infection a large amount of fibrillar material was observed which was suggested to be phage DNA. The photosynthetic lamellae are no longer completely surrounding the nucleoplasm but no invagination was observed. At six hours after infection many phage precursors were seen, such as empty-head vesicles, "helical" structures, which may represent the phage DNA, darkly staining hexagonal particles, which may be the filled head, and complete phages containing both heads and tails. These represent the process of phage assembly.

Sherman studied the fate of host DNA after infection by first labeling a culture of S. cedrorum for twenty-four hours with ^3H -uracil and subsequently chasing with non-radioactive uracil for twenty-four hours, followed by infection with cyanophage AS-1M. From measurements of the NaOH stable, TCA-insoluble material there was the indication that degradation of the host DNA begins almost immediately. This process continues until about two and a half to three

hours when 40% of the DNA has been degraded to an acid soluble form. The radioactivity in the soluble pool is then slowly incorporated into phage DNA.

DNA synthesis in infected S. cedrorum was measured by the cumulative incorporation of ^3H -uracil into AS-1M DNA. During the first one and a half hours post infection there is little incorporation; however, incorporation begins to increase between two and a half and three hours post infection and continues until lysis.

Sherman also studied the effect on AS-1M DNA synthesis and host DNA breakdown of chloramphenicol (CAP), an antibiotic that blocks protein synthesis by binding to 70S ribosomes, and rifampicin, an antibiotic that stops RNA transcription by binding to the β subunit of the DNA-dependent RNA polymerase. It was found that when CAP is added at or before two hours after infection, no phage DNA is synthesized. At three hours after infection 25-30% of the normal amount of incorporation takes place. At four hours 75% of normal incorporation was obtained. At five hours after infection the amount of DNA was the same as the control. Therefore, it was concluded that protein synthesis after infection is required for DNA synthesis, and that translation of the requisite enzymes takes place between two and five hours after infection. The addition of rifampicin at any time before two hours completely stops DNA synthesis as found in CAP treatment. Addition at three, four and

five hours allows 43, 82 and 92% of the amount of DNA synthesis compared to the control. These results indicate that transcription of the DNA synthesizing enzymes takes place after two hours and is completed by five hours after infection.

The protein composition of AS-1M was characterized from purified ^{35}S labeled AS-1M, which was analyzed by SDS-acrylamide gel electrophoresis and yielded about thirty distinct bands (138). Among these proteins four bands which have molecular weights of 77,000, 33,000, 17,000 and 16,000 respectively contain 70% of the total radioactivity. The 33,000 molecular weight band, which represents about 50% of the total radioactivity is most likely the major head protein.

The time course for protein synthesis after AS-1M infection was determined by analyzing ^{35}S -pulse-labeled extracts of infected cells on SDS-acrylamide gels. The autoradiographs obtained from such an experiment were compared with the proteins labeled during a one hour pulse of uninfected cells and with the AS-1M structural proteins. The protein labeling pattern is observed to continue relatively unaffected during the one to two hour pulse. Host protein continues to be synthesized through the three to four hour pulse, but many of the species present are quantitatively greatly reduced. However, by four hours after infection host protein synthesis is either completely

shut down or severely decreased. Therefore, it was concluded that cessation of host protein synthesis begins by about two hours after infection and is essentially complete by four hours.

The proteins synthesized after phage infection are divided into three classes: early, middle and late. Early protein synthesis is initiated between one and three hours after infection and is terminated by four hours. The molecular weight of these proteins are 76,000, 62,000, 52,000, 39,500, 22,000 and 15,500. All these proteins are present in small amounts. Synthesis of middle proteins begins at about three hours after infection and continues throughout the infective cycle. The times of synthesis are varied; some proteins are synthesized at three hours while others were not noticeable until four hours. The times of synthesis termination also differ: some proteins stop being synthesized by seven to eight hours, while some continue to be synthesized until lysis. Late protein synthesis is initiated at three hours after infection, increases greatly between four and five hours after infection, and continues throughout the infective cycle.

The production of enzymes necessary for phage DNA synthesis, host DNA breakdown and structural protein synthesis were determined by using the inhibitor of RNA synthesis, rifampicin (138). Rifampicin (Rif) was added to an infected culture at two, three, four and five hours after infection.

After a one hour incubation period the cells were washed, resuspended in low sulfate medium and pulsed with $\text{H}_2^{35}\text{SO}_4$ for one hour. From these experiments it was concluded that synthesis of the middle proteins required phage directed RNA transcription that begins after two hours and is completed by four hours. Synthesis of the late proteins requires transcription that begins after three hours and is completed by five hours after infection. Thus, synthesis of the middle and late proteins requires post-infection RNA transcription. AS-1M has approximately thirty structural proteins which represents about 75% of the coding capacity of the phage genome.

D. Cellular Metabolism and Phage Development

1) The Role of Photosynthesis in Phage Development

Photosynthetic requirements during the growth of AS-1/AS-1M were determined by measuring O_2 evolution and CO_2 fixation (136,56). The results indicated that CO_2 fixation and O_2 evolution continued at high levels throughout the latent period, but decreased precipitously immediately prior to lysis. Thus, it was concluded that the growth of cyanophage AS-1/AS-1M depends on host photosynthesis. The studies on phage production by Blashka (24) indicated that the most critical time period for photosynthesis and/or photophosphorylation is the first three to four hours post infection. The requirement of light during AS-1/AS-1M

growth was also studied and it was found that the burst size in the dark was decreased to 2% of that observed in the light. Infected cells must be illuminated for at least thirteen hours to produce a maximum burst (8). It has also been noticed that low light intensity retarded the development cycle (24), and growth of AS-1M in the dark resulted in a total cessation of phage development. These findings are in contrast to what has been suggested for the LPP-1 system. An increase in the final yield of cyanophage was found in the dark pretreatment in LPP-1 (135).

Similar results were obtained when photosynthesis was perturbed by DCMU, an inhibitor of photosystem II, CCCP, an uncoupler of photosynthetic electron transport chains, and CO₂ starvation. The presence of DCMU and CCCP completely abolishes phage production, although some reports indicate that DCMU only reduces the burst size to 27% (102). Since DCMU has a detrimental effect on phage growth, it was concluded that the photosynthetic requirement is not only for ATP but also for a supply of carbon.

2) Photoheterotrophic Growth and AS-1 Development

The host strain Anacystis nidulans is a photoautotroph. The main energy yielding pathway in this organism is the pentose phosphate pathway. Stainer et al. tentatively concluded that the lack of heterotrophy could be due to the absence of a glucose permease (154). Nevertheless,

the infected cells do contain a limited facultative heterotrophic capability. About 10% of the burst size relative to the control is obtained when the cells are supplemented with 1% glucose in the dark or in the presence of DCMU (136). Thus, under these conditions, the infected cell is acting as a facultative heterotroph. With or without the use of chloramphenicol the incorporation of glucose by infected or uninfected cells remains the same. It is therefore suggested that the host has a small quantity of glucose permease but that the rate of ATP generation, in the absence of photosynthesis, is normally not sufficient to meet the growth requirement of the cell (136,154).

IV. Rationale for Thesis

It is evident that all the biochemical mechanisms involved in AS-1 replication and phage development have not yet been clearly elucidated, but their investigation has begun. The study of cyanophage in general is still in a primitive stage. Further studies are required in order to achieve a detailed understanding of the pattern of AS-1 DNA metabolism.

A multifaceted approach has been taken in order to study the mechanism of viral-host interaction in the AS-1/A. nidulans infective system.

First, direct evidence was needed to demonstrate the quantitative existence of PIL-DNA in the infective system. Preparative amounts of this material have therefore been collected. Second, after purification, this material has been further characterized using such techniques as thermal denaturation, base analysis etc. Third, to study the DNA metabolism in the infective system and to ascertain the physiological role of PIL-DNA attempts have been made to identify the origin of PIL-DNA. There are several possibilities as to its source. It may originate by the modification of some preexisting host or phage DNA, or it may be synthesized de novo during the early hours of the lytic cycle utilizing solely photoreduced metabolites. From the work presented in this thesis, it appears that both these possibilities are involved in the formation of PIL-DNA.

Finally, the application of certain DNA inhibitors such as hydroxyurea, nalidixic acid and UV-irradiation has been undertaken to further increase our knowledge of the role of DNA synthesis in the development of cyanophage and the formation of PIL-BNA. In addition, the effect of these inhibitors upon the growth and infectivity of cyanophage AS-1 have been determined.

V. Microorganism DNA Modification

Further characterization of PIL-DNA has been made and several possibilities as to its source have been suggested. The results accumulated from several different analytical methods favor the suggestion that the formation of PIL-DNA is due to a modification of host or phage DNA.

Modification has been reported in some animals (56b), plants (56b) and microorganisms. In general, only N⁶-methyldeoxyadenosine has been detected in animal DNA, and only 5-methyl-deoxycytidine in plant DNA. Both of these nucleosides occur as trace components of bacterial DNA (56b). In bacteriophage the distribution of modified nucleosides in the DNA is somewhat unique. These nucleosides can occur in minor amounts, as they do in the RNA and DNA of bacteria and other organisms, or they can completely replace one of the four major nucleoside components. For example, 5-hydroxymethyl-deoxycytidine replaces deoxycytidine in the DNA of the T-even bacteriophages (176), 5-methyl-deoxycytidine replaces deoxycytidine in the DNA of a bacteriophage that infects Xanthomonas oryzae, and 5-hydroxymethyldeoxyuridine or deoxyuridine replaces thymidine in the DNA of certain bacteriophages that infect Bacillus subtilis (61,164).

One function definitely ascribed to modification is the creation of a form of molecular "fingerprinting." Such a mechanism, suggested by Srinivasan and Borek in 1966 (151b),

allows for the modification of a cell's DNA in such a way that it is resistant to degradation by homologous endonucleases and yet is unimpaired with respect to the utilization of the encoded genetic information. Billen (1968) and Lark (1968) (226, 746) studied the mechanism of in vivo methylation of the DNA of E. coli 15T and found that the nascent polynucleotide strand is methylated as the DNA is replicated. The replication of the methyl-deficient DNA stops at the point where the normal methylation pattern is located. These findings further support the hypothesis suggested by Srinivasan et al.

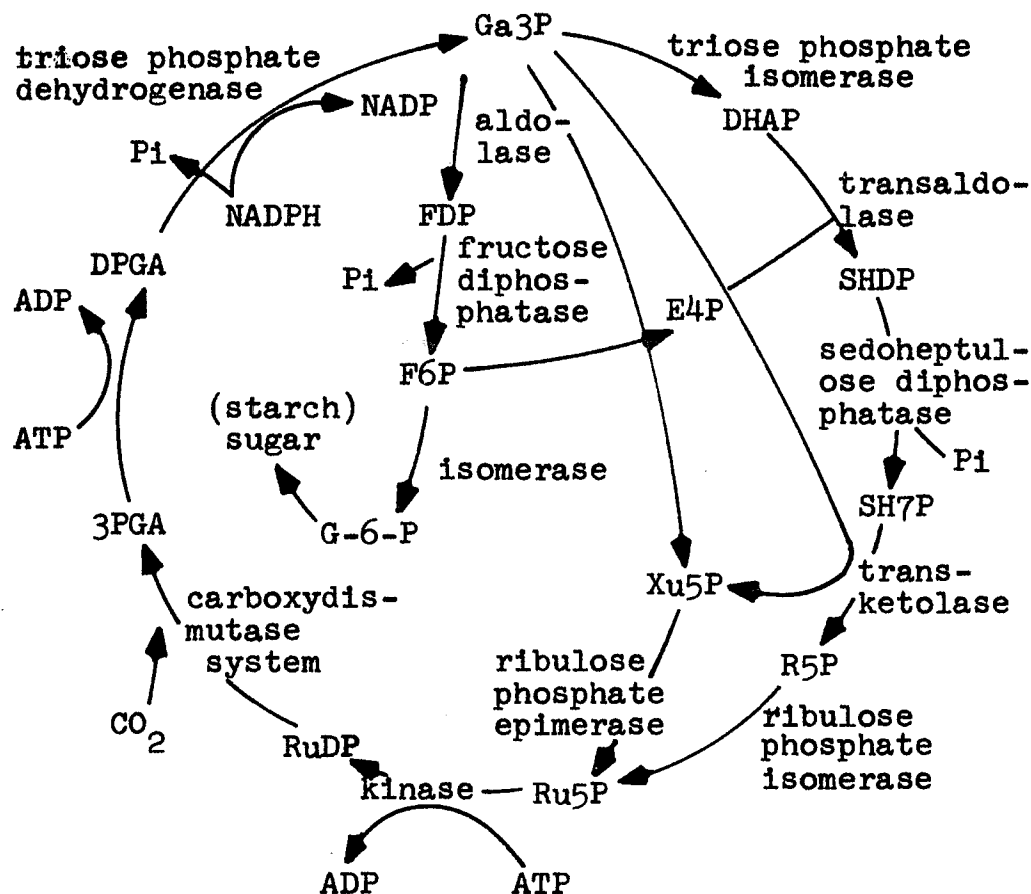
With respect to modified bases it is not clear whether cyanophage or cyanobacteria contain any modified bases typical of many bacteriophages or whether there is any host modification and restriction. Recently, a new cyanophage, S-2L, morphologically similar to SM-2, that lyses some strains of unicellular cyanobacteria Synechococcus has been studied and reported. In phage S-2L adenine is completely substituted by 2,6-diaminopurine (65,69). Although the substituted base is quite different from those found in bacteriophages, this finding demonstrates convincingly that the same phenomenon can exist in some cyanophage/cyanobacteria infective systems.

VI. Involvement of the Intermediatory Metabolism
of Blue-Green Algae in the Development
of Cyanophage AS-1

It is known that the carbon source for blue-green algae is the photoassimilation of CO_2 . Reduction of CO_2 and its subsequent incorporation into DNA either during host cell growth or during the phage replicative cycle is primarily by photosynthesis. However, other mechanisms can and do contribute to the assimilation of CO_2 into DNA.

A. Pentose Phosphate Pathway

The fixation of carbon dioxide in blue-green algae is mainly via the reductive pentose phosphate pathway. Carbon dioxide is added onto a 5-carbon acceptor ribulose diphosphate (RuDP), which splits to yield two molecules of a 3-carbon substance, phosphoglyceric acid (PGA). PGA is reduced to a triose sugar phosphate from which, by a series of sugar phosphate interconversions, the acceptor for carbon dioxide, RuDP, is regenerated. This cycle can be visualized as producing one glucose molecule for every six carbon dioxide molecules fed into it, but in actual fact the fixed carbon can be removed in a variety of forms ((i.e., ribose-5-phosphate can serve as a precursor for deoxyribonucleotide biosynthesis). The scheme of the reductive pentose phosphate cycle is as follows:



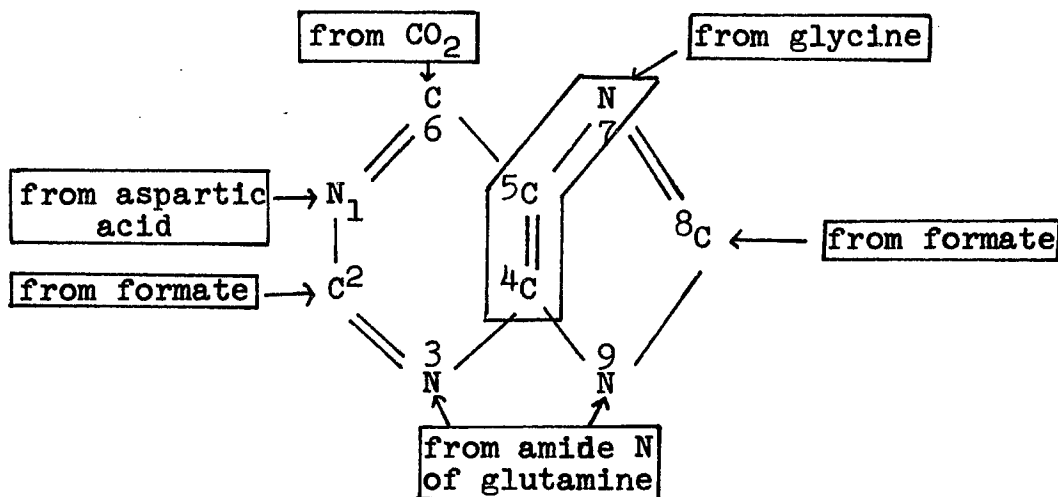
RuDP	ribulose 1,5-diphosphate
3PGA	3-phosphoglyceric acid
DPGA	1,3-diphosphoglyceric acid
Ga3P	glyceraldehyde-3-phosphate
DHAP	dihydroxyacetone phosphate
FDP	fructose-1,6-diphosphate
F6P	fructose-6-phosphate
G6P	glucose-6-phosphate
E4P	erythrose-4-phosphate
SHDP	sedoheptulose-1,7-diphosphate
SH7P	sedoheptulose-7-phosphate
Xu5P	xylulose-5-phosphate
R5P	ribose-5-phosphate
Ru5P	ribulose-5-phosphate

B. The Biosynthesis of Nucleotides

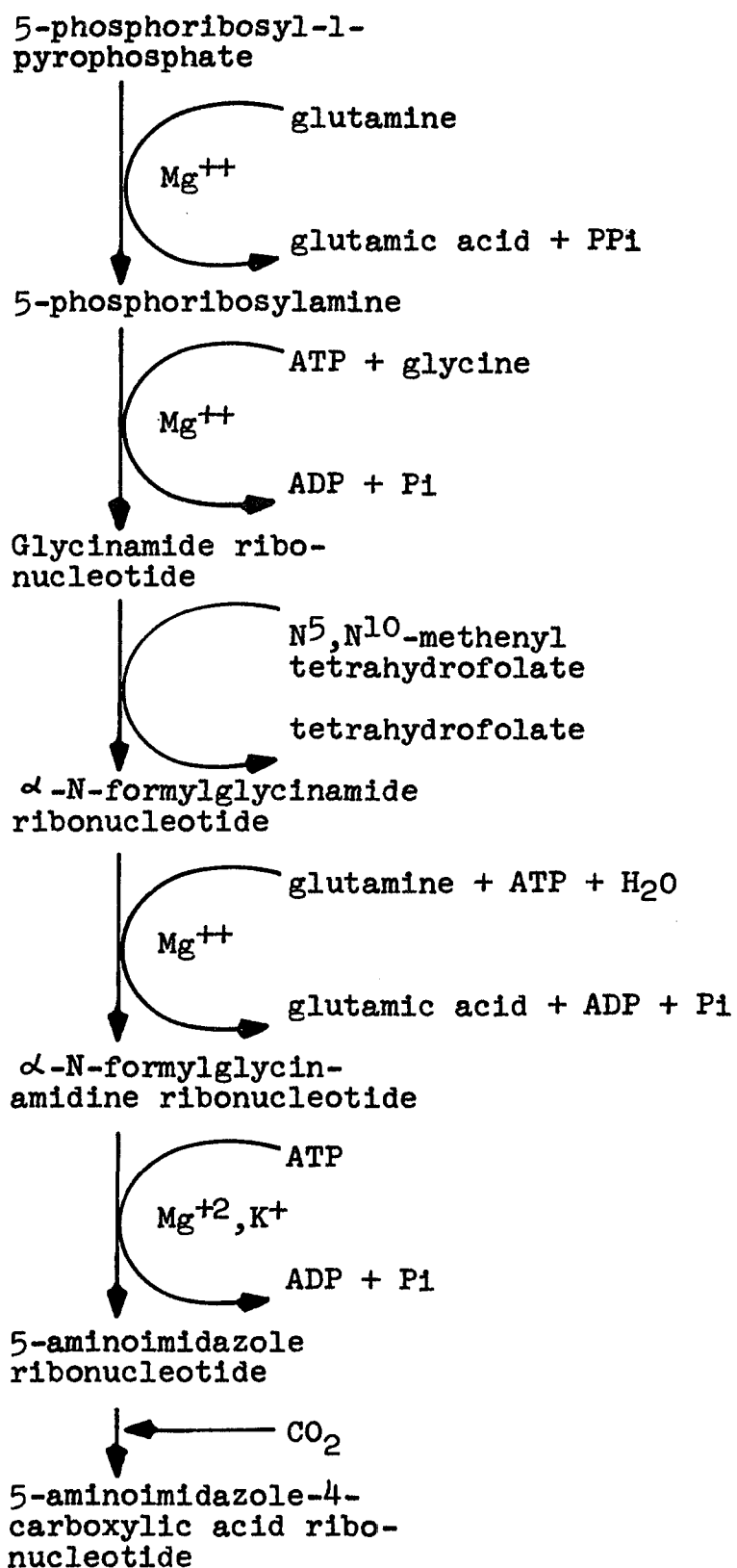
It has been shown that CO_2 also participates in a carboxylation reaction during the de novo synthesis of the purine and pyrimidine nucleotide precursors.

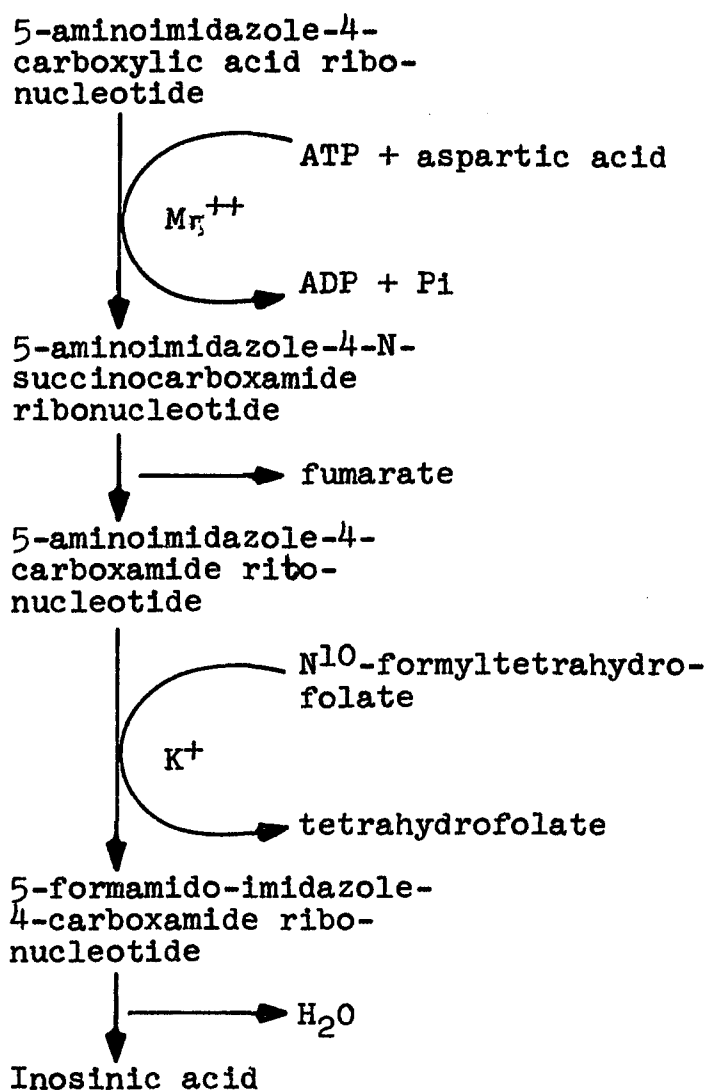
1) Purine Biosynthesis

Experiments with radioisotopes have shown that the nine atoms of the purine nucleus are derived from five different precursors, each precursor contributing the atoms indicated in the following diagram:



The starting point for purine biosynthesis is the 5-phospho-compound ribosyl-1-pyrophosphate (PRPP) which is obtained from ATP and ribose-5-phosphate. The steps of biosynthesis of inosinic acid are as follows:



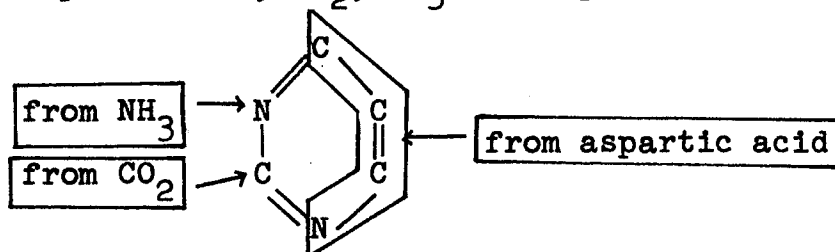


The carbon atom at position six of the purine skeleton is formed by carboxylation of the imidazole nucleus with CO₂.

After subsequent interconversions from inosinic acid two purine nucleotides AMP and GMP are found.

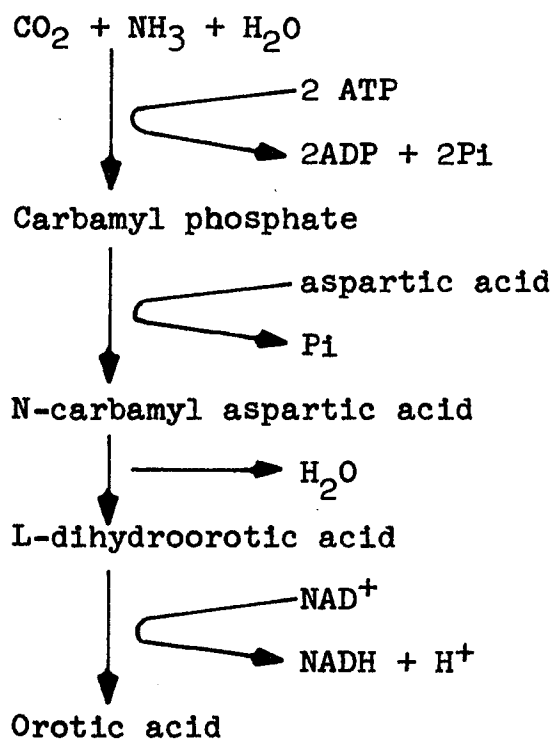
2) Pyrimidine Biosynthesis

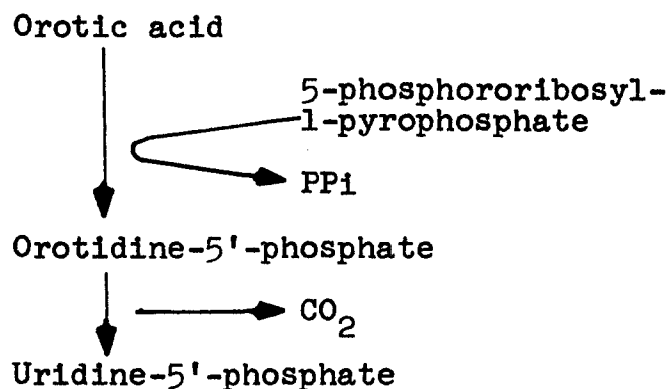
The atoms of the pyrimidine nucleus are derived from three simple precursors, CO_2 , NH_3 and aspartic acid.



The biosynthetic pathway differs from purine biosynthesis in that the D-ribose moiety is attached after the pyrimidine ring has been formed from its open chain precursors.

The biosynthesis of uridylic acid is as follows:





The uridine-5'-phosphate is first phosphorylated to UTP, and then undergoes an amination reaction on the four position of the pyrimidine ring to yield cytidine-5'-triphosphate (CTP).

3) Methylation System

In a methylation system the ultimate methyl donor can be S-adenosylmethionine. The methyl group can be derived from CO₂ fixation by the pathway involving serine biosynthesis from 3-phosphoglycerate. The transfer is made of the hydroxymethyl group from serine to give N²⁵, N¹⁰-methylene-tetrahydrofolate with further transfer of the methyl group to homocysteine giving methionine. This pathway would give a fairly direct transfer of photosynthetic CO₂ fixation to DNA.

MATERIALS AND METHODS

1) Chemicals

DNA of calf thymus # D-1501 (Lot 126C-9600), SDS (Sodium dodecyl sulfate) # L-5750 (Lot 57C-0403), Poly-L-lysine # P-1886 (Lot 87C-5044), Hydroxyurea # H-8627 (Lot 27C-0221) and Nalidixic acid # N-8878 (Lot 86C-0253), and Folin & Ciocalten's phenol Reagent # F-9252 (Lot 68C-5046) were purchased from the Sigma Chemical Co., St. Louis, Missouri.

Deoxyribonuclease I # DN-100 (Lot 37C-0153) (specific activity-1940 units/mg solid); Ribonuclease A # R-5500 (specific activity-100 Kunitz units/mg); Lysozyme # L-6876 (Lot 74C-8041, specific activity-40,800 units/mg solid); Lipase # L-1754 (Lot 112C-0580); and Phosphodiesterase # P-3134 (Lot 92C-1531-9) were obtained from the Sigma Chemical Co., St. Louis, Missouri.

Cesium Chloride high purity grade (Lot 1363-111) was purchased from Kawecky Berylco Industries, Inc., Boyertown, Pennsylvania. Sarkosyl (Sodium lauryl sarcosinate) detergent (Lot NL-97) was purchased from Geigy Industrial Chemicals, Ardsley, New York. Kieselguhr was purchased from Johns-Manville Celite Division, New York. Sephadex G-200 (Lot 1680) was purchased from Pharmacia Fine Chemicals Inc., Piscataway, New Jersey.

Radioactive $\text{Ba}^{14}\text{CO}_3$ (specific activity -55.7 Ci/m mole) and 2- ^3H -adenine (specific activity -28.2 Ci/m mole), and 8- ^3H -thymidine (specific activity -20.2 Ci/m mole) were purchased from New England Nuclear Corp., Boston, Massachusetts. S-adenosyl-L-methionine (methyl ^3H) (specific activity 12 Ci/m mole) was purchased from ICN of Irvine, California.

2) Maintenance of Algal Growth and Induction of Synchronization

In order to maintain the Anacystis nidulans (Indiana 625) obtained from Safferman in Indiana University, the algae were grown in conical tubes (about 200 ml) containing Mauro's Modified Medium (3M) (71) at pH 7.5 (see Appendix A) at a temperature of 30-34°C. A filter-sterilized air stream containing 0.5% CO_2 was pumped into the conical tubes via a network of glass tubing to agitate the culture. Continuous illumination was provided by a series of "cool white" fluorescent tubes at light density of 3600 lux. Cultures were maintained in the exponential growth stage by transferring to a new flask containing sterile medium every 2-5 days. Growth of the algal cultures was monitored by either a direct determination of cell numbers obtained with a Petroff-Houser counting chamber in conjunction with a microscope equipped with phase optics, or an indirect turbidometric method utilizing a Gilford spectrophotometer set at 750 nm.

The algal cultures were systematically monitored for possible contamination by plating 0.1 ml aliquots onto nutrient agar plates and observing the plates after 2-3 days' incubation. The algal stocks were maintained on cyanophycean agar slants (see Appendix B).

Synchronization of the algal culture was achieved by two alternative cycles of the imposition of a 12 hour dark and 12 hour light phase upon an exponentially growing culture and subsequent exposure to the light for one hour prior to the conduction of further experiments (11).

3) Viral Maintenance, Titer and Growth Characterization

Cyanophage AS-1 was kindly supplied by Dr. R. Safferman. The virus was cultured under aseptic conditions in flasks containing exponentially growing Anacystis nidulans (MOI=5). Prior to infection a sterile NaCl solution was added to the algal culture at a resultant concentration of 0.1 M to facilitate adsorption of virus onto the algal cell surface. The infected culture was incubated at 31-34°C for 24 hours under a continuous illumination provided by a series of "cool white" fluorescent tubes. A longer incubation period (e.g., longer than 48 hours) inevitably led to as much as a 10-fold loss of viable virus. The lysis ability was found to be satisfactorily correlated with a decrease in turbidity, and accompanied by a reduction in algal cell numbers as indicated by microscopic observation of the infected cultures. The lysate was centrifuged

at 10,000 rpm for 10 min. to remove the unlysed cells and any cellular debris. The viral suspension was then collected by passing the supernatant through a sterile Nalgene Filter Unit.

Quantitative determination of AS-1 was achieved by utilizing a phage technique (56) modified for blue-green algal virus studies (122). The viral sample was diluted serially at 100-fold intervals in sterile TM buffer solution, inverted and illuminated with fluorescent light at 30-33°C. The plaques induced by the virus were counted with an American Optical Corp. counting apparatus equipped with a red filter. The titer varied, but a concentration of 10^8 - 10^9 plaque forming units per ml (PFU/ml) was generally obtained.

Growth characteristics of cyanophage AS-1 were determined by modified one-step growth experiments, as conducted by Safferman (40,126), in which the preparation of host algae for viral adsorption was carried out as described above. Final algal concentrations were approximately 1×10^8 cells/ml and final viral concentrations about 1×10^7 PFU/ml.

Adsorption was allowed to proceed for 1 hour, after which the cultures were diluted 10^{-2} and 10^{-4} times in 3M medium. Small aliquots were withdrawn and titered at varying time intervals for a period of 24 hours after infection.

4) Preparation of $\text{NaH}^{14}\text{CO}_3$

Utilizing a specially designed Warburg trapping apparatus barium carbonate ($\text{Ba}^{14}\text{CO}_3$) was converted to sodium bicarbonate ($\text{NaH}^{14}\text{CO}_3$) as described by Baye (20). A known amount of barium carbonate ($\text{Ba}^{14}\text{CO}_3$) was placed onto the base of a 25 ml Warburg flask. One ml of 10 N sodium hydroxide (NaOH) was then carefully pipetted into a center well situated on the base of the flask. Into the sealed sidearm 1 ml of lactic acid ($\text{CH}_3\text{CHOHCOOH}$) was introduced via a disposable capillary pipette that had been bent by heat to an angle of 45° . To insure full collection of the released $^{14}\text{CO}_2$ gas, a General Electric White Silicone Seal was applied to the glass surface surrounding the flask cap interface. After a period of three days had elapsed, the contents of the center well, now containing trapped $^{14}\text{CO}_2$ in the form of $\text{NaH}^{14}\text{CO}_3$, were removed with a capillary pipette and diluted with water to give a final concentration of 3 N NaOH . This served as the concentrated stock for $\text{NaH}^{14}\text{CO}_3$. A small amount of stock $\text{NaH}^{14}\text{CO}_3$ was diluted and adjusted to pH 7.5 for experimental purposes.

5) The Uptake of the Radioactively Labeled Materials

^3H -adenine, ^{14}C -glucose-6-phosphate (final concentration of 0.5-1.0 $\mu\text{Ci/ml}$) and $\text{NaH}^{14}\text{CO}_3$ (final concentration of 0.05-0.1 $\mu\text{Ci/ml}$) were added singly to three uninfected and three AS-1 infected cultures ($\text{MOI}=5$). Metabolite

incorporation was determined by indirectly checking the radioactive count remaining in the supernatant, and testing for the extent of acid insolubility of the exogenously supplied radioactive metabolite.

A 1 ml sample was removed from each flask at different time intervals and spun down at 10,000 rpm for 5 minutes to create a supernatant and pellet. From each supernatant triplicate samples of 100 λ were removed and directly spotted onto Whatman GF/A paper. The samples used to test for the presence of $\text{NaH}^{14}\text{CO}_3$ were subsequently converted to the more stable $\text{Ba}^{14}\text{CO}_3$ by addition of a saturated $\text{Ba}(\text{OH})_2$ solution to the filter.

Each pellet then was treated with 1 ml of 20% tri-chloroacetic acid and incubated at 4°C for at least 30 minutes. The samples were then collected by Whatman GF/A glass fiber filters (2.4 cm), washed three times with cold 5% TCA, and rinsed once with cold 95% ethanol. All the filters were gently dried and inserted into 4 ml of a scintillation cocktail composed of a toluene-based scintillant (Liquorfluor, New England Nuclear). Their radioactivity was measured in a Beckman LS-150 Liquid Scintillation Counter.

6) Preparation of Crude Lysate for DNA Extraction

- a) The uninfected and AS-1 infected Anacystis nidulans cultures and E. coli

The exponentially growing E. coli, Anacystis nidulans,

and Anacystis nidulans which had been infected by AS-1 for 5 hours, were all centrifuged at 5,000 rpm for 10 minutes in a Sorvall SS34 rotor, washed once with a saline-EDTA buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0) and resuspended in the same solution. Lysozyme (final concentration of 2 mg/ml) was added to the concentrated cell suspensions which were then incubated at 37°C for 12 hours. At the end of this time, the spheroplasts were gently centrifuged and resuspended in saline-EDTA buffer. The samples were incubated for 15 minutes at 60°C following the addition of SDS detergent (at a final concentration of 1%) to complete the lysis.

b) The cyanophage AS-1

A large volume (1-4 liters) of a lysed infected culture was centrifuged at 10,000 rpm for 10 minutes in a Sorvall SS34 rotor to remove unlysed algal cells and any remaining cellular debris. The clarified supernatant solution was then concentrated by G-200 Sephadex (water gained 20 ± 2 g/g). The final volume of 300 ml was sterilized by filtration through a 0.45 μ sterile Nalgene Filter unit and transferred to cellulose nitrate centrifuge tubes. After subjection to centrifugation at 25,000 rpm in a 60 Ti rotor for 1 hour at 19°C in a Beckman L3-50 ultracentrifuge, the pelleted viruses were resuspended in 5 ml of TM buffer. DNase, at a final concentration of 100 μ g/ml, was added to digest the DNA fragments remaining in the buffer outside the viruses, and the sample was left for 1 hour at 37°C. It was

then dialyzed against TM buffer (10 mM Tris, 250 mM NaCl, 0.1 mM EDTA) overnight at 4°C. Sarkosyl detergent (10%) was added to the dialyzed virus solution which was incubated for 15 minutes at 60°C to completely lyse the virus.

7) DNA Extraction

DNA of crude lysates prepared as described above may be extracted by using a modified Marmur procedure (86). An equal volume of phenol (distilled and saturated with 0.1 x SSC buffer) was added to the detergent treated lysates. The mixtures were put into small flasks, gently swirled on a gyratory shaker (New Brunswick, Model G2) for 10-15 minutes. This was followed by a phase separation aided by centrifugation at 7,500 rpm for 10 minutes. The aqueous phase was recovered and combined with an equal volume of chloroform-isoamyl alcohol (24:1, v:v) for further deproteinization. It was swirled and then centrifuged into two separate phases as described above. The phenol and chloroform treatments were performed repeatedly until a clear interface free of denatured protein was obtained.

The aqueous phase was then collected and added to two volumes of cold 95% ethanol. The DNA was recovered by spooling onto a thin glass rod. The spooled DNA was dried and dissolved in 0.1 x SSC (standard saline citrate, 0.15 M NaCl, 0.015 M Na citrate, pH 7.0). Following ribonuclease A (final concentration 100 ug/ml) digestion for 0.50-0.75

hours at 37°C and protease (final concentration 100 ug/ml) digestion for 2 hours at 50°C, the solution was reextracted with chloroform-isoamyl alcohol as above. The DNA was precipitated by addition to two volumes of cold 95% ethanol, spooled, and then dissolved in 0.1 x SSC, dialysed overnight against 0.1 x SSC and stored at 4°C over chloroform.

8) Preparation of "prelabeled algae"

$\text{NaH}^{14}\text{CO}_3$ was added to an exponentially growing algal culture at a final radioactivity concentration of 1 uCi/ml. A control with cold sodium bicarbonate substituted for $\text{NaH}^{14}\text{CO}_3$ was subjected to the same protocol as the radioactive culture.

The radioactivity of the culture, supernatant and TCA-precipitable material was determined as described in "The Uptake of the Radioactively Labeled Materials." When approximately half of the total radioactivity was incorporated into TCA-precipitable materials, the culture was terminated as described in "Preparative CsCl Density Gradients."

In order to remove the radioactivity from the intracellular pools of the labeled cultures, chase procedures were performed as follows. The labeled cells were centrifuged daily at 5,000 rpm for 10 minutes, washed twice with sterile 3M medium and resuspended in 3 M medium supplemented with sodium bicarbonate (final concentration of 0.01 M). These cultures were then allowed to grow under standard

conditions and the radioactivity of pellets, supernatant solutions and TCA-precipitable materials was assessed as before. The intracellular pools were judged to be devoid of radioactivity when the level of radioactivity incorporated into acid-precipitable components approached the level of radioactivity incorporated into the pelleted cells with adjustments for the radioactivity remaining in the medium

The $\text{NaH}^{14}\text{CO}_3$ pulse chase labeled algal cultures were used as the "prelabeled" cells in further studies.

9) Preparation of Prelabeled Phage

The crude viral lysates were collected and concentrated as described in "Preparation of Crude Lysate for DNA Extraction." The concentrated viral lysates were added to logarithmically growing cells ($\text{MOI}=5$), supplemented with ^3H -adenine at a concentration of 0.5 uCi/ml , under conditions identical to those described in "Viral Stock Preparations." The infection proceeded for a period of 20 hours and the lysates were then collected by centrifugation at $10,000 \text{ rpm}$ for 10 minutes to remove the cellular debris. The radioactively labeled crude lysates were dialyzed three times against TM buffer, then sterilized by passage through a 0.45 u sterile Nalgene Filter unit. The crude viral lysates and the dialyzed viral lysates were spotted directly onto Whatman GF/A filter paper to measure the total radioactivity, or were treated with an equal volume of 20% TCA for 1 hour at 40°C to measure the radioactivity incorporated into TCA-

precipitable materials. For different protocols the radioactively labeled lysates were concentrated or directly used as the "Prelabeled Virus" in further experiments.

10) Preparation and Photoreactivation Studies of UV-irradiated Cells and UV-irradiated Phage

a) UV-irradiated cells

(i) UV-irradiated dark survival curve

An aliquot of an exponentially growing cell culture was placed in a petri dish placed on a speed-controlled shaker in the UV-irradiated chamber. Irradiation took place in the dark at a distance of 45 cm from a germicidal lamp, emitting UV-irradiation primarily at 254 nm. At different times following irradiation, an 0.1 ml sample was removed, diluted, and plated out on the cyanophycean agar plates. The plates were completely wrapped with aluminum foil and incubated in the dark for approximately 24 hours to prevent photoreactivation and thus minimize excision repair (12). Samples were then exposed to light and the colonies were counted after 4-6 days. The dosage causing a 99% loss in viability of the cells was used to prepare the damaged cells necessary for further experiments.

(ii) Photoreactivation study of UV-irradiated cells

UV-irradiated algal cultures were diluted and plated out in four sets of cyanophycean agar plates. One set was

illuminated immediately after UV-irradiation. The other three sets were kept in the dark for 4, 8 and 24 hours respectively and then exposed to light.

A set of controls was prepared for each experimental condition. The plates were illuminated on a fluorescent light bank and the colonies were counted after 4-6 days had elapsed.

b) UV-irradiated phage

(i) UV-irradiated lytic ability curve

The phage lysate was collected as described in "Maintenance of Phage." The virus solution was irradiated with UV-light under the same conditions as described in "UV-irradiated Cells."

Following irradiation, at different time periods, 2 ml of virus solution were removed and added to exponentially growing cells. The infected cultures were exposed to the light for 16-17 hours. The lytic ability was then determined by monitoring the decrease in turbidity of the infected culture at OD_{750nm}. Phage samples that had lost 99% of their lysing ability were used as the "damaged phage" in further studies.

(ii) The photoreactivation of UV-irradiated AS-1 cyanophage and UV-irradiated AS-1 in Anacystis nidulans

In order to examine the photoreactivation of AS-1

cyanophage, UV-irradiated phage were kept in the dark for a period of 0, 4, 8, or 24 hours, added to the exponentially growing cells and analyzed for lytic ability as before.

To examine the photoreactivation of AS-1 in its host, Anacystis nidulans, UV-irradiated phages were added to the exponentially growing cells and the infected cultures were then kept in the dark for 0, 4, 8, and 24 hours. At the end of the dark period the infected cultures were exposed to the light. The extent of phage development was ascertained both by the number of viable phage produced (PFU/ml) determined as described in "Viral Titer", and by culture lysis during the infection cycle which was measured by turbidity change at 0.D.750 nm.

11) Preparation of DNA Inhibitors

a) Hydroxyurea

The stock solution of hydroxyurea was always freshly prepared by dissolving the drug in 3M medium at a concentration of 5 mg/ml. It was sterilized by filtration through a 0.45 u Millipore filter.

b) Nalidixic acid

Fresh stock solutions of nalidixic acid (5 mg/ml) were prepared as follows (81): 100 mg of nalidixic acid powder was dissolved in 0.8 ml of 1 N NaOH, and 3M medium added to a final volume of 100 ml. The solution was sterilized by

Millipore filtration as above.

12) Preparation of CsCl Density Gradients

Preparation of CsCl density gradients was in accordance with the method of Blamire (23). For UV-absorption studies DNA samples of Anagystis nidulans, AS-1, and A. nidulans infected with AS-1 for 5 hours were prepared using the modified method of Marmur's described in "DNA Extractions."

For radioactive pulse labeling experiments, samples were centrifuged at 5,000 rpm for 10 minutes at the end of the "pulse" period. Pelleted cells were washed twice with saline EDTA, lysed in 1.0 ml of termination solution (saline-EDTA, 0.01 M NaN₃, 0.05 M NaCN and 3 mg/ml of lysozyme), and incubated at 37°C overnight.

Lysates of 1.0 ml each were transferred to cellulose nitrate centrifuge tubes containing 4 ml of saturated CsCl solution (130 g of CsCl in 70 ml of 0.01 M Tris buffer, pH 6.8). The density of each tube was adjusted with saline-EDTA to 1.705 g/ml using a Bausch and Lomb refractometer. When all the tubes had been so adjusted, mineral oil was used to completely fill the tubes, which were then inserted into the Spinco 50 Ti rotor and centrifuged in a Beckman L3-50 ultracentrifuge at 33,000 rpm for 42 hours at 19°C. Fractions of approximately 0.2 ml were collected dropwise from each gradient by piercing the bottom of each tube with a 22 gauge 1.5 inch needle.

For those gradients which were to be monitored for UV-absorbing material, fractions were transferred to quartz micro-cuvettes and the absorbances at 260 nm were measured. For gradients to be monitored for radioactively labeled material, fractions were treated according to the following protocol: 0.2 ml of 1 M KOH was added to each fraction and the fractions were then incubated at 60°C for 2 hours (or at room temperature for 24 hours). Following the alkaline hydrolysis the fractions were cooled, and 0.1 ml of bovine serum albumin (1000 ug/ml) and 0.7 ml of 20% TCA were added to each fraction. The fractions were allowed to remain at 40°C for at least 30 minutes. Samples were collected on a multiple sample precipitator (47), washed twice with 100 ml of cold 5% TCA, and once with cold 95% ethanol. The radioactivity was then determined as previously described in "Uptake of Radioactively Labeled Materials."

13) Preparative Collection of ^{14}C -PIL-DNA and ^3H -labeled DNA from an AS-1 Infected Anacystis nidulans Culture

An AS-1 viral lysate was added to a large synchronous algal culture (MOI=5) to a final volume of 2 liters. Two hours after infection, 50 ml of infected culture was withdrawn and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ (1 uCi/ml). Five hours after infection, 50 ml of the infected culture was removed and radioactively labeled with ^3H -adenine (1 uCi/ml). Five hours post infection, the $\text{NaH}^{14}\text{CO}_3$ labeled 50 ml culture was collected together with 950 ml of the original infected

culture by centrifugation at 5,000 rpm for 10 minutes. Eight hours after infection, the ^3H -adenine labeled 50 ml culture was centrifuged together with the remainder of the original infected culture.

The 2-5 hour, $\text{NaH}^{14}\text{CO}_3$ -labeled and the 5-8 hour, ^3H -adenine-labeled cultures were then separately washed twice with saline EDTA and terminated in the termination solution (saline EDTA, 0.01 M NaN_3 , 0.05 M NaCN and 3 mg/ml of lysozyme). The ^3H -adenine-labeled culture, $\text{NaH}^{14}\text{CO}_3$ -labeled culture and two samples of a combination of these cultures were transferred to the nitrocellulose tubes and run on CsCl density gradients at 33,000 rpm for 42 hours at 19°C in a L3-50 ultracentrifuge. The procedures were described in detail in the section entitled "Preparation of CsCl Density Gradients."

The radioactivity of each fraction collected from the CsCl density gradient was determined by KOH hydrolysis and TCA precipitation to 50λ from each fraction, and counting it in a scintillation counter.

The ^3H -adenine-containing fractions and ^{14}C -containing fractions were pooled and collected separately and then dialyzed against three changes of 0.1 x SSC buffer. The dialyzed samples were purified by re-ultracentrifugation in a CsCl density gradient. The radioactively labeled fractions of ^3H -adenine and ^{14}C - NaHCO_3 were again pooled and dialyzed against three changes of 0.1 x SSC buffer. The dialyzed DNAs of the ^{14}C fractions and the ^3H -adenine fractions were

spooled onto a glass rod following the addition of two volumes of cold 95% ethanol. These were then dissolved in 0.1 x SSC buffer and stored. The ^{14}C -labeled DNA which was collected at 5 hours after infection had a lighter density in the CsCl density gradient compared to the cell or phage DNA. This was named PIL-DNA (phage induced light DNA). The ^{14}C -PIL-DNA and ^3H -adenine DNA were collected, purified as above and used in further experiments.

14) Poly-L-lysine Kieselguhr Chromatography

Separation of different DNA species using poly-L-lysine Kieselguhr (PLK) chromatography was effected by a modification of the methods of Ayad and Blamire (16).

Samples for the PLK columns were prepared using either purified DNA or crude lysates. Purified DNA was isolated using the modified Marmur procedure described in "DNA Extraction." The crude lysates were prepared by breaking the infected or non-infected cells with a Braun Homogenizer for 1 minute, after which the suspensions were treated with excess RNase for 0.5 to 0.7 hours at 37°C . To prepare the column material, 2.5 gm of refined kieselguhr was suspended in 12.5 ml of 0.4 M buffered saline (0.4 M NaCl, 0.02 M KH_2PO_4 , pH 6.8) and boiled briefly to remove the trapped air. The cooled suspension was then mixed and 0.4 ml of a 10 mg/ml poly-lysine solution was added with gentle stirring. Samples (about 1 mg DNA) were then put into the poly-L-lysine kieselghur slurry. Air pressure was used to

accelerate the packing of the column material into a glass tube (1 x 20 cm) on the bottom of which was a small amount of glass wool. The loaded columns were washed free of unbound material using 15 ml of 0.4 M buffered saline.

Linear gradients of 0.4-4.0 M buffered saline (or 1-2.5 M saline buffer) were generated using a multichannel peristaltic pump and 45 drop fractions were collected using a Gilson fraction collector. The flow rate was about 20 ml/hour. The linearity of the salt gradient was checked by monitoring the refractive index of each fraction with a Bausch and Lomb refractometer. The salt concentration of the individual fractions was determined by comparison with the standard solution. The extinction of the fractions was measured at 260 nm using a Gilford spectrophotometer. The radioactivity of fractions in pulse-labeling experiments was determined as previously described in "Uptake of Radioactively Labeled Materials" and "Preparation of CsCl Density Gradients."

15) Thermal Denaturation

The purified DNAs of AS-1, Anacystis nidulans, PIL-DNA, and E. coli (serving as a standard) were collected as previously described and diluted to a final concentration of 50 ug/ml with 0.1 x SSC. The diluted DNA samples were dialyzed against three changes of 0.1 x SSC. Two ml of each DNA sample was then transferred to a silica cuvette. Several drops of mineral oil were applied to the surface of the

sample and the cuvette was sealed with a polypropylene stopper in order to eliminate evaporation. The absorbance at 260 nm was recorded using a Beckman Acta CIII Spectrophotometer, during which time the increase in temperature was held at a constant rate of $1^{\circ}\text{C}/5$ minutes by a Haake circulating polyethylene glycol bath. The temperature was monitored with a YSI model 42 SC telethermometer. The absorption values were subsequently corrected for the volume expansion of the liquid (85).

The results were graphically presented by plotting relative absorbance at 260 nm against temperature. The temperature responsible for 50% of the total hyperchromatic shift was determined to be the T_m . The apparent G + C content was determined from the T_m by use of the equation provided by Mandel and Marmur (85).

16) DNA Composition Analysis

The DNA of AS-1 and Anacystis nidulans was extracted as described in "DNA Extraction," and PIL-DNA was collected as described in "Preparative Collection of PIL-DNA." The DNA samples were dialyzed at 4°C against three changes of a solution containing 0.01 M Tris HCl (pH 7.6) -5 mM MgCl_2 , freeze-dried by a lyophilizer, and treated in one of the following ways according to the method of Rae and Hanawalt (110) and Rae (108).

a) Nucleotide analysis

The DNA was dissolved at a concentration of 1 mg/ml in 0.01 M Tris HCl (pH 7.6) -5 mM $MgCl_2$.

Enzymatic degradation to mononucleotides was accomplished by incubation for 5 hours at 37°C with 0.02 ml of pancreatic deoxyribonuclease at 1 mg/ml (in 0.0001 M NaCl and 1 mg/ml bovine serum albumin), followed by incubation at pH 8.5 for 5 hours at 37°C with 0.01 ml of venom phosphodiesterase at 4 mg/ml (in 0.001 M NaCl and 4 mg/ml bovine serum albumin). The resultant nucleotide solutions were evaporated to dryness under vacuum at 20°C and redissolved in 0.05 ml of distilled water. Aliquots (20-30 μ l) of the nucleotide samples were then spotted directly on 20 x 20 cm thin-layer chromatography plates (TLC) composed of cellulose (cellulose MN 300, 250 μ).

Two-dimensional chromatography on TLC plates with the solvent front moving 15 cm from the origin in each direction was performed with isobutyric acid-water-ammonia, 60:20:1, and with saturated ammonium sulfate-1M sodium acetate-isopropanol (80:18:2). Twenty to thirty μ g of 5'dAMP, 5'dGMP, 5'dCMP, and 5'dTMP spotted separately or in combination upon the cellulose plates served as standards and the plates were developed two-dimensionally along with the samples. Spots on the TLC plates that absorb ultraviolet light can be made visible after development by exposure to a high intensity UV-lamp (Model UVs-54, Fisher).

An autoradiograph of ^{14}C -labeled PIL-DNA was made in order to identify the ^{14}C -containing nucleotides. Kodak

X-ray film was overlaid on the chromatogram and a press was used to insure continuous contact between the film and the thin-layer chromatography plate. The chromatogram was then stored in the dark room for 2-3 weeks. The X-ray film was developed by a 5 minute immersion in Kodak X-ray film developer, rinsed with H_2O and then fixed in Kodak acid fixer for 10-20 minutes.

b) Base analysis

One mg of each DNA sample was hydrolyzed with 0.5 ml of 88% formic acid at 175°C for 40 minutes. After lyophilization, the residue was dissolved in 0.1 ml of 1 N HCl. Twenty to thirty μ l of each sample was spotted on CEL 300 plates, and 5-10 μ g each of thymine, adenine, cytosine, guanine, and certain modified bases were spotted separately or in combination alongside. The plates were developed with isopropanol-concentrated HCl-water (85:20.5:19.9). UV-absorbing spots were detected and autoradiographs performed as described earlier.

17) DNA Determination

DNA determination was carried out according to the method of Burton et al. (28). Ten ml of infected or non-infected algal cells were centrifuged at 5,000 rpm for 10 minutes. DNA extraction from the pellets was performed in two stages (35).

The pellets were initially incubated in 1 ml of 10%

TCA for a period of 1 hour at 37°C. The osmotically lysed cells were then treated with 3 ml of 0.5 M perchloric acid (HClO₄) and incubated at 70°C for 1 hour. After cooling the acidified reaction mixture to ambient temperature, 4 ml of diphenylamine reagent (DPA) was added (1.5 g of diphenylamine was dissolved in 100 ml of glacial acetic acid and 1.5 ml of concentrated H₂SO₄). The reagent was stored in the dark at 4°C. On the day of use, 0.1 ml of aqueous acetaldehyde (16 mg/ml) was added for each 20 ml portion of reagent required. Following vigorous agitation, the reaction mixture was incubated at 30°C for 24-30 hours. Any particulate matter was then separated from the solution by centrifugation of the samples in a Fisher International Clinical Centrifuge at top speed for 5 minutes. The absorbance of the supernatant solution was measured at 600 nm in a Gilford spectrophotometer. Blanks were prepared using acid-extracted DNA from algal cells that had been subjected to identical experimental protocols with the exception of 4 ml of distilled water added in lieu of DPA treatment. Calf thymus DNA was utilized as the standard for the colorimetric determination of the DNA.

18) Protein Determination

Protein determination was performed according to the method of Lowry (77). Five ml of infected or non-infected cells were centrifuged at 5,000 rpm for 10 minutes. Four and a half ml of 0.1 N NaOH were added to the pellets, and

the samples were mixed well and boiled for 20 minutes, cooled to room temperature, and recentrifuged as before. To 1 ml aliquots of the supernatant solution, 3 ml of freshly prepared reagent C (reagent A: 49 ml of 2% Na_2CO_3 in 0.10 N NaOH + Reagent B: 1 ml 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 2% sodium potassium tartrate) were added; the samples were then mixed well and allowed to stand for 10 minutes at room temperature. Three tenths of 1 ml of Folin reagent (Folin and Ciocalteu's phenol reagent 2 N diluted 1:1 with distilled water) was rapidly added and mixed immediately. After a period of at least 30 minutes, the samples were read in a spectrophotometer at 750 nm. Bovine serum albumin (BSA) was used as a standard.

19) Chlorophyll Determination

Chlorophyll content was determined by a modification of the method of Arono (10). Ten ml of the uninfected or infected cells were centrifuged at 5,000 rpm for 10 minutes. The supernatant solution was decanted and 5 ml of 90% methanol and a minute amount of magnesium carbonate (MgCO_3) were added to the sediment. The samples were incubated at 75°C for 10 minutes, vortexed for 30 seconds to release the chlorophyll from the cells. After cooling to room temperature, the samples were recentrifuged as before. The absorbance of the supernatant solutions were measured at 663 nm on a Gilford spectrophotometer.

The chlorophyll concentration in Anacystis nidulans

was determined by using the following conversion:

$$A_{663} \times 12.2 \times \text{dilution factors} = \text{ug of chlorophyll/ml}$$

RESULTS

I. The Growth of *Anacystis nidulans* and the Development of Cyanophage AS-1 in *Anacystis nidulans*

A. Growth Curve of *Anacystis nidulans*

Figure 1 shows a typical growth curve for cells of *A. nidulans*. Two parameters were used to monitor the culture: direct cell counts and turbidity measurement at 750 nm. As can be seen, the two curves closely correspond one another with only slight divergence at the upper and lower ends of each curve.

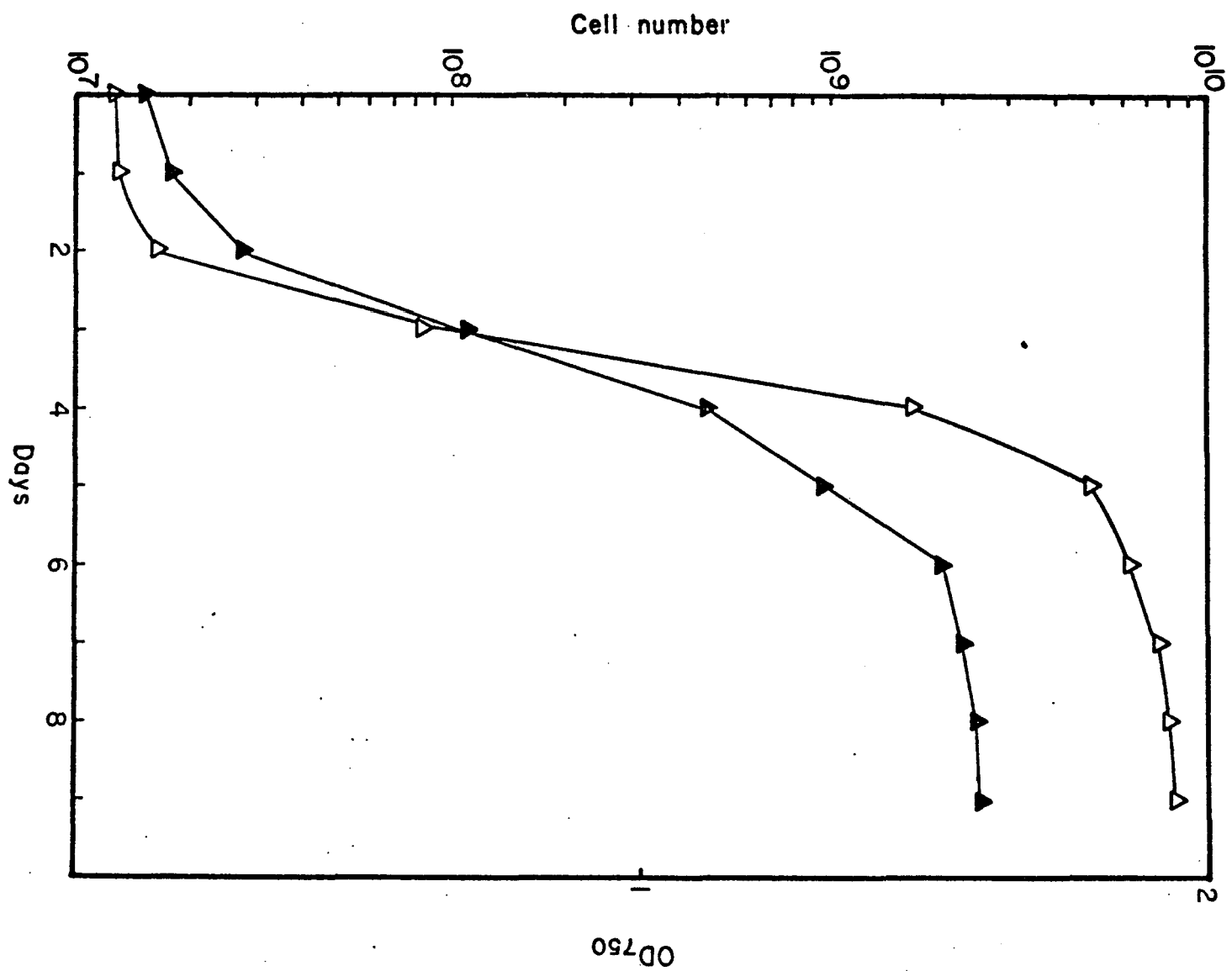
Since it was considered important that the infective process be followed in cells of more or less identical physiological state for each experiment, standard growth conditions were adopted as follows:

Cultures were inoculated to give 1.5×10^7 cells per ml of *Anacystis nidulans* and placed under optimal conditions of light, temperature and agitation. In addition it was found (data not shown) that a constant pH and an adequate supply of carbon dioxide were important. Under these conditions a newly inoculated culture has a lag phase of less than one day when followed by turbidity changes. This is in turn followed by an exponential growth phase of about five days, with a generation time of approximately 12 hours, and eventual leveling off into a typical stationary phase.

Figure 1. The Growth Curve of Anacystis nidulans as Monitored by Changes in Turbidity and Cell Number

Ten ml of exponentially growing Anacystis nidulans was inoculated into 200 ml of sterile 3M medium (pH 7.5). The culture was illuminated continuously with fluorescent light and aerated with 0.5% CO₂ at a temperature of 30-34°C as described in MATERIALS AND METHODS. Triplicate 2 ml aliquots were sterilely removed daily from the growing cultures. The turbidity of the aliquots was measured using a Gildord spectrophotometer at 750 nm and the cell number was determined using a Petroff-Hauser counting chamber.

▲ — ▲ cell number/ml
△ — △ OD_{750 nm}



A heavier inoculum enhances the overall rate at which the culture grows and reaches stationary phase but does not alter the generation time. For all routine cell growth and to prepare cells for infection these standard conditions were used, and each experiment was followed by monitoring the turbidity changes at 750 nm.

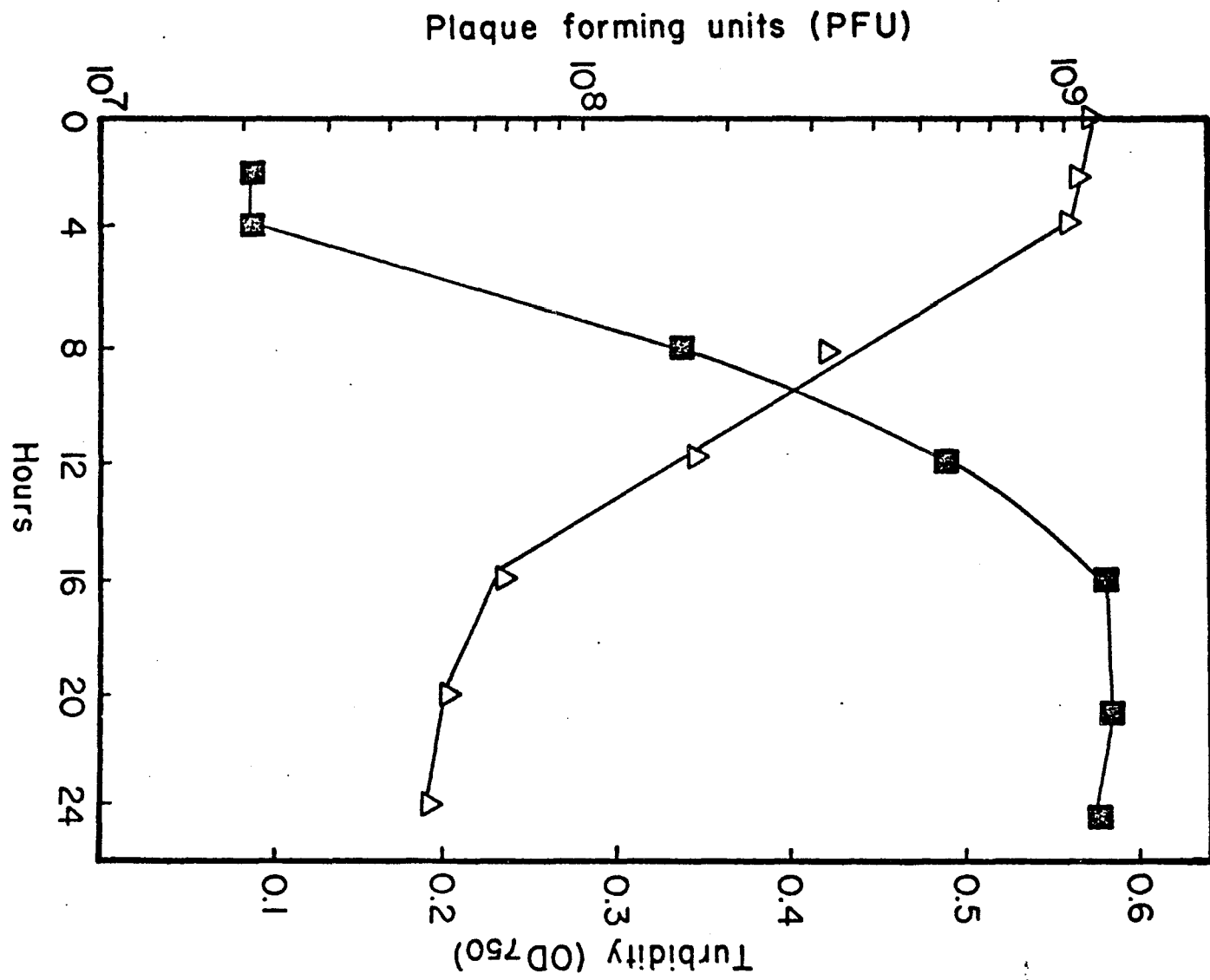
B. Growth of Cyanophage AS-1

Phage development in the AS-1/Anacystis nidulans infective system is characterized by both viable phage production and host lysis. A rapid measure of cell lysis during infection cycles can be obtained by observing the decrease in turbidity of the culture. However the number of phage produced (PFU/ml) can only be determined by the less immediate standard bacteriophage plating techniques. Figure 2 shows the correlation between optical density measurements and phage growth, using a culture grown under continuous light and infected with an MOI of 0.1. Both a decrease in turbidity and an increase in plaque-forming units are observed at 8 hours after infection; the lytic cycle is completed by 16 hours, as seen by the leveling off of both curves. Continuous illumination, proper temperature range (30-35°C) and the exponentially growing cells are necessary for the cyanophage developmental cycle (24,136). Under optimal conditions, the average burst size is about 50. Essentially identical results to those shown in Figure 2 were obtained on at least five different occasions.

Figure 2. Cyanophage AS-1 Development in Anacystis nidulans

An AS-1 infected culture (MOI=0.1) was incubated under standard conditions. At the time intervals of 0, 2, 4, 8, 12, 16 and 24 hours post infection, triplicate 2 ml aliquots were sterilely removed from the infected culture and the extent of algal lysis obtained by measuring the change in turbidity of the samples at 750 nm. These same aliquots were also analyzed for the number of phage produced by a modification of the plating assay described in MATERIALS AND METHODS. The modification was the dilution of the infected culture immediately after the one hour adsorption period.

△—△ OD_{750 nm}
■—■ PFU/ml



II. DNA Metabolism in the AS-1/*Anacystis nidulans* Infective System

- A. Analysis of the Pattern of DNA Metabolism during the Infection of *Anacystis nidulans* by the Cyanophage AS-1 using CsCl Density Gradients
- 1) The Pattern of the Radioactive Precursor Incorporation into DNA during the Lytic Cycle

The unicellular blue-green alga, *Anacystis nidulans*, is relatively impermeable to reduced carbon precursors (105). However, it has been observed (24) that alteration of host permeability takes place following phage infection, and that infected cells will incorporate all of the nucleic acid precursors, some amino acids and two sugars. Radioactive nucleic acid precursors such as adenine were used to study the changing patterns of DNA synthesis in the infected cells.

The photosynthetic capability of the blue-green algae allows for the photoreduction of $^{14}\text{CO}_2$ into ^{14}C metabolites. Therefore $\text{NaH}^{14}\text{CO}_3$ was used in the infective system as a general metabolic precursor and ^3H -adenine was utilized as a specific nucleic acid precursor.

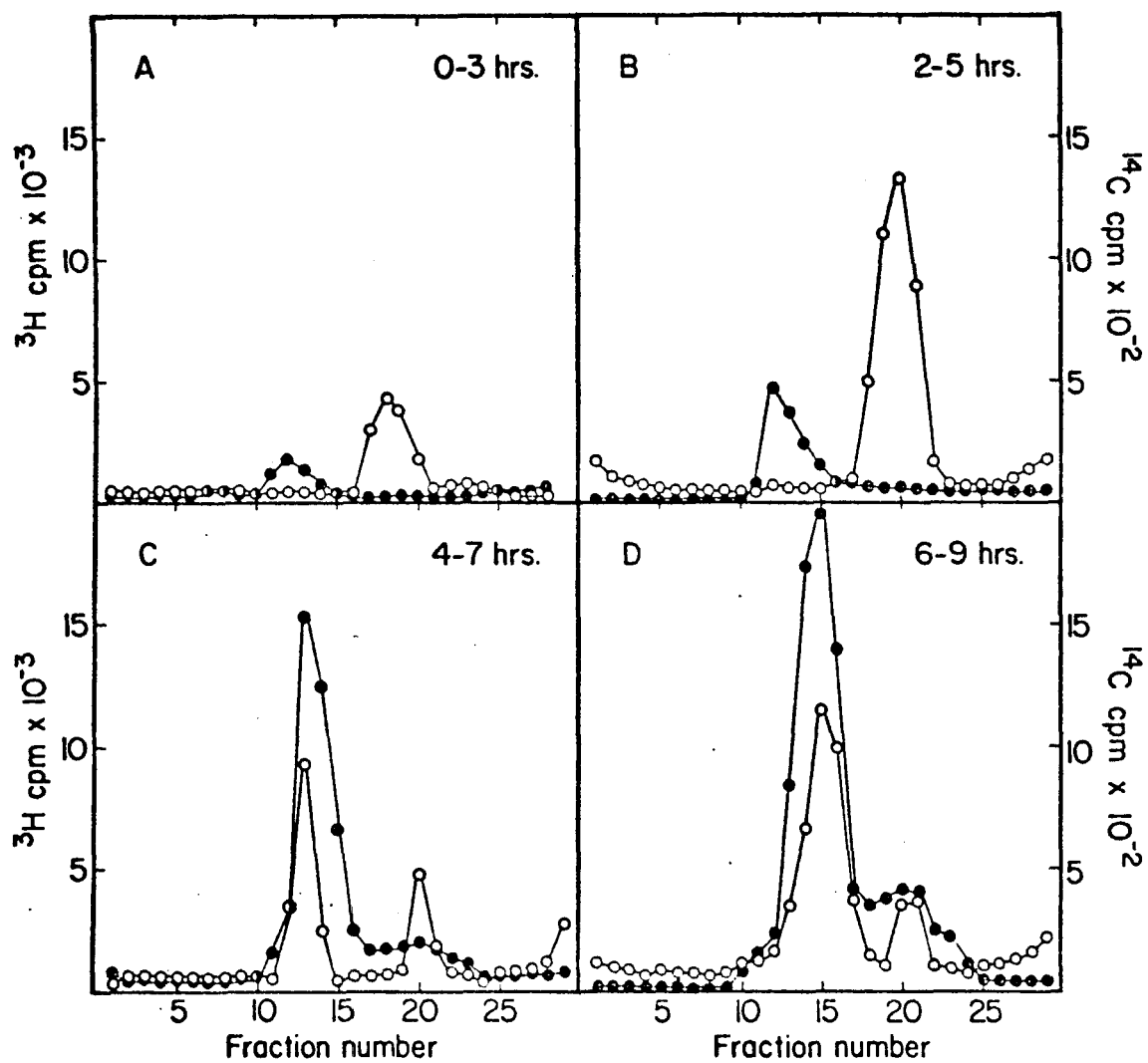
Blashka (1977) (24) found that two species of DNA were present during the lytic cycle when whole cell, radioactively labeled lysates were analyzed using CsCl density gradients. These experiments used one hour pulse labeling periods at various times following infection and utilized both radioactive ^{14}C bicarbonate and ^3H -adenine. Figure 3 shows the

Figure 3. Fraction Profiles from a CsCl Gradient of AS-1 Infected Anacystis nidulans Simultaneously Pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ and 2- ^3H -adenine

AS-1 was added to an A. nidulans culture which had been synchronized as described in MATERIALS AND METHODS, and supplemented with NaCl to give a final concentration of 0.1 M. The infected culture was divided into 5 small flasks, each containing 10 ml aliquots, which were kept at 30-35°C under constant illumination from a bank of white fluorescent lamps. At various times post infection, ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ were added to different flasks at final concentrations of 5 $\mu\text{Ci/ml}$ and 1 $\mu\text{Ci/ml}$ respectively. Three hours after the exposure to the ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$, the samples were collected by centrifugation at 5,000 rpm for 10 min, washed twice with saline-EDTA, and then resuspended in termination solution. Following overnight incubation at 37°C, the samples were treated with the detergent sarkosyl (1%) and sedimented by CsCl density gradient ultracentrifugation. Following preparative ultracentrifugation each gradient was fractionated and each fraction monitored for alkaline stable, acid precipitable radioactivity as described in MATERIALS AND METHODS. The radioactivity incorporation profile of ^3H -adenine and ^{14}C from different gradients representing different time intervals post infection are presented in the individual panels.

A. 0-3 hr; B. 2-5 hr; C. 4-7 hr; D. 6-9 hr

●——● ^3H ○——○ ^{14}C



results of an experiment performed using the same protocols as per Blashka except that the time exposure to the radioisotope has been extended. The results indicate that the incorporation patterns of ^3H and ^{14}C during the infective cycle are quite different. The two radioactive precursors are incorporated into two distinct DNA bands with different buoyant densities. At 0-3 hours post infection, a significant amount of ^{14}C is found incorporated into DNA of lighter density. This incorporation increases continuously until 5 hours after infection. At the end of 7 hours post infection, ^{14}C is also found to have been incorporated into the heavier DNA whose buoyant density is equal to that of the normal phage DNA. The amount of ^{14}C incorporated into the light DNA decreases and eventually becomes insignificant. In the late period of the infection cycle (at the end of 9 hours) the ^{14}C incorporating peak is coincident with the peak from ^3H -adenine.

^3H -adenine incorporation, on the other hand, is very slow during the early period following infection; however, by the end of 7 hours, a significant amount of ^3H -adenine is rapidly incorporated into a DNA species which bands at a buoyant density coincident with the phage DNA. The results presented in Figure 3 have been corroborated on at least ten separate occasions. Thus, pulse-labeling experiments analyzed by CsCl density gradients appear to indicate that two different species of DNA are generated during the lytic cycle. These DNA species exhibit three distinctive charact-

eristics: a) different buoyant densities; b) a possible preference for different metabolic precursors; and c) different timing for incorporation of the radioisotope precursors. These results are consistent with the conclusions drawn by Blashka. The emergence of a light density DNA band in CsCl gradients which only occurs following AS-1 infection and which solely incorporates photosynthetically reduced carbon becomes one of the most fascinating features of viral development. This light peak material has been named PIL DNA (phage induced light DNA) (24).

2) Direct Evidence Demonstrating the Existence of PIL-DNA

The qualitative existence of PIL-DNA in the early hours (2-5) following viral infection has been demonstrated by the double pulse-labeling experiments. However, in order to establish whether or not the ^{14}C associated with it reflects a quantitative amount of DNA or whether it is solely due to an unusually high specific activity of ^{14}C incorporation into some minor DNA species, it was necessary to examine the AS-1/Anacystis nidulans infective system by a method independent of radioactivity.

DNA was extracted from uninfected logarithmically growing Anacystis nidulans, concentrated cyanophage, AS-1, and blue-green algae that had been infected with cyanophage for 5 hours, using a modified Marmur procedure (see Materials and Methods). Samples were taken at 5 hours post infection because the PIL-DNA phenomenon was seen maximally at 2-5

hours after infection. These extracted DNAs were analyzed individually by preparative CsCl density gradients ultracentrifugation. Quantitative detection of the nucleic acids in each fraction from a gradient was accomplished by measurement of their absorption at OD₂₆₀ nm. The banding profiles of DNA from Anacystis nidulans, AS-1 and the AS-1/Anacystis nidulans infective systems from CsCl gradients are shown in Figures 4, 5 and 6. ³H-adenine labeled E. coli DNA was included in each CsCl gradient and used as a known buoyant density marker. The linearity of each gradient was determined from refractive index measurements of each fraction. Both the Anacystis nidulans and AS-1 DNAs band at heavier buoyant densities than the E. coli marker. In addition, there is only one species of DNA seen in both the Anacystis nidulans and AS-1 samples; however, two distinct species of DNA can be seen in the AS-1 infected cell samples. One of these species bands in a position heavier than the E. coli DNA marker and has a similar relative buoyant density to that of the cell and phage DNA. However, the other species of DNA bands at a buoyant density lower than that of the E. coli DNA marker in a position where PIL-DNA had previously been observed. These results indicate that a significant amount of light, UV-absorbing material exists in the infected cell samples collected after 5 hours of AS-1 infection, and furthermore, that it can only be found in the infective system. The concentration of this lighter material relative to the total DNA present is about 50%. The above experiments

Figure 4. The Elution Profile from a CsCl Gradient of Purified Anacystis nidulans and E. coli DNA

The exponentially growing A. nidulans and 2-³H-adenine-labeled E. coli cultures were collected, washed twice, and resuspended in saline-EDTA. The crude lysates for DNA extraction of A. nidulans and 2-³H-adenine E. coli were then prepared by treating the concentrated cell samples with lysozyme (2mg/ml, 12 hr, 37°C) and SDS detergent (1%, 15 min, 60°C). DNA of different samples was extracted by using a modified Marmur procedure as described in MATERIALS AND METHODS. The purified DNAs of A. nidulans and 2-³H-adenine E. coli were then subjected to a CsCl preparative ultracentrifugation as delineated in MATERIALS AND METHODS. The linearity of the gradient was checked by the refractive index of CsCl. The fractions were monitored spectrophotometrically for UV-absorbing material at OD₂₆₀ nm. An aliquot of 20 of each fraction was used to assay for alkaline stable, acid-precipitable radioactivity.

- refractive index
- △—△ absorbance of A. nidulans at 260 nm
- ³H-E. coli DNA

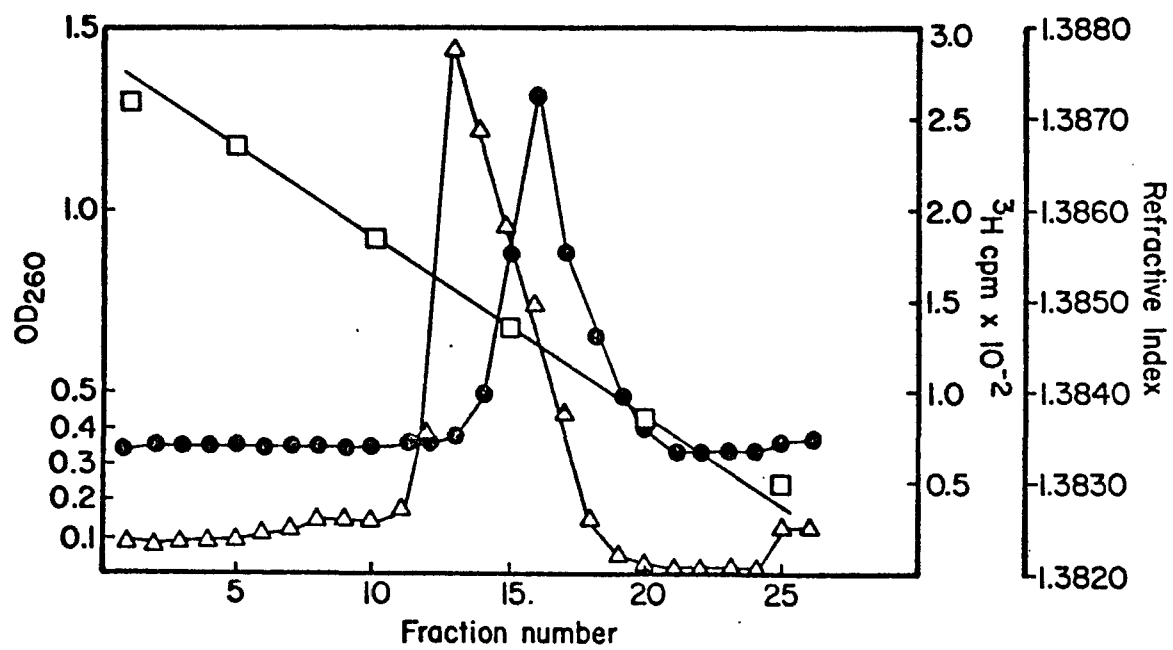


Figure 5. The Elution Profile from a CsCl Gradient of Purified AS-1 DNA and E. coli DNA

The clarified AS-1 suspension was achieved by centrifugation of a large volume (1-4 l) of a lysed culture at 10,000 rpm for 10 min to removed unlysed algal cells and any remaining cellular debris. The AS-1 suspension was concentrated using sephadex G-200, sterilized by filtration, and then subjected to high speed ultracentrifugation to pellet the virus. The harvested virus was resuspended in 5 ml TM buffer. DNase digestion (100 ug/ml, 1 hr, 37°C) was utilized to remove the cellular DNA fragments in the buffer. After overnight dialysis against 0.1 x SSC the suspension was treated with sarkosyl detergent and the DNA extracted using the modified Marmur procedure as described in MATERIALS AND METHODS. The purified DNA of AS-1 and 2-³H-adenine E. coli were subjected to CsCl preparative ultracentrifugation as described in MATERIALS AND METHODS. The linearity of the gradient was checked by the refractive index of CsCl. The fractions were monitored spectrophotometrically for UV-absorbing material at OD₂₆₀ nm. An aliquot of 20 from each fraction was taken and assayed for alkaline stable, acid precipitable radioactivity.

□—□ refractive index
 △—△ absorbance at 260 nm of AS-1
 ●—● ³H-E. coli DNA

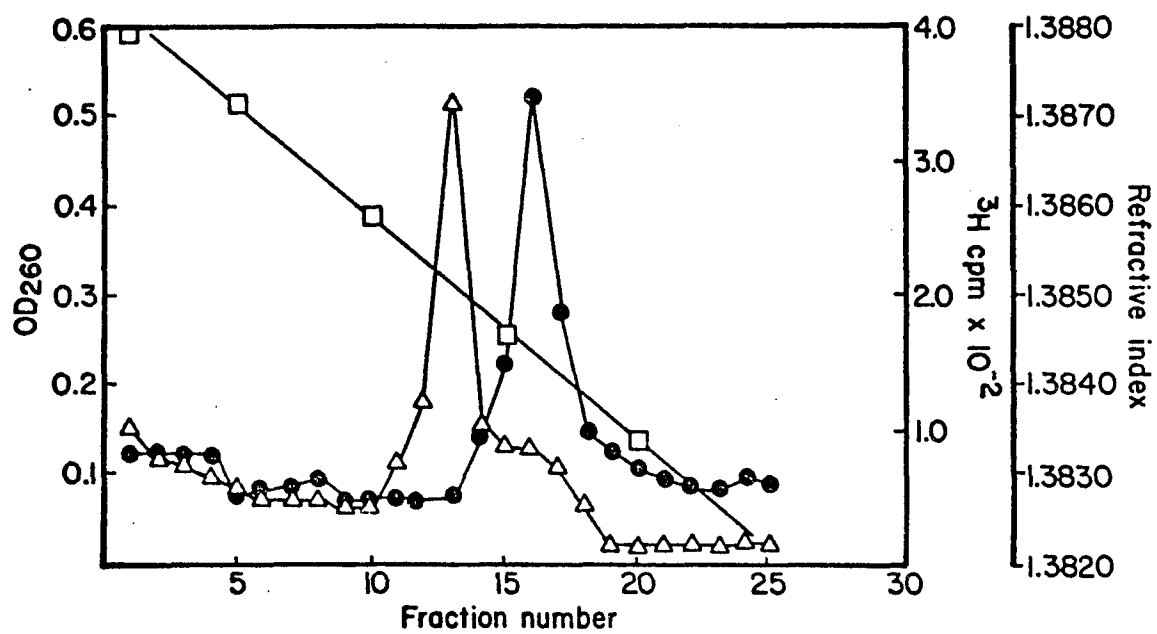
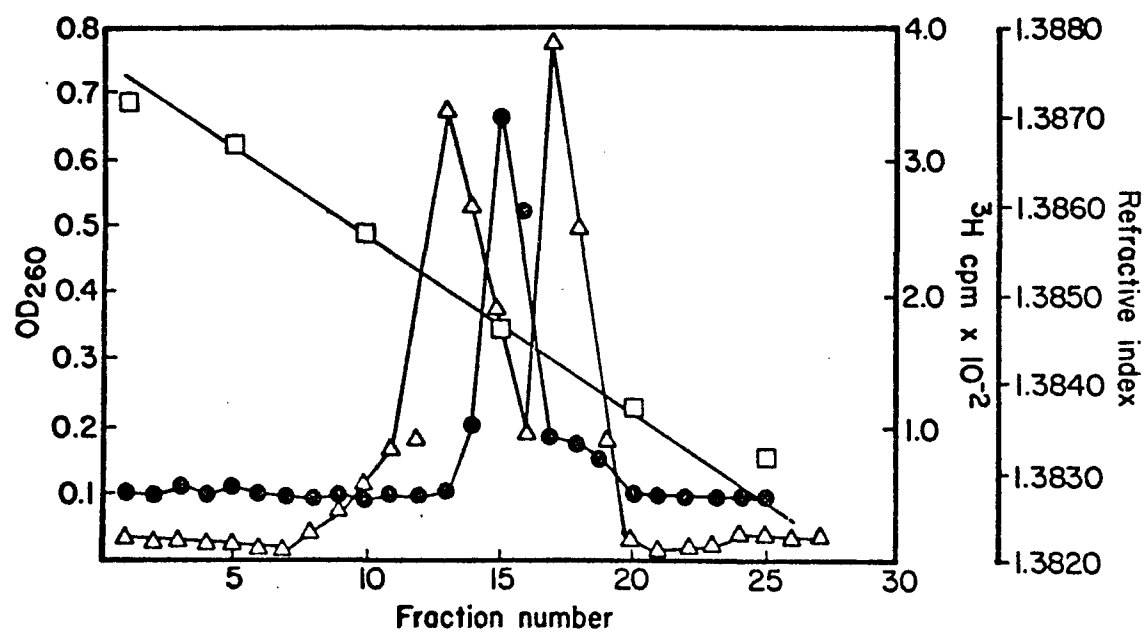


Figure 6. The Elution Profile from a CsCl Gradient of DNA Purified from Anacystis nidulans Infected with AS-1

An exponentially growing synchronized culture of A. nidulans was infected with AS-1 for 5 hr. The preparation of crude lysates from infected cells and extraction of DNA from the crude lysates were as described in the legend to Figure 4. The purified DNAs of infected A. nidulans and 2-³H-adenine E. coli were then subjected to a CsCl preparative ultracentrifugation as described in MATERIALS AND METHODS. The linearity of the gradient was checked by the refractive index of CsCl. The fractions were monitored spectrophotometrically for UV-absorbing material at OD₂₆₀. An aliquot of 20 of each fraction was used to monitor for alkaline stable, acid precipitable radioactivity.

- refractive index
- △—△ absorbance at 260 nm of infected A. nidulans
- ³H-E. coli DNA



provide the direct evidence for the coexistence of normal cell or phage DNA and a species of light, UV-absorbing material which is only detected during the early hours (2-5) of the infective cycle.

3) Isolation and Purification of ^{14}C -PIL DNA and ^3H -adenine DNA

Once direct evidence had been obtained (as described above) that demonstrated the existence of quantitative amounts of UV-absorbing, lighter density material, it was necessary to isolate and purify this material for further characterization. To achieve this the following protocols were used.

a) Isolation and identification of ^{14}C -PIL DNA and ^3H -adenine DNA

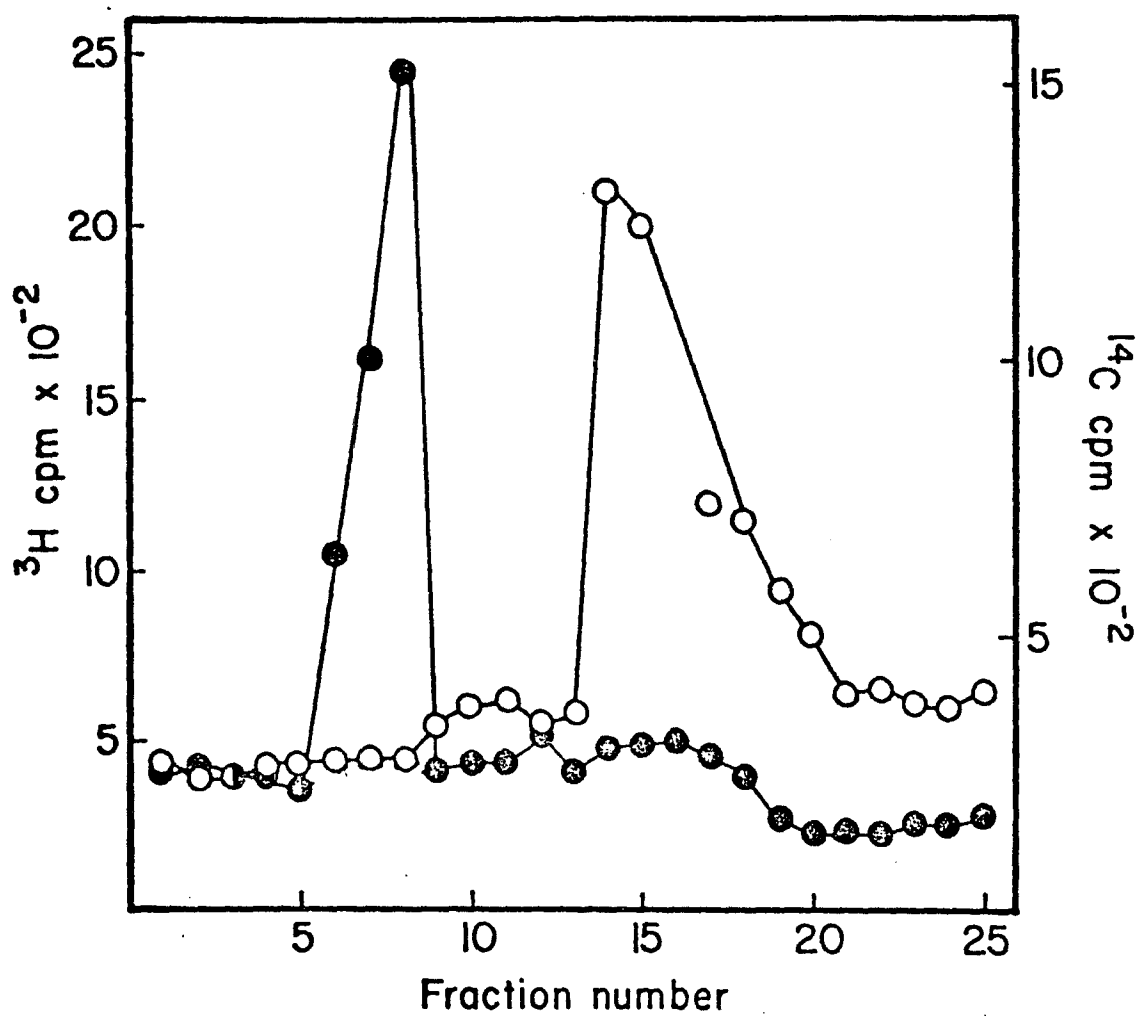
Using the information gained earlier that there were different times following infection at which isotope incorporation into the two species of DNA took place maximally, samples of ^{14}C -bicarbonate cultures treated between 2-5 hours and ^3H -adenine labeled samples treated between 5-8 hours were preparatively collected from large AS-1 infected cultures.

The relative buoyant densities of the various DNA species in the two cultures were compared by combining a small aliquot of each labeled sample and separating the DNA molecules on a preparative CsCl density gradient. The results are shown in Figure 7. It can be seen that the ^{14}C and the ^3H have been incorporated into two distinct and

Figure 7. Fractionation Profile from a CsCl Gradient of a Mixture of the DNAs Labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine within the Time Intervals of 2-5 Hours and 5-8 Hours Respectively During the Lytic Cycle of AS-1 in Anacystis nidulans

AS-1 viral lysate was added to a large synchronous algal culture (MOI=5) to a final volume of 2 l. At the start of the 2-5 hr interval 50 ml of infected culture was removed and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ (1 uCi/ml). At the start of the 5-8 hr interval another 50 ml of infected culture was removed and pulse-labeled with ^3H -adenine (1 uCi/ml). At the end of 5 hr following infection the 50 ml of $\text{NaH}^{14}\text{CO}_3$ labeled culture was added to 950 ml of the original infected culture and collected by centrifugation at 5,000 rpm for 10 min. The pellet was washed twice with saline-EDTA and terminated in 2.2 ml of the termination solution. At the end of 8 hr the 50 ml of the ^3H -adenine labeled culture was collected together with the remainder of the original infected culture, centrifuged, washed and terminated in 2.2 ml of the termination solution. A 0.1 ml sample of each of the two aliquots was then removed and the combination of these samples run on a CsCl density gradient as described in MATERIALS AND METHODS. This protocol was performed twice. The fractions were monitored for alkaline stable, acid precipitable radioactivity.

●—● ^3H -adenine for 5-8 hr
 ○—○ $\text{NaH}^{14}\text{CO}_3$ for 2-5 hr



separable peaks of material as would have been expected from the previous time course experiments. This experiment also confirmed that a large amount of material is labeled during the 2-5 hour period post infection and that this material preferentially incorporates ^{14}C -bicarbonate and bands at a lighter buoyant density in CsCl density gradients to that of the normal ^3H -adenine labeled phage DNA.

b) Purification of ^{14}C -PIL DNA and ^3H -adenine labeled DNA

The ^{14}C labeled (2-5 hours) material and the ^3H -adenine labeled (5-8 hours) material were fractionated separately on preparative CsCl density gradients under the standard conditions described in Materials and Methods. Fractions were collected from each gradient and 20 μ aliquots of each fraction used to monitor alkaline stable, acid precipitable radioactivity. Fractions containing radioisotope were pooled and after dialysis against three changes of 0.1 x SSC, the purity of the different DNA species was tested using their UV absorption properties. For both ^{14}C -labeled and ^3H -labeled materials, the ratio of O.D.260 nm/O.D.280 nm was 1.72.

Further purification was accomplished by resubjecting the ^{14}C and ^3H -labeled material to separate CsCl density gradients. The radioisotope incorporating fractions of ^{14}C and ^3H were determined and collected as above. After dialysis against 0.1 x SSC the ultraviolet absorption of the

two different samples was determined between 280 nm and 210 nm. The optical densities at different wavelengths are shown in Table 1. In ^3H -adenine incorporated DNA the ratio of O.D.260 to O.D.280 is 1.95 and the ratio of O.D.260 to O.D.230 is 1.91. In ^{14}C -labeled material the ratio of O.D.260 to O.D.280 is 1.94 and the ratio of O.D.260 to O.D.230 is 1.83.

c) Analysis of purified material

While base composition is the main property which determines the banding position of a DNA species in CsCl density gradients other factors can affect the buoyant density. Two of these factors which may be responsible for the PIL DNA properties on CsCl density gradients are: (a) the ^{14}C -incorporated material found in the lighter density region on the CsCl density gradients might be due to certain polysaccharides that band in CsCl in regions similar to the DNA of blue-green algae (40); or (b) the location might be due to some unusual properties of the DNA capable of shifting the DNA density on CsCl gradients, such as the presence of unusual bases (67).

To determine the biochemical properties of the ^{14}C -incorporated material and compare them with ^3H -adenine DNA, separate equal aliquots of ^{14}C -incorporated and ^3H -incorporated materials were respectively treated with RNase A (100 ug/ml, 0.75 hours, 37°C), DNase I (100 ug/ml, 1 hour, 37°C), protease (100 ug/ml, 2 hours, 50°C) and KOH hydrolysis (1 M,

TABLE I

Ultraviolet absorption of ^{14}C -incorporated material
and ^3H -adenine DNA in 0.1 x SSC
at different wavelengths

Wave- length	Optical density readings of ^{14}C -incorporated materials*	Optical density readings of ^3H -adenine DNA**
280	1.023	1.289
270	1.595	1.938
260	1.983	2.512
250	1.935	2.473
240	1.606	1.998
230	1.084	1.312
220	2.877	above 3
210	above 3	above 3

* For ^{14}C -incorporated species

$$A_{260}/A_{280} = 1.94$$

$$A_{260}/A_{230} = 1.83$$

** For ^3H -adenine DNA species

$$A_{260}/A_{280} = 1.95$$

$$A_{260}/A_{230} = 1.91$$

2 hours, 60°C). The treated and untreated samples were then precipitated with cold 20% TCA and monitored for radioactivity. The effects of these treatments on ^{14}C -incorporating material and ^3H -incorporating DNA are shown in Table 2.

In both ^{14}C - and ^3H -labeled samples there are no significant changes in the radioactive counts in RNase and protease digested samples or in the KOH hydrolyzed samples. However, both ^{14}C and ^3H -incorporated materials are sensitive to DNase digestion. After one hour at 37°C in DNase treatment, approximately 90% of the radioactive counts are no longer precipitable by TCA. These results indicate that both ^{14}C -incorporated material and the ^3H -adenine incorporated material known to be phage DNA are sensitive to DNase digestion but insensitive to all the other treatments. Thus the major components of both materials collected from CsCl density gradients is DNA. From these results the ^{14}C -incorporated material, which has a lighter relative buoyant density than ^3H -adenine DNA, is termed PIL-DNA (24).

To investigate the possibility that the PIL-DNA phenomenon is due to a density shift caused by DNA that is attached to proteins, lipids or lipoproteins, etc., the following experiments were performed: identical 10 $\times$ aliquots of a purified PIL-DNA sample were subjected to (a) protease treatment (100 ug/ml, 2 hours, 50°C); (b) lipase treatment (100 ug/ml, 1 hour, 37°C); (c) first protease treatment (100 ug/ml, 2 hours, 50°C), cooled, then lipase treatment (100 ug/ml,

TABLE II

The effects of various treatments on ^{14}C -incorporated material and ^3H -adenine incorporated material

Type of treatment***	Absolute level of incorporation (cpm)*	
	^{14}C -incorporated material**	^3H -adenine incorporated material**
DNase ¹	36.0	45.0
RNase ²	376.0	680.0
Protease ³	363.0	712.0
KOH ⁴	320.0	703.0
Control ⁵	402.0	728.0

* expressed as CPM of reaction mixture. The results represent average of three samples precipitated with cold 20% TCA

** 5 μ of ^{14}C -PIL-DNA and ^3H -adenine DNA was removed to do each treatment

*** 1. DNase 100 ug/ml 1 hr 37°C
 2. RNase 100 ug/ml 0.75 hr 37°C
 3. Protease 100 ug/ml 2 hr 50°C
 4. KOH 1M 2 hr 60°C
 5. Control sample was left untreated

1 hour, 37°C); (d) first lipase treatment (100 ug/ml, 1 hour, 37°C), cooled, then protease treatment (100 ug/ml, 2 hours, 50°C). The treated samples plus an untreated control were then ultracentrifuged on separate CsCl density gradients. In each gradient a 10 λ aliquot of the purified ^3H -adenine DNA was used as a standard buoyant density marker. The banding profiles obtained from each of the CsCl gradients for all the treated samples were virtually identical to those obtained for the untreated samples. The relative positions of the ^3H and ^{14}C -incorporated peaks on the CsCl gradient are identical to those in Figure 7. Whether enzymatically treated or untreated, the PIL-DNA has a lighter density on CsCl than the ^3H -adenine DNA. These experiments would seem to suggest that PIL-DNA contains only pure DNA, and its density shift is unrelated to the attachment of other macromolecules.

From all the experiments conducted so far, the following conclusions may be reached: The preparative amount of ^{14}C -PIL-DNA and ^3H -adenine phage DNA can be isolated from CsCl density gradients and purified by recentrifugation on CsCl density gradients. The purified PIL-DNA and ^3H -phage DNA are DNase sensitive, but protease and RNase have no effect. Both purified DNAs have a relatively high ratio of O.D.₂₆₀ to O.D.₂₈₀. The purification procedures were not responsible for the density shift of the PIL-DNA (data not presented). These preparatively collected ^{14}C -PIL-DNA and ^3H -phage DNAs will be utilized for future investigation.

B. Other Analytical Methods: Poly-L-lysine Kieselguhr Chromatography for Studying the DNA Pattern of the AS-1/Anacystis nidulans Infective System

Nucleic acids have been shown to bind to the poly-L-lysine coated kieselguhr (PLK) columns and in turn to be eluted by an increasing gradient of buffered saline. The column fractionates DNA primarily on the basis of base composition and to a lesser extent on other parameters (16). Poly-L-lysine kieselguhr has also been shown capable of removing nucleic acids selectively from the supernatants of mechanically disrupted cells which have undergone no prior purification (23). These properties make PLK chromatography one of the best methods for preparative collection of DNA and the analysis of the changing DNA patterns, largely because of the rapidity and simplicity with which crude cell lysates may be analyzed following their infection with cyanophage AS-1.

1) Characterization of Anacystis nidulans DNA and Cyanophage AS-1 DNA on PLK columns

In order to characterize the fractionating properties of PLK columns with regard to Anacystis nidulans DNA, exponentially growing cells were broken mechanically, the supernatant of a crude cell lysate was loaded onto a standard PLK column, washed with 0.4M buffered saline and eluted with a linear gradient of 0.4-4.0 M buffered saline (100 ml of each). Fractions (40 drops, 1.5-2.0 ml) were collected and monitored for u.v. absorbance at 260 nm. The elution

profile obtained is shown in Figure 8. A large amount of ultraviolet-absorbing material was not retained by the column and was either unabsorbed or washed off with the initial 0.4 M buffered saline wash. Elution by the salt gradient distinguished several peaks of ultraviolet-absorbing material, starting at about 0.8 M NaCl (refractive index 1.3260) and continuing until about 2 M NaCl (refractive index 1.3360).

In order to identify the DNA material in these regions crude lysates of Anacystis nidulans were either treated with RNase or their DNA was extracted and purified by a modified Marmur procedure. The crude lysates and purified DNA were then analyzed separately on standard PLK columns. The elution profile from RNase-treated lysates (Figure 9) indicates that only one peak, the one eluting at about 1.9 M NaCl, is RNase-resistant; this material is classified as DNA. Similar findings were also obtained using purified Anacystis nidulans DNA. Therefore, the mechanically broken crude lysates, treated with RNase and washed with excess 0.4 M buffered saline were used for further analysis of DNA by PLK.

The fractionating properties of cyanophage AS-1 DNA were also characterized by PLK column chromatography. Cyanophage AS-1 can not be easily broken mechanically, nor may detergent be used since it has been reported (23) that small traces of detergent eliminate the binding of nucleic acids to PLK. Therefore, the DNA was collected and purified from cyanophage AS-1 according to Marmur's procedure. ³H-adenine

Figure 8. The Elution Profile from a Poly-L-lysine Kieselguhr Chromatograph (PLK) of Nucleic Acids of Anacystis nidulans

The exponentially growing A. nidulans cultures were centrifuged at 5,000 rpm for 10 min, washed twice, resuspended in 10 ml saline-EDTA, and then put into a Braun Homogenizer vessel containing 37 g of 0.5 mm glass beads. The cells were broken by a Braun Homogenizer for 60 sec. Following collection of the samples the crude lysates were ready for analysis on PLK columns. The poly-L-lysine kieselguhr column materials were prepared and packed as described in MATERIALS AND METHODS. The loaded columns were washed free of unbound material using 15 ml of 0.4 M buffered saline. The flow rate of the linear gradient of 0.4-4.0 M buffered saline used for the elution was maintained at 20 ml/hr by a multichannel peristaltic pump and fractions of 40 drops each were collected. The fractions were monitored for UV-absorbance at 260 nm. The linearity of the salt gradient was checked by a refractometer. The salt concentration of the individual fraction was determined by comparison with the standard solution.

△—△ A₂₆₀
□—□ refractive index

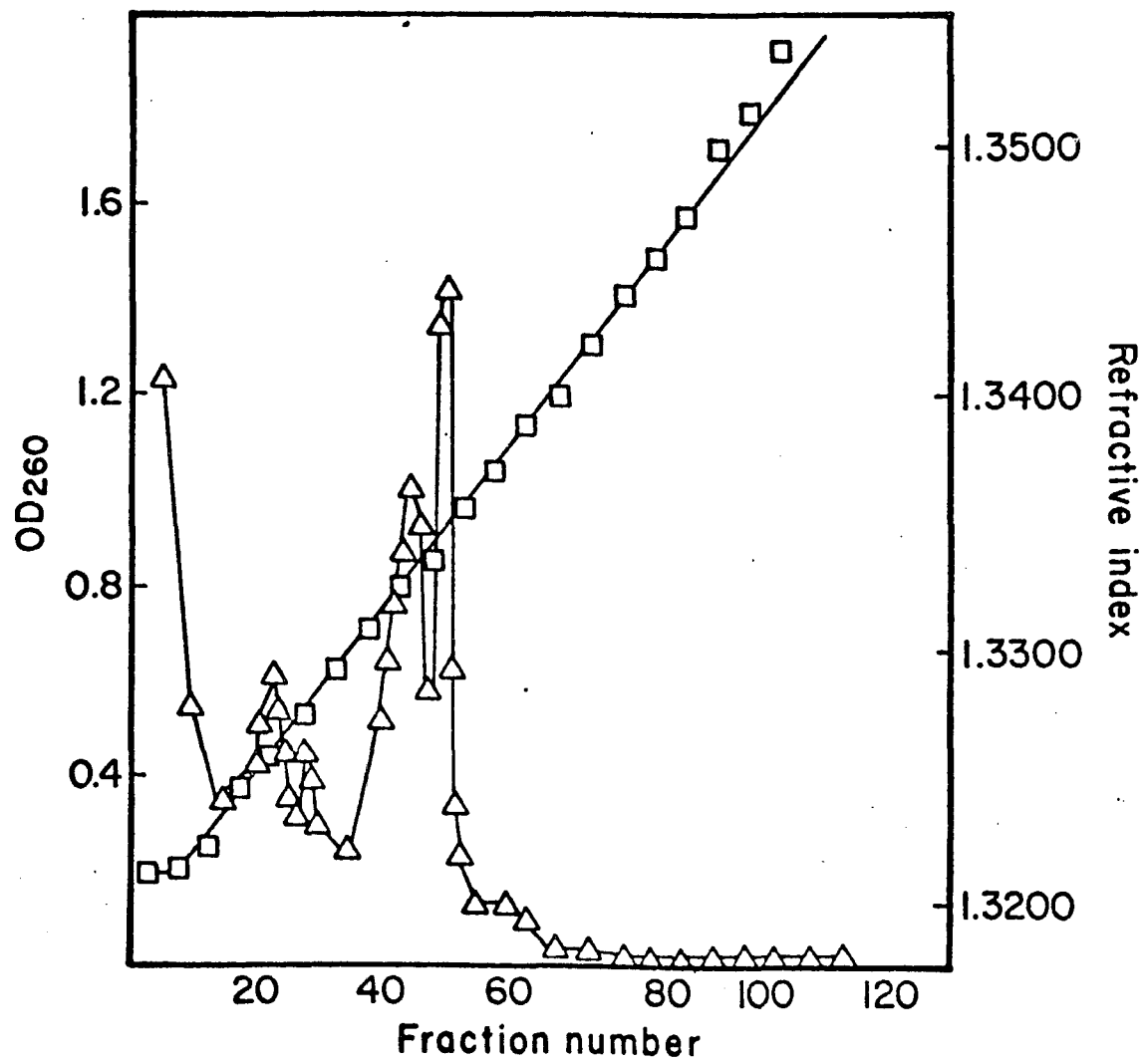
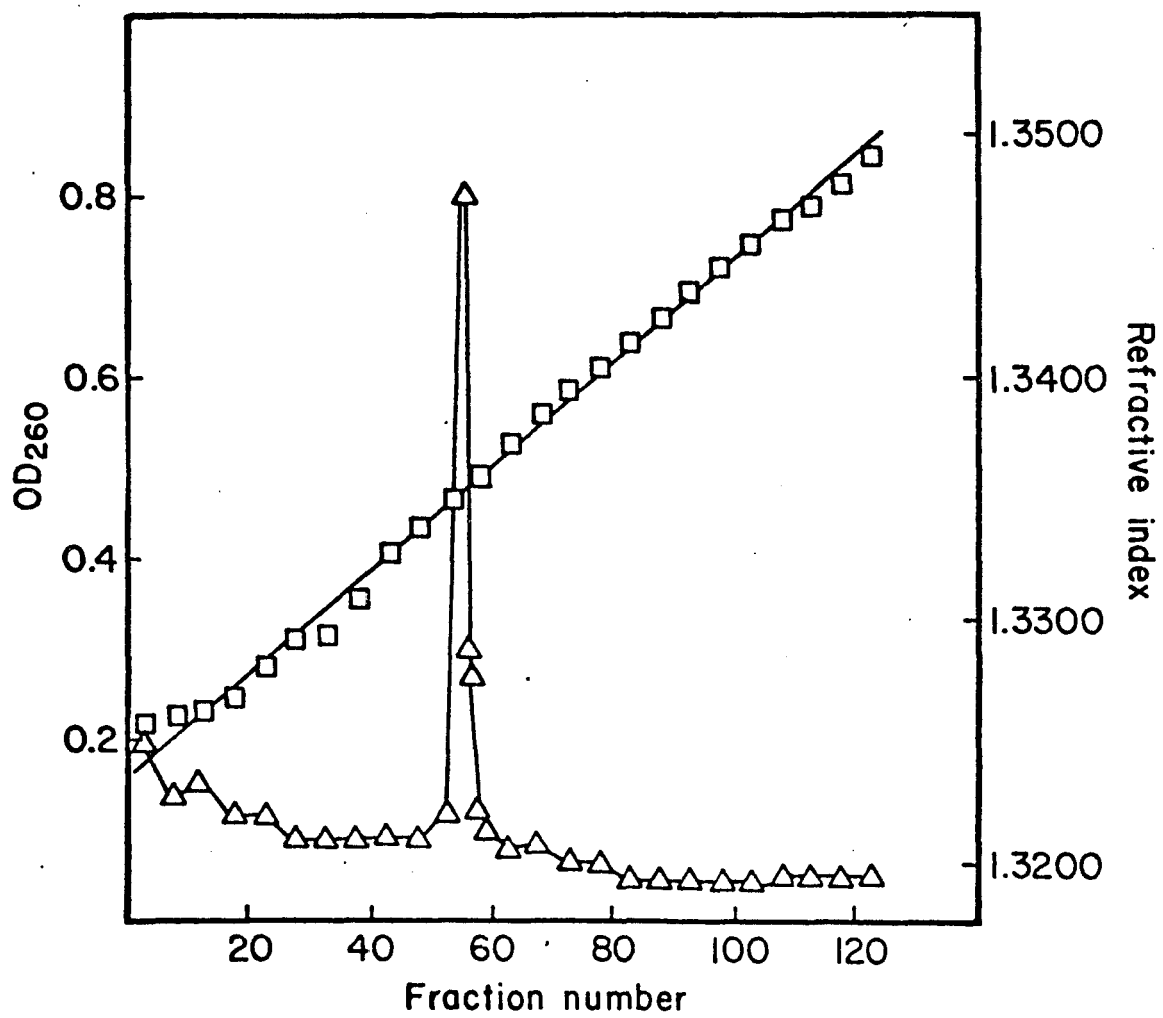


Figure 9. The Elution Profile from a PLK Column of Deoxyribonucleic Acid from Anacystis nidulans

The exponentially growing A. nidulans cultures were collected and broken by the Braun Homogenizer as described in the legend to Figure 8. The crude lysates were treated with excess RNase (200 ug/ml) for 0.75 hr at 37°C. The RNase-treated lysates were analyzed on PLK columns. The experimental protocols were as described in MATERIALS AND METHODS, and the Figure 8 legend. The fractions were monitored for UV-absorbance at 260 nm and the linearity of the gradient was checked by refractive measurements of each fraction.

△—△ A₂₆₀
□—□ refractive index



labeled phage DNA was prepared, mixed with a known amount of carrier calf thymus DNA (used also as a standard) and fractionated on a standard PLK column. The fractions containing calf thymus DNA were detected using ultraviolet absorbance at A₂₆₀ and the presence of cyanophage DNA in each fraction determined by ³H-adenine incorporation. Figure 10 shows two distinct peaks eluting at different salt concentrations. The UV-absorbing material elutes at the lower salt concentration of about 1.7 M (1.3335 ± 0.0003), characteristic of calf thymus DNA (with a buoyant density of 1.699 g/cm³). A second radioactive band of DNA elutes at a higher salt concentration of about 1.9 M (1.3355 ± 0.0005) characteristic of AS-1 DNA (with a buoyant density of 1.715 g/cm³) (126).

The elution profile of cellular DNA (Figure 9) and cyanophage AS-1 DNA (Figure 10) may thus be compared. Both Anacystis nidulans DNA and AS-1 DNA are eluted from the column sharply with no satellite DNA found on either side of the peaks. The concentration of the buffered saline which elutes both DNAs is very similar. Both DNAs are eluted at about 1.9 M NaCl. These results imply that the DNAs of Anacystis nidulans and AS-1 are very similar with regard to base composition and agree with the results reported by Safferman (126).

From these results it can be seen that DNA species which have similar buoyant densities and thus similar base compositions cannot be resolved on PLK columns, whereas DNAs such as calf thymus and AS-1, which differ in both buoyant

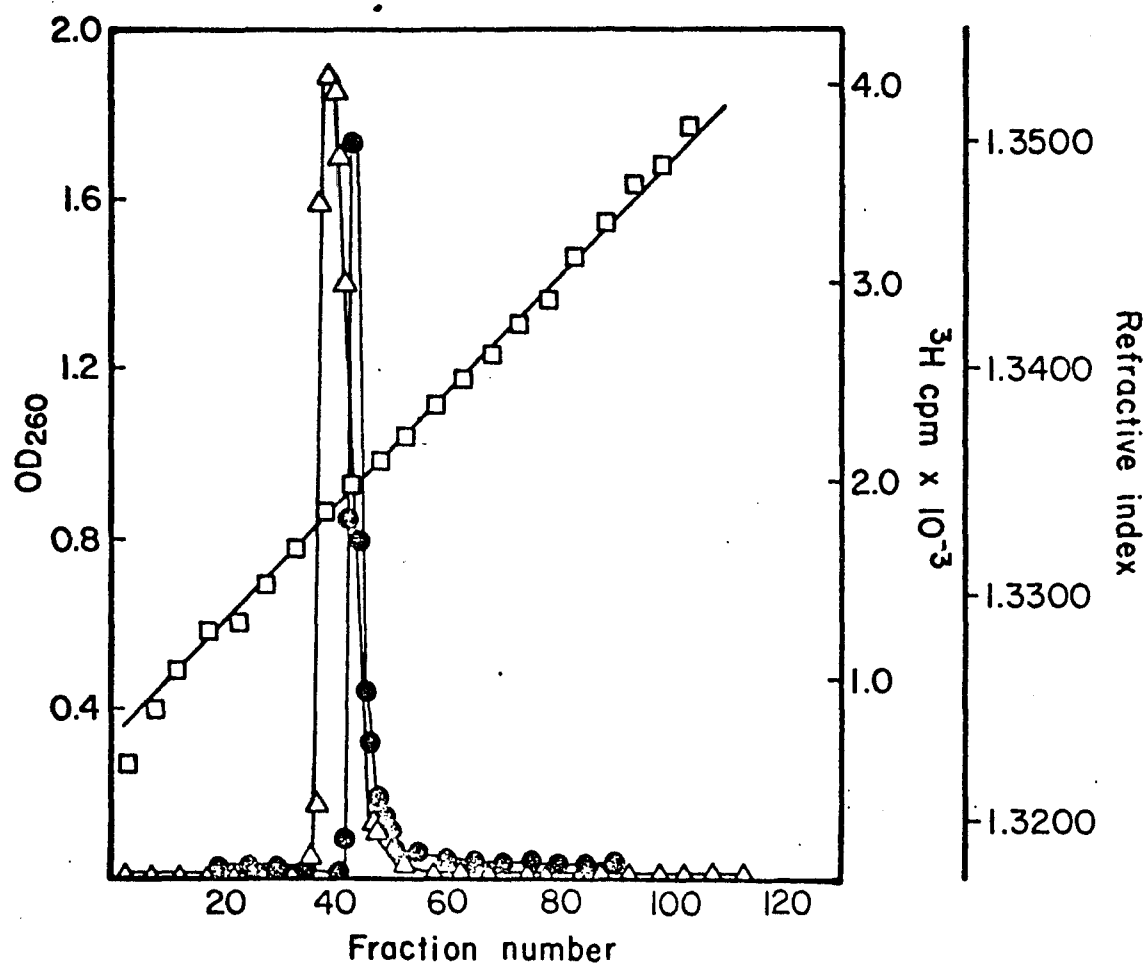
Figure 10. The Elution Profile from a PLK Column of Purified DNA of Cyanophage AS-1

Synchronously grown A. nidulans cultures were infected with cyanophage AS-1 (MOI=5). ^3H -adenine (1 uCi/ml) was added to the infected culture. At 24 hr following infection the ^3H -adenine labeled viral lysates were clarified by centrifugation at 10,000 rpm for 10 min and then pelleted by ultracentrifugation at 25,000 rpm for 1 hr. The pelleted viruses were resuspended in TM buffer (10 mM Tris, 250 mM NaCl and 0.1 mM EDTA). DNase (100 ug/ml) was added for 1 hr at 37°C to digest the DNA fragments left in the buffer solution outside the viruses and the samples were then dialyzed against TM buffer overnight at 4°C. Sarkosyl detergent (10%) was added to the dialyzed virus solution at 60°C for 15 min to lyse the virus. The DNA was extracted by using a modified Marmur procedure as described in MATERIALS AND METHODS. Aliquots of 10 of purified ^3H -adenine phage and 0.5 ml of calf thymus DNA (1 mg/ml) were applied together onto a PLK column. The experimental procedures were identical to those described in MATERIALS AND METHODS and the Figure 8 legend.

Figure 10 continued

The linearity of the gradient was checked by refractive index measurements. The fractions were monitored for calf thymus DNA at 260 nm and the radioactivity of fractions containing the ^3H -phage DNA was determined as described in "Preparation of CsCl Density Gradients" and in MATERIALS AND METHODS.

●—●	^3H -adenine
△—△	A_{260}
□—□	refractive index



density and base composition can be separated. The PLK column therefore provides an independent measure of whether or not differences in buoyant density truly reflect differences in base composition, or whether the difference in buoyant density between two species of DNA is due to some other factor such as base modification (see Introduction). If two DNA species show different buoyant densities but elute from PLK columns at the same salt molarity, then it is possible that both of them have the same or very similar base compositions but that one of them is showing some form of modification.

PIL-DNA has a buoyant density of 1.701 g/cm^3 , very different to that of A. nidulans and AS-1 DNA with buoyant densities of 1.715 g/cm^3 . If the elution salt molarity of PIL-DNA from PLK columns also differs significantly from that of the host and phage DNAs then it would be reasonable to suspect that this difference in buoyant density was due to base compositional differences. If, however, the PIL-DNA eluted from a PLK column at the same salt concentration as the host and phage DNAs then the implication would be that PIL-DNA has the same base composition as the host and phage, but also contained some type of modification.

- 2) The DNA Incorporation Patterns for the Radioactive Precursors into Whole Cell DNA during the Lytic Cycle of the AS-1/A. nidulans System as Analyzed by PLK Chromatography.

Cyanophage infected synchronized cell cultures were

pulse-labeled with ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ at 2.5-4.5 hours and at 6-8 hours following infection. The uninfected cells were used as controls for the pulse-labeled samples at both time points. At the end of each pulse-labeling period, the infected or uninfected cells were collected by centrifugation, washed, and resuspended in saline EDTA. They were then broken in a Braun homogenizer, treated with RNase and applied to a PLK column. After washing the column with excess 1 M buffered saline, the samples were eluted with a 1-2.5 M salt gradient (commercial calf thymus DNA was used as a standard). It has been reported (23) that the resolution of DNA elution from PLK columns may be increased by altering the shape of the salt gradient. The standard 0.4-4.0 M buffered saline gradient was therefore replaced by a shallower gradient of 1.0-2.5 M in these and future experiments.

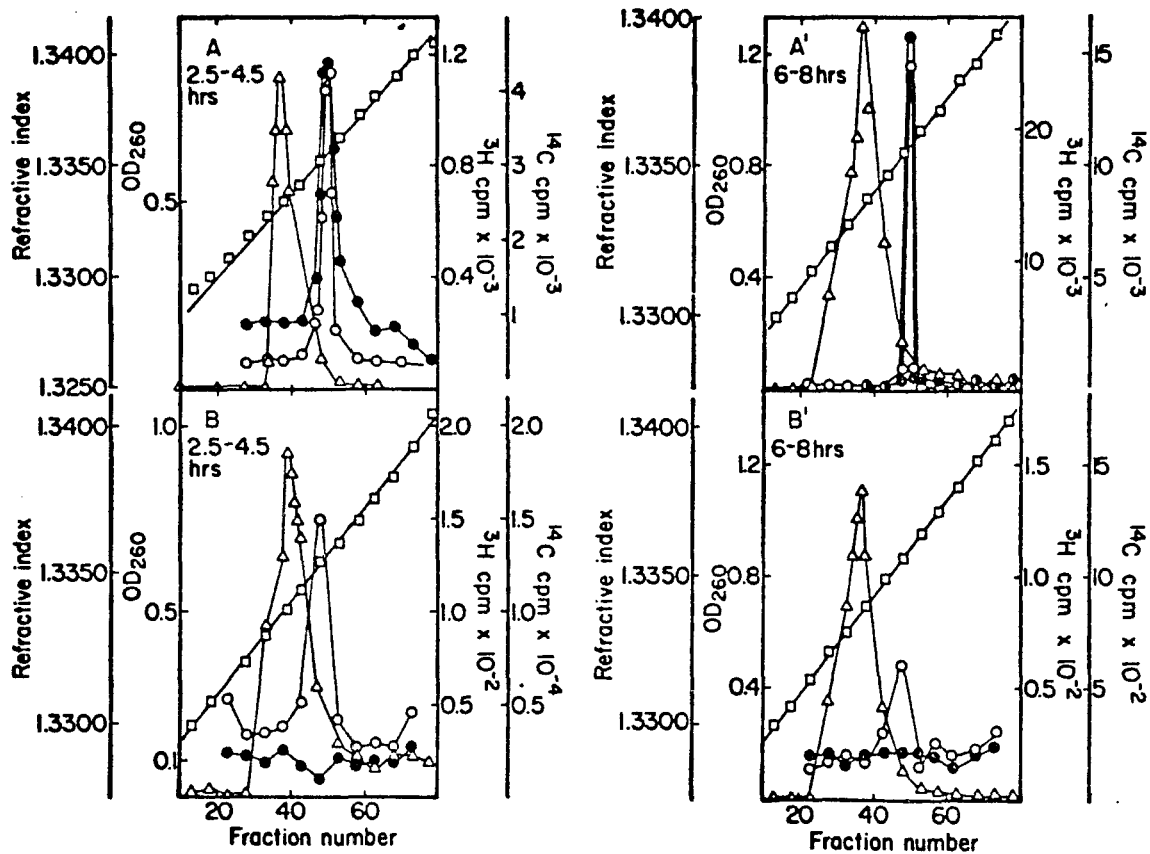
Figure 11 and Figure 12 demonstrate that by using the shallower gradient (1.0-2.5 M) the resolution between the calf thymus DNA marker and radioactivity incorporated DNA has been improved. In the uninfected (control) samples there has been no significant amount of ^3H -adenine incorporation in either the 2.5-4.5 hours or the 6-8 hours pulse-labeled periods. This demonstrates the impermeability of the Anacystis nidulans cells to ^3H -adenine (24,71). There is, however, significant incorporation of ^{14}C in both the 2.5-4.5 hours and 6-8 hours infected samples. Although there is slight incorporation of ^3H -adenine in the 2.5-4.5 hours samples, it is in the latter period of infection (6-8 hours)

Figure 11. The Elution Profile from a PLK Column of Extracts from Anacystis nidulans Infected with AS-1 (Panel A) and Uninfected A. nidulans (Panel B), Pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine at 2.5-4.5 Hours and 6-8 Hours.

From a synchronized A. nidulans culture half the volume was removed and infected with cyanophage AS-1 (MOI=5), with the other half remaining uninfected. At 2.5 hr ^3H -adenine (1 uCi/ml) and $\text{NaH}^{14}\text{CO}_3$ (1 uCi/ml) were added to half of the infected and uninfected cultures. At 6 hr ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ were added to the remaining infected and uninfected cultures. After 4.5 hr and 8 hr the cells in the cultures were broken in the Braun Homogenizer as described in the legend to Figure 8. The crude lysates were treated with excess RNase (200 ug/ml) for 0.75 hr at 37°C. The RNase-treated lysates were then combined with 0.5 ml calf thymus DNA (1 mg/ml), which served as an A_{260} marker, and analyzed by PLK column chromatography individually. The experimental protocols were similar to those described in MATERIALS AND METHODS and the Figure 8 legend, except that the loaded columns were washed free of unbound material using 15 ml of 1.0 M buffered saline, and the DNA was eluted by linear gradients of 1.0-2.5 M buffered saline. The linearity of the gradients was checked by refractive index measurements. The fractions were monitored for calf thymus DNA at OD_{260} . The radioactivity of each fraction was determined as described in "Preparation of CsCl Density Gradients."

- Panel A Anacystis nidulans infected with AS-1 and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine at 2.5-4.5 hr.
- Panel A' As above, except pulse-labeling for 6-8 hr.
- Panel B Uninfected A. nidulans pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine at 2.5-4.5 hr.
- Panel B' As Panel B, except pulse-labeling for 6-8 hr.

●—●	^3H -adenine
○—○	^{14}C
△—△	A_{260}
□—□	refractive index



that the incorporation of ^3H -adenine increases dramatically. This phenomenon is similar to the results from the CsCl gradients: ^3H -adenine incorporation is slow at the early times post infection, while a significant amount of ^3H -adenine is rapidly incorporated into DNA in the later time periods.

The elution profiles of radioactivity for ^{14}C and ^3H -adenine are very similar in both 2.5-4.5 hours and 6-8 hours pulse-labeled infected cells. The ^{14}C and ^3H -adenine are eluted at the same salt concentration, 1.9 M, which is the concentration used to elute both phage and cell DNA.

When the 2.5-4.5 hours pulse-labeled samples were analyzed on CsCl density gradients, a phage-induced light peak having a lighter density than cell/phage DNA was found. However, on PLK columns, no distinct peak of ^{14}C -incorporated DNA is found to exist between the calf thymus DNA marker and ^3H -adenine at 2.5-4.5 hours post infection.

In a second series of experiments a large infected culture of Anacystis nidulans was prepared. At the end of 5 hours post infection, half of the volume of the culture was combined with a smaller $\text{NaH}^{14}\text{CO}_3$ pulse-labeled (2-5 hours) culture, collected and broken mechanically. At 8 hours post infection the remainder of the original infected culture was combined with a second smaller ^3H -adenine pulse-labeled (5-8 hours) culture, collected and disrupted in the Braun homogenizer. The two crude lysates were combined and fractionated on a PLK column. Figure 12 indicates that all the different

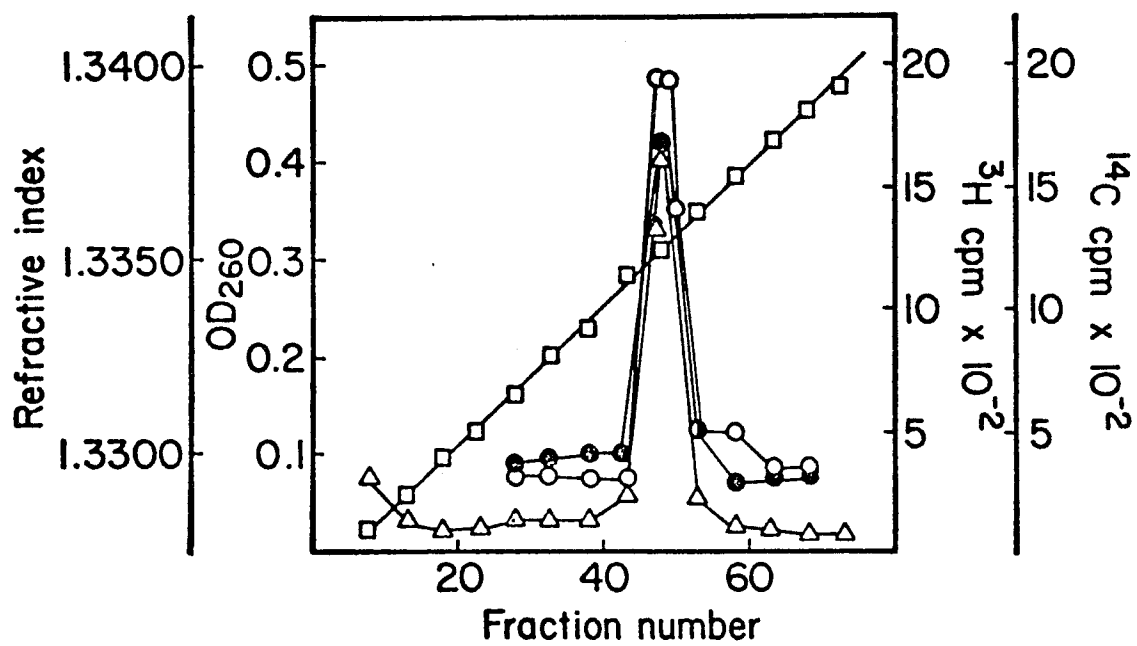
Figure 12. The Elution Profile from a PLK Column of the Combined Extracts from Identically Infected Anacystis nidulans Cultures Separately Pulsed with either $\text{NaH}^{14}\text{CO}_3$ for 2.5-5 Hours or ^3H -adenine for 5-8 Hours

An AS-1 viral lysate was added to a large synchronized algal culture (MOI=5) to a final volume of 1 liter. Two and a half hours after infection 50 ml of the culture was removed and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ (1 uCi/ml). Five hours after infection the $\text{NaH}^{14}\text{CO}_3$ labeled culture was terminated together with 400 ml of the original culture by breaking the cells with the Braun Homogenizer. Five hours after infection another 50 ml of the large infected culture was removed and radioactively labeled with ^3H -adenine (1 uCi/ml). At 8 hr post infection the ^3H -adenine labeled culture was terminated together with the rest of the original culture by breaking the cells with the Braun Homogenizer. The ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ labeled lysates were combined and treated with excess RNase (200 ug/ml) for 0.75 hr at 37°C . The RNase treated mixture was then analyzed on a PLK column as described in MATERIALS AND METHODS, and the DNAs eluted by a linear gradient of 1.0-2.5 M buffered saline.

Figure 12 continued

The linearity of the gradient was checked by refractive index measurements. The fractions were monitored for UV-absorbance at OD₂₆₀, ³H-adenine incorporation and ¹⁴C incorporation. The radioactivity of the fractions was determined as described in "Preparation of CsCl Density Gradients."

●—●	³ H-adenine
○—○	¹⁴ C
△—△	A ₂₆₀
□—□	refractive index



infected cell DNA species, as monitored by UV absorbance at 260 nm, ^{14}C -incorporating DNA at 2-5 hours of infection, and ^3H -incorporating DNA at 5-8 hours of infection are eluted at the same salt concentration (1.9 M) which is the concentration for cell and phage DNA elution. Therefore, using the PLK column, there is no spectrophotometrically measurable separate peak of the PIL-DNA at 2-5 hours of infection, although its existence is evident by CsCl density gradient centrifugation.

The results of Figures 11 and 12 indicate that PLK column analysis can not resolve the PIL-DNA from the cell/phage DNA during the early hours of the infection cycle.

3) Analysis of ^{14}C -PIL-DNA combined with purified Anacystis nidulans DNA on PLK column

Since termination solution and detergent (2% sarkosyl) were both used during the preparation of crude cell lysates for CsCl gradient analysis, a different protocol was required for crude lysate preparation for PLK fractionation. Use of the Braun homogenizer to mechanically break the cells was therefore employed, since the possibility exists that the different elution profiles of the two nucleic acids from PLK columns may be due to different treatments of the lysates. The mechanically broken crude lysates of dual pulse-labeled infected cultures (^3H -adenine and ^{14}C at 2-5 hours and 6-8 hours) were analyzed by CsCl density gradient ultracentrifugation. The banding profiles are very similar to those

obtained from CsCl gradients described in the previous section (Figure 3). A distinct ^{14}C -incorporated PIL-DNA exists in the 2-5 hours post infection.

The Anacystis nidulans DNA was extracted and purified from an exponentially growing culture according to Marmur's procedure. A synchronized cell culture was infected by AS-1 and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ during the 2-5 hour interval following infection. The ^{14}C -PIL-DNA was collected at 5 hours and purified by a CsCl density gradient. The purified DNA was then dialyzed to remove the salt and precipitated with two volumes of cold ethanol. The precipitated samples was dissolved in 0.1 x SSC and dialyzed against three changes of 0.1 x SSC. The purified ^{14}C -PIL-DNA and A. nidulans DNA were then loaded together on a PLK column.

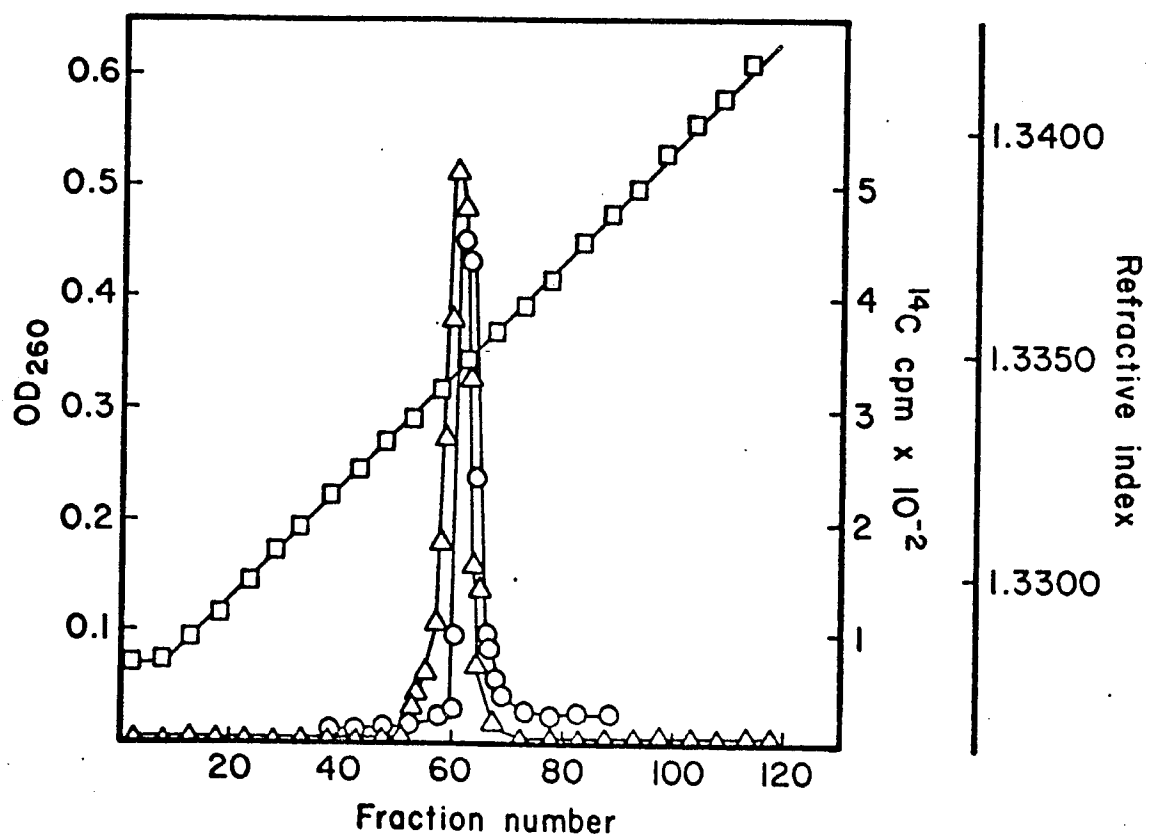
The elution profile for A. nidulans DNA, which is monitored by UV absorbance at A_{260} , and ^{14}C -PIL-DNA, which is monitored for radioactivity of ^{14}C , are shown in Figure 13. It is clear that both purified DNAs elute at an almost identical salt concentration. There is no resolution between the ^{14}C -PIL-DNA and A. nidulans DNA. The total recovery for both DNAs from one PLK column is about 90%. This high recovery indicates that purified DNA will bind with poly-L-lysine and elute at a salt concentration appropriate with their G + C content.

From all the experiments discussed above, there appears to be no distinction between ^{14}C -incorporating DNA

Figure 13. The Elution Profile from a PLK Column of Separately Purified and then Combined Anacystis nidulans DNA and ^{14}C -PIL-DNA

The A. nidulans DNA was extracted from an exponentially growing culture according to a modified Marmur procedure as described in MATERIALS AND METHODS. The crude lysate of ^{14}C -PIL-DNA was collected from a synchronized A. nidulans culture that had been infected with AS-1 and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ (1 $\mu\text{Ci}/\text{ml}$) for 2-5 hr and then terminated by the termination solution for 12 hr at 37°C . The ^{14}C -PIL-DNA was identified on and collected from a CsCl density gradient and was then purified on another CsCl density gradient. The procedures were described in detail in "Preparative Collection of ^{14}C -PIL-DNA." One ml (150 $\mu\text{g}/\text{ml}$) of purified A. nidulans and 50 λ of ^{14}C -PIL-DNA were applied together onto a PLK column as described in MATERIALS AND METHODS. The DNAs were eluted by a linear gradient of 1.0-2.5 M buffered saline. The linearity of the gradients was checked refractometrically. The fractions were monitored for A. nidulans DNA at OD_{260} . The radioactivity of the fractions was determined as described previously.

○—○	^{14}C
△—△	A_{260}
□—□	refractive index



and ^3H -adenine incorporating DNA in this system. Both radioactivities are eluted at the same or very similar salt concentrations. PLK chromatography would normally be expected to resolve the cell/phage DNA from that of ^{14}C -PIL-DNA if their widely divergent buoyant densities on a CsCl gradient were due to a difference in base composition. The inability of the PLK column to resolve the cell/phage DNA and PIL-DNA indicates a probable similarity in their base ratios. Thus the lighter buoyant density of PIL-DNA might instead be due to a post infection modification of either cell DNA, phage DNA, or some other species.

III. The Study of Phage Induced Lighter Density DNA (PIL-DNA)

A. Characterization of PIL-DNA

PIL-DNA was characterized by the determination and analysis of thermal denaturation patterns and base composition of Anacystis nidulans, phage AS-1 and PIL-DNA.

1) DNA Thermal Denaturation

The data presented in Figure 14 shows the results of an experiment designed to determine the thermal denaturation profile of PIL-DNA. The sharpness of the transition profile would seem to indicate that the major nucleic acid species of PIL-DNA is double-stranded DNA. The temperature of the midpoint of the transition (T_m) is 76.3°C in $0.1 \times \text{SSC}$.

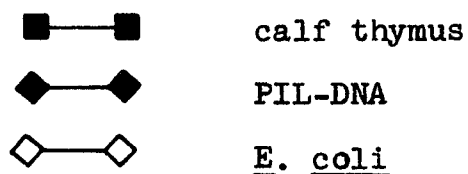
E. coli and calf thymus DNA were used as two known T_m markers. The T_m s of E. coli and calf thymus in $0.1 \times \text{SSC}$ were found to be 73.7°C and 70.5°C respectively. The hyperchromicity value at 260 nm is $35 \pm 5\%$.

Using the equation derived by Mandel and Marmur: $\text{GC} = (T_m - 53.9) 2.44$ (130), the T_m s of PIL-DNA, calf thymus and E. coli with this solvent system correspond to a G-C content of 54.6%, 40.5%, and 48.3% respectively.

The thermal denaturation profiles of PIL-DNA, phage AS-1 DNA and Anacystis nidulans DNA are compared in Figure 15. The melting temperature of PIL-DNA, phage AS-1, and A. nidulans DNA are 76.3°C , 76°C and 76.7°C respectively. A

Figure 14. Thermal Denaturation of PIL-DNA

PIL-DNA was collected from AS-1 infected cells after 5 hr of infection and purified by preparative ultracentrifugation in CsCl as described in MATERIALS AND METHODS. The purified DNA was diluted with 0.1 x SSC and dialyzed against three changes of 0.1 x SSC to achieve a final concentration of 50 ug/ml. It was then transferred into a stoppered silica cuvette. 50 ug/ml of both calf thymus and E. coli DNA, prepared by a modification of Marmur's method described in MATERIALS AND METHODS and dialyzed against the same 0.1 x SSC, were placed in the second and third silica cuvettes. These three samples, along with a fourth cuvette containing only 0.1 x SSC were placed in a Beckman Acta CIII spectrophotometer. The program of temperature increase (1°C/5 min) was achieved using a Haake circulating bath containing polyethylene glycol. The absorbance at 260 nm was recorded and the absorption values were subsequently corrected for the volume expansion of the liquid. From the comparison of the melting profile of the PIL-DNA and the known T_m of calf thymus E. coli DNA, the T_m of PIL-DNA in 0.1 x SSC was determined.



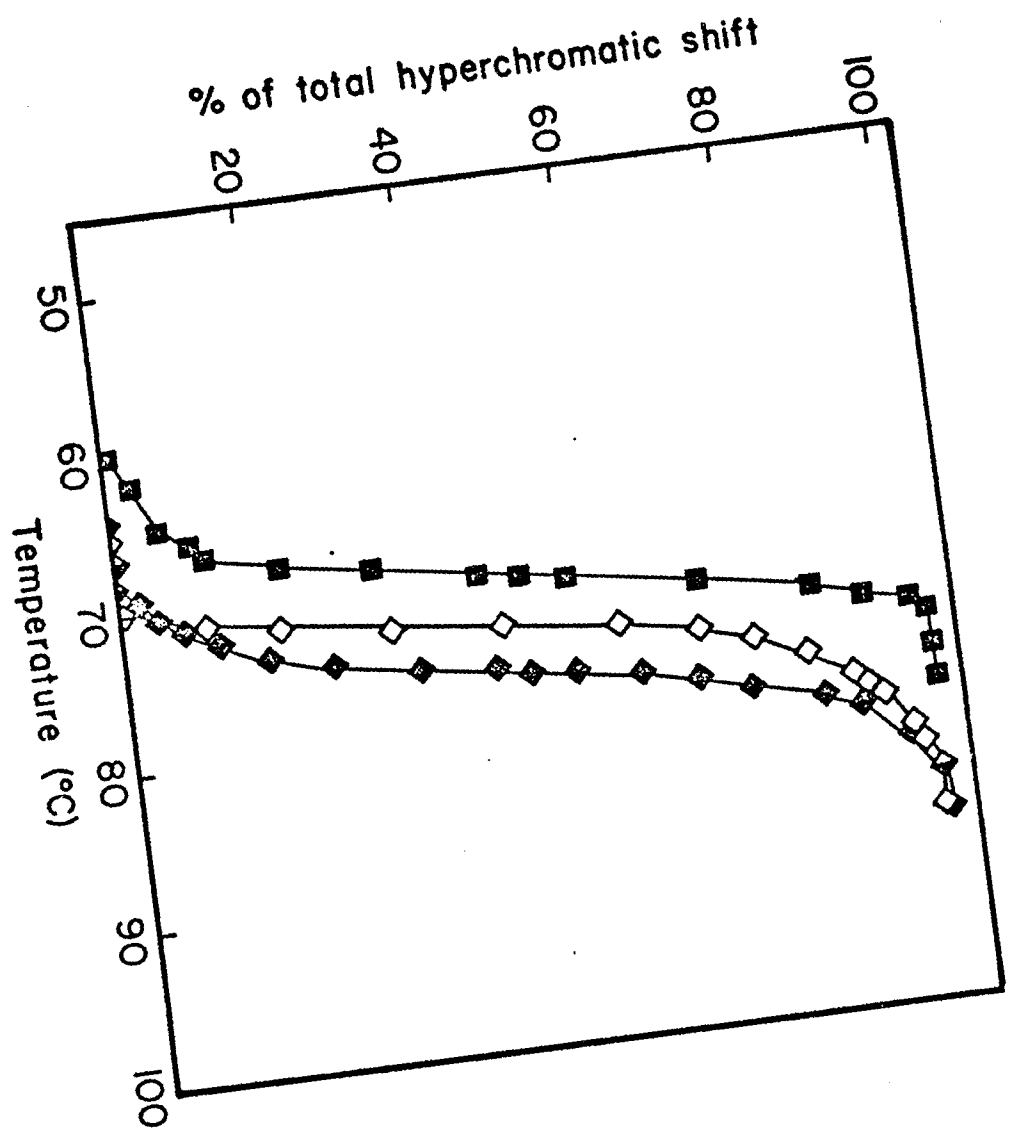
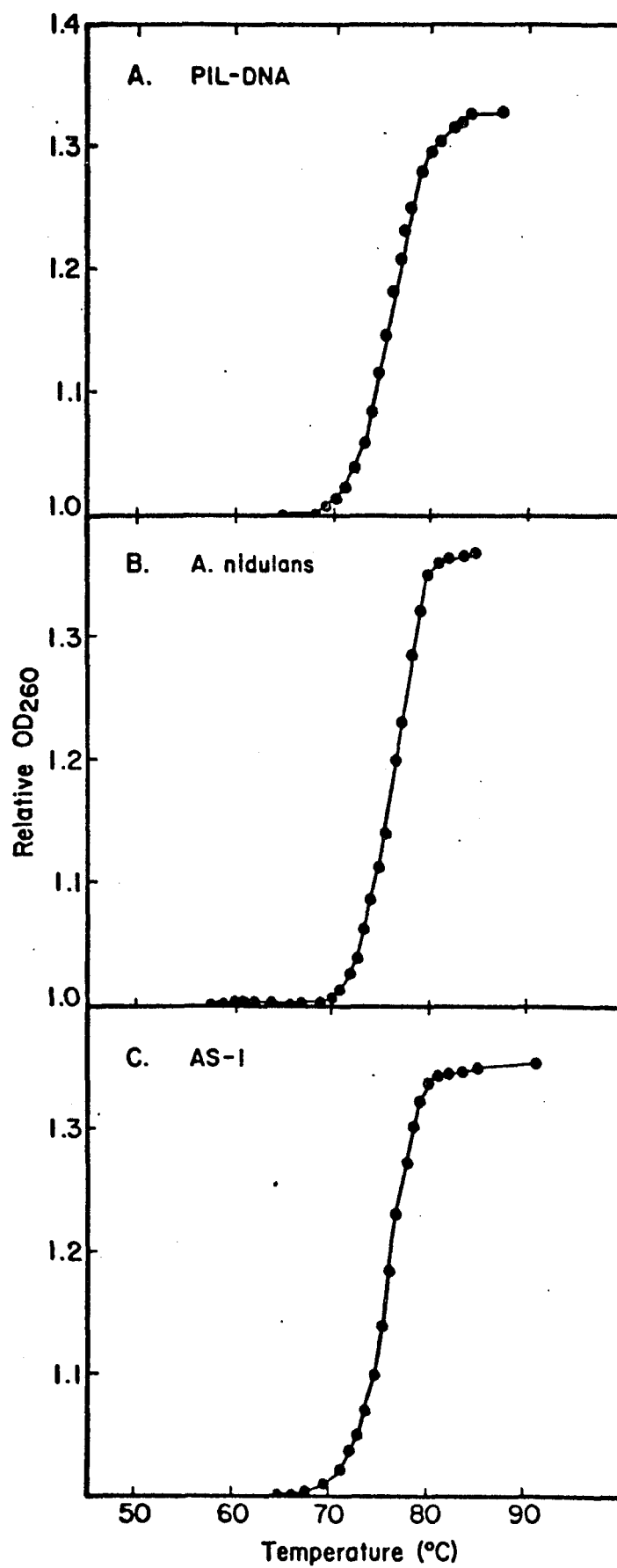


Figure 15. Comparison of the DNA Thermal Denaturation Patterns of PIL-DNA with those of Anacystis nidulans and Phage AS-1

Preparation of purified PIL-DNA, Anacystis nidulans DNA, and phage AS-1 DNA was described in detail in MATERIALS AND METHODS. These three samples, along with a blank (0.1 x SSC buffer) were placed in four identical cuvettes. The experimental protocol followed that of Figure 14. The melting profiles of the PIL-DNA, A. nidulans DNA, and AS-1 DNA are compared.

- Panel A thermal denaturation of PIL-DNA
- Panel B thermal denaturation of A. nidulans
- Panel C thermal denaturation of cyanophage AS-1



very similar T_m is obtained for each of these three different DNAs. The transition midpoint range corresponds to a G-C content of $54 \pm 1\%$ using the above equation. Calculating from the Schildkraut et al. formula, PIL-DNA with a buoyant density of 1.701 ± 0.001 g/ml should have a G-C content of $42 \pm 1\%$ (24). These results would tend to confirm the conclusions gained from the PLK fractionation described previously. The discrepancy in G-C content of PIL-DNA as determined from the buoyant density and PLK chromatography or thermal denaturation patterns may be explained by a modification of the DNA. Perhaps a large amount of an unusual base is present in PIL-DNA (61). Modifications of DNA such as methylation and glucosylation which cause a discrepancy between the melting-out data and the buoyant density data have been observed for many bacteriophages (56b).

2) Base Composition - Direct Determination

The previously used procedures of isopycnic centrifugation, PLK column chromatography, and thermal denaturation are all indirect measurements of base composition. Given a discrepancy among the results from these techniques, such as described above, it is necessary to utilize a procedure that can give a more direct indication of the presence of any anomalous components. Such a procedure is the hydrolysis of DNA into its monomeric constituents (either mononucleotides or free bases) and their complete separation and identification by thin layer chromatography.

The results from the application of this technique are presented in Figure 16. Mononucleotide analysis was performed by hydrolyzing each DNA species with pancreatic DNase and snake venom phosphodiesterase followed by resolution using two dimensional thin layer chromatography. The developed chromatogram was inspected for the presence and position of UV-absorbing spots and compared with the appropriate mononucleotide standards (dAMP, dCMP, dGMP and dTMP).

From all of the three samples (A. nidulans, A8-1 and PIL) four clear spots are discernible whose relative mobilities are coincident with those of the standards. Thus, it is possible to conclude that the four major DNA bases are present in these three DNAs. However, for PIL-DNA additional weakly UV-absorbing spots were also observed. Unfortunately the level of UV absorption in these regions is very low and unambiguous identification is proving difficult.

Another way of visualizing the separated nucleotides on a TLC plate is to use autoradiography. For these types of studies radioactive labeling of the PIL-DNA with ^3H -adenine is impractical since the evidence of the time course experiments described earlier indicated that this precursor is not incorporated during the 2-5 hour period post infection. However, ^{14}C -bicarbonate is an efficient label of this DNA species, so PIL-DNA- ^{14}C was taken and analyzed.

Two results were obtained, one of which was somewhat unexpected (see Figure 18A). First, all the nucleotides resolved on the TLC plate showed ^{14}C incorporation. This

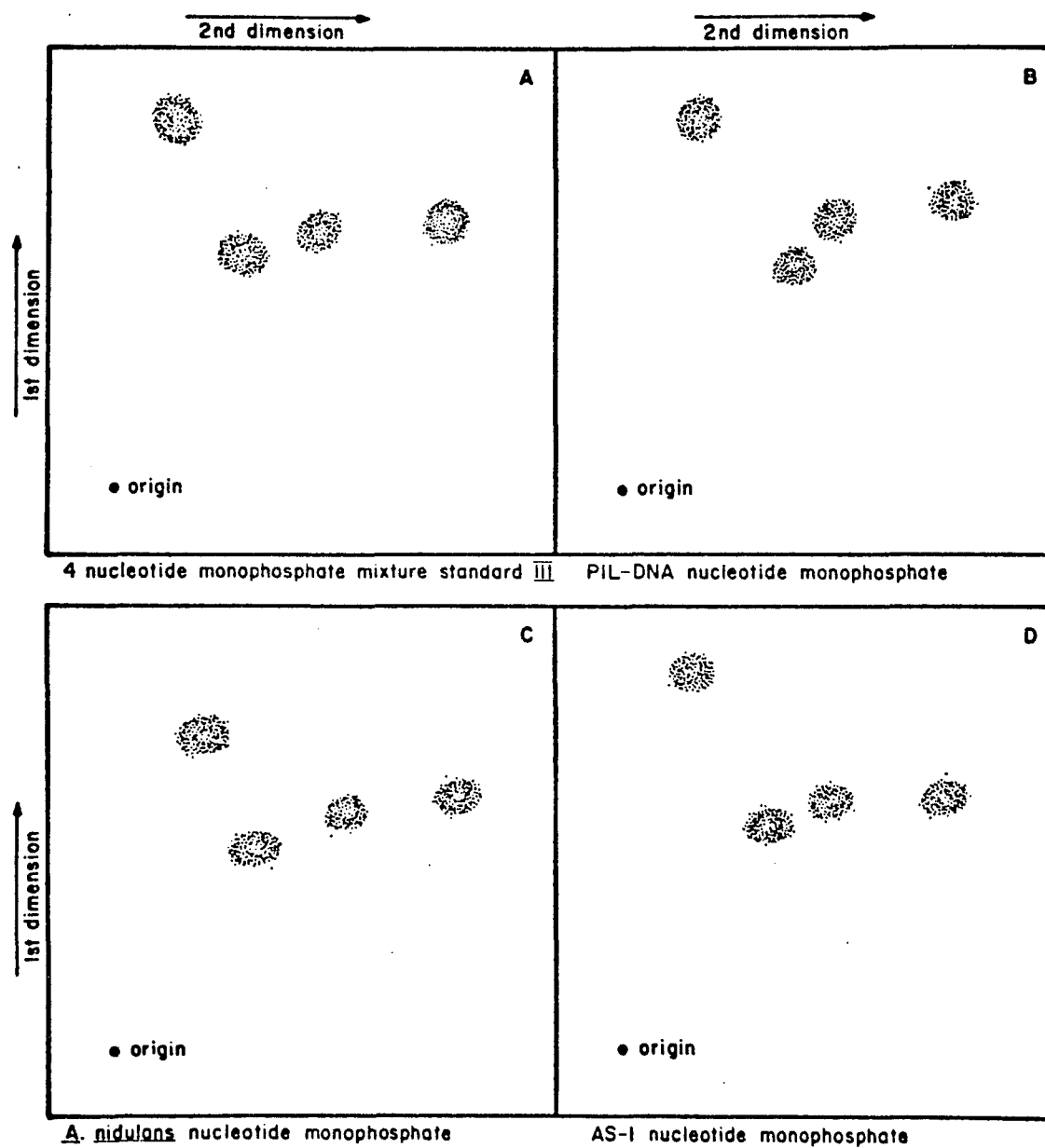
Figure 16 Analysis of UV-absorbing nucleotides from Anacystis nidulans, AS-1 and PIL-DNA by Cellulose Thin-layer Chromatography

The DNA of A. nidulans, AS-1, and PIL-DNA were extracted and purified as described in MATERIALS AND METHODS. The DNA samples were dialyzed at 4°C against three changes of a buffer containing 0.01 M Tris HCl (pH 7.6) and 5 mM MgCl₂, freeze-dried with a lyophilizer, and dissolved at a concentration of 1 mg/ml in 0.01 M Tris HCl (pH 7.6) - 5 mM MgCl₂. Enzymatic degradation to mononucleotides was accomplished by incubating the samples for 5 hr at 37°C with 0.02 ml of pancreatic deoxyribonuclease (1 mg/ml) followed by incubation at pH 8.5 with 0.01 ml of venom phosphodiesterase (4 mg/ml) for 5 hr at 37°C.

The resulting nucleotides were evaporated to dryness and then redissolved in 0.05 ml of distilled H₂O. Aliquots (20-30 μ l) from each sample were spotted separately upon 20x20 cm thin-layer chromatography plates (TLC) of cellulose (cellulose MN 300, 250 μ).

Two-dimensional chromatography on TLC plates with the solvent front moving 15 cm from the origin in each direction was performed with an isobutyric acid-water-ammonia solvent in the ratios 60:20:1 and with a saturated ammonium sulfate-1 M sodium acetate-isopropanol solvent (80:18:2). A plate spotted with 20-30 ug of 5' dAMP, 5' dGMP, 5' dCMP and 5' dTMP served as a standard plate.

- Panel A 4 standard nucleotide monophosphate mixture
- Panel B PIL-DNA nucleotide monophosphate
- Panel C A. nidulans nucleotide monophosphate
- Panel D AS-1 nucleotide monophosphate



might not have been predicted on the basis that the ^{14}C should have been incorporated only into the modified base or nucleotide. Second, in addition to the clearly identifiable major nucleotides, a fifth radioactively labeled spot appeared. This unidentified spot migrated behind dCMP in the first dimension and almost coincident with dCMP in the second dimension. This could be the expected modified nucleotide.

These results, while showing the presence of a fifth base in PIL-DNA, raised an additional question. Was the ^{14}C being incorporated into all the nitrogenous bases of the DNA? To answer this question the DNA was hydrolyzed to its constituent free bases using 88% formic acid and then these bases separated from one another using one dimensional TLC chromatography.

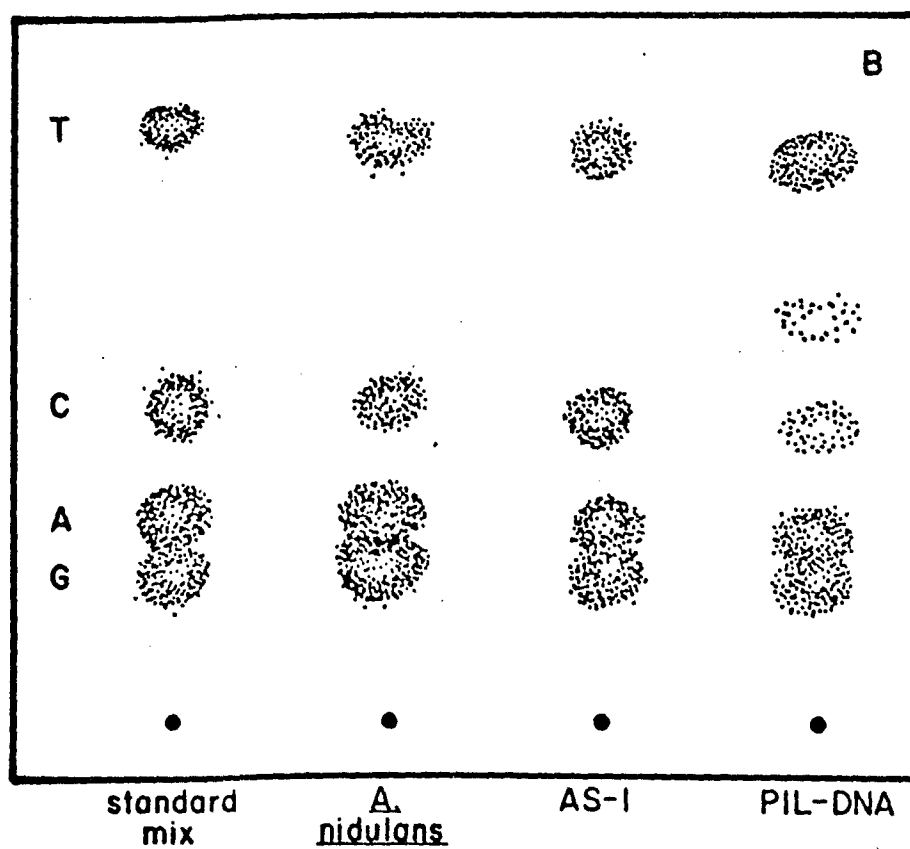
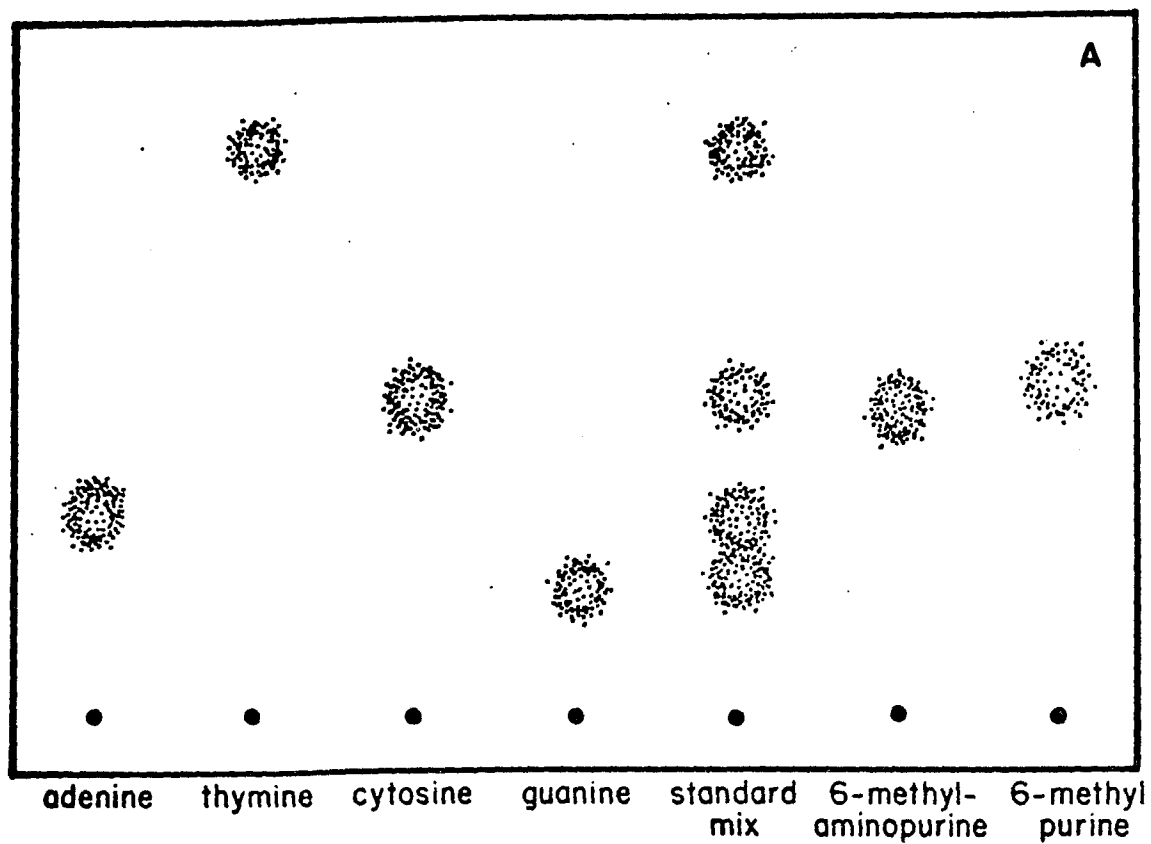
The results are shown in Figures 17A and B and 18B. In Figure 17 it can be seen that the UV-absorbing spots obtained from A. nidulans and AS-1 DNA are identical to those obtained from the commercially prepared standards adenine guanine cytosine and thymine. However, the base composition of PIL-DNA, analyzed in this way, is somewhat different. The UV-absorption of the spot corresponding to cytosine is somewhat lighter than that of the other major bases, and in addition a fifth, faint, but distinct spot appears and migrates ahead of the cytosine but behind the thymine. The migration of this spot does not correspond to either 6-methyl purine or 6-methyl amino purine also included with the stand-

Figure 17. Analysis of Bases from Anacystis nidulans, AS-1 and PIL-DNA by Cellulose Thin-layer Chromatography

The DNAs of A. nidulans, AS-1 and PIL-DNA were extracted and purified as described in MATERIALS AND METHODS. The DNA samples were hydrolyzed with 0.5 ml of 88% formic acid at 175°C for 40 min. After lyophilization the residue was dissolved in 0.1 ml of 1 N HCl. Twenty to thirty μ l of each sample was spotted on CEL 300 plates, and 5-10 μ g each of thymine, adenine, cytosine, guanine and some modified bases were spotted separately or in combination. The plates were developed in isopropanol-concentrated HCl-water (80:20.5:19.5).

Panel A known bases

Panel B base analysis of standard mixture, A. nidulans, AS-1, PIL-DNA



ards.

In Figure 18B the free bases from ^{14}C -labeled PIL-DNA have been separated using identical one dimensional TLC chromatography then visualized using autoradiography. Two things can be seen. First, all of the bases in PIL-DNA have become radioactively labeled by the $\text{NaH}^{14}\text{CO}_3$, but the incorporation into the spot corresponding to cytosine is lower than that found for the adenine, thymine and guanine. Second, a fifth very light radioactive spot is found migrating between cytosine and thymine, in an analogous position to that seen for the UV-absorbing material.

The results indicate that at least part of the radioactivity of the nucleotides seen in two dimensional TLC analysis comes from DNA nitrogenous bases. This implies that the non-specific metabolite ^{14}C is incorporated into DNA bases at an early hour of AS-1 infection (2-5 hours) and further suggests that the formation of PIL-DNA may be partially due to de novo synthesis.

B. The Origin of PIL-DNA

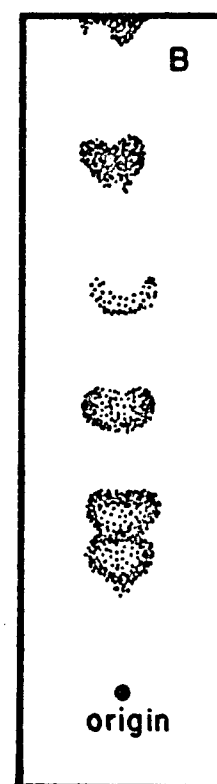
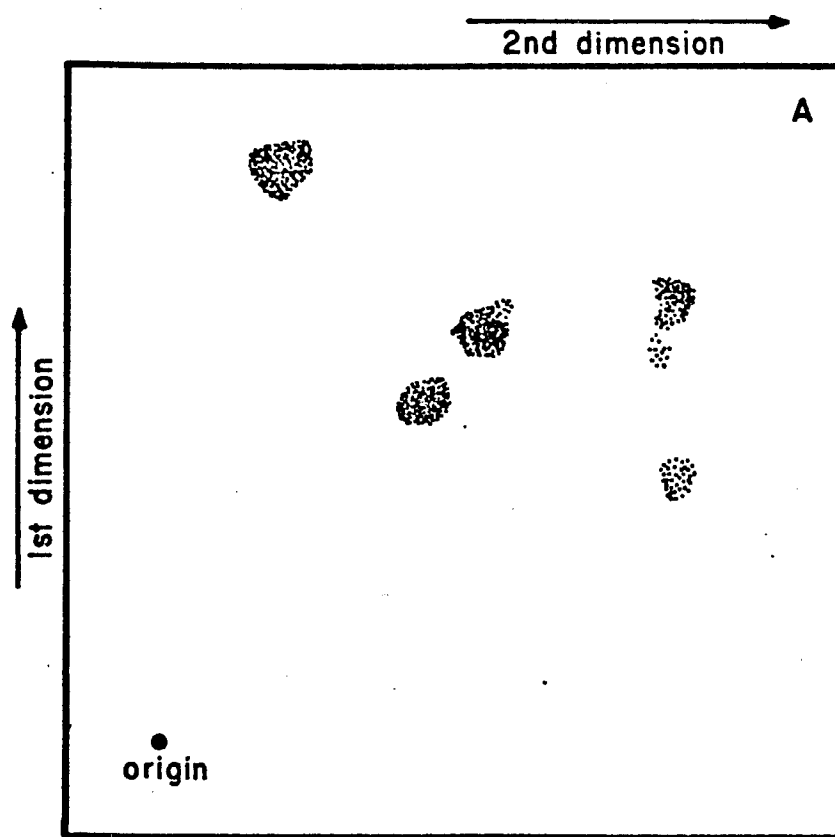
The data derived from CsCl density gradients, PLK chromatography, and T_m and Base composition studies have supplied information vital to our understanding of PIL-DNA and DNA metabolism during the infection of Anacystis nidulans by the cyanophage AS-1. However, the origin of the PIL-DNA species has remained a mystery. Thus far, three distinct possibilities for the source of PIL-DNA have arisen:

Figure 18 Autoradiograph of ^{14}C Nucleotides and ^{14}C
DNA bases from PIL-DNA Separated by Cellulose
Thin-layer Chromatography

The nucleotides of PIL-DNA were subjected to two-dimensional TLC chromatography as shown in Figure 16. The DNA bases of PIL-DNA were developed on a TLC plate one-dimensionally, as depicted in Figure 17. Autoradiography of ^{14}C -labeled PIL-DNA was performed in order to identify the ^{14}C -containing nucleosides and bases. Kodak X-ray film was overlaid on the developed chromatographs and a press was used to insure continuous contact between the film and the thin-layer chromatography plates. The chromatographs were stored in a dark room for 2-3 weeks. The X-ray film was developed as described in MATERIALS AND METHODS.

Panel A Autoradiograph of PIL-DNA nucleotide monophosphate

Panel B Autoradiograph of PIL-DNA bases



(a) PIL-DNA represents a modification of some preexisting host DNA; (b) PIL-DNA might be synthesized de novo during the early hours of the lytic cycle, utilizing solely photo-reduced metabolites; and (c) the viral DNA acts as a donor for the modification leading to PIL-DNA.

To investigate whether PIL-DNA contains host DNA, phage DNA or a combination, experiments utilizing prelabeled cells and prelabeled phages were performed. The prelabeled cells were prepared by growing the cells in a $\text{NaH}^{14}\text{CO}_3$ -containing medium for 24 hours, by which time approximately half of the total radioactivity had been incorporated into TCA-precipitable materials. The cells were then centrifuged and resuspended in sterile 3 M medium. Removal of the radioactivity from the intracellular pools was accomplished by growing the cells in a medium containing excess cold NaHCO_3 for an additional 60 hours. By this time the level of radioactivity incorporated into acid-precipitable materials approached the level of radioactivity incorporated into the pelleted cells (Table 3A). The prelabeled phage was prepared by adding ^3H -adenine into an infected cell culture; the crude lysate was then collected, dialyzed and concentrated (Table 3B). These prelabeled cells and phages were then used in the following series of experiments:

a) Following infection of ^{14}C -prelabeled cells with unlabeled phage, half of the culture was pulse-labeled with ^3H -adenine at 2-5 hours, and the remainder pulse-labeled with ^3H -adenine at 4-7 hours (Figures 19, 20).

TABLE III

Stage of preparation of ^{14}C -prelabeled cells
and ^3H -adenine prelabeled phage

A. The preparation of ^{14}C -prelabeled cells

Hours	50 λ sample	Control*	^{14}C -radioactivity CPM**
Pulse-labeled procedures:			
	0 hours	9	1720
	culture	8	1715
24 hr	supernatant	12	348
	TCA ppt	13	660
Chase procedures:			
	culture	9	382
48 hr***	supernatant	11	30
	TCA ppt	10	357
	culture	8	293
60 hr***	supernatant	10	12
	TCA ppt	12	284

* For the control, cold NaHCO_3 was substituted. All other conditions were identical to those of the $\text{NaH}^{14}\text{CO}_3$ labeled cells

** The ^{14}C -radioactivities are obtained from the average counts of triplicate samples

*** Every 12 hours, the $\text{NaH}^{14}\text{CO}_3$ radioactively labeled cells were centrifuged, washed and resuspended in sterile 3M medium. NaHCO_3 was added to the final conc. of 0.01M. The samples were counted at 48 hr and 60 hr.

B. Preparation of ^3H -adenine prelabelled phage

Stage of preparation**	^3H radioactivity (cpm per 50 λ)*
Aliquots	5921
Crude lysate	1933
Dialyzed lysate	322
Concentrated lysate (5x)	1675
TCA ppt of concentrated lysate	1537

* The ^3H -adenine radioactivities are obtained from the average counts of triplicate samples

** Lysates collected after 20 hours of infection

Figure 19. Fractionation Profiles of CsCl Gradients of
 Prelabeled Anacystis nidulans Infected with
 AS-1 and Pulse-labeled with ^3H -adenine

An exponentially growing culture of Anacystis nidulans was radioactively labeled by growth in a 3M medium containing $\text{NaH}^{14}\text{CO}_3$ (1 uCi/ml) for 24 hr. The cells were collected, washed twice and resuspended in 3M medium that had been supplemented with sodium bicarbonate to a final concentration of 0.01 M, in which it was grown for at least 60 hr to "chase" the radioactive metabolites from the intracellular pools. These $\text{NaH}^{14}\text{CO}_3$ pulse-chased cells are the so-called "prelabeled" cells. AS-1 was added to the prelabeled cells at a MOI=5. At 2 hr after infection half of the infected culture was withdrawn, pulse-labeled with ^3H -adenine for 3 hr, and then terminated in the termination solution. At 4 hr post infection the remainder of the infected culture was pulse-labeled with ^3H -adenine for 3 hr and then terminated. Following overnight incubation at 37°C the samples were treated with 1% sarkosyl and ultracentrifuged on CsCl gradients for 42 hr. The fractions of each gradient were monitored for KOH-stable, acid precipitable radioactivity as described in MATERIALS AND METHODS.

Panel A The radioactivity incorporation profile of AS-1 infected ^{14}C -labeled A. nidulans that had been pulse-labeled with ^3H -adenine for 2-5 hr

Panel B As Panel A, except for pulse-labeling for 4-7 hr

● — ● ^3H ; ○ — ○ ^{14}C

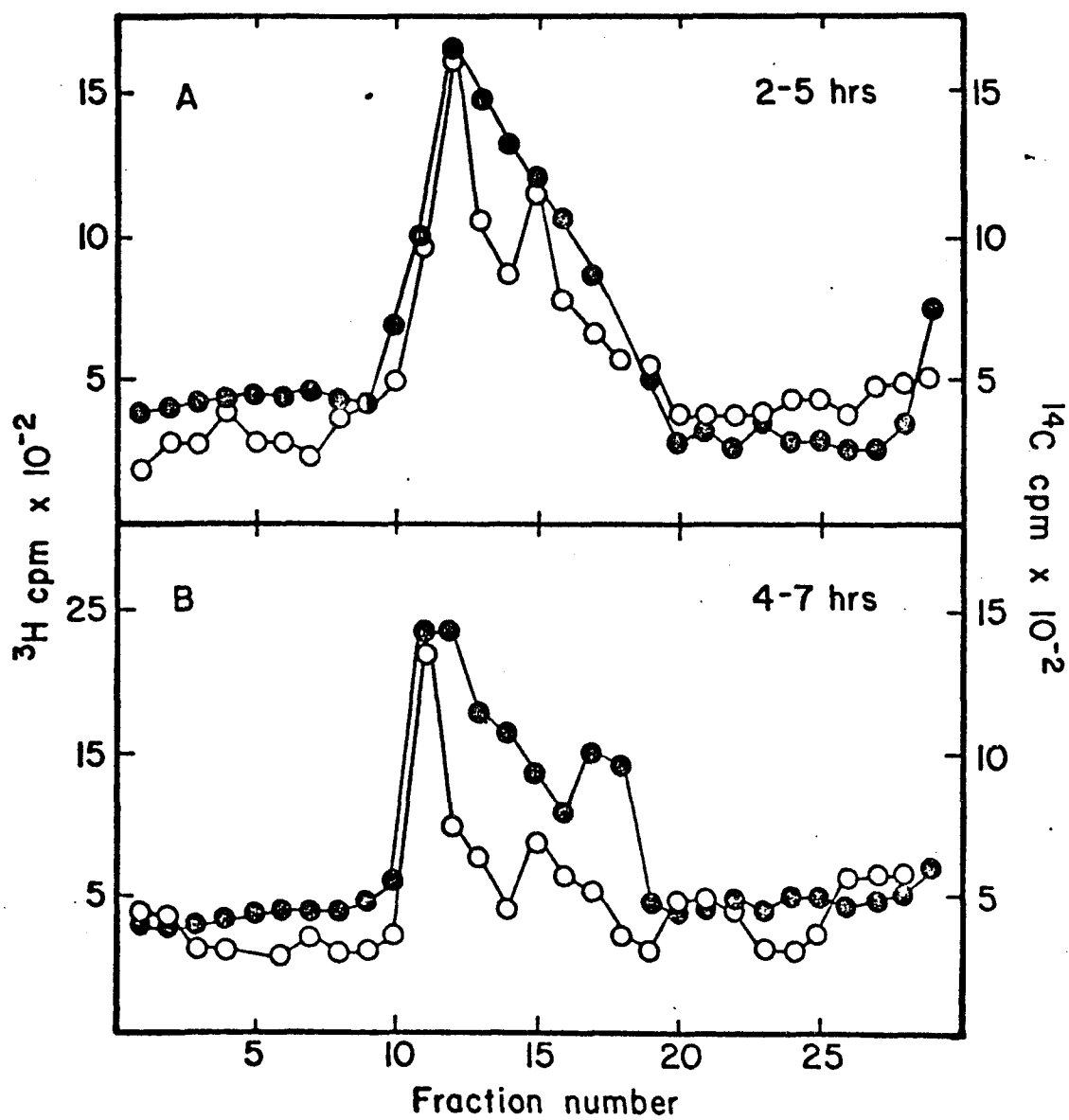
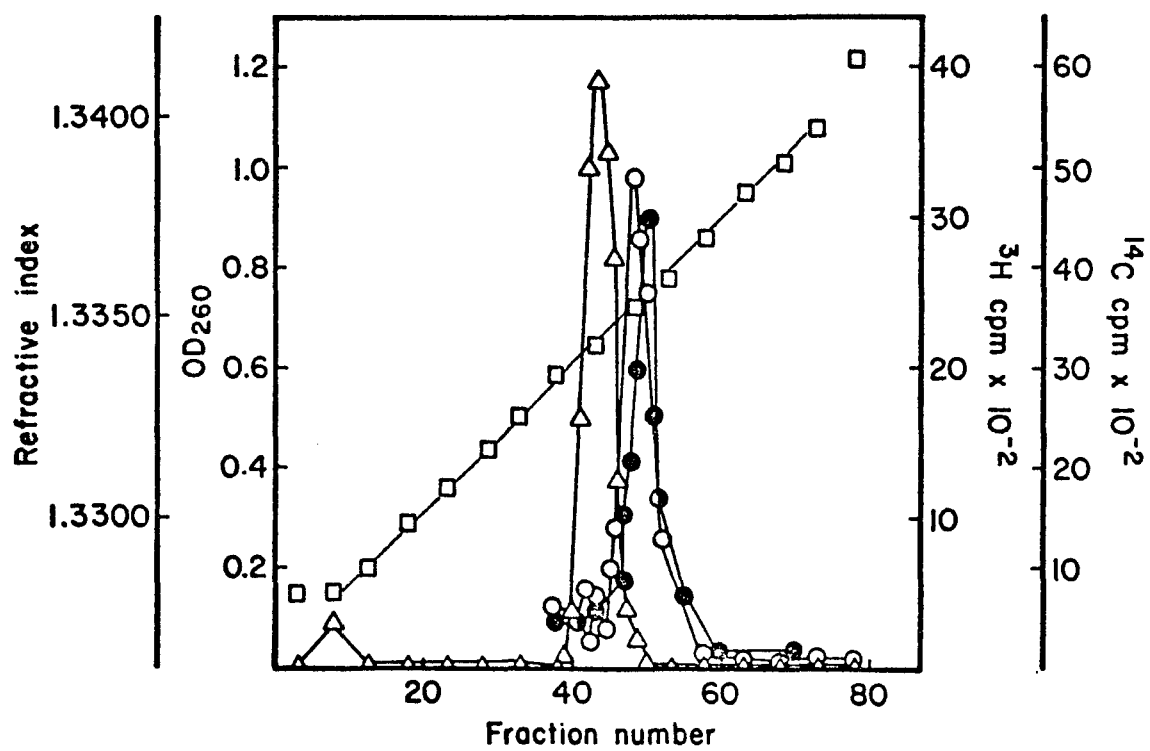


Figure 20. The Elution Profile from a PLK Column of an Extract of ^{14}C -labeled Anacystis nidulans Infected with AS-1 and Pulse-labeled with ^3H -adenine for 2-5 Hours

The preparation of ^{14}C - "prelabeled" cells was described in MATERIALS AND METHODS. The prelabeled cells were infected with cyanophage AS-1 (MOI=5). 2- ^3H -adenine (1 uCi/ml) was added at the 2 hr point to the infected culture. At the end of 5 hr the infected culture was centrifuged, washed, and broken by the Braun Homogenizer as described in the legend to Figure 8. After RNase (200 ug/ml) treatment the lysate, together with 0.5 ml of a 1 mg/ml calf thymus DNA solution was analyzed on a PLK column. (Calf thymus DNA served as an OD_{260} marker). The DNAs were eluted out by a linear gradient of 1-2.5 M buffered saline. The linearity of the gradient was checked by a refractometer. The fractions were monitored for calf thymus DNA at 260 nm. The radioactivity of the fractions due to ^3H -adenine and ^{14}C was determined as described in "Preparation of CsCl Density Gradients" in MATERIALS AND METHODS.

●—●	^3H -adenine
○—○	^{14}C
△—△	A_{260}
□—□	refractive index



b) The ^3H -adenine pre-labeled phage was used to infect unlabeled cells, and the culture was then separated into two flasks. Pulse labeling was performed with $\text{NaH}^{14}\text{CO}_3$ at 2-5 hours in one flask and at 4-7 hours in the other (Figures 21, 22).

c) The ^3H -adenine pre-labeled phage was used to infect the ^{14}C -pre-labeled cells. The samples were then collected at 5 and at 7 hours after infection (Figure 23).

d) The unlabeled phage was used to infect unlabeled cells, and the culture was then dually pulse-labeled with ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ at either 2-5 hours or 4-7 hours. These samples served as controls.

When all of the experiments had been terminated, their DNA was analyzed on CsCl density gradients and PLK columns.

In previous dual pulse-labeling experiments, analyzed on CsCl density gradients, the emergence of ^{14}C -PIL-DNA occurred at 2-5 hours after infection, after which time it decreased until lysis. If some preexisting host DNA does indeed act as a donor for modification to PIL-DNA, then the radioactivity of the ^{14}C -pre-labeled cells should be partially present at an early stage of the lytic cycle in the gradient position occupied by the PIL species.

The results obtained from CsCl density gradient analysis (experiment a) in the As-1 infected ^{14}C -pre-labeled cells are shown in Figure 20. At 2-5 hours, a significant amount of ^{14}C -pre-labeled algal DNA has migrated into the lighter region. At 4-7 hours, the amount of ^{14}C incorporation into

Figure 21. Fractionation Profiles of CsCl Gradients of Unlabeled Anacystis nidulans Infected with ^3H -adenine Prelabeled AS-1 and then Pulse-labeled with $\text{NaH}^{14}\text{CO}_3$

A crude AS-1 lysate was added to exponentially growing cells at an MOI=5. The infected culture was then supplemented with ^3H -adenine to a final concentration of 1 uCi/ml. After infection had proceeded for 20 hr the crude lysates were collected by centrifugation at 10,000 rpm for 10 min to remove the cellular debris and were dialyzed against three changes of Tm buffer. The radioactively labeled lysates were concentrated by Sephadex G-200 and passed through a 0.45 u sterile Nalgene Filter Unit. The ^3H -adenine-labeled AS-1 was then added to an exponentially growing culture (MOI=5). Two hours after infection half of the infected culture was removed, pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ for 3 hr and then terminated in the termination solution. At 4 hr after infection the remainder of the infected culture was pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ for 3 hr and terminated. The samples were analyzed on CsCl gradients and the protocols for determination of the radioactivity present in each fraction were as previously described in the legend to Figure 20.

Panel A Radioactivity incorporation profile of A. nidulans infected with ^3H -adenine-labeled AS-1 and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ for 2-5 hr.

Panel B As Panel A; pulse-labeled for 4-7 hr.

●—● ^3H -adenine; ○—○ ^{14}C

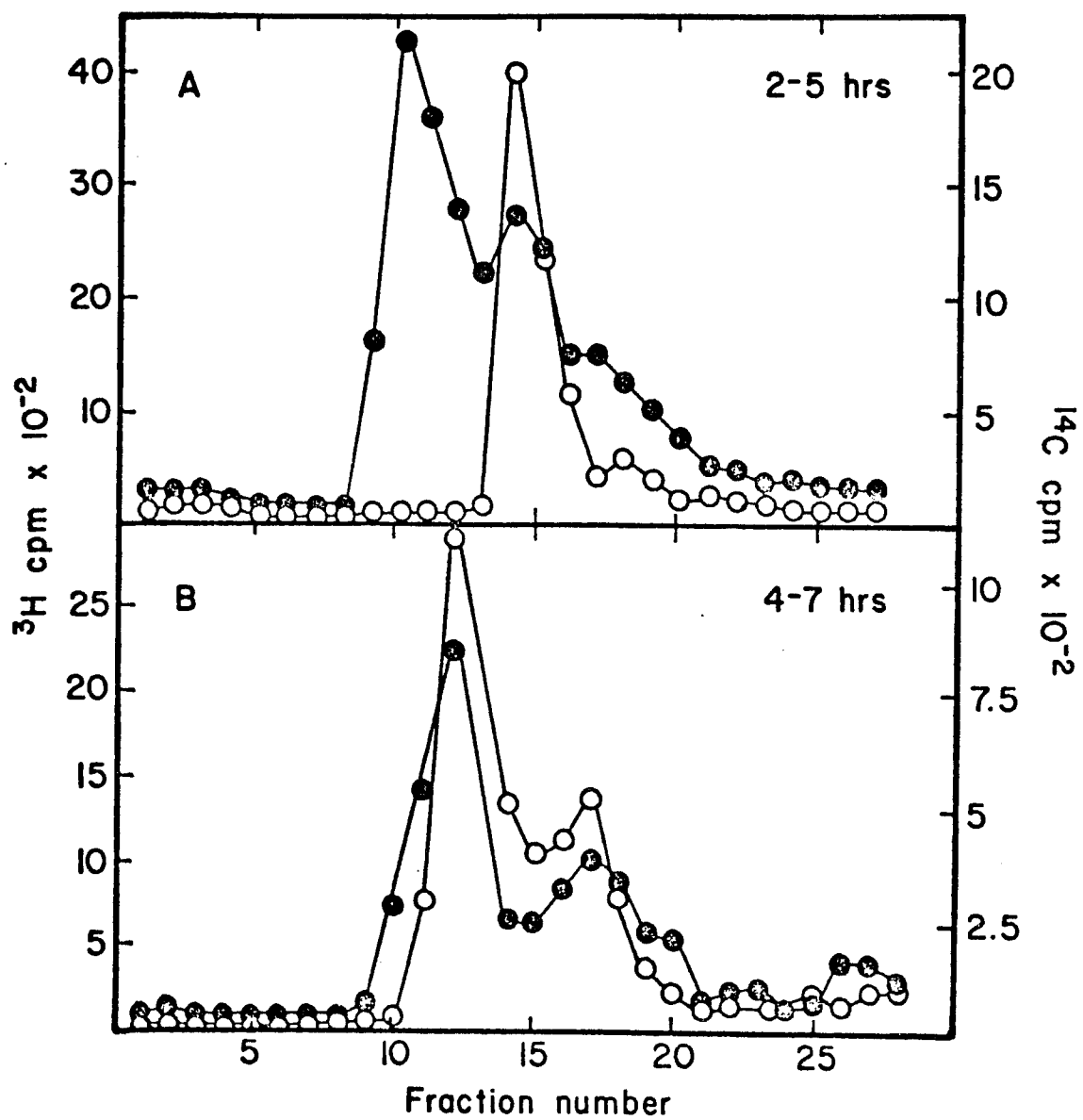


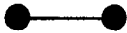
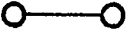
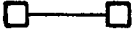
Figure 22. The Elution Profiles from PLK Columns of Extracts from Anacystis nidulans Infected with ^3H -adenine-labeled AS-1 and Pulse-labeled with $\text{NaH}^{14}\text{CO}_3$

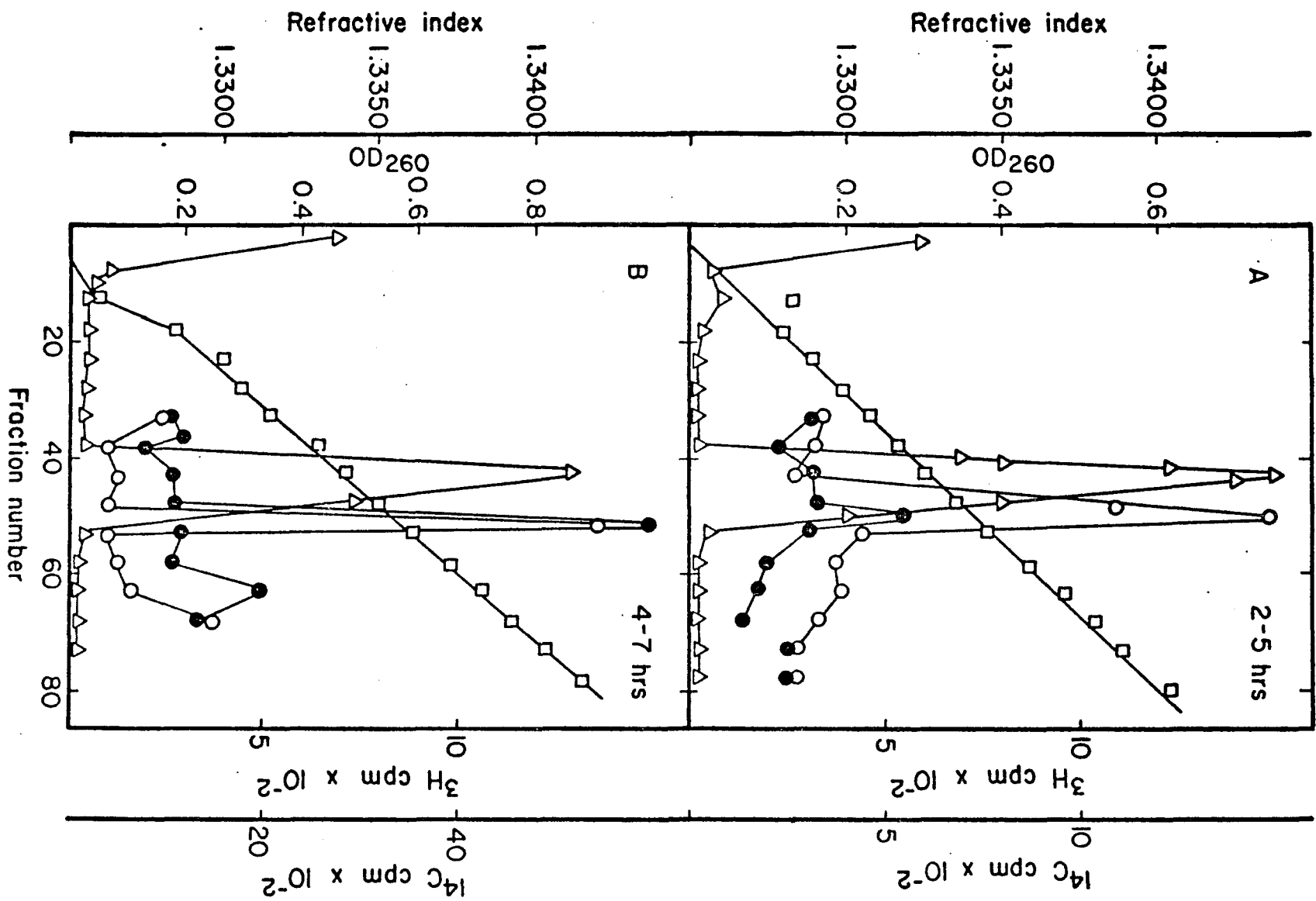
The preparation of ^3H -adenine "prelabeled" phage AS-1 was described in MATERIALS AND METHODS. The lysate of ^3H -adenine AS-1 was added to a synchronized A. nidulans culture (MOI=5). The infected culture was divided into two parts, one of which was labeled with $\text{NaH}^{14}\text{CO}_3$ (1 uCi/ml) at 2-5 hr and then terminated by breaking the cells in a Braun Homogenizer as described in the legend to Figure 8. The other half was labeled with $\text{NaH}^{14}\text{CO}_3$ (1 uCi/ml) from 4-7 hr and was terminated in the Braun Homogenizer. The crude lysates were treated with excess RNase for 0.75 hr at 37°C. Following RNase treatment each lysate, together with 0.5 ml of a 1 mg/ml calf thymus DNA solution was analyzed on a PLK column. (Calf thymus DNA served as an OD₂₆₀ marker). The DNAs were eluted out by a linear gradient of 1-2.5 M buffered saline. The linearity of the gradients was monitored refractometrically. The fractions were monitored for calf thymus content DNA at 260 nm. The radioactivities of fractions for the ^3H -adenine and ^{14}C were determined as described in "Preparation of CsCl Density Gradients" in MATERIALS AND METHODS.

Figure 22 continued

Panel A The radioactivity incorporation profile of A. nidulans infected with ^3H -adenine pre-labeled AS-1 and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ for 2-5 hr.

Panel B Same conditions as in Panel A except pulse-labeling for 4-7 hr.

	^3H -adenine
	^{14}C
	A_{260}
	refractive index



the lighter region has decreased but is still significant. In both infective intervals some minor discrepancies are also noted. This may be due to a random breakdown of the host DNA following AS-1 infection with only part of the broken host DNA reincorporated into the newly synthesized phage DNA.

The PLK chromatography results for the AS-1 infected ^{14}C -prelabeled cells are indicated in Figure 21. The elution profile from the PLK column is very similar to the ^3H -adenine, $\text{NaH}^{14}\text{CO}_3$ dual pulse-labeling experiments. There is no distinguishable shift of ^{14}C -incorporated material. Relative to the known density of the calf thymus DNA both ^3H -adenine and ^{14}C are eluted out at the salt concentration similar to that for cell or phage DNA.

If the infecting viral DNA is involved in the formation of PIL-DNA, it would be expected that some of the ^3H -adenine prelabeled phage DNA would become incorporated in the PIL-DNA region at the early hours of infection (experiment b). CsCl ultracentrifugation analyses of a culture of unlabeled cells infected with ^3H -adenine prelabeled AS-1 and then pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ at 2-5 hours and at 4-7 hours are shown in Figure 21. It is clear that at 2-5 hours post infection ^{14}C incorporates exclusively into a region of density lighter than that of the major ^3H -adenine-incorporated peak. Although at 4-7 hours post infection the major amount of ^{14}C has been incorporated into the heavy density, a significant amount of ^{14}C is still incorporated into the

lighter density region. These phenomena are identical to those observed for the dual pulse-labeling experiments described previously. By comparison to the ^{14}C radioactivity peak's light density location, it was established that a significant portion of radioactivity derived from the infecting ^3H -adenine prelabeled phage DNA migrates to the lighter density occupied by the PIL species.

Results derived from the PLK chromatography analysis of A. nidulans infected with prelabeled phage are identical to all other PLK analyses done. ^3H -adenine and ^{14}C -incorporated DNAs are eluted at the same salt concentrations (Figure 22).

In an attempt to trace the paths of pre-infection phage DNA and original host DNA during the lytic cycle a dual DNA-prelabeling experiment was performed with ^3H -adenine phage DNA and ^{14}C -Anacystis nidulans DNA (experiment c). The results are depicted in Figure 23.

The fraction profiles from CsCl gradients of these samples at 5 and 7 hours post infection imply that both pre-existing host DNA and original infecting viral DNA are involved in the formation of PIL-DNA. While this assessment can be readily made, the preliminary results from these experiments are far from clear-cut. The origin of the PIL-DNA cannot be totally attributed to either a host or a phage modification phenomenon. Perhaps its formation represents an intermediate step in phage replication, a process which utilizes both host and phage DNAs.

Host DNA degradation and subsequent reincorporation

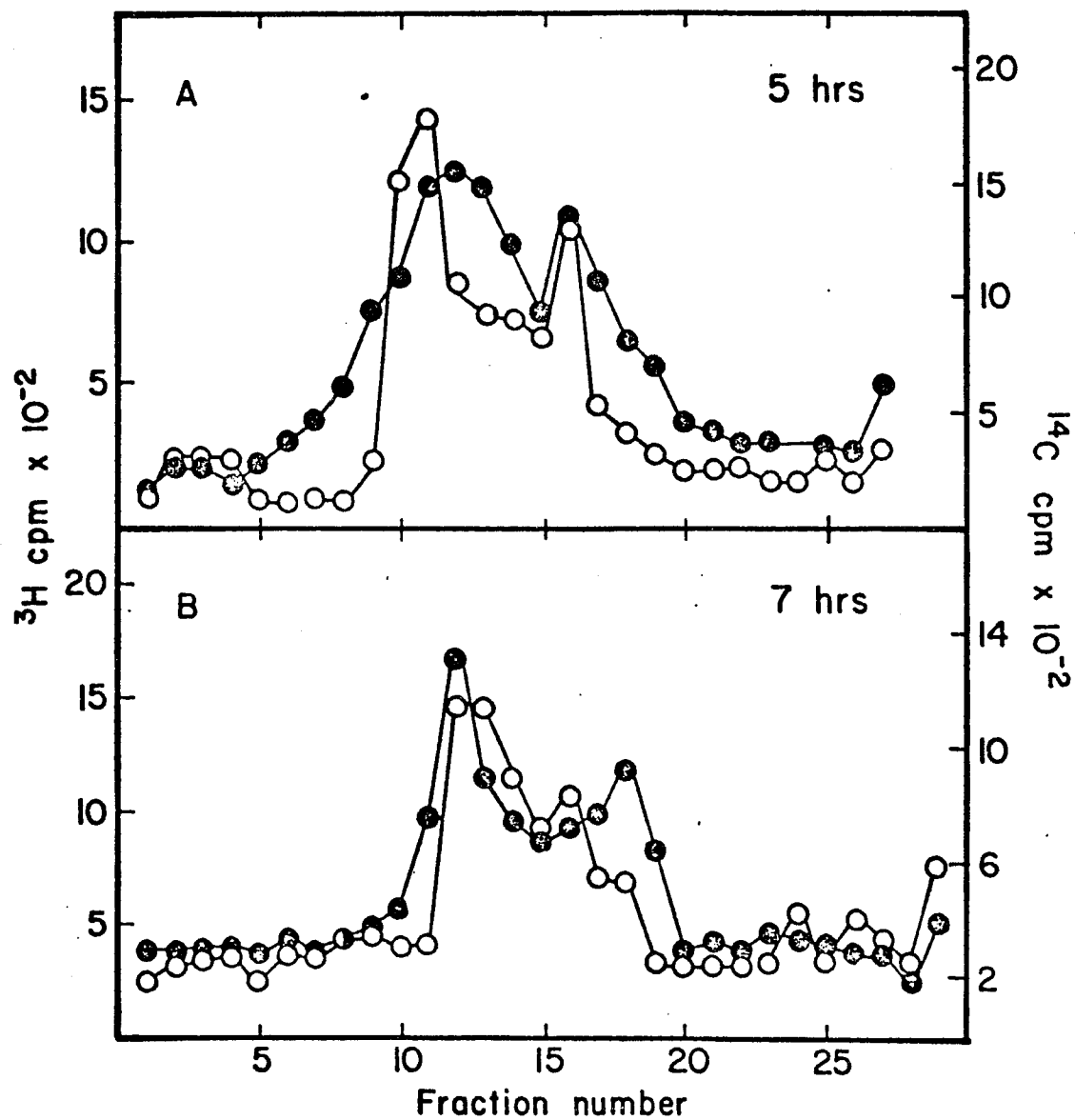
Figure 23. Fractionation Profiles from CsCl Gradients of Anacystis nidulans Prelabeled with $\text{NaH}^{14}\text{CO}_3$ and then Infected with ^3H -adenine Prelabeled AS-1 Stock

The "prelabeled viruses" and "prelabeled cells" were prepared as described in the legends to Figure 19 and Figure 21. Five hours following the addition of ^3H -adenine prelabeled AS-1 to $\text{NaH}^{14}\text{CO}_3$ prelabeled cells ($\text{MOI}=5$), half of the infected culture was withdrawn and terminated in the termination solution. After 7 hr of infection the remainder of the infected culture was collected and terminated. The samples were analyzed on CsCl gradients and determination of the radioactivity incorporation in each fraction followed the protocol described in the legend to Figure 19.

Panel A The radioactivity incorporation profile of ^{14}C -prelabeled A. nidulans infected with ^3H -adenine prelabeled AS-1 for 5 hr.

Panel B Same conditions as Panel A; infection for 7 hr.

● — ● ^3H -adenine
○ — ○ ^{14}C



into the lighter density DNA is suggested as a hypothesis for the role of host DNA in PIL-DNA formation by the report of Sherman and Pauw (138). They observe an immediate breakdown of the Anacystis nidulans DNA following infection by AS-1. Furthermore, they have discovered a slow incorporation of this degraded DNA into newly synthesized phage DNA beginning at 2.5-3 hours after infection, and increasing until the 7 hour point. However, the possibility of a host DNA modification contributing to the formation of PIL-DNA, rather than an actual degradation cannot be ignored. This possibility, in fact, provides the likeliest explanation for the role of phage DNA in PIL-DNA formation.

From the data obtained in this study of PIL-DNA's origin, a tentative hypothesis may be suggested. Perhaps the PIL-DNA is an intermediate in the production of new viral DNA that utilizes the modified infecting phage DNA as a template, and the degraded host DNA as a precursor source. The presence of ^{14}C -radioactivity in the PIL-DNA may be due either to a direct utilization of the precursor in a de novo synthesis or to its role in the modification of the infecting phage DNA that serves as the PIL-DNA template. Further experimentation is required, however, before any of the above proposals may gain acceptance as the true explanation for the nature and origin of PIL-DNA.

IV. The Effect of Certain DNA Inhibitors on the Development of AS-1 in *Anacystis nidulans*

Hydroxyurea, a DNA synthesis inhibitor, has been shown to specifically inhibit DNA synthesis without necessarily causing death of the treated cells (118). Nalidixic acid is also known to have complex effects on bacteria, including inhibition of DNA replication and the degradation of DNA (34). These two agents might be especially valuable in elucidating the role of DNA synthesis in PIL-DNA formation since they differ from the other known inhibitors of DNA synthesis by being bacteriostatic rather than bactericidal. Since the concentration range over which these compounds are effective varies depending upon the organism, preliminary experiments must be performed in order to define the effect of different concentrations of each inhibitor on the growth pattern of a synchronized algal population and on the development of AS-1 in *Anacystis nidulans*. Once determined, the proper concentration of inhibitor may be used for subsequent experiments.

A. The Effect of Inhibitors on the Growth of *A. nidulans*

A series of experiments was performed utilizing various concentrations of hydroxyurea and nalidixic acid. The data, summarized in Figure 25 and Figure 26, indicate that both hydroxyurea and nalidixic acid are bacteriostatic rather than bactericidal. The turbidity of the cultures in the presence of both agents at concentrations ranging from 0 ug/ml to

500 ug/ml continued to increase, although the increases were less at the higher concentration of inhibitors (Panel A in Figure 24, Figure 25). Even prolonged exposure (24 hr) of the algae to hydroxyurea or nalidixic acid did not have a lethal effect*. DNA synthesis (panel B), on the other hand, was drastically curtailed by exposure of the algae to higher concentration of both inhibitors. In the culture containing 500 ug/ml of hydroxyurea DNA synthesis is completely inhibited since no significant change in DNA content appears within the entire exposure time (24 hr). Similarly, the cultures containing nalidixic acid at concentrations of 100 ug/ml, 250 ug/ml and 500 ug/ml exhibit a constant level of DNA. At the end of 24 hours, a slight decrease in DNA content is detected which may be due to partial degradation of DNA after prolonged exposure. The data presented thus far indicates that the inhibition of algal replication is accompanied, however, by a continued increase in cultural turbidity. This indicates the possibility of unbalanced growth and with protein and other macromolecular synthesis proceeding in the absence of DNA synthesis and cyanobacterial division. This suggestion is supported by protein and chlorophyll measurements on the cultures.

In the presence of hydroxyurea or nalidixic acid in the concentrations ranging from 0 ug/ml to 500 ug/ml a continuous

* Preliminary experiment, with the culture at 24 hours exposure, turbidity increases, but the cell number remains constant. In addition, the cultures were shown to recover their growth upon removal of inhibitors and transfer to fresh medium.

increase in protein content (panel C, Figure 24, 25) indicates that protein synthesis, as compared to DNA synthesis, is relatively insensitive to inhibition. The effect of hydroxyurea on chlorophyll synthesis is similar to its effect on protein synthesis (panel D, Figure 24). A slight decrease in chlorophyll content is observed following 12 or more hours exposure to nalidixic acid at a concentration of 500 ug/ml (panel D, Figure 25). These results may be due to the bleaching effect on chlorophyll of nalidixic acid at high concentration. The inhibition induced by these agents apparently does not involve structures participating in the translation of genetic information into the synthesis of protein and other macromolecules. The highly specific mode of action of these two inhibitors, especially hydroxyurea, makes them an ideal agent for determining the role of DNA synthesis in the system we are studying.

A concentration of 500 ug/ml of both hydroxyurea and nalidixic acid were used in subsequent experiments for the reason that this concentration stops the DNA synthesis completely, but does not stop the other growth parameters.

B. The Effect of Inhibitors on the AS-1/A. nidulans
Infective System and on the Development of Cyanophage
AS-1

The inhibitors, hydroxyurea and nalidixic acid, were added to the AS-1 infected cultures (MOI=5) to give a final concentration of 500 ug/ml. The effect of these agents on phage lytic ability and on DNA, protein and chlorophyll

Figure 24. Effect of Various Concentrations of Hydroxyurea on Algal Culture Turbidity Change, DNA, Protein and Chlorophyll Synthesis

Algal cultures were synchronized and the stock of hydroxyurea was prepared as described in MATERIALS AND METHODS. The synchronized cultures were divided into five flasks and hydroxyurea was added to give final concentrations of 0 ug/ml, 50 ug/ml, 100 ug/ml, 250 ug/ml and 500 ug/ml respectively. At different time intervals, 35 ml of the cultures were withdrawn from each flask and analyzed for turbidity change (determined spectrophotometrically at OD₇₅₀), and the content of DNA, protein and chlorophyll. The methods for DNA, protein and chlorophyll determination were described in MATERIALS AND METHODS.

Panel A Effect of various concentrations of hydroxyurea on the cultural turbidity change.

Panel B Effect of various concentrations of hydroxyurea on DNA synthesis.

Panel C Effect of various concentrations of hydroxyurea on protein synthesis.

Panel D Effect of various concentrations of hydroxyurea on chlorophyll synthesis.

●—●	control (0 ug/ml)
○—○	50 ug/ml hydroxyurea
△—△	100 ug/ml hydroxyurea
■—■	250 ug/ml hydroxyurea
□—□	500 ug/ml hydroxyurea

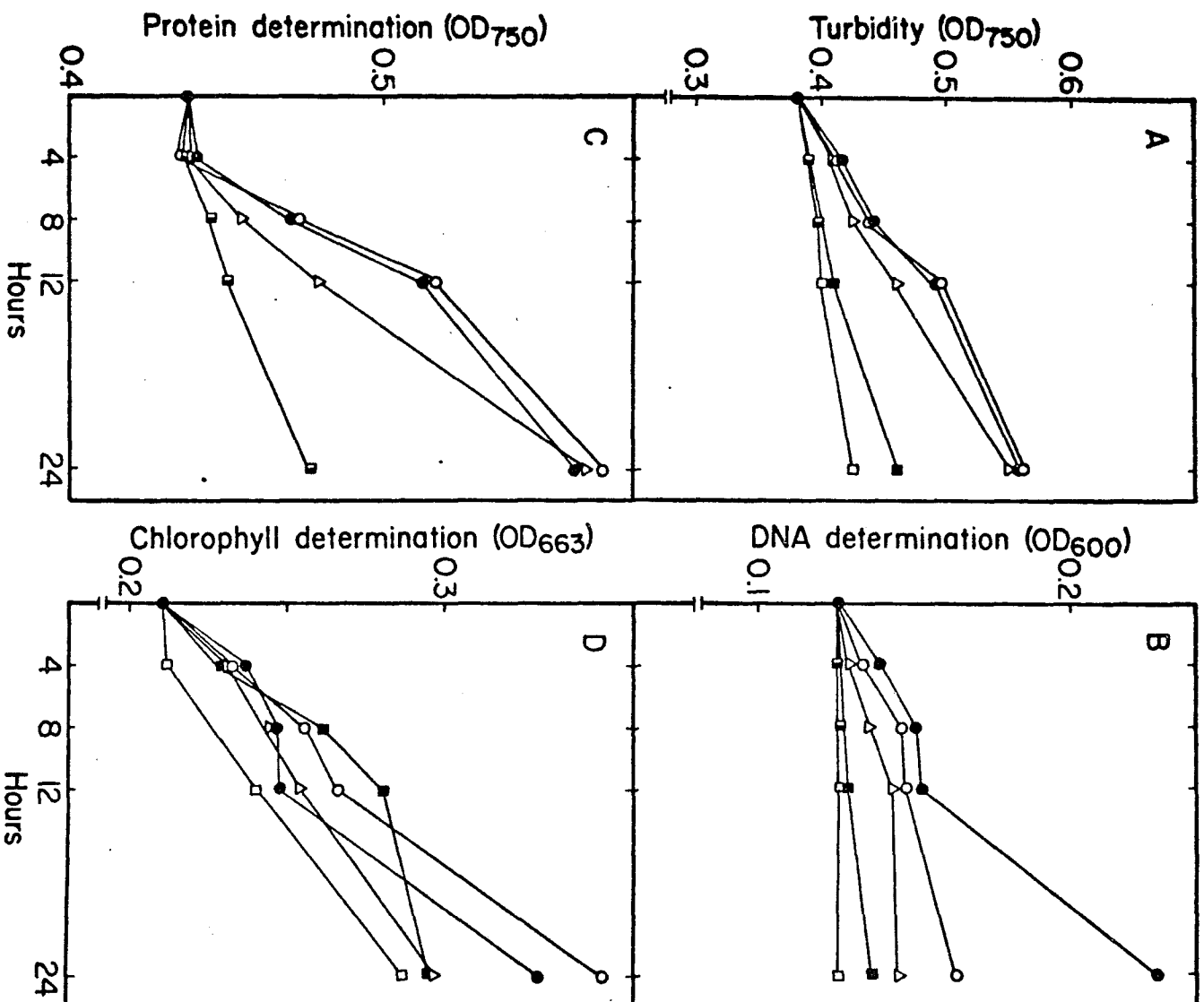


Figure 25. Effect of Various Concentrations of Nalidixic Acid on Algal Cultural Turbidity Change, DNA, Protein and Chlorophyll Synthesis.

Algal cultures were synchronized and the stock of nalidixic acid prepared as described in MATERIALS AND METHODS. The experimental protocols and turbidity, DNA, protein and chlorophyll determinations were identical to those of Figure 24.

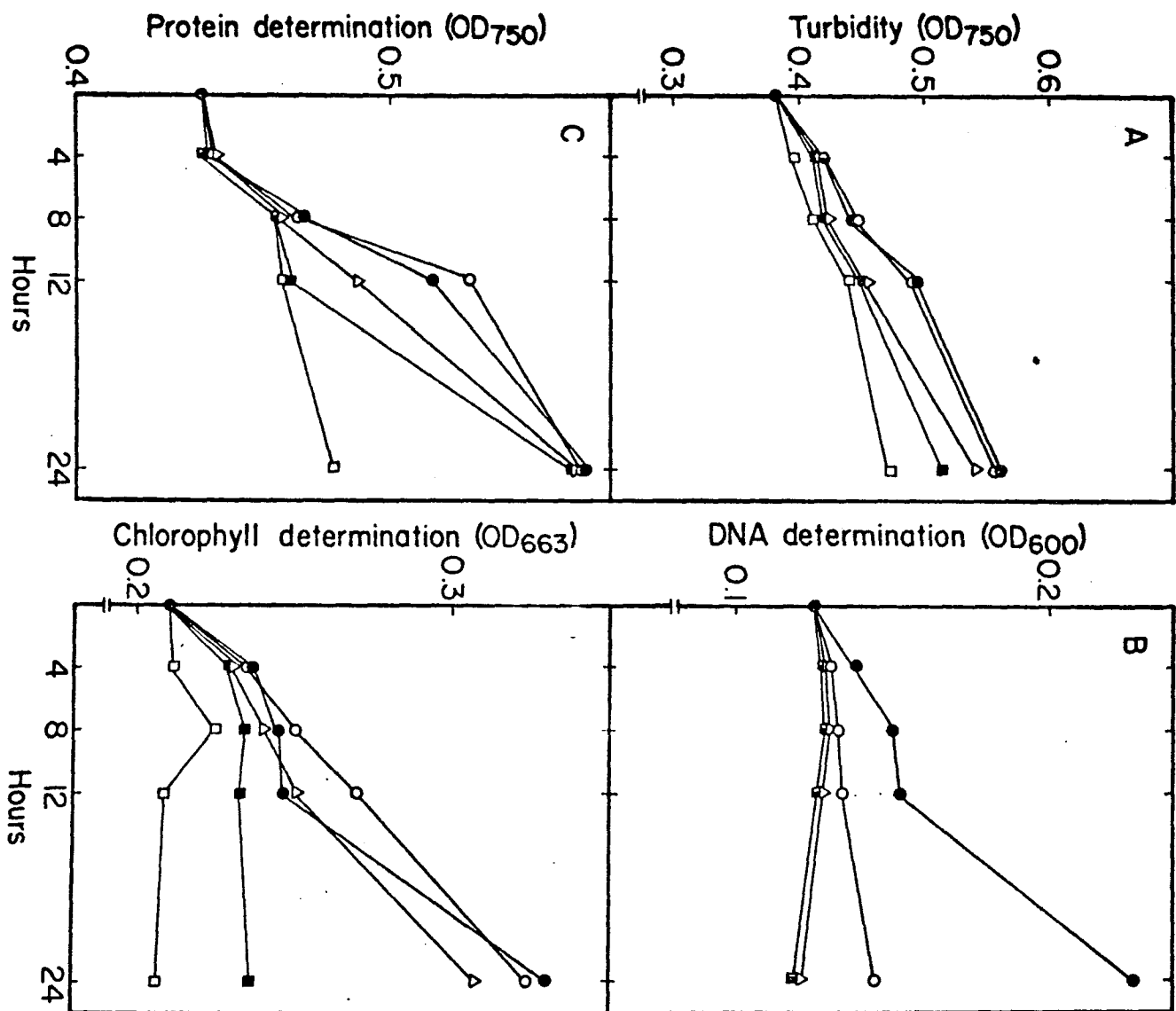
Panel A Effect of various concentrations of nalidixic acid on the cultural turbidity change.

Panel B Effect of various concentrations of nalidixic acid on DNA synthesis.

Panel C Effect of various concentrations of nalidixic acid on protein synthesis.

Panel D Effect of various concentrations of nalidixic acid on chlorophyll synthesis.

- control (0 ug/ml)
- 50 ug/ml of nalidixic acid
- △—△ 100 ug/ml of nalidixic acid
- 250 ug/ml of nalidixic acid
- 500 ug/ml of nalidixic acid



content in the infected cultures are summarized in Figure 26. A decrease of turbidity is found in the presence of the inhibitors, especially hydroxyurea, but not in the normal infective system at four hours post infection (panel A, Figure 26). This may be due to the combined effect of inhibitors and cyanophage on the growing algae. At 24 hours the lytic cycle should be complete in the normal infective system; approximately 70% of lysis relative to the normal infective system was found in the infected culture with hydroxyurea. However, in the nalidixic acid containing infected culture only 30% lysis was detected.

Similar results were obtained from the DNA and protein determination. Nalidixic acid shows a potent effect on the AS-1/A. nidulans infective system. The DNA content was decreased in all three conditions (normal infective systems, infective system with the presence of hydroxyurea, and the infective system with the presence of nalidixic acid) at four hours of infection. This may be the result of the breakdown of the host DNA before the initiation of the phage DNA synthesis.

With respect to protein content, the control culture shows a peak of protein content at eight hours post infection. The same peak in protein content was observed in the cultures containing hydroxyurea and nalidixic acid. Hydroxyurea causes an increase in protein content relative to the control while nalidixic acid cause a decrease.

With respect to chlorophyll content, the three exper-

Figure 26. The Effect of Hydroxyurea and Nalidixic Acid on Phage Lysis and DNA, Protein and Chlorophyll Synthesis in the AS-1/A. nidulans Infective System

Cyanophage was added to a synchronized A. nidulans culture (MOI=5) and the infected culture was continuously illuminated throughout the lytic cycle. After infection hydroxyurea was added to one third of the infected culture and nalidixic acid was added to another third to give a final concentration of 500 ug/ml for each inhibitor. The remainder served as the control. At 0, 4, 8, 12 and 24 hr after infection 35 ml from each culture was removed, and lysis and the synthesis of DNA, protein and chlorophyll were determined as described in MATERIALS AND METHODS.

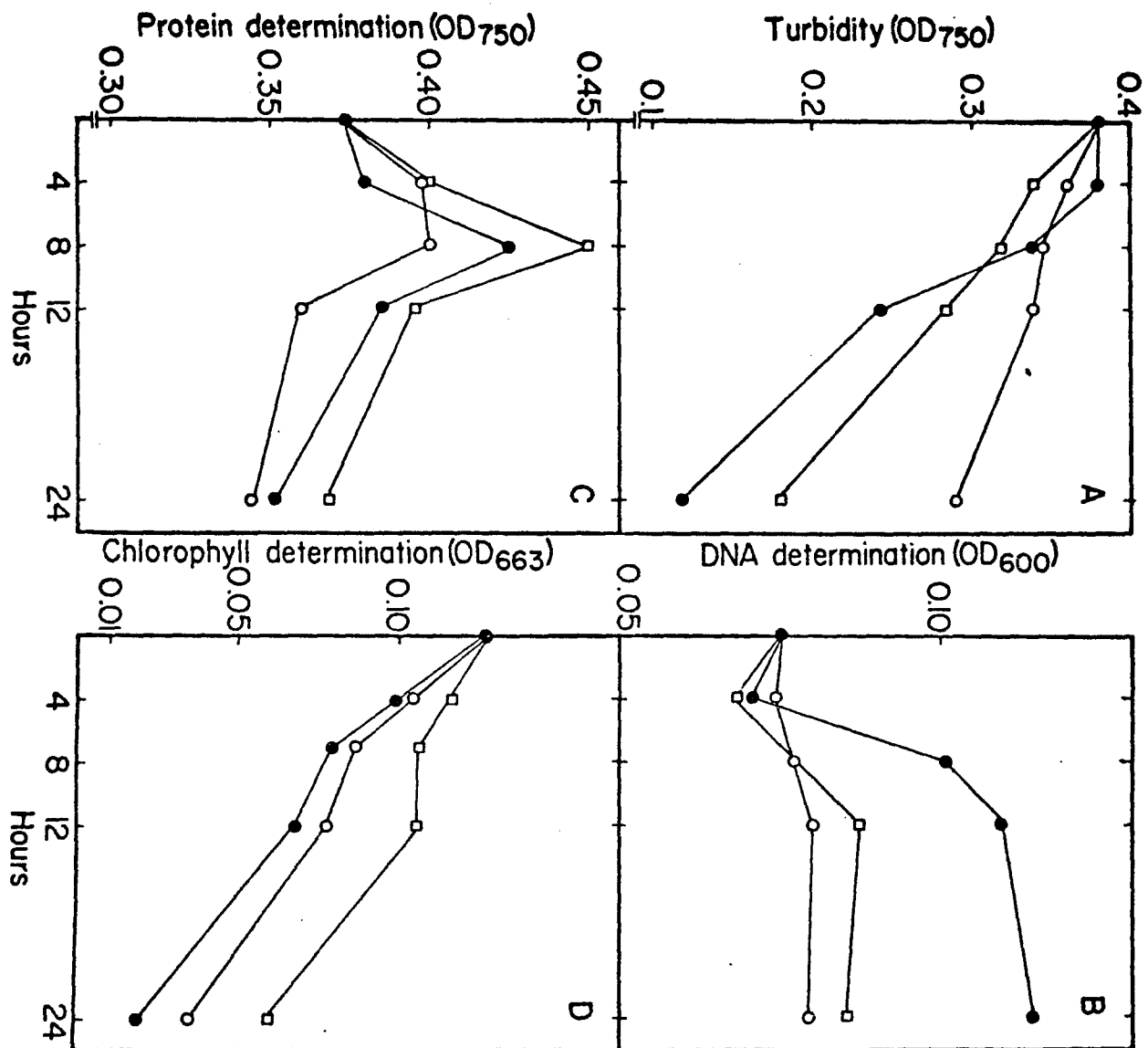
Panel A Lysis

Panel B DNA synthesis

Panel C Protein synthesis

Panel D Chlorophyll synthesis

●—● control, algae + phage
 □—□ algae + phage + hydroxyurea
 ○—○ algae + phage + nalidixic acid



imental conditions give similar results. In the presence of the inhibitor, especially hydroxyurea, the decrease of chlorophyll is less than the control samples. This suggests that cyanophage infection causes the decrease in chlorophyll content. From the turbidity, protein and DNA determinations the inhibitors have been shown to affect the development of cyanophage with nalidixic acid having the stronger effect.

The effect of nalidixic acid and hydroxyurea on the production of cyanophage as determined by plaque formation is represented by the data in Figure 27 and Figure 28. It is clear that the burst size is drastically decreased in the presence of the inhibitors. In the infected culture containing hydroxyurea a burst size of eight is obtained. Compared with its control which has a burst size of about fifty, the formation of cyanophage is about 12% of the normal infective system. In the infected culture containing nalidixic acid the phage formation was seriously inhibited. A burst size of 2.6 is obtained which is about 5% of the normal infective system.

From all the data accumulated thus far, it can be concluded that hydroxyurea and nalidixic acid have an effect on the AS-1/A. nidulans infective system and on the formation of the cyanophage. Seventy percent and 30% of lytic ability were found with the hydroxyurea and nalidixic acid respectively, but only 12% and 5% of the burst size relative to the normal infective culture. This suggests that, although the number of the cells lysed by the phage is less, the main

Figure 27. The Effect of Hydroxyurea on the Formation of Cyanophage AS-1

Cyanophage AS-1 was added to a synchronized A. nidulans culture and the infected culture was continuously illuminated throughout the lytic cycle. After one hour of infection the infected cells were centrifuged and washed to remove any unadsorbed phage and equally apportioned resuspended into two flasks which contained equal volumes of fresh sterile 3M medium. Hydroxyurea was added to one of the infected cultures to a final concentration of 500 ug/ml; the other served as a control. At 3, 6, 9, 12, and 20 hr after infection 1 ml aliquots were removed from each culture and the formation of the cyanophage AS-1 was determined by viral titer (PFU/ml) as described in MATERIALS AND METHODS.

●—● control
○—○ hydroxyurea (500 ug/ml)

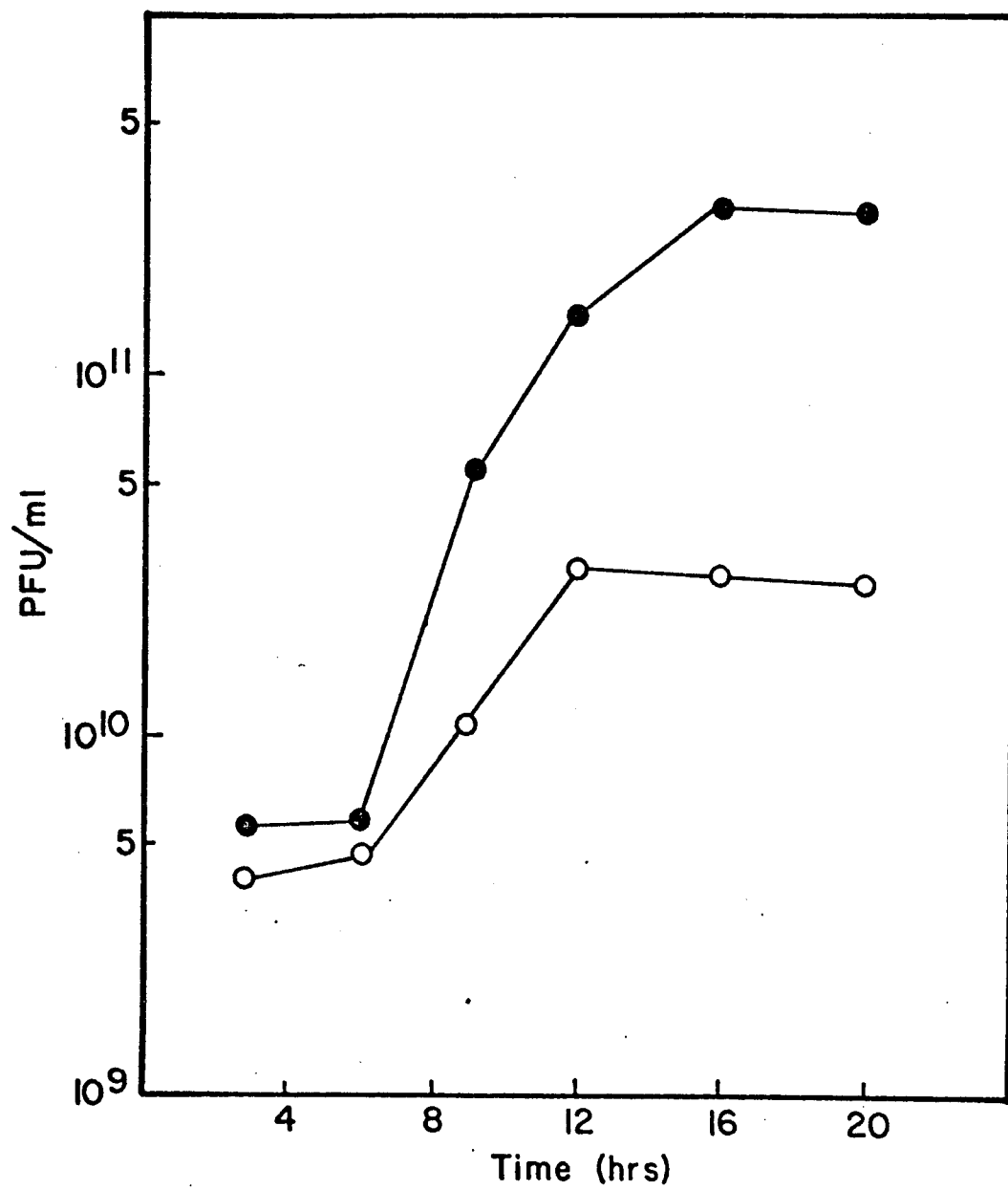
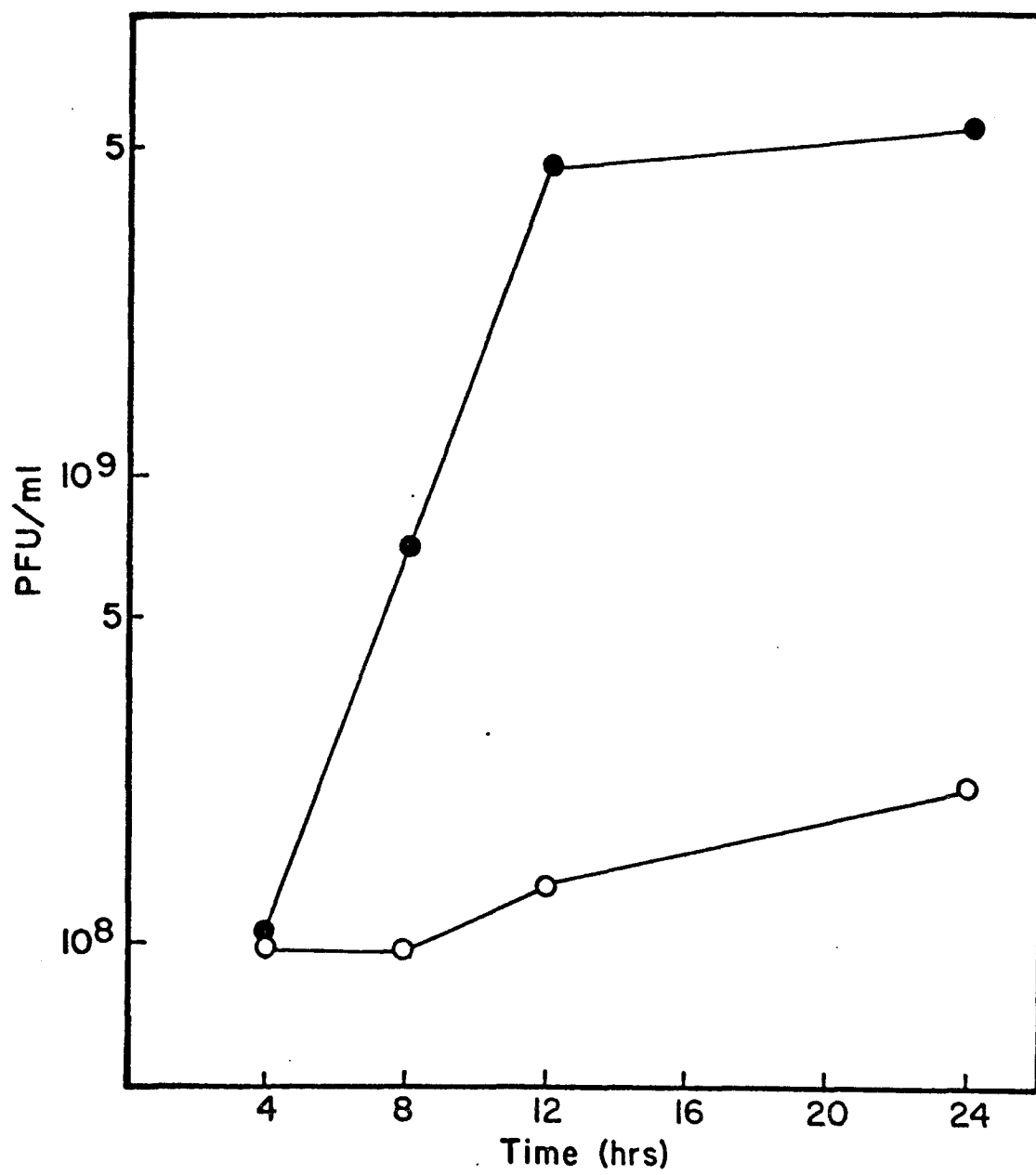


Figure 28. The Effect of Nalidixic Acid on the Formation of Cyanophage AS-1

Cyanophage AS-1 was added to a synchronized A. nidulans culture and the infected culture was continuously illuminated throughout the lytic cycle. After one hour of infection the infected cells were centrifuged and washed to remove any unadsorbed phage and equally apportioned resuspended into two flasks which contained equal volumes of fresh sterile 3M medium. Nalidixic acid was added to one of the infected cultures to a final concentration of 500 ug/ml; the other served as a control. At 4, 8, 12, and 24 hr after infection 1 ml aliquots were removed from each culture and the formation of the cyanophage AS-1 determined by viral titer (PFU/ml) as described in MATERIALS AND METHODS.

●——● control
○——○ nalidixic acid (500 ug/ml)



effect of these agents is on the development of the cyanophage in the infected cells. It has been shown that these inhibitors can inhibit algal DNA synthesis; from these preliminary studies, however, the exact mode of action on the infective system is still far from clear.

C. CsCl Gradient Analysis of the Changing Patterns of DNA Synthesis in the Presence of Different Inhibitors in the AS-1/A. nidulans Infective System

The effect of the two different inhibitors on the DNA synthesis patterns was analyzed on CsCl gradients and compared with the normal infective system. The DNA banding profiles obtained at different time intervals post infection are presented in Figure 29 (for details, see figure legend).

In the hydroxyurea treated infective system a minute amount of the ^{14}C -incorporating PIL-DNA is found at 0-3 hours post infection and continuously increases until 2-5 hours. At this time interval part of the ^{14}C is also found at the heavy phage DNA density. At 4-7 hours, most of the ^{14}C is incorporated at the phage DNA density, although a small amount still exists at the PIL-DNA density. This incorporation pattern is very similar to the normal infective system except that the ^{14}C incorporation occurs earlier. These results parallel the findings from the plaque formation studies which indicated that the cyanophage exhibits a shorter lytic cycle in the presence of hydroxyurea. Although hydroxyurea has no significant qualitative effect on PIL-DNA formation, the incorporation of ^3H -adenine into DNA of phage

Figure 29 Fractionation Profiles of CsCl Gradients of Anacystis nidulans Infected with AS-1 in the Presence of Hydroxyurea and Simultaneously Pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine at Different Time Intervals Post Infection

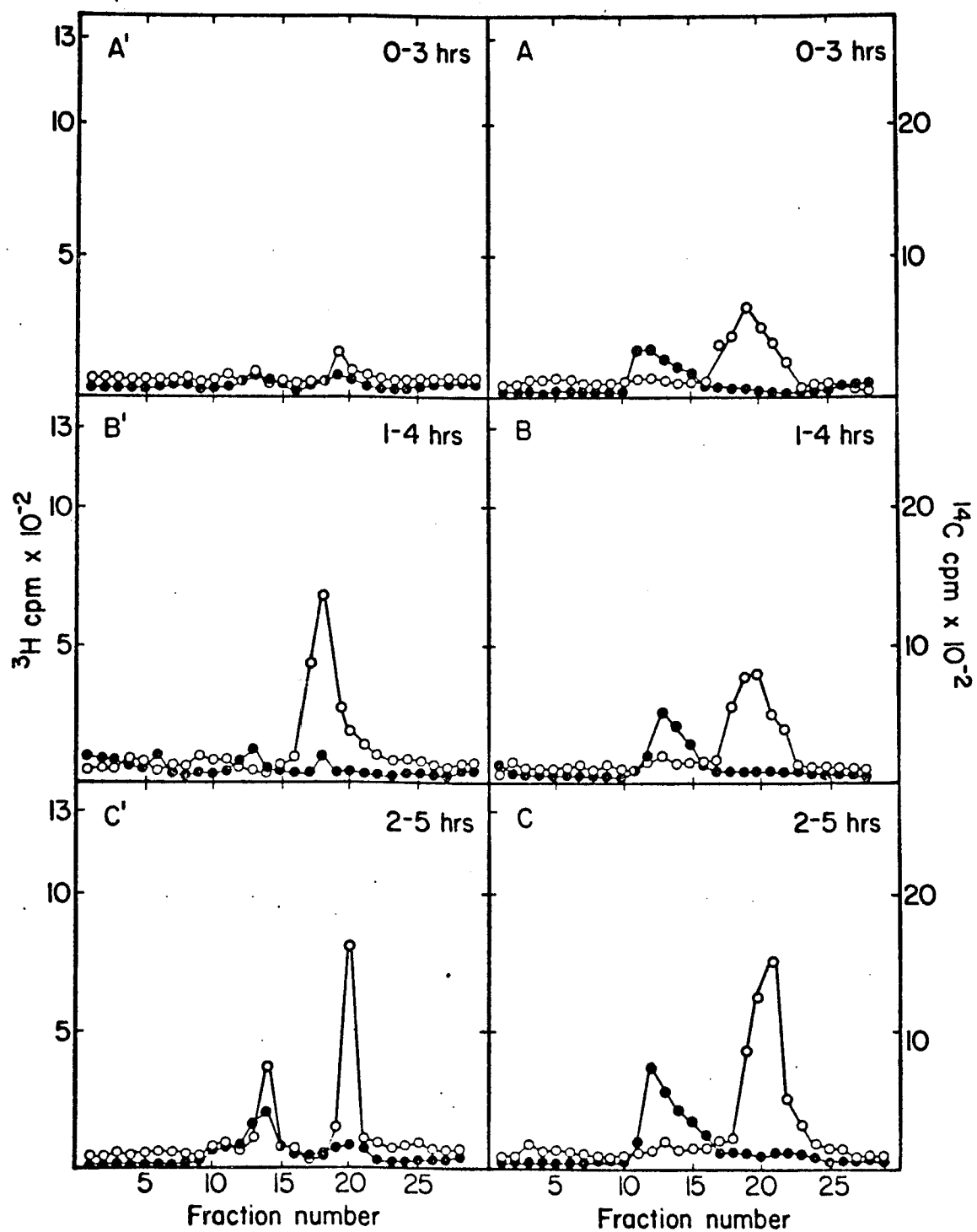
AS-1 was added to a synchronized A. nidulans culture (MOI=5), supplemented with NaCl to give a final concentration of 0.1 M. Hydroxyurea (Hu) was added to half of the infected culture to a final concentration of 500 ug/ml. The remainder of the infected culture under the identical conditions served as the control. From each of the infected cultures 10 ml was removed at 10 min, 1 hr, 2 hr, 3 hr and 4 hr and double-labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine for 3 hr. At the end of each pulse-labeling period (i.e. 3 hr, 4 hr, 5 hr, 6 hr and 7 hr respectively), the samples were collected and terminated in a termination solution as described in MATERIALS AND METHODS.

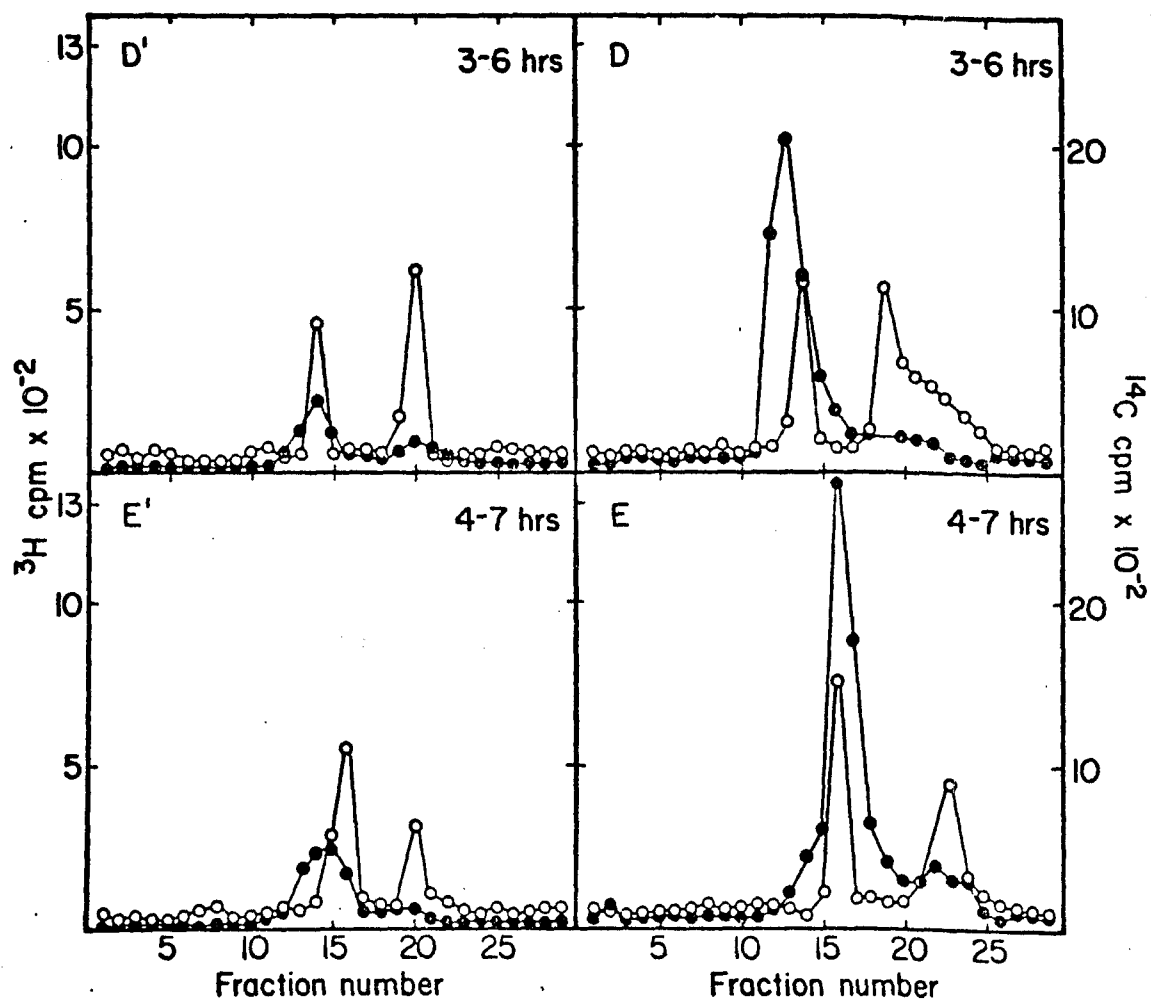
Each sample was subjected to preparative ultracentrifugation on a CsCl density gradient. Each gradient was fractionated and each fraction was monitored for alkaline stable, acid precipitable radioactivity as described in MATERIALS AND METHODS. The radioactivity incorporation profile of ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ from different gradients are presented in the individual panels.

Panels A', B', C', D', E' Post infection with hydroxy-
urea (500 ug/ml)

Panels A, B, C, D, E Post infection without hydroxy-
urea (control)

● — ● ^3H -adenine
○ — ○ ^{14}C





DNA density is much less than the control. This is especially evident at the later hours of infection (3-6, 4-7 hours) where the uptake of ^3H -adenine increases rapidly in the normal system, but not in the hydroxyurea containing system. Previous experiments showed that hydroxyurea inhibited host DNA synthesis and the above experiments show that it also inhibits phage DNA synthesis but not the formation of PIL-DNA.

The fractionation profiles from CsCl gradients of the infected culture in the presence of nalidixic acid are quite different from the controls and from the hydroxyurea treatments (Figure 30). Both the formation of PIL-DNA and the incorporation of ^3H -adenine are strongly inhibited by this agent. Therefore, nalidixic acid blocks mechanisms required for both PIL-DNA synthesis and DNA precursor incorporation.

Interpretation of the above results is strongly dependent on a knowledge of the mechanism of action of these inhibitors in the host-phage system.

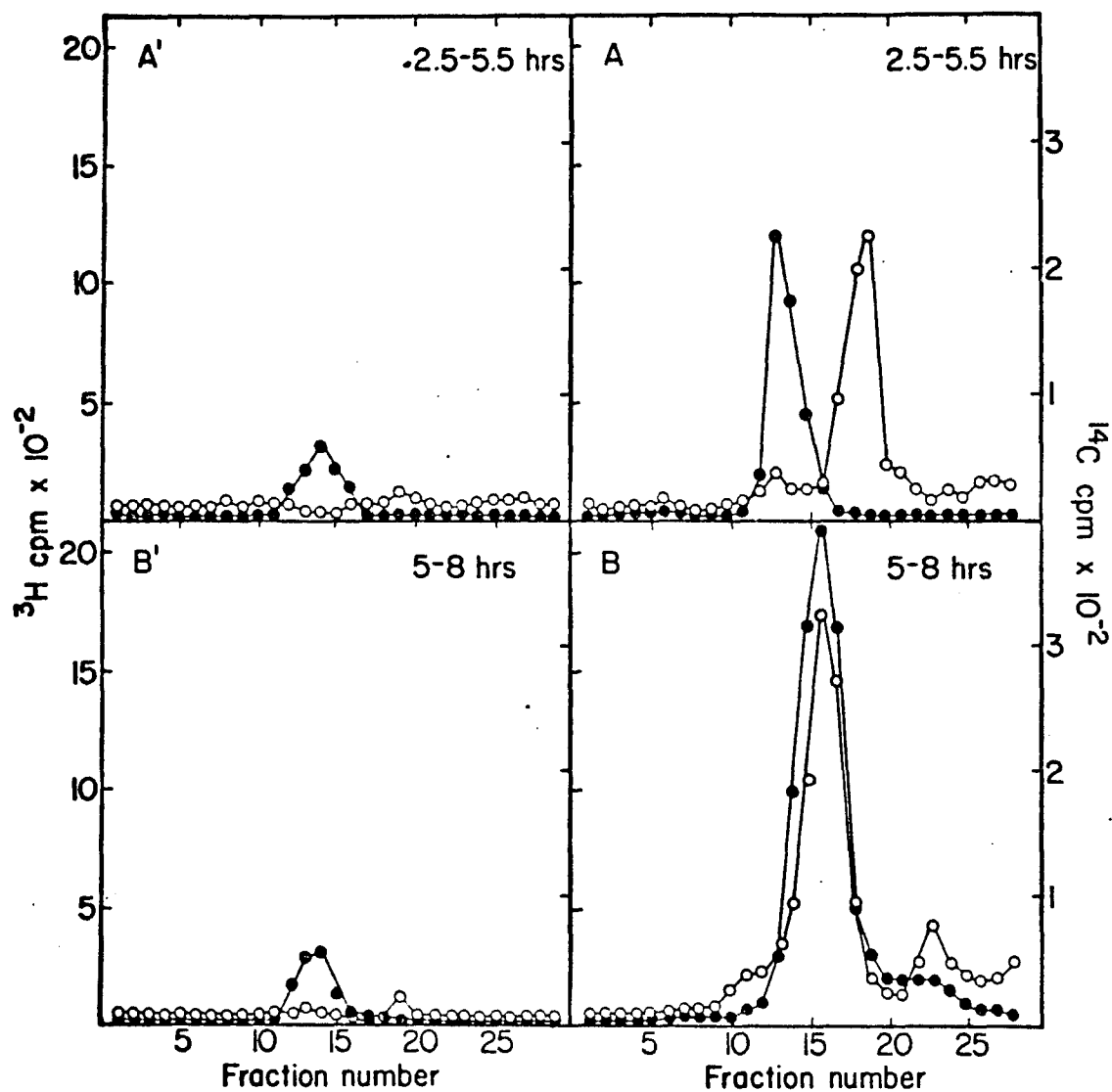
Figure 30. Fractionation Profiles from CsCl Gradients of Anacystis nidulans Infected with AS-1 in the Presence of Nalidixic Acid and Simultaneously Pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine

AS-1 was added to a synchronized A. nidulans culture (MOI=5) supplemented with NaCl to give a final concentration of 0.1 M. Nalidixic acid (NA) was added to half of the infected culture to a final concentration of 500 ug/ml. The remainder of the infected culture under the identical conditions served as the control. From each of the infected cultures 10 ml was removed at 2.5 hr and 5 hr and double-labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine for 3 hr. At the end of the pulse-labeling period (i.e. 5.5 hr, 8 hr) the sample was subjected to preparative ultracentrifugation on a CsCl density gradient. Each gradient was fractionated and fraction monitored for alkaline stable, acid precipitable radioactivity as described in MATERIALS AND METHODS. The radioactivity incorporation profile of ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ from different gradients are presented in the individual panels.

Panel A', B' post infection with nalidixic acid (500 ug/ml)

Panel A , B post infection without nalidixic acid, served as controls

● — ● ^3H -adenine
○ — ○ ^{14}C



V. The Effect of UV-irradiation on *Anacystis nidulans*
and AS-1, and the Development of AS-1
in *Anacystis nidulans*

Both hydroxyurea and nalidixic acid are DNA synthesis inhibitors as revealed by the inhibition studies discussed in the previous section. However, their effects were not seen to be specific for either AS-1 or *A. nidulans* DNA synthesis during infection. These inhibitors, therefore, could not be utilized in experiments where a specific cessation of cellular DNA synthesis or phage DNA synthesis was desired. Results collected from inhibitor experiments so far indicate that the effect of the inhibition is felt by the infective system as a whole. In an attempt to specifically interfere with either the host or the phage DNA metabolism, and hence separate the effects of new phage DNA synthesis from the effects of host cell breakdown and PIL-DNA formation, UV irradiation of either the host cell or the intact phage particle prior to infection was performed.

It has been established that under normal conditions of UV irradiation, almost all of the biologically effective damage is due to pyrimidine dimer formation in polynucleotide chains. This dimer acts as a block for DNA synthesis. It is also known that the effects of UV irradiation on DNA synthesis may be reversed by the action of visible light in some microorganisms (131). Prior to the preparation of UV-damaged *A. nidulans* and cyanophage AS-1, the extent of

photoreactivation of cells, phage and the infective system following UV-irradiation must be determined, and the condition necessary for maximum prevention of this photorecovery established. With determination of photoreactivation, UV-damaged cells and phage which have lost 99% of their viability or lytic ability may be obtained and utilized in further experiments to analyze the DNA metabolic patterns in the infective systems.

A. The Effect of UV-irradiation on Anacystis nidulans

1) Studies of Photoreactivation in UV-irradiated Anacystis nidulans

The property of photorecovery from ultraviolet (UV) radiation damage has been demonstrated to exist in cyanobacteria (12,19,175). This fact necessitated a determination of the extent of photorecovery by UV-irradiated A. nidulans cells, exposed to various light or dark periods, before their use in studies of the AS-1/A. nidulans infective system.

A UV-exposure time of four minutes was utilized for the photo reactivation studies. This period is sufficient to cause a 50% loss in algal cell viability in the presence of continuous light following the UV-irradiation period. Subsequent to the exposure the culture was separated into two flasks. One was illuminated immediately after UV-irradiation (UVCL), while the other was incubated in the dark. At the 0, 4, 8 and 24 hour intervals, aliquots were removed from both flasks, diluted and plated out onto cyanophycean

agar plates, which were then incubated in the light for 5-6 days. Exponentially growing non-irradiated cells served as the control system (CL, CD) for UVCL and UVCD conditions. The viable counts of the different samples and the percentage of photoreactivation are recorded in Table IV and Figure 31. The viability of the controls subjected to each condition was considered 100%. Comparison of the continuously illuminated UV-irradiated cells (UVCL) with the control (CL) revealed a 51% viability for those samples plate out immediately after UV-exposure. Viability measurements of 56%, 62% and 74% were obtained for the samples that had been incubated in light for 4, 8 and 24 hours prior to plating. Because of the greater chance for photoreactivation, increases in light incubation before plating are accompanied by a higher algal viability. From the UV-irradiated samples kept in the dark (UVCD) for 4, 8 and 24 hours before plating, 42%, 21% and 0.008% of the cells were viable. This rapid decrease in cell population is correlated with the increase of the dark incubation period before plating of UVCD samples. The results indicate that a rapid and efficient repair mechanism exists in the UV-irradiated Anacystis nidulans cells that is light requiring for its catalysis. Dark incubation results in a decrease in this photorecovery function. Almost no repair occurred in the algal cells which were kept in the dark for 24 hours before illumination. This 24 hour dark incubation period is utilized later in the study of the dark survival ability of A. nidulans.

Table IV Studies of Photoreactivation as Assayed by
Viable Counts of UV-irradiated Anacystis
nidulans Cultures

Exponentially growing Anacystis nidulans cultures were irradiated with UV-light as described in MATERIALS AND METHODS. After 4 min of irradiation, half of the culture was immediately exposed to light (UVCL). The remainder was wrapped in aluminum foil and incubated in the dark (UVCD). At 0, 4, 8 and 24 hr of incubation triplicate 0.1 ml samples of the UVCL and UVCD cultures were removed, diluted and plated onto cyanophycean agar plates and incubated over a fluorescent light bank. A set of non-irradiated samples which were collected under identical conditions to those above were used as controls (CD, CL). The colony forming units were counted after incubation for 4-6 days.

TABLE IV

Time in light ⁵	CL ¹ Viable cells/ ml ⁶	UVCL ² Viable cells/ ml	UVCL/CL x 100%
0	1.75×10^7	8.96×10^6	51%
4	1.70×10^7	9.85×10^6	56%
8	1.80×10^7	1.14×10^7	62%
24	1.96×10^7	1.45×10^7	74%

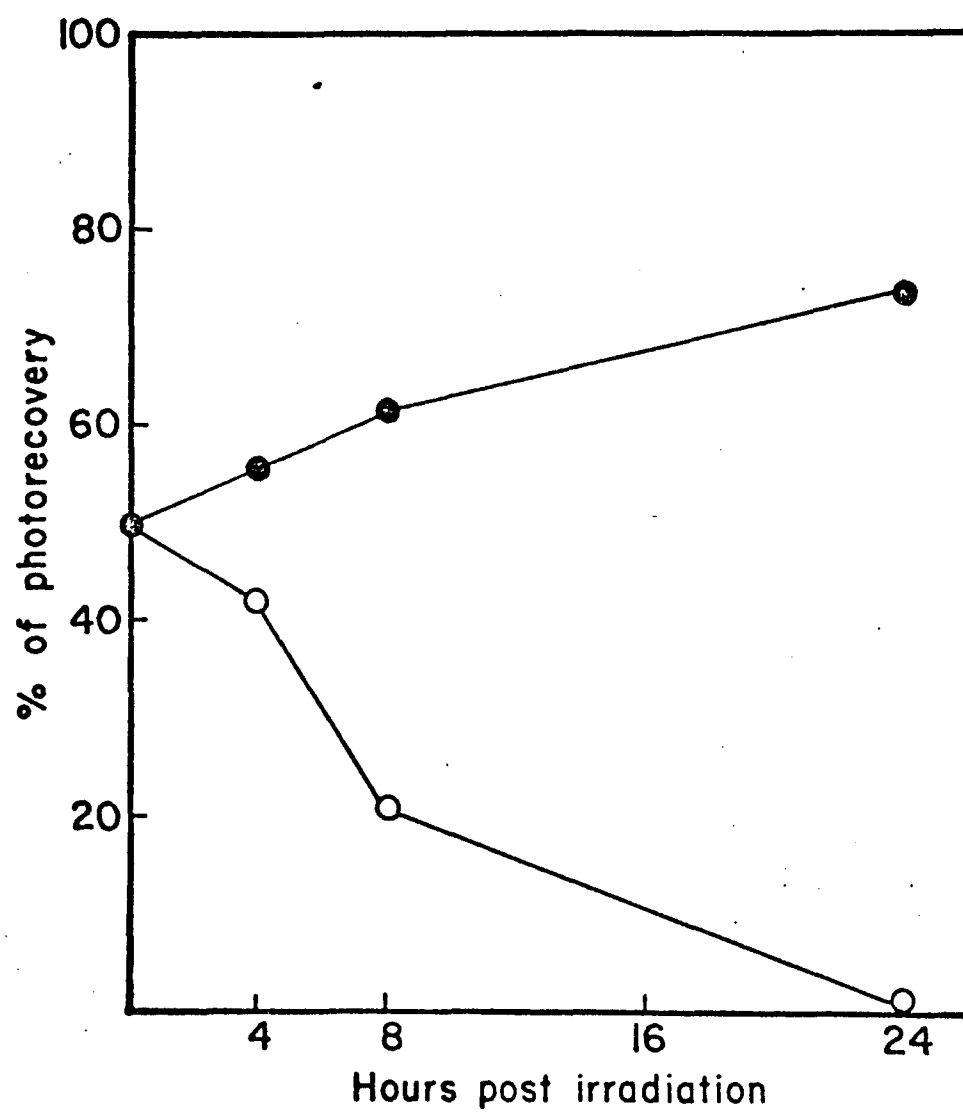
Time in dark ⁵	CD ³ Viable cells/ ml	UVCD ⁴ Viable cells/ ml	UVCD/CD x 100%
0	1.75×10^7	8.96×10^6	51%
4	1.40×10^7	6.00×10^6	42%
8	1.47×10^7	3.12×10^6	21%
24	1.27×10^7	1.10×10^5	0.008%

- 1 CL: cells kept in light continuously
- 2 UVCL: UV-irradiated cells kept in light continuously
- 3 CD: cells kept in the dark
- 4 UVCD: UV-irradiated cells kept in the dark
- 5 Time in light: CL, UVCL kept in the light for different time periods and then plated out
Time in dark: CD, UVCD kept in the dark for different time periods and then plated out
- 6 Each cells/ml represents an average of three samples

Figure 31 Photoreactivation Studies as Observed by
Percentage Survival of UV-irradiated Anacystis
nidulans Cultures

Exponentially growing Anacystis nidulans cultures were exposed to UV-irradiation as described in MATERIALS AND METHODS. The preparation and plating of different UVCL and UVCD samples were detailed in the legend to Table 4. The percentage survival for different experimental conditions was derived from the ratio of UV-cell and control cell.

● — ● UVCL
○ — ○ UVCD



2) Dark Survival Curve of UV-irradiated Anacystis nidulans

An exponentially growing A. nidulans culture was exposed to UV-irradiation for 5 minutes. Following each 30 second exposure interval aliquots were removed, plated and maintained in the dark for 24 hours. A subsequent light incubation period of 4-6 days was imposed and a dark survival curve of UV-irradiated cells represented by colony counts and percentage of viability was obtained. This is presented in Figure 32. The colony counts obtained from a non-irradiated exponentially growing cell sample served as the standard for 100% survival. At one minute of irradiation, more than 50% of the cells lose their viability. At 3 minutes only 1% of the cells are viable, while at 5 minutes almost all the cells are damaged. It is clear that when the UV-exposure time is increased, the population of viable cells decreases rapidly and exponentially. Since a 3 minute irradiation causes a 99% loss in cellular viability, this exposure time was utilized to prepare UV-damaged cells for further experiments.

B. The Effect of UV-irradiation on Cyanophage AS-1

1) The Survival Curve of UV-irradiated AS-1 Measured by Lytic Ability and Plaque Formation

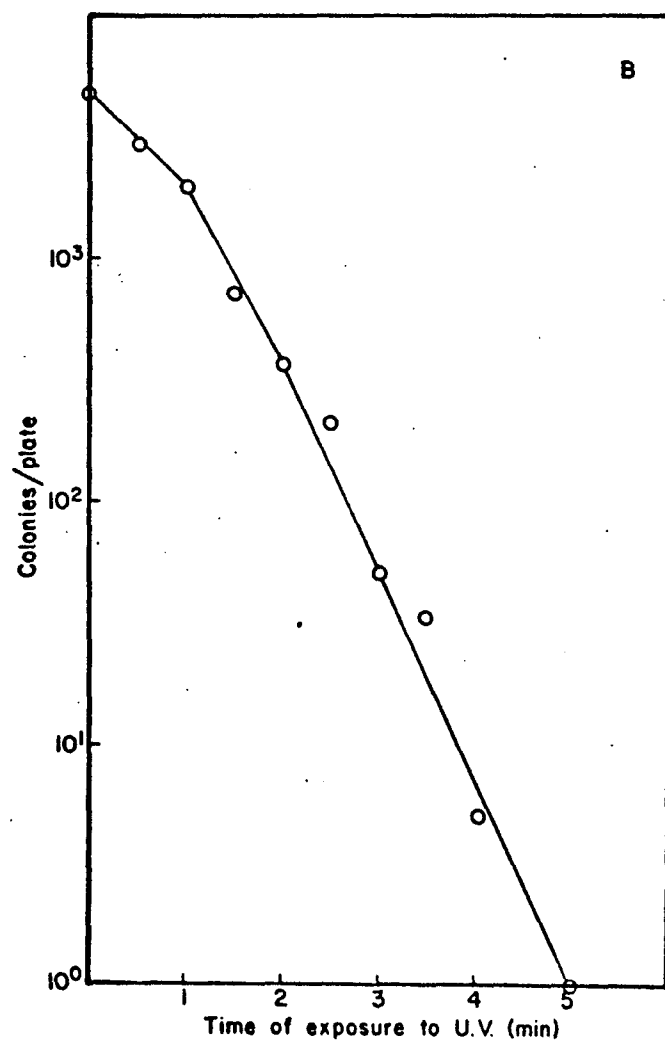
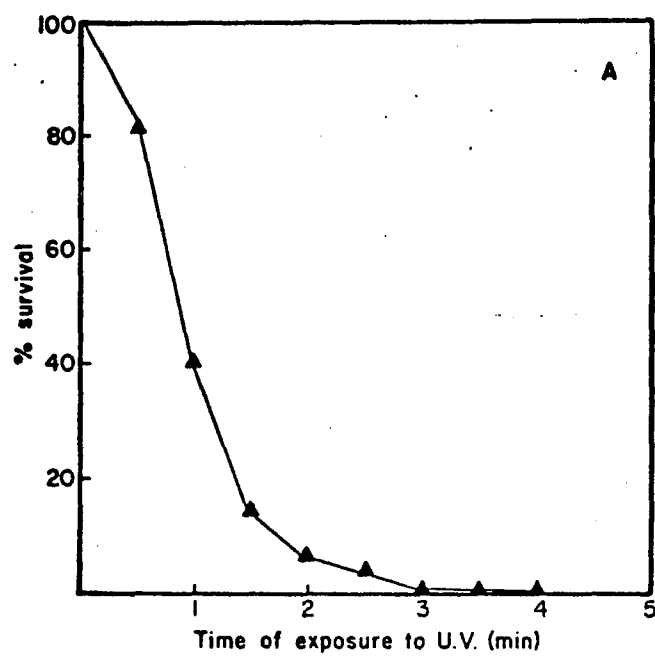
The crude AS-1 lysates were exposed to UV-irradiation for different time periods. Following each exposure time, aliquots were removed and assayed for lytic ability and

Figure 32 Dark Survival Curve of UV-irradiated Anacystis nidulans as Represented by Colony Counts and Percentage of Viability

An exponentially growing cell culture was placed in a UV-irradiation chamber equipped with a germicidal lamp emitting at 254 nm as described in MATERIALS AND METHODS. The irradiation took place in the absence of any other light. Every 30 seconds of a 5 minute UV-exposure period, triplicate 0.1 ml samples were removed, diluted, and plated onto cyanophycean agar plates. The plates were wrapped with aluminum foil, incubated in the dark for 24 hours, and then exposed to light. Colonies were counted after 4-6 days. Percent viability was obtained by comparison of the UV-irradiated samples with the non-irradiated controls.

▲—▲ Panel A: percentage of viability

○—○ Panel B: colony counts



plaque formation as depicted in Figure 33 and Figure 34. The decrease observed in both lytic ability and plaque formation was proportional to the UV-exposure time. The survival curve obtained from both assays were well correlated. A non-irradiated phage stock was used as a control and its lytic ability was regarded as 100%. During the first four minutes of UV-irradiation, a rapid and exponential decrease in lytic ability and in PFU/ml is found. Approximately 50% of the phage lost their viability after four minutes of UV exposure. In the range of 5 to 20 minutes of UV irradiation, however, the damage of the UV-light to phage is not uniformly related to the dosage of radiation as was previously observed in the 4 minute range. The inactivation curve is still an exponential one, but it possesses a much less negative slope. During the 20-40 minutes of UV exposure time the number of survivors decreases dramatically and exponentially as the time of UV exposure increases. At the end of this period almost no viable phage may be detected.

The survival curve of cyanophage AS-1 is a discontinuous one. Initially it decreases exponentially, and then, over a broad dosage range, the rate of decrease though still exponential, is diminished. At very high dosages it drops as before. The existence of this type of curve could be explained by the presence of a number of more resistant particles which do not contribute significantly to the rate of cell death until they become a large fraction of the surviving population. With increasing dosage of UV-irradiation, the

Figure 33 The Lytic Ability Curve of UV-irradiated Cyanophage AS-1

The crude phage lysates were collected as described in MATERIALS AND METHODS. After transfer to a sterile petri dish the lysates were exposed to UV-light in a UV-irradiated chamber as described in the legend to Figure 32. At different time intervals triplicate 2 ml aliquots of the irradiated phage suspensions were removed and added to exponentially growing Anacytis nidulans cultures (MOI=5). Non-irradiated phage lysates were added to algal cultures as controls. The turbidities of the cultures infected with either UV-irradiated or non-irradiated phage were monitored spectrophotometrically by reading a 1 ml aliquot of each culture at OD_{750 nm} immediately. Following infection cultures were kept in continuous light at 31-34°C for 24 hr. The lytic abilities were then determined by decreases in turbidity of the infected cultures at OD_{750 nm}. The % of lytic ability was obtained by comparison of the turbidity change in cultures infected with UV-irradiated phage to that in the control.

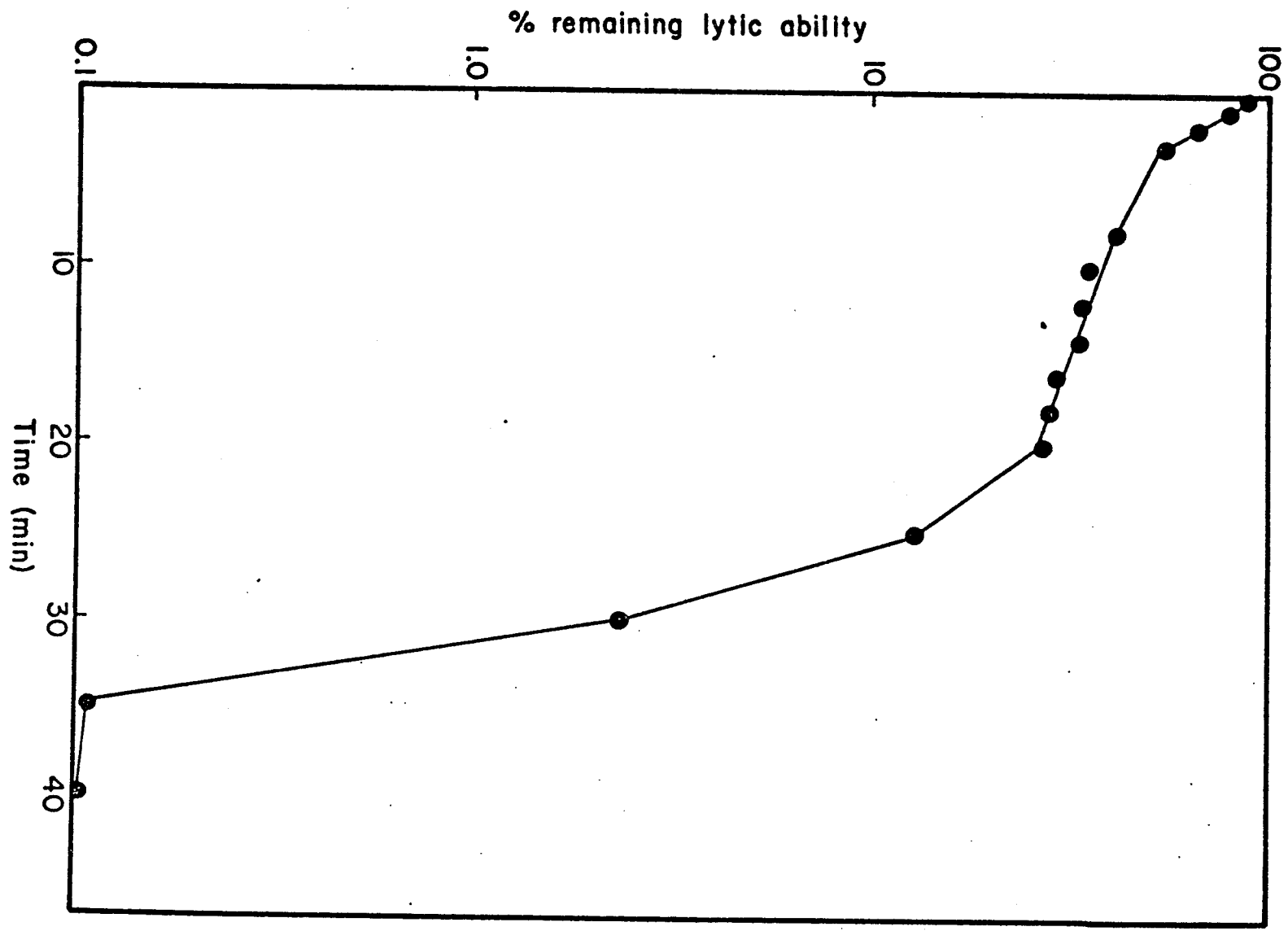
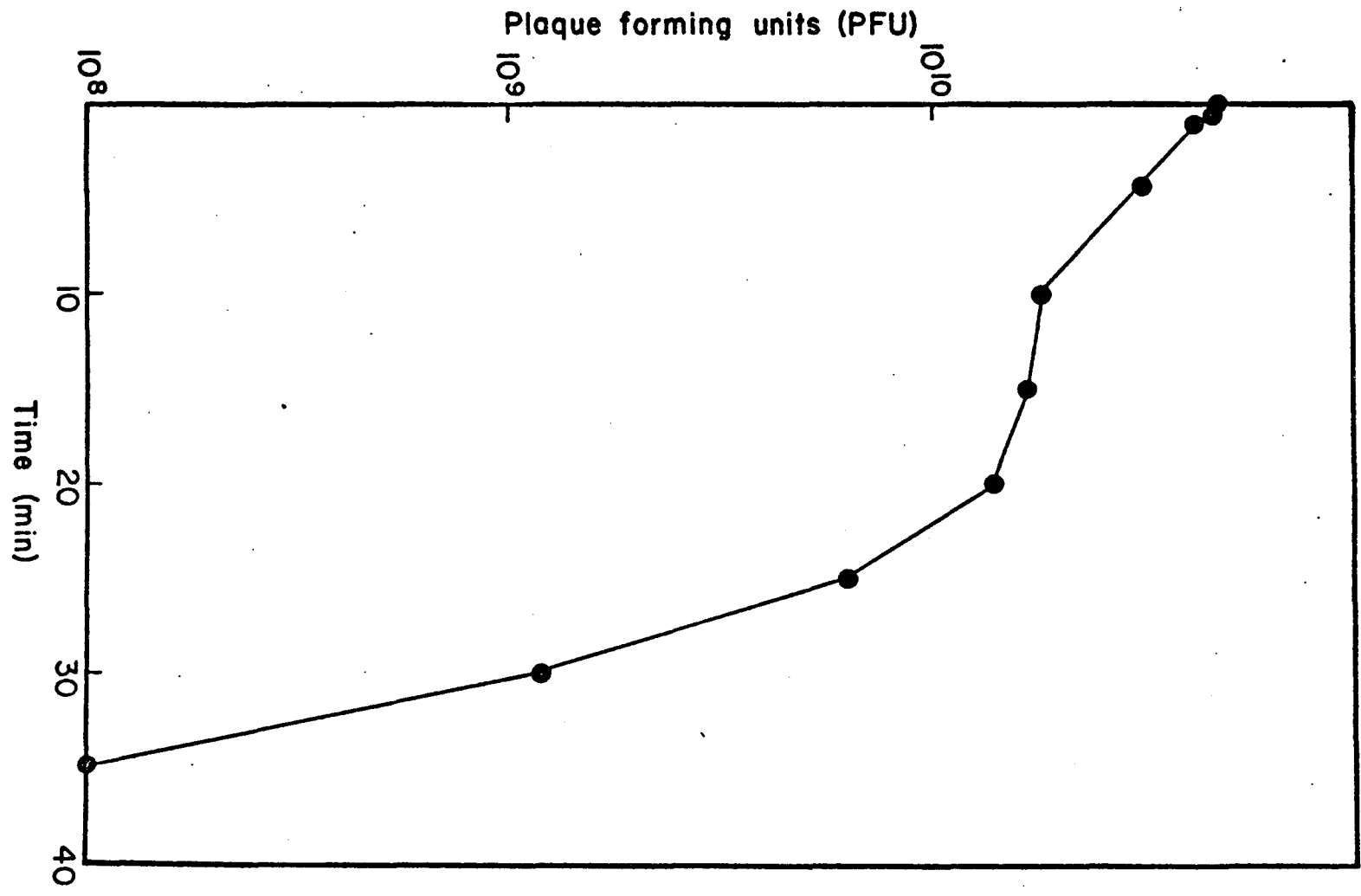


Figure 34 Survival of UV-irradiated Cyanophage AS-1 as
Determined by Plaque Forming Ability

The crude phage lysates were collected as described in MATERIALS AND METHODS. After transferring to a sterile petri dish the lysates were exposed to UV in an UV-irradiation chamber as described in the legend to Figure 32. At different time intervals of UV-exposure, 1 ml of each irradiated phage suspension was removed. The production of cyanophage AS-1 in different samples was measured by the titer techniques described in MATERIALS AND METHODS. The non-irradiated phage lysates served as the control.



sensitive particles rapidly disappear and the more resistant population can now be observed to decline exponentially at their own rate. With exposure to a dosage beyond all phage resistance capacities, any difference between members of the population is eliminated and the particles present again show a rapidly decreasing curve.

A more likely explanation of the above results is to be found from an examination of the experimental protocol in which the phage were introduced to viable cells in the light directly after irradiation. Under these circumstances the strong photorepair mechanism that exists in the host cells might be responsible for reactivating the incoming phage genome.

Although a small portion of UV damage is irreversible, the major effects may be repaired, resulting in a decrease in the inactivation caused by the UV-exposure and a shallower survival curve. After more than 20 minutes of UV-exposure, the UV-dosage is so great that complete repair is rendered impossible and the curve displays the steep drop observed previously.

Because 35 minutes of UV-irradiation causes a 99% loss of viability in the cyanophage AS-1, this exposure time is used for the experiments in the preparation of UV-damaged phage.

2) Photoreactivation Study of Cyanophage AS-1

a) Photorecovery of UV-irradiated phage

The crude phage lysates were exposed to UV-light for 35 minutes, after which time part of the samples were kept in light and the remainder incubated in the dark. Following the different incubation periods, the samples were added to the exponentially growing cells and illuminated continuously. The lytic ability and plaque forming units (PFU/ml) for the individual samples are shown in Figure 35 and Figure 36. Controls for each condition were prepared by using a healthy phage infective system. The turbidity change at OD_{750nm} of the infected culture was utilized as a measure of the phage lytic ability. The UV-irradiated phage samples kept in light or dark for 0 and 8 hours are found to display a slight decrease in turbidity. For the samples incubated 24 hours in the light or dark prior to infection no decrease in turbidity was found. At this time the effect of algal growth and lysis are probably balanced. It is known that the crude phage lysates lose some of their infectivity if they are kept too long either in light or darkness before infection, and this phenomenon is displayed in the control samples which were kept in the dark for different periods of time before infection. Since only a slight turbidity change is detected in the UV-irradiated phage samples incubated in light or dark for 0 and 8 hours, no turbidity change in the samples of longer incubation period (24 hours) may be

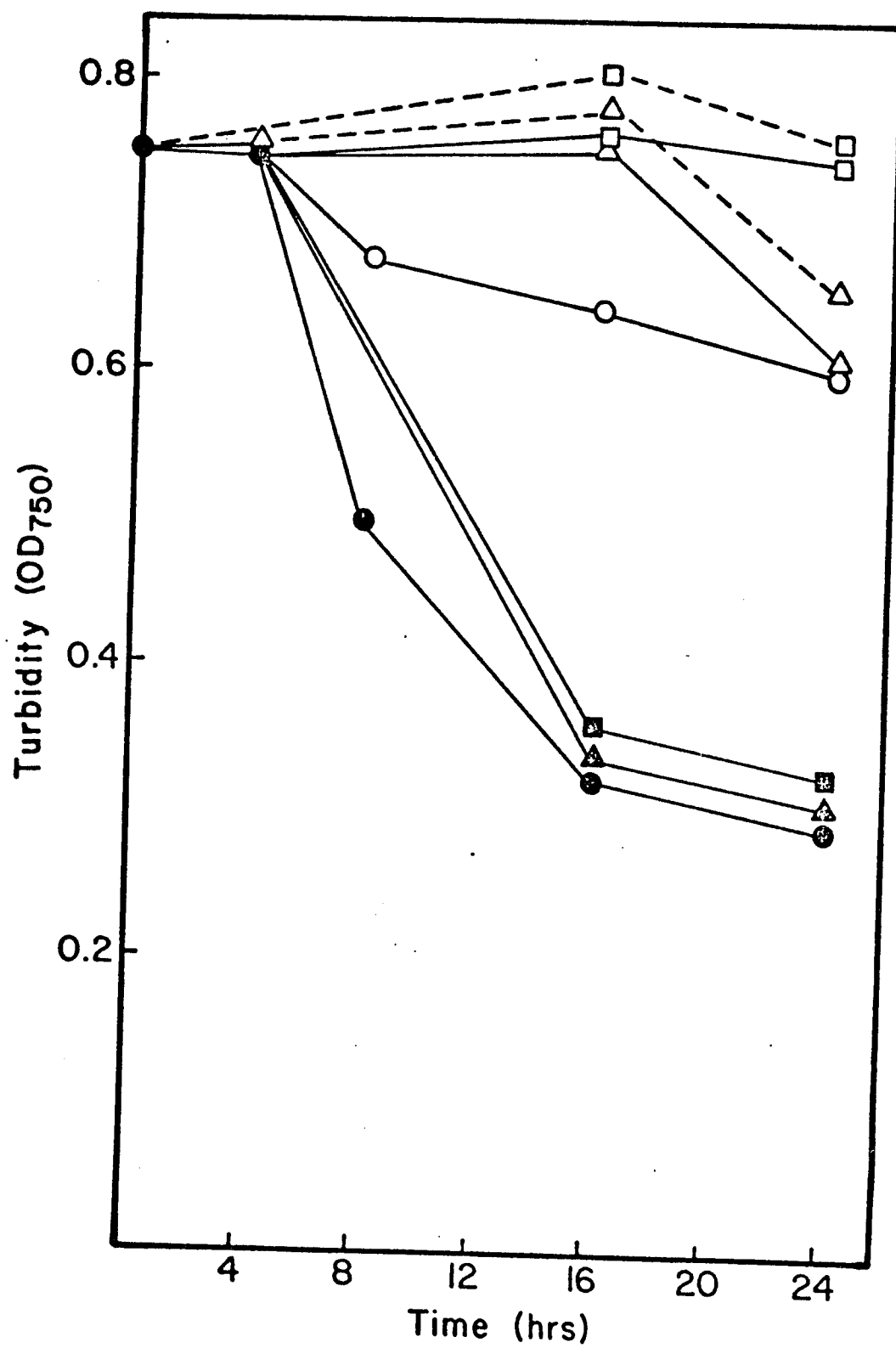
Figure 35 Photoreactivation of UV-irradiated Cyanophage AS-1 as Determined by Lytic Ability

The crude phage lysates were prepared and exposed to UV-light as described in MATERIALS AND METHODS and the legend to Figure 32. After 35 min of UV-irradiation, one third of the samples were added to an exponentially growing Anacystis nidulans culture and were illuminated by a fluorescent light bank. The remaining samples of UV-irradiated AS-1 were wrapped with aluminum foil and incubated in the dark. After an 8 hr dark period half of the samples were added to an exponentially growing algal culture and then exposed to light. After a total of 24 hr in the dark the remaining samples were added to a third exponentially growing algal culture and then exposed to light. For a control, non-irradiated AS-1 was used to infect the algal culture under the various conditions described above.

Figure 35 continued/

At different time periods after exposure of the infected culture to the light the turbidities of the cultures infected with either UV-irradiated or non-irradiated phage were monitored spectrophotometrically at OD_{750 nm}.

- phage (0 hr dark) + algae
- ▲—▲ phage (8 hr dark) + algae
- phage (24 hr dark) + algae
- UV-phage (0 hr dark) + algae
- △—△ UV-phage (8 hr light) + algae
- △—△ UV-phage (8 hr dark) + algae
- UV-phage (24 hr light) + algae
- UV-phage (24 hr dark) + algae



expected.

Plaque formation by UV-irradiated phage maintained under identical conditions to those just mentioned is presented in Figure 36. For UV-damaged phage kept either in light or dark for 0 or 8 hours, a similar amount of phage production was obtained. Similar PFU/ml but smaller burst size are found when phage samples are subjected to either light or dark incubation for 24 hours prior to infection.

According to the data presented above, similar lytic and plaque forming abilities are obtained from the UV-damaged phage whether incubated in the light or in the dark for 0-8 hours prior to infection. This indicates that no photo-reactivation of the cyanophage itself occurs in the presence of light. The low PFU/ml and the absence of any turbidity change were found in both 24 hour light- and dark-incubated samples. This may be due merely to a decrease in infectivity resulting from the long incubation period. Since a strong repair mechanism is found in the UV-irradiated Anacystis nidulans, the possibility that photorepair of UV-damaged phage can occur inside the infected cells must be investigated.

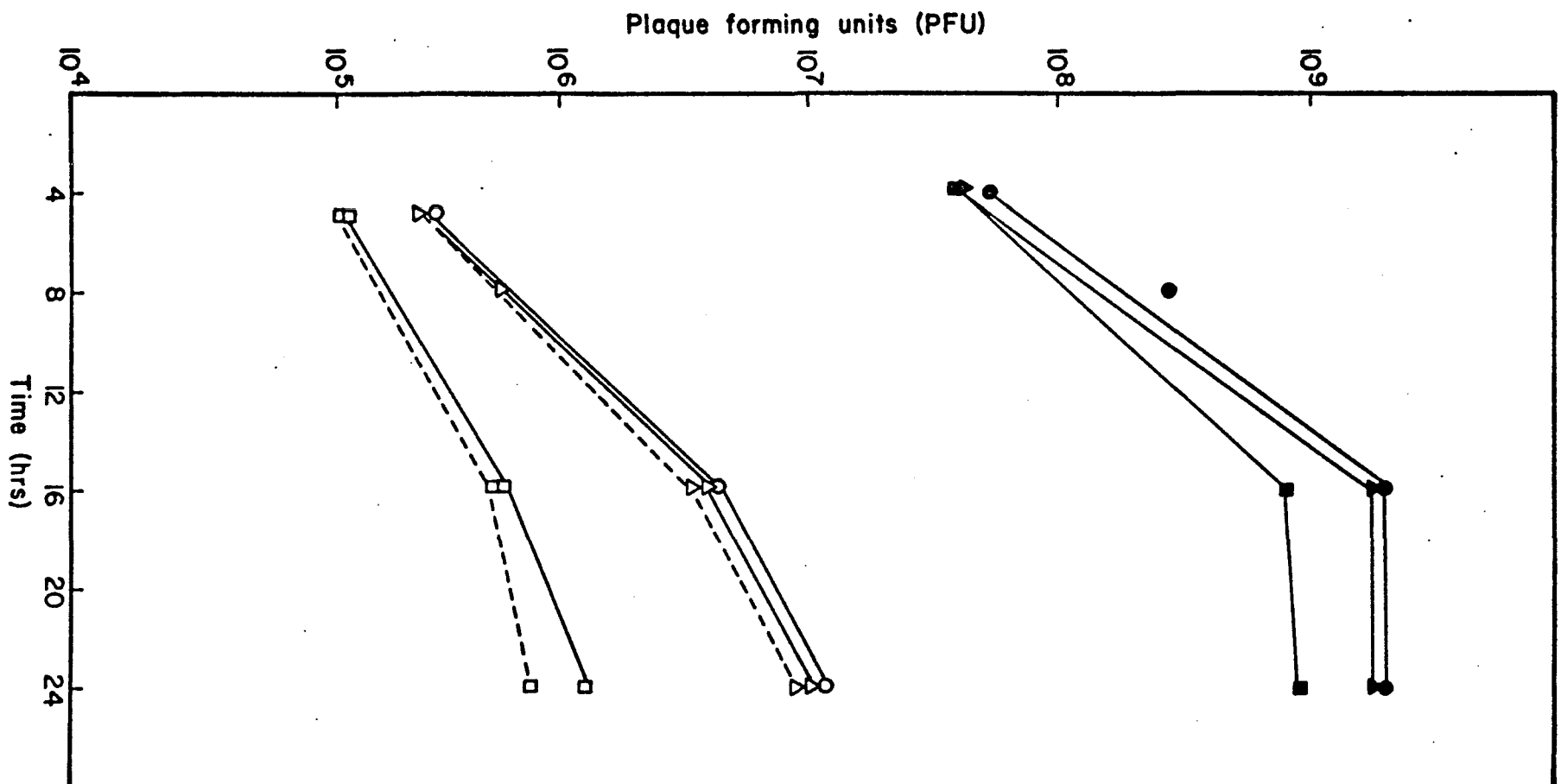
- b) The study of photorecovery of UV-irradiated AS-1 inside the infected Anacystis nidulans

Cyanophage AS-1, which had been exposed to UV-irradiation for 35 minutes, was added to exponentially growing cells. After 0, 8, or 24 hours of incubation in the dark, the

Figure 36 Photoreactivation of UV-irradiated Cyanophage AS-1 as Determined by Phage Production

The crude phage lysates were prepared and exposed to UV as described in MATERIALS AND METHODS and the legend to Figure 32. After 35 min of UV-irradiation the phage AS-1 lysates were separated into three flasks and were kept in the dark for 0, 8, or 24 hr. At each time point the phage were added to exponentially growing algal cultures which were then subjected to continuous illumination. Algal cultures infected with identically incubated non-irradiated phage served as controls. At different time periods after exposure of the infected culture to light, the phage production of each culture infected with either UV-irradiated or non-irradiated phage was monitored by titering.

- phage (0 hr dark) + algae
- ▲—▲ phage (8 hr dark) + algae
- phage (24 hr dark) + algae
- UV-phage (0 hr dark) + algae
- △—△ UV-phage (8 hr light) + algae
- △--△ UV-phage (8 hr dark) + algae
- UV-phage (24 hr light) + algae
- UV-phage (24 hr dark) + algae



infected cultures were then illuminated continuously. The lytic ability and phage production (PFU/ml) were determined and the data are presented in Figure 37 and Figure 38. A parallel set of cultures using non-irradiated phage served as controls.

Prior to studying the photorecovery of UV-damaged phage inside the cells, the effect of dark incubation of the infected culture on the development of cyanophage AS-1 had to be determined. It was determined that there are no significant changes in turbidity and in phage titer in the infected culture after 8 or 24 hours of dark incubation. This indicates that the complete lytic cycle of AS-1 cannot take place in the dark; thus no lysis of the host occurs. Phage was found to be adsorbed onto algal cells in the dark and this phenomenon is in agreement with the data of Allen et al. (8)

After continuous illumination for a complete lytic cycle (about 16 hours), the pre-dark incubated samples have less lytic ability and plaque formation than the continuously illuminated samples. A lysis of 84% and 70% of the continuously illuminated samples are obtained in 8 and 24 hours pre-dark incubated samples respectively. Similar results are also evident in the PFU studies. Therefore, the longer the dark pre-incubation period, the less the lytic ability and PFU/ml that are obtained. At the end of a 24 hour exposure to continuous light the same PFU/ml are obtained regardless of prior dark treatment. The control culture, which was not

Figure 37 The Photoreactivation of UV-irradiated Cyanophage AS-1 Inside Anacystis nidulans as Determined by Lytic Ability

The preparation of UV-irradiated phage AS-1 and the experimental protocols are identical to those described in the legend to Figure 32. The UV-irradiated phage infected cultures were kept in the dark for 0, 8, and 24 hr and then subjected to continuous illumination. At different time periods after illumination the turbidity of the culture infected with either UV-irradiated or non-irradiated phage was determined spectrophotometrically at OD_{750 nm}.

- algae + phage with 0 hr dark treatment
- algae + phage with 8 hr dark treatment
- algae + phage with 24 hr dark treatment
- algae + UV-phage with 0 hr dark treatment
- algae + UV-phage with 8 hr dark treatment
- algae + UV-phage with 24 hr dark treatment

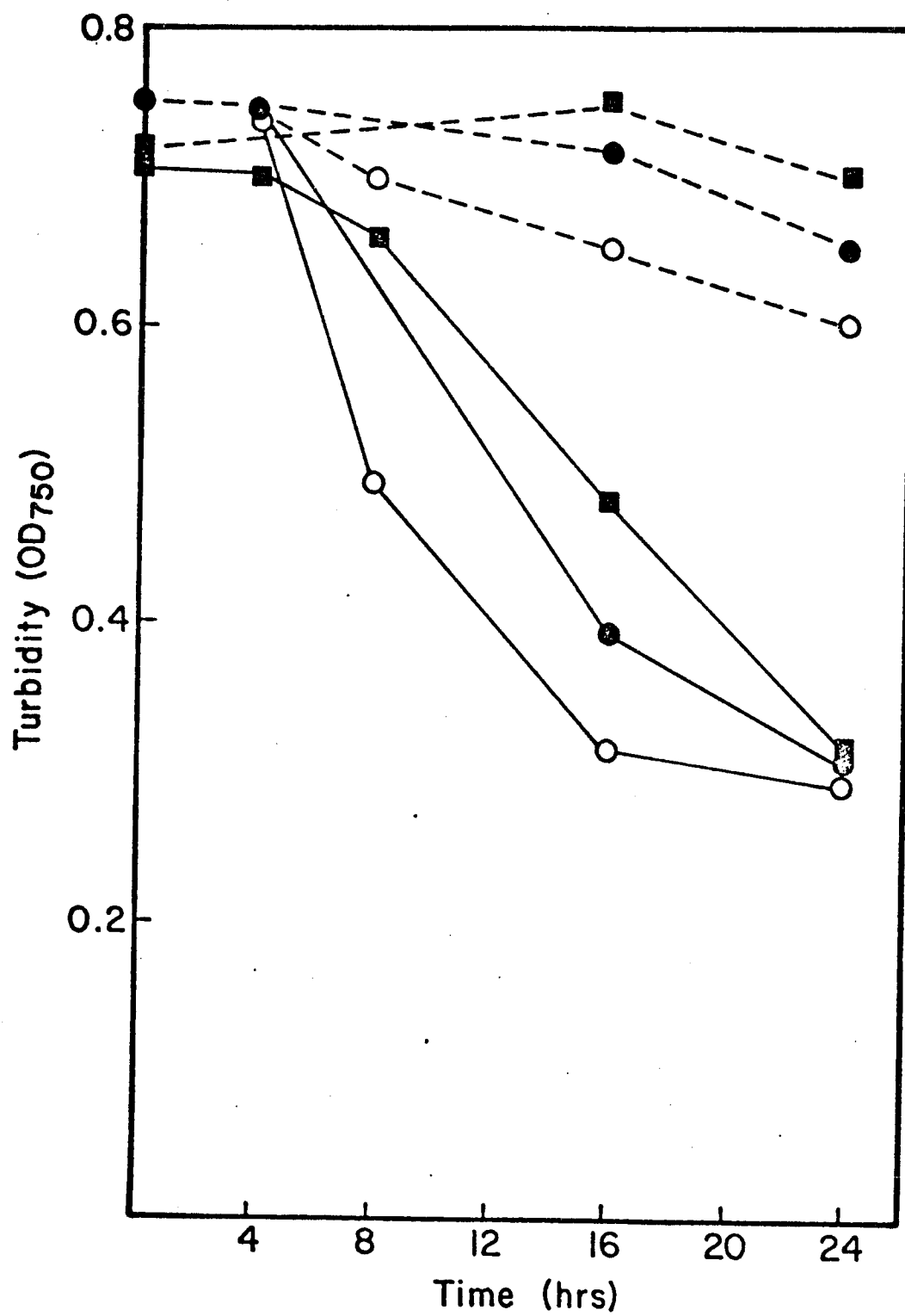
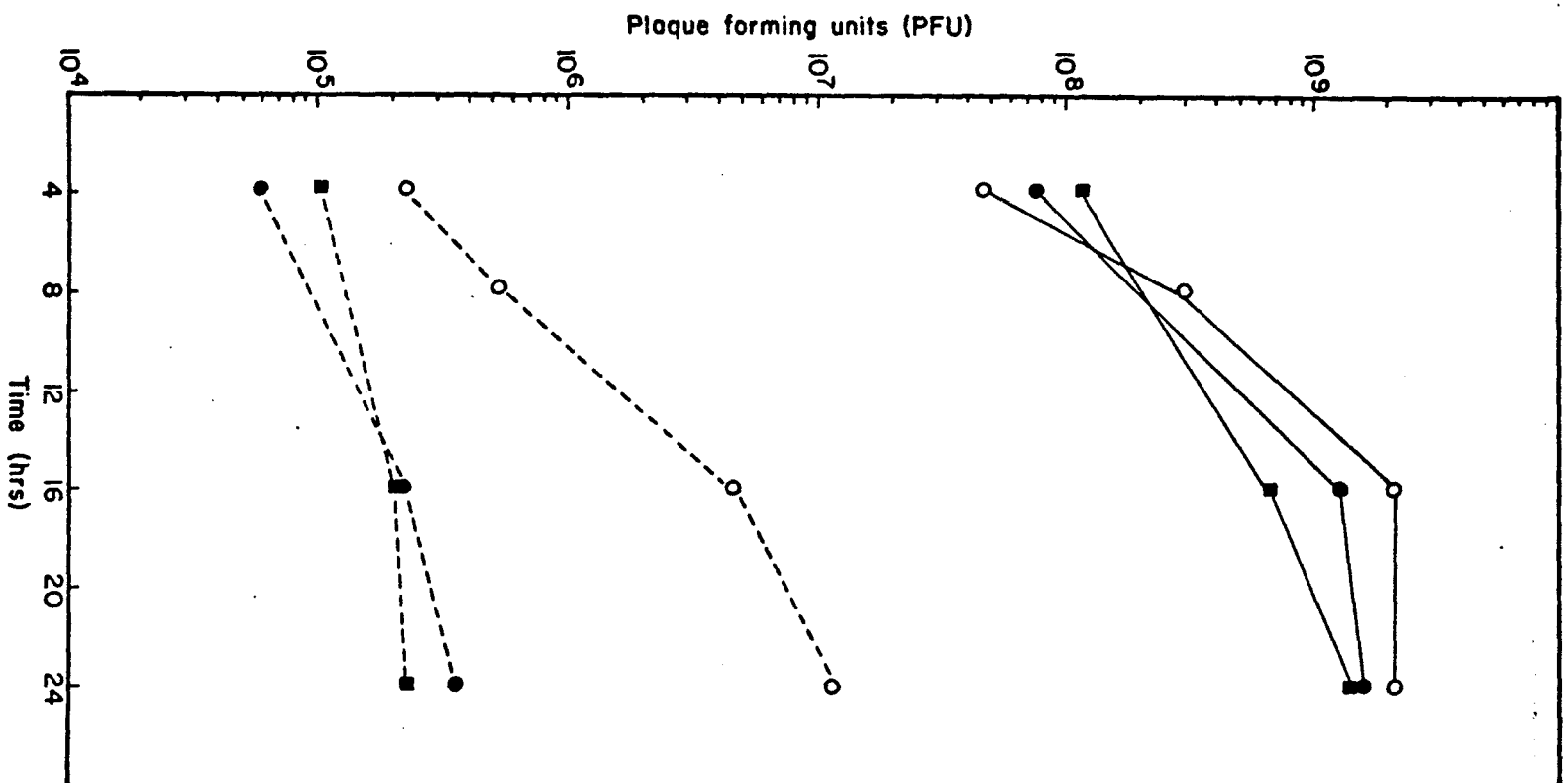


Figure 38 Photoreactivation of UV-irradiated Phage AS-1 after Infection into the Host Anacystis nidulans as Determined by Phage Production.

The crude phage lysates were prepared and exposed to UV-light as described in MATERIALS AND METHODS. After 35 min of UV-irradiation the cyanophage AS-1 was added to the exponentially growing A. nidulans culture and then kept in the dark for 0, 4, 8, and 24 hr. At each time point 20 ml of the infected culture was removed and subjected to continuous illumination. Algal cultures infected with non-irradiated phage (under identical conditions as irradiated phage) served as controls. At different time periods after exposing the infected culture to light, the phage production of each culture infected with either UV-irradiated or non-irradiated phage was monitored by titers.

- algae + phage with 0 hr dark treatment
- algae + phage with 8 hr dark treatment
- algae + phage with 24 hr dark treatment
- algae + UV-phage with 0 hr dark treatment
- algae + UV-phage with 8 hr dark treatment
- algae + UV-phage with 24 hr dark treatment



exposed to any dark pre-incubation exhibited a normal lytic cycle with a latent period of 4 hours and a lytic period of 12 hours (4-16 hours post infection). Beyond 16 hours post infection, very little increase in phage titer is observed. A continuous decrease in turbidity and concomitant increase in PFU/ml between 16 and 24 hours post illumination is found in the samples which had been preincubated in the dark for 8 and 24 hours. This may be due to reinfection of the remaining cells by dark-induced early burst phage or to a prolonged lytic cycle resulting from the dark pre-incubation.

Photorecovery of the UV-damaged phage inside the host was determined by incubation of the infected cells for different dark periods. A lytic ability of 25%, 5% and 2% were obtained from the infected cultures which had been incubated in the dark for 0, 8 or 24 hours respectively. It appears that the lytic ability of UV-irradiated phage decreases significantly as compared to the control. A burst size of 52.4, 48 and 24 is found in the controls for 0, 8 and 24 hours of dark preincubation. Dark incubation reduces the burst size to 90% for 8 hour incubation and 50% for 24 hour incubation when compared to the infected culture under normal conditions. This result indicates that prolonged dark incubation after infection affects the production of cyanophage AS-1 as in the normal infective system. A burst size of 25, 8 and 3 is obtained in the 0, 8 and 24 hour dark preincubation of the UV-irradiated phage infected

cultures. The UV-damaged phage-infected culture under continuous light has a burst size 3.1 times greater than the one incubated in the dark for 8 hours and more than 8 times as great as the one incubated in the dark for 24 hours. Comparison of the burst size ratio among different dark incubated samples in both the normal infective system and UV-damaged phage infective system, indicates a much stronger effect of dark preincubation on the UV-irradiated phage samples. The shorter the dark incubation period, the larger is the burst size. This suggests that illumination is more important to the UV-irradiated phage than to the healthy phage infective system. From the data accumulated thus far, we can conclude that the UV-damaged phage can be repaired after infection in the presence of light. Since the cyanophage itself has no photoreactivation system, as described previously, the utilization of a host photorepair mechanism is suggested.

C. The Effect of UV-irradiated Anacystis nidulans or Cyanophage AS-1 on the Incorporation Pattern of Different Precursors into AS-1/A. nidulans Infective System

It has been reported that phage infection alters the host permeability and thus permits the study of various metabolic mechanisms using radioactive precursors in the AS-1/A. nidulans infective system.

In order to follow the changing pattern of synthesis in the UV-damaged infected systems (UV-damaged cell or UV-

damaged phage, or both), the permeability and incorporation patterns of these systems had to be determined. ^3H -adenine, ^{14}C -glucose-6-phosphate and $\text{NaH}^{14}\text{CO}_3$ were used to study the incorporation into different infective systems which were set up as follows:

- (a) healthy phage infecting healthy Anacystis nidulans (algae + phage)
- (b) UV-irradiated phage infecting healthy A. nidulans (algae + UV-phage)
- (c) healthy phage infecting UV-irradiated A. nidulans (UV-algae + phage)
- (d) UV-irradiated phage infecting UV-irradiated A. nidulans (UV-algae + UV-phage)
- (e) Anacystis nidulans (algae)
- (f) UV-irradiated A. nidulans (UV-algae)
- (g) cyanophage AS-1 (phage)
- (h) UV-irradiated phage (UV-phage)

The UV-irradiated cells and UV-irradiated phage were prepared by exposing the cells or phage to UV-irradiation for 3 minutes and 35 minutes respectively as described in previous sections. At 0 hour ^3H -adenine was added to one set of flasks containing combination from (a) to (h). ^{14}C -glucose-6-phosphate and $\text{NaH}^{14}\text{CO}_3$ were likewise added to their respective sets. At 0 hour and every 2 hours thereafter, up to 8 hours, aliquots of 1 ml were removed from each flask and centrifuged. The pellets and the supernatant solution were separated and the radioactivity found in the supernatant

Figure 39 The Incorporation of Radioactivity from ^{14}C -glucose-6-phosphate, ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ Separately into Acid Precipitable Materials of Various Combinations of Phage-infected or Non-infected Anacystis nidulans Cultures (I)

The UV-irradiated phage AS-1 (UV-phage) and UV-irradiated A. nidulans (UV-algae) were prepared as described in Figures 32 and 33, and MATERIALS AND METHODS. Following the preparation of UV-algae and UV-phage the different combinations of samples were set up as follows:

- (a) Three flasks, each containing 15 ml of healthy cells infected with healthy phage AS-1 (MOI=5)---algae + phage;
- (b) Three flasks, each containing 15 ml of healthy cells infected with UV-irradiated phage AS-1 (MOI=5)---algae + UV-phage;
- (c) Three flasks, each containing 15 ml of UV-irradiated cells infected with healthy phage AS-1 (MOI=5)---UV-algae + phage;
- (d) Three flasks, each containing 15 ml of UV-irradiated cells infected with UV-irradiated phage AS-1 (MOI=5)---UV-algae + UV-phage;
- (e) Three flasks, each containing the same volume of healthy cells as above with sterile 3M medium added to replace the phage lysates to give a final volume of 15 ml---algae;
- (f) Three flasks, each containing the same volume of UV-irradiated cells as above with sterile 3M medium added

Figure 39 continued/

to replace the phage lysates to give a final volume of 15 ml---UV-algae.

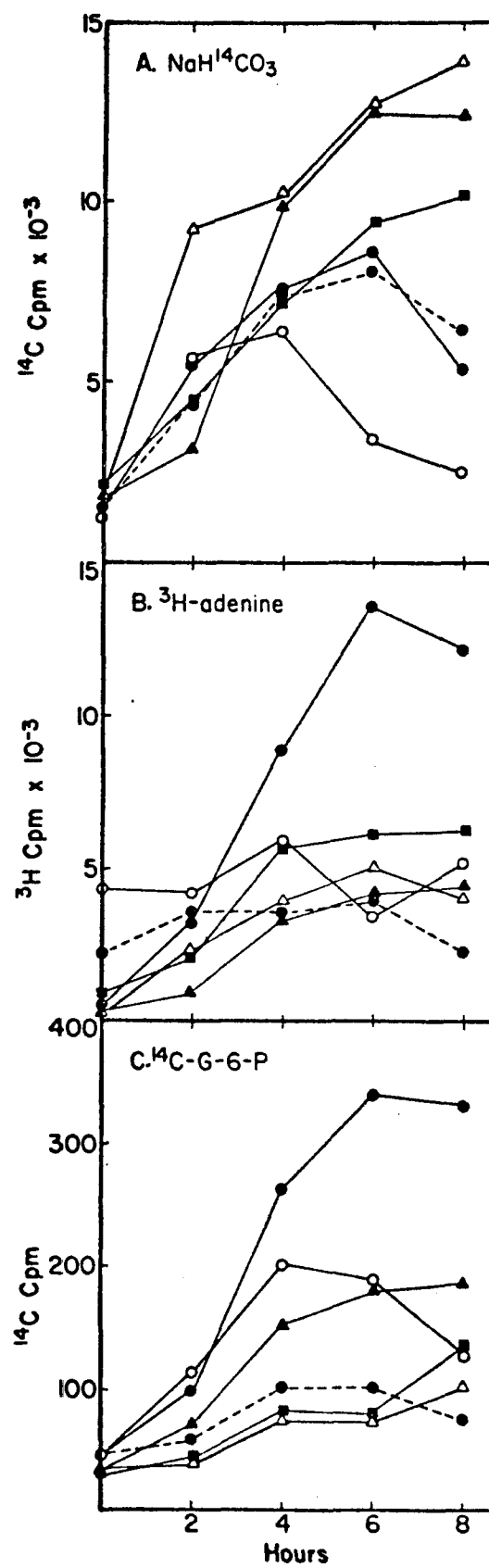
^3H -adenine (0.5 uCi/ml), ^{14}C -6-phosphate (1 uCi/ml) and $\text{NaH}^{14}\text{CO}_3$ were added separately into three different flasks of each combination as described above at 0 hr of infection. One ml was removed from each flask at 0, 2, 4, 6, and 8 hr post infection and then centrifuged at 10,000 rpm for 10 min and the supernatant was decanted. Each pellet was treated with 1 ml of 20% TCA and incubated at 40°C for at least 30 min. The samples were then collected on Whatman GF/A filters, washed three times with cold 5% TCA, and rinsed once with cold ethanol. All the filters were dried and inserted into scintillation fluid and the radioactivity of each filter was counted in a scintillation counter.

Panel A incorporation patterns of $\text{NaH}^{14}\text{CO}_3$

Panel B incorporation patterns of ^3H -adenine

Panel C incorporation patterns of ^{14}C -glucose-6-phosphate

- (a) algae + phage
- ▲—▲ (b) algae + UV-phage
- (c) UV-algae + phage
- (d) UV-algae + UV-phage
- △—△ (e) algae
- (f) UV-algae



solution and in the TCA precipitable material of the pellets were determined (Figure 39). The incorporation patterns of the different radioactive precursors are discussed separately:

1. The Incorporation of ^3H -adenine

The amount of radioactive incorporation of ^3H -adenine for different combinations are presented in order of decreasing incorporation: algae + phage, UV-algae + phage, UV-algae, algae, algae + UV-phage and UV-algae + UV-phage. These data indicate that a significant incorporation of ^3H -adenine, relative to the cells alone, is found in combinations in which healthy phage is the infective agent. This includes those combinations with healthy and UV-irradiated cells (algae + phage, UV-algae + phage), with a higher incorporation exhibited by the algae + phage sample compared to the UV-algae + phage sample. Lower ^3H -adenine incorporation with respect to the cells alone is exhibited by combinations in which UV-irradiated phage is the infective agent for either healthy or UV-damaged cells (algae + UV-phage, UV-algae + UV-phage). In addition, greater incorporation is found for the UV-irradiated cells than the non-irradiated cells. This may be due to a permeability change caused by UV-irradiation.

2. The Incorporation of ^{14}C -glucose-6-phosphate

The incorporation patterns of ^{14}C -glucose-6-phosphate into different samples following infection are very similar to those for ^3H -adenine, as shown in Figure 39. The amount

of ^{14}C -glucose-6-phosphate incorporated into different samples is presented in decreasing order: algae + phage, UV-algae + phage, algae + UV-phage, UV-algae + UV-phage, UV-algae, and algae. An increased incorporation, compared to the cells alone, is found in all the infective systems, including algae + phage, algae + UV-phage, UV-algae + phage, UV-algae + UV-phage, although lower incorporation of ^{14}C -glucose-6-phosphate is found for the UV-irradiated phage infective system than for the normal infective system.

3. The Incorporation of $\text{NaH}^{14}\text{CO}_3$

The decreasing order for incorporation of $\text{NaH}^{14}\text{CO}_3$ by different combinations is as follows: algae, algae + UV-phage, UV-algae, algae + phage, UV-algae + UV-phage, UV-algae + phage. The healthier the cell, the greater is the incorporation of $\text{NaH}^{14}\text{CO}_3$. A lower $\text{NaH}^{14}\text{CO}_3$ incorporation is found in both UV-irradiated cells and phage infected cells.

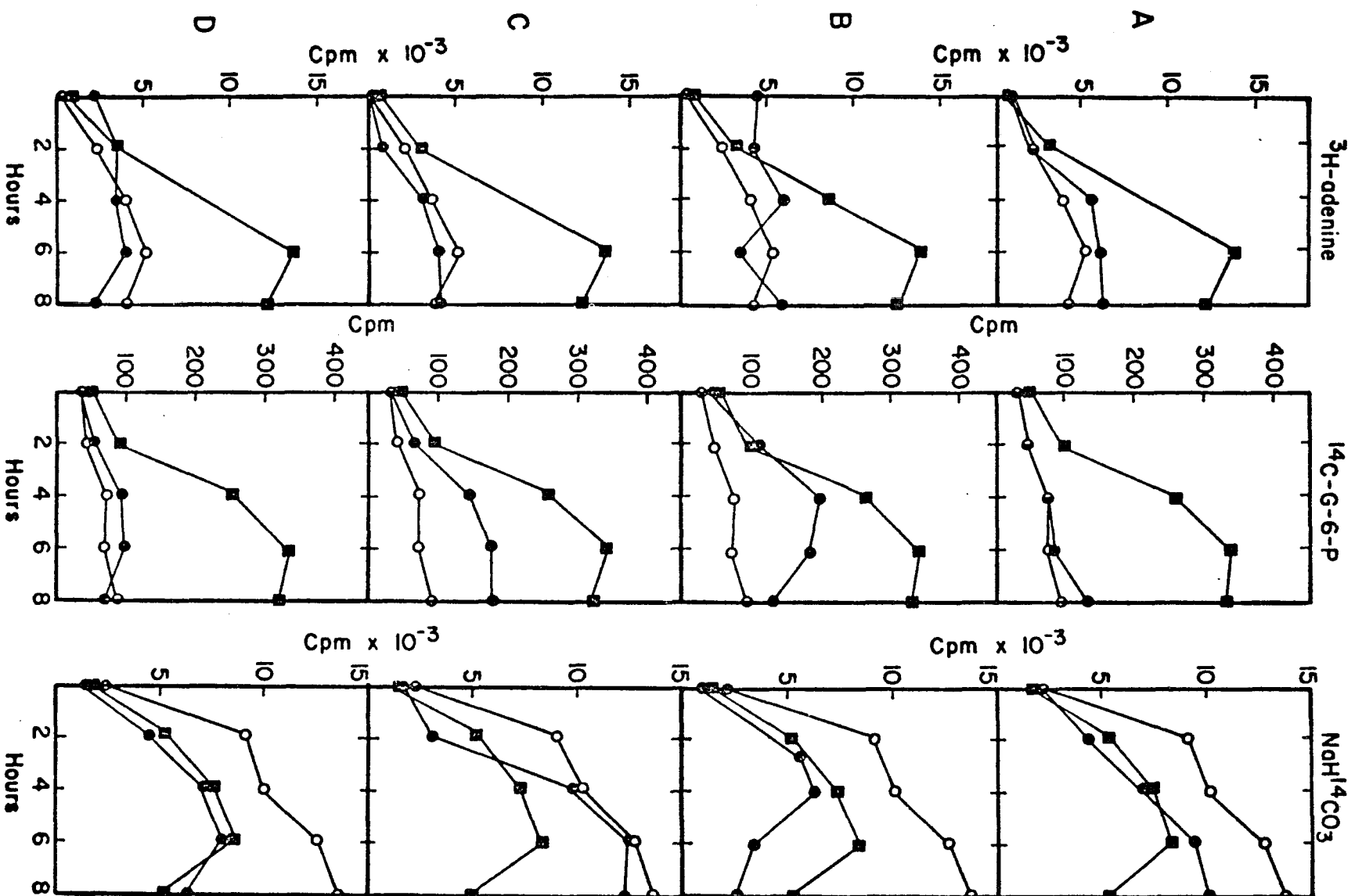
4. The Interrelationship of the Incorporation of $\text{NaH}^{14}\text{CO}_3$, ^{14}C -glucose-6-phosphate and ^3H -adenine in Different Combinations

The incorporation patterns of different precursors into UV-treated samples (algae or phage or both) and the comparison of these patterns with those of the normal infected systems and healthy cells are shown in Figure 40. It is found that a very similar pattern of ^{14}C -glucose-6-phosphate and ^3H -adenine occurs for the various combinations. The

Figure 40 The Incorporation of Radioactivity from ^{14}C -glucose-6-phosphate, ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ Separately into Acid Precipitable Materials of Differently Treated Phage-infected or Non-infected *Anacystis nidulans* Cultures at Different Time Intervals (II)

The UV-irradiated phage AS-1 (UV-phage) and UV-irradiated (UV-algae) *A. nidulans* were prepared as described in Figures 32 and 33 and MATERIALS AND METHODS. Different samples were set up and radioactively labeled with ^{14}C -glucose-6-phosphate, ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ as in Figure 39. At 0, 2, 4, 6, and 8 hr after radioactive labeling, the samples were collected and the radioactivities of the pellets were determined as described in Figure 39. Comparison of the incorporation patterns of UV-irradiated cells or phages of the infected cultures with the normal infected culture and healthy cells are indicated in different panels.

- Panel A UV-algae (●—●) with algae + phage (■—■) and algae (○—○)
- Panel B UV-algae + phage (●—●) with algae + phage (■—■) and algae (○—○)
- Panel C algae + UV-phage (●—●) with algae + phage (■—■) and algae (○—○)
- Panel D UV-algae + UV-phage (●—●) with algae + phage (■—■) and algae (○—○)



radioactive incorporation in all the systems is greater than that of the cells themselves. The highest incorporation of both ^{14}C -glucose-6-phosphate and ^3H -adenine is found in the normal infective system. UV-irradiation of either host or phage decreases the radioactive incorporation.

An inverse relationship exists in $\text{NaH}^{14}\text{CO}_3$ incorporated patterns relative to those of ^3H -adenine and ^{14}C -glucose-6-phosphate in different samples. It appears that both phage infection and UV-irradiation cause a decrease of $\text{NaH}^{14}\text{CO}_3$ incorporation. An increase of ^3H -adenine and ^{14}C -glucose-6-phosphate incorporation, however, is found in those systems as described previously. This may be due to the ^{14}C -glucose-6-phosphate partially substituting for the metabolite which could normally be formed in CO_2 fixation, and ^3H -adenine acts as a direct precursor for DNA synthesis in the infected cells after the alternation of cell permeability. Furthermore, the rate of incorporation of $\text{NaH}^{14}\text{CO}_3$ is the highest during the first 2 hours of infection (0-2 hours) in all the samples. The highest rate of incorporation of ^{14}C -glucose-6-phosphate and ^3H -adenine, however, is found between 2-4 hours post infection. This phenomenon is especially significant in the infective system. The early high rate of incorporation of $\text{NaH}^{14}\text{CO}_3$ is indirectly correlated with the findings that cyanophage AS-1 is dependent upon host photosynthesis during the early hours of infection (136). The high rate of ^{14}C -glucose-6-phosphate and ^3H -adenine utilization occurring later may be due to the direct utilization of

precursors for cyanophage biosynthesis which are initiated at this time period.

The maximum incorporation of different precursors into the TCA precipitable material in the pellets was examined for the different samples (Appendix E). In the combinations of algae + phage and UV-algae + UV-phage, where the cell and the phage were either both healthy or UV-irradiated, the incorporation of these three substrates peaked at 6 hours post infection and then dropped at 8 hours post infection. In the system of algae + UV-phage, the incorporation of all the substrates peaked at 8 hours post infection. In the system of UV-algae + phage, however, the incorporation of all the substrates peaked at 4 hours post infection and then dropped at 6 hours post infection. Complementary results were obtained by examination of the radioactivity in the supernatant, i.e., an increase of a substrate in the pellet is concomitant with a decrease of that substrate in the supernatant and vice versa. This indicates that a decrease of the substrates inside the cells is due to the release of these substrates into the media. The fact that a decrease of radioactive incorporation in the pellet of the normal infective system at 8 hours is in close agreement with those findings from one step growth experiments indicated that the release of new phage is initiated at this time period. A delayed lysis is suggested in the system of algae + UV-phage, and an early lysis is suggested in the UV-algae + phage samples. However, the precise information about the develop-

ment of cyanophage AS-1 inside the cells under different conditions must be derived from a study of the lytic ability and phage production (PFU/ml) and will be described in the following section.

D. The Effect of UV-irradiated Anacystis nidulans or Cyanophage AS-1 on the AS-1/A. nidulans Infective System during the Lytic Cycle

The UV-damaged cells and UV-damaged phage were prepared as described previously. Four different combinations were tested: algae + phage (MOI=0.2), algae + UV-phage, UV-algae + phage, and UV-algae + UV-phage. The growth characteristics of the phage for each combination was determined by one-step growth experiments (Figure 41) and by lysis as measured by the turbidity change of the cultures (Figure 42).

A rapid measure of cell lysis during infection cycles can be obtained by following the changes in the turbidity of cultures (8). Turbidity readings give an indication of the extent of infection and/or the extent of lysis but these measurements provide no indication of the number of phage that are produced. This latter information can only come from the measurement of plaque forming units (PFU/ml).

For the normal infective system (algae + phage), a decrease in turbidity and a concomitant increase in plaque forming units occurs after 8 hours of infection (Figure 41 and Figure 42). The lytic cycle of AS-1 has a burst size of 45 and is completed in 16 hours as evidenced by the leveling off of both curves. Comparing UV-damaged cells infected with

Figure 41 The Effect of UV-irradiation on the Formation of Cyanophage AS-1

The UV-irradiated cells (UV-algae) were prepared by exposing the exponentially growing cell cultures to UV for 3 min and then keeping them in the dark for 24 hr. The UV-irradiated AS-1 (UV-phage) were prepared by exposing the crude phage lysates to UV for 35 min. The protocols for UV-irradiation and the collection of crude phage lysates were described in detail in MATERIALS AND METHODS.

Following the preparation of UV-algae and UV-phage, four different combinations of phage-infected cell samples were set up as follows:

- (a) Healthy AS-1 infecting the UV-irradiated cells (UV-algae + phage);
- (b) UV-irradiated AS-1 infecting the exponentially growing cells (algae + UV-phage);
- (c) UV-irradiated AS-1 infecting the UV-irradiated cells (UV-algae + UV-phage);
- (d) Healthy AS-1 infecting the exponentially growing cells (algae + phage).

The MOI is about 0.2 in all the testing systems. Adsorption was allowed to proceed for 1 hr, after which time half volumes of each sample were removed and diluted to 10^{-2} , 10^{-4} , and 10^{-6} -fold.

Figure 41 continued/

The PFU/ml of AS-1 in each sample was assayed at 0, 2, 4, 8, 12, 16, 20, and 24 hr after infection using the titer technique as described in MATERIALS AND METHODS.

- algae + phage
- △—△ algae + UV-phage
- ▲—▲ UV-algae + phage
- UV-algae + UV-phage

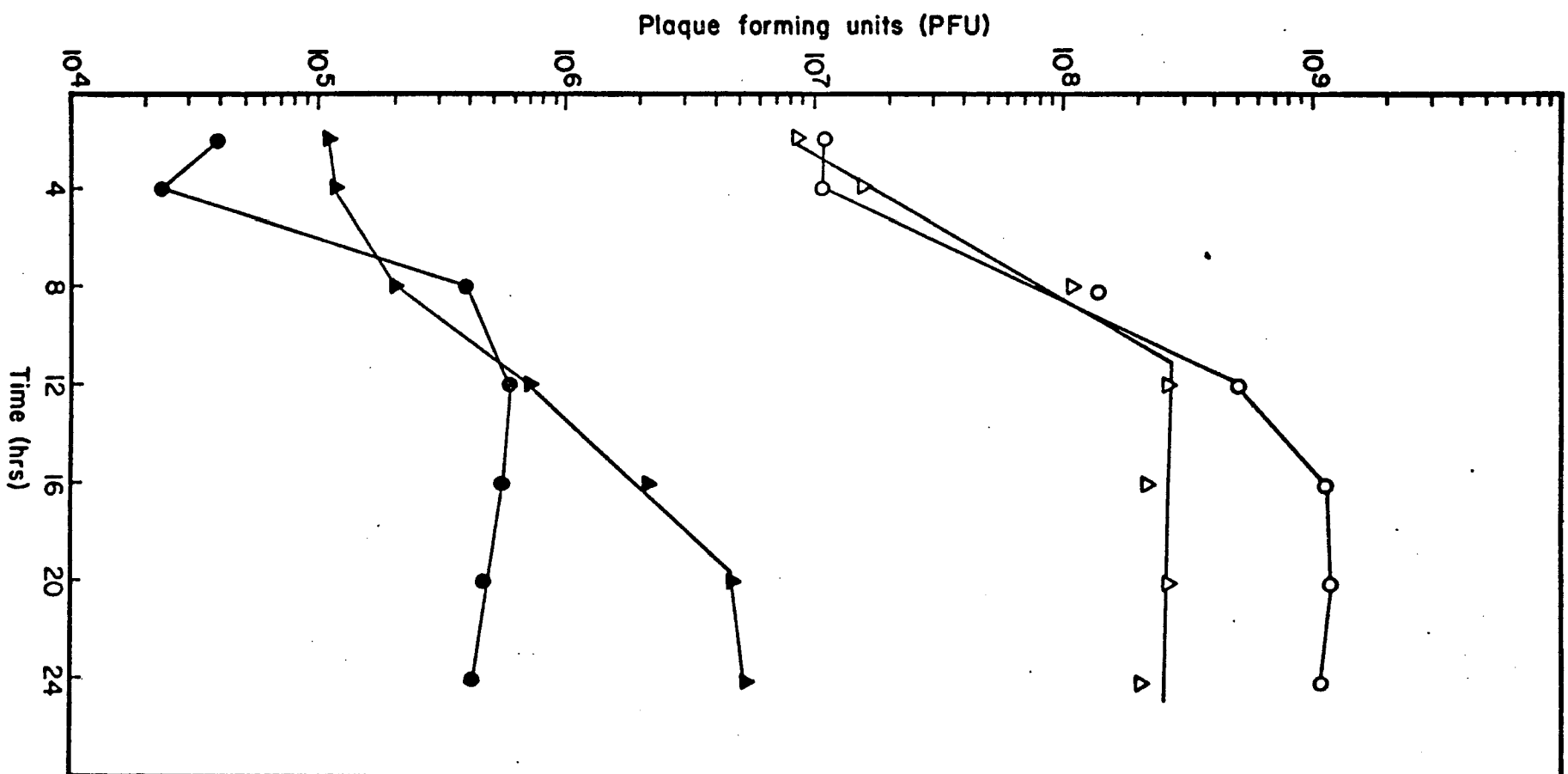
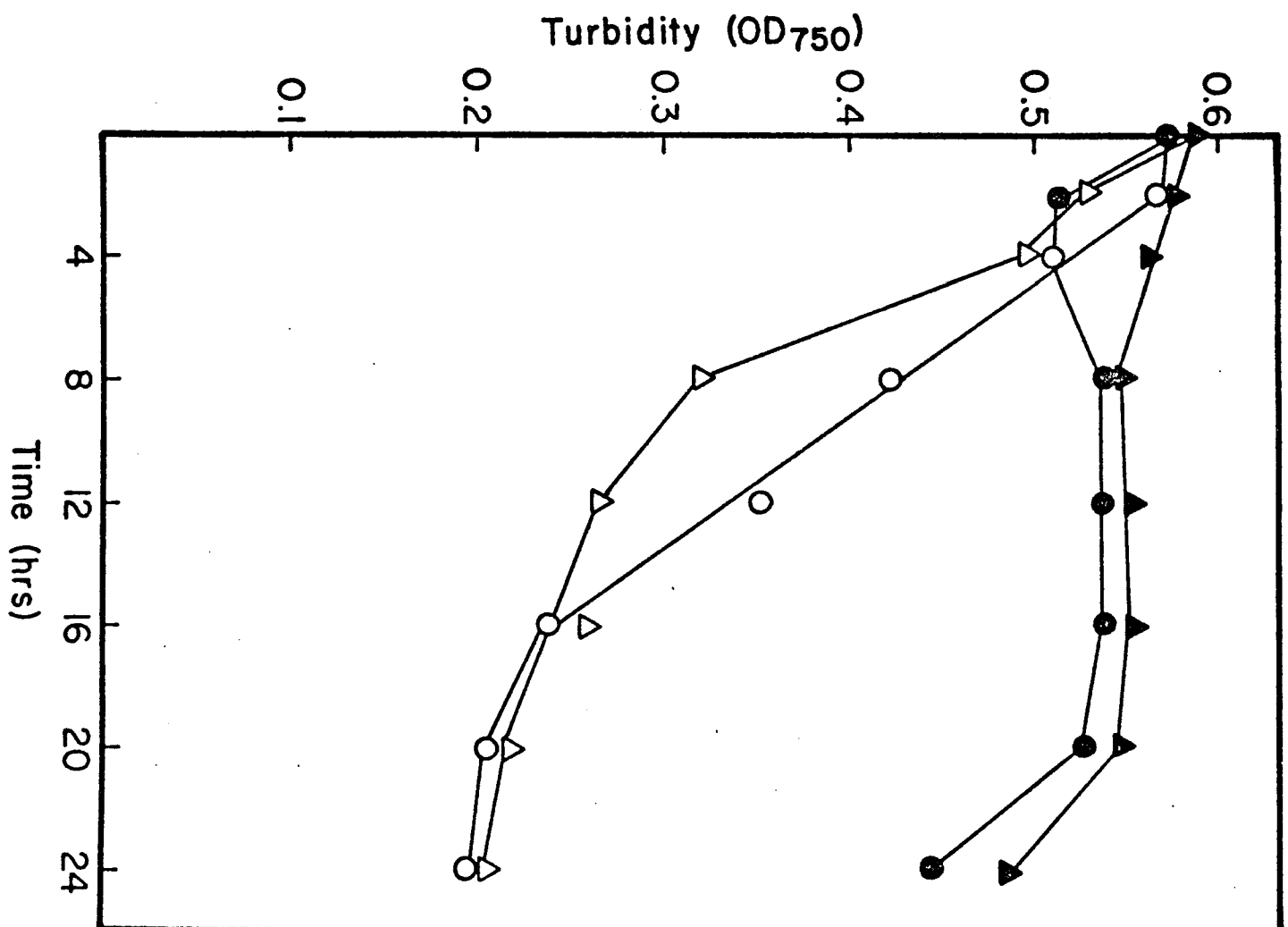


Figure 42 The Effect of UV-irradiation on the Lysis Ability of Cyanophage AS-1

The experimental protocols and the preparation of different samples (algae + phage; algae + UV-phage; UV-algae + phage; UV-algae + UV-phage) are identical to those described in the legend to Figure 41. Half volumes of each sample were removed after 1 hr of infection to assay the PFU/ml of AS-1 (Figure 41). The remainder of each sample was read spectrophotometrically at OD₇₅₀ nm in order to study the turbidity change of different infected cultures at different time points after infection.

- algae + phage
- ▲—▲ algae + UV-phage
- △—△ UV-algae + phage
- UV-algae + UV-phage



healthy phage (UV-algae + phage) with the normal infective system, an earlier decrease in turbidity is found for the UV-algae + phage samples, although both curves level off similarly. From the study of phage formation (PFU/ml) in UV-algae + phage samples, the developmental cycle is completed in 12 hours instead of the normal 16 hours and the burst size is reduced to 21% of the control in the UV-damaged algae (~ 9.5). These results indicate that the number of cells infected by AS-1 are very similar in both combinations; however, earlier lysis and fewer phage production occur when UV-damaged cells are the host. When the healthy cells are the host infected with the UV-damaged phage (algae + UV-phage), there is no significant decrease in turbidity during the entire interval of a normal lytic cycle (0-16 hours). The decrease in turbidity is less than 10% at the end of 16 hours post infection, and is about 20% at the end of 24 hours post infection. A titer of 2×10^5 PFU/ml is obtained from the infective system infected with UV-damaged phage at 0 hour instead of a titer of 2.7×10^7 PFU/ml from the normal infective system, indicating that 99% of lethality results from the UV-irradiation. The surviving AS-1 ($\sim 1\%$) exhibits a prolonged lytic cycle of about 20 hours with a burst size of 24 which is about 50% of the standard burst size.

The combination of both UV-damaged cells and UV-damaged phage produces a similar pattern of turbidity change to that of the combination of healthy cells infected with UV-damaged phage. However, the phage formation pattern is quite

different. A 12 hour lytic cycle is found, instead of a delayed burst, along with a burst size of about 6 which is only 10% of the standard burst size.

From all the data accumulated thus far, a conclusion can be derived and summarized in Table V. A shorter lytic cycle with a smaller burst size is found in all the combinations when a UV-damaged cell is used as host. This may be due to a more rapid breakdown of the host DNA after phage infection. A much larger burst size but similar pattern of turbidity change is found in the system of algae + UV-phage, compared with the system of UV-algae + UV-phage. This may result from the photorecovery of the UV-damaged phage inside the healthy cells as suggested by the data discussed in the previous section. On the other hand, the UV-damaged algae have hardly any photoreactivation capacity since the samples were held in the dark for 24 hours before illumination and infection. Even though the UV-damaged phage had lost 99% viability, the strong repair mechanism existing in the cells allowed the photoreactivation to take place after infection. The prolonged lytic cycle in the system of algae + UV-phage may be due to the time for photorecovery of the phage after penetrating into the cells.

Table V Summary of the Effect of UV-irradiation on
the Development of Cyanophage AS-1

The UV-damaged algal cells and UV-damaged phage were prepared as described in Figures 32 and 33. These were mixed together in four different combinations: algae + phage; algae + UV-phage; UV-algae + phage; UV-algae + UV-phage. The plaque formation and lytic ability were determined as shown in Figures 41 and 42. The burst size, % of standard burst size, complete lytic cycle and the morphology of the plaques are presented.

TABLE V

<u>Infective system</u>	<u>OD₇₅₀</u>	<u>Burst size</u>	<u>% standard burst size</u>	<u>Complete lytic cycle</u>	<u>Morphology of plaques</u>
algae + phage	1.correlated with PFU/ml 2.decreases at 8 hr 3.levels off at 16 hr	45	100%	16 hr	large homogeneous round-shaped plaques
UV-algae + phage	1.pattern similar to algae + phage (control) 2.decreases more at 8 hr than algae + phage (control) 3.levels off at 12 hr	9.5	21%	12 hr	heterogeneous sized plaques (large and small)
algae + UV-phage	1.no significant turbidity change seen 2.less than 1% of infectivity seen in algae + phage (control)	24	56%	20 hr	some large round shaped and some tiny plaques
UV-algae + UV-phage	similar to algae + UV-phage	6	10%	12 hr	tiny plaques

E. CsCl Density Gradient Analysis of the DNA Synthesis Patterns during the Infective Cycle of AS-1 in Anacystis nidulans in which Different Combinations of UV-damaged Cells and/or UV-damaged Phage were used

1) Anacystis nidulans Infected with UV-damaged Phage

Synchronized cells were infected with UV-damaged phage and double-pulse labeled with ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ at different time intervals following infection. The DNA pattern was analyzed on CsCl density gradients and the data from these gradients are presented in Figure 43.

From our previous knowledge about the temporal pattern of PIL-DNA synthesis, we would expect to observe an appreciable quantity of ^{14}C being incorporated into the lighter density relative to the ^3H -adenine incorporated at the viral DNA density during the early hours of infection (2-5 hours). However, a minor discrepancy in density is noted in the ^{14}C incorporation after infection in this UV-damaged phage infected system at both the 0-3 and 2-5 hours pulse labeled periods. The peak attributed to ^{14}C DNA does not correspond to that shown in the earlier data; this ^{14}C peak is situated at a density very close to the ^3H -adenine incorporating DNA. A significant amount of ^{14}C is incorporated into a lighter density similar to that of the PIL-DNA; however, this appears at 4-7 hours post infection. This delayed appearance of PIL-DNA correlates well with the earlier findings that the cells infected with the UV-damaged phage have a prolonged lytic cycle (~ 20 hours). The density

Figure 43 Fractionation Profiles of CsCl Gradients of Anacystis nidulans Infected with UV-treated Cyanophage AS-1, Pulse-labeled with ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ at Different Time Intervals Following Infection (Algae + UV-Phage)

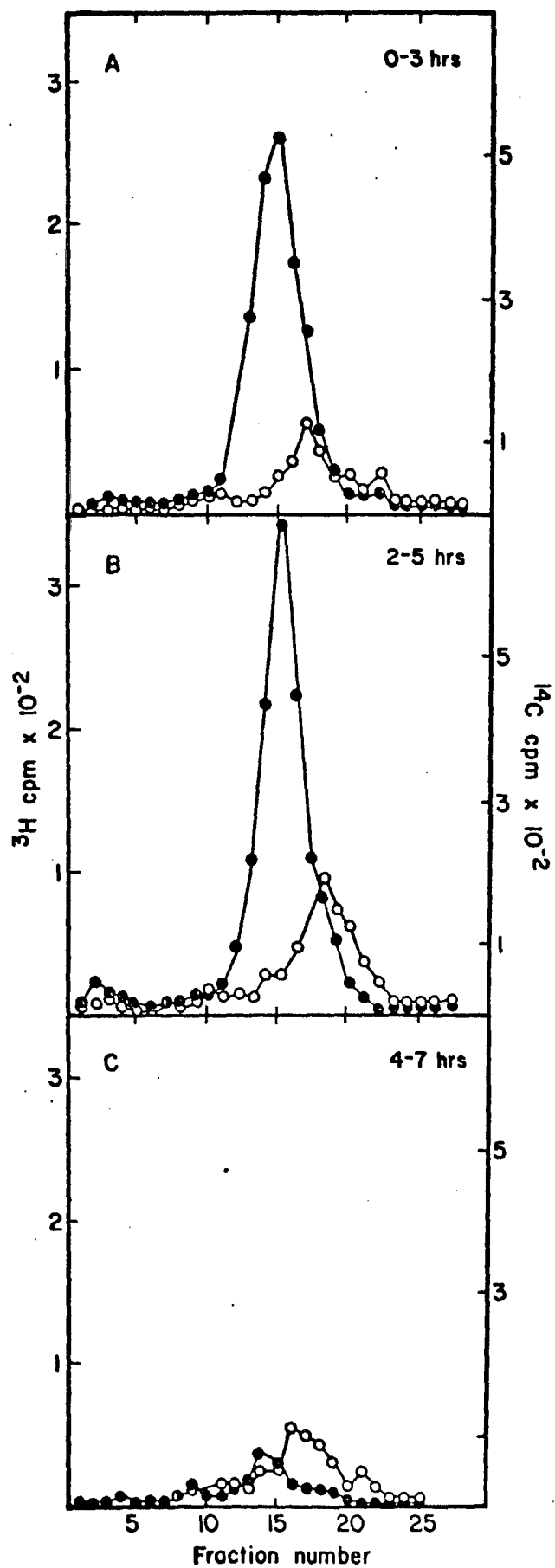
The crude viral lysates were prepared and irradiated with UV-light for 35 min as described in MATERIALS AND METHODS and the legend to Figure 33. Exponentially growing cell cultures were synchronized as described in MATERIALS AND METHODS. Following a 1 hr interval of illumination the UV-irradiated phage was added to the synchronized culture to give a MOI of 5. The infected cultures were divided into three parts and the experimental protocol was identical to that described in Figure 46.

Panel A Radioactive incorporation pattern of A. nidulans infected with UV-damaged phage AS-1 and pulse-labeled for 0-3 hr.

Panel B As above, except for pulse-labeling for 2-5 hr.

Panel C As above, except for pulse-labeling for 4-7 hr.

● — ● ^3H -adenine
○ — ○ ^{14}C



fluctuation of ^{14}C -incorporating DNA in the early post infection period (0-5 hours) is not as extensive as that seen in the normal infective system. However, at later hours there does appear a significant shift, which may be due to the repair of UV-damaged viral DNA utilizing the photorecovery mechanism provided by the algal cells before the initiation of phage biosynthesis with its attendant PIL-DNA formation.

In order to determine if the preexisting algal DNA is involved in the minor discrepant ^{14}C -incorporating DNA, $\text{NaH}^{14}\text{CO}_3$ prelabeled cells with a ^3H -adenine pulse label following infection were used in this particular infective system. The banding profile from CsCl gradients are shown in Figure 44. At 2-5 hours after infection, there is no significant portion of ^{14}C -prelabeled DNA migrating to the lighter density, the ^{14}C is incorporated into a single peak. At 4-7 hours of infection, ^{14}C is incorporated into a major peak along with a broad shoulder at the lighter density site. No distinct peak exists where the PIL-DNA is located; it is clear, however, that part of the preexisting host DNA migrates to lighter densities. From this preliminary study, it might be suggested that the host prelabeled DNA may not be involved in the formation of the minor density shifted peak which appears in the UV-damaged phage infective system at the early hours of infection. Part of the preexisting host DNA distributed in a heterogeneous density range at later hours of infection may simply be due to the breakdown of the host DNA.

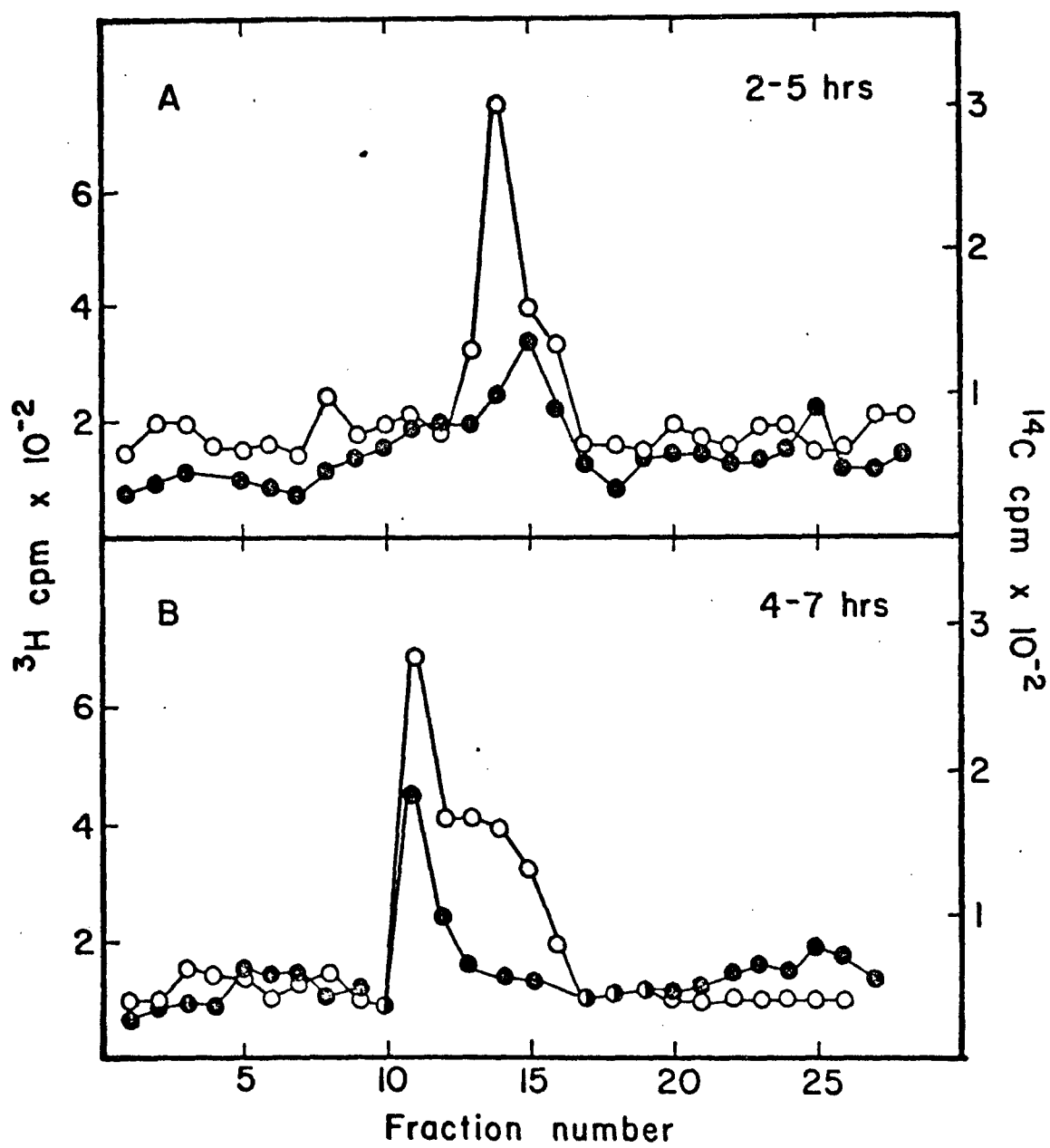
Figure 44 Fractionation Profiles of CsCl Gradients of $\text{NaH}^{14}\text{CO}_3$ Prelabeled Anacystis nidulans Infected with UV-damaged Unlabeled Phage AS-1 and Pulse-labeled with ^3H -adenine at Different Time Intervals (Algae + UV-Phage)

The prelabeled A. nidulans was prepared as described in the legend to Figure 19. The UV-damaged phage AS-1 was prepared by exposing the phage to UV-irradiation for 35 min as described in Figure 33. The UV-damaged phage was added to the "prelabeled" cells. At 2 hr after infection half of the infected culture was removed, pulse-labeled with ^3H -adenine for 3 hr, and then terminated in the termination solution. At 4 hr post infection the remainder of the infected culture was pulse-labeled with ^3H -adenine for 3 hr and then terminated. The samples were analyzed on CsCl gradients and the protocols of determination of the radioactivity incorporation in each fraction followed those previously described in the legend to Figure 3.

Panel A The radioactive incorporation of $\text{NaH}^{14}\text{CO}_3$ pre-labeled A. nidulans infected with UV-damaged phage and pulse-labeled with ^3H -adenine for 2-5 hr.

Panel B As above, except pulse-labeling for 4-7 hr.

● — ● ^3H -adenine
○ — ○ ^{14}C



In order to find out if phage DNA is involved in the formation of the minor discrepant DNA, further experiments utilizing ^3H -adenine prelabeled UV-damaged phage in this infective system were performed, and the results are presented in Figure 45. The results show that at 2-5 hours post infection pulse labeled $\text{NaH}^{14}\text{CO}_3$ is incorporated into a DNA of a density very close to that of the phage DNA instead of being incorporated into PIL-DNA density. Part of the ^3H -adenine prelabeled phage DNA migrates to a heterogeneous range of DNA densities with a small peak at a lighter density coincident with the PIL-DNA. At 4-7 hours post infection the major portion of ^3H -adenine prelabeled phage DNA, as well as the ^{14}C , co-fractionates to the PIL-DNA density. This suggests that the preexisting phage DNA is involved in the formation of discrepant DNA and that it also is involved in the formation of PIL-DNA.

All CsCl density gradient studies of cells infected with UV-damaged phage suggest that in the UV-damaged phage infective system a DNA with slightly lighter density than host or phage DNA is involved as an intermediate. This observation, that only the original phage DNA but not the pre-existing host DNA migrates to this intermediate density, indirectly supports the suggestion that there is a photo-repair mechanism for UV-damaged phage inside the cells.

2) UV-damaged Anacystis nidulans Infected with Cyanophage AS-1

The UV-damaged cells were prepared as described

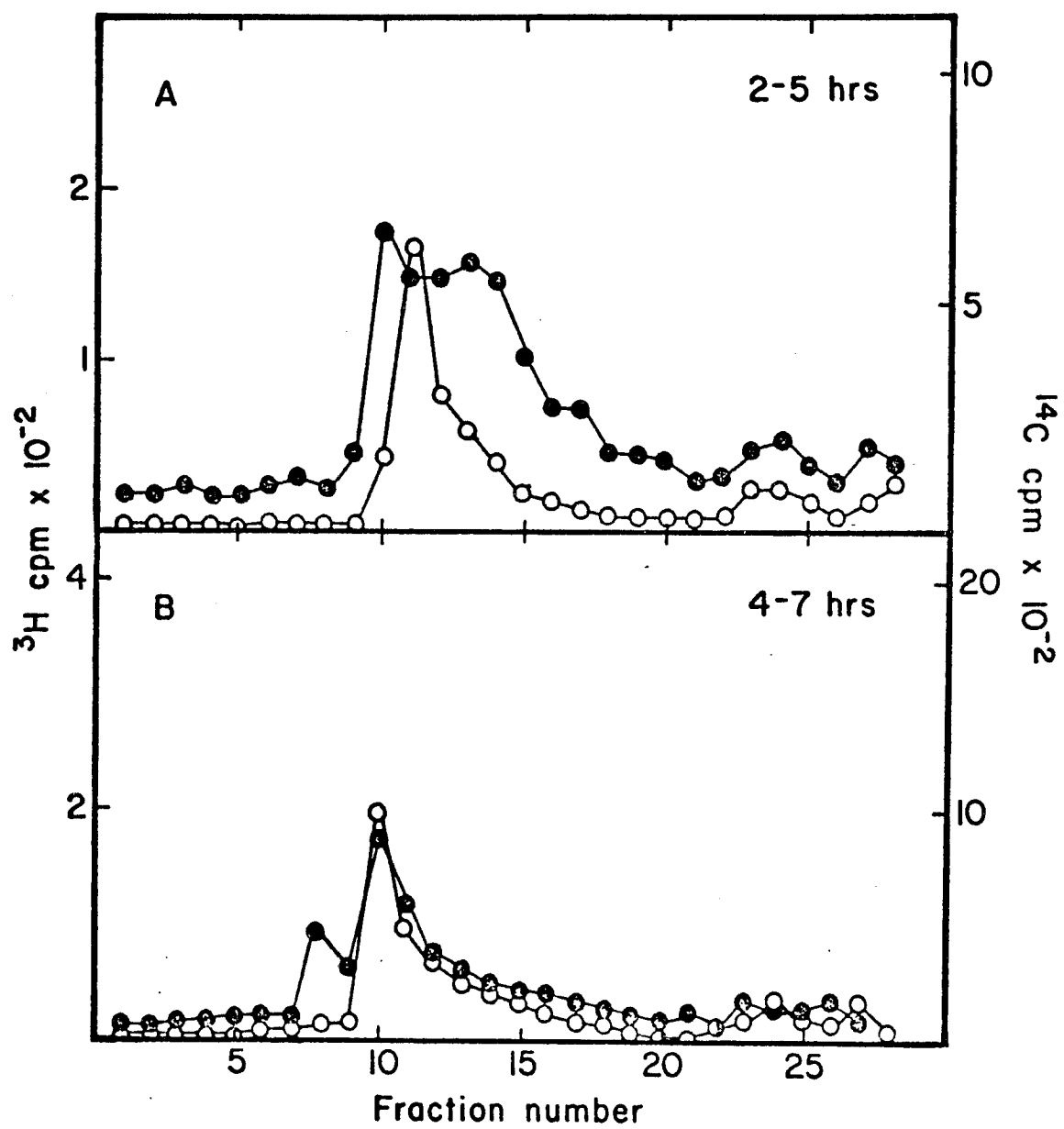
Figure 45 Fractionation Profiles of CsCl Gradients of Anacystis nidulans Infected with ^3H -adenine Prelabeled UV-damaged Phage, Pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ Following Infection (Algae + UV-Phage)

The ^3H -adenine prelabeled phage was prepared as described in MATERIALS AND METHODS. The UV-damaged phage was prepared by exposing the phage to UV-irradiation for 35 min as described in Figure 33. The ^3H -adenine prelabeled UV-damaged phage was added to the exponentially growing A. nidulans culture and the experimental protocols were identical to those described in Figure 44.

Panel A Radioactive incorporation of A. nidulans infected with ^3H -adenine prelabeled UV-damaged phage and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ for 2-5 hr.

Panel B Same conditions as in Panel A except pulse-labeling for 4-7 hr.

● — ● ^3H -adenine
○ — ○ ^{14}C



previously and was infected with AS-1. The samples were dual pulse labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine and removed from the infective system at different time intervals after infection, followed by analysis on CsCl density gradients. The DNA banding profiles from these CsCl gradients are presented in Figure 46.

It is obvious that $\text{NaH}^{14}\text{CO}_3$ starts to be incorporated into a DNA of lighter density relative to the cell or phage DNA at the early hours of infection (0-3 hours), and continues until 4-7 hours. At the end of 7 hours post infection, part of the $\text{NaH}^{14}\text{CO}_3$ is found to be incorporated into the heavy DNA density which is the normal cell or phage DNA. A small amount of ^3H -adenine is incorporated into phage DNA at the early hours of infection; however, by 7 hours there has been a significant amount of incorporation. These findings are identical to that of the healthy AS-1/A. nidulans infective system, however, a small amount of ^3H -adenine is incorporated in the PIL-DNA density in this case. Therefore, it is concluded that the UV-irradiation of Anacystis nidulans has no effect on the formation of PIL-DNA.

The ^3H -adenine prelabeled phage and $\text{NaH}^{14}\text{CO}_3$ prelabeled UV-damaged cells were used individually to study the phage development in the AS-1/UV-damaged A. nidulans infective system, and the results are shown in Figure 47 and Figure 48. The fractionation profile at 2-5 hours post infection of UV-damaged cells, infected with ^3H -adenine prelabeled phage and then pulse labeled with $\text{NaH}^{14}\text{CO}_3$, is similar to the normal

Figure 46 Fractionation Profiles from CsCl Gradients of Ultraviolet Treated Anacystis nidulans Infected with Cyanophage AS-1, Pulse-labeled with ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ at Different Time Intervals Following Infection³(UV-Algae + Phage)

Exponentially growing cultures of A. nidulans were exposed to UV-irradiation for 3 min as described in MATERIALS AND METHODS, and then kept in the dark for 24 hr. Following 1 hr of illumination the UV-irradiated cell cultures were infected with cyanophage AS-1 (MOI=5). The infected cultures were divided into three parts and each was dual pulse-labeled with ^3H -adenine and $\text{NaH}^{14}\text{CO}_3$ at 0-3, 2-5, and 4-7 hr separately. The normal AS-1 infected A. nidulans culture under identical conditions served as a control. At the end of each pulse-labeled period the samples were centrifuged, washed with saline-EDTA and then resuspended in termination solution. Following overnight incubation at 37°C each sample was treated with 1% sarkosyl and added to CsCl density gradients. After preparative ultracentrifugation the gradients were fractionated and an assessment of the level of alkaline-stable, acid-precipitable radioactivity was made as described in MATERIALS AND METHODS.

Panel A Radioactive incorporation pattern of UV-damaged A. nidulans infected with cyanophage AS-1 and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine for 0-3 hr.

Figure 46 continued/

Panel B Same conditions as in Panel A except pulse-labeling for 2-5 hr.

Panel C Same conditions as in Panel A except pulse-labeling for 4-7 hr.

●—● ^3H -adenine
○—○ ^{14}C

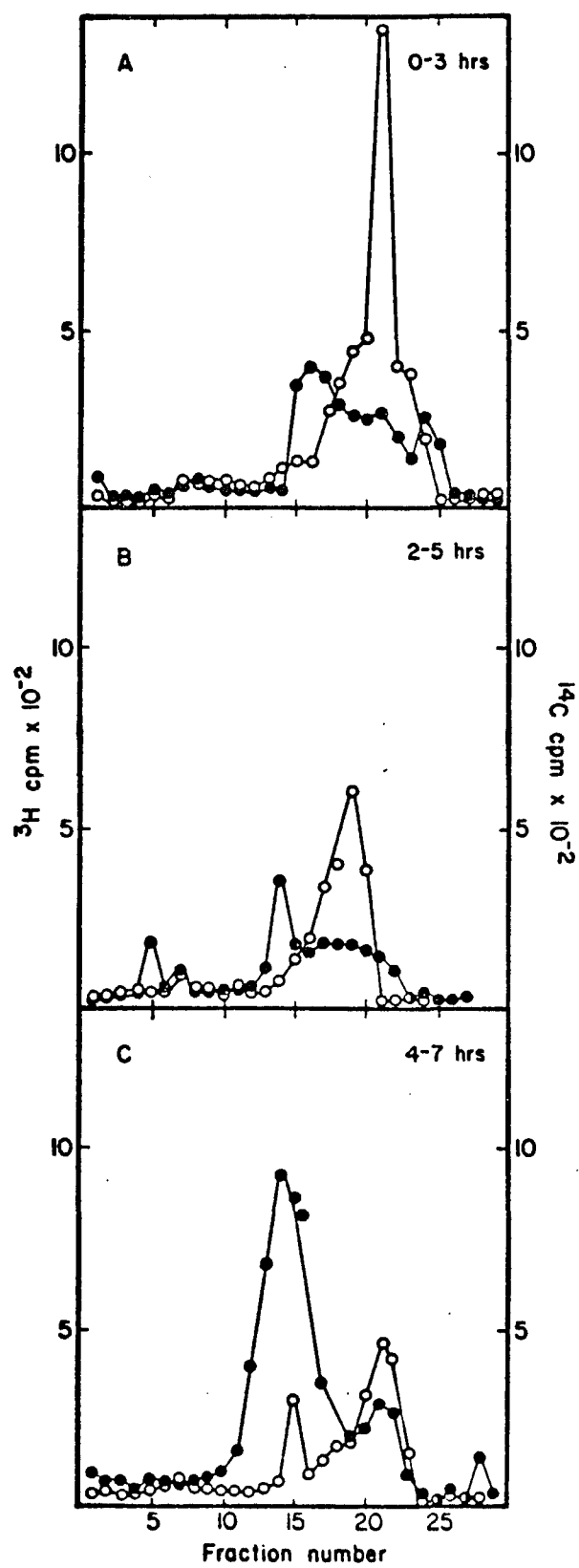


Figure 47 Fractionation Profiles of CsCl Gradients of UV-irradiated Anacystis nidulans Infected with ^3H -adenine Prelabeled Cyanophage AS-1, Pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ Following Infection (UV-Algae + Phage)

The ^3H -adenine prelabeled phage was prepared as described in MATERIALS AND METHODS. The UV-irradiated cells were prepared by exposing the cells to UV-irradiation for 3 min and then kept in the dark for 24 hr. After 1 hr of illumination the ^3H -adenine prelabeled phage was added to the UV-damaged cell cultures and the experimental protocols were identical to those described in the legend to Figure 46.

Panel A Radioactive incorporation of UV-damaged A. nidulans infected with ^3H -adenine prelabeled phage and pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ for 2-5 hr.

Panel B Same conditions as in Panel A; pulse labeling for 4-7 hr.

● — ● ^3H -adenine
○ — ○ ^{14}C

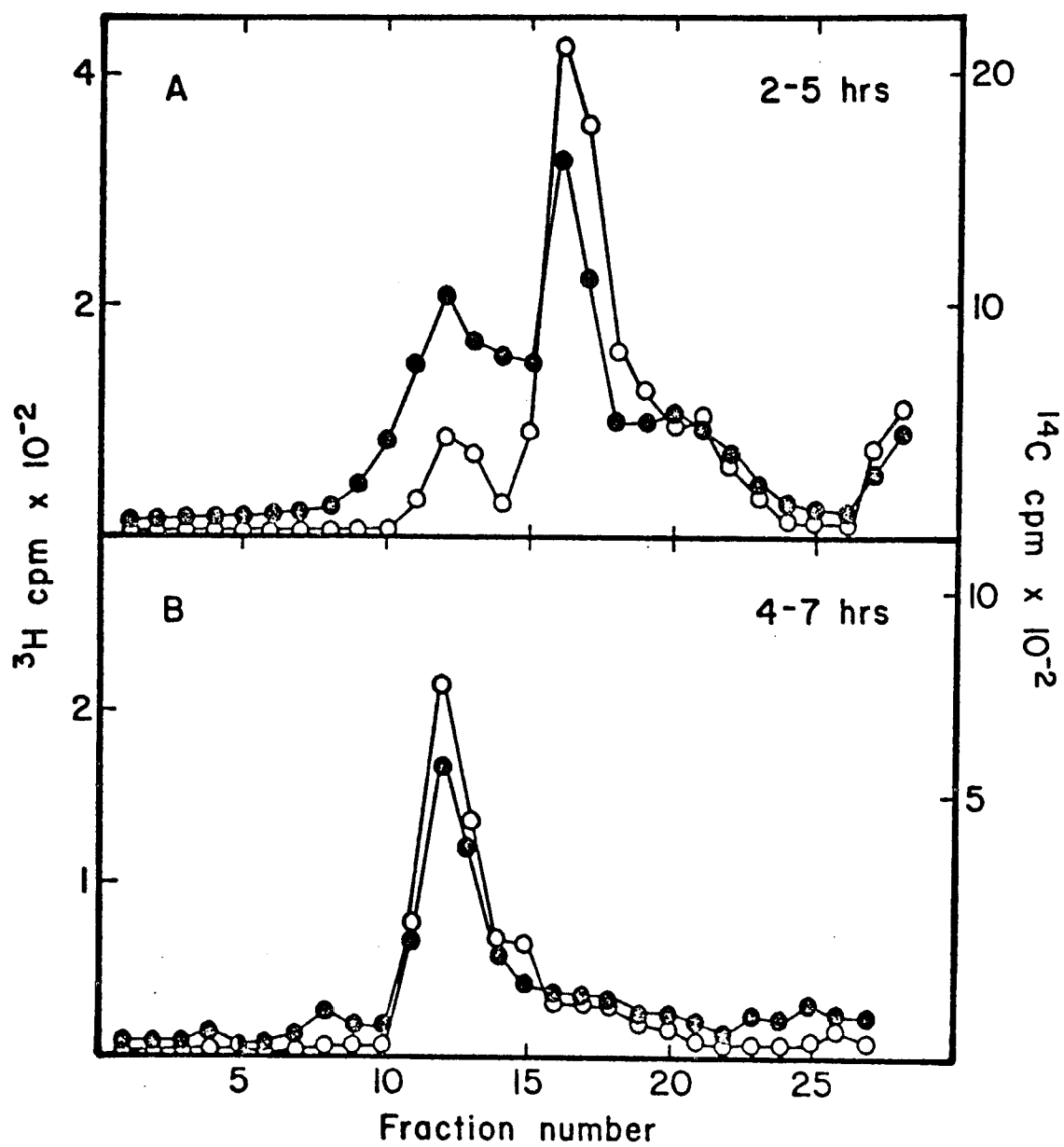


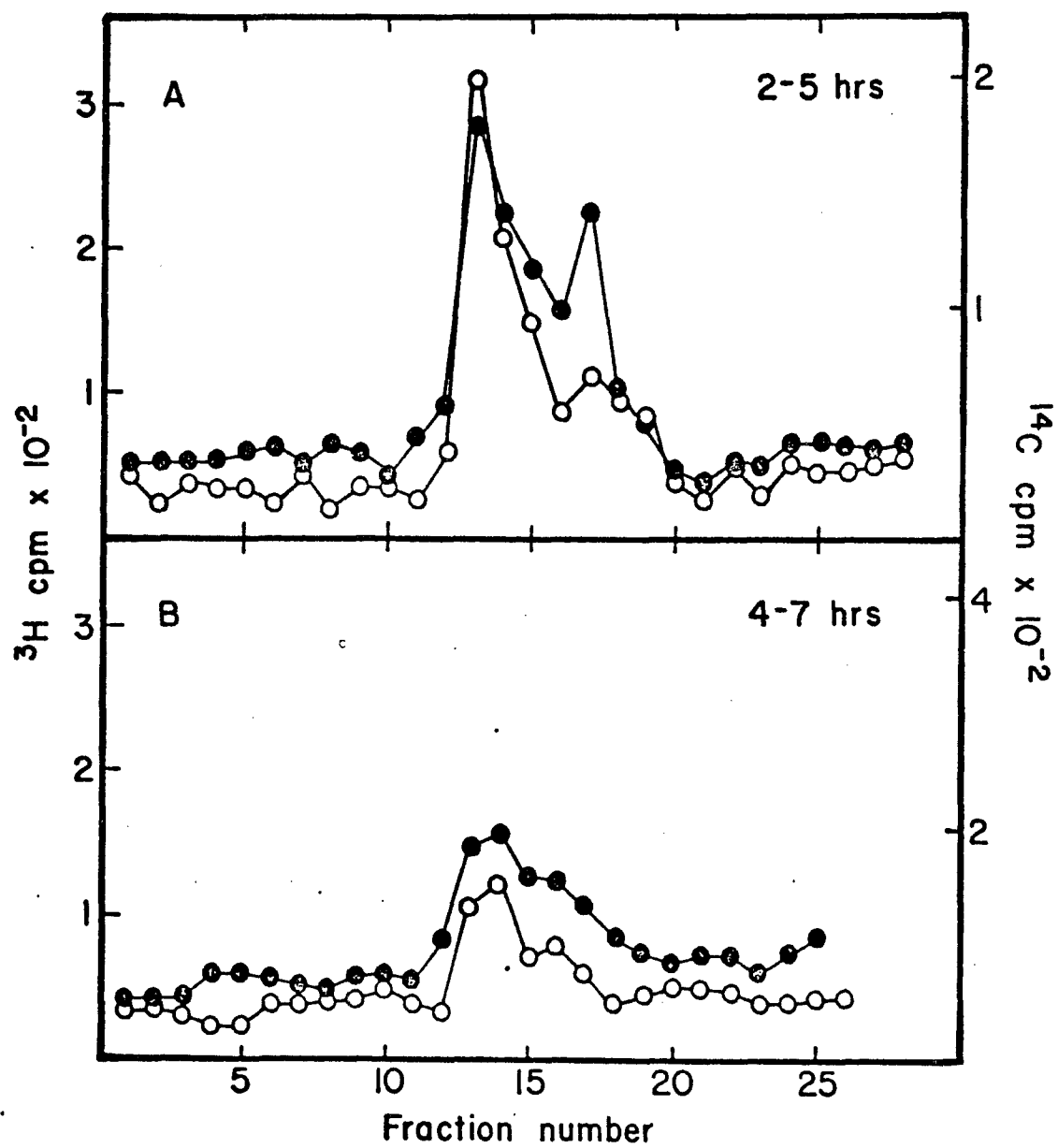
Figure 48 Fractionation Profiles of CsCl Gradients of $\text{NaH}^{14}\text{CO}_3$ Prelabeled and then UV-irradiated Anacystis nidulans Infected with Cyanophage AS-1, Pulse-labeled with ^3H -adenine following Infection (UV-Algae + Phage)

The $\text{NaH}^{14}\text{CO}_3$ prelabeled cells were prepared as described in MATERIALS AND METHODS. The prelabeled cells were then exposed to UV-irradiation for 3 min and then kept in the dark for 24 hr. After 1 hr of illumination cyanophage AS-1 was added to the $\text{NaH}^{14}\text{CO}_3$ prelabeled, UV-damaged cells and the experimental protocols were identical to those described in the legend to Figure 46.

Panel A Radioactive incorporation of $\text{NaH}^{14}\text{CO}_3$ prelabeled, UV-damaged A. nidulans infected with phage and pulse-labeled with ^3H -adenine for 2-5 hr.

Panel B Same conditions as in Panel A; pulse labeling for 4-7 hr.

● — ● ^3H -adenine
○ — ○ ^{14}C



infective system as shown in Figure 21. Parts of the original phage DNA has moved to the lighter PIL-DNA density. A significant amount of $\text{NaH}^{14}\text{CO}_3$ is found at this density with a small portion at the phage DNA density. At 4-7 hours post infection both $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine are associated with DNA of a density which is that of phage DNA. Comparison of these results with the normal prelabeled phage infective system (Figure 21) reveals a shorter lytic cycle of phage development in the UV-damaged cell. This result further supports the findings of the one-step growth experiment that an early burst occurs in this infective system.

From the fractionation profile of ^{14}C -prelabeled UV-damaged cells infected with cyanophage AS-1 and pulse-labeled with ^3H -adenine, it can be seen that a small portion of the preexisting host DNA is found at the PIL-DNA density. The pulse-labeled ^3H -adenine is also found partially incorporated into the PIL-DNA species. These results further support the findings from the dual pulse labeling experiments in the same infective system.

Several conclusions can be derived from these CsCl density gradients studies of the effect of UV-irradiation on the development of cyanophage and the formation of PIL-DNA. The combination of the UV-damaged cells infected with cyanophages does not change significantly the DNA metabolic pattern inside the infected cell. The formation of PIL-DNA still takes place normally at the early hours of infection, although an early burst is found as indicated before. The incorpor-

ation of ^3H -adenine is slightly different from the normal infective system. In three differently designed experiments, a small amount of ^3H -adenine is found with the PIL-DNA species. This may suggest that a compartmentation breakdown inside the cells has taken place due to UV-irradiation. In the UV-damaged phage infected system there is no PIL-DNA formation at early hours of infection. $\text{NaH}^{14}\text{CO}_3$ is incorporated into a minor discrepant DNA species relative to the host or phage DNA, and preexisting phage DNA is involved in this phenomenon. It is only at the later hours of infection that the PIL-DNA species seems to occur. A prolonged lytic cycle is suggested. Therefore, the UV-damaged phage affects the formation of PIL-DNA. This suggests that healthy phage is needed for the formation of PIL-DNA and this indirectly supports the hypothesis discussed previously that phage DNA serves as a template.

DISCUSSION

The work presented in this thesis can be discussed under four categories: (I) The characterization of the growth conditions for the host Anacytis nidulans and for the AS-1/A. nidulans infective system; (II) The analysis of the DNA synthesis patterns during the AS-1 infective cycle and the unequivocal demonstration of PIL-DNA as a unique form of DNA resulting from infection; (III) The development of methods for the preparative collection of PIL-DNA and phage DNA and the comparison of the physical and chemical properties of DNA of A. nidulans, AS-1, and PIL-DNA; (IV) The probing of the metabolic origin and development of PIL-DNA by the use of inhibitors of DNA synthesis. From this discussion it is intended that an understanding of the composition and metabolic origin of PIL-DNA can be achieved.

I. Determination of Conditions for Establishment of the Infective System and Characterization of Cyanophage AS-1 Development in Anacytis nidulans

A. Parameters for Growth of A. nidulans

The physiology of the host has been shown to affect the development of virus in all infective systems. Elucid-

ation of conditions necessary for optimal blue-green algal growth becomes a prerequisite for studying the infective system of cyanophage AS-1/Anacystis nidulans. The medium producing most satisfactory growth is Mauro's Modified Medium (3M), and it was the standard culture medium for all experiments performed.

Photosynthetically reduced CO₂ is the main carbon source and the algal growth rate can be increased by bubbling air enriched with up to 0.5-5% CO₂ through the culture suspension. Most blue-green algae grow best in the pH range 7.5-9.0 (71), and A. nidulans can tolerate even a much higher pH value with no apparent ill effects on its growth. The temperature range suitable for the growth of A. nidulans is significantly wider than that for other algae: the optimal temperature is near 34°C. A. nidulans is noted for phenotypic variation in pigmentation under different conditions. When it is grown in red light of wave-length greater than 660 nm the chlorophyll content of its cells is only about a quarter of that of cells grown under normal light, although phycocyanine and carotenoid levels show little change (92). Also, growing A. nidulans in the presence of 1% CO₂ cuts the concentration of phycocyanin to about half of what it is for cells grown in air alone (42). It has been reported that the maximum relative growth rate of A. nidulans has a doubling time of about two hours at 3600 Lux at 41°C (71). In our system the

generation time was approximately twelve hours under similar light density, 0.5% CO₂ and a temperature range of 30-34°C.

B. Synchronous Growth of Anacystis nidulans and its Application to the AS-1 Infective System

Although infection by the virus is itself a synchronizing event (44,168), phage replication processes have been shown to be influenced by the host cell cycle-mediated events which modify the state of subcellular pools (97). This is especially so in the first few hours of infection, while there exists a strong dependence on the continued metabolism of the host cells (8). Studies which aim to examine the physiological and biochemical changes occurring during the lytic cycle of the cyanophage depend on the successful synchronization of the host culture. Synchronization of A. nidulans has been achieved by two different techniques. Lorenzen and Ven Kataraman (76) utilized temperature shifts as a synchronizing factor in a cycle consisting of an eight hour period at 26°C and a six hour period at 32°C, both in continuous light. Herdman et al. (59) induced synchrony in an exponentially growing culture by termination of illumination and the supply of carbon dioxide. In both techniques the degree of synchrony gradually decreases and is entirely lost after several generations. In 1970 Asato and Folsome (11) devised a simple but more efficient method of synchrony by placing the exponentially

growing cultures in the dark for twelve hours and then exposing them to continuous light. Recently, this synchrony method has been further analyzed to characterize the change in cell number, DNA content, etc., and has been used for the induction of synchrony (20). Indeed the data presented on cell and DNA studies with this synchrony technique still exhibit less than perfect synchrony. This method was modified for our experiments by growing the algae for forty-eight hours with alternating light and dark cycles of twelve hours each. The start of each light cycle was coupled with dilution of the algal suspension to a constant cell concentration. In order to restore the depleted metabolic pools necessary for viral maturation, the synchronized culture requires illumination for at least one hour prior to viral infection. A larger burst size is obtained from the cyanophage developed in synchronized cultures (24).

C. Phage Adsorption

Complete adsorption of the phage AS-1 occurs in approximately sixty minutes (126). Since dark conditions affect the total amount of phage adsorption (36,128), the adsorption process was conducted under illumination for one hour in all the experiments.

We found, in addition, that lysis-from-without is evident at a high multiplicity of infection ($\text{MOI} > 15$),

which causes a decrease in overall burst size. However, at a MOI of from 0.1 to 10.0 under a variety of physiological conditions, a burst size of 50-55 PFU/ml was routinely obtained. Therefore, in all the experiments performed for this thesis a MOI of 0.1 and 5 was used, depending on the experimental purposes.

D. The Effect of Temperature on the Lytic Cycle

Temperature can affect the length of the lytic cycle (24). A normal sixteen hour lytic cycle was usually obtained at 30-34°C. Lower temperatures (26°C, 28°C) have the effect of lengthening the lytic cycle (18 hours); higher temperatures (35°C, 36°C) shorten the lytic cycle of AS-1 to twelve to fourteen hours. Sherman and Pauw have isolated AS-1M (138), a cyanophage identical to AS-1 in morphology, seriological test, host range, and biochemical, and biophysical characteristics. The only difference between the two phages is that AS-1M has a slightly shorter lytic cycle of twelve hours duration. Since AS-1M was studied at 35°C (138) but AS-1 was studied at 26°C (126), this temperature difference might explain why the lytic cycle of AS-1M reported by Sherman (twelve hours) is shorter than that of AS-1 as reported by Safferman (sixteen hours).

II. The Study of DNA Metabolism in AS-1/*Anacystis*
nidulans--Analysis of DNA Synthesis Patterns,
Proof of the Existence of PIL-DNA

Viral development of cyanophage AS-1 in the infected host *Anacystis nidulans* can be measured by monitoring the production of viral DNA. CsCl density gradient ultracentrifugation has been used to resolve the changing pattern of phage and host DNA during infection in bacteriophage/bacteria and LPP-1/*Plectonema boryanum* infective systems (133). Blashka, in 1978, was the first to use this technique to trace the DNA synthesis pattern with radioactive pulse labeling in the AS-1/A. *nidulans* infective system. Using the one hour pulse labeling with $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine method to study the infective sample following infection, he observed that newly photoreduced carbon was incorporated into a lighter density DNA relative to the host or phage DNA during the early hours of infection (two to five hours). The DNA metabolism in this infective system has been further studied in this thesis to demonstrate unequivocally the existence of this PIL-DNA. Dual pulse-labeling with longer time periods (three hours) following infection clearly demonstrates that $\text{NaH}^{14}\text{CO}_3$ is incorporated into the lighter density during the early hours of infection (two to six hours). At the end of the latent period a decrease of ^{14}C incorporated into PIL-DNA and an increase of ^{14}C incorporated into newly synthesized phage DNA was

observed. A normal precursor of polynucleotides, ^3H -adenine, is destined for incorporation into new phage DNA molecules rather than PIL-DNA. This incorporation does not become significant until the very end of the latent period. The fact that the ^{14}C -PIL-DNA only occurs early in the latent period and that the incorporation of ^3H -adenine is only found late in the period of infection suggests that a compartmentalization of precursor sources exists at the early hours of infection. The incorporation of newly photoreduced carbon into PIL-DNA implies that it is involved in the simultaneous production of nucleotide precursors along with the production of DNA. At a later time in the infective cycle the compartmentalization seems to change and ^3H -adenine can be utilized as a polynucleotide precursor for phage DNA. Although these dual pulse labeled experiments are convenient, accurate and sensitive, the use of radioisotopes alone can be misleading. The apparent preponderance of ^{14}C -PIL-DNA in the early latent period may be due to the incorporation of high specific activity ^{14}C into a small amount of a specific sequence of DNA. Thus, a quantitative study of the DNA changing patterns independent of radioactive labeling during infection is required. From the results obtained from experiments in which the CsCl gradients were monitored for 260 nm absorbing material, it is evident that in A. nidulans and AS-1 there is only one species of DNA. This DNA with a buoyant density of $1.714 \pm$

0.001 is heavier than the marker E. coli DNA (1.710 g/ml) (35,126). However, two species of DNA were detected on CsCl density gradients after the host was infected for five hours. One species is similar in buoyant density to that of the host or phage DNA and the other is a new species of DNA which is present in a significant amount in the infected culture. This DNA has a lighter density than the E. coli marker and has the same relative density as the ^{14}C -incorporating species. These results provide direct evidence of the existence of the lighter density UV-absorbing material which is known to be a PIL-DNA. However, the role of ^{14}C in this lighter density material cannot be elucidated from the above experiments. It might be directly involved in the synthesis of DNA or only involved in some macromolecules which attach to DNA or in a form of polysaccharide which migrates to the lighter density on CsCl gradients (40). All of these possibilities had to be tested.

Since this material is RNase and protease resistant and DNase sensitive, the possibility that this is a polysaccharide is eliminated. It has been reported that some macromolecules such as protein, lipoprotein, etc., attached to DNA can cause a DNA density shift in CsCl gradients. PIL-DNA was treated either with DNase, RNase and protease, or digested by protease and then lipase or digested by lipase and then protease, then subjected individually to the CsCl gradient centrifugation with ^3H -adenine phage DNA

as a marker. The elution profile for all the enzyme digestive samples except the DNase treated samples were similar to the purified PIL-DNA. These results indicate that the lighter density of PIL-DNA is not due to some unusual non-nucleic acid properties of the DNA which caused a shifting of the DNA density on CsCl gradients. The nature of this material is DNase sensitive, but RNase, protease and protease + lipase, and lipase + protease resistant. Thus, this is evidence that PIL-DNA is only DNA. Further experiments using dual pulse labeling with ^{35}S -methionine and ^3H -adenine to determine if any amino acid or short polypeptides are involved in the PIL-DNA formation were conducted, and the results also proved negative (Appendix D). This is further evidence that the PIL-DNA is a pure DNA species. It can therefore be concluded that there are two DNAs, phage DNA and PIL-DNA, that are synthesized after AS-1 infection of A. nidulans. They exhibit distinctive characteristics in four different respects: (1) buoyant density: phage DNA has a buoyant density of 1.714 g/ml and PIL-DNA has a buoyant density of 1.701 g/ml (24); (2) temporal synthesis: $\text{NaH}^{14}\text{CO}_3$ -incorporating PIL-DNA builds up from two hours post infection with maximum synthesis around five hours and declining synthesis thereafter, while the ^3H -adenine-incorporating peak only builds up at a later time in the infection cycle (later than five hours post infection); (3) precursors sources: ^3H -adenine is the major precursor

for the synthesis of new phage DNA and only photoassimilated ^{14}C is involved in the formation of PIL-DNA; (4) fate: phage DNA normally exists in the mature AS-1 particles while PIL-DNA is a phage induced DNA that exists only in the host after phage infection.

Although the nucleic acids can be separated very accurately according to their different G + C contents and base composition on CsCl density gradients, the complete process for this analytical method is complicated and time-consuming. Another analytical method, Poly-L-lysine Kieselguhr Chromatography (PLK), fractionates DNA primarily on the basis of base composition and less on other parameters, and has been reported to be applicable for the isolation of nucleic acids rapidly and simply from crude cell lysates, which have undergone no prior purification. This method is therefore a good candidate for preparative collection of PIL-DNA.

Poly-L-lysine kieselguhr chromatography has been used to fractionate nucleic acids (16,150). The interaction is due to hydrogen bonding between the $\epsilon\text{-NH}_3^+$ group of lysine and the PO_2^- of the nucleotides. The complex formed between double stranded DNA occurs in a lysine to nucleotide ratio of 1:1. The precipitates formed by reaction of DNA with polylysine can be redissolved in a NaCl solution of 1-2 M. Secondary forces influence the binding of the poly-L-lysine to the polynucleotides; however, the exact nature of these

forces is not certain (18). Thus, PLK columns fractionate DNA primarily on the basis of base composition and are less affected by molecular weight and secondary structure properties of the DNA. Although RNA can also bind to the PLK, the interactive forces between PLK and RNA are such that they allow for the elution of all RNA from the PLK at a lower salt concentration. Blamire et al. reported that all nucleic acids bound and subsequently eluted from PLK are free of protein and that the DNA has no visible contamination by any UV-absorbing materials (23). All the above-mentioned properties of PLK chromatography make it an excellent choice for the preparative collection and purification of different species of DNA from the AS-1/A. nidulans infective system (AS-1 DNA, PIL-DNA, and A. nidulans DNA).

Total DNA from A. nidulans was complexed with PLK, washed free of unbounded materials, and eluted by a linear gradient of 0.4-4.0 M buffered saline. The salt gradient eluted several peaks of ultraviolet-absorbing material as is shown in Figure 8. Nucleic acids are known to be eluted in the following order: degraded RNA, tRNA, rRNA and DNA (17). After excess pancreatic RNase treatment to remove the RNA species, the elution profile exhibited a single DNA species free of RNA. The complete removal of RNA by the RNase allows for the study of the DNA changing patterns in the AS-1/A. nidulans infective system. It has been reported that DNA with a low G + C content is eluted at

a lower NaCl concentration and DNA with higher G + C content elutes with increasingly higher NaCl concentrations (23). The buoyant density in CsCl of PIL-DNA has been found to be 1.701 g/ml (24) and the AS-1 DNA and A. nidulans DNA have been reported to be about 1.714 g/ml (40,126). The PLK chromatography should resolve the host/phage DNA from that of PIL-DNA, if the reason for their widely divergent buoyant densities is base composition.

For the purpose of separation and preparative collection of different DNAs in this infective system, some modification of the standard method of PLK chromatography was made. Altering the shape of the salt gradient by narrowing the salt concentration range from 0.4-4.0 M (Figure 9) to 1.0-2.5 M (Figure 11) was necessary in order to have better resolution among DNAs with similar buoyant densities. In addition, it has been noticed that after application of crude lysate to the PLK column, cellular debris trapped on the top of the column or large amounts of DNA bound at the top of the PLK matrix decreased the flow rates. Therefore, mixing the crude lysate and PLK together in a beaker before leading the complex into the column effected the uniform binding of nucleic acid throughout the PLK and prevented the slowing in flow rate. With this modification larger amounts of DNA can be loaded and purified. ^3H -adenine, $\text{NaH}^{14}\text{CO}_3$ dual pulse labeled samples collected from AS-1-infected A. nidulans at different time intervals were analyzed by PLK

chromatography and the results (Figure 11) indicate that ^{14}C and ^3H -adenine always eluted at the same salt concentration regardless of time in the infective cycle. The conclusion was obtained using preparative amounts of DNA from infected cultures. Furthermore, the failure to separate purified ^{14}C -PIL-DNA, collected from CsCl gradients, from purified A. nidulans DNA, collected by the use of Marmur's procedure, on PLK columns gave direct evidence that there is no difference between base composition between PIL-DNA and host/phage DNA. Therefore, the possibility that ^{14}C -PIL-DNA is an A-T-rich new species of DNA can be eliminated. The PLK chromatography analysis favors the suggestion that the formation of PIL-DNA is due to a modification of host or phage DNA.

III. The Development of Methods for the Preparative
Collection of PIL-DNA and Phage DNA and Comparison
of the Physical and Chemical Properties of
Anacystis nidulans, AS-1 and PIL-DNA

In order to further characterize the PIL-DNA, methods for the preparative collection and purification of this DNA had to be devised. For purifying significant amounts of PIL-DNA, large infected cultures were combined, after five hours of infection, with a small parallel ^{14}C -labeled culture. The radioactive labeling acted as a probe for easy tracing of the location of the PIL-DNA. A parallel set-up with the same experimental protocol was performed utilizing ^3H -adenine as a radioactive probe for the new phage DNA, but collected at eight hours instead of five hours after infection in order to obtain ^3H -adenine phage DNA. After each step of PIL-DNA purification a small amount of purified DNA was ultracentrifuged on a CsCl density gradient with the ^3H -adenine DNA serving as a known density DNA reference. This precaution was to detect whether the purification process caused any DNA density change. Between each ultracentrifugation the samples were dialyzed to remove the CsCl salt. After collection and purification of this PIL-DNA on two CsCl density gradients, reasonably high absorbance ratios (260nm/280nm 1.94, and 260nm/230nm 1.83) were obtained. The purified PIL-DNA was finally recovered by spooling following addition to two volumes of cold 95%

ethanol.

A method based on the precipitation of virus particles by polyethylene glycol developed by Yamamoto et al. (178) was usually used to concentrate and purify the crude lysate. However, after it was applied to the concentrates of large amounts of virus, a significant decrease in infectivity was found and viability dropped rapidly during storage. This concentration technique was inadequate for the purpose of generating active phage in the high concentrations necessary for some experimental protocols of the infective system. In all the experiments sephadex G-200 was used to concentrate the crude lysate. The water regain of sephadex particles was so accurate that it was easy to control the extent of phage density during the concentration process. By checking the decrease of cell number and plaque formation, it was determined that the concentrated phage still retained high lytic ability and normal burst size. The percent viability is similar to that of the non-treated phage even after prolonged storage.

For the purpose of isolation and purification of viral DNA, the phage was first concentrated, then treated with DNase in the presence of Mg^{++} to digest the DNA fragments in the crude lysate which were outside the phage particles. It was found that the presence of the detergent sarkosyl can inactivate the DNase activity. It is therefore very convenient to extract phage DNA starting with sarkosyl to

break open the capsid without knowing that there would be no DNase hydrolysis of the intact phage DNA (data not shown). With this treatment and following Marmur's procedures , highly purified phage DNA was obtained.

The fact that the PIL-DNA cannot be resolved from the phage DNA by PLK chromatography and that there is no density shift following different enzymatic digestions such as protease, lipase, etc., eliminates the idea of an A-T rich DNA or one with macromolecular attachments (protein, lipid, membrane fragments) and leaves open the possibility of modification or the existence of specific replicating sequences of bases in PIL-DNA. From previous experiments modification seems to be the most likely explanation for the lighter density. However, the possibility that specific sequences of DNA exist cannot be ruled out, since it has been reported that T4 bacteriophage DNA precursors contain unit lengths of phage genome which arise by recombination between molecules of ordinary size bearing terminal repetitions of specific nucleotide sequences. The production of normal phage DNA molecules will occur at a later step when unit portions are chopped off and packaged (31,46).

In order to further characterize and understand the nature of PIL-DNA, thermal denaturation experiments were performed. The T_m in 0.1 x SSC of PIL-DNA was calculated to be 76.3°C, which corresponds to a G + C content of 54.6%. However, from CsCl gradient analysis, a density of

1.701 g/ml was obtained, which represents a G + C content of 42%. It has been suggested by many researchers that, for double-stranded DNA, any discrepancy between the melting data and the buoyant density data is an indication of the presence of a modified component.

Based on numerous studies, the changes in physical properties of DNAs with modified bases from various organisms can be divided into two classes according to the resultant buoyant density shift. In cases where deoxyuridine exists instead of thymidine or 5-hydroxymethyl-uracil instead of thymine, or 2-amino-adenine replaces adenine or where hydroxymethylcytosine replaces cytosine an increase in buoyant density by CsCl gradient analysis is observed. On the other hand, 5-methylcytosine instead of cytosine causes a decrease in buoyant density. The methyl group at the 5-position in the pyrimidine ring was suggested to be responsible for lowering the buoyant density of DNA (41). Kirk (68) calculated that the lowering of the buoyant density of DNA upon methylation at the 5-position is due to an increase in molar volume, which is disproportionate to the increase in weight. This methylation can also stabilize the double helix, resulting in a higher T_m than is expected. Since PIL-DNA has a lighter buoyant density and a high T_m (identical to the host and phage DNA), the most obvious covalent modifications which might give rise to our results are: the conversion of adenine to methyl adenine, cytidine

to methyl cytidine, or guanine to methyl guanine. It has been found that for bacteriophage SP8 hexose is attached to predominantly one strand of its DNA, which results in a shift to a lighter buoyant density. The possibility of the presence of hexose cannot be ruled out (56b).

In order to study the base composition of A. nidulans, AS-1 and PIL-DNA, the simplest and most widely used method, thin-layer chromatography (TLC), was used to analyze the base composition of DNA. The TLC data for both nucleotide analysis and base analysis of PIL-DNA, AS-1 DNA and A. nidulans DNA has been presented in the RESULTS. There are four major spots in all the samples and their relative mobilities are very similar to the four standards which co-chromatographed in the same solvent system at the same time. During base analysis with a strong acidic solvent, adenine, cytosine and thymine appear as dark-blue spots whereas guanine fluorescence appears brilliant light-blue. Only in PIL-DNA an additional unclear spot was also detected. It seems that PIL-DNA contains the four major bases, although some modified base might also be present. However, a small amount of modification should not cause such a large discrepancy between the data from T_m and CsCl gradient analysis. It has been reported that on cellulose thin-layers the separation of some groups of nucleotides is poor (98), especially the separation between 5-methylcytosine and cytosine. A similar mobility has always been obtained

(56b) and for these two bases from both enzymatically digested and acid hydrolyzed samples. Recently, Khudyakov et al. (65) failed to resolve a special modified base 2,6-diaminopurine in S-2L DNA by cellulose TLC in the same solvent system as we use for base analysis. Since methylation of the base can lower the buoyant density, 5-methylcytosine becomes one of the most possible factors for modification in PIL-DNA. However, with this technique, convincing results cannot be obtained. Further experiments with improved methods to analyze the DNA base composition are necessary. It has been reported that nucleotides can be separated very sharply on PEI-cellulose (polyethyleneimine) ion exchange TLC (98,109), and that the DNA bases can be successfully separated on cation-exchange thin layer such as commercially prepared Ionex-25 SA, Fixion 50-X8 plates which are developed one dimensionally using strong acid (165). Conclusive data will hopefully be obtained in the future by the utilization of these techniques to analyze the base composition of PIL-DNA.

The incorporation of ^3H -methyl group of L-(^3H -methyl) methionine into 6-methylaminopurine and 5-methylcytosine has been demonstrated by Kaye et al. (62). In order to detect the possible presence of methylated bases in PIL-DNA, ^3H -methyl-adenosyl-methionine was added in the AS-1 infected A. nidulans culture. Information about the total amount of radioactivity in the culture, the amount of radio-

activity incorporated into nucleic acids and the amount of radioactivity appearing in DNA has been obtained from the results of direct count, TCA precipitation and KOH + TCA precipitation, respectively. Since a significant amount of radioactivity appearing in the KOH + TCA precipitation samples was found (data not presented), experimental analysis on CsCl density gradients should be performed to determine the location of the ^3H -methyl group (in the host/phage DNA peak or in PIL-DNA, or both). If the ^3H -methyl group is incorporated into the PIL-DNA, base analysis and autoradiography of ^3H -methyl labeled PIL-DNA should also be performed using several known modified DNAs. Identification of the species of the methylated bases should be easily accomplished.

The autoradiographs of the thin layer nucleotide analysis of hydrolyzed ^{14}C -PIL-DNA showed four clear and one faint radioactive spots. The location is exactly the same as the UV-visualized spots indicating that ^{14}C is incorporated into all the nucleotides although the location of the ^{14}C in the nucleotides cannot be identified. The fact that ^3H -adenine is not involved in the formation of PIL-DNA and that some ^{14}C is incorporated into dAMP, suggests that the radioactive labeling is in the deoxyribose portion of the nucleotide. However, the results from autoradiographs of base analysis of ^{14}C -PIL-DNA indicate that all the bases are radioactively labeled. This suggests that at least part

of the ^{14}C is incorporated into all the bases. Since photoassimilation of CO_2 is mainly through a pentose phosphate pathway in blue-green algae, there is a possibility that sugar phosphate is synthesized through this metabolic pathway and participates in nucleotide production. Blashka studied this possibility by using ^{14}C -glucose-6-phosphate as a precursor (24) and reported that ^{14}C -glucose-6-phosphate is incorporated into AS-1 DNA instead of PIL-DNA. These findings indirectly support our results that the ^{14}C incorporated into PIL-DNA is not derived from the pentose phosphate pathway. In order to further probe this phenomenon, alternative radioactive precursors such as ribose-5-phosphate and ribulose-1,5-diphosphate should be used as possible precursors of carbon flow into PIL-DNA. In the meantime, other biosynthetic pathways resulting in CO_2 fixation, including those alternative methods of CO_2 fixation to that of photosynthetic CO_2 photoassimilation, must be examined. Since both pyrimidines and purines are radioactively labeled in PIL-DNA, the biosynthesis of the precursors of purines and pyrimidines should be studied. It has been shown that CO_2 participates in the de novo synthesis of these nucleotides. It involves a direct carboxylation of 5'-phosphoribosyl-5-aminoimidazole to 5'-phosphoribosyl-5-aminoimidazole-4-carboxylic acid in the biosynthetic pathway of inosinic acid, a purine nucleotide precursor. CO_2 is also involved in a carboxylation reaction in the syn-

thesis of carbamyl phosphate, a precursor in the pyrimidine synthesis pathway. In the future, studies should be conducted using $\text{NaH}^{14}\text{CO}_3$ to trace these biochemical pathways. Valuable information might be obtained with respect to the formation of PIL-DNA and the role of CO_2 in the PIL-DNA.

The mode of PIL-DNA formation has been limited to three possibilities: de novo synthesis, modification of host DNA and modification of AS-1 DNA. For the purpose of studying the origin of PIL-DNA, the DNA prelabeled host, DNA prelabeled phage or both prelabeled were prepared in the AS-1/A. nidulans infective system. Data obtained from these studies indicate that both preexisting host DNA and preexisting phage DNA are involved in the formation of PIL-DNA. A tentative hypothesis has been proposed in the RESULTS: "Perhaps the PIL-DNA is an intermediate in the production of new viral DNA that utilizes the modified infecting phage DNA as a template, and the degraded host DNA as a precursor source." The non-specific metabolite $^{14}\text{CO}_2$ incorporated into this PIL-DNA might occur directly as a result of nucleotide biosynthesis. However, further evidence from other approaches is needed to clear this puzzle. A DNA-DNA hybridization technique could be used to hybridize the PIL-DNA with DNA extracted from A. nidulans and from purified AS-1. From the degree of sequence similarity among them, some further information may be gained with regard to the origin of PIL-DNA. Restriction and

modification systems which attack foreign DNA have been described extensively (87,150). A restriction endonuclease recognizes specific nucleotide sequences of foreign DNA and subsequently cleaves this DNA. Alternatively, a modification enzyme recognizing the same nucleotide sequences is able to modify these sequences by methylation. A modified DNA is in this way protected against cleavage. Thus, the restriction/modifications system has become an invaluable tool in research in physical mapping of DNA and DNA-sequencing. Analysis of restriction fragments of PIL-DNA, AS-1 DNA and A. nidulans DNA should be performed. Using the agarose horizontal slab gel electrophoresis technique described by McDonell et al. (89), a comparison of the fragmented patterns may be obtained. In this manner direct information about the nature and origin of PIL-DNA can be elicited.

IV. The Effect of the DNA Inhibiting Agents
Hydroxyurea, Nalidixic Acid and UV-irradiation
on the Development of Cyanophage AS-1 and
Formation of PIL-DNA

The inhibitors, which inhibit DNA synthesis, such as hydroxyurea and nalidixic acid appear to be especially valuable in the elucidation of the role of DNA synthesis in PIL-DNA formation. These agents add to our knowledge of the regulation of nucleic acid biosynthesis in the infective system and PIL-DNA metabolism.

Hydroxyurea ($\text{H}_2\text{N}-\overset{\text{O}}{\underset{\text{H}}{\text{C}}}-\text{N}-\text{OH}$) inhibits DNA synthesis in several prokaryotes (114) and eukaryotes (103). It does not affect the synthesis and metabolism of RNA and protein, and it allows for an increase in turbidity of a bacterial culture. This is known as unbalanced growth (119). The DNA from hydroxyurea-treated cells is not degraded nor does it appear to be cross-linked (114), and the inhibition is reversible. Thus, it is sometimes chosen as an agent for the induction of synchronized division (157). A comparison was made between the effects of hydroxyurea and those of ultraviolet light. It has been shown that cellular DNA is the structure primarily affected by ultraviolet radiation and that DNA synthesis is inhibited in UV-treated cells. The action of hydroxyurea and UV-light differed in this respect. It is reported that hydroxyurea affects neither deoxynucleoside nor deoxynucleotide kinase nor DNA poly-

merase (117). Some investigators have presented good evidence that the primary action of hydroxyurea is ribonucleotide reductase, the enzyme which reduces the four common ribonucleotides to their corresponding deoxyribonucleotides (14,43,70,90). Others, however, using different cell systems have reached different conclusions (106, 120,179,180). The rapidity with which a metabolic inhibitor affects DNA synthesis may reflect its mode of action. The effect may be immediate, or it may allow for a cycle of DNA synthesis to be completed. In this study, a synchronized algal culture was used and our results indicate that hydroxyurea-induced inhibition of DNA synthesis is immediate. This suggests that hydroxyurea interferes with either a rate-limiting enzymatic reaction that is concerned with DNA synthesis or that it reacts rapidly with the cellular DNA, thus preventing it from serving as a primer for further DNA synthesis. These observations give no precise information as to the specific site of action. In order to ascertain whether the ribonucleotide reductase is blocked by hydroxyurea, further experiments might be planned. If this enzyme is the site of action of hydroxyurea, one may expect that supplementing the treated culture with deoxyribonucleotides would bypass the hydroxyurea-induced blockage and allow the cyanobacteria infective system to metabolize normally.

The effect of hydroxyurea on virus development has

also been studied (88,115). It is reported that hydroxyurea in appropriate concentration inhibits the formation of mature infectious particles of Vaccinia virus without preventing the synthesis of viral proteins. The action of hydroxyurea on the development of Vaccinia virus was suggested to have an effect on the metabolism of bacteria, wherein it inhibits DNA synthesis without altering the formation or function of mRNA and specific enzymes. Margarett et al. found that hydroxyurea also inhibited the formation of infective virus in the case of bacteriophage T4 (88). Our results from the study of the development of cyanophage AS-1 in A. nidulans in the presence of hydroxyurea agrees very well with the observations described above. An enhancement of lytic activity was noted with high concentrations of hydroxyurea; however, a very low level of plaque forming units was revealed. These results are very similar to those obtained from Mitomycin C treated infected cells by Baye (20). It was suggested that high concentrations of the inhibitor either induces the de novo synthesis of specific viral-coded lysozyme molecules or alternatively reflects the activation of certain lytic enzymes already present in the cytoplasm of the host. Lysis would therefore occur and the resultant viral yield would be diminished (59). Recently, Ove Berglund et al. (21) reported that the effect of hydroxyurea on T4 bacteriophage is to inhibit the induction of a new viral ribonucleotide

reductase. Since the AS-1/Anacystis nidulans infective system is still at a level of preliminary study, the enzymes induced by AS-1 are not completely understood. In the future identification and isolation of AS-1 ribonucleotide reductase (if it exists) must be achieved in order to resolve the exact action of hydroxyurea in the infective system.

A similar ^{14}C incorporation pattern but much less ^3H -adenine uptake was found in hydroxyurea treated AS-1 infected cells relative to untreated infected cells. These data were derived from CsCl gradient analysis of dual pulse labeled experiments. This suggests that the formation of PIL-DNA is not affected by this agent. However, it has been reported that a culture of E. coli exposed to hydroxyurea for a prolonged period possessed an additional component of reduced buoyant density (116). This abnormal component did not arise from preexisting DNA, but was presumably made de novo in the presence of hydroxyurea (118). The delayed effect of hydroxyurea was suggested to be caused by carbamoyloxyurea, an oxidation product of hydroxyurea (119). This agent resulted in degradation of DNA and further in cellular death. In order to eliminate the possibility of PIL-DNA formation being due to the delayed effect of hydroxyurea in the treated samples uninfected hydroxyurea treated A. nidulans should be analyzed by the same experimental protocol. If no extra band of lower density is found in this

sample, the PIL-DNA is not a result of the degradation of hydroxyurea treated DNA.

Nalidixic acid (1,4-dihydro-1-ethyl-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid) is reported to be a specific inhibitor of DNA synthesis of a growing bacterial culture (54). It does not inhibit DNA polymerase, endonuclease I or exonuclease I, II, and III from E. coli (103a). In addition to the inhibition of DNA synthesis (34), single stranded breaks accumulate in the DNA (60) and some of the DNA is degraded to an acid soluble material at high concentration (34,55). Protein synthesis is also depressed and morphological abnormalities are detected (34) after extended exposure to nalidixic acid. It has recently been reported (58,160) that nalidixic acid prevents DNA gyrase from introducing superhelical turns into circular DNA. This may result in the inhibition of DNA synthesis. The interaction of nalidixic acid with DNA gyrase cannot, however, be solely responsible for its bactericidal effect (38). In our studies an amount of less than 100 ug/ml of nalidixic acid did not affect growth and DNA synthesis of A. nidulans. A concentration of 500 ug/ml strongly inhibited DNA synthesis and was found after longer exposure times to result in DNA degradation. Bleaching of the cells was also detected. This bleaching effect is similar to the findings reported by Pienkos et al. for Euglena chloroplasts (104). It was suggested that this effect was due to degradation of

DNA. The effect of nalidixic acid on green algae is enhanced by light and is said to be related to the functioning of the photosynthetic electron transport system (81). The site of action of nalidixic acid may be more complicated in photosynthetic organisms than it is in bacteria. The mechanism of action is still far from clearly understood.

Studies of the development of cyanophage AS-1 in the AS-1/Anacystis nidulans infective system show that nalidixic acid at a concentration of 500 ug/ml effectively inhibits phage production (2% of control). Whether this is caused by a DNA injection defect or by interference with DNA synthesis of cyanophage AS-1 similar to that reported by Wysocki et al. (177) in studies of T7 bacteriophages is unknown. Nalidixic acid was also found to strongly affect the elution profile from CsCl density gradients of the changing DNA pattern in AS-1 infected A. nidulans cultures. There was no significant formation of PIL-DNA or incorporation of ³H-adenine. This may be due to the potent inhibition and degradation of DNA in the presence of too high a concentration of nalidixic acid. Further experiments with lower concentration, which only inhibits DNA synthesis without causing the degradation, should be performed to study DNA metabolism and the development of cyanophage inside the infected cells. Then the dependence of DNA synthesis for the formation of PIL-DNA and the development of cyanophage can be determined.

It is known that ultraviolet irradiation affects both colony formation and DNA synthesis in bacteria (131). The inhibition of DNA synthesis following irradiation of bacteria almost always results from the formation of thymine dimers. The thymine dimers inhibit DNA synthesis in a polymerase system. DNA polymerization along a "primer" proceeds until it is blocked by a thymine dimer, then it stops. The almost complete inhibition of DNA synthesis after radiation may be reversed by the action (63) of a photoreactivating enzyme, which in the presence of visible light splits thymine dimers (169). Photoreactivation of UV-irradiation damage has also been demonstrated in cyanobacteria (12,19,175), although the details remain obscure. In our experiments the effects of UV-irradiation on the unicellular blue-green algae Anacystis nidulans have been examined in terms of dark survival and photoreactivation. UV-irradiated samples were kept in the dark for different periods of time prior to growth in the light. If samples are exposed to light before twenty-four hours of dark incubation, the lethal process can be immediately stopped and the remaining damaged sites can be photorepaired (Figure 31). For all the experiments the interval of dark treatment of the algae was standardized at twenty-four hours since following this interval they can no longer be repaired.

The dark survival curve demonstrates the response of A. nidulans to increased doses of UV-light in the absence

of the photoreactive process. It displays a lag, followed by an exponential decay. The rate of photosynthesis in A. nidulans after UV-irradiation was measured by the incorporation of $\text{NaH}^{14}\text{CO}_3$ (i.e., CO_2 fixation). A gradual decay in photosynthesis rate rather than an immediate stoppage of photosynthesis was found. In the coccoid blue-green algae, Agmenellum quadruphicatum, it has been reported that the same UV-doses that affect survival also damage the photosynthetic machinery. It is suggested that photosynthetic damage is the cause of cellular death (19). A highly effective photoreactivation was observed when studying the UV-irradiated Anacystis nidulans. The blue-green algae are an ancient group, which must have developed a means to cope with harmful irradiation. It is therefore not surprising to find a powerful photoreactivation system in them. The photoreactivation system in A. nidulans can completely negate a much longer UV-dosage than can the one in E. coli and possibly contains two photoreactivation processes. One route could be the photoreactivation of pyrimidine dimers (127,174) and the other would be related in some manner to photoreactivation of the photosynthetic apparatus (19).

The effect of UV-irradiation on cyanophage AS-1 was also studied in order to prepare the UV-damaged phage. The kinetics of inhibition after various doses of UV-irradiation were multihit with an extrapolation to a number of two.

Four minutes of UV-irradiation caused the number of viable phage to be reduced by 50%; however, thirty-five minutes of exposure was needed to cause 99% damage. There is a long lag period which has a relatively high tolerance to ultraviolet radiation. This suggests that some recovery mechanism might be taking place. Photoreactivation of UV-irradiated DNA bacteriophage was discovered by Delbecco (37) and later observed for photosynthetic microorganisms (13,175). These studies show that the dark plaque formation curves for free cyanophage AS-1 and phage within host cells are remarkably similar. Apparently little or no protection of cyanophage DNA is afforded by the host cell. When, however, a UV-irradiated phage infected culture was exposed to light continuously, an increase in plaque-forming units was seen. The data presented in the RESULTS leaves no doubt that suitable illumination of infected cultures of the A. nidulans can photoreactivate the DNA of the virus AS-1. The photoreactivation of UV-induced lesions occurs in viral DNA after it has penetrated the host cell. The host provides for the photorecovery of UV-damaged phage DNA and might be involved in the photorepair of pyrimidine dimers. It is not unreasonable to expect a photoreactivation enzyme in the algae to be responsible for both the photoreactivation of UV-irradiated host DNA and phage DNA. This suggestion, however, needs further evidence for support. Preliminary studies have to be performed in order to

determine the extent to which the survival of the irradiated virus and host cell depend on the quality of the illuminating light. If the wavelengths that restore algae and virus replication are the same, then the mechanisms involved in photorepair of viral and algal nucleic acids are probably similar. Efforts should emphasize isolation and characterization of UV-sensitive mutants and mutants of the photorecovery system. These should provide a means for analyzing the repair of UV-irradiation damage in Anacystis nidulans.

In order to study the development of cyanophage inside the host cell and the effect on the formation of PIL-DNA, ultraviolet damaged algae or cyanophage were used in different combinations in the AS-1/A. nidulans system (i.e., algae + UV-phage, UV-algae + phage, UV-algae + UV-phage, and algae + phage). In the sample of UV-irradiated cells infected with healthy phage it appears that UV-irradiated cells do not cause significant changes in phage infectivity and phage DNA metabolism inside the host, although a reduction of burst size and a shortening of the lytic cycle is detected. However, the healthy cells infected with UV-irradiated phage are quite different from the normal infective system in terms of infectivity, DNA metabolic pattern, burst size and complete lysing period. The UV-damaged phage lost most of their infectivity and a very small burst size was obtained with a longer lytic cycle. Instead of

the formation of PIL-DNA, a DNA with a slightly lighter density than the cell or phage was found at early hours of infection. At a later hour of infection PIL-DNA seemed to appear again.

From the studies of UV-irradiated prelabeled cells or phage, it is found that the formation of PIL-DNA is independent of healthy host DNA, but highly dependent on the undamaged phage DNA. A minor discrepant DNA species (slightly lighter density than the cell or phage, but heavier than PIL-DNA) is also observed in UV-irradiated prelabeled phage systems. The data accumulated thus far indicates that the host DNA, whether normal or damaged, does not affect the formation of PIL-DNA. Thus, it is unlikely that it is the template for PIL-DNA formation. Less tolerance to phage infection, early shut-down of host metabolism, and fewer materials for the phage production occur if the host cells are UV-damaged. This might explain the smaller burst size and shorter lytic cycle. Remarkably, the UV-irradiated phage (99% damaged) inhibits the formation of PIL-DNA at the time it is supposed to be synthesized. Later on, PIL-DNA does appear. This may be due to the photoreactivation of the cyanophage inside the host. This photoreactivation mechanism repairs the damaged phage, which then goes through a normal DNA metabolic pathway. These findings further support the previously suggested mode of formation of PIL-DNA. The UV-damaged phage was prepared by exposing the

phage to UV for thirty-five minutes. A long lag period exists in the phage survival curve. It is necessary to learn more about the response of irradiated phage at the initial time point of this lag period (four minutes exposure time and 50% of damage). From preliminary studies (data not shown) it is apparent that the UV-damaged phage at this dosage did not cause significant changes in phage characteristics during infection, (e.g., infectivity, lytic cycle and burst size, etc.), and that there is no effect on the formation of PIL-DNA. This further implies that photoreactivation is strong enough to repair this range of damage in the phage DNA allowing it to function normally. In order to understand the effect of UV-irradiation on the AS-1/Anacystis nidulans infective system, further experiments have to be performed. The UV-irradiated infective system can be prepared by infecting the cell with cyanophage AS-1, then exposing it to UV-irradiation in order to analyze the effect of different doses of UV on the infected culture, the extent of virus photoreactivation, which depends on the physiological state of the host, and the cyanophage growth characteristics in the damaged infective system.

APPENDIX A

Mauro's Modified Medium (3M)

per liter:

0.7500 gm sodium nitrate
0.2500 gm potassium nitrate
0.1000 gm potassium phosphate dibasic
0.0870 gm potassium phosphate monobasic
0.0750 gm septahydrated magnesium sulfate
0.0250 gm sodium chloride
0.0250 gm calcium chloride dihydrate
0.0040 gm septahydrated ferric sulfate
0.0500 gm EDTA
0.0310 gm potassium hydroxide
2.8600 mg boric acid
1.8100 mg manganese chloride tetrahydrated
0.2220 mg septahydrated zinc sulfate
0.0717 mg molybdate (85%)
0.0790 mg copper sulfate pentahydrate

The pH of the prepared medium is adjusted to 7.5 prior to autoclaving

APPENDIX B

Cyanophycean Agar

per liter:

5.0 gm	KNO_3
0.1 gm	K_2HPO_4
0.05 gm	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
10 drops	1% Fe Ammonium Citrate
1000 ml	H_2O
15 gm	Bactoagar (1.5%-Hard Agar)
-or-	
9 gm	(0.9%-Soft Agar)

preparation:

mix reagents with 500 ml H_2O

mix agar with 500 ml H_2O

autoclave each solution separately

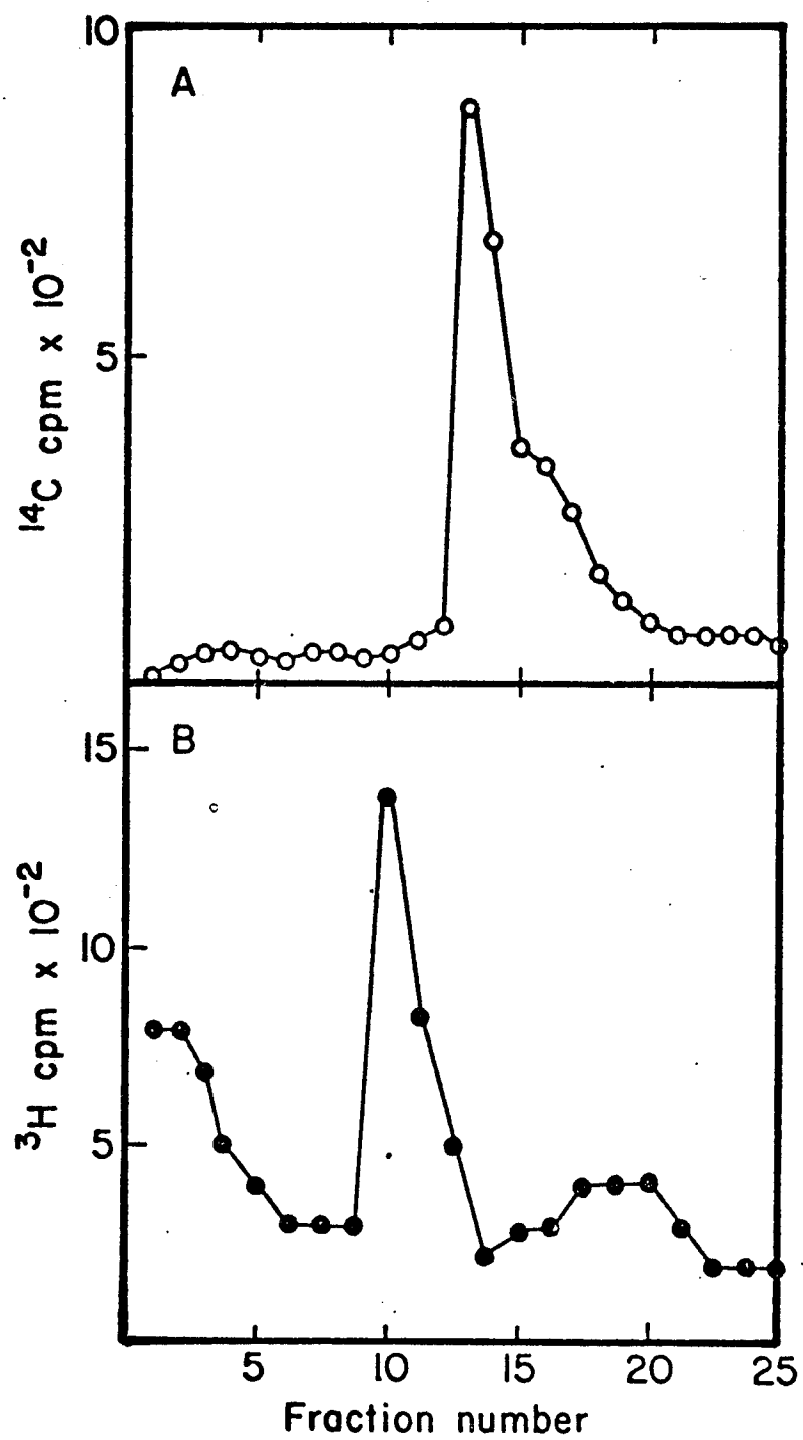
mix both solutions while hot

pour into plates or tubes for titer

APPENDIX C

Fractionation Profile from a CsCl Gradient of AS-1 Infected Anacystis nidulans Pulse-labeled with $\text{NaH}^{14}\text{CO}_3$ during the 2-5 hours Time Interval (Panel A) and Pulse-labeled with ^3H -adenine during the 5-8 hour Time Interval (Panel B)

AS-1 viral lysate was added to a large synchronized algal culture (MOI=5) to a final volume of 2 l. The collection of 50 ml of a $\text{NaH}^{14}\text{CO}_3$ pulse-labeled (2-5 hours) culture together with a large volume of cold infected culture and 50 ml of ^3H -adenine pulse-labeled (5-8 hours) culture together with a large volume of cold infected culture was described in detail in the legend to Figure 7. The terminated $\text{NaH}^{14}\text{CO}_3$ and ^3H -adenine labeled cultures were separately subjected to CsCl density gradient ultracentrifugations. The radioactivity of each fraction was monitored by alkaline stable, acid precipitable radioactivity.



APPENDIX D

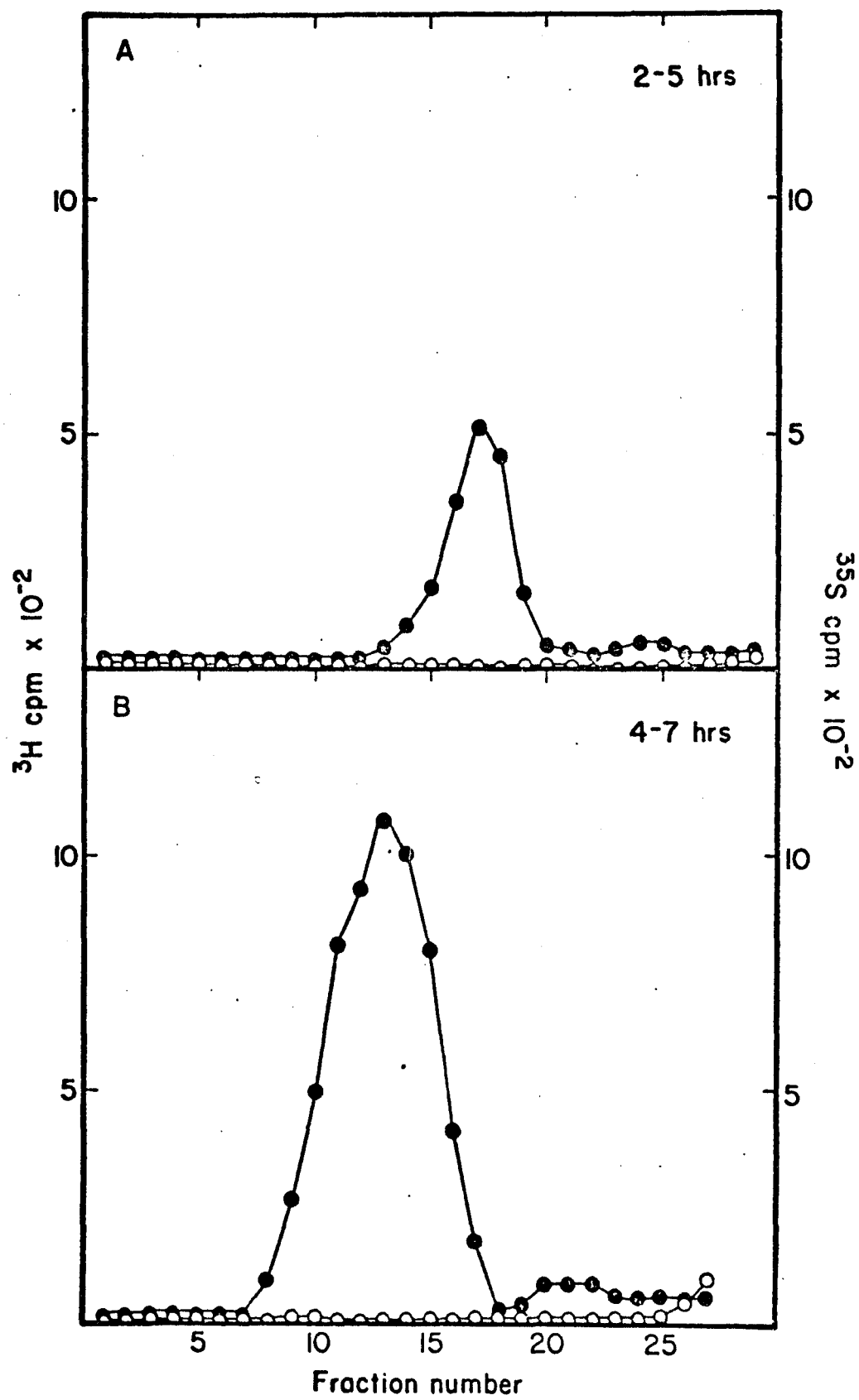
Fractionation Profiles from a CsCl Density Gradient
of AS-1 Infected Anacystis nidulans Simultaneously
Pulse-labeled with ^{35}S -methionine and ^3H -adenine

AS-1 was added to a synchronized Anacystis nidulans culture and the infected culture was divided into two small flasks and illuminated continuously. At 2 and 4 hours post infection the cultures were dual pulse-labeled with ^3H -adenine and ^{35}S -methionine at final concentrations of 5 uCi/ml and 1 uCi/ml respectively. The samples were collected at 5 and 7 hours (i.e. three hour pulse-labeling period) and terminated in the termination solution. The cultures were separately subjected to CsCl density gradient ultracentrifugation. The radioactivity of each fraction was monitored by alkaline stable and acid precipitable radioactivity.

Panel A: dual pulse-labeled with ^3H -adenine and ^{35}S -methionine at 2-5 hours

Panel B: at 4-7 hours

● — ● ^3H
○ — ○ ^{14}C



APPENDIX E

Hours of Maximum Incorporation Peaks of
 ^3H -adenine, ^{14}C -glucose-6-ph. and
 $\text{NaH}^{14}\text{CO}_3$ in Different Treated Samples

	^3H -adenine	^{14}C -glucose- 6-ph	$\text{NaH}^{14}\text{CO}_3$
algae + phage	6h	6h	6h
algae + UV-phage	8h	8h	6 or 8h
UV-algae + phage	4h	4h	4h
UV-algae + UV-phage	6h	6h	6h
UV-algae	8h	8h	8h
algae	6h	8h	8h

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