PHAGE-ALGAL INTERACTIONS IN THE CYANOPHAGE AS-1/BLUE-GREEN ALGA *ANACYSTIS NIDULANS* INFECTIVE SYSTEM

by

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ABSTRACT

The effects of cyanophage AS-1 infection on synchronized cultures of the blue-green alga Anacystis nidulans were studied with regard to a variety of phage-algal interactions as influenced by several environmental parameters of control. The duration of the lytic cycle has been demonstrated to be a function of temperature. In addition, viral induced macromolecular synthesis as well as maximal cyanophage production are shown to be strongly dependent on continued host photosynthesis particularly during the first 3-4 hours of the lytic cycle. The regimentation of the algal host into a synchronous state was the third environmental factor investigated with regard to maximizing cyanophage biosynthesis and replication.

The interactions between the infecting cyanophage and the algal host under defined experimental conditions were investigated with regard to both viral induced macromolecular synthesis as well as perturbations of host physiology resulting from infection. Infection of Anacystis nidulans by cyanophage AS-1 produced an altered algal permeability to a wide variety of reduced carbon substrates. The incorporation of 22 organic metabolites into synchronized uninfected and AS-1 infected A. nidulans was followed. These included sixteen amino acids, four nucleic acid precursors and two sugars. Thirteen of the amino acids, and all the nucleic acid precursors and sugars showed increased incorporation into the infected cells relative to the non-infected cells. The three remaining amino acids evidenced somewhat higher incorporation into the uninfected alga. The basis for the increased incorporation of the metabolites is seen to be (in part)

an altered host permeability with infection by a single cyanophage particle being sufficient to induce altered permeability in an alga.

The increased incorporation of nucleic acid precursors into cyanophage infected cells as compared to the uninfected alga engendered a study of DNA synthesis in AS-1 infected Anacystis nidulans. DNA extracted from cyanophage AS-1 infected A. nidulans grown at 26°C was analyzed using analytical equilibrium density centrifugation in CsCl. A new macromolecular species having a lower buoyant density in CsCl than the DNA of Anacystis nidulans was observed commencing at four hours post infection. A characterization of the new macromolecular species revealed DNase sensitivity, RNase insensitivity and a buoyant density in CsCl of 1.701 ± 0.001 g/cc. The new DNA species is tentatively identified as being phage induced light DNA (PIL-DNA) since its buoyant density in CsCl is lighter than that of the DNA of cyanophage AS-1M as well as being lighter than the DNA of the host alga, Anacystis nidulans. The incorporation of nucleic acid precursors (adenine, thymidine, uracil) into DNA was followed by exposing AS-1 infected algal cultures to the tritiated nitrogenous base or nucleoside. Preparative isopycnic centrifugation in CsCl indicated that the nucleic acid precursors incorporated into a DNA species having a buoyant density in CsCl of 1.714/1.715 g/cc. The utilization of $^{14}CO_2$ in nucleic acid metabolism in AS-1 infected A. nidulans was examined in light of the continued host photosynthetic capacity during the lytic cycle of the cyanophage. In contrast to exogenous nucleic acid precursor incorporation into DNA of buoyant density 1.714/5 g/cc, photoreduced ^{14}C incorporated into DNA is found

at a buoyant density of 1.701 $\pm$ 0.001 g/cc. The temporal pattern of incorporation of tritiated adenine, thymidine and uracil varied from that of the incorporation of photoreduced ^{14}C. At 32°C incorporation of adenine, thymidine and uracil into DNA begins from two to four hours post infection and accumulation continues until lysis. Photoassimilated ^{14}C found in DNA incorporates with a periodicity peaking between four and six hours post-infection. DNA extracted from PIL-DNA was analyzed using thermal denaturation and sedimentation velocity techniques. The Tm in 0.1X SSC was 70.9°C, the Tm in 1X SSC was 85.8°C and the G-C content was calculated to be 40.9 $\pm$ 0.6% from these values. The G-C content as calculated from buoyant density determinations (ρ = 1.701 $\pm$ 0.001 g/cc) was 41.8%. The molecular weight of the macromolecule was determined to be 138 x 10^6 daltons.

The fate of PIL-DNA was followed in a series of pulse chase experiments. Packaging of this DNA into viral particles was confined to material synthesized early in the latent period, the bulk of PIL-DNA remaining unpackaged in the infected cell. This contrasts to DNA of buoyant density 1.714/1.715 g/cc which is packaged primarily in the latter half of the latent period.

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Abbreviations

CCCP	Carbonyl Cyanide m-Chlorophenylhydrazone
DCMU	3 - (3, 4 Dichlorophenyl) -1, 1 Dimethyl Urea
FCCP	Carbonyl Cyanide p-trifluoromethoxyphenylhydrazone
G-C	Guanine Cytosine
MOI	Multiplicity of Infection
PIL-DNA	Phage Induced Light DNA
SSC	0.5M NaCl, 0.015 Sodium Citrate, pH 7
Saline - EDTA	0.5M NaCl, 0.1M EDTA, pH 8
Tm	The Temperature at which the Helix-Coil Transition of DNA is 50% complete
ts	Temperature Sensitive
TCA	Tricarboxylic Acid
$U^{14}C$	Uniformly Labeled with ^{14}C

Table of Contents

List of Tables

List of Figures

INTRODUCTION

The Cyanophyta (blue-green algae), a group of procaryotic, photoautotrophic microorganisms, are of particular interest in studying a wide variety of phenomena both at the molecular and ecological levels. Since the Cyanophyta occupy an extraordinary position, being at an evolutionary branch point between the procaryotes and the photosynthetic eucaryotes, studies of these organisms could provide insights into the mechanisms of control and development of chloroplasts and the molecular nature of photosynthesis. In fact it has been proposed in the endosymbiont theory of organelle evolution (56) that blue-green algae may be the ancestors of chloroplasts. The Cyanophytes have a demonstrated capacity for symbiosis with other algae and with fungi, and endosymbiosis is certainly feasible. The cyanelles found in species of Glaucocystis (39) and Cyanophora (38) are inclusions resembling unicellular blue-green algae and can be cultured separately after extraction from the cell (68). Even though definite identification of these structures as being of blue-green algal origin has not, as yet, been established due to various modifications found in the cyanelle as compared to cyanophyte structure (38,39) the possibility of the chloroplast being the end product of an evolutionary chain beginning with endosymbiotic blue-green algae still remains.

While the photosynthetic machinery and the products of photosynthesis found in the blue-green algae are similar to those found in higher plants, the cyanophytes are of a procaryotic nature with regard to their cellular organization. In this respect they may

be simpler and more readily controllable than eucaryotic organisms. In addition, blue-green algae, along with various non-photosynthetic procaryotes, have nitrogen fixing capability. At present, this metabolic activity has been found only in the filamentous members of the cyanophytes including both heterocyst and nonheterocystic forms (30,104). The combined capacity of cyanophytes for photosynthesis, nitrogen fixation and growth under aerobic conditions demonstrates another unique aspect of this group, and one having the potential for great ecological impact. It is interesting to note the almost contradictory effects of these organisms in different regions. Blue-green algae can and have played roles in contributing to the soil fertility of certain habitats such as rice paddy fields (20,106,114). On the other hand, algal toxins and eutrophication caused by algal blooms have proven detrimental to many bodies of fresh water with their associated flora and fauna as well as to the olfactory sensibilities of the surrounding residents (37,42,92).

Information derived from studies of the Cyanophyta could be directed at the ecological level to control their growth and to deter the noxious qualities they may impart to fresh waters. Conversely, research can lead to the generation of varieties as improved forms of natural fertilizer. In any event, research into the growth and metabolism of this group is requisite for understanding them. In the past decade, reports on cyanophyte metabolism have shown that certain aspects of their metabolism are unique as compared to other procaryotes. For example, in Anabaena variabilis (a filamentous species) and in Anacystis nidulans (a coccoid form) it has been shown

that the activity of enzymes involved in glycolysis, the pentose phosphate, glyoxylate, and interrupted tricarboxylic acid pathways were not affected by the inclusion of a major substrate of these pathways into the growth medium (61-64). This lack of enzyme induction implies a different type of control mechanism in the blue-green algae for gene regulation than that found in bacteria such as E. coli. However, some of the greatest modifications of gene activity takes place when a host cell is infected by a virus. The use of bacteriophages as probes in the elucidation of macromolecular mechanisms such as RNA transcription (8,111), translation (45,112,113), enzyme induction (18,28) and DNA biosynthesis (46) has resulted in a considerable body of knowledge related to these processes. The relatively simple, homogenous, and controllable nature of the interaction between a procaryotic host and its virus has allowed for many advances in the field of molecular biology.

The existence of cyanophages, viruses infecting blue-green algae, was first reported in 1963 by Safferman and Morris (77). It was suggested that cyanophages might prove to be a useful tool for control of algal growth (43,79). In addition, these viruses offered a new method 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. In fact, Safferman et al. (43) have shown the feasibility of Plectonema bloom control with cyanophage LPP-1, while others have investigated the debilitating effects of cyanophages on Plectonema (79) and Microcystis (75) both

in nature and in large scale experiments. It has also been observed that susceptible strains of algae are never dominant in ponds where the appropriate cyanophages appear in significant amounts (59,80). As an example, Lyngbia, Plectonema, and Phormidium, despite their universal distribution, are not known to form blooms in nature. While cyanophages may not be the only factor influencing the lack of bloom formation in these organisms, it has been suggested (19) that they may be as important as the availability of nutrients and light intensity. As a class, the cyanophages are ranked among the bacteriophages (13) and have been compared morphologically and biochemically with the coliphages in particular (2,49). Table 1, adapted from Padan and Shilo (59), and revised and updated, presents various parameters on a comparative basis among the cyanophages and selected bacteriophages. While there is a tremendous amount of data available from bacteriophage systems, especially from those phages infecting E. coli, with regard to viral host interactions (5,8,18,28, 45,46,50,112,113), this type of study has only recently begun with the cyanophages. Investigations of viral host interactions and cyanophage developmental processes have been reported in depth for the LPP-1/P. boryanum (15,35,36,58,78,87-90,96-98,117) and AS-1/AS-1M A. nidulans/S. cedrorum (3,65,81,84,86,91,110) infective systems with less extensive reports on other systems (1,52). For both LPP-1 and AS-1M infection of their respective hosts, the patterns of protein synthesis are analogous to those found in E. coli infected with some of the T series of coliphages (41,105). Early, middle, and late classes of phage directed proteins can be

Table 1
Morphological, Physicochemical, and Physiological Characteristics of Cyanophages and Some Selected Bacteriophages

Phage group	LPP	SM	N	AS	T even	T odd
Members of group	LPP-1, LPP-2	SM-1, SM-2	N-1	AS-1, AS-1M	T2,T4,T6	T3,T7
Phage characteristics Head	Polyhedron, 6 sided outline [a]	Icosahedron [a]	Polyhedron, 6 sided outline [a]	Polyhedron, 6 sided outline [a]	Prolated Polyhedron [a]	Icosahedron [a]
Head, diameter (nanometers)	60±2	67±1.8	55	90	65 x 95 [b]	47 [b]
Tail	Short, non-contractile [a]	Very short collar with thin appendage [a,b]	long contractile [a]	long contractile [a]	long contractile [c]	short, non-contractile [c]
Tail; length, width (nm)	20,15	-	110,16	243, 22.5	100, 25 [b]	15,10 [b]
Sedimentation coefficient (S20,w)	555 [b]	1.021,1.029 [c]	539 [a]	754 [b]	890±15 [d]	453±8 [d]
Bouyant density of phage in CsCl (g/cm^3)	1.48 [a]	1.48 [a]	1.498 [b]	1.49 [b]		1.50 [e]
pH range of greatest stability	5-11 [c]	5-11 [c]		4-10 [a]	T6: 4.6-8.9 [e]	T7: 5-8 [f]
Length of latent period and lytic cycle (minutes)	360; 960 [d,e]	1800; 3000 [d]	420; 1080 [c]	180-240 [a,b]	21-25; [f,g]	T3: 30° 20;30 [g]
Average burst	300-400 [d,e]	100 [d]	100-300 [c]	50-80 [a,c,d]	150-400 [f,g]	300 [g]
Phage dependence on photosynthesis	- [f,g]	+ [e]	- [c]	+ [c,d]	-	-
DNA characteristics molecular weight (daltons x 10^6)	27 [a,h]	56-62 [a]	38 [a]	57±3 [b]	105.7±3.8 [d]	25.5-1 [d]
Buoyant density in CsCl	1.714(25°) [a,i]	1.725 [a]	1.696(20°) [a]	1.714 [b]	T2,1.700 T4,1.698;T6,1.707 [h]	T7,1.705 T3,1.712
G+C content	53 [a,h]	66-67 [a]	37 [a]	53-54 [a,b]	35 [h]	48 [h]
Phage host DNA characteristics	*P. boryanum*	*S. elongatus*	*N. muscorum*	*S. cedrorum*/ *A. nidulans*	*E. coli*	*E. coli*
Buoyant density in CsCl	1.706(25°) [i]	1.73(25°) [f]	1.702(25°) [a]	1.715 [e]	1.710 [h]	1.710 [h]
G+C content (%)	48 [i]	71.4 [f]	43 [d]	55/56 [e,f]	50 [h]	50 [h]

The references pertaining to each phage group are given directly below each listing. The numbers given refer to the literature cited portion of this thesis.

REFERENCES:

a: 49	a: 50a	a: 1a	a: 81	a: 4	a: 4
b: 36	b: 31	b: 1b	b: 85	b: 116	b: 116
c: 78	c: 82	c: 1	c: 65	c: 13	c: 13
d: 60	d: 51	d: 24	d: 86	d: 21	d: 21
e: 87	e: 52		e: 24	e: 69	e: 49
f: 35	f: 101		f: 101	f: 23	f: 44
g: 90				g: 25	g: 25
h: 35a				h: 83	h: 83
i: 24					

distinguished. Protein synthesis is required throughout the cycle and DNA synthesis, if interrupted results in a reduced yield of free phage. Within the cyanophage infective systems that have been studied, a fundamental difference has been noted with regard to the effect of infection on host photosynthesis. Whereas those cyanophages infective for filamentous blue-green algae induce the rapid cessation of CO_2 photoassimilation (35,58,117), AS-1 and AS-1M, viruses infective for some unicellular species of the genera Anacystis and Synechococcus, cause relatively little disruption until or just prior to lysis (65,84,86). A similar series of events, in which photosynthesis continues at a reduced rate as compared to uninfected levels, is true for SM-1, which is infective for some species of unicellular cyanophytes (51,52). Although CO_2 photoassimilation was not disrupted, it seemed possible in an infective system such as AS-1/A. nidulans that the patterns of carbon flow and fixation might be altered as a result of cyanophage infection.

Both in LPP-1 infected Plectonema boryanum and in AS-1M infected Synechococcus cedrorum it has been shown that host DNA is degraded with subsequent utilization of the breakdown products in the synthesis of cyanophage DNA (88,91). In the LPP-1 infective system, however, the breakdown is incomplete and host DNA is seen to be capable of incorporating exogenously supplied nucleotides as late as 7 hours post infection (88). Sherman (91) found for the AS-1M/S. cedrorum infective system that some 15% of host DNA is solubilized in the first hour post infection with 40% degraded by 2.5-3.0 hours.

This latter time corresponds to the time when disruption of the host nucleoplasm is evident as visualized in the electron microscope. Additionally, Udvardy et al. (110) found that after AS-1 infection of Anacystis nidulans, the level of DNase increases 15-20 fold, and the development of this activity is dependent on host photosynthesis for the first two hours post infection, since treatment with DCMU just after infection inhibited the induction of this DNase activity.

In order to follow the changing patterns of synthesis of host and cyanophage DNA, as well as to determine the sources of precursors, it is necessary at some point to be able to separate the two types of DNA and be able to quantitate the level of synthesis of each of the species. One of the best ways of achieving this is to label the newly synthesized DNA with radioisotopes and separate the two types of DNA on CsCl isopycnic density gradients. This requires that the host cells be permeable to nucleic acid precursors and that host and viral DNA's be of sufficiently different base composition (G-C content) to allow for their resolution on CsCl. However, it has been shown in uninfected Anacystis nidulans that the incorporation of organic metabolites, under near saturating light conditions, occurs at low levels (47,95,99) and that this alga is relatively impermeable to several nucleic acid precursors (67,72). Since studies from E. coli infected with T even/odd viruses (to which the AS-1/ Anacystis nidulans system can be compared) show that the incorporation of many metabolites is lower after phage infection as compared to the uninfected cell (22,34,70), it was questionable

whether nucleic acid precursors could be introduced into AS-1 infected A. nidulans. Although Sherman (91) has shown incorporation of adenine and uracil into AS-1M infected Synechococcus cedrorum, the basis for this incorporation remained unclear.

In addition, thermal denaturation studies by both Safferman (81) and Sherman (86) on DNA extracted from purified virus indicated that the G-C content was 53.5 $\pm$ 1.5%. This corresponds to a buoyant density in CsCl of a value essentially equal to that of 1.714 g/cc obtained by Sherman using equilibrium density centrifugation of isolated, purified AS-1M DNA. Since the DNA of Anacystis nidulans has a G-C content of 55% (ρ = 1.715 g/cc) (24), the feasibility of a complete separation of phage DNA from host DNA during the infectious cycle seemed unlikely. However, as presented in this thesis, the incorporation of nucleic acid precursors, along with a variety of organic metabolites, is seen to be possible in Anacystis following infection by cyanophage AS-1. A novel aspect of this work is the identification of a phage induced light DNA (PIL-DNA) which utilizes newly synthesized precursors containing photoreduced carbon and which has a buoyant density in CsCl considerably different from that of host DNA or the bulk of the DNA found packaged in cyanophage AS-1. This difference allows for the discrimination between the various species of DNA found in the infected alga during the latent period and for kinetic studies of the phage induced light DNA synthesis.

In order to clarify the sequence of morphological and biochemical events occurring following infection of Anacystis nidulans by

cyanophage AS-1, the following review is presented. Following adsorption of AS-1 by the host, there is a three to four hour eclipse period during which the infectivity associated with the parental AS-1 cyanophage is lost; no infective phage particles can be found in infected algae artificially lysed within this period (86). During the eclipse portion of the latent period, host DNA breakdown commences. This event has been correlated with electron microscopic studies showing disruption of the algal nucleoplasm commencing at two hours post infection (65,86). During the latent period cyanophage AS-1 DNA synthesis begins. Sherman (91) followed AS-1M DNA synthesis by incorporation of tritiated nucleic acid precursors into infected cultures of S. cedrorum. He found a rise in incorporation of ^{3}H adenine or ^{3}H uracil beginning about three hours post-infection with continued incorporation until lysis. Prior to and concomitant with the events of DNA metabolism described, cyanophage specific protein synthesis and/or induced activity increases (91,110). The activity of a cyanophage induced nuclease increases some fifteen fold during the first hour post infection (110). This can be related to the breakdown of host DNA and to the progressive decrease in host protein synthesis which ceases by about the fourth hour post infection (91). At least three classes of cyanophage AS-1M proteins have been noted: early, middle and late proteins with the latter class being primarily structural (91). It is believed that these proteins serve functions similar to the three classes of proteins found after T4 infection of E. coli (91).

Development of cyanophage AS-1 and AS-1M has been shown to be

dependent on continued host photosynthesis (3,65,84,86). Morphologically, this is borne out by the lack of invagination of the photosynthetic lamellae as compared with the LPP-1/ P. boryanum system in which this invagination is concurrent with cessation of CO_2 photoassimilation. Inhibition of algal photosynthesis results in a corresponding inhibition in the biosynthesis of cyanophages AS-1 and AS-1M. A limited heterotrophic potential for development of the virus is seen, however, by the utilization of glucose to support limited amounts of viral growth, even in the dark or in the presence of DCMU (3,84). The latent period, during which the biochemical changes described above occur, extends (for AS-1) to 8-9 hours post infection (81). This is followed by a period during which free virions can increasingly be found. The full burst size is 50-80 plaque forming units/infected cell (65,81,86), a factor reflecting the extremely large size of this cyanophage relative to its host. Electron micrographs of thin sectioned AS-1 infected Anacystis nidulans show that matured AS-1 fill the host cell so that the low burst size is presumably not due to premature lysis (65,86).

The studies developed in this thesis present a multifaceted approach to the study of the cyanophage AS-1/blue-green alga Anacystis nidulans infective system. I. Environmental parameters of control over the infective process were examined to determine their effect on the quality and duration of cyanophage biosynthesis and replication. These included the regimentation of the algal cultures into a synchronized state prior to infection, the duration

of the lytic cycle as a function of temperature and perturbations of algal photosynthesis with a consequent effect on cyanophage metabolism. II. The cyanophage infective process was examined from the aspect of the alteration of host metabolism, in particular, the altered sensitivity after infection to reduced carbon substrates. It is suggested that the relative incorporation of these compounds in the infected cell offers clues as to the composition of cyanophage specified biosynthesis of (macro)molecules and the changeover of metabolic pathways from host to cyanophage biosynthesis. III. The third area of investigation involved the identification and characterization of a hitherto unreported phage induced species of DNA (PIL-DNA) found in Anacystis nidulans and Synechococcus cedrorum by any of the known AS type cyanophages. IV. DNA synthesis in AS-1 infected Anacystis nidulans was examined with respect to the kinetics of AS-1 DNA and PIL-DNA biosynthesis as well as the unique compartmentalization of precursor sources for each of these species of DNA.

Materials and Methods

Chemicals

DNA of Micrococcus lysodeikticus, purchased from the Sigma Chemical Co., St. Louis, Mo., and preparatively purified through CsCl, was a kind gift of Dr. R. Eckhardt. CsCl was purchased from the Harshaw Chemical Co., Solon, Ohio, and from the Kawecki-Berylco Co., Boyertown, Pennsylvania. Sarkosyl (Sodium lauryl sarcosinate) was purchased from Geigy Industrial Chemicals, Ardsley, New York. FCCP and DCMU were purchased from the Worthington Biochemical Co., New York, N.Y.

The ^{14}C L-amino acids: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, proline, serine, threonine and valine were all 50 mCi/mMole and were purchased from Schwarz-Mann, Orangeburg, N.J. Also from Schwarz-Mann were the ^{3}H L-amino acids methionine (2.6 Ci/mMole), tyrosine (37.8 Ci/mMole) and ^{14}C uridine (48 mCi/mMole). From New England Nuclear Corp., Boston, Mass., were purchased ^{14}C L-leucine (273 mCi/mMole), ^{14}C L-phenylalanine (409 mCi/mMole), ^{3}H adenine (28.2 ci/mMole), ^{3}H thymidine (20.2 Ci/mMole), and ^{3}H uracil (41.5 Ci/mMole). $Ba^{14}CO3$ was also purchased from New England Nuclear and converted to $NaH^{14}CO_3$ (76). From ICN of Irvine, Cal. were purchased $U^{14}C$ glucose (45mCi/mMole) and $U^{14}C$ glucose 6-P (220 mCi/mMole).

Algal Growth

Axenic cultures of Anacystis nidulans (Indiana 625) or

Synechococcus cedrorum (Indiana 1191) were grown in Bristol's medium pH 8.0 (102), without soil extract, on a gyrorotatory shaker under continuous illumination provided by 6 fluorescent growth lamps at a distance of 25 cm from the flask surface. Algal stocks were monitored for contamination by plating 0.1 ml aliquots on nutrient agar plates.

Cultures were maintained in exponential growth by transfer to fresh medium every 2-5 days. Growth was monitored turbidimetrically using a Klett Somerson colorimeter equipped with a green filter or by direct count in a Petroff Hauser counting chamber using a microscope equipped with phase optics. Large volume algal cultures were grown in 10 l carboys with aeration provided aseptically by passing air through a sterilized cotton filled glass tube. 10 l carboys were illuminated by nine 15 watt Sylvania FIST8D lamps placed around the carboy.

For experiments volume small algal cultures (50-100 ml) were transferred to a gyrotory shaker in a Puffer Hubbard incubator where the temperature was controlled and illumination provided by a bank of 4 Sylvania grow lux bulbs at a distance of 15-20 cm from the flask surface.

Induction of Synchrony via Dark Regimentation

Stock algal cultures were maintained at 30-33^{o}C in a Puffer Hubbard incubator with constant agitation on a gyrotory shaker at 125 rpm and illumination provided as described in Algal Growth.

Approximately twelve hours prior to experiments (6) flasks containing algal cultures were wrapped with aluminum foil. After the period of dark regimentation, experiments were conducted.

Viral Maintenance and Titer

Cyanophage AS-1 was kindly supplied by Dr. Robert Safferman and Cyanophage AS-1M was provided by Dr. Louis Sherman. These phages were routinely maintained by transferring lysates to logarithmically or synchronously growing algal cultures every 1-2 days. Prior to infection the algal stocks were made 0.1M in NaCl from a sterile stock solution (81). Phage titer was determined in the following manner. Lysates were centrifuged at 5000 x g for 10 minutes to remove unlysed cells and cellular debris. An aliquot of the clarified supernate was serially diluted into Tm buffer (10 mM Tris, 250 mM NaCl, 0.1 mM EDTA) (65) adjusted to pH 8.0. From each dilution 100 µl was transferred to 2 ml of a concentrated algal suspension of exponentially growing cells containing 2-10 x 10^8 cells/ml. Adsorption of Cyanophage AS-1 was allowed to proceed for 1 hour in the light. Following adsorbtion, 2.5 ml of liquified 1% cyanophycaen agar (102) maintained at 45^oC was added to the infected cell suspension and the resulting suspension was overlayered onto 1.5% cyanophycaen agar in petri plates. Following solidification of the overlay the petri plates were inverted and placed on plexiglass sheets over a light bank. Plaques were counted using an American Optical Corp. counting apparatuus equipped with a red filter.

Measurement of Metabolite Incorporation

Radioisotope was added to 10 ml each of uninfected and AS-1 infected cultures (5×10^7 cells/ml) with ^{14}C metabolites added to a concentration of 0.05 - 0.1 µCi/ml and ^{3}H compounds added to a final concentration of 0.5 - 1.0 µCi/ml. Metabolite incorporation was followed by removing triplicate 250 µl aliquots at hourly intervals from each culture and testing for the extent of acid insolubility of the exogenously supplied radioactive metabolite. Following acidification with an equal volume of 20% TCA and incubation at $4^{o}C$ for at least 20 minutes, the samples were collected onto Whatman GF/A glass fiber circles (2.4 cm), washed 3 x with cold 5% TCA and rinsed once with cold 95% ethanol. Samples were gently dried, placed in glass vials, covered with 5 ml of scintillation cocktail 4 g Omnifluor (New England Nuclear)/l toluene, and the radioactivity measured in a Nuclear Chicago liquid scintillation counter.

Radioactive Labeling of DNA

The introduction of radioactive nucleic acid precursors to either uninfected cells or infected cells for selected times during the lytic cycle was accomplished by the addition of 2-^{3}H adenine, ^{3}H thymidine or ^{3}H uracil to give final concentrations of radioactivity of 3.5 - 10.0 µCi/ml. For the introduction of $H^{14}CO_3^-$ an aliqot from a stock solution of $NaH^{14}CO_3$ in 0.1M Tris pH 8.0 was diluted into the cell suspension to give a final concentration of 4 - 10 µCi/ml. In pulse-chase type experiments, the radioactively labeled infected cells were collected by centrifugation at 5000 x g for 10 min, washed and resuspended in 0.4 original volume of Bristol's medium pH 8.0 made 0.1M in $NaHCO_3$ and to which 200 ug/ml non-radioactive nucleic acid

precursor was added. The cultures were allowed to proceed until lysis, or about twelve hours, at which point the lysate was clarified by low speed centrifugation (4000 x g, 20 min). Cyanophage AS-1 was collected from the clarified lysate by centrifugation for 45 - 60 min at 30,000 rpm using a Spinco 60 Ti rotor in a Beckman L3-50 ultracentrifuge. The remaining pellet was either immediately frozen or resuspended in 1 ml of saline-EDTA, treated with 2-3 drops of 30% Sarkosyl NL97 followed by incubation at 60°C for 15 minutes. The cyanophage containing lysate and the lysed, resuspended pellets were placed onto CsCl for preparative ultracentrifugation.

In all experiments termination was achieved by harvesting the cells by centrifugation at 5000 x g for 10 min and resuspension of the cells in a small volume of lysing buffer (0.15M NaCl, 0.1M EDTA, 0.05M NaCN, 0.01M NaN_3, 3 mg/ml lysozyme). These cyanide azide killed cells were maintained in the lysing buffer overnight at 37°C followed by the addition of three drops of a 30% aqueous solution of the detergent Sarkosyl NL 97 and incubated at 60°C for 15 min. The samples were either frozen or placed into CsCl for preparative ultracentrifugation.

Preparation of Material for DNA Purification

Preliminary to the extraction of DNA from either uninfected or AS-1 infected Anacystis nidulans, the cells were collected by centrifugation at 5000 x g for 10 min and resuspended in saline-EDTA (0.15M NaCl, 0.1M EDTA, pH 8.0). To this concentrated cell suspension (5 - 10 x concentration) lysozyme was added to a final concentration

of 2 mg/ml and the suspension incubated overnight at 37°C. Lysates of large (8 - 10 l) AS-1 infected Anacystis cultures were prepared for total DNA extraction in the following manner. The lysate was first made free of unlysed cells and cellular debris by centrifugation through a "Szent-Gyorgyi-Blum" continuous flow system (Sorvall KSB), made 8% in polyethylene glycol 6000 (Carbowax 6000) (118), and maintained at 4°C for several hours. The precipitate induced by this treatment was collected by centrifugation at 10,000 x g for 20 min and redissolved in saline EDTA.

DNA Purification

DNA was purified from samples prepared as described above using a modification of the method of Marmur (54). The samples dissolved in saline-EDTA were made 1% in SDS, heated for 15 min at 60°C, cooled, and made 1 M in either $NaClO_4$ or NaCl. To this high salt detergent mixture was added an equal volume of chloroform-isoamyl alcohol (24:1, v:v) and the mixture shaken for 1 hr followed by phase separation aided by centrifugation at 7000 x g for 10 min. The aqueous phase was recovered and mixed with an equal volume of 90% phenol. This mixture was shaken for 30 min and the phases separated using centrifugation. The aqueous phase was recovered and gently mixed with 0.5 volume of ether. Following centrifugation, two volumes of cold 95% ethanol were added to the aqueous phase and the DNA recovered by spooling onto a glass rod. This DNA was dissolved in 0.1 X standard saline citrate (SSC) (0.15 M NaCl, 0.015 M Na citrate, pH 7), the solution made 100 µg/ml with respect to Ribonuclease A concentration, and incubated for

30 - 45 min at 37°C. The solution was then made 100 µg/ml with respect to protease concentration and incubated at 50°C for 2 hr. The solution was then re-extracted with phenol followed by reprecipitation of the DNA with ethanol. The precipitated DNA was spooled onto a glass rod, dissolved in 0.1 X SSC, dialyzed overnight against 1 X SSC and then stored at 4°C over chloroform.

Analytical CsCl Density Gradients

Analytical ultracentrifugation was performed in a Beckman Model E ultracentrifuge equipped with ultraviolet optics set at 260 nm and a scanning attachment. Samples containing 2.5 - 4 ug of DNA were made 1.700 g/cc in neutral CsCl and loaded into double sector cells. The cells were placed in an AN-G rotor and centrifuged at 44,750 rpm for 20 hr at 25°C (57).

Preparative CsCl Density Gradients

Preparative CsCl density gradients were performed according to the procedure of Blamire et al. (12). Lysates of 1.0 ml were transferred to cellulose nitrate ultracentrifuge tubes followed by addition of 4.1 ml of a saturated CsCl solution (130 g/70 ml, 0.10 mM Tris pH 8). The density of each tube was adjusted to 1.700 g/cc using a Bausch & Lomb refractometer. The CsCl solution was overlaid with mineral oil to fill the tube. Tubes were placed into a Spinco 50 Ti rotor and centrifuged at 33,000 rpm for 42 - 65 hr at 19°C Fractionation of the gradients was accomplished by piercing the bottom of each tube with a 22 gauge needle and collecting 7 - 10 drop aliquots (0.18 ml). Each gradient yielded approximately 30 fractions.

For gradients which were to be monitored for UV absorbing material, each fraction was diluted with 0.2 ml of 0.1 X SSC, transferred to quartz micro-cuvettes and the absorbance at 260 nm measured. Gradients to be monitored for radioactive DNA were fractionated as described above and each fraction was made 0.5 N in KOH and incubated at 60°C for 2 hr. Following this alkaline hydrolysis the fractions were cooled to 4°C, 100 µg of bovine serum albumin was added to each fraction and the KOH was neutralized with 20% TCA. Excess TCA was then added to a final concentration of 10% and the fractions incubated at 4°C for 20 min. The precipitates from the TCA treatment were collected onto glass fiber filters (Whatman GF/A), washed three times with cold 5% TCA and once with cold 95% ethanol. The filters were then gently dried, placed in glass vials to which 5 ml of scintillation cocktail was added (4 g Omnifluor, New England Nuclear/l toluene) and the radioactivity measured in a Nuclear Chicago liquid scintillation Counter.

Thermal Denaturation

AS-1 and Anacystis nidulans DNA's extracted by the modification of the Marmur method described above were further purified by isopycnic banding in separate preparative CsCl density gradients. The initial density of the gradients was adjusted so that the DNA would band towards the center of the gradient, thus avoiding any possible remaining protein or RNA contamination. The samples were centrifuged and fractionated as described in Preparative CsCl Density Gradients. The DNA samples from the entire peak were pooled and dialyzed against

a total of 3 changes of 0.1 X or 1 X SSC. The optical melting curves were obtained using a Gilford 2400S recording spectrophotometer. The temperature of the cuvette chamber was adjusted in the transition range by $2^{o}C$ a minute using a Haake circulating water bath. The G-C content of the DNA's tested was determined by the formula of Mandel and Marmur (53) for 0.1 X SSC and by the formula of Marmur and Doty (55) for 1 X SSC. (See appendix D for calculations.)

Molecular Weight Determination

Rate zonal sedimentation performed according to the method of Blamire et al. (11) to determine the molecular weight of PIL DNA. A 2 ml sample, composed of equal volumes of lysate and a detergent mixture (2% sarkosyl, 3% sodium deoxycholate, 5% sodium dodecyl sulfate in 0.02 M EDTA, 0.01 M Tris pH 8.0, sterile filtered was gently layered with a wide tipped pipette onto the surface of a 36 ml 5 - 25% sucrose gradient containing 0.1 M NaCl and 0.02 M EDTA pH 7.0. Centrifugation was for 16 hrs at 14,000 rpm in a SW 27.1 rotor using a Beckman L3-50 ultracentrifuge. Fractions (1.0 - 1.1 ml) were collected from the top using a Buchler Co. gradient device and an LKB multiperpex pump. The determination of the radioactivity in the fractions was performed as described in the section on Preparative CsCl Density Gradients. As an internal marker ^{3}H labeled DNA from coliphage T7 was added [(MW = 25.5 x 106 daltons (33)] and the calculation of Freifelder (33) applied to determine the molecular weight of PIL DNA. (See appendix D for the calculations.)

Base Composition Analysis

Base composition studies of PIL DNA were performed on DNA made radioactive with ^{14}C following AS-1 infection of Anacystis nidulans in the presence of $H^{14}CO_3^-$. Lysates of infected cells were prepared as described in the section Radioactive Labeling of DNA and then centrifuged in preparative CsCl density gradients. After the gradients were fractionated as described in Preparative CsCl Density Gradients, 10 µl aliquots were removed from each fraction to assay for the position of the ^{14}C labeled DNA in the gradient. The enzymatic hydrolysis of the DNA to nucleotides and subsequent thin layer chromatography were performed according to the method of Rae (71). The ^{14}C containing peak was pooled and dialyzed against three changes of a solution containing 1mM NaCl and 5mM $MgSO_4$. One ml of DNA was incubated with 50 µl DNase I (stock solution, DNase I, 1 mg/ml; bovine serum albumin 1 mg/ml; 1mM NaCl; 5mM $MgSO_4$, pH 7.0) for 5 hrs at 37°C. The pH of the solution was adjusted to 8.5 using pH paper (pHydrion paper, Micro Essential Laboratory, Inc., Brooklyn, N.Y.) followed by incubation with 50 µl of a solution of snake venom phosphodiesterase (4 mg/ml with bovine serum albumin 4 mg/ml; 1mM NaCl; pH 8.7) for 5 hrs at 37°C. Aliquots (20 - 40 µl) of the hydrolysate along with 20 - 30 µg each of 5' dAMP, 5' dGMP, 5' dCMP, and 5' dTMP were spotted on a 20 cm x 20 cm thin layer chromatography plate (cellulose MN 300, 500 µ), and chromatographed in two dimensions, each to within 2.5 cm from the top of the plate. The solvent for the first dimension contained isobutyric acid: H_2O: ammonia (66:20:1) while the solvent for the second dimension was composed of saturated ammonium sulfate: 1M sodium acetate and

isopropanol (80:18:2). Autoradiography was then performed in order to identify the ^{14}C containing nitrogenous bases. Kodak "no screen" 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 at -70oC in a Revco freezer for 5 - 10 days. The x-ray film was developed by immersion for 5 minutes in Kodak x-ray film developer, rinsed with H_2O, and then fixed in Kodak acid fixer for 10 - 20 minutes.

Results

I. The Physiology of Phage-Algal Interactions

A. Phage Initiated Alterations in Host Permeability

1. Changes in Permeability to a Variety of Substrates

Populations of Anacystis nidulans infected with cyanophage AS-1 were exposed to a wide variety of radioactively labeled organic substrates including nucleic acid precursors, amino acids and sugars. The incorporation of these substances into infected Anacystis populations was determined as a function of the time of infection by measuring the radioactivity accumulated into the TCA insoluble fraction resulting from the addition of TCA to aliquots of infected cells. As a control, uninfected cells were exposed to the radioactively labeled organic substrates and the incorporated radioactivity was determined as above.

The patterns of incorporation of four nucleic acid precursors in infected and uninfected Anacystis nidulans are shown in figure 1. The data for adenine and thymidine show dramatically increased incorporation in infected relative to non-infected cells, while those for uracil and uridine while showing increased incorporation over uninfected levels, show lesser percentage increments as compared to the two other nucleic acid precursors tested (see Table 2). The incorporation patterns for 16 amino acids by infected and uninfected cells are included in figures 1 and 2. Within this group, differential patterns of incorporation in infected and uninfected cells are apparent. Arginine, histidine, leucine, lysine, methionine, phenylalanine, serine, tyrosine and valine show greater

Figure 1

Metabolite Incorporation by AS-1 Infected and Uninfected *Anacystis nidulans*. I.

A culture of *Anacystis nidulans* ($8x10^7$ cells/ml) was infected at a multiplicity of 4 and apportioned to 25 ml erlenmeyer flasks to which radioactive metabolites had been added. The ^{14}C metabolites were added to give 0.05-0.1 μCi/ml and the ^{3}H compounds were added to give 0.5-1.0 μCi/ml. At hourly intervals triplicate 0.25 ml aliquots were removed and assayed as described in Materials and Methods for incorporation of radioactivity into TCA precipitable material. As six hours post infection incorporation in CPM for infected (●) and uninfected (o) cells was as follows: adenine, $1.43x10^5$, $2.25x10^4$; thymidine $9.95x10^3$, $1.10x10^3$; glucose $5.95x10^3$, $1.50x10^2$; glucose 6-P $2.9x10^4$, $4.5x10^2$; uracil $5.85x10^4$, $3.71x10^4$; uridine $7x10^4$, $2.65x10^4$; histidine $2.50x10^4$, $8.60x10^3$; phenylalanine $4.50x10^3$, $1.38x10^3$; valine $6.38x10^3$, $3.70x10^3$; and, alanine $2.14x10^4$, $2.34x10^4$.

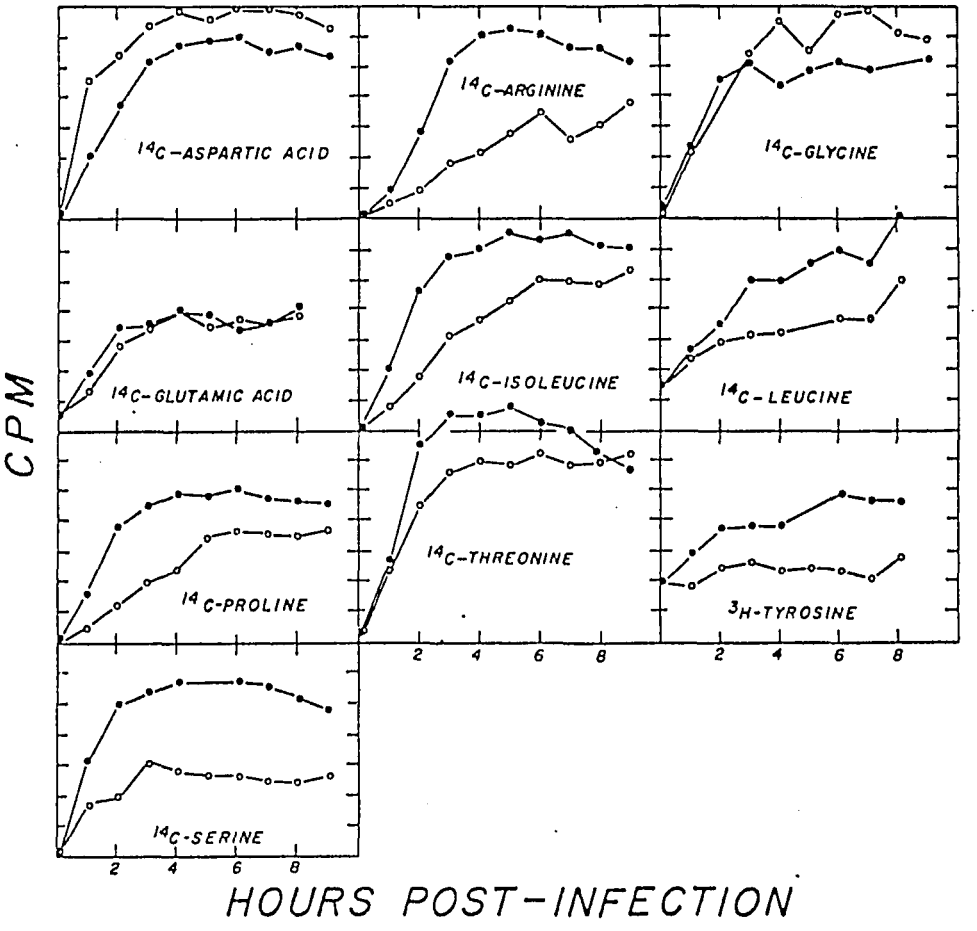

CPM
^{14}C-ASPARTIC ACID
^{14}C-ARGININE
^{14}C-GLYCINE
^{14}C-GLUTAMIC ACID
^{14}C-ISOLEUCINE
^{14}C-LEUCINE
^{14}C-PROLINE
^{14}C-THREONINE
^{3}H-TYROSINE
^{14}C-SERINE
2
4
6
8
HOURS POST-INFECTION

Figure 2

Metabolite Incorporation by AS-1 Infected and Uninfected

Anacystis nidulans. II.

Metabolite concentrations and incorporation assays were performed as described in the legend of Figure 1. At six hours post-infection, incorporation in CPM for infected (●) and uninfected (o) cells were as follows: aspartic acid 2.4×10^4; 2.8×10^4; arginine 2.44×10^4, 3.69×10^4; isoleucine 2.56×10^4; leucine 3×10^4, 1.88×10^4; proline 2.05×10^4; threonine 2.92×10^4, 2.52×10^4; tyrosine 4.9×10^2, 2.35×10^2; and serine 2.32×10^4, 1.08×10^4.

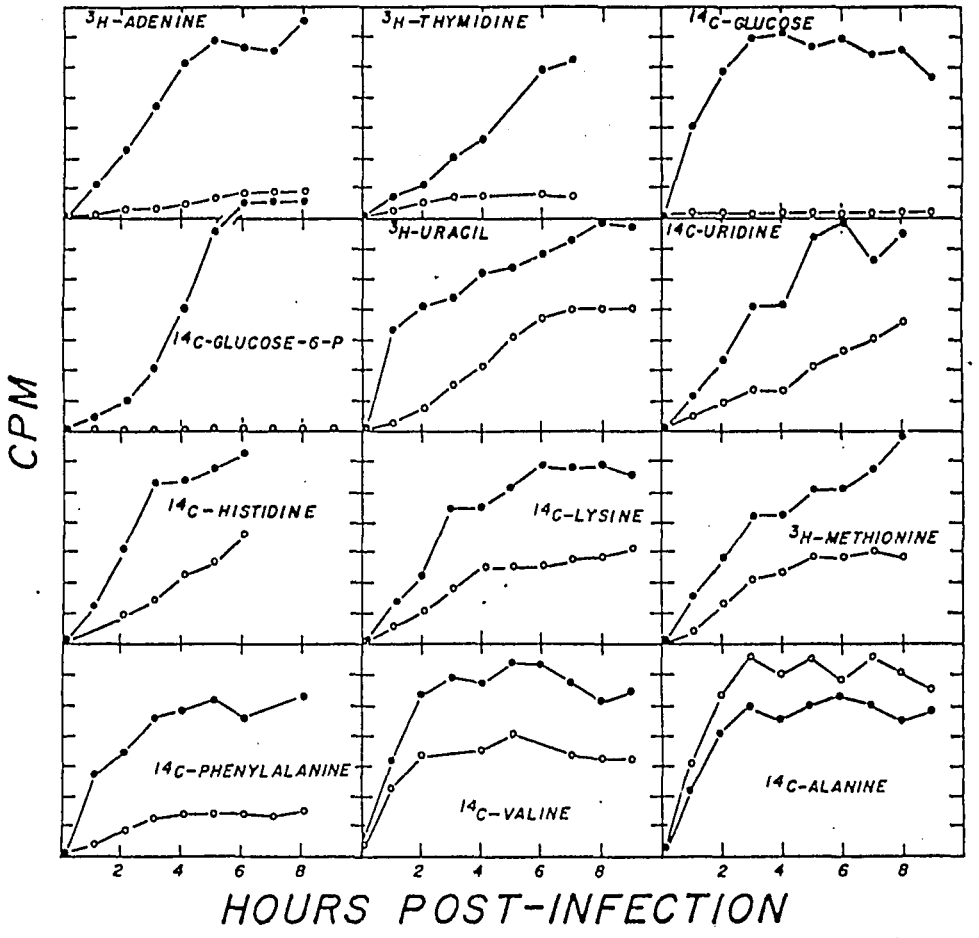

^{3}H-ADENINE
^{3}H-THYMIDINE
^{14}C-GLUCOSE
^{14}C-GLUCOSE-6-P
^{3}H-URACIL
^{14}C-URIDINE
^{14}C-HISTIDINE
^{14}C-LYSINE
^{3}H-METHIONINE
^{14}C-PHENYLALANINE
^{14}C-VALINE
^{14}C-ALANINE
2
4
6
8
CPM
HOURS POST-INFECTION

Table 2

Metabolite incorporation into AS-1 infected Anacystis nidulans. The values of radioisotope incorporation into TCA insoluble material are for the 6th hour post infection (cpm x 10^{-3}/ml). The data was taken from figures 1 and 2.

Nucleic Acid Precursors	Infected	Uninfected	I/U
Adenine	143	22.5	6.35
Thymidine	9.95	1.10	9.05
Uracil	58.5	37.1	1.58
Uridine	70.0	26.5	2.64
Sugars			
Glucose	5.95	0.15	39.7
Glucose 6-P	29.0	0.45	64.4
L-Amino Acids			
Alanine	21.3	23.4	0.91
Arginine	24.4	14.0	1.74
Aspartic Acid	24.0	28.0	0.86
Glutamic Acid	33.8	26.9	1.26
Glycine	20.7	27.1	0.76
Histidine	25.0	14.3	1.75
Isoleucine	25.6	20.1	1.27
Leucine	30.0	18.8	1.60
Lysine	23.8	10.4	2.29
Methionine	15.5	8.60	1.80
Phenylalanine	4.5	1.38	3.26
Proline	20.5	15.0	1.37
Threonine	29.2	25.2	1.16
Tyrosine	0.49	0.24	2.04
Serine	23.2	10.8	2.15
Valine	6.38	3.70	1.72

incorporation in the infected cells. Alanine, aspartic acid, glutamic acid, glycine, isoleucine, proline and threonine show relatively equal incorporation by infected and non-infected cells. However, alanine, aspartic acid and glycine show somewhat higher incorporation in uninfected cells.

The incorporation patterns of the two sugars used in this study are shown in figure 1. It is indicated from the data that these two substrates are incorporated into AS-1 infected Anacystis, even in the micro-molar quantities present, as compared to a distinct lack of such incorporation in the uninfected cells.

2. Dependency of Substrate Incorporation on the Extent of Phage Infection

In order to determine the dependence of metabolite incorporation on the extent of phage infection, Anacystis was infected at various multiplicities of infection with AS-1. When viruses are added to a population of cells, the number of infected cells is statistically related to the number of viral particles added. According to the approximation of the Poisson distribution

where bo is the fraction of cells that received no viral particles and m is the multiplicity of infection (103). Figure 3 shows the relationship between the infection of Anacystis at various multiplicities of infection of AS-1 and the incorporation of ^{3}H-adenine into the infected cells. In Figure 4, a linear relationship is perceived when the log of the CPM of the incorporated radioactivity is plotted vs. the multiplicity of infection. From Figure 3 it can be seen that the bulk of the ^{3}H-adenine

Figure 3

Incorporation of ^{3}H-adenine by AS-1 Infected Anacystis nidulans as a Function of the Multiplicity of Infection. I.

A culture of Anacystis nidulans of $5x10^{7}$ cells/ml was made 5 μCi/ml in 2-^{3}H-adenine and 10 ml apportioned into 25 ml erlenmeyer flasks. Each flask was infected with AS-1 at the desired multiplicity of infection and maintained at 33^{o}C with continuous agitation and illumination. At hourly intervals post infection duplicate 0.25 ml aliquots were removed and processed as described in Materials and Methods for determination of incorporation into TCA precipitable material. The incorporation at 6 hours is presented as a function of multiplicity of infection.

Figure 3

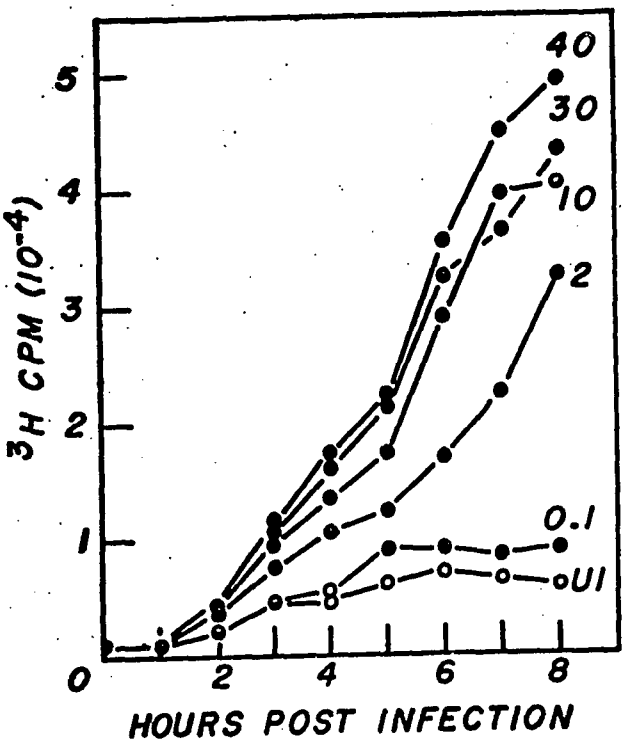
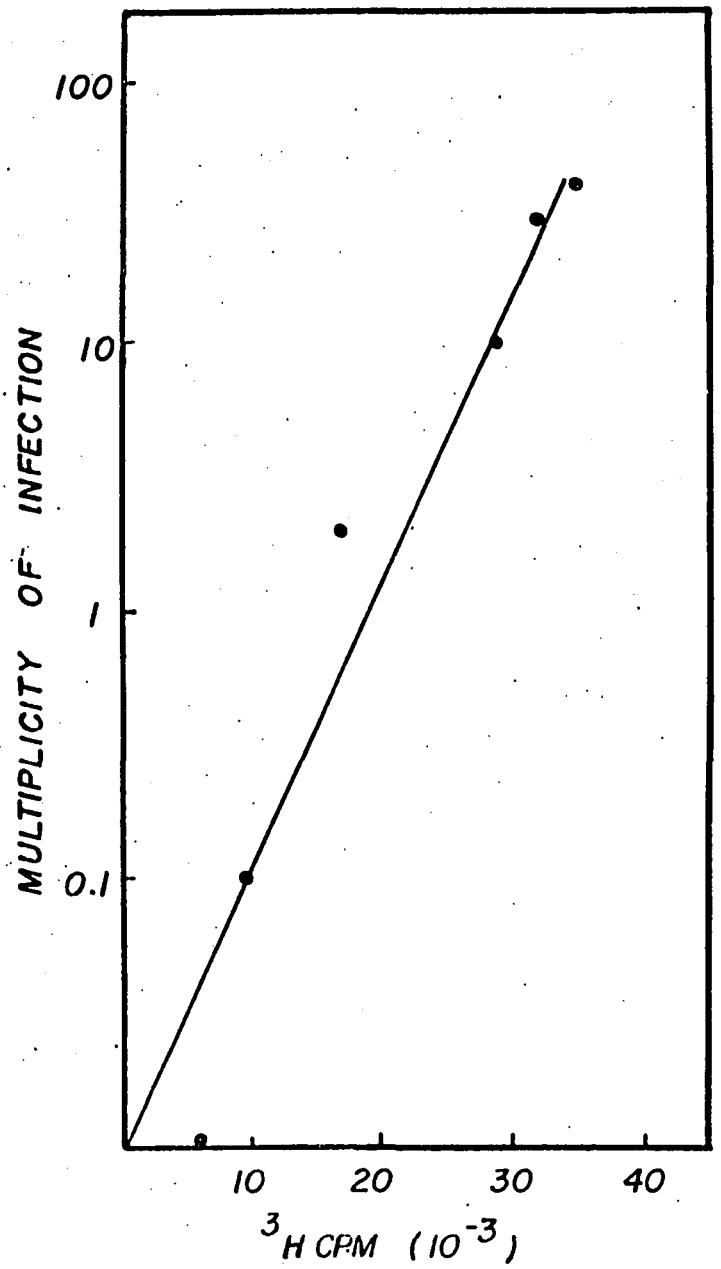


Figure 4

Incorporation of ^{3}H-adenine by AS-1 Infected Anacystis nidulans as a Function of the Multiplicity of Infection. II.

The data from the six hour time point of figure 3 has been replotted on a semi-log scale to show the linearity of the function between incorporated radioactivity and MOI.

Figure 4



incorporation has occurred between MOI = 2 and MOI = 10. Increasing the multiplicity of infection above levels at which all available cells are infected (MOI 5) does not substantially increase the amount of incorporated radioactivity. The small increase that is seen (Figure 3,MOI's 30, 40) may reflect the increased number of replicative phage genomes present within those cells that have been multiply infected at the very high multiplicities of infection.

B. Environmental Effects on the AS-1/Anacystis nidulans Infective System

1. Synchronization of the Algal Host

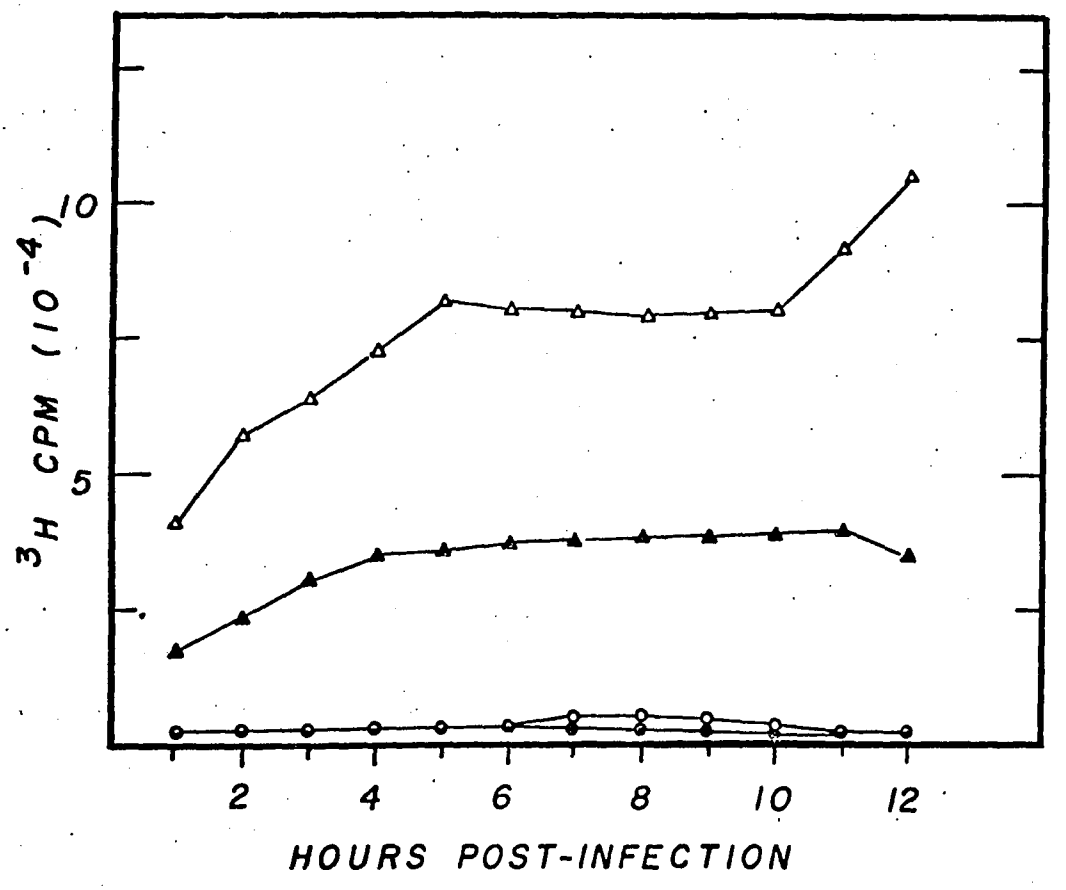
Weppelman and Brenton (115) Have demonstrated the relationship between the sensitivity of Pseudomonas aeruginosa to phage infection and the bacterial division cycle. In order to determine the effects of cyanophage infection on a uniform algal population a culture of Anacystis nidulans was synchronized according to the light-dark regime of Asato and Folsome (6). In Figure 5, the effect of AS-1 infection on the incorporation of ^{3}H-adenine into synchronized algal population and an asynchronous control are shown along with the incorporation levels into the uninfected algae. Substantially greater incorporation of ^{3}H-adenine is seen into the pre-synchronized infected culture as compared to the culture infected in the asynchronous state. Essentially no difference is seen between the negligable incorporation levels of ^{3}H-adenine into the uninfected cultures, whether synchronized or not.

Figure 5

The Effect of AS-1 Infection of the Incorporation of ^{3}H-adenine into Synchronous and Asynchronous Populations of Anacystis nidulans.

^{3}H-adenine (5μCi/ml) was added to equal volumes of synchronous or asynchronous cultures of Anacystis nidulans (5 x 10^{7} cells/ml). Anacystis was synchronized by incubation in the dark by wrapping the culture with aluminum foil for 11 hrs. (6). Each culture was divided and one half of each was infected with AS-1 at a MOI of 5. Triplicate 0.25 ml aliquots were removed hourly and precipitated at $4^{o}C$. Measurement of ^{3}H incorporation was as described in Materials and Methods (Δ- synchronous infected; ▲-asynchronous infected; 0-synchronous uninfected; ●-asynchronous uninfected).

Figure 5



2. The Effect of Light Duration

Cyanophage infecting unicellular blue-green have been shown to have a light requirement for the maintenance of their lytic cycle (3,52,84) and this is reflected by relatively little diminution of host photosynthesis until or just prior to lysis (3,65,84,86). In order to determine whether this light requirement was constant throughout the infectious cycle or was limited with some period of phage replication being light independent, infected cultures were subjected to one of three separate treatments designed to impair the light utilization by AS-1 infected A. nidulans. At various times post infection cultures were completely enclosed with electrical tape, exposed to the electron transfer inhibitor DCMU or exposed to the uncoupler FCCP. The data from these experiments are presented in Figure 6. It can be seen that the most critical period of time with respect to the requirement for photosynthesis and/or phosphoylation is the first 3-4 hours post infection and that beyond five hours into the lytic cycle the system demonstrates a high degree of independence from photosynthesis for maximum phage production.

Nucleic acid metabolism in AS-1 infected Anacystis nidulans was determined as a function of the time into the lytic cycle that the inhibitors DCMU and FCCP were added by measuring the incorporation of ^{3}H-adenine into the cell lysate fraction that was alkali insensitive and precipitable by cold TCA. These experiments (Figures 7,8) complement those determining phage titer as a function of light impairment and corroborate the findings that

Figure 6

The Effect of Darkness, DCMU, and FCCP on the Replicative Cycle of AS-1 in Anacystis nidulans.

A culture of anacystis nidulans of 5 x 10^7 cells per ml was infected with AS-1b at an MOI of 5. At hourly intervals following infection aliquots of 1 ml were transferred to tubes either made dark (▲) by wrapping in electrical tape, containing 5 nanomoles DCMU (●), 100 nanomoles in FCCP (o), or untreated (△). The tubes were maintained at 30°C and agitation was by a roller drum adapted to provide for continuous illumination. Twelve hours after infection all tubes were centrifuged to remove cells and cell debris and triplicate aliquots removed from each tube for viral titer as described in Materials and Methods.

Figure 6

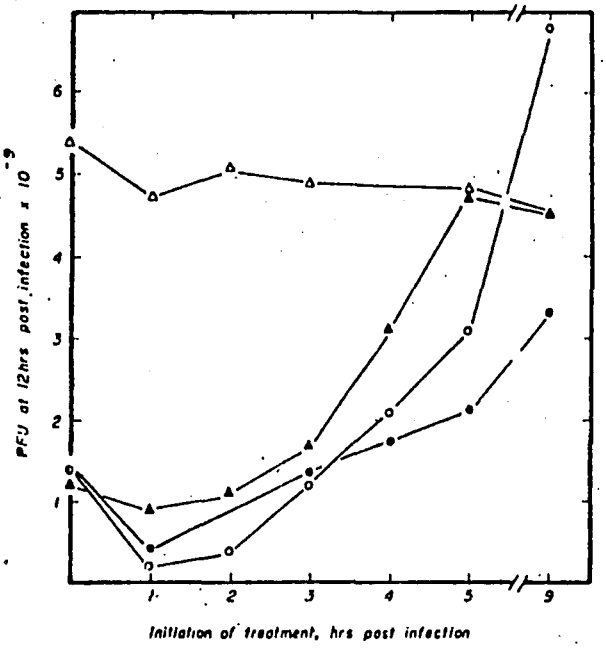

PFU at 12hrs post infection x 10⁻⁹
6
5
4
3
2
1
1
2
3
4
5
9
Initiation of treatment, hrs post infection

Figure 7

The Effect of DCMU on the Incorporation of ^{3}H-adenine Into Macromolecules in AS-1 Infected Anacystis nidulans.

A culture of Anacystis nidulans of 5 x 10^{7} cells per ml was infected at an MOI of 5 and made 10μ Ci/ml with respect to ^{3}H adenine. The culture was apportioned into 25 ml erlenmeyer flasks and each flask assayed hourly (duplicate 0.25ml samples) for incorporation of radioactivity into macromolecules as described in Materials and Methods. At selected times flasks were made 10μm in DCMU and sampling for ^{3}H incorporation continued.

Figure 7

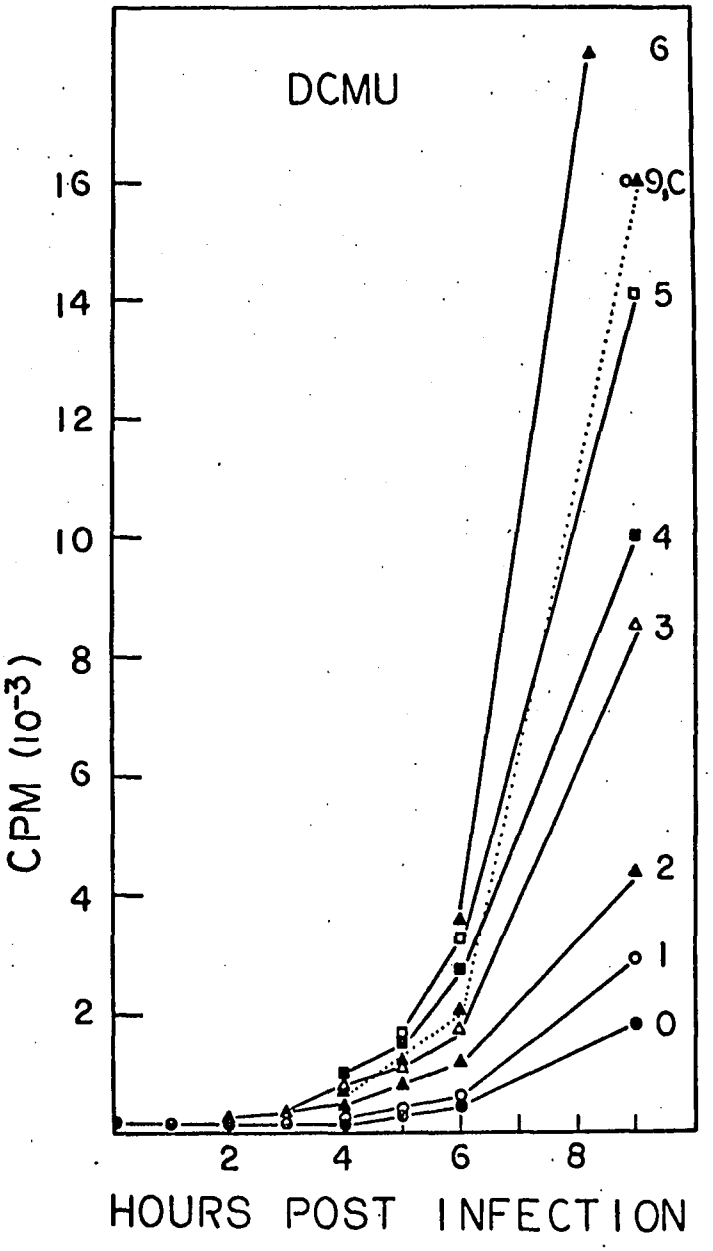
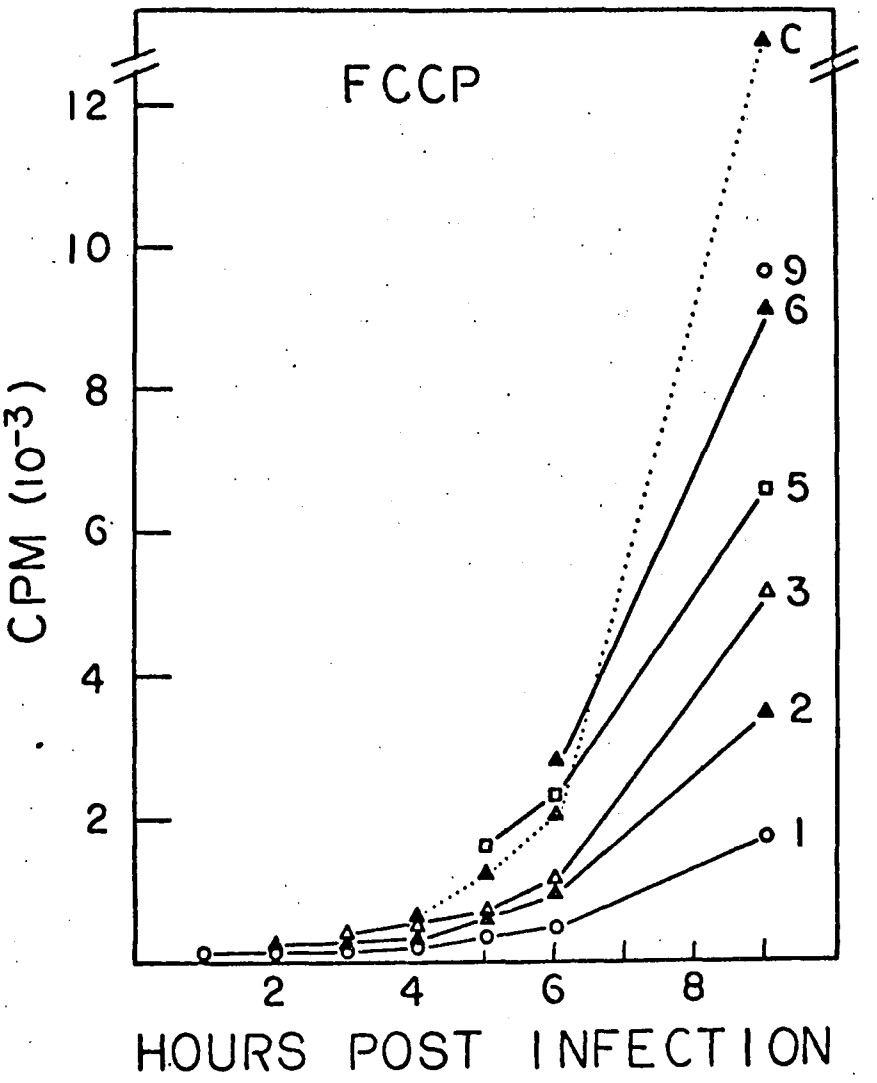

DCMU
6
9,C
5
4
3
2
1
0
16
14
12
10
8
6
4
2
CPM (10⁻³)
2
4
6
8
HOURS POST INFECTION

Figure 8

The Effect of FCCP on the Incorporation of ^{3}H-adenine Into Macromolecules in AS-1 Infected Anacystis nidulans.

A culture of Anacystis nidulans (5×10^{7} cells/ml) was infected at a MOI = 5 and made 10μCi/ml with respect to ^{3}H-adenine. The culture was apportioned into 25 ml erlenmeyer flasks and each flask was assayed hourly (duplicate 0.25 ml samples) for incorporation of radioactivity as described in Materials and Methods. At selected times the flasks were made 100 μM in FCCP and sampling for ^{3}H incorporation continued.

Figure 8



relative light insensitivity occurs beginning approximately 5-6 hours post-infection. The exact timing of the above-described phenomena, whether relating to metabolite incorporation or the period of light requirement for continued phage development, is variable and can be seen to be temperature dependent as seen from the following data.

3. Temperature Effects on the Lytic Cycle of AS-1

A third environmental parameter affecting the AS-1/A. nidulans infective cycle that was studied was that of temperature. A culture of Anacystis cells was synchronized by a 12 hour dark regimen and infected at 26°C. The culture was subdivided and aliquots were incubated in the light at several different temperatures. At hourly intervals samples were withdrawn from each culture and assayed for the remaining intact host cells by direct cell counts using a Petroff-Hauser counting chamber. In Figure 9, the effect of 5 temperatures ranging from 26°C-34°C on the AS-1 lytic cycle, is shown. The dependence of the length of the lytic cycle on temperature is clear. The trend is for a shortening of the lytic cycle with temperatures up to 34°C. At the higher temperatures (30-34°C) lysis is seen to commence earlier than at 26°C or 28°C with the times at which 50% of the infected algal cells are still surviving post infection are 26°C, 9.7 hours; 28°C, 9.0 hours; 30°C, 7.3 hours; 32°C, 7.1 hours; 34°C, 5.4 hours. Temperatures higher than 40°C are inhibitory to phage development (data not shown).

Figure 9

The Effect of Temperature on the Lytic Cycle of AS-1

A culture of Anacystis nidulans of 5 x 10^7 cells per ml was divided in half. One half was infected with AS-1 at an MOI of 10 and the other remained uninfected. These two cultures were then apportioned into 25 ml erlenmeyer flasks, and one set (infected and uninfected) maintained at each of the desired temperatures with constant agitation. All cultures were maintained under identical light conditions (4 Sylvania Gro-lux lamps 15 cm from the flask surface). The progress of the lytic cycle was determined by monitoring the decrease in cell number due to lysis. Cell counts were made using a Petroff Hauser counting chamber and a microscope equipped with phase optics. ([],26°C; Δ ,28°C; ▲ ,30°; o, 32°C; ●,34°C)

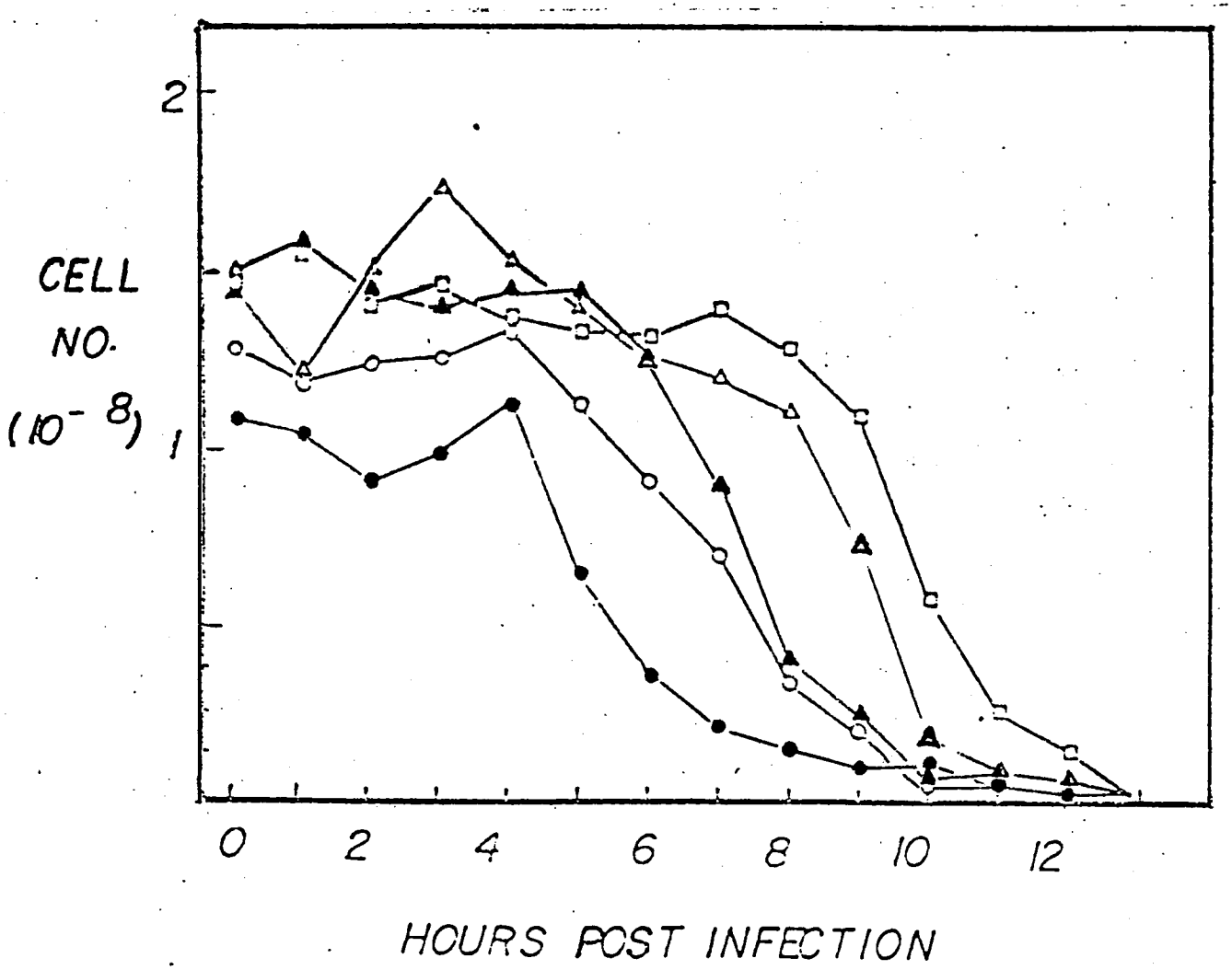


Figure 9

II. Formation of a New Cyanophage AS-1 Induced DNA Species

1. Isolation of a Phage Induced Light DNA (PIL DNA)

Phage infection generates a new sequence of biosynthetic events in the infected host. In the following experiments, the parameter of DNA biosynthesis was chosen to follow the dynamics of macromolecular synthesis in cyanophage AS-1 infected Anacystis nidulans. Figure 10 shows the results of a time course experiment in which 7 1 of logarithmically growing Anacystis cells ($5x10^7$ cells/ml) were infected with AS-1 at a MOI of 4 with 600 ml aliquots being withdrawn at hourly intervals. The total DNA was extracted and purified from each of these samples and the DNA centrifuged in analytical CsCl density gradients as described in Materials and Methods. It can be seen from these UV (260 nm) scans of the centrifuge cells at equilibrium, that for the first three hours post-infection DNA of only one buoyant density species was present in the infected cells. However, commencing with four hours post-infection, a second peak of UV absorbing material is evident at a lighter buoyant density than that of Anacystis nidulans DNA (ρ = 1.715, ref. 24).

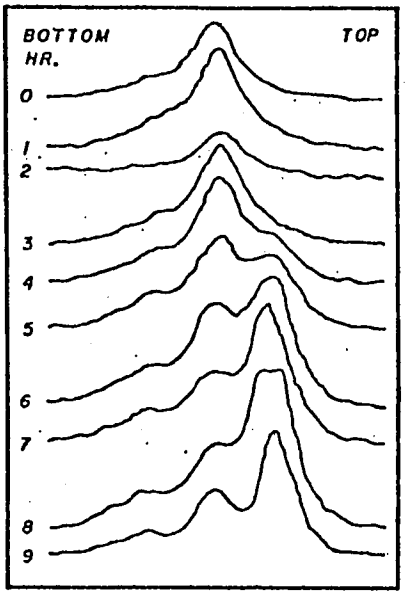
Edelman et al. (24) have shown that various blue-green algae contain polysaccharides that band in CsCl similar to that of the DNA of the algae. In order to ascertain the nature of the new UV absorbing material identified in the time course experiment, the material was subjected to nuclease treatment. Equal aliquots were treated with RNase A(200 ug/ml, 60 min, 37°C) or DNase I (200 µg/ml, 60 min, 37°C). Figures 11b (RNase treatment) and 11C (DNase

Figure 10

The Temporal Pattern of DNA Occurrence in Anacystis nidulans Infected with the Cyanophage AS-1

A 7 l exponentionally growing culture of Anacystis nidulans (Indiana 625) was infected with cyanophage AS-1 at a multiplicity of infection of 4. At hourly intervals after infection aliquots of 600 ml were removed, centrifuged and the pelleted cells resuspended in a small volume of saline-EDTA which was 50mM in NaCN and 10 nM in NaN_3. Each sample was made 2 mg/ml in lysozyme and protoplasts generated by incubation with the enzyme for 12 hours at 37°C. Each sample was subsequently treated with sodium dodecyl sulfate (1% final concentration) and the DNA purified for each sample according to the modified method of Marmur as described under Materials and Methods. Following the ethanol precipitation and spooling of the DNA from each sample onto a glass rod the DNA was redissolved in 0.1X SSC and then made 1X in SSC. Analytical ultracentrifugation was performed using an AN-G rotor as described under Materials and Methods. The numbers next to each gradient scan represent the hour at which the aliquot was removed from the large infected culture.

Figure 10



treatment) depict scans of the UV material present in the analytical CsCl gradients after equilibrium had been reached. Figure 11a (untreated) and 11b (RNase treated) are identical in showing a peak of UV absorbing material. The sensitivity to DNase seen in Figure 11C and the relatively light buoyant density seen in Figures 10 and 11 lead to terming the new material "phage induced light DNA" (PIL DNA).

The buoyant density in CsCl of this new macromolecular species was more accurately determined by purifying DNA from one of two sources; I. Total DNA samples from a temporal sequence of DNA synthesis experiment were centrifuged in preparative amounts through preparative CsCl gradients to separate the two species; II. DNA was extracted using the modification of the Marmur method (54) described in Materials and Methods from the polyethylene glycol precipitate (118) of a clarified supernate of a lysate of AS-1 infected Anacystis nidulans. One of these DNA's was then centrifuged in an analytical CsCl gradient along with the DNA's of Anacystis nidulans (ρ=1.715 g/cc) and Micrococcus lysodeikticus (ρ=1.731 g/cc). Material extracted by either method I or II gave essentially the same results. In Figure 11, the results of such an analytical ultracentrifugation, including PIL-DNA extracted by Method II are shown. The buoyant density in CsCl, based on three determinations was 1.701 ± 0.001 g/cc. The G-C content of the DNA was calculated by the formula of Schildkraut et al. (83) to be 40.9-0.1%

Figure 11

Treatment of "New" DNA with RNase and DNase

"New" DNA which had been purified from lysates of large infected cultures by first precipitation in the presence of 8% polyethylene glycol 6000 followed by DNA extraction using the modified Marmur method described in Materials and Methods, was adjusted to a concentration of 50 μg ml and divided into 3 samples. One sample was untreated, a second was made 2 mg/ml in RNase A, and a third was made 2 mg/ml in DNase I. After a period of incubation of 1 hr at 37°C a volume originally contained 4 μg of nucleic acid was removed from each sample and analytical ultracentrifugation in neutral CsCl performed as described under Materials and Methods. Each panel represents the UV scan of the cell containing the sample of each treatment. Panel A, untreated; B, RNase treated; and C, DNase treated.

Figure 11

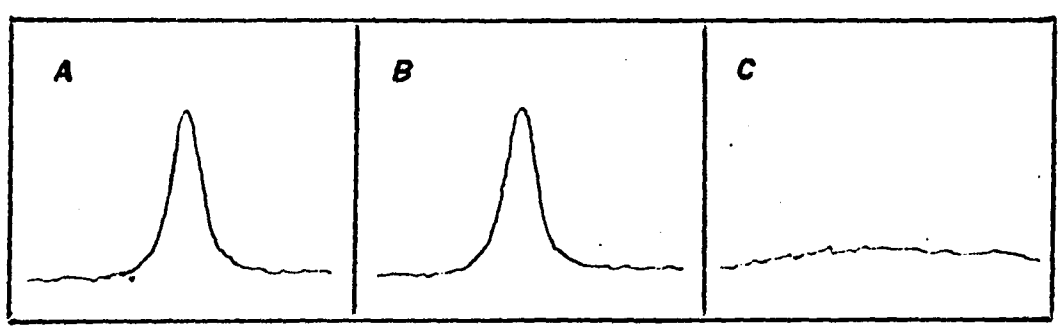
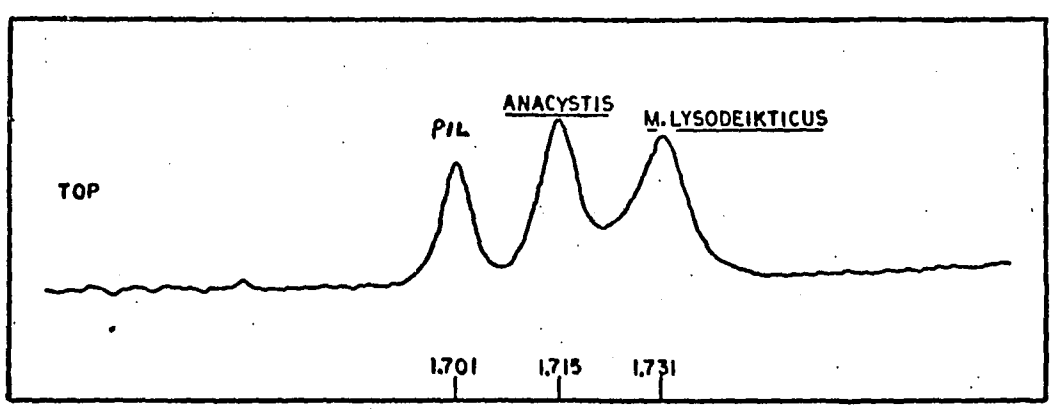


Figure 12

Sedimentation Equilibrium of PIL-DNA in CsCl

PIL-DNA was isolated using the modification of Marmur's method (54) described in Materials and Methods from the polyethylene glycol precipitate (118) obtained from treating a clarified lysate (5000xg, 30 min) of a 5 l culture of AS-1 infected A. nidulans. PIL-DNA was added along with the DNA's of A. nidulans (ρ=1.715 g/cc) and M. lysodeikticus (ρ=1.731 g/cc) to a common analytical CsCl gradient. The sample was placed in a double sector cell in an AN-G rotor and centrifuged in a Beckman model E analytical ultracentrifuge equipped with a UV scanner set at 260 nm. The ultracentrifugation was conducted at 44,770 rpm for 20 hours at 25°C.

Figure 12



2. The Incorporation of Radioactive Precursors into DNA in AS-1 Infected Anacystis nidulans

In order to study the biosynthesis of AS-1 DNA and PIL-DNA, the metabolic fate of exogenously supplied radioisotopes into both species of DNA was followed as a function of the lytic cycle. The increased permeability of AS-1 infected Anacystis to a variety of organic substrates including 4 nucleic acid precursors was previously demonstrated (Figures 1 and 2). In addition the photoautotrophic nature of the blue-green algae enhances the photo-reduction of $^{14}CO_2$ into ^{14}C metabolites for use in the infective system. Aliquots of infected cultures were treated with ^{3}H adenine or $H^{14}CO_3$ for one hour durations at hourly intervals throughout the infectious cycle. These aliquots were then harvested, lysed and the distribution of radioactivity was analyzed following preparative CsCl centrifugation. The results of the sequential one hour exposures to ^{3}H adenine are shown in Figures 13 and 14. Most of the radioactivity is seen to incorporate into DNA of a buoyant density heavier than that of E. coli DNA (ρ=1.710 g/cc) which was added as an internal standard in each of the preparative CsCl gradients. The equation of Sueoka (107) shows the relationship of 2 DNA's in a gradient when the buoyant density of one is known.

$$\rho = \rho_o + 4.2\omega^2(r^2 - r_o^2) \times 10^{-10} \text{ g/cc}$$

where ρ = buoyant density of the unknown DNA species

ρ_o = buoyant density of the marker species of DNA

r = distance of the unknown DNA from the center of rotation

r_o = distance of the marker DNA from the center of rotation

ω = speed of rotation in radians/sec

Figure 13

The Incorporation of ^{3}H-adenine Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans. I.

To a logarithmically growing A. nidulans culture which had been synchronized as previously described (see legend of figure 5) was added AS-1 to give 5 x 10^{8} phage/ml, sufficient NaCl to give a concentration of 0.1 M and a final cell concentration of 5 x 10^{7} cells/ml in a final volume of 50 ml. At hourly intervals after infection, 5 ml aliquots were withdrawn and added to flasks containing sufficient ^{3}H adenine to give a concentration of radioactivity of 10 μCi/ml. All flasks were agitated at 100 rpm and maintained at 32^{o}C under constant illumination provided by a bank of 4 24 watt cool white fluorescent lamps. One hour after the exposure to the ^{3}H adenine the sample was collected by centrifugation at 5000 x g for 15 min and resuspended in lysing buffer. Following overnight incubation at 37^{o}C each sample was treated with the detergent Sarkosyl NL97 and added to 4.1 ml of CsCl stock solution. Following preparative ultracentrifugation, each gradient was fractionated and each fraction monitored for alkaline hydrolyzable, acid precipitable radioactivity as described in Materials and Methods. Panel A is the radioactivity incorporation profile for the gradient from a sample exposed to ^{3}H adenine between hours 1 and 2 post infection; B is 3-4 hr; C is 4-5 hr. The vertical line in each panel is the midpoint of the peak for the DNA of E. coli (ρ=1.710 g/cc) included in each gradient.

Figure 13

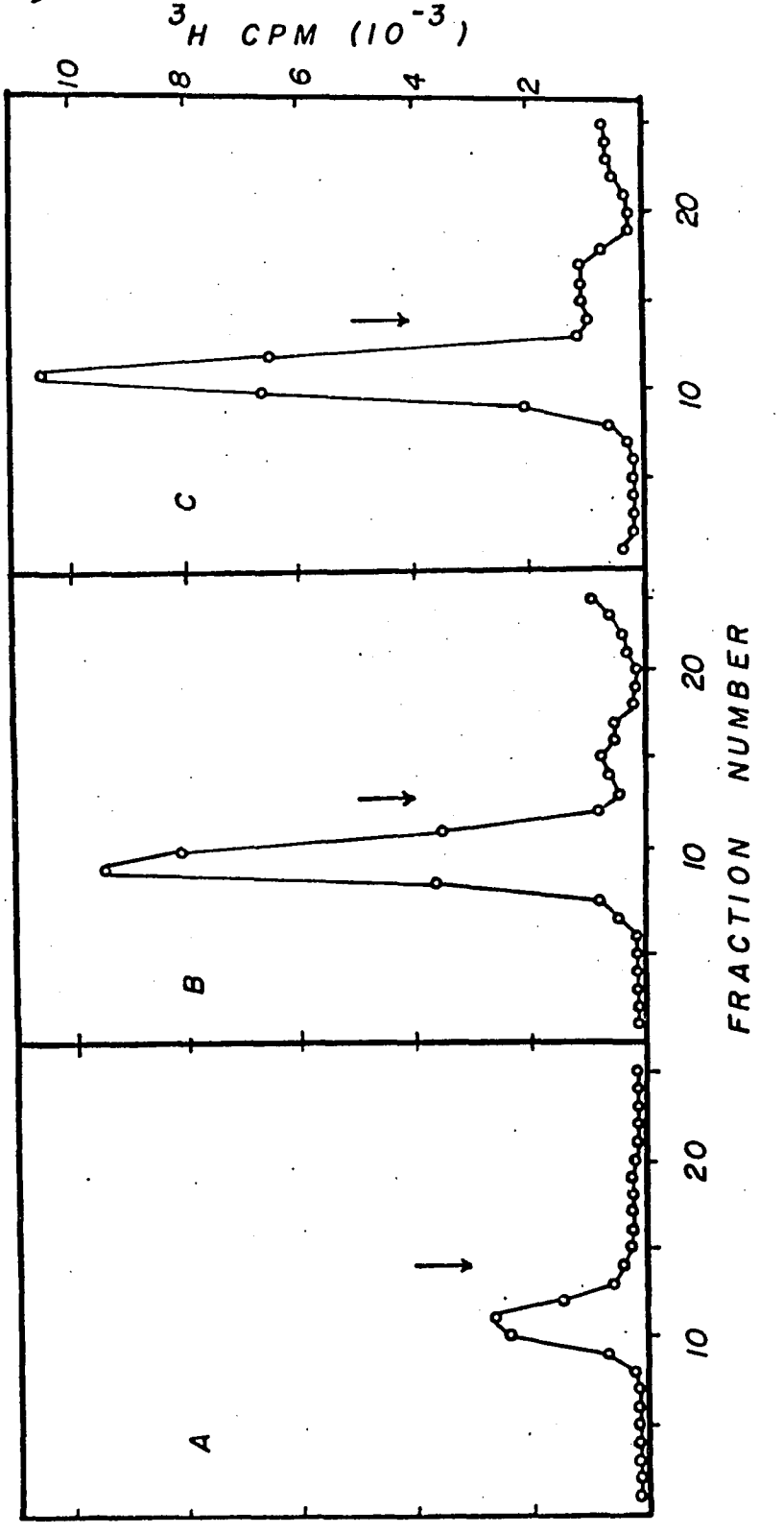
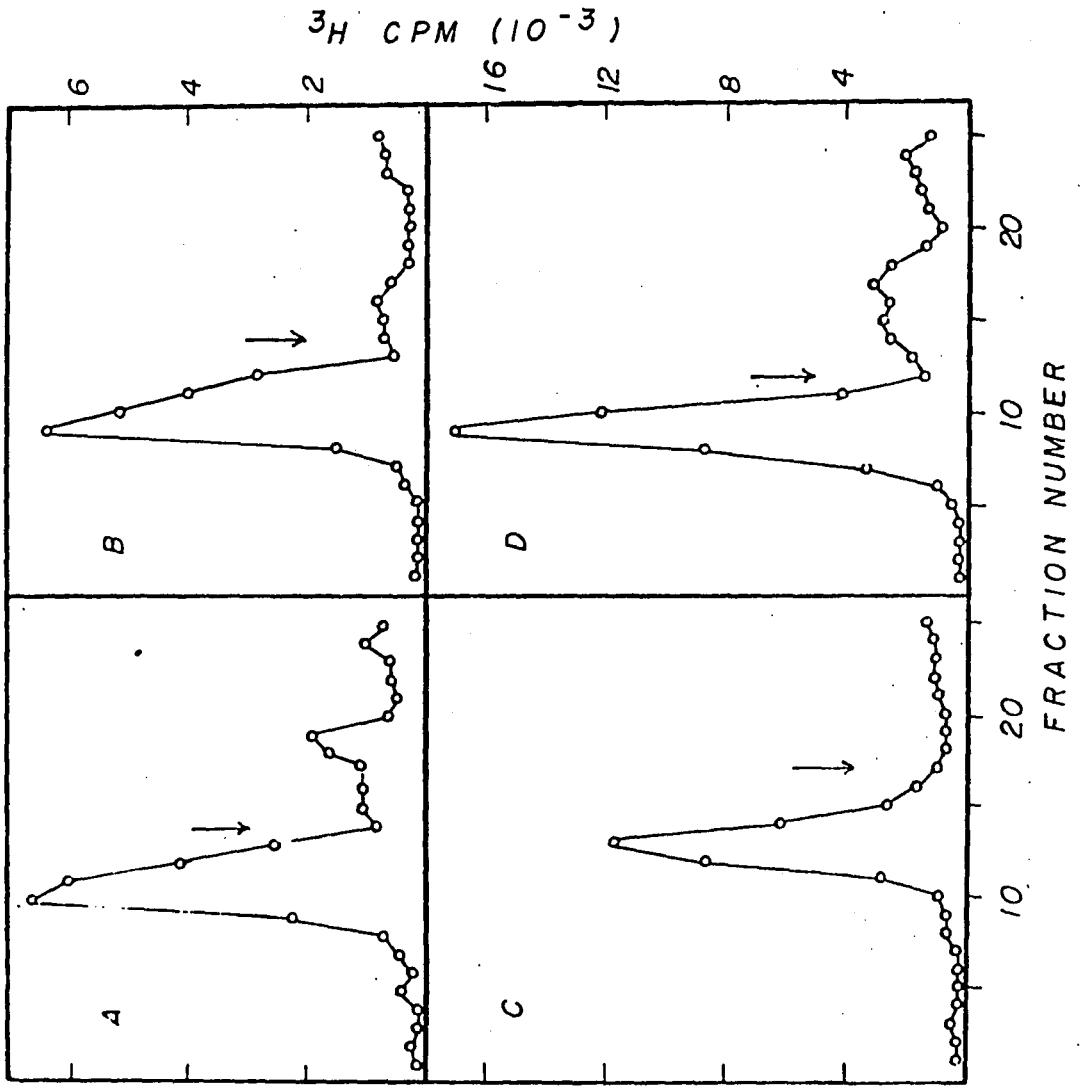


Figure 14

The Incorporation of ^{3}H-adenine Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans. II.

^{3}H-adenine was added to AS-1 infected Anacystis nidulans (MOI=10, cell concentration 5 x 10^{7} cells/ml) at hourly intervals post infection and 5 ml aliquots were lysed, centrifuged in preparative CsCl gradients and analyzed as described in the legend of figure 13. Panel A is the radioactivity incorporation profile for the sample exposed to ^{3}H-adenine 5-6 hours post infection, B) 6-7 hrs., C) 7-8 hrs., D) 8-9 hrs. DNA of E. coli (ρ=1.710 g/cc) added as reference DNA to the gradients is represented by the arrow in the panels.

Figure 14



The buoyant density of the ^{3}H containing peak was calculated to be 1.714 g/cc, a value equivalent to that derived by Sherman (85) for AS-1M DNA extracted directly from the purified phage.

When $H^{14}CO_3$ was used as the source of radioactive label for the infected cells, ^{14}C counts are found to band at a buoyant density in CsCl of 1.703/4 g/cc (Figure 15), a value approximating that of PIL-DNA. The contrasting results of the experiments in which radioactivity was added as a nucleic acid precursor (^{3}H adenine) or as a non specific metabolite ($H^{14}CO_3$) were examined by using a protocol in which both sources of radioactivity were added simultaneously to AS-1 infected cells in a series of one hour exposures during the lytic cycle. The samples from this dual isotope experiment were harvested, lysed, and the DNA analyzed as before in preparative CsCl density gradients. From the data presented in Figures 16-18 it can be seen that two species of DNA are generated after AS-1 infection of Anacystis nidulans. The two DNA's exhibit distinctive characteristics with regard to buoyant density in CsCl and temporal synthesis. Early in the latent period (Fig 16b, 1-2 hours post infection), one DNA species is seen, containing both ^{3}H (derived from ^{3}H adenine) and ^{14}C (newly photoassimilated). By 2-3 hours post infection (Fig. 16c), two separate varieties of DNA are evident, one heavy (ρ=1.714/5 g/cc) and one light (ρ=1.701$\pm$0.001 g/cc). In addition, each of these DNA's characteristically contains primarily one or the other of the radioactive materials added during the pulse. The heavy DNA (ρ=1.714/5 g/cc) contains mostly ^{3}H while the light DNA (ρ=1.701$\pm$0.001

Figure 15

The Incorporation of $NaH^{14}CO_3$ Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans.

The experimental protocol was identical to that described in figure 13 except that: the temperature was 26°C; the culture was adjusted to pH 8 using a pH meter; the radioactive compound was $NaH^{14}CO_3$ buffered to pH 8; and, the concentration of radioactivity was 10 uCi/ml. The vertical line in each panel is the midpoint of the peak for the DNA of B. subtilis (ρ=1.7035 g/cc) included in each gradient.

Figure 15

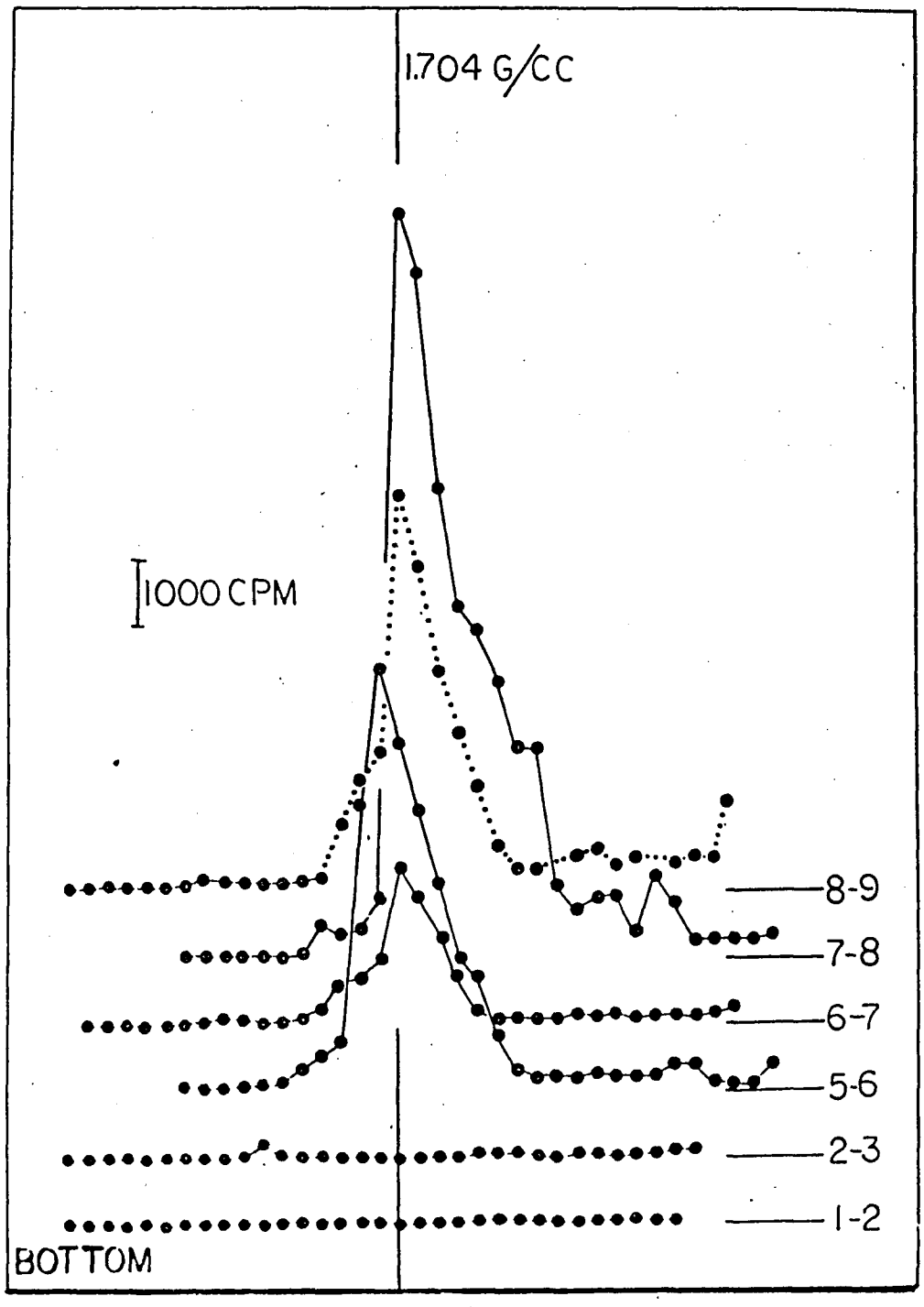


Figure 16

The Simultaneous Incorporation of ^{3}H-adenine and $NaH^{14}CO_3$ Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans. I.

The experimental protocol was identical to that described in figure 13 except that the culture pH was adjusted to 8 using a pH meter and each sample was exposed to both $NaH^{14}CO_3$ and ^{3}H adenine to give 8 and 3.5 uCi/ml respectively. Panel A is 0-1 hr.; B, 1-2 hrs.; C, 2-3 hrs.; and D, 3-4 hrs. (-o-, ^{3}H, -●-, ^{14}C)

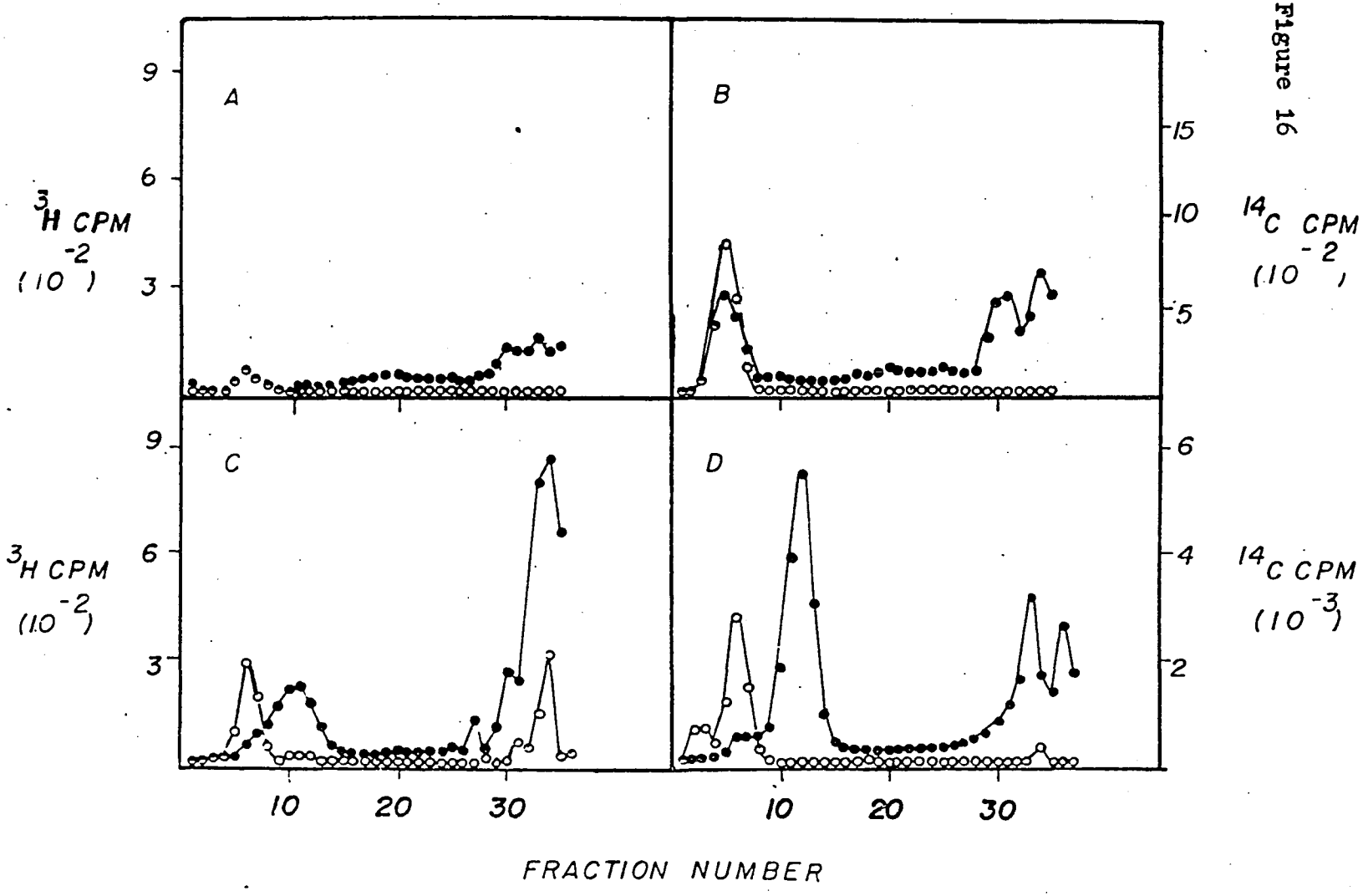

A
B
C
D
9
6
3
15
10
5
6
4
2
10
20
30
^{3}H CPM (10^{-2})
^{14}C CPM (10^{-2})
^{3}H CPM (10^{-2})
^{14}C CPM (10^{-3})
FRACTION NUMBER

Figure 16

Figure 17

The Simultaneous Incorporation of ^{3}H-adenine and $NaH^{14}CO_3$ Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans. II.

The experimental protocol is identical to that described in figure 13 except that the culture pH was adjusted to 8.0 using a pH meter and each sample was exposed to both $NaH^{14}CO_3$ and ^{3}H adenine to give 8.0 and 3.5 uCi/ml respectively. Panel A is 4-5 hrs., B) 5-6 hrs., C) 6-7 hrs. (-o-, ^{3}H, -●-, ^{14}C)

Figure 17

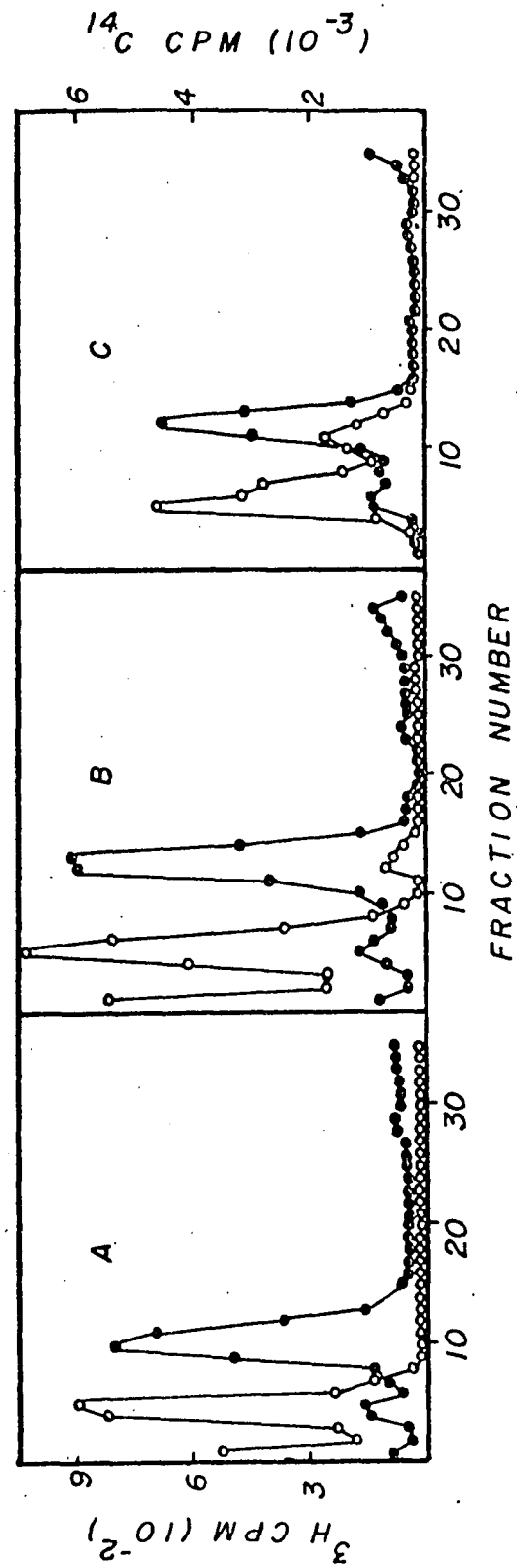
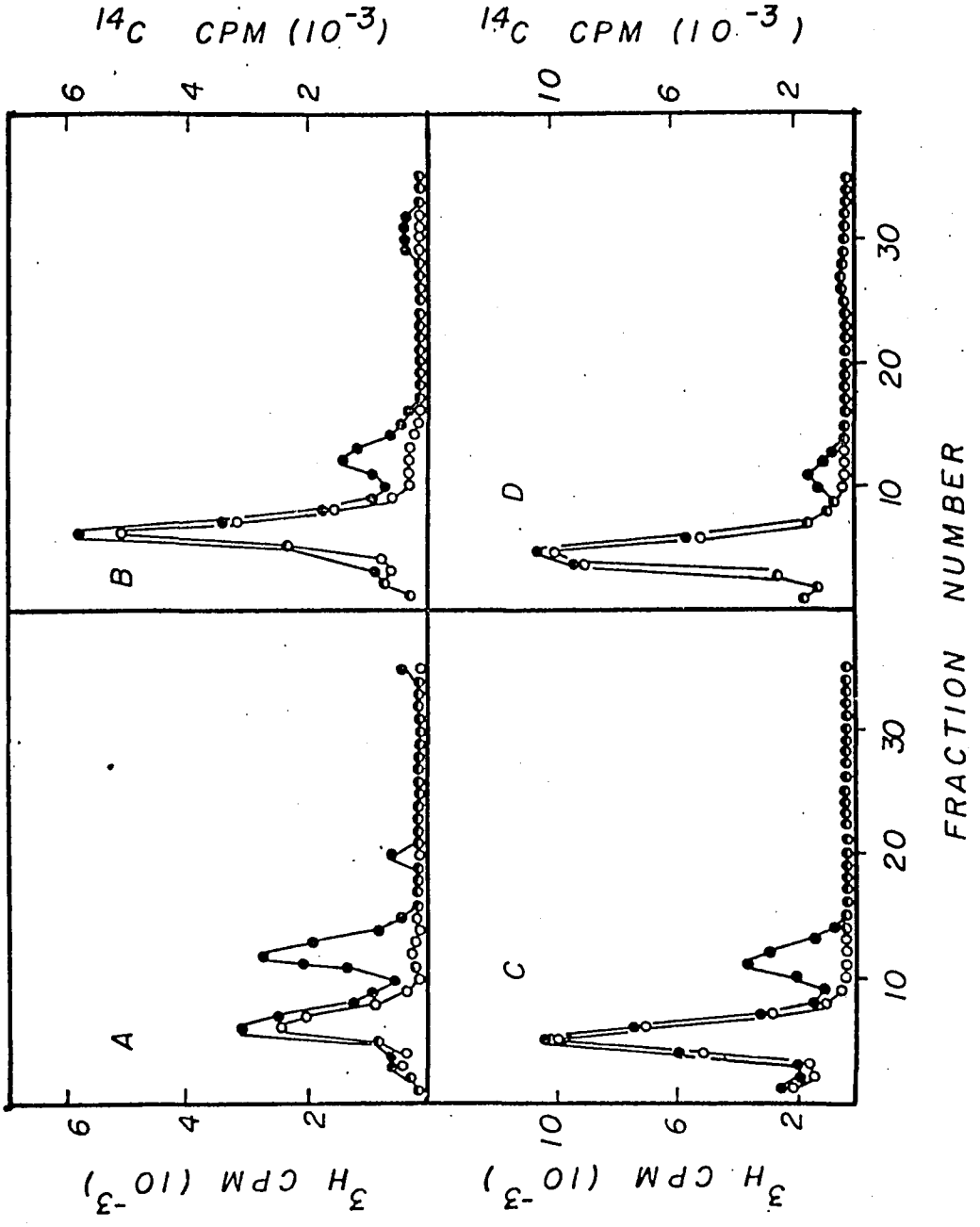


Figure 18

The Simultaneous Incorporation of ^{3}H-adenine and $NaH^{14}CO_3$ Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans. III.

The experimental details are identical to those in figures 16 and 17. Panel A is 7-8 hrs., B) 8-9 hrs., C) 9-10 hrs., D) 10-11 hrs. (-o-, ^{3}H, -●-, ^{14}C)

Figure 18


^{14}C CPM (10^{-3})
^{14}C CPM (10^{-3})
A
B
C
D
^{3}H CPM (10^{-3})
^{3}H CPM (10^{-3})
FRACTION NUMBER

g/cc) contains almost exclusively ^{14}C. Only at times late in the infectious cycle (Figure 18a, beginning at 7-8 hours post infection) does the compartmentalization of precursor sources seem to break down with the increasing incorporation of ^{14}C being seen in the heavy (ρ=1.714/5 g/cc) peak. Another observation regarding the two species of DNA is their distinct sequence of synthesis. ^{14}C is seen to incorporate into the light peak (ρ=1.701$\pm$0.001 g/cc) beginning 2-3 hours post infection (Fig. 16c, reaches a maximum between 4-6 hours post infection (Fig. 17) with ^{14}C incorporation into the light peak declining after 6 hours (Fig. 18). ^{3}H adenine incorporation, however, begins slowly as early as 1-2 hours post infection with more substantial incorporation of this nucleic acid precursor being evident in the middle and late portions of the infectious cycle (See Figures 17 and 18).

III. Cyanophage Induced DNA Synthesis with Alternative Precursors and Hosts

1. Use of Alternative precursors for Cyanophage DNA Synthesis

The question of nucleic acid incorporation into phage DNA of buoyant density 1.714/5 g/cc was extended since it was not known if the effect of adenine could be considered the norm for incorporation of these compounds. The nucleic acid precursors thymidine and uracil were chosen to make this determination. Figures 19, 20 and 21, 22 show the results of dual isotope experiments in which ^{3}H thymidine or ^{3}H uracil were added along with $H^{14}CO_3$ to uninfected or AS-1 infected Anacystis nidulans in sequential 1 hour

Figure 19

The Simultaneous Incorporation of ^{3}H-thymidine and $NaH^{14}CO_3$ Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans. I.

To a logarithmically growing A. nidulans culture which had been synchronized as previously described (see figure 5) was added AS-1 to give a phage concentration of 5×10^8 phage/ml, sufficient NaCl to give a concentration of 0.1 M and a final cell concentration of 5×10^7 cells/ml in a final volume of 50 ml. Immediately infection, the pH of the culture was adjusted to 8 using a pH meter. At hourly intervals after infection, 4 ml aliquots were withdrawn and added to flasks containing sufficient ^{3}H thymidine and $NaH^{14}CO_3$ buffered to pH 8 to give final concentrations of radioactivity of 5 and 8 μCi/ml respectively. All flasks were agitated at 100 rpm and maintained at 32°C under constant illumination provided by a bank of 4 24 watt cool white fluorescent lamps. One hour after the exposure to the radioactive compounds the cells from each sample were collected by centrifugation at 5000 g for 10 min and resuspended in lysing buffer. Following overnight incubation at 37°C each sample was treated with the detergent Sarkosyl NL97 and added to CsCl as described previously (figure 13). Following preparative ultracentrifugation, each gradient was fractionated and each fraction monitored for alkaline hydrolyzable, acid precipitable radioactivity as described in Materials and Methods. In figure 19 Panel A is the radioactivity incorporation profile for the gradient from a sample exposed to the radioactivity between hr 1 and 2 post infection; B is 2-3 hrs.; C, 3-4 hrs.; and D, 4-5 hrs. (-o-, ^{3}H, -●-, ^{14}C.)

Figure 19

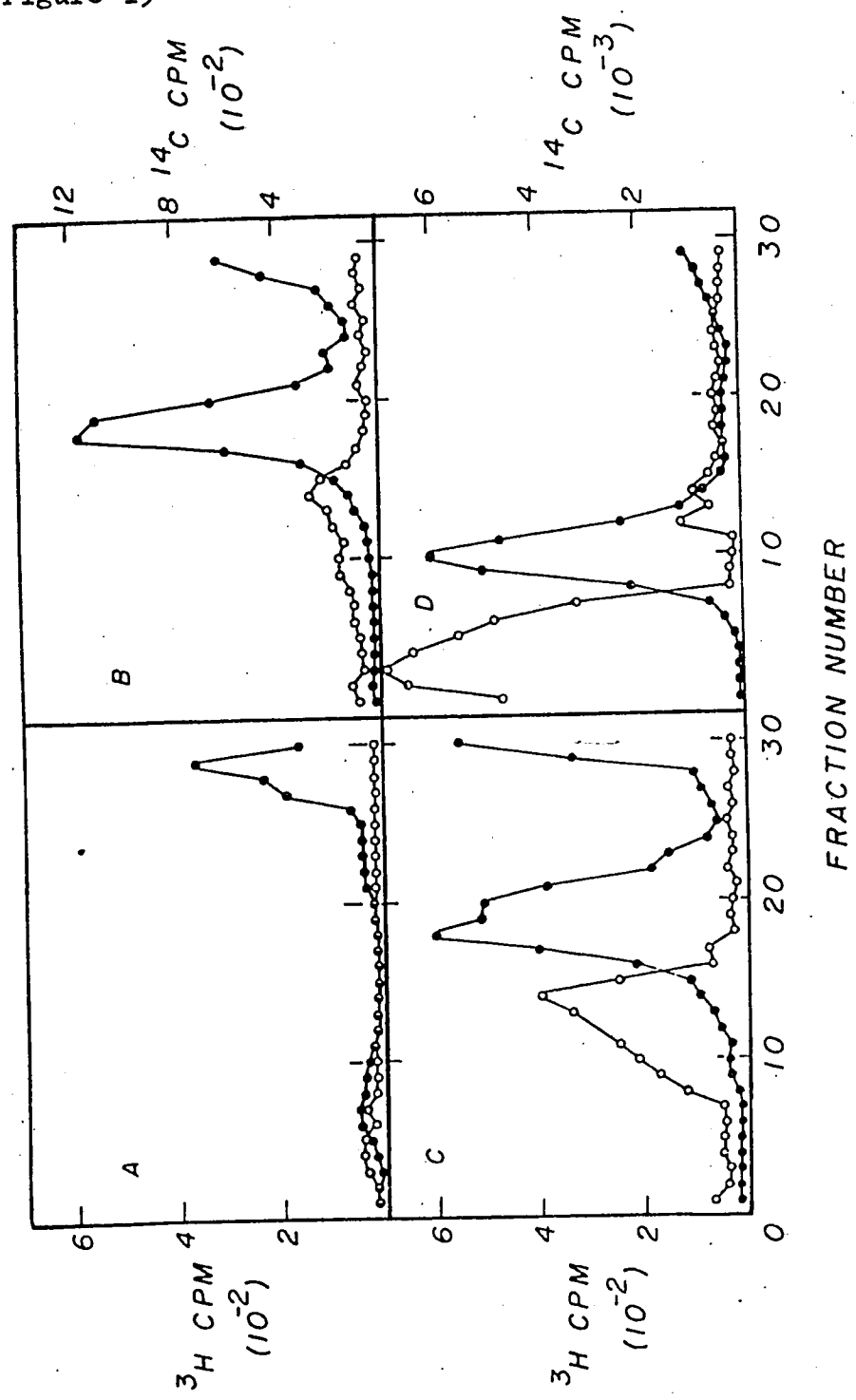
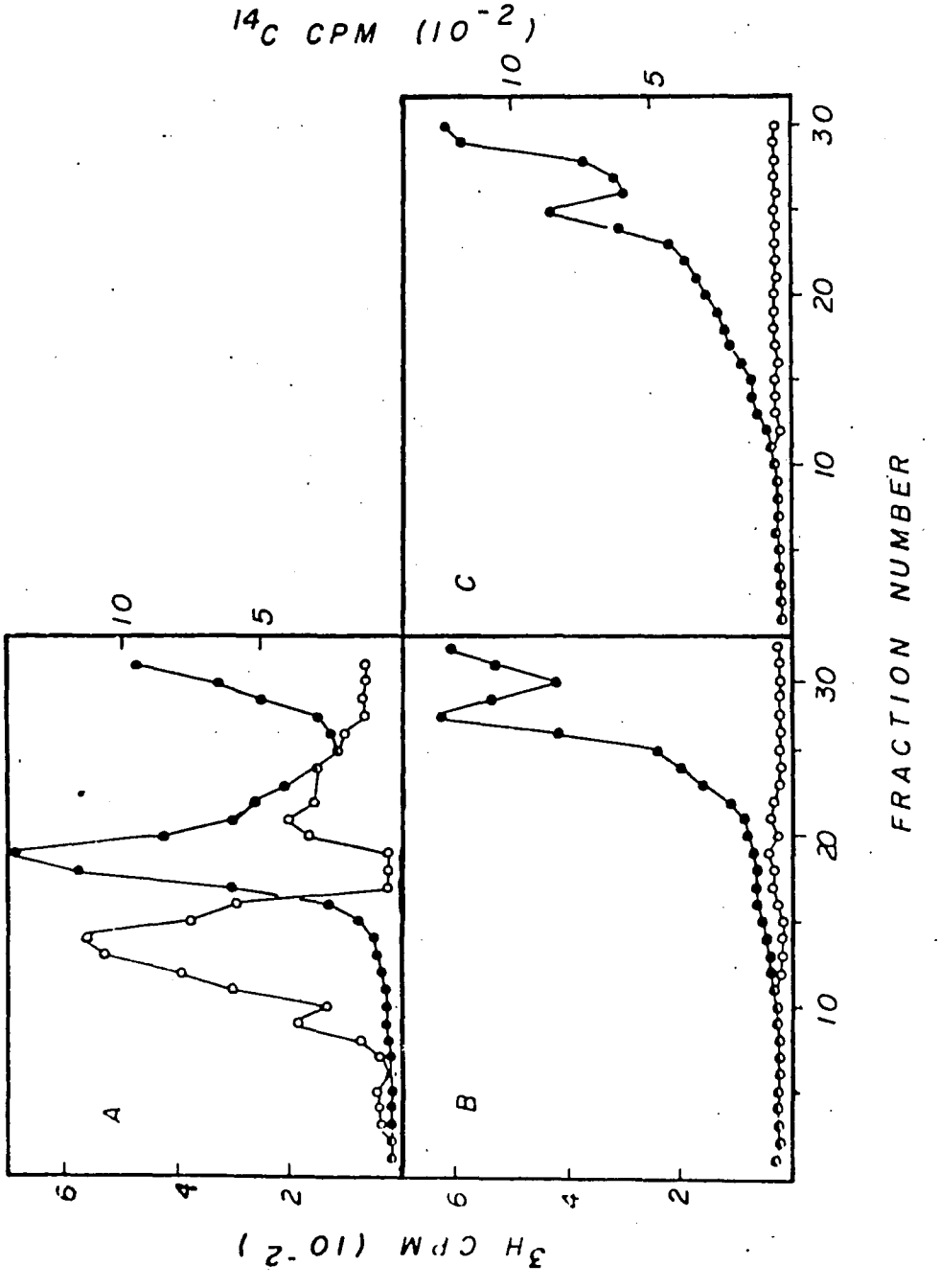

A
B
C
D
^{3}H CPM (10^{-2})
^{14}C CPM (10^{-2})
^{3}H CPM (10^{-2})
^{14}C CPM (10^{-3})
FRACTION NUMBER

Figure 20

The Simultnaeous Incorporation of ^{3}H-thymidine and $NaH^{14}CO_3$ Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans. II.

The experimental protocol was identical to that of figure 19. Panel A represents the radioactivity incorporation profile for an infected sample exposed to the radioactivity 5-6 hrs. post infection; B represents incorporation into an uninfected sample taken 1-2 hrs. after the parallel culture was infected; C uninfected 5-6 hrs. (-o-, ^{3}H, -●-, ^{14}C.)

Figure 20



pulses during the lytic cycle. The cells were harvested, lysed and the distribution of radioactivity into each of the two species of DNA (phage DNA and PIL-DNA) analyzed following centrifugation in preparative CsCl density gradients. In the experiment with ^{3}H thymidine and $H^{14}CO_3^-$, ^{14}C is associated with PIL-DNA by 2-3 hours post infection, reaches the maximum of synthesis by 4-5 hours post infection with reduced synthesis thereafter. The ^{3}H thymdine, however, is first seen incorporated into phage DNA some 3-4 hours post infection, slightly later than seen in the dual isotope experiments involving ^{3}H adenine and $H^{14}CO_3^-$. Figures 21 and 22 show the results of ^{3}H uracil and $H^{14}CO_3^-$ simultaneously added to AS-1 infected A. nidulans as in the above described experiment. The compartmentalization of radioactivity, with ^{3}H found in buoyant density of 1.714/5 g/cc and ^{14}C localized in PIL-DNA (1.701 ± 0.001 g/cc) is identical to the patterns identified in the dual isotope experiments using either adenine or thymidine and $H^{14}CO_3^-$. However, some tritium is seen to associate with PIL-DNA in middle and later time periods examined. This may reflect the modifications necessary to convert uracil from an RNA precursor to utilization for DNA synthesis.

The use of adenine, thymidine and uracil in nucleic acid biosynthesis is obvious. However, since $^{14}CO_2$ can be reduced into a variety of metabolites, the nature of the ^{14}C radioactively labeled material incorporating into PIL-DNA was open to question. Glucose 6-phosphate is an early product of carbon fixation and was shown (Figure 1), when supplied exogenously, to only incorporate into

Figure 21

The Simultaneous Incorporation of ^{3}H-uracil and $NaH^{14}CO_3$ Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans. I.

The experimental protocol was identical to that described in figures 19 and 20. The following are the radioactivity incorporation profiles for infected cells exposed to the radioactivity at the given times post infection. A) 1-2 hrs.; B) 2-3 hrs.; c) 3-4 hrs.; D) 4-5 hrs. (-o-, ^{3}H, -●-, ^{14}C.)

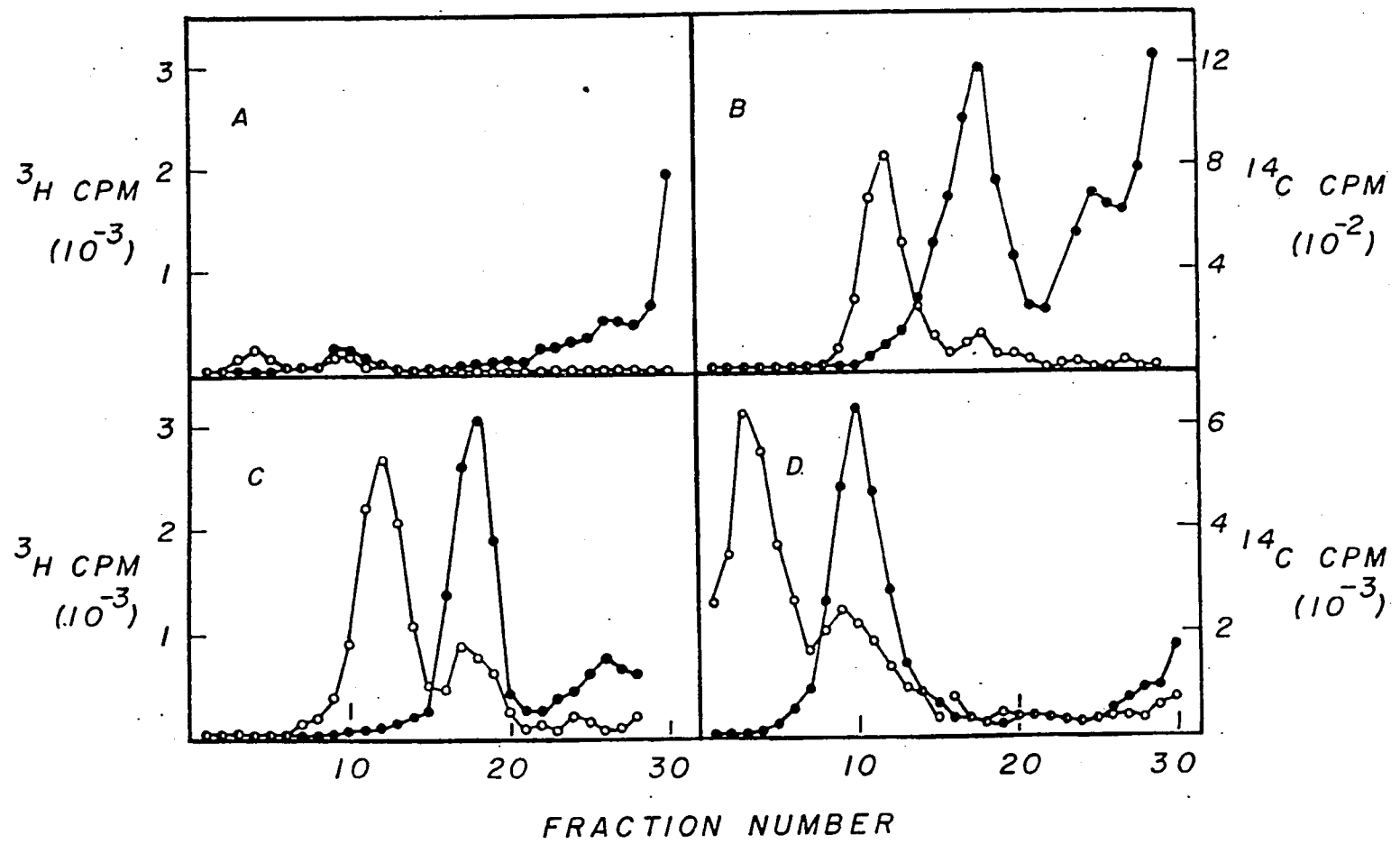

A
B
C
D
^3H CPM (10^{-3})
^{14}C CPM (10^{-2})
^3H CPM (10^{-3})
^{14}C CPM (10^{-3})
FRACTION NUMBER

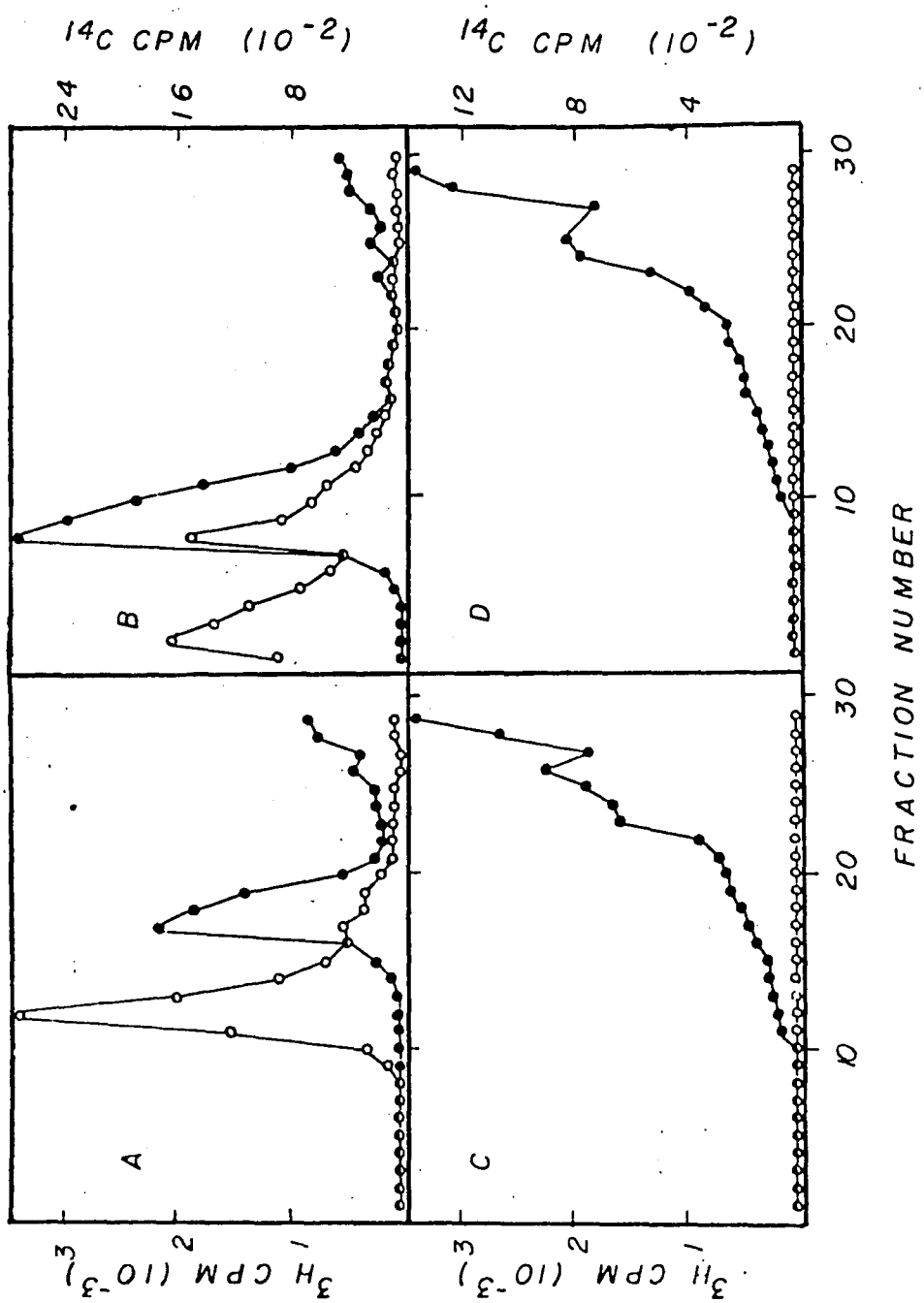
Figure 21

Figure 22

The Simultaneous Incorporation of ^{3}H-uracil and $NaH^{14}CO_3$ Into DNA Within Specific Hourly Intervals During the Lytic Cycle of AS-1 in Anacystis nidulans. II.

The experimental protocol was identical to that described in figures 19, 20 and 21. The following are the radioactivity incorporation profiles for infected and uninfected cells exposed to the radioactivity at given times following the infection of one of the two parallel cultures. A) Infected, 5-6 hrs.; B) Infected 6-7 hrs.; C) Uninfected, 1-2 hrs; D) Uninfected, 6-7 hrs. (-o-, ^{3}H, -●-, ^{14}C.)

Figure 22


14C CPM (10-2)
14C CPM (10-2)
24
16
8
12
8
4
A
B
C
D
3H CPM (10-3)
3H CPM (10-3)
1
2
3
0
10
20
30
FRACTION NUMBER

AS-1 infected Anacystis nidulans with essentially no incorporation into the uninfected algae. The results of a single 2 hr exposure using ^{14}C glucose 6-phosphate and ^{3}H adenine which were simultaneously added 3-5 hours post infection followed by preparative CsCl gradient centrifugation of the lysate are shown in Figure 23. The radioactivity from both tritium and ^{14}C are found associated with the density of the newly synthesized AS-1 DNA and not the PIL-DNA.

2. Alternative Hosts for Cyanophage Infection

Cyanophages AS-1 and AS-1M form together with the host algae Anacystis nidulans 625 and Synechococcus cedrorum 1191, a related but distinct group of phage:host infective systems (81,85,101). In order to determine if the synthesis of PIL-DNA was restricted to infection of Anacystis nidulans by our laboratory strain of AS-1, both hosts were paired with a fresh isolate of AS-1 provided by Dr. R. Safferman, AS-1M provided by Dr. L. Sherman and the laboratory strain of AS-1 temporarily designated AS-1b to differentiate between the cyanophages. In a comparative experiment in which phage and PIL-DNA were examined, ^{3}H adenine and $H^{14}CO_3$ were simultaneously added for a one hour duration to cultures in which each phage was paired with each host along with an uninfected control for each. The DNA species were separated in preparative CsCl gradients and the distribution of radioactivity was analyzed. The results are shown in Figures 24 and 25. Although the net incorporation of radioactivity into DNA is somewhat different, the qualitative results are similar among the cyanophages. For both AS-1M and the original isolate of AS-1, photoreduced ^{14}C is found of a

Figure 23

The Simultaneous Incorporation of ^{3}H-adenine and ^{14}C-glucose-6-phosphate Into DNA During the Lytic Cycle of AS-1 in Anacystis nidulans

AS-1 was added at a MOI=5 to a logarithmically growing A. nidulans culture (5×10^{7} cells/ml). The flask was aerated by gyrorotation at 100 rpm and maintained at 32°C under constant illumination provided by a bank of 4 24 watt cool white fluorescent lamps. ^{3}H-adenine (10μCi/ml) and ^{14}C glucose 6-phosphate (1 μCi/ml) were added 3-5 hours post infection. Following the exposure to the radioactive compounds (5 hours post infection) a 5ml aliquot of cells was collected by centrifugation at 4000 x g for 20 minutes and resuspended in lysing buffer (saline-EDTA, 5mM NaCN + 1mM NaN_3 + 3mg/ml lysozyme). Following overnight incubation at 37°C the sample was treated with 3 drops of sarkosyl NL-97 and heated at 60°C for 15 minutes. The lysate was added to 4.1 ml of a stock saturated CsCl solution and centrifuged in a 50Ti rotor at 33,000 rpm and 19°C for 42 hours using a Beckman L3-50 ultracentrifuge. Following preparative ultracentrifugation each gradient was fractionated and each fraction monitored for alkaline hydrolyzable, acid precipitable radioactivity as described in Materials and Methods. (—— = ^{14}C, ... = ^{3}H).

Figure 23

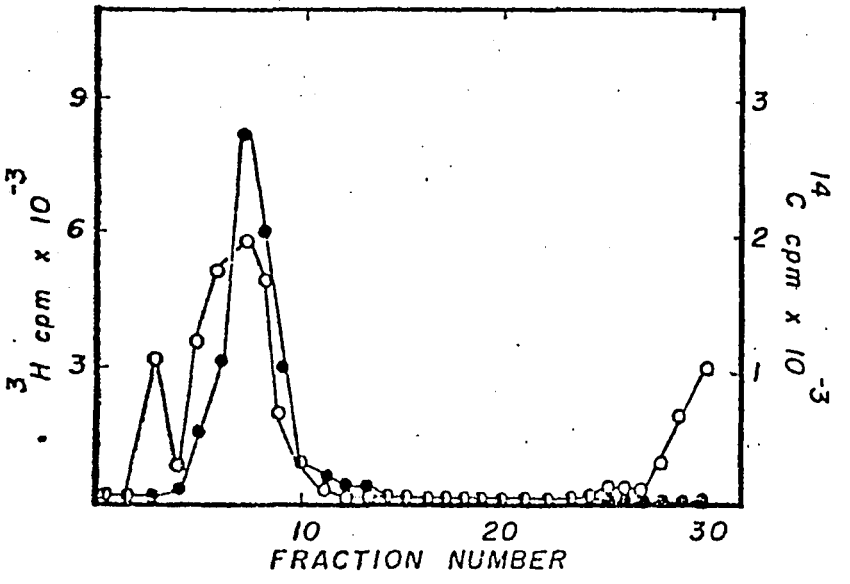


Figure 24

Comparative DNA Biosynthesis in Anacystis nidulans 625 Infected With Various Cyanophages.

Separate 25 ml aliquots were removed from a stock culture of Anacystis nidulans 625 (6×10^7 cells/ml) growing at $32^{\circ}C$ and synchronized as described in Fig. 5 and parallel infections with AS-1, AS-1M and AS-1b (the in house laboratory strain of AS-1) were performed, all at a MOI=5. A 5ml aliquot of uninfected or cyanophage infected culture was removed and ^{3}H-adenine (5 μCi/ml) and $NaH^{14}CO_3$ (4 μCi/ml) were added 3-4 hours post infection. At four hours post infection the cells were collected by low speed centrifugation followed by lysis of the infected cells and preparative CsCl centrifugation, all as described in the legend of figure 23. (-●-, ^{14}C, -o-, ^{3}H). A) AS-1M, B) AS-1, C) AS-1b, D) Uninfected A. nidulans 625.

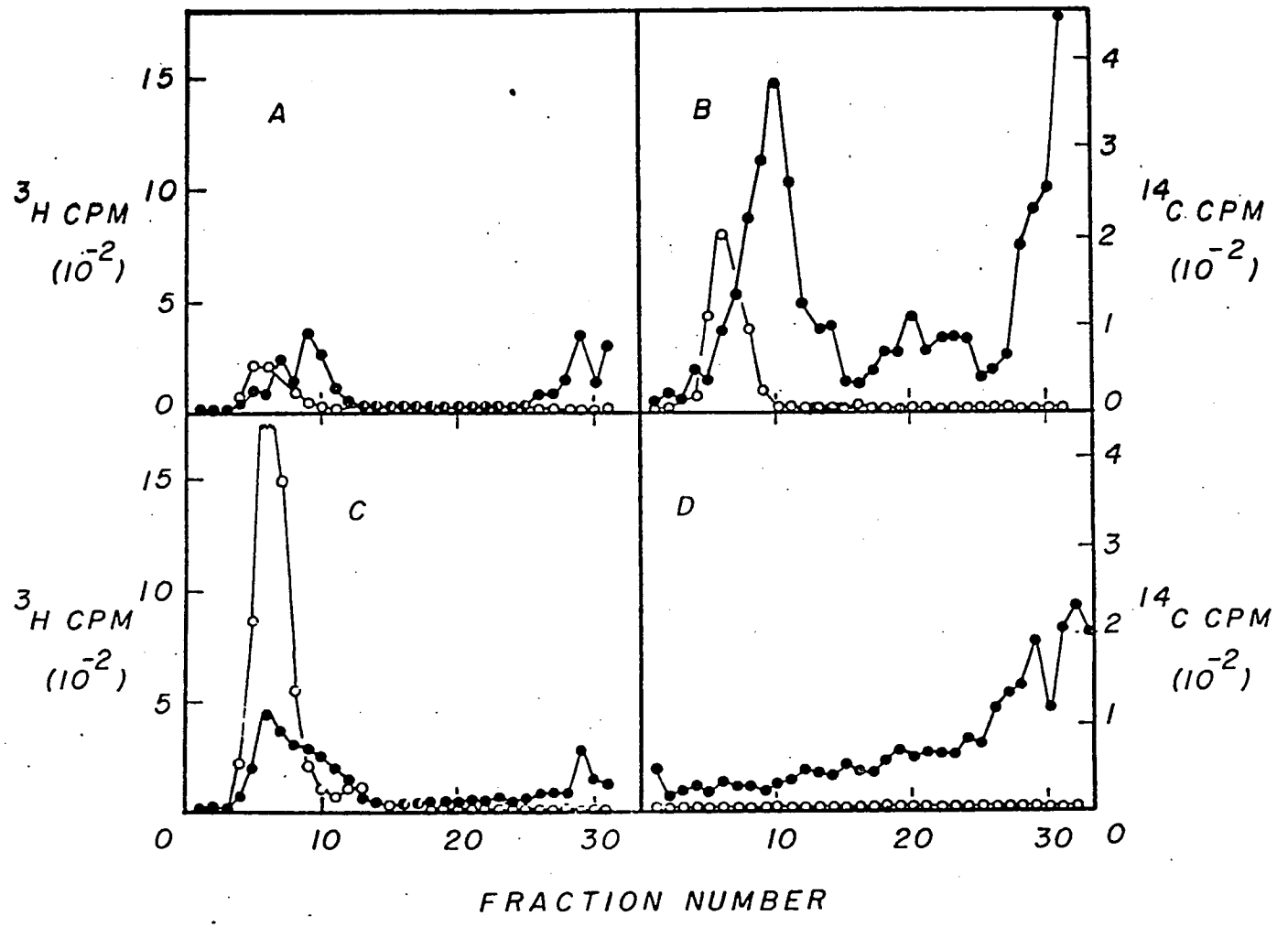


Figure 24

Figure 25

Comparative DNA Biosynthesis in Synechococcus cedrorum 1191 Infected With Various Cyanophages.

Separate 25 ml aliquots were removed from a stock culture of Synechococcus cedrorum 1191 (6 x 10^7 cells/ml) growing at 32^oC, synchronized as described previously (fig. 5) and parallel infections with AS-1, AS-1M, and AS-1b (the in- house laboratory strain of AS-1) were performed, all at a MOI=5. A 5 ml aliquot of uninfected or cyanophage infected culture was removed and ^3H-adenine (5 uCi/ml) and $NaH^{14}CO_3$ (4 uCi/ml) were added 2-3 hours post infection. At three hours post infection the cells were collected and treated as described in the legend of figure 23. (-●-, ^{14}C, -o-, ^3H.) A) AS-1M, B) AS-1, C) AS-1b, D) Uninfected S. cedrorum 1191.

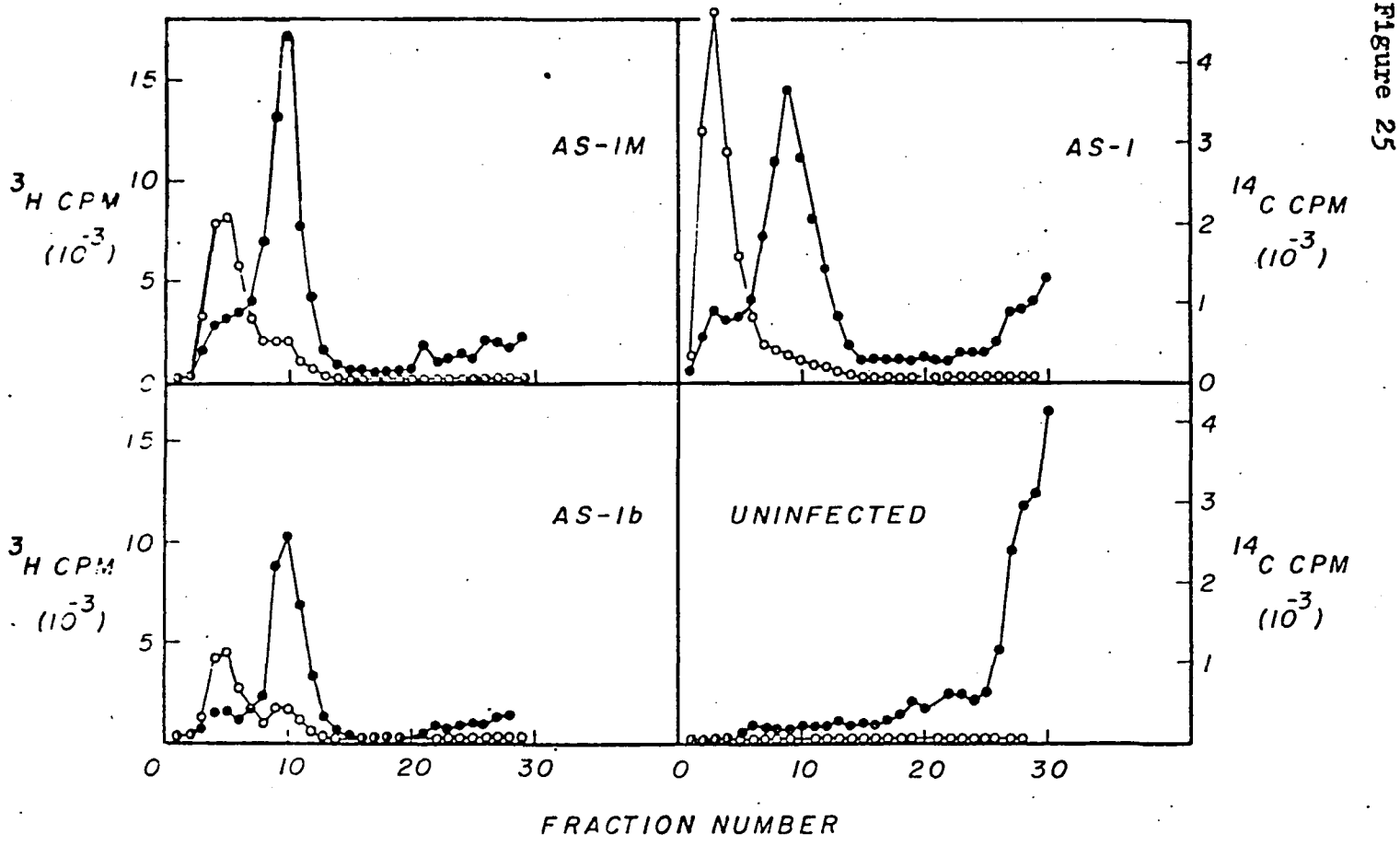

AS-1M
AS-1
AS-1b
UNINFECTED
^{3}H CPM (10^{-3})
^{14}C CPM (10^{-3})
FRACTION NUMBER
0
10
20
30
5
15
1
2
3
4

Figure 25

lighter buoyant density, equivalent to that of the PIL-DNA found in the Anacystis system, than that of the peak of incorporated ^{3}H adenine which corresponds to the newly synthesized AS-1 DNA.

IV. The Fate of PIL-DNA

In the experiments monitoring the kinetics of DNA synthesis in cyanophage infected Anacystis nidulans which were described in the preceding two sections of this thesis, the data that were presented reflected all the DNA present in the infected cells at the time of lysis, whether induced by lysozyme or the lytic effects of the phage. To determine if a DNA species was protected at the time of lysis, presumably by packaging of the DNA into a phage head, treatment of the lysate with DNase was performed. Unprotected DNA was hydrolyzed while packaged DNA was protected. The nuclease was inactivated by the addition of the detergent sarkosyl which caused the rupture of the phage and release of the DNA into the buffer prior to preparative CsCl gradient centrifugation. Figures 26-29 show the results of an experiment in which $H^{14}CO_3^-$ and ^{3}H adenine were added to cultures of AS-1 infected A. nidulans in a series of one hour pulses at selected early, mid and late portions of the lytic cycle. Figure 26 presents those DNA's which have been synthesized during the period of the radioactive pulse and have become radioactively labeled with ^{3}H or ^{14}C. Following the addition of radioisotope, the infected algae were resuspended in fresh medium containing excess non-radioactive adenine and $NaHCO_3$ and were incubated with shaking until 13 hours post infection. At $32^{o}C$, the temperature of this experiment, lysis should have been complete by 13 hours (Figure 9).

Figure 26

Pulse and Chase of ^{3}H-adenine and $NaH^{14}CO_3$ in AS-1 Infected Anacystis nidulans. I.

^{3}H-adenine (3 uCi/ml) and $NaH^{14}CO_3$ (5 μCi/ml) were added to a culture of Anacystis nidulans (4×10^{7} cells/ml) growing at 32°C and infected with AS-1 at a MOI=5 at a variety of times post infection. After the period of growth in the presence of the radioactivity (one hour exposures) the cells were collected by centrifugation at 4000 x g for 20 minutes and resuspended in 1 ml of lysing buffer consisting of Saline-EDTA + 5mM NaCN + 1mN NaN_3 + 3mg/ml lysozyme. Following overnight incubation at 37°C, lysis was completed by addition of 3 drops of 30% Sarkosyl NL-97 and heating at 60°C for 15 minutes. The lysate was added to 4.1 ml of a stock saturated CsCl solution and centrifuged in a 50 Ti rotor at 33,000 rpm and 19°C for 42 hours in a Beckman L3-50 ultracentrifuge. Following preparative ultracentrifugation each gradient was fractionated and each fraction monitored for alkaline hydrolyzable acid precipitable radioactivity as described in Materials and Methods. The radioactivity was added, A) 2-3 hrs., B) 4-5 hrs, C) 6-7 hrs., D) 8-9 hrs. -o-, ^{3}H, -●-, ^{14}C.

Figure 26

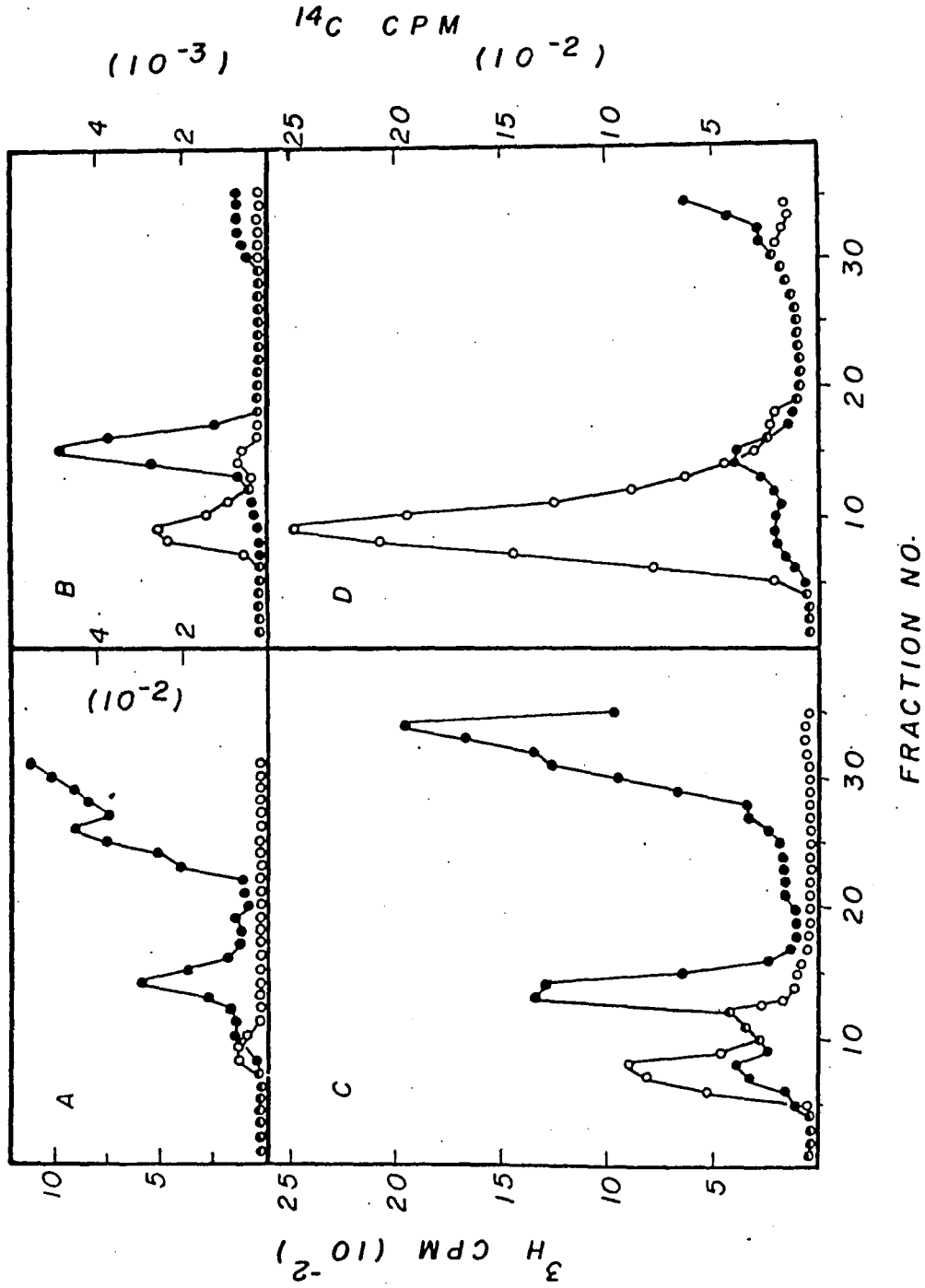


Figure 27

Pulse and Chase of ^{3}H-adenine and $NaH^{14}CO_3$ in AS-1 Infected *Anacystis nidulans*. II.

^{3}H-adenine and $NaH^{14}CO_3$ were added to AS-1 infected *A. nidulans* as described in the legend of figure 26. After the pulse, 12 ml of cells were collected, resuspended in 4 ml fresh Bristol's medium with excess $NaHCO_3$ (0.1M) and adenine (200 µg/ml) and the cultures incubated until 13 hours post infection. The unlysed cells were collected by low speed centrifugation (4000 x g, 20 minutes), lysed, centrifuged in CsCl gradients and analyzed as described in the legend of figure 26. Addition of radioactivity was, A) 2-3 hrs., B) 4-5 hrs., C) 6-7 hrs., D) 8-9 hrs. -o-, ^{3}H, -●-, ^{14}C.

Figure 27

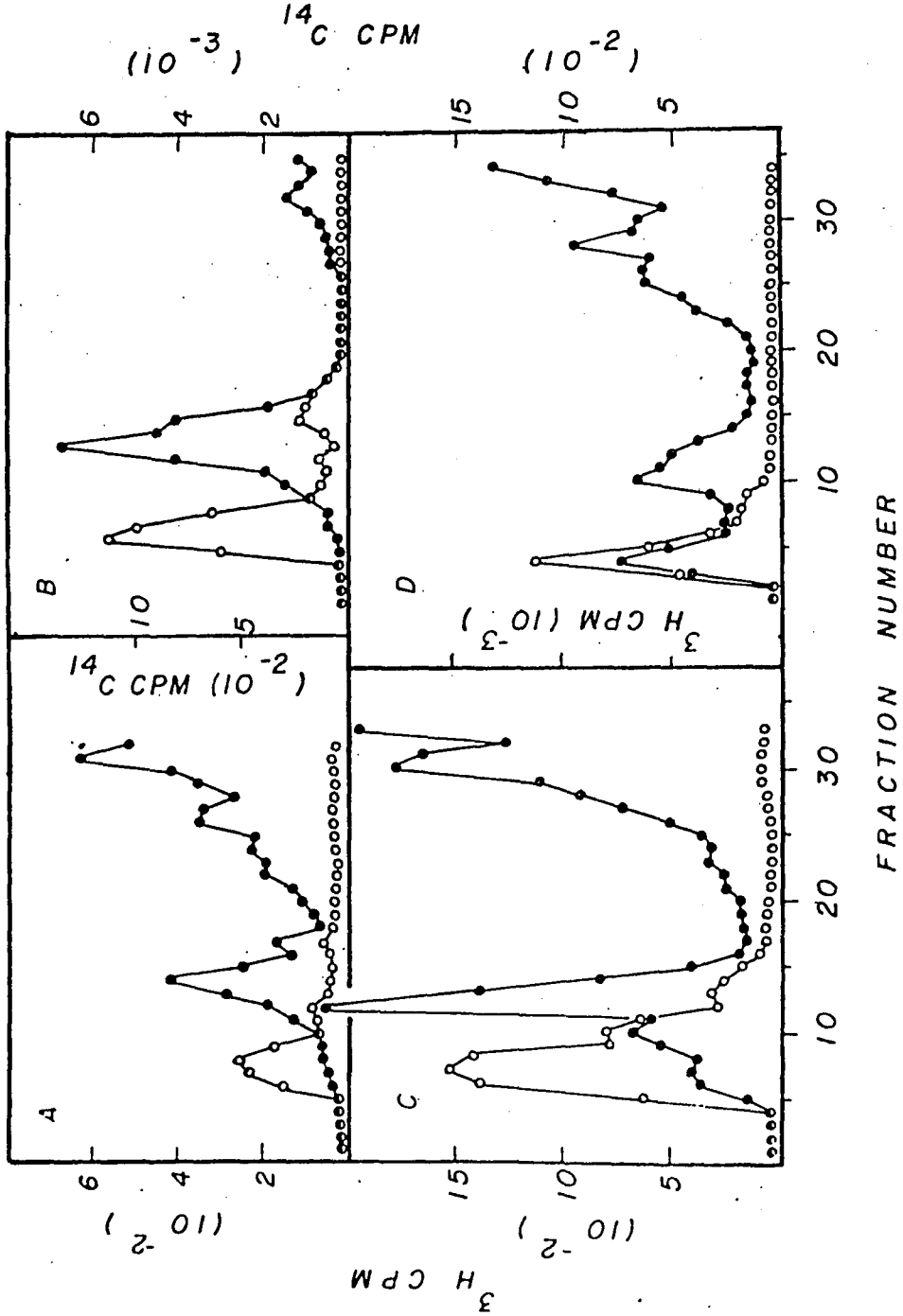

14C CPM (10-3)
14C CPM (10-2)
3H CPM (10-2)
3H CPM (10-3)
A
B
C
D
FRACTION NUMBER

Figure 28

Pulse and Chase of ^{3}H-adenine and $NaH^{14}CO_3$ in AS-1 Infected *Anacystis nidulans*. III.

The concentrated AS-1 infected *Anacystis* culture described in the legend of figure 27 was centrifuged at low speed (4000 x g, 20 min.) at 13 hours post infection. A 1.5 ml aliquot was removed and subjected to CsCl density gradient centrifugation and analyzed as described in Materials and Methods. The hours that are described refer to the addition of the radioactive label in hours post infection. A) 2-3 hrs., B) 4-5 hrs., C) 6-7 hrs., D) 8-9 hrs. -o-, ^{3}H; -●-, ^{14}C.

Figure 28

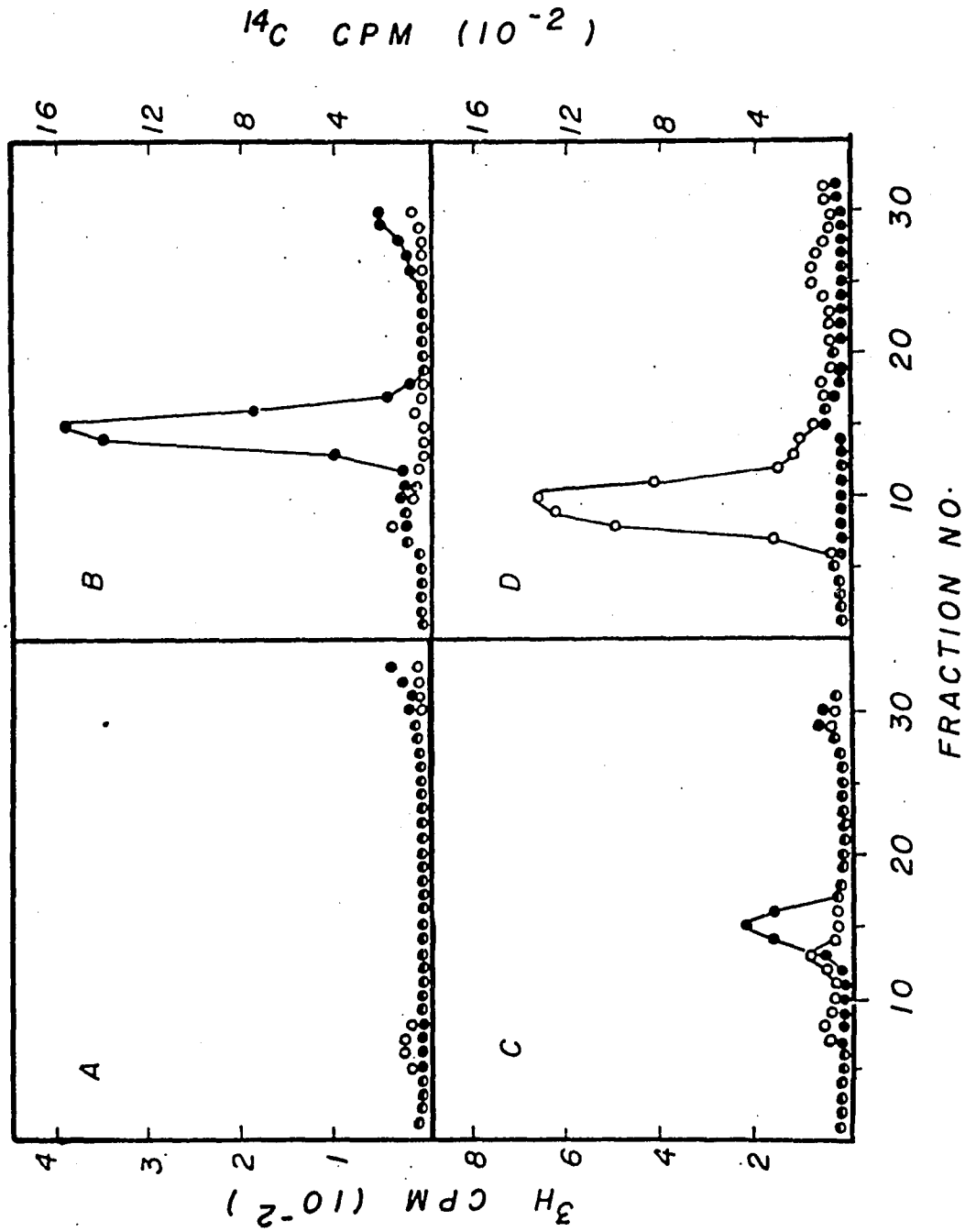

^{14}C CPM (10^{-2})
16
12
8
4
B
D
A
C
4
3
2
1
8
6
4
2
^{3}H CPM (10^{-2})
10
20
30
FRACTION NO.

Table 3

Pulse and chase of $NaH^{14}CO_3$ and ^3H adenine in AS-1 infected Anacystis nidulans. The experimental protocol is that described in the legend of Figure 26. The data presented in this table is derived from Figures 26-29.

A. Infected cells collected and lysed immediately after pulse.

B. Infected cells resuspended in Bristol's medium (without soil extract) with excess $NaHCO_3$ (0.1M) and adenine (200 ug/ml) after the pulse. The values given are for the DNA species present in those cells which remained unlysed 13 hours post infection.

C. Supernate resulting from low speed (4000 x g, 20 min) centrifugation of infected culture 13 hours post infection. No DNase was added.

D. Supernate of low speed (4000 x g, 20 min) centrifugation of infected culture 13 hours post infection (same as C. above) treated with DNase I (45 min, 37°) followed by high speed centrifugation (75,000 x g, 60 min) to pellet the phage.

	A*		B		C		D	
	I**	II‡	I**	II‡	I**	II‡	I**	II‡
	^3H, ^{14}C	^3H, ^{14}C	^3H, ^{14}C	^3H, ^{14}C	^3H, ^{14}C	^3H, ^{14}C	^3H, ^{14}C	^3H, ^{14}C
Time of pulse (hours post-infection)								
2-3	92, 34	1, 178		19, 785	16, 5	5, 5	5, 6	0, 78
4-5	316, 190	70, 2640	87, 105	30, 1308	20, 52	3, 900	7, 4	0, 36
6-7	604, 138	260, 441	361, 157	46, 272	43, 16	42, 264	33, 9	5, 10
8-9	1998, 115	410, 160	1458, 119	263, 147	563, 10	46, 56	157, 7	- -

* All values normalized to cpm/ml of infected culture.

**I = "heavy" DNA peak (p = 1.714/5 g/cc).

‡II = "light" DNA peak (p = 1.701 ± 0.001 g/cc).

Figure 29

Pulse and Chase of ^{3}H-adenine and $NaH^{14}CO_3$ in AS-1 Infected *Anacystis nidulans*. IV.

The supernate of the centrifuged concentrated AS-1 infected *Anacystis* culture described in the legend of figure 28 was treated with DNase I (100 µg/ml, 37°C, 45 min.) followed by ultracentrifugation (75,000 x g, 45 min.) to pellet the phage. The pelleted phage were resuspended in 1.0 ml Saline-EDTA and lysed with 3 drops of 30% Sarkosyl at 60°C for 15 min. The lysed phage solution was subjected to CsCl density gradient centrifugation and analyzed as described in Materials and Methods. The time periods referred to below reflect the addition of the radioactivity to the infected cultures. A) 2-3 hrs., B) 4-5 hrs., C) 6-7 hrs., D) 8-9 hrs. -o-, ^{3}H; -●-, ^{14}C.

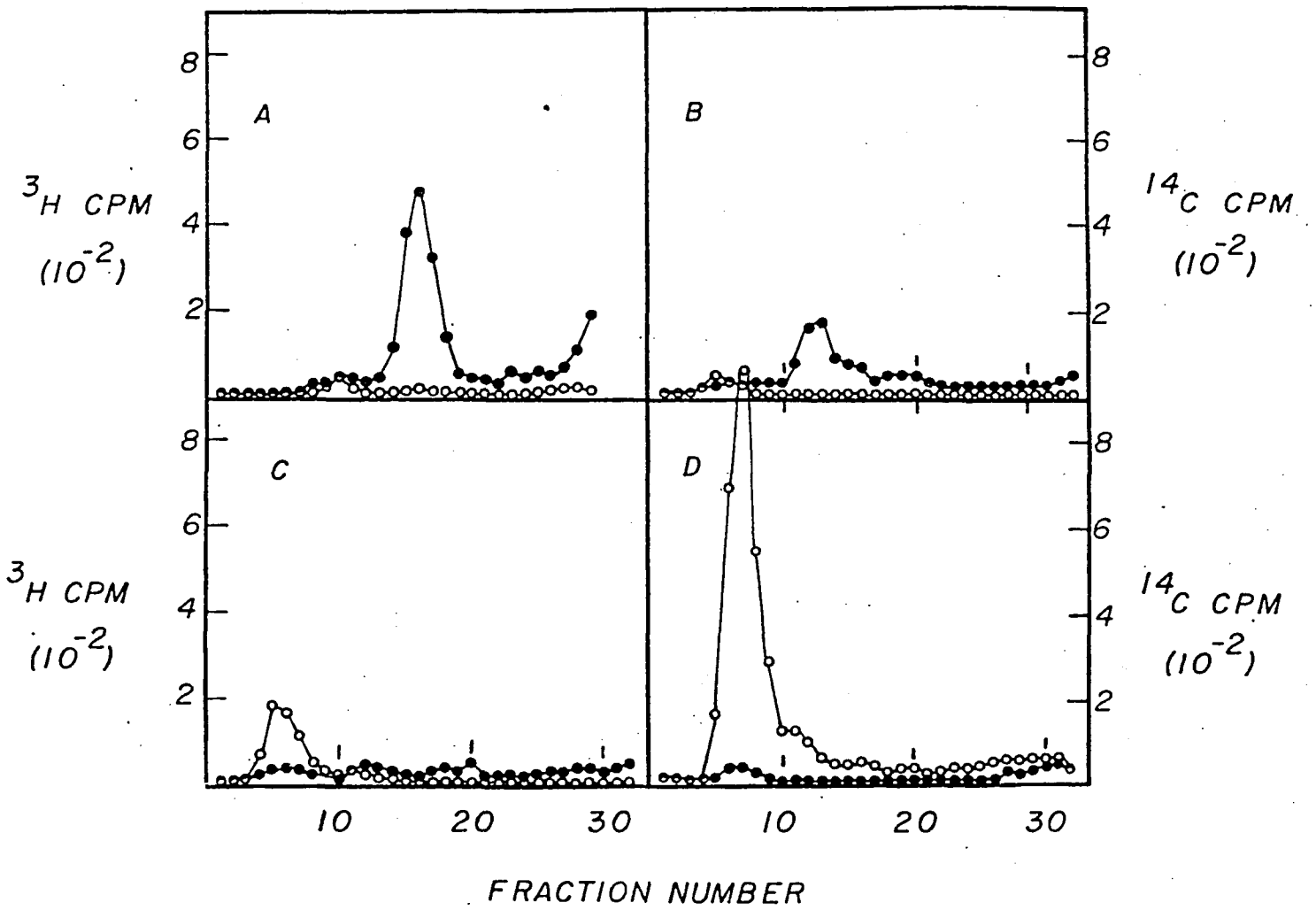

A
B
C
D
^3H CPM (10^{-2})
^{14}C CPM (10^{-2})
^3H CPM (10^{-2})
^{14}C CPM (10^{-2})
2
4
6
8
10
20
30
FRACTION NUMBER

Figure 29

However, possibly due to shock after washing and resuspension of the cells in fresh medium, only a portion of the infected cells lysed by this time. Figure 27 represents the total radioactively labeled DNA present in those cells which remained unlysed at the end of the experiment. The total DNA that is present in the balance of the culture medium (i.e. the supernate of the centrifuged infected culture at the end of the experiment), which contains DNA from lysed cells and that which is packaged in phage, is shown in Figure 28. Table 3 and Figure 29 present the amounts of incorporation of ^{3}H adenine and ^{14}C into AS-1 DNA (ρ=1.714/5 g/cc) and PIL-DNA ($\rho = 1.701 \pm 0.001$ g/cc). It is seen that ^{14}C pulsed in early (2-3 hours post infection) is incorporated into PIL-DNA that is protected at the time of lysis (Figure 29) from the action of DNase added to the culture. ^{14}C pulsed in later was progressively less protected and presumably was not incorporated into packaged DNA. The incorporation of ^{3}H adenine into DNA of buoyant density 1.714 g/cc is seen to increase with substantial incorporation occurring late in the infectious cycle. It can be seen (Table 3) that not all of the DNA of buoyant density 1.714 g/cc is protected at the time of lysis, presumably because lysis occurred before packaging of the DNA, rendering it susceptible to the nuclease treatment.

In a further attempt to characterize the nature of PIL-DNA, an experiment was performed in which $H^{14}CO_3^-$ was added to Anacystis nidulans for 24 hours, followed by washing and resuspending the cells in fresh medium with excess $NaHCO_3$. After a synchronizing

regimen (6), the cells were infected and ^{3}H adenine was added in one hour pulses at a variety of selected early, middle and late times post infection. It can be seen (Figure 30) that $H^{14}CO_3^-$ added to uninfected Anacystis nidulans is photoassimilated and some ^{14}C metabolites that are synthesized are incorporated into the algal DNA (ρ=1.715 g/cc). The question then to be answered was, would this DNA be degraded and reutilized in PIL-DNA synthesis as well as in AS-1 DNA synthesis as was shown by Sherman (91). The gradients described in Figures 31-33 are derived from a series of experiments in which ^{3}H adenine was added in one hour pulses to an AS-1 infected culture of Anacystis nidulans prelabeled with $H^{14}CO_3^-$ (the DNA extracted from an aliquot of this culture was shown in Figure 30). Figure 31 depicts the total radioactively labeled DNA present in the remaining unlysed cells at the end of the various exposures to ^{3}H adenine. The temporal pattern of utilization of ^{3}H adenine in AS-1 DNA is similar to experiments described earlier in this thesis monitoring incorporation of ^{3}H adenine into AS-1 infected Anacystis cultures (Figures 13, 14, 16-18). The position of the ^{14}C peak is offset from the peak of tritium but by a lesser separation than previously seen in dual isotope experiments. Figure 32 shows the total DNA present after the ^{3}H adenine had been removed from the culture by collection of the cells by centrifugation and resuspension in fresh medium containing excess non-radioactive adenine. The cells were then allowed to incubate until 12 hours following the infection. The 2-3 hour time point (Figure 32b) shows the presence of two peaks of DNA, one composed of

Figure 30

Incorporation of ^{14}C from $NaH^{14}CO_3$ Into DNA in Anacystis nidulans.

To a 40 ml culture of exponentially growing Anacystis nidulans was added $NaH^{14}CO_3$ (15 μCi/ml). The culture was incubated for 3 days at 30°C under Sylvania gro-lux lamps at a distance of 15 cm from the flask surface. The cells were collected and resuspended in 30 ml of fresh Bristols medium with excess $NaHCO_3$ (0.1M) for 12 hours, resuspended again as above and incubated in the dark for 12 hours. The culture was divided with half being infected with AS-1 (MOI=5) and the balance used as the uninfected control. A) 2-3 hours and B) 8-9 hours after addition of AS-1 to one of the cultures, 4 ml aliquots were removed, the cells collected, lysed, centrifuged in CsCl gradients and analyzed as described in Materials and Methods. ^{3}H DNA from E. coli (ρ=1.710 g/cc) was included in the gradient as a marker. The ^{14}C banded at buoyant density 1.714/5 g/cc.

Figure 30

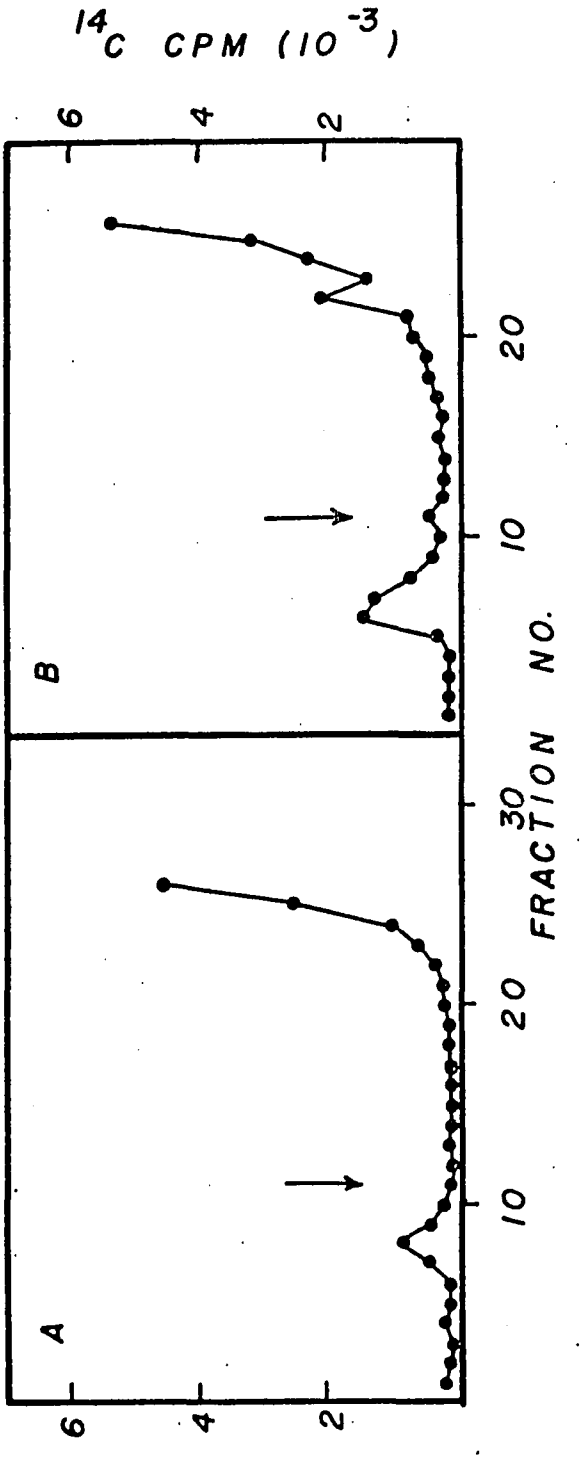


Figure 31

^{3}H-adenine Pulse and Chase in AS-1 Infected, ^{14}C Labeled *Anacystis nidulans*. I.

^{3}H-adenine (final conc. 5 μCi/ml) was added for a variety of times post infection A) 2-3 hrs., B) 4-5 hrs., C) 6-7 hrs., D) 8-9 hrs. to a 75 ml culture of *A. nidulans* (1 x 10^{8} cells/ml) radioactively labeled with ^{14}C (as described in the legend of figure 30), infected with AS-1 at a MOI=5 and growing at 32°C. After each one hour pulse a 5 ml aliquot of infected cells was collected by low speed centrifugation (4000 x g, 20 min.) and the cells were resuspended and lysed in one ml of a Saline-EDTA buffer also containing 3 mg/ml lysozyme, 0.05M NaCN and 0.01M NaN_3 for 12 hours at 37°C. The lysate was added to 4.1 ml of a stock saturated CsCl solution and centrifuged in a 50 Ti rotor for 42 hours at 33,000 rpm using a Beckman L3-50 ultracentrifuge, and analyzed as described in Materials and Methods. (-o-, ^{3}H; -●-, ^{14}C).

Figure 31

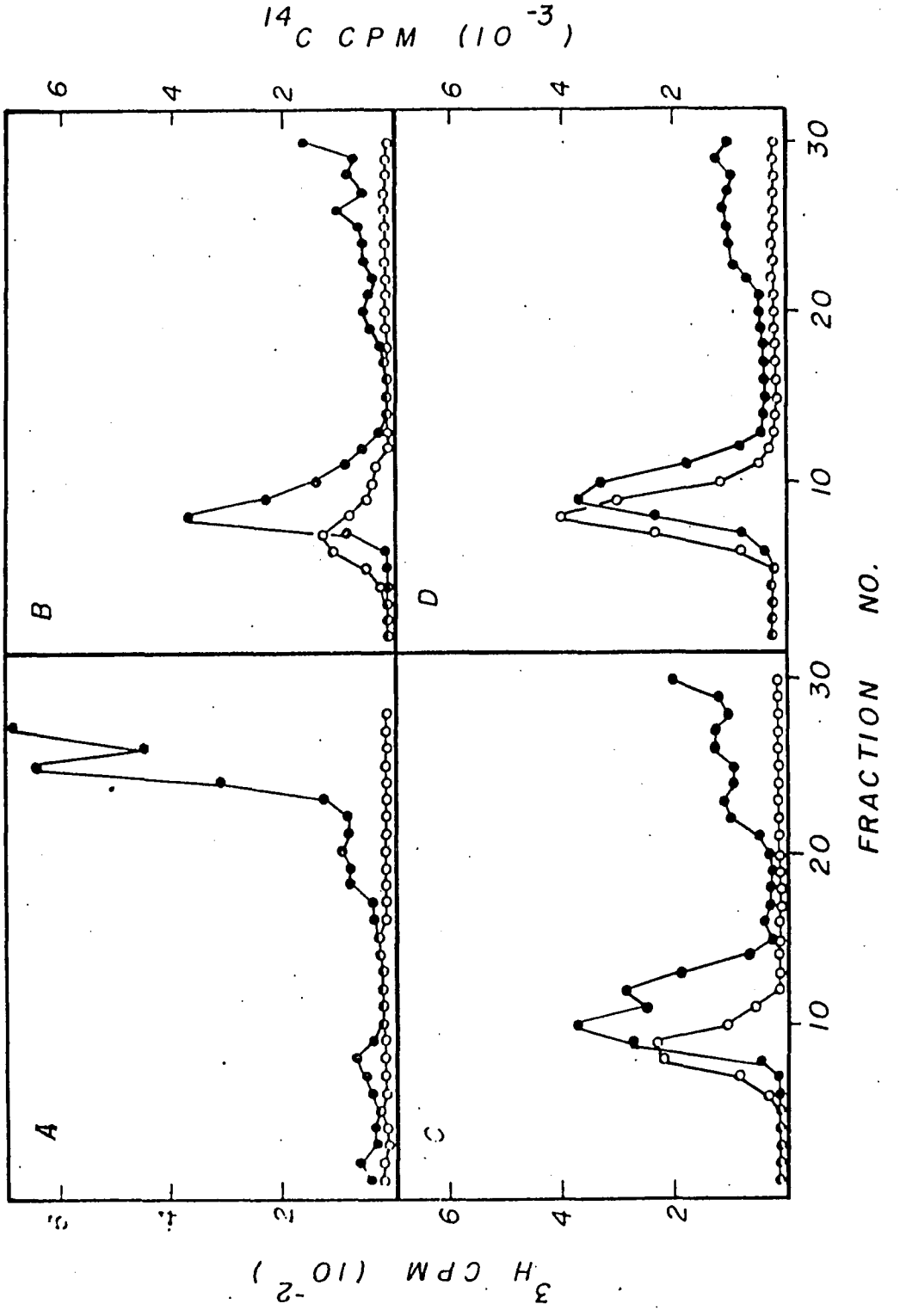
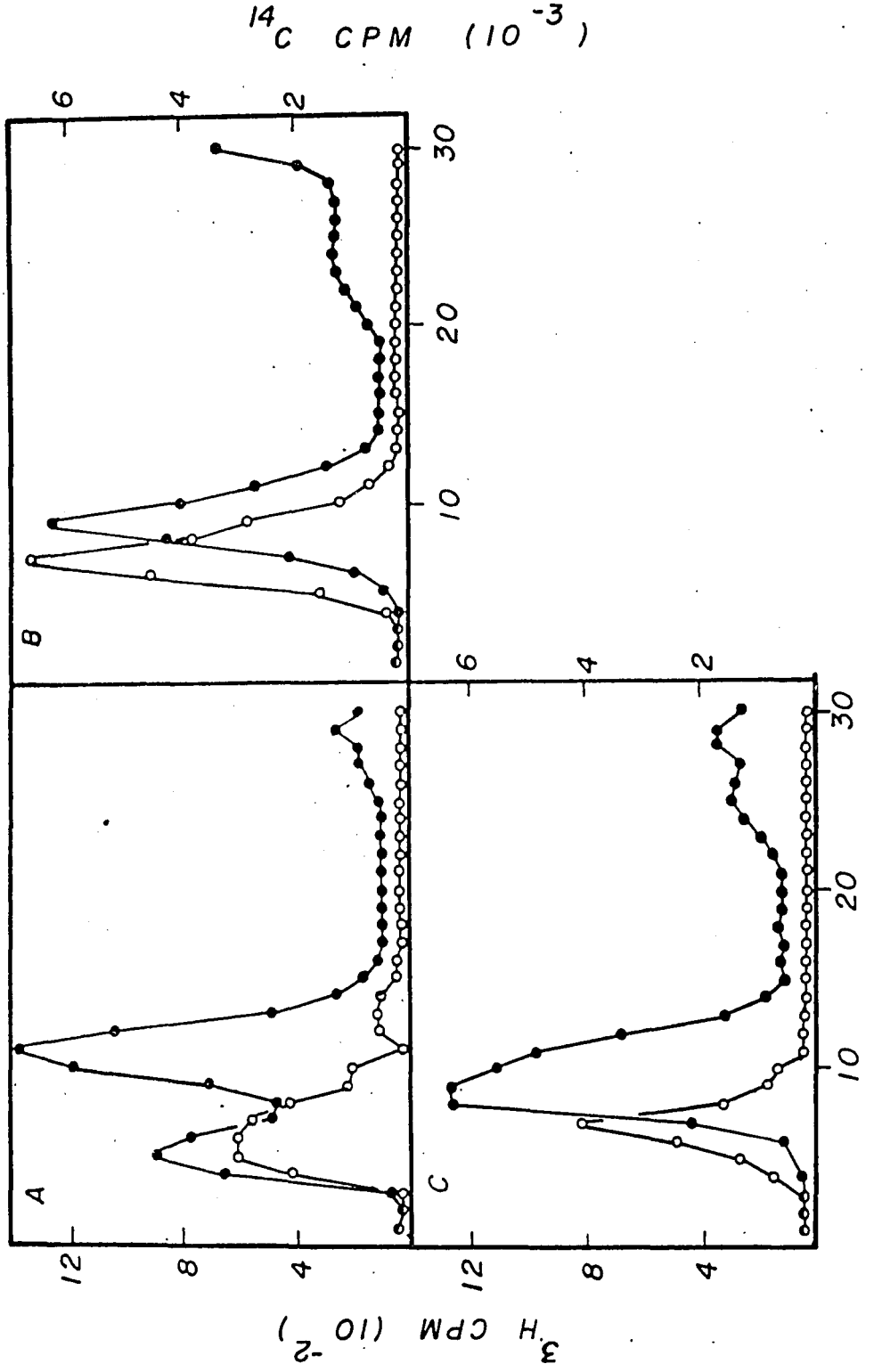


Figure 32

^{3}H-adenine Pulse and Chase in AS-1 Infected, ^{14}C Labeled *Anacystis nidulans*. II.

^{3}H-adenine was added to a 12 ml culture of AS-1 infected *A. nidulans* as described in the legend of figure 31. A) 2-3 hrs., B) 4-5 hrs., C) 8-9 hrs. After the one hour radioactive pulse the cells were collected by low speed centrifugation (4000 x g, 20 min.) and resuspended in 4 ml of fresh Bristol's medium containing excess adenine (200 µg/ml) and the cultures incubated until 12 hrs. post infection. The remaining unlysed cells were collected by low speed centrifugation (4000 x g, 20 min.) and then lysed and centrifuged in CsCl density gradients as described in the legend of figure 31. (-o-, ^{3}H; -●-, ^{14}C).

Figure 32



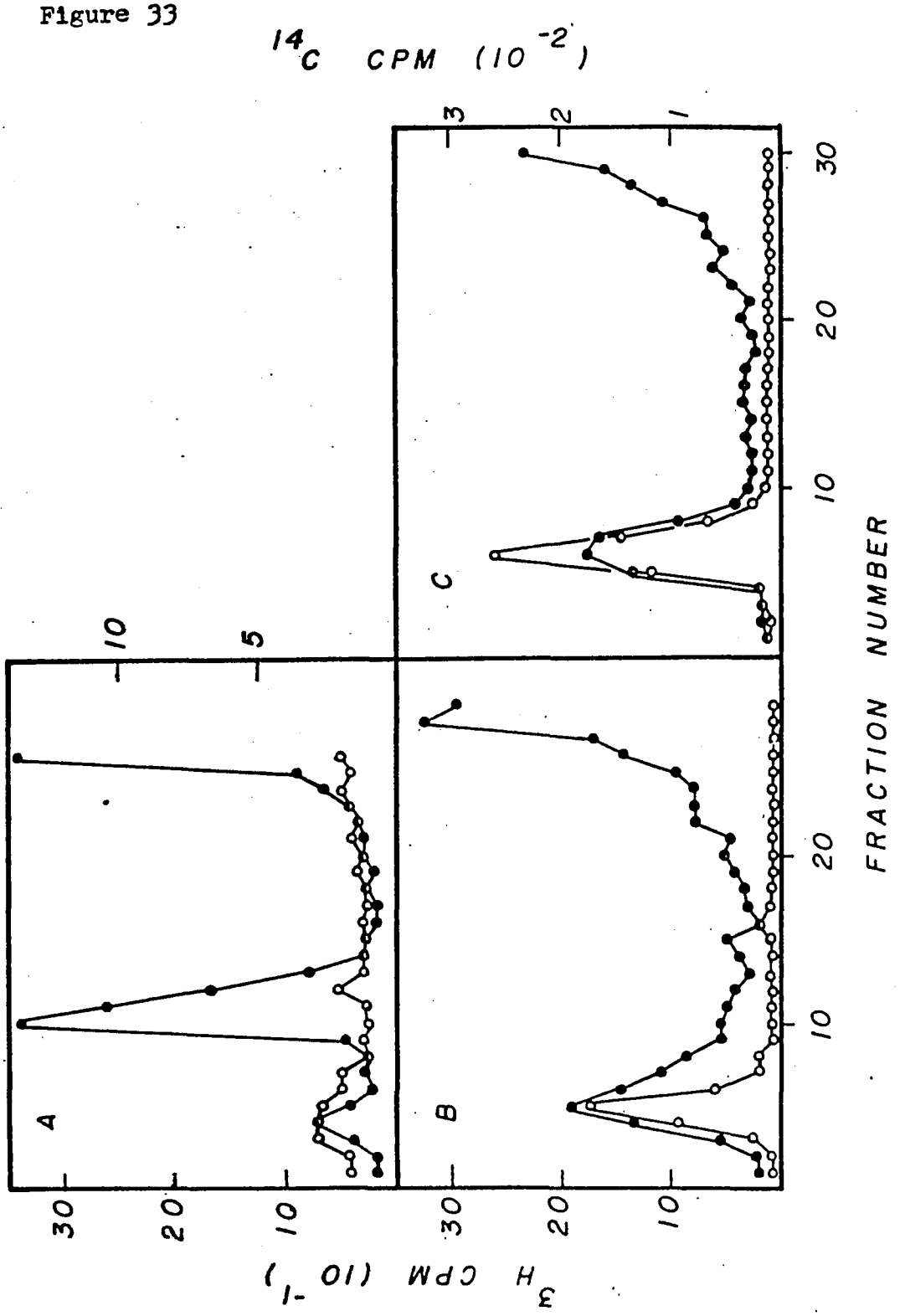
both ^{3}H and ^{14}C, the other, lighter one of ^{14}C alone. In the later time points (Figure 32 B,C) 4-5, and 8-9 hours post infection, the discrimination between the peaks is less clear with the ^{14}C containing peak being offset from the ^{3}H containing one by only one or two fractions (equal to a difference in buoyant density of approximately 0.0015-0.003 g/cc; see equation 1). In Figure 33, that DNA which has been packaged in phage 12 hours after the initial infection of ^{14}C labeled A. nidulans which was exposed to ^{3}H adenine at selected times post infection (33A, 2-3 hrs; 33B, 6-7 hrs; 33C, 8-9 hrs), is shown. Again in this experiment it is evident that lysis was inhibited after the resuspension in the fresh medium, since at the temperature the experiment was performed, $32^{o}C$, lysis should have been more than 90% complete by 12 hours (Figure 9). Due to the lysis inhibition, the greater bulk of the radioactively labeled DNA remained inside the cell rather than being found in the supernate of a low speed centrifugation at the termination of the experiment.

Figure 33

^{3}H-adenine Pulse and Chase in AS-1 Infected ^{14}C Labeled *Anacystis nidulans*. III.

Twelve hours post infection the AS-1 infected culture described in the legend of figure 31 was subjected to low speed centrifugation (4000 x g, 20 min.). A 2 ml aliquot was removed from the supernate and treated with DNase I (100 µg/ml, 45 min., 37°C). The DNase treated supernate was then centrifuged in a 60 Ti rotor at 75,000 x g, 45 min.) using a Beckman L3-50 ultracentrifuge to pellet the phage. The pelleted phage was resuspended in 1.0 ml Saline-EDTA and lysed using 3 drops of 30% Sarkosyl NL97 for 15 min. at 60°C. The lysed phage solution was subjected to CsCl density gradient centrifugation and analyzed as described in the legend of figure 30. The panels are from the cultures pulsed with ^{3}H-adenine at the following times post infection, A) 2-3 hrs., B) 6-7 hrs., C) 8-9 hrs., -o-, ^{3}H; -●-, ^{14}C.

Figure 33



Discussion

The data presented in this thesis details an array of physiological and biochemical characterizations of various aspects of a cyanophage/cyanophyte infective system with the universality of the phenomena described being shown to exist for the different phages and algae under study. The particular infective system most examined in these studies was that of cyanophage AS-1/blue-green alga Anacystis nidulans.

In any infective system, the physiology of the host often is a factor in the nature of the developmental process of the phage. As an example, it has been noted (K. Blashka and R. McGowan, unpublished observations) that algal cultures approaching stationary phase are not infected as easily nor display the same extent of new biosynthesis as younger, logarithmic phase cultures. The blue-green algae are particularly interesting as hosts because they are photoautotrophic, capable of producing their substrate and metabolite requirements through the process of photoassimilation of CO_2. Blue-green algae can, however, incorporate a variety of metabolites when included in the growth medium (73,95). It is the relative extent of this incorporation (in Anacystis nidulans and other of cyanophyta) that differs widely among the compounds (47,61-64,67,72,99,100). In addition, this assimilation is to a very great extent light dependent (47).

There are, however, many compounds which are not assimilated by intact Anacystis, and this as well as the poor incorporation of the other compounds has been attributed to a combination of factors

including the impermeability of the cells to certain organic compounds and disjunctures in cyanobacterial metabolism which prevent heterotrophic growth on reduced carbon sources (66,99,100).

Examples of the relative impermeability of Anacystis to organic compounds include most of the nucleic acid precursors (67,72). Restaino and Frampton (72) have shown that both thymidine phosphorylase and thymidine kinase activities are absent in cell free extracts of Anacystis nidulans. Pigott and Carr (67) have shown that various nucleic acid precursors, with the exception of uracil, incorporate poorly into uninfected Anacystis. Upon treatment of this alga with lysozyme, which removes the cell wall resulting in the formation of protoplasts, the incorporation of all the precursors increased with thymidine incorporation increasing from 0.2% after 20 hours in the intact cells to 32.5% after three hours in the protoplast.

In the experiments presented here (Figures 1 and 2, Table 2), there is an increase in incorporation of a wide variety of substrates, similar to that seen following the lysozyme treatment of the alga. The data, however, indicate (Figures 3 and 4) that the altered incorporation is not merely a "hole-punching" phenomenon generated by the cyanophage infection. Infection by a single phage particle is seen to be sufficient to cause the increased incorporation of exogenously supplied substrates, presumably due to the alteration of host permeability as well as subsequent utilization of the various metabolites by a variety of phage specific biosynthetic pathways in the infected cell.

The effect of cyanophage infection on the assimilation of the two sugars used in this study, glucose and glucose 6-phosphate, is extremely interesting. Pelroy et al. (66) and Stanier (100) have tentatively concluded that the non-utilization of glucose in uninfected cells may be due to a lack of glucose permease. In Anacystis nidulans, glucose supplied in the medium at a 5mM concentration results in the assimilation of 42 mM/mg dry weight increase while an exogenous concentration of 25 mM results in the incorporation of 195 nM/mg dry weight increase (99). Smith et al. (99) have shown the effect of concentration on the differential rates of incorporation of several organic compounds in Anacystis nidulans during photosynthetic growth. It is obvious that the 0.01 to 1 μM concentration of metabolites used in this study are far below saturating levels and that the generally hyperbolic curves for incorporation may well be due to the turnover and usage of the introduced substrate, especially in the case of the infected cells. The function of the experiments presented here was to maintain in vivo conditions avoiding perturbations of the host pool size with the specific aim of indicating basic differences between the infected and uninfected cell. Since the molar concentration of glucose added to both infected and non-infected cells in this study was 1.1 μM, an amount yielding negligable incorporation in uninfected cells (99), the substantial increase in the incorporation of this substrate (as seen in Figure 1 and Table 2) in infected cells lends credence to a cyanophage induced alteration of algal permeability.

In the blue-green algae that have been studied, the pentose phosphate pathway is the major, if not the sole, route of glucose dissimilation (16). Since it is indicated in another section of this thesis that this pathway is operative in Anacystis nidulans after AS-1 infection (see Section III, 1. Use of Alternative Precursors for Cyanophage DNA Synthesis), it is unlikely that the sugars used in this study are integrated per se into any phage-specific product. The results presented here imply that the increased glucose assimilation upon infection should allow for heterotrophic support of viral synthesis. In fact, Sherman (84) has shown that glucose, when added to infected cultures and even those incubated in the dark or in the presence of DCMU, is capable of supporting some viral development. The uptake of glucose 6-phosphate may be more interesting in that no transport system for sugar phosphates has been described among the blue-green algae. In addition, the incorporation pattern suggests utilization at a specific time of phage development, reminiscent of the patterns of some of the nucleic acid precursors already presented in this study (see Figure 1.) The studies presented here have not determined whether glucose 6-phosphate has been incorporated as an intact molecule or whether it has been dephosphorylated prior to uptake. However, Rubin (76) has presented evidence that glucose 6-phosphate can be taken up as an intact molecule in Anacystis nidulans.

The incorporation of radioactive precursors into AS-1 infected A. nidulans was of importance in the determination of phage induced metabolic activity. However, since the temporal sequence of

biosynthetic events following infection was of even greater interest than the absolute identification of phage biosynthesis, it became mandatory to regulate certain variables affecting either the host alone or the infective system as a whole. It became evident that an optimal system would include host cultures in which all the cells were at the same stage of the cell cycle in order to account for the host macromolecular synthesis. It was shown (10) that the synchrony regimen of Asato and Folsome (6) met these requirements. Following the dark period, the culture displayed logarithmic growth for the next 5-8 hours (6,10). It has been shown (6,40,40a) that DNA synthesis occurs in the latter 0.5-0.67 of the cell cycle of synchronized Anacystis. If infection were to occur after the development of increased nucleotide pools within the host in anticipation of algal DNA synthesis, a large untapped pool of nucleotides would be available for phage DNA biosynthesis if infection occurred prior than midway through the cell cycle of a synchronized host culture. The increased amount of ^{3}H adenine incorporation seen in the synchronized cells relative to the asynchronous culture (Figure 5) may reflect the uniform susceptibility to infection of a synchronous population as compared to a variety of physiological conditions of cells in an asynchronous population. This latter condition has been reported by Weppelman and Brenton (115) with regard to the relationship between the sensitivity of Pseudomonas aeruginosa to phage infection and the bacterial division cycle.

An environmental factor affecting the AS-1/A. nidulans infective system is the requirement for light during the lytic cycle (xxx). The studies presented here (Figures 6-8) examined the effect of light on the infective system as a function of phage replication and release (Figure 6) and to DNA synthesis, measured by precursor incorporation in the infective system. The studies presented here tested for the differential effects of a variety of inhibitory treatments, physical deprivation of light, electron transport inhibition by DCMU (9) and uncoupling of phosphorylation by FCCP (9), when these treatments were effected on AS-1 infected cultures at a variety of times post infection. The data on phage production indicate (Figure 6) that the most critical period of time with respect to the requirement for photosynthesis and/or phosphorylation is the first 3-4 hours post infection. The data (Figures 7,8) on the incorporation of ^{3}H adenine into DNA indicate a sensitivity of incorporation to inhibition by FCCP and DCMU at any point during the infectious cycle with the sensitivity to inhibition principally occurring when DCMU is added during the first two hour post infection and with less severe effects being seen when the inhibitors were added at later times.

These results show similarities to those presented by Sherman (84) for AS-1M infection of Synechococcus cedrorum. Sherman followed the effects of CCCP and DCMU on protein syntthesis in uninfected and infected cells as well as on viral burst size. His data indicates that CCCP is a somewhat more potent inhibitor than DCMU for protein synthesis with both uninfected and infected cells. In uninfected S.

cedrorum 10 μM CCCP decreased protein synthesis to 6.8% while the two inhibitors tested resulted in a corresponding inhibition for infected cells. CCCP at 10 μM caused a decrease of protein synthesis to 3% of noninhabited levels while the same concentration of DCMU reduced synthesis to 7.6% of the control. Both CCCP and DCMU at 10 μM completely eliminated any viral burst. However, in Sherman's report the inhibitors CCCP and DCMU were added at, or immediately after, the time of infection. Therefore, the entire developmental cycle of the phage was affected. In the studies presented here the examination of the lytic cycle by treatment at various periods of time post infection with agents inhibitory to light utilization lead to a consequent clarification of the requisite period of lighting for the infective system.

Although the data from Figures 6 - 8 corroborate each other, some variability can be seen regarding the exact timing of the effects of the inhibitory treatments. These two experiments were performed at different temperatures with the experiment followed phage production performed at 30°C, while those testing the inhibitors DCMU and FCCP were performed at 26°C. This lower temperature would have the effect of lengthening the lytic cycle. The difference in the exact timing of the inhibitory effects could thus be temperature related. This temperature effect is also most likely to be the cause of the discrepancies noted by other workers (65,81,86) with regard to the length of the lytic cycle, with Safferman describing the lytic cycle as a 16-18 hour event at 26°C, Pearson et al. (65) reporting the infectious cycle to be 16 hours at 25°C or

35°C, while Sherman (86) noted that the lytic cycle of AS-1M was 12 hours. It is not known, however, which if any of the temporal sequence of events is temperature dependent.

The process of definition of various physiological and environmental parameters relating to and affecting the AS-1 infective process allowed a detailed sequence of biochemical determinations to be performed in which a level of standardization was achieved to relate sometimes varied results. The major thrust of this thesis relates to the isolation and characterization of a phage induced light DNA (PIL-DNA) and the sequence and mode of its synthesis in the AS-1/A. nidulans infective system.

Viral development in an infected host can be measured by monitoring the production of a number of virus specific products including DNA, RNA and protein. However, since cyanophage AS-1 is known to contain only DNA and protein, and some phage specific protein produced is not found in the free virion (91), the accumulation of phage DNA seemed a reasonable method for monitoring cyanophage development. Equilibrium density centrifugation in CsCl and Cs_2SO_4 has been used to resolve the DNA's of phage and host during infection with other bacteriophage systems (26,108) including separation in CsCl of the DNA's of cyanophage LPP-1 and its host, Plectonema boryanum (88). However, the potential applicability of density gradient centrifugation in cesium salts was complicated by data given in the initial report of Safferman et al. (81) on the isolation of AS-1. In this report, thermal denaturation studies of AS-1 DNA were presented to show a G-C content of 53-54%. Calculating from

the equation of Schildkraut et al. (83)

$$\rho = 1.660 + 0.098\ G\text{-}C \qquad (2)$$

the buoyant density in CsCl of this DNA was found to be 1.713 g/cc, a value too close to that of Anacystis DNA to be completely separated in CsCl density gradients (26,108). Nonetheless, since this was the only report in the literature at the time concerning AS-1 DNA, appropriate experiments were undertaken to either confirm or modify the experimental results of Safferman. The results presented in Figure 9 showed the temporal pattern of DNA synthesis in AS-1 infected Anacystis nidulans. A new peak of DNA was seen at a different buoyant density in CsCl than that expected from the results of Safferman et al. (81). The concentration of the newly synthesized DNA relative to the total DNA present was at (hours post infection) 4 hr, 31.7%; 5 hr, 49.6%; 6 hr, 57.4%; 7 hr, 60.2%; 8 hr, 65.1%; 9 hr, 64%. The initial presumption was that this DNA was that of the cyanophage AS-1 and that there was an unexplained discrepancy between these results and those of Safferman et al. (81). The report of Sherman and Connelly (85) showed that DNA extracted from isolated AS-1M had a buoyant density in CsCl of 1.714 g/cc. This value obtained by measurement in CsCl essentially confirmed the results of Safferman et al. (81). Based on its characteristics, being synthesized only after AS-1 infection and being light in CsCl the DNA was termed phage induced light DNA (PIL-DNA). It was evident that there were two varieties of DNA synthesized following infection of Anacystis nidulans with AS-1. The biosynthesis of the phage DNA (AS-1) and the new DNA (PIL-DNA) was followed by monitoring

the incorporation of radioactive isotopes into both species as a function of time. Commonly such studies are performed using specific DNA precursors. However, in addition to the specific precursor approach, a considerably more general radioactive precursor, $^{14}CO_2$ was used. The rationale for using such a general radioactive label is threefold: first, blue-green algae are strongly phototrophic and CO_2 represents a more natural substrate; second, the particular technique employed in generating and analyzing the preparative CsCl gradients eliminates some of the normal difficulties encountered when using a general label. Specifically, much of the radioactivity associated with protein is eliminated before the centrifugation process since the buoyant density of protein in CsCl is such that it "floats" on top of the gradient and can be removed manually. Third, it has been shown by Pearson et al. (65) and by Sherman (86) that photosynthesis continues until or just prior to lysis in the host following infection by AS-1 or AS-1M. Since host macromolecular biosynthesis is "shut-down" following AS-1 infection (Sherman and Pauw (91)), the photosynthetic reduction of $H^{14}CO_3^-$ could result in metabolites used in cyanophage biosynthesis. In fact, this supposition was substantiated as seen from the data of Figure 15 in which the incorporation of ^{14}C into PIL-DNA resulted from the photoassimilation of ^{14}C from the general radioisotope $H^{14}CO_3^-$ which was added to the medium of the infected cultures.

Although intact Anacystis was found to incorporate nucleic acid precursors (except for uracil) at very low levels (67), it was shown earlier in this thesis (Figure 1) that the infected cell incorporated

substantial amounts of these metabolites. The metabolic fate of exogenously supplied nucleic acid precursors supplied during the infectious cycle was explored in the experiments depicted in Figures 13 and 14. In this case the radioactivity associated with the ^{3}H adenine migrated to a buoyant density of 1.715 g/cc in preparative CsCl gradients. Very little of the ^{3}H adenine ever migrated to a density commensurate with that of the new PIL-DNA, except on occasion at later time periods of the lytic cycle.

In examining the experiments following DNA synthesis using $H^{14}CO_3^-$ and ^{3}H adenine as the source of radioisotope, some differences were seen regarding the timing of synthesis of AS-1 DNA and PIL-DNA. However, these experiments were run at different temperatures, 26°C for $H^{14}CO_3^-$ and 32°C when using ^{3}H-adenine. It was shown earlier (Figure 8) that the lytic cycle was greatly accelerated at 32°C, therefore a more accurate comparison of the patterns of synthesis of AS-1 DNA and PIL-DNA was performed as seen in Figures 15-17. Both radioisotopes were added simultaneously to infected cultures in order to elucidate the nature of DNA biosynthesis in AS-1 infected A. nidulans. A comparison of the data obtained from the individual isotope experiments (Figures 13-15) and the dual isotope experiment (Figures 16-18) show several interesting phenomena. The two DNA's that are synthesized after AS-1 infection of A. nidulans exhibit distinctive characteristics with regard to precursor sources and temporal synthesis. There is a marked difference in the patterns of incorporation of radioactivity into DNA when a nucleic acid precursor is used as compared to the patterns obtained using a general non

specific precursor. Each DNA, AS-1 or PIL, characteristically contains primarily one or the other of the radioactive materials added. Adenine is used in the synthesis of AS-1 DNA molecules, similar to the results of Sherman and Pauw (91) but for the most part is excluded from the lighter buoyant density PIL-DNA molecules. Only at times late in the infectious cycle (Figure 18, starting from 7-8 hours post-infection) does the compartmentalization of precursor sources seem to break down with the increasing incorporation of ^{14}C being seen in the heavy DNA peak (1.714/5 g/cc) associated with AS-1 DNa. In addition a slight reversal in the utilization of ^{3}H adenine can be seen by some incorporation of this precursor as seen in in Figure 17C, some 6-7 hours post infection as well as the small peaks of incorporation seen at times in the single isotope experiment using ^{3}H adenine (Figures 13 and 14) at a buoyant density lighter than the E. coli marker added (ρ=1.710 g/cc) with the ^{3}H adenine presumably incorporating into PIL-DNA.

Another observation regarding the two species of DNA is their distinct sequence of synthesis. The dynamics of PIL-DNA synthesis as revealed by the single isotope ($H^{14}CO_3^-$) and dual isotope experiments suggest that $H^{14}CO_3^-$ incorporation builds up from three hours post infection with maximum synthesis around six hours post infection (at 32°C) with declining synthesis thereafter. Incorporation of ^{3}H adenine into AS-1 DNA commences as early as 1-2 hours post infection with incorporation continually increasing until very late in the lytic cycle. In addition, in examining the data from a series of experiments (Figures 15-18, also see 20A, 15C

incorporation vs 19 and 22A,B vs 21), it was noted that the amount of ^{14}C found in DNA synthesized late in the infectious cycle, relative to that made early, was not constant. It was found that the rate of CO_2 fixation after infection (data not presented) was somewhat variable; in some experiments there was a high rate of fixation up until lysis, in others there was a progressive decline during the latent period. This latter case is similar to that of cyanophage SM-1 infection of the blue-green alga NRC-1 (51,52,82) which, like Anacystis nidulans, is a unicellular blue-green alga. In order to understand why there might be variability in the net amount of ^{14}C fixation in Anacystis nidulans after AS-1 infection, other biosynthetic pathways resulting in CO_2 fixation, including those alternative mechanisms of CO_2 fixation to that of CO_2 photoassimilation, must be examined. In the biosynthetic pathway of inosinic acid, a purine nucleotide which can be converted either to adenylic acid (AMP) or guanylic acid (GMP) the conversion of 5' phosphoribosyl 5-aminoimidazole to 5' phosphoribosyl 5 aminoimidazole 4-carboxylic acid, involves a carboxylation reaction (29). An additional mechanism of CO_2 fixation found in blue-green algae is that of the urea cycle. In this case there is a carboxylation reaction in the synthesis of carbamyl phosphate, a precursor in the pryimidine synthetic pathway, as well as those of citrulline and agrinine. The variability in carbon fixation noted above may also relate to slightly different physiological condition of the host cell at the time of infection. As it was noted earlier in this thesis, algal cultures approaching stationary phase do not

display the same extent of new biosynthesis as younger logarithmic phase cultures.

From the data discussed thus far, it is seen that there is cyanophage induced synthesis of a new species of DNA (PIL-DNA). This DNA has different precursor sources, temporal sequence of synthesis and a different buoyant density (ρ=1.701 -0.001 g/cc vs 1.714 g/cc) as compared to AS-1 DNA. In addition an additional piece of information can be deduced from the data concerning the incorporation of ^{14}C into PIL-DNA. The presence of newly photoreduced carbon in this DNA after the addition of $H^{14}CO_3^-$ for only a fraction of the latent period indicates the simultaneous production of nucleotide precursors along with the production of DNA, a situation not previously described for cyanophage infection and only recently described in T4 infected E. coli (17).

The use of alternative precursors and hosts as subjects for study of cyanophage induced DNA synthesis was designed to test the universality of the system described above. Thymidine was chosen since it is a direct precursor of DNA and in the dual isotope experiment described in Figures 18 and 19 it is seen to behave in a fashion analagous to that of adenine. The sequestering of thymidine for use in AS-1 DNA production and the assimilation of ^{14}C into PIL-DNA confirms those patterns presented earlier (Figures 15-17). Uracil, on the other hand, is not normally a precursor of DNA. This nucleic acid precursor is, however, seen to be used (Figures 20,21), presumably after modification, as a precursor for phage DNA is this system. It can be seen that some tritium appears to become associated with PIL-DNA, (Figures 21C, 21D, 22B). It is possible that the modifications necessary for converting uracil for use in DNA may affect the sequestration process in which exogenously supplied nucleic acid precursors are almost exclusively used in AS-1 DNA synthesis. Alternatively, in the conversion process the ^{3}H may have been removed from the uracil and in an exchange process may have become associated with a precursor of PIL-DNA.

The photoassimilation of CO_2 leads to the biosynthesis of a number of metabolic products. Various metabolic pathways continue to function after AS-1 infection. The pentose phosphate pathway is of major metabolic importance in the cyanophyta (16) and would be valuable in supplying sugar phosphates to participate in nucleotide production. The use of glucose 6-phosphate as a precursor for DNA synthesis in AS-1 infected Anacystis was an attempt to determine if

this early product of carbon fixation would become associated with AS-1 DNA or would be sequestered for the biosynthesis of PIL-DNA. A clue as to the outcome could be garnered from the data of Figure 1. The timing of incorporation of glucose 6-phosphate was reminiscent of that of the nucleotide thymidine, a DNA specific precursor. For glucose 6-phosphate to be used by AS-1 infected cells in the production of DNA it must be either used directly in the modification (glucosylation) of the DNA or converted by the pentose phosphate pathway to a deoxyribose sugar. Since no evidence has been found for glucosylation, the conversion by the pentose phosphate pathway (indicating continued operability post infection) most likely occurred. Figure 23 showed that when ^{3}H-adenine and ^{14}C glucose 6-phosphate were added to AS-1 infected cultures, the radioactivity from both metabolites were found associated with AS-1 DNA and not with PIL-DNA. This would suggest that radioactivity from glucose 6-phosphate enters the pathway for DNA synthesis after the point of discrimination for PIL-DNA.

The use of alternative hosts in an infective system is often an attempt to expose subtle differences in metabolism in comparing phage effects in two related though distinct systems. While Stanier (101) considers both Anacystis nidulans 625 and Synechococcus cedrorum 1191 to be identical, classifying both as strain 6301, some differences have been noted between Anacystis 625 and Synechococcus 1191. Sherman (personal communication) noted that S. cedrorum yielded increased titers of cyanophage as compared to A. nidulans and also claimed higher photosynthetic capacity (CO_2 photoassimilation) for Synechococcus. The data presented in figures 24 and 25 tend to support in part both theories. Both algal hosts support the growth of cyanophages AS-1 and AS-1M and after cyanophage infection both AS-1 and PIL-DNA is synthesized. These factors imply a very close phylogenetic relationship between the two algal hosts used in this study. However, it can also be seen in examining the relative incorporation of ^{14}C into PIL-DNA in cyanophage infected A. nidulans 625 and S. cedrorum 1191 that the amount of ^{14}C radioactively incorporated in the Synechococcus culture was an order of magnitude greater than for the Anacystis culture. Since both algal stocks contained equal numbers of early logarithmic phase cells, the indication is that Synechococcus is more competent in CO_2 photosassimilation than Anacystis, therby confirming Sherman's above mentioned observation.

Sherman's study of AS-1M infection of S. cedrorum indicated little incorporation of ^{3}H adenine or uracil into the DNA in infected cells until after 3 hours post infection (91). The results

presented here indicate substantial incorporation into DNA before then. Similarly, in a study of DNA synthesis in LPP-1 infected P. boryanum, Sherman and Haselkorn (88) found incorporation of ^{3}H adenine into viral DNA early in infection prior to the bulk of DNA synthesis. They claimed that this incorporation was not completely explained by precocious synthesis in an asynchronously infected population, believing rather that the incorporation of label at times before bulk replication of DNA was due to either recombination or repair. Their experiments employed a multiplicity of infection (MOI) of 10, a condition in which many cells are multiply infected and which offers the potential for recombination between the genomes of the infecting viruses. Repair was suggested because blue-green algae are very resistant to both UV and x-rays and contain very active repair systems (88).

The main thrust of the data presented until this point is that there is a phage induced light species of DNA (PIL-DNA) synthesized following AS-1 infection of Anacystis nidulans which uses different precursor sources and has a different buoyant density in CsCl than that of AS-1 DNA. Some fundamental questions regarding this DNA including those resolved in this thesis as well as some unexplored areas are listed below.

1. Are there physiological requirements for the synthesis of this DNA such as the necessity for light? Since the photoassimilation of $^{14}CO_2$ into PIL-DNA is one of its distinguishing hallmarks, would PIL-DNA be synthesized in AS-1 infected cultures incubated in the dark?

2. What is the mode of synthesis of PIL-DNA? Does this DNA represent:
   a.) de novo synthesis (and if so, what is the template?), or
   b.) the unregulated proliferation of a host plasmid, or
   c.) modification of host DNA, or
   d.) modification of AS-1 DNA (either the parental infective molecule or subsequently synthesized AS-1 DNA).
3. Does the physical biochemistry of the molecule offer clues as to the "lightness" in CsCl of this DNA or to some unusual base composition including possibly modified bases.
4. What is the fate and possible function of such a molecule?

It has been suggested in this thesis that the pentose phosphate metabolic pathway remains functional in Anacystis following AS-1 infection and that metabolic derivatives of glucose 6-phosphate may be disseminated via this path in the cyanophage infected system. In the experiment (figure 23) describing the disposition of the ^{14}C radioactive label from ^{14}C glucose 6-phosphate (in which ^{14}C cosedimented with ^{3}H label from tritiated adenine - diagnostic for AS-1 DNA in the CsCl density gradients described in this thesis), the standard lighting conditions described in Materials and Methods were used. Under conditions of light deprivation glucose 6-phosphate is turned over rapidly, therefore the utilization of this sugar in DNA synthesis by the infective system could be used as one of a number of probes to examine the nature of PIL-DNA. Specifically, it might be possible to show whether this DNA represented new macromolecular biosynthesis (via semi-conservative replication) or whether the sugars used in this study would be metabolized and some resultant derivative

have shown that glucose can be used to support the growth of AS-1 infected algal cultures incubated in the dark so that the resulting burst is some 10% of a normal burst. In this system AS-1 DNA is obviously synthesized. Not so obvious is whether PIL DNA would be made in such a system. Ostensibly, PIL DNA synthesis requires the presence of light since it has been shown that this DNA preferentially accumulates newly photoassimilated $^{14}CO_2$. The experimental methodology to make this determination would entail the addition of ^{14}C glucose 6-phosphate to AS-1 infected cultures incubated in the dark. If PIL DNA was found at levels similar to those seen in infected cultures exposed to normal lighting conditions the implication would be that PIL DNA is a modification of some preexisting DNA molecule rather than representing de novo biosynthesis. Alternatively, a lack of PIL DNA or levels reduced below the proportionate amount of AS-1 DNA synthesized in the dark incubated system might imply new biosynthesis with metabolite derived from glucose 6-phosphate providing some of the necessary precursors.

There are several pieces of data that pertain to the second question asked, that of the mode of PIL DNA synthesis. Modification of DNA such as methylation and glucosylation could account for the "light" nature of a DNA molecule. In fact, figure 36, representing an enzymatic digest of PIL DNA, showed the potential for such modification in that there were some unidentified loci on the chromatogram. In addition, Baye (10) has presented preliminary evidence using an AS-1/A. nidulans infective system in which the DNA of the infecting cyanophage was radioactively labeled with tritium

showing that some of the tritium equilibrates at the position of PIL DNA in a CsCl density gradient. There is however, some difficulty reconciling this data with other work presented here. Figures 34 and 35 present the thermal denaturation profiles of PIL DNA in 0.1 X SSC and 1 X SSC. This data reveals that modification of PIL DNA is unlikely since the G-C estimates are compatible with those derived from CsCl density gradient centrifugation. In addition, it was shown in Figure 10, using an MOI=4 that some 65% of the total DNA in infected cells could be PIL DNA. It is unreasonable to assume that all of this DNA represents the modified infecting parental cyanophage DNA since AS-1 DNA has a molecular weight of 57-60 x 10^6 daltons (85) while Anacystis contains some 3 x 10^9 daltons of DNA.

In order to determine if PIL DNA does represent de novo synthesis, the following experiment is proposed. Add $NaH^{14}CO_3$ and ^{15}N as nitrate salts to an AS-1 infected Anacystis culture growing at 30°C, just after infection and incubate until 3 or 4 hours post infection. Under these conditions lysis should have not yet occurred and PIL DNA synthesis should be evident. The initially synthesized PIL DNA should contain ^{14}C, a heavy as well as radioactive label. If these infected cells were shifted into a ^{15}N containing media (nitrate salts comprise part of Bristol's medium used for growth of Anacystis) and if de novo synthesis was occurring, the ^{14}C radioactive label should be seen to shift to a heavier density in CsCl.

The third question - that of the physical characterization of the molecule to reveal aspects of its composition, does in fact refer back to the question of modification of some preexisting DNA. the

preliminary results presented here (Figure 36) indicated that some modification is likely and it is highly possible that a small degree of modification could reconcile the identification of a modified base as seen in figure 36 and the equality of G-C estimates from buoyant density and thermal denaturation techniques.

Some insight into the fate and nature of PIL DNA was presented in this thesis. It has been shown (table 3) that some PIL-DNA synthesized early in the infectious cycle does become packaged. However, the bulk of this DNA remains in the cell after synthesis. An initial belief was that this DNA might be a modified precusor of DNA that was eventually packaged. In fact, it has been shown (figures 16-18) that incorporation of ^{14}C into PIL-DNA declines at later periods in the infectious cycle with a corresponding increase of the ^{14}C radioactivity into AS-1 DNA. In addition, the molecular weight of PIL-DNA has been found (see appendix C) to be more than twice that of the reported value for DNA extracted from the purified cyanophage AS-1M (85). However, PIL-DNA fails to meet the criterion of being a precursor in that little of this material was found packaged in phage at periods late in the infective cycle (table 3, figures 26-29). In coliphage T4 (32) and other bacteriophages (109), precursor DNA has been found to be several unit lengths of phage genome with portions being clipped off and packaged. In the studies reported here the entire molecule is packaged early with little, if any, being packaged from material synthesized later in the infectious cycle.

An alternate method for examining the nature of PIL-DNA entailed prelabeling the DNA of the algal host with ^{14}C. Since Anacystis nidulans is a photoautotroph, the vast majority of all metabolites within the cell are derived from the photosynthetic process of CO_2 assimilation. When $NaH^{14}CO_3$ is added to the medium of a growing Anacystis culture, some photoreduced ^{14}C should be found in nucleic acid precursors and in the algal DNA either in the (deoxy)ribose sugar moiety or directly in the purine or pyrimidine ring structure. The incorporation of photoreduced ^{14}C into cyanophycean DNA was shown in figure 30. If PIL-DNA synthesis involved only the utilization of newly formed precursors immediately preceding or simultaneous with DNA synthesis, it would seem likely that little if any PIL-DNA would be discernable following AS-1 infection of a ^{14}C prelabeled Anacystis culture. In this case the sole source of ^{14}C should be a result of the breakdown products of host DNA. Sherman (91) has shown the reincorporation of breakdown products from Synechococcus DNA into the DNA of cyanophage AS-1M. The experiments described in figures 31-33 tested this hypothesis and essentially confirmed what was theorized above. In these experiments ^{3}H adenine was added to ^{14}C prelabeled Anacystis culures which were infected with AS-1. The incorporation of ^{3}H adenine into DNA was to be used as a marker since in all other experiments described in this thesis (figures 16-33) the ^{3}H adenine was found to incoporate into DNA of buoyant density 1.714/5 g/cc. As expected, ^{3}H adenine incorporation into DNA increases at the later time points of the infectious cycle. An unusual feature noted is that the ^{14}C containing peak at

2-3 hours post infection is considerably smaller than those seen 4-5,6-7 and 8-9 hours post infection. It is possible that the chase of ^{14}C metabolites from their pools in the uninfected cells was incomplete since the chase lasted about one generation (12 hours in these experiments). Cyanophage biosynthesis could have utilized some of the remaining labeled metabolites which would account for the greater amount of ^{14}C containing DNA at the later time periods. However, it has been recently noted (10) that cultures of Anacystis nidulans radioactively labeled with $NaH^{14}CO_3$ and chased for 4-8 generations prior to infection exhibited similar patterns to those described above. Additionally, it is noted that a large amount of ^{14}C containing material is found at the top of the gradient of the 2-3 hour time period but that later in the infectious cycle the ^{14}C seems to have migrated to a buoyant density of almost 1.714/5 g/cc. This may represent utilization by cyanophage metabolism of breakdown products of host glycoproteins which are known to band at the top of CsCl equilibrium density gradients. A final observation is that the midpoint of the ^{14}C containing peak falls at a buoyant density of 1.712 g/cc which is heavier than that described in the previous experiments for ^{14}C containing DNA. This buoyant density is sufficiently close to that of AS-1 DNA and different from PIL-DNA to conjecture that only AS-1 and not PIL-DNA uses breakdown products of host DNA. This has been confirmed by Baye (10) who showed an identical overlap of ^{14}C and ^{3}H containing DNA peaks in experiments similar to those described above.

The experiment which examined the nature of the DNA present, packaged in phage found in the supernate at the end of the experiment (figure 33) at first glance seems inconsistent. Theoretically only the amount of ^{3}H should vary based on the time period during which the ^{3}H adenine was pulsed in. Therefore the presence of a light ^{14}C peak seen in figure 32a could not be explained. However, it was shown in Figure 29 and Table 3 that ^{14}C incorporated into DNA early after infection is quickly packaged into phage particles. These phage (presumably a minor portion of the total phage to be liberated) are postulated to be released early. The infected cells that are resuspended at later times after the pulse no longer have the PIL-DNA present to any great extent and that which is left isn't packaged and is therefore sensitive to DNase. This would explain the observation of only one ^{14}C containing peak in those gradients of the phage which are found in the supernate at the end of the experiment.

In the discussion above some evidence and various experiments were presented concerning the nature of PIL DNA synthesis. Taken in its entirety the data presented in this thesis concerning PIL DNA lead me to favor the theory of modification as a source of this DNA. The single most compelling piece of data, although scant in relation to the many CsCl density gradients presented here, seems to be the chromatographed digest of PIL DNA (figure 36). De novo synthesis, of necessity, would have generated ^{14}C radioactive label in each of the nucleotides if the sole source of carbon for this DNA was derived from newly photoassimilated sources. A final unanswered question remains - what is modified? To provide this answer one must continue experimentation along some of the lines suggested in the discussion and in the conclusion of this thesis.

Conclusion

The data presented in this thesis details an array of biophysical and biochemical characterizations of various aspects of the cyanophage AS-1/blue-green alga Anacystis nidulans infective system. Cyanophage AS-1 is seen to exhibit characteristics similar to other large bacteriophages such as the T even coliphages with regard to a variety of parameters. However, the (obligate) photoautotrophic nature of the host allows a greater variety of phage-algal interactions to be studied than is possible in a heterotrophic host. Similarities to other bacteriophages include the pattern on one-hit kinetics in the inactivation and lysis of the host. In addition, the developmental processes of the phage are seen, comparable to other bacteriophages, to be sensitive to environmental conditions such as temperature. The studies in the first section of this thesis were investigations into the alteration of algal permeability and the incorporation of organic metabolites as a consequence of cyanophage infection. The alteration of host permeability is seen to be a result of particular alterations of the algal permeability, probably due to changes in the cell membrane, rather than a generalized hole punching phenomena caused by the phage injecting its DNA into the host cell. A major difference between the studies presented here and previous work with bacteriophages is that of the greater emphasis placed on continued host metabolic phenomena as opposed to solely following phage related biosynthetic events. The studies of metabolite incorporation give clues as to the amino acid composition of phage proteins and the

pattern of incorporation of various nucleic acid precursors gave indications of the temporal sequence of DNA synthesis which was confirmed in the more detailed biochemical studies presented in the latter parts of the thesis. Additionally, the incorporation of the two sugars used in this study, glucose and glucose 6-phosphate, are of particular interest in the study of potential heterotrophic capacity of this blue-green alga. Sherman (84) has shown that glucose is capable of supporting some development of cyanophage AS-1M even in the presence of DCMU which is an inhibitor of photosystem II. (9). Since Anacystis nidulans is an (obligate) photoautotroph and cyanophage AS-1 (AS-1M) has been shown to be dependent on continued host photosynthesis, the apparent heterotrophic potential for growth on glucose is particularly exciting. Further experimentation in which cyanophage AS-1 would be used as a vector to induce heterotrophy in Anacystis nidulans could possibly be quite revealing with regards to the mechanisms of control of autotrophy and heterotrophy. An example of such an experiment might entail infection of Anacystis with temperature sensitive mutants of AS-1 at a permissive temperature followed by rapid inactivation of phage development by exposure to non-permissive temperatures. Presumably, the alteration of host permeability, an early phenomenon resulting from phage infection, would remain. If the host remained functional,the altered permeability would allow the cell to benefit, by heterotrophic growth, from exogenously supplied glucose.

Another interesting physiological factor examined in this thesis involved utilization of the continued functionality of the host photosynthetic apparatus as an instrument in the production of reduced carbon which incorporated into DNA of buoyant density 1.701 $\pm$ 0.001 g/cc (PIL-DNA). The compartmentalization of nucleotide sources for the PIL-DNA (which is almost exclusively derived from newly reduced photosynthetic products) as compared to the precursors used in the synthesis of AS-1 DNA of buoyant density 1.714/1.715 g/cc (derived from breakdown products of host DNA or from exogenous sources) presents a phenomenon heretofore not identified in any other bacteriophage system. One of the experiments presented (Figure 23) sheds some light on this unusual metabolic phenomena. The pentose phosphate pathway is of major metabolic importance in blue-green algae with glucose 6-phosphate being involved in an early step of the pathway. When ^{14}C glucose 6-phosphate was supplied to AS-1 infected cultures of Anacystis nidulans, ^{14}C was found to cosediment with DNA at buoyant density of 1.714/1.715 g/cc. This implies that photoreduced ^{14}C (from $NaH^{14}CO_3$ in the medium) initially enter pathways forming nucleic acid precursors before the production of glucose 6-phosphate or is involved in a totally different metabolic pathway since photoreduced ^{14}C primarily is found in DNA of buoyant density 1.701 $\pm$ 0.001 g/cc.

The predominant utilization of only one pool of nucleic acid precursors in the synthesis of PIL-DNA (ρ=1.701$\pm$0.001 g/cc) is only one of the many unusual aspects of this macromolecule. For example, the molecular weight determination studies indicated that the mass of

this DNA was 138 x 10^6 daltons (see Appendix C). DNA of this extremely large size has previously only been identified in the T even series of coliphages. However, since Sherman (85) has identified the DNA present in isolated AS-1M to have a buoyant density of 1.714 g/cc and it was shown (Figure 29, Table 3) that only the earliest synthesized PIL-DNA (1.701 ± 0.001 g/cc) gets packaged, the question of the nature and function of this DNA remains only partially answered.

A salient feature of this thesis has been the biosynthetic and biochemical characterization of a unique species of DNA which is synthesized in AS-1 infected Anacystis nidulans and Synechococcus cedrorum. There are, however, many unusual features of this DNA which require further examination. Some of the basic questions that are incompletely resolved as described above, regard the composition, function and origin of this DNA. The following model systems are proposed as possible explanations for the phenomena described regarding the unique "light" phage induced DNA (PIL-DNA).

The unique DNA (PIL) was noted in the thesis as being "light" in CsCl. The buoyant density in CsCl was determined to be 1.701 ± 0.001 g/cc. This is considerably lighter than the DNA extracted by Sherman (85) from the free phage (ρ=1.714 g/cc). For a species of DNA to be "light" in CsCl one of a number of conditions must be met: A) The DNA must be rich in adenine-thymine base pairs; B) the DNA must be modified such as is found in glucosylated or methylated DNA's or C) a combination of A and B. The questions asked when examining the data in this thesis with regard to the physical aspect of the DNA are twofold. 1) What are the physical factors involved in making the

PIL-DNA light -- is it solely the greater number of A-T base pairs or is modification involved? 2) Why is there a "light" as well as a heavy AS-1 induced DNA in the infected host cell?

To answer the first question, the studies related to the physical characterization of the PIL-DNA must be examined (see Appendices A, B, C). The buoyant density of this DNA in CsCl was found to be 1.701 ± 0.001 g/cc and the G-C molar ratio was calculated from these values to be $41.8 \pm 1\%$. The determination of G-C content from thermal denaturation studies yielded a value of $40.9 \pm 0.6\%$ (see Appendix A). When the values of G-C content derived from these two methods of determination are compatible, it is usually indicative that no modification of the DNA is present. However, as was shown in Figure 36, photoreduced ^{14}C found in DNA constituents is seen to be present in one and possibly two precursors that do not correspond to the standard four nucleotides found in DNA as well as the ^{14}C found in AMP. It is possible, however, that if there were only small amounts of modified bases present in the PIL-DNA, but still enought to be detectable by the sensitivity of the radioactive labeling, the buoyant density value would not be altered and allow for the equality of the G-C molar ratios as determined by thermal denaturation and ultracentrifugation in CsCl. As an example, in classical modification/restriction systems, protection of phage DNA against host restriction enzymes is afforded by methylation at several recognition sites. T_3 DNA contains about 5-7 cleavage sites for endonuclease R. Eco B in vivo (48); the corresponding recognition sites are either methylated or lacking such protection, trigger the

cleavage mechanism. Correspondingly phage T_7 has been shown to have (in vitro) 4-5 recognition sites (27). Only a small number of molecules is seen to be necessary for protection against restriction, a factor that would lessen the possibility of an altered buoyant density of the DNA in CsCl.

If modification does not satisfactorily answer why the PIL-DNA is light, one must look to the G-C (or A-T) molar ratios for a clue. It has been shown (Figure 36) that ^{14}C from photoreduced sources does incorporate preferentially into adenine in the PIL-DNA. However, if this PIL-DNA does have a higher A-T (and lower G-C) content than does DNA extracted from isolated phage, the second question, asked initially regarding why such a DNA is present, comes into the fore. It was demonstrated that there are different temporal patterns of synthesis of the PIL-DNA and the phage DNA which is primarily found packaged in free phage. It is possible that this PIL-DNA represents the DNA of a (defective) lysogenic phage carried by Anacystis nidulans which may be induced by infection with cyanophage AS-1. If the site of synthesis of this DNA is close to the membranes of the photosynthetic lamellae, the region for DNA synthesis could be compartmentalized which would explain why precursors for this DNA would contain newly photoreduced carbon as compared to AS-1 which develops in the nucleoplasm of the host and therefore uses breakdown products of host DNA or exogenously supplied DNA precursors (65, 86). Since AS-1 is a particularly virulent phage, its biosynthetic pathways and packaging may overshadow those of the hypothetical lysogen, which would explain why so little of this DNA is packaged. That the PIL-DNA

which is synthesized early after the onset of infection by AS-1 is packaged, may represent the utilization of early AS-1 proteins to initiate the lysogens maturation cycle followed by packaging of the (defective) lysogen's DNA.

A final, and possibly less satisfying model that is proposed is that the PIL-DNA is not of cyanophage origin at all. In other systems (14), satellite DNA's containing relatively high amounts of A-T base pairs, have been found. In Agmenellum quadruplicatum, another blue-green alga, plasmid DNA's consisting of covalently closed circles having molecular weights ranging from 3×10^6 - $65\text{-}80 \times 10^6$ daltons have been found (74). The buoyant density in neutral CsCl of these DNA's has been determined to be 1.7026 g/cc with a sharp shoulder at 1.699 g/cc. The values fall quite near the range determined for the PIL-DNA presented in these studies. In addition, Agmenellum has been classified by Stanier (101) to be in the same genus as Anacystis -- placing them both in the genus Synechococcus. However, the total amount of this DNA (in Agmenellum) has been found to be about 5% of the total cellular DNA, while in these studies (Figure 10) the PIL-DNA comprises as much as 65% of the DNA present in AS-1 infected Anacystis nidulans.

It is possible that this information, in conjunction with other data presented in this thesis and elsewhere, may lend credence to the endosymbiont theory of evolution in which blue-green algae are considered to be the ancestors of chloroplasts(56). The following facts have been noted. It has been shown that newly synthesized DNA is associated with membranes. Both chloroplasts and the

photosynthetic lamellae of blue-green algae are highly membranous and in addition chloroplasts contain their own DNA. It has been shown in this thesis that PIL-DNA primarily incorporates DNA precursors that contain newly photoreduced carbon. The possibility arises that PIL-DNA is an evolutionary precursor of chloroplast DNA. If PIL-DNA really is a "pre-chloroplast" DNA found in Anacystis nidulans, located at or near the photosynthetic lamellae, the supply of nucleic acid precursors could be localized to that area. Since photosynthesis isn't compartmentalized into chloroplasts in blue-green algae, the control of the synthesis of this DNA could still presumably be from the main algal genome. After AS-1 infection, however, the host genome is degraded while photosynthesis continues. The PIL-DNA ("pre-chloroplast" DNA) may be protected from degradation by an association with the membranes of the photosynthetic site. The loss of regulatory control over this DNA could account for the increase of this DNA to as much as 65% of the total DNA in the infected cell as noted in this thesis (Figure 10). This compares ot the 5% of plasmid DNA found in Agmenellum (74). PIL-DNA can also be compared to the plasmid DNA found in Agmenellum in terms of its molecular weight. The plasmid DNA of Agmenellum was in a variety of sizes, the smallest being 3×10^6 daltons with the largest being between 65-80 $\times 10^6$ daltons. The value of 138 $\times 10^6$ daltons calculated for PIL-DNA (see Appendix C) was based on its being a linear molecule. Closed covalent circles, and especially supercoiled molecules sediment much more rapidly than open linear molecules. The true molecular weight of PIL-DNA could then be much

closer to the value derived for Agmenellum. This model could be tested for by means of DNA hybridization. By hybridizing PIL-DNA with DNA extracted from Anacystis nidulans and from purified AS-1 some evidence could be gained with regard to the cyanophycean or cyanophage origins of this DNA. An additional means of testing for the origin of this DNA would be to attempt to stimulate the generation of PIL-DNA in the absence of functional infective AS-1. Lysogens may be induced by means of such agents as UV light or reduced glutathione. Alternatively, if this DNA was solely a host phenomenon, infection using a ts mutant of AS-1 at permissive temperatures followed by incubation at non-permissive temperatures would inhibit AS- development but might permit the (potentially) induced stimulation of the PIL-DNA.

The studies presented in this thesis were designed to explore a variety of phage-algal interactions. In the course of the research as many new questions arose as were answered. It is hoped that the work presented here is sufficiently tantalizing to entice others into further exploration of the problems of molecular biology in the cyanophage AS-1/blue-green alga Anacystis nidulans infective system.

Appendix A

Thermal Denaturation of PIL-DNA

The thermal denaturation properties of PIL-DNA were examined in order to determine the degree of correspondence between the values obtained for the G-C content of this DNA by the formulas of Schildkraut et al. (ref. 83, determined from ultracentrifugation in CsCl) and Marmur and Doty (ref. 55, determined from the thermal denaturation properties of the macromolecule.). Calculating from the Schildkraut et al. (83) formula, PIL-DNA with a buoyant density of 1.701 ± 0.001 g/cc has a G-C content of $41.8 \pm 1\%$. Thermal denaturation of PIL-DNA in 0.1X SSC was performed as described in the legend of Figure 34. The Tm of PIL-DNA in 0.1X SSC was found to be $70.9^{\circ}C$ and the Tm in 1X SSC (Figure 35) was $85.8^{\circ}C$. The relationship derived by Mandel and Marmur (53) equating G-C content and Tm in 0.1X SSC is:

$$G\text{-}C = 2.44\ (Tm - 53.9) \qquad (3).$$

The G-C content based on the calculations from 0.1X SSC is 41.5%. Since PIL-DNA of buoyant density 1.701 ± 0.001 g/cc was used for the thermal denaturation in 0.1X SSC, the G-C estimate obtained from this method is essentially identical to that derived from CsCl equilibrium density centrifugation. The slope of the transition shown in Figures 34 and 35 indicates that this DNA is double stranded according to Sinsheimer (93,94) and would fit Bradley's prediction that viruses with contractile tails contain double stranded DNA (13). As seen in Figure 35, using 1X SSC as the buffer for thermal denaturation a Tm of

Figure 34

Thermal Denaturation of PIL-DNA in 0.1 X SSC Buffer

PIL-DNA was extracted by the modification of the Marmur method described in Materials and Methods and purified by preparative isopycnic ultracentrifugation in CsCl. 30 µg of the purified DNA (in 1 ml 0.1 X SSC) was placed in a stoppered quartz semi-micro cuvette. 30 µg of Anacystis nidulans DNA, prepared as above was placed in an identical cuvette. These two samples, along with a third cuvette containing only 0.1 X SSC were placed in a Gilford 2400 S recording spectrophotometer in which the chamber temperature was adjusted by 2°C a minute using a Haake circulating water bath. From the observed melting profile of the PIL DNA and the known Tm of Anacystis nidulans DNA the Tm of PIL DNA in 0.1X SSC was determined to be 70.9°C.

Figure 34

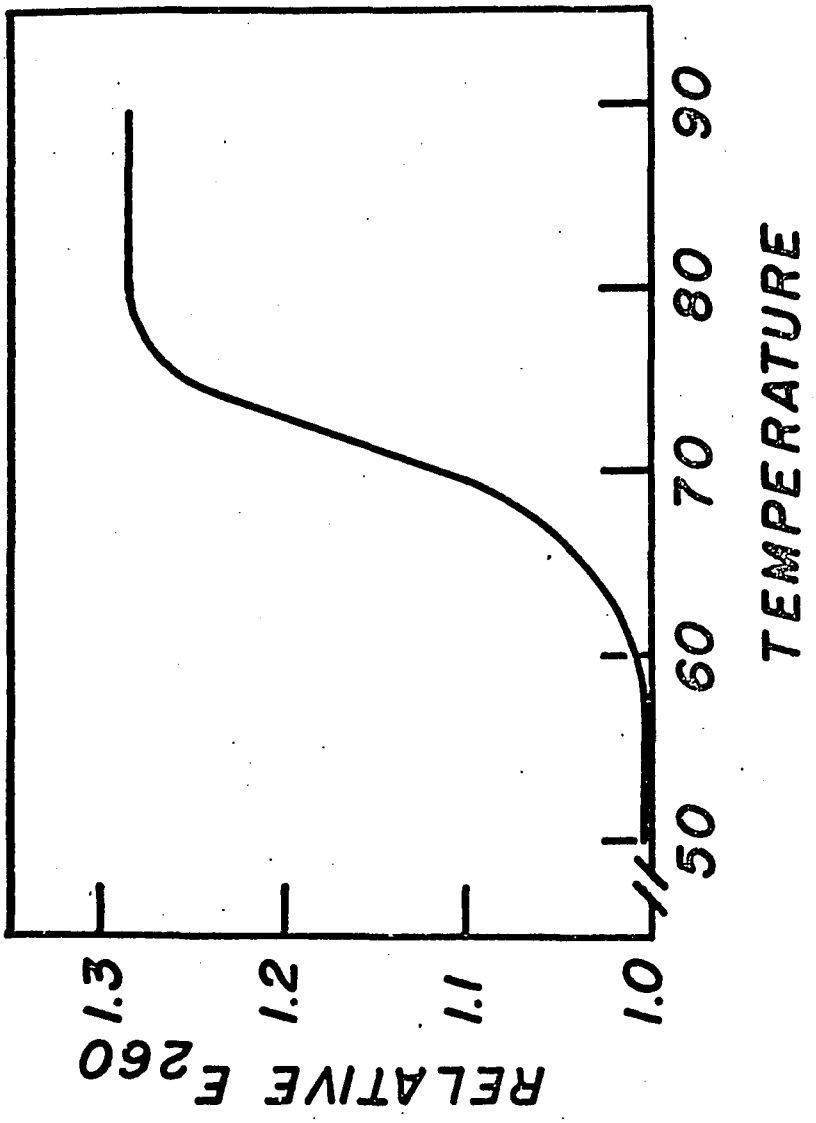
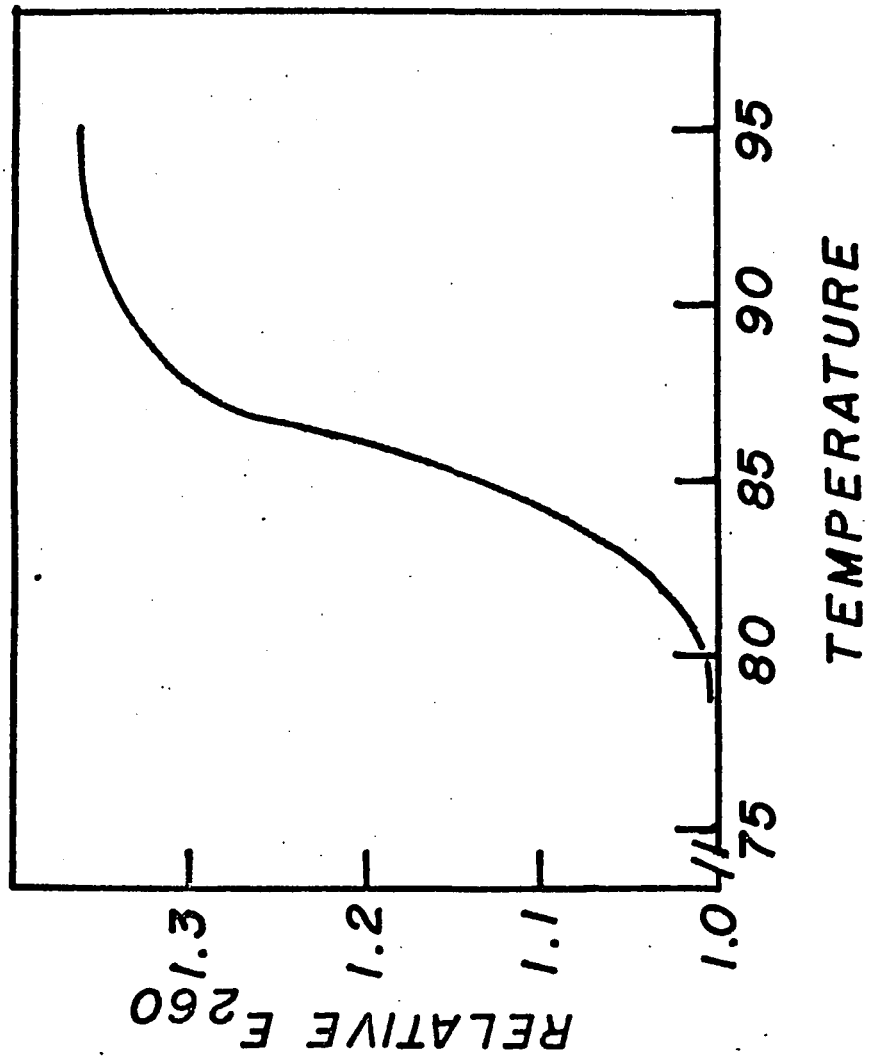


Figure 35

The experimental protocol was identical to that of figure 34 with the exception that 1X SSC was the buffer employed in the DNA containing and control samples. The Tm of PIL DNA in 1X SSC was determined to be 85.8^{o}C.

Figure 35



85.8°C was obtained. According to the equation of Marmur and Doty (55) for 1X SSC:

$$Tm = 69.3^{\circ} + 0.41\ (G+C) \qquad (4).$$

The G-C estimate using this equation is calculated to be 40.3%. This value, although slightly low, is in reasonable agreement with the values calculated from 0.1X SSC and the buoyant density determinations. Since modifications of DNA such as methylation and glucosylation are known to alter the buoyant density of DNA in CsCl without altering the thermal denaturation properties of the macromolecule (108), the correspondence of the G-C estimates between the values derived from the thermal denaturation and buoyant density determinations was indicative that there was little, if any modification present in PIL-DNA.

Appendix B

Thin Layer Chromatography and Autoradiography of Nuclease Treated PIL-DNA

The photosynthetic reduction of $NaH^{14}CO_3$ by Anacystis nidulans has been shown to result in the incorporation of ^{14}C metabolites into PIL DNA (Figures 15-22). In order to determine whether the ^{14}C radioactive label was uniformly distributed among the nucleotides an experiment was performed in which AS-1 infected A.nidulans was exposed to $NaH^{14}CO_3$ at 3 hours post infection and collected at 6 hours post infection (the period of maximal synthesis of PIL DNA). A lysate of this material was then subjected to CsCl equilibrium density centrifugation (data not shown). The ^{14}C containing peak was collected and after dialysis, subjected to RNase A, DNase I, and snake venom phosphodesterase treatment. An aliquot of the hydrolysate was applied to a thin layer chromatography plate along with 25 μg each of 5' dAMP, 5' dGMP, 5' dCMP and 5' dTMP. After the chromatography in the second dimension the thin layer plate was subjected to autoradiography. The results of this study are shown in Figure 36. The non-radioactive nucleotides were visualized by ultraviolet absorbance at 2554 A using a hand held ultraviolet lamp. The x-ray film showed two major regions of exposure, one corresponding to 5' dAMP. A third minor spot was also observed. It is possible that the major region of ^{14}C not associated with any of the added nucleotides may be that of UMP. Since no dialysis was performed following the nuclease treatment of the ^{14}C

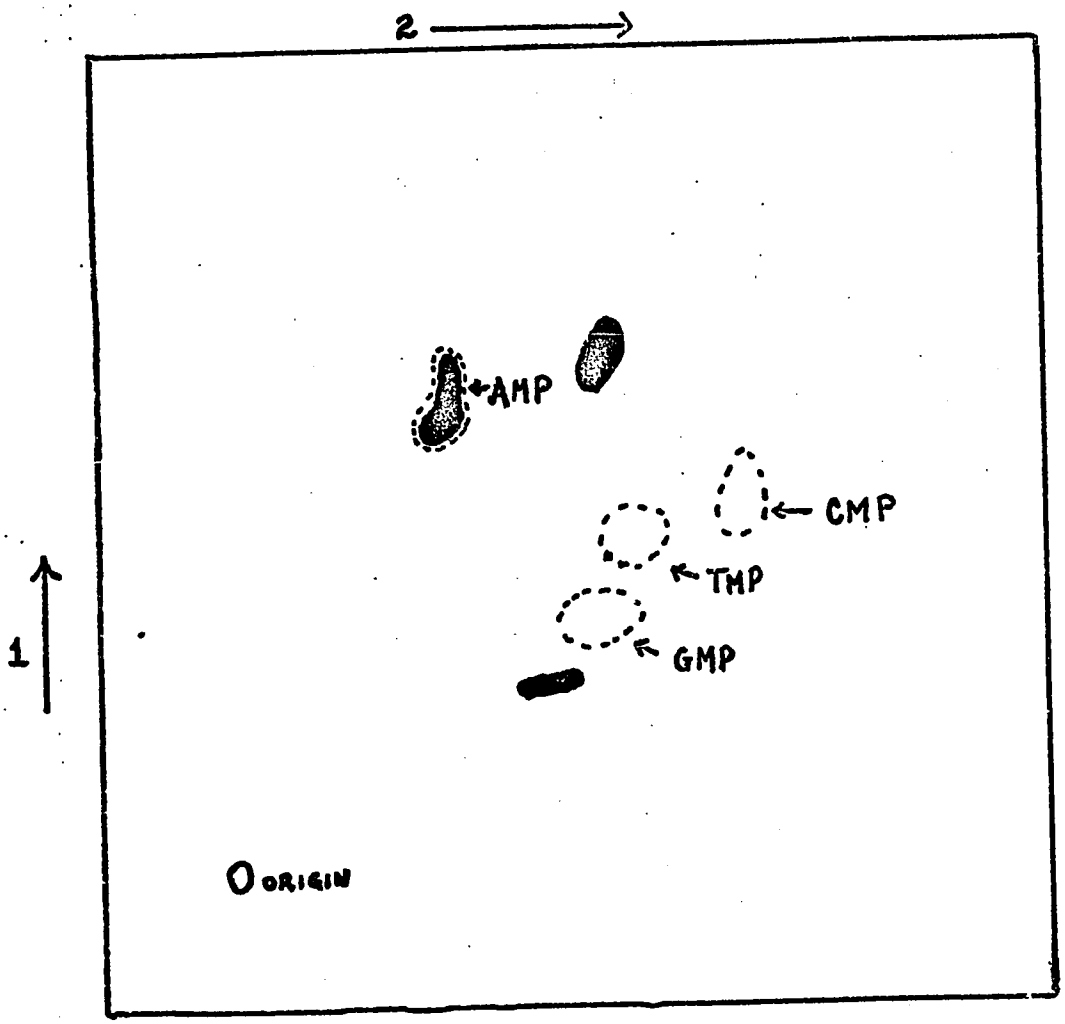
Figure 36

Thin Layer Chromatography and Autoradiography of Nuclease Digested ^{14}C Labeled PIL-DNA

$NaH^{14}CO_3$ (5 μCi/ml) was added at the time of infection to a 20 ml culture of Anacystis nidulans (7×10^7 cells/ml) infected with AS-1 at a MOI=5 growing at 30°C. Five hours post infection the cells were collected by low speed centrifugation (4000 x g, 20 min.) and resuspended in a Saline-EDTA buffer containing 3 mg/ml lysozyme, 5mM NaCN and 1mM NaN_3. The suspension was incubated for 12 hours at 37°C and lysis was completed by treatment with 3 drops of 30% Sarkosyl NL97 for 15 minutes at 60°C. The lysate was then added to 4.1 ml of a stock saturated CsCl solution. Preparative ultracentrifugation and analysis of the distribution of the radioactivity were as described in Materials and Methods. After the position of the radioactive peak was determined the fractions containing the radioactivity were collected and dialyzed against 3 changes of a solution containing 1mM NaCl and 5 mM $MgSO_4$. One ml of DNA was incubated with 50 μl DNase I (stock solution, DNase I, 1 mg/ml; bovine serum albumin 1 mg/ml; 1mM NaCl; 5 mM $MgSO_4$, pH 7.0) for 5 hours. The pH of the solution was adjusted to 8.5 with pH paper followed by incubation with 50 μl of a solution of snake venom phosphodiesterase (4 mg/ml, with bovine serum albumin 4 mg/ml, 1mM NaCl; pH 8.7) for 5 hours, all at 37°C. An Aliquot (25 μl) of the hydrolysate along with 25 μg each of 5' dAMP, 5' dGMP, 5' dCMP and 5' dTMP, was spotted on 20 cm x 20 cm thin layer chromatography plates (cellulose MN 300, 500u), and chromatographed in 2 dimensions, each to within 2.5 centimeters from the top of the plate. The solvent

for the first dimension contained isobutyric acid: H_2O: ammonia (66:20:1) while the solvent for the 2nd dimension was composed of saturated ammonium sulfate, 1M sodium acetate, and isopropanol (80:18:2). Kodak "no-screen" 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 at -70°C in a Revco freezer for 5-10 days. The film was developed for 5 min. in Kodak X-ray film developer, washed for 1 min with H_2O, treated for 20 min. with Kodak acid fixer and hardener and then washed for at least 30 min. with H_2O.

Figure 36



containing peak, any RNA present would have been hydrolyzed and the ribose nucleotides would have been chromatographed along with the deoxyribose nucleotides resulting from PIL-DNA hydrolysis. The significance of the localization of ^{14}C only in the region of the UV absorbing spot associated with 5' dAMP and the 3rd minor region is unclear.

Appendix C

Molecular Weight Determination of PIL-DNA

Further characterization of PIL DNA was accomplished by means of rate zonal sedimentation to determine its molecular weight. $NaH^{14}CO_3$ was added at the time of infection to an AS-1 infected Anacystis culture growing at 34°C. At this temperature lysis of over 80% of the infected cells has occurred by 7 hrs. (see Figure 9). At seven hours the culture was clarified by low speed centrifugation (4000 x g, 20 min.) and a 1 ml aliquot of the clarified supernate was treated with lysing buffer and sedimented in a 5-25% neutral sucrose gradient (Figure 37). ^3H labeled DNA of coliphage T_7 was included in the gradient by lysing this phage directly on the gradient. According to the equation of Freifelder (33):

$$D1/D2 = (M1/M2)^{0.38} \qquad (5).$$

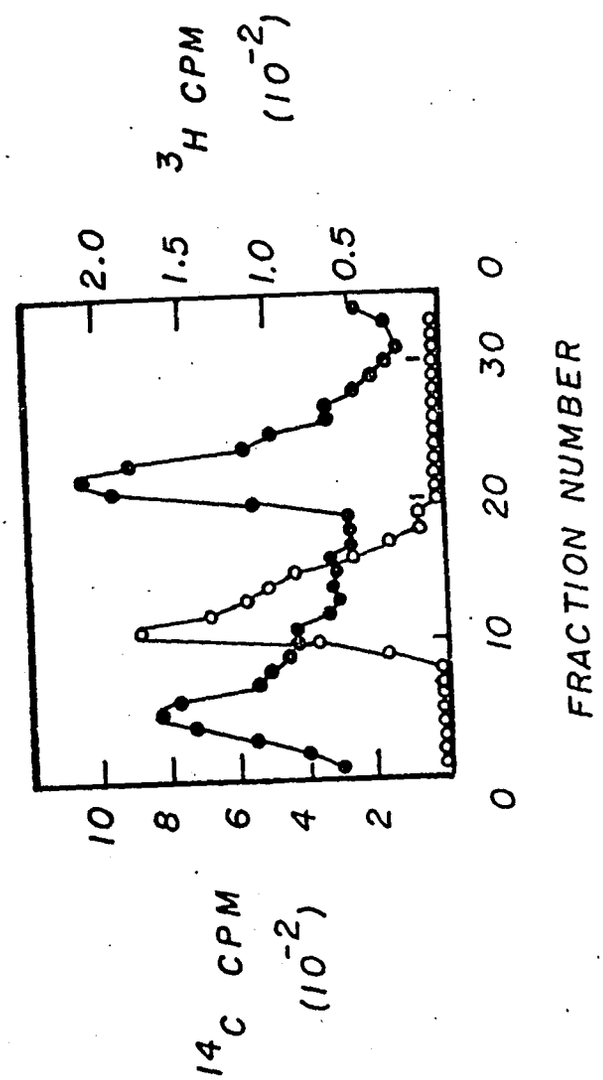
The calculations for this equation are shown in Appendix D. The molecular weight of the ^{14}C labeled PIL-DNA was computed (based on the average of three determinations) to be 13.8×10^7 daltons. The ^{14}C containing peak seen in Figure 37 which is lighter than T_7 DNA is most likely protein (which is also radioactively labeled in the regimen employed) with an alternate possibility being degradation products of PIL-DNA The average molecular weight of this smaller peak is 3.25×10^6 daltons.

Figure 37

Rate Zonal Sedimentation in Sucrose of PIL-DNA

$NaH^{14}CO_3$ (3 μCi/ml) was added to a culture of Anacystis nidulans (5 x 10^7 cells/ml) infected with AS-1 (MOI=4) growing at 34°C. Five hours post infection the cells were collected by low speed centrifugation (4000 x g, 20 min) and a 1.0 ml aliquot of the supernate was added to 1.0 ml of a detergent mixture (2% Sarkosyl, 3% sodium deoxycholate, 5% sodium dodecyl sulfate in 0.02M EDTA, 0.01M Tris pH 8.0, sterile filtered) and the mixture was gently layered onto a 36 ml 5-25% (w/v) sucrose gradient containing 0.1M NaCl and 0.02M EDTA, pH 7.0. Tritiated DNA of coliphage T_7 (MW=25.5 x 10^6 daltons) was included in the gradient as a reference marker. The samples were centrifuged using a SW 27.1 rotor for 16 hours at 14,000 rpm in a Beckman L3-50 ultracentrifuge. Fractions (1.0-1.1 ml) were collected from the top using a Buchler co. gradient device and a LKB multiperpex pump. The determination of the radioactivity in the fractions was performed as described in Materials and Methods. The calculations showing the determination of the molecular weight values are presented in Appendix D.

Figure 37


^{3}H CPM
(10^{-2})
2.0
1.5
1.0
0.5
0
30
20
10
0
FRACTION NUMBER
10
8
6
4
2
^{14}C CPM
(10^{-2})

Appendix D

Calculations

Formula 1 Reference No. 107

$$\rho = \rho_o + 4.2\omega^2(r^2 - r_o^2) \times 10^{-10} \text{ g/cc}$$

ρ = buoyant density of unknown DNA species

ρ_o = buoyant density of marker species of DNA

r = distance of unknown DNA from center of rotation

r_o = distance of marker DNA from center of rotation

ω = speed of rotation in radians/sec

To determine ω, multiply rpm of centrifuge run by $2\pi/60$, thus in preparative ultracentrifuge runs described in this thesis, W = 33,000 rpm x $2\pi/60$ = $66{,}000\pi/60$ = 1100π = 3456

$$\omega^2 = 1.194 \times 10^7$$

$$4.2\omega^2 = 5.016 \times 10^7$$

The determination of r and r_o is based on the distance from the center of rotation of the rotor. The angulation of tubes inserted in the Spinco 50 Ti rotor is 26^o while the radius to the midpoint of the bottom of the centrifuge tube from the center of rotation is 7.7 cm. The hypotenuse of this triangle can be trigonometrically derived from the formula sin x = opposite/hypotenuse. Sin 26^o = .4384 = 7.7/x = 17.564 cm.

The standard preparative CsCl gradient contained 4.1 ml CsCl solution and 1.0 ml of lysate. The total volume filled the centrifuge tube 3.5 cm from the bottom of the tube. To determine the position of a DNA species in a gradient the total number of fractions in the

gradient is divided into the total distance of the gradient. Thus using panel A of Figure 17 (Simultaneous incorporation of ^{3}H-adenine and $NaH^{14}CO_3$ into DNA within specific hourly intervals during the lytic cycle of AS-1 in A. nidulans, 4-5 hrs. post infection), there were 35 fractions in the gradient, dx = 0.1. The midpoint of the first peak (^{3}H containing) equilibrated at fraction 5 while that of the 2nd peak (^{14}C containing) equilibrated at fraction 10. Thus the first peak moved to a distance of 0.5 cm from the bottom of the centrifuge tube while the second peak moved to a distance of 1 cm from the bottom. To determine r (the radius of the unknown = ^{14}C containing peak) and r_o [the ^{3}H containing DNA being the marker DNA in this case since it was shown in Figures 13 and 14 that ^{3}H-adenine incorporates into AS-1 DNA which has a buoyant density of 1.714/5 g/cc (references 81 and 85)], the values of 1 and 0.5 cm respectively are subtracted from the standard hypotenuse of 17.564 cm to the center of the bottom of the centrifuge tube. The new values hyp = 16.564 and hyp = 17.064 are used in the equation sin 26^{o} = opposite (=radius)/hypotenuse. Thus,

$$r = .4384 = x/16.564 = 7.262$$

$$r^2 = 52.73$$

$$r_o = .4384 = x/17.064 = 7.481$$

$$ro^2 = 55.963$$

$$\rho = \rho_o + 4.2\omega^2 (r^2-ro^2) \times 10^{-10} \text{ g/cc}$$

$$\rho = 1.715 + 5.015 \times 10^{7} (52.73-55.96) \times 10^{-10} \text{ g/cc}$$

$$= 1.715 + 5.015 \times 10^{-3} (3.23)$$

$$= 1.715 + (-.016)$$

$$= 1.699$$

Formula 2 Reference No. 83

$\rho = 1.660 + 0.098$ G-C

$= 1.660 + 0.098\ (0.54)$

$= 1.660 + 0.0529$

$= 1.713$

Formula 3 Reference No. 53

G-C $= 2.44\ (Tm-53.9)$

$= 2.44\ (70.9-53.9)$

$= 2.44\ (17)$

$= 41.5$

Formula 4 Reference No. 55

$Tm = 69.3 + 0.41$ (G+C)

$85.8 = 69.3 + 0.41$ (G+C)

$16.5 = 0.41$ (G+C)

G+C $= 40.3$

Formula 5 Reference No. 33

$D1/D2 = (M1/M2)^{0.38}$ D1 = distance traveled by T_7 DNA

D2 = distance travelled by PIL-DNA

M1 = molecular weight of T_7 DNA

M2 = molecular weight of PIL-DNA

ln D1-ln D2 = .38 lnM1 - .38 ln M2

D1/D2 = .526 (average of 3 determinations)

ln .526 = -.642

M.W.T_7 DNA 25.5×10^6 Daltons

-.642 = .38 (17.054) - .38ln M2

M2 = 138,000,000 daltons

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