Bispecific, Cleavable, Protein DNA Crosslinker, Psoralen-Diol-Nitroveratrole. A Probe of Bacteriophage Structure.

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To My Parents

Abstract

The synthesis of a cleavable, photochemically activated, bispecific protein-DNA crosslinking reagent, psoralen-diol-nitroveratrole (**PDN**) is described. The reagent crosslinks the capsid proteins to the packaged DNA in bacteriophage T7. The SDS dissociated crosslinked phage appears under the electron microscope as a rosette with the phage head at the center. DNA from the crosslinked phage does not enter agarose gels in the absence of SDS. Treatment of the crosslinked phage with proteinase K or cleavage of the crosslink with sodium periodate restores the gel mobility of the DNA to that of the non-crosslinked phage DNA. No evidence for protein-protein crosslinking was obtained when the protein composition of crosslinked and non-crosslinked phage was compared by polyacrylamide gel electrophoresis. No evidence for DNA-DNA crosslinking was obtained when the frequency of crossed BglI restriction fragments for the crosslinked and non-crosslinked phage was compared by electron microscopy. An attempt was made to analyze the distribution of protein crosslinked to intraphage DNA. It was not possible to carry out this analysis by electron microscopy as the non-crosslinked phage gave too high a background of phage heads attached to the DNA. Sodium periodate treatment of the crosslinked DNA-protein complex failed to give detectable levels of protein on a silver stained polyacrylamide gel.

The phage head of bacteriophage λ was also crosslinked to the DNA by treatment with **PDN** and irradiation at long wavelength (> 360 nm). The crosslink was cleaved by sodium periodate. However, proteinase K

treatment did not remove the protein from the DNA. Evidence is presented indicating that the λ phage head is exceptionally stable to dissociation. Preliminary crosslinking results are presented for simian virus 40.

TABLE OF CONTENTS

Page

CHAPTER 1. INTRODUCTION

I. Protein-Nucleic Acid Crosslinking........................ 2

II. Bispecific Cleavable Protein-DNA Crosslinking Reagent... 7

III. Manipulation of Protein - DNA Complexes................. 11

IV. References.. 13

CHAPTER 2. METHODS

I. Synthesis of PDN... 19

a) 8-hydroxypsoralen (9)................................ 19

b) 8(6-bromo-1-hexoxy)psoralen (10)..................... 19

c) diethyl-1,6-hex-3-enedioate (2)...................... 20

d) 1,6-hex-3-enediol (3)................................ 20

e) 6(2-methoxy-4-nitrophenoxy)hex-3-ene-1-ol (9)....... 20

f) 1(2-methoxy-4-nitrophenoxy)6-phthalimidohex-
3-ene (5).. 21

g) 1(2-methoxy-4-nitrophenoxy)6-phthalimido-
3,4-hexanediol (6)................................... 21

h) 1(2-methoxy-4-nitrophenoxy)-6-amino-3,4-
hexanediol (7)....................................... 22

i) Psoralen-diol-nitroveratrole (11)................... 22

II. Biochemical Methods..................................... 27

a) Materials.. 27

b) Buffers.. 27

c) Crosslinking procedure............................... 27

d) Phenol extraction.................................... 28

e) Lyophilization.. 28

f) Sepharose chromatography............................ 28

g) Agarose gel electrophoresis......................... 29

h) Polyacrylamide gel electrophoresis.................. 31

i) Silver staining/polyacrylamide gels................. 33

j) Electron microscopy................................. 35

III. References... 39

CHAPTER 3. RESULTS

I. Synthesis of Psoralen-Diol-Nitroveratrole.............. 43

II. Bacteriophage T7 43

a) Background.. 43

b) Results... 47

1) Protein-DNA crosslinking/agarose gel............ 47

2) Protein-DNA crosslinking/electron microscopy.... 47

3) Reversal of protein-DNA crosslink............... 51

4) Assay for protein-protein crosslinking.......... 52

5) Assay for DNA-DNA crosslinking.................. 52

6) Crosslinked protein-DNA complex/gel mobility.... 55

7) Protein release and detection/silver stained polyacrylamide gel.............................. 55

c) Discussion.. 59

d) References.. 62

III. Bacteriophage 63

a) Background....................................... 63

b) Results... 65

1) Protein-DNA crosslinking/agarose gel........... 65

2) Protein-DNA crosslinking/electron microscopy .. 66

3) Reversal of protein-DNA crosslink.............. 66

4) Crosslinked protein-DNA complex/gel mobility... 69

5) Electron microscopy/phage...................... 71

6) Protein release and detection/silver stained polyacrylamide gel............................ 74

c) Discussion.. 76

d) References.. 78

IV. Simian Virus 40.................................... 80

a) Background 80

b) Results... 81

1) Protein-DNA crosslinking/agarose gel.......... 81

2) Protein release and detection/silver stained polyacrylamide gel............................ 82

c) Discussion 82

d) References.. 85

V. Summary... 87

CHAPTER 1

INTRODUCTION

I. PROTEIN-NUCLEIC ACID CROSSLINKING

Protein nucleic acid complexes play a fundamental role in living systems. The DNA or RNA of viruses is protected and delivered to the cell with a protein coat (1). The 0.34 meters of DNA in the nucleus of the human cell is packaged on a protein matrix (2,3). The E. coli ribosome is an RNA-protein complex comprising 3 pieces of RNA and 55 proteins (4). DNA replication and RNA synthesis are controlled by specific DNA protein interactions (5). Protein nucleic acid crosslinking reagents are powerful tools for determining the structure of these complexes. Some examples of systems that have been studied follow:

a) Lac Repressor-Lac Operator and RNA Polymerase-Promoter: When DNA containing the modified based, bromouridine is irradiated (= 254 nm) strand cleavage occurs at the bromouridine site. If a protein is nearby, crosslinking occurs. This technique has been used to map the protein-DNA contacts between lac repressor and lac operator (6) and between RNA polymerase and its promoter (7,8)

b) t RNA-Aminoacyl-tRNA Synthetase: Schimmel has crosslinked a number of tRNA's to their cognate and noncognate aminoacyl-tRNA synthetases by direct irradiation and identified the crosslinked sites on the tRNA (9).

c) Nucleosome: Treatment of DNA with dimethylsulfate depurinates DNA exposing an aldehyde functionality. Mirzabekov has mapped the DNA-protein contacts in the nucleosome by crosslinking partially depurinated nucleosomal DNA to the histone proteins (10). More recently, the nucleosome has been crosslinked with <u>trans</u>-$Cl_2(NH_3)_2Pt$ (11).

d) Bacteriophage: Bacteriophage λ has been crosslinked with formaldehyde (12) and with a water soluble carbodiimide (13). Phages Sd (14) and MS2 (15) have been crosslinked with bisulfite. This crosslink can be reversed with bisulfite and O-methylhydroxylamine

e) Ribosome: RNA has been crosslinked to protein in the ribosome by direct irradiation (16) and with a variety of crosslinking reagents. Proteins S7, L4 and L2 are crosslinked to RNA when the ribosome is exposed to low doses of UV radiation. Higher doses result in an intractable complex (17). The sites of crosslinking of S7 and L4 have been identified by using a series of proteolytic and RNAse digestions. Tyrosine 35 of L4 is crosslinked to uridine 615 of the 23S RNA (18) and methionine 114 of S7 is crosslinked to uridine 1239 of the 16S RNA (19-21). The number of proteins that can be photochemically crosslinked to the ribosomal RNA is limited. Therefore recent research has concentrated on the development of new protein-RNA crosslinking reagents(22-37). Table I shows the structure of the reagents that have been explored up to the present.

Reagents 3, 9, 11 and 18 have generated useful structural information. The aldehyde formed by periodate cleavage of the 2',3'-diol on the 16S RNA reacts with proteins S1 and S21 (31). Reagent 18 was used to locate the binding site of S1 between bases 861 and 889 on the 16S RNA (36). The binding sites for proteins L4, L6, L21, L23, L27, and L29 on the 23S RNA have been identified by using reagent 11 (33).

TABLE I. Protein-RNA Crosslinking Reagents

	REAGENT	SYSTEM CROSSLINKED	PROTEINS CROSSLINKED	REF.
1.	HCHO	Ribosome	Several	22
2.		Ribosome	S3, S4, S5, S9/S11, S13, L1, L2	23
3.		Ribosome	Several	24,25
4.	R—N=C=N—R	30S subunit	S4, S5, S7, S13, S18	26
5.		30S subunit	S4, S7, S9	27
6.		30S subunit 50S subunit	S3, S4 L2	28
7.		Ribosome		29
8.		30S subunit		30

TABLE I.

9.	30S subunit	S1, S21	31
10.	L24 & 23S RNA	S1, S21	32
11.	50S subunit	Several	33
12.	30S subunit	S2, S3, S4, S5, S7, S12	34
13.	Ribosome		35
14.	Ribosome subunits	Several	35

TABLE I

15.	Ribosome		35
16.	Ribosome		35
17.	Ribosome		35
18.	30S subunit	S1	36
19.	23S RNA + Ribosomal Proteins		37

II. BISPECIFIC CLEAVABLE PROTEIN-DNA CROSSLINKING REAGENTS

We set out to design a protein-DNA crosslinking reagent to probe bacteriophage structure. The protocol we decided to develop is shown in Figure 1. This approach requires that the reagent should be cleavable and bispecific, i.e. protein specific on one end and DNA specific on the other to avoid problems associated with protein-protein and DNA-DNA crosslinking. Sodium bisulfite catalyzed protein-DNA crosslinking fulfills these requirements. Unfortunately this reagent failed to introduce crosslinks into phage λ (62). None of the other crosslinking reagents listed in Table I fulfill these criteria. Their most serious limitation is that they all function as efficient protein-protein crosslinkers. This problem is minimized in ribosome studies by using very low levels of crosslinking and by detecting crosslinked protein-RNA complexes with P^{32} labeled RNA. This problem is more serious in the bacteriophage system where the double stranded DNA is considerably less reactive than the relatively accessible single stranded RNA of the ribosome. In our approach we want to generate a sufficiently high level of crosslinking to be able to analyze the crosslinked protein on silver stained polyacrylamide gels without forming crosslinked protein-protein-DNA or protein-DNA-DNA complexes. With this technique, we could detect 10% crosslinking of proteins P13, and P14, 1% crosslinking of proteins P16, P15, P12, P17, P8, P9 and Q and 0.1% crosslinking of protein P10 (the major capsid protein) in a 20 μg sample of bacteriophage T7, assuming 100% reversibilty of the crosslink.

Suitable chromophores for specific covalent attachment to DNA,

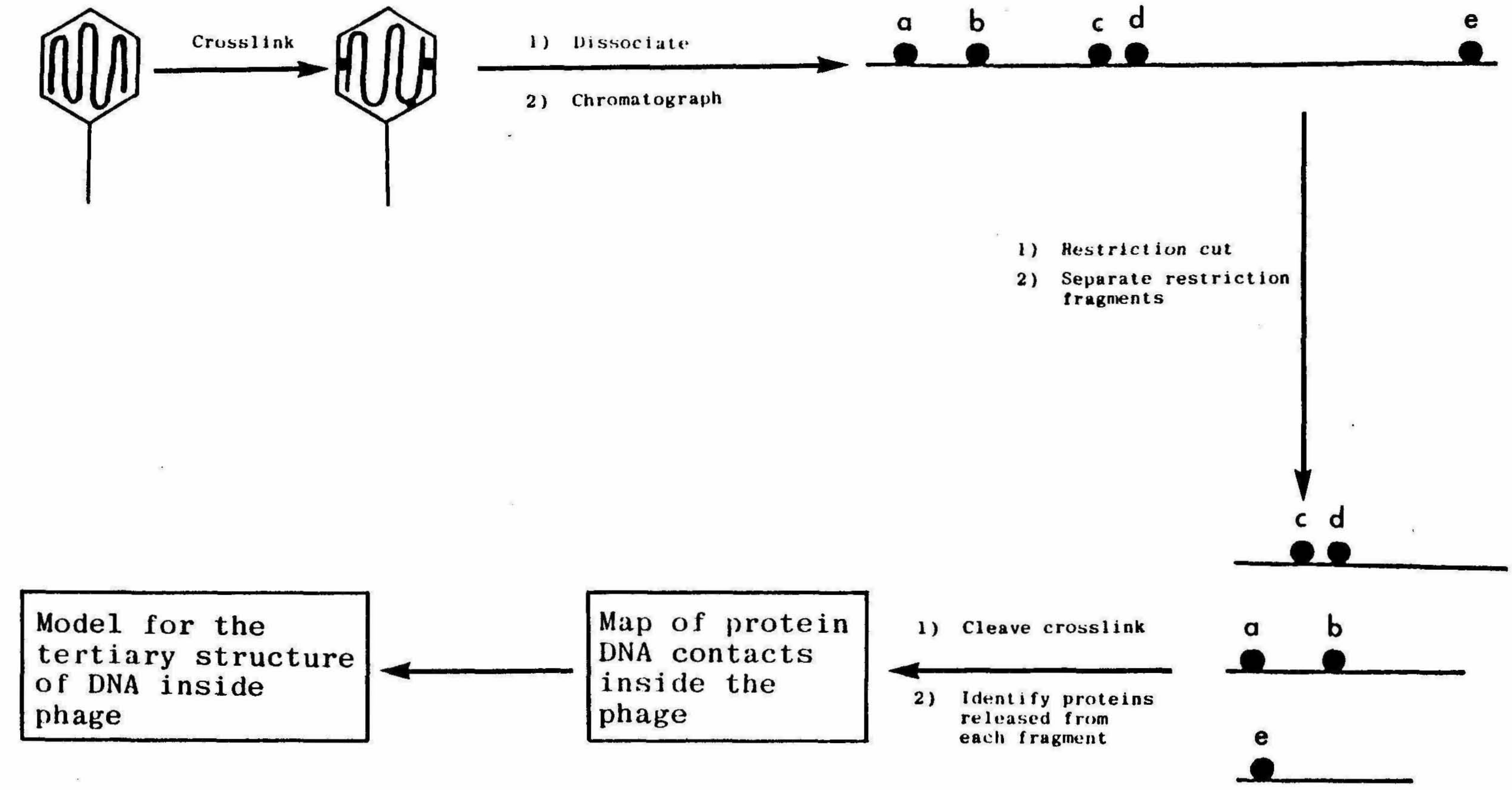

Figure 1. Protocol for probing bacteriophage structure by protein-DNA crosslinking.

cleavable tethers, and functionality suitable for covalent attachment to proteins are shown in Table II. The reagent that we synthesized is shown in Figure 2.

PDN

Figure 2 Photochemically activated, bispecific, cleavable protein-DNA crosslinking reagent.

Psoralen derivatives react photochemically with double stranded nucleic acids. The pyran or the furan double bond adds to the 5,6 double bond of thymine or uracyl (Figure 3). The major adducts between 4,5',8-trimethylpsoralen and DNA has been isolated and charcterized.(47,48) This reaction is highly specific for double stranded nucleic acids(49) and it has been extensively used to probe their structure. (38,50) Psoralen derivatives have been used to inactivate bacteriophages λ and T7. (51)

Table II. Suitable functional groups for a cleavable, bispecific protein-DNA crosslinking reagent.

DNA specific groups	Cleavable center	Protein specific groups
O, O, O (38,50)	S–S (41)	$\overset{+}{N}H_2\overset{-}{Cl}$, OCH_3 (45)
N_3, NH_2, $\overset{+}{N}$ (39)	N=N (42)	O, O–N, C, O, O (45)
N, N_3, H–N–R (40)	HO, OH (43)	NO_2, O, O (46)
	O, S (44)	

Figure 3 Major addition product between 4,5',8-trimethyl psoralen and DNA (48).

1,2-dimethoxy-4-nitrobenzene was chosen as the protein specific reagent because it is chemically inert yet undergoes nucleophillic substitution of the 2-methoxy group upon direct irradiation (360 nm). (63) A malimide-4-nitroveratole reagent was previously used for protein-protein crosslinking. (46)

Both chromophores are activated by irradiation at long wavelengths (>360 nm). Photochemical damage to the phage would be minimized under these conditions. The 1,2-diol would be readily cleaved by periodate treatment. (43) In addition, the amine and the 1,2-diol contribute to the water solubility of the reagent.

III. MANIPULATION OF PROTEIN-DNA COMPLEXES

One of the critical practical issues we will face in this project is the manipulation of crosslinked protein-DNA complexes. The manipulation of nucleic acid and proteins is well worked out methodology. What is less well understood is the behavior of protein-DNA complexes.

DNA-protein complexes can be isolated from an SDS sucrose gradient (52), from a guanidine hydrochloride caesium chloride gradient (53), or by sepharose chromatography (54).

Proteins bound to DNA can be seen under the electron microscope if they are amplified by binding to avidin spheres (55) or to antibodies (56). DNA- protein complexes show a range of behaviors on gels. Small pieces of single stranded DNA (≈ 200 bases) crosslinked to histone proteins will enter an SDS urea polyacrylamide gel (57). SV40 DNA (5200 base pairs) with the major capsid protein (MW = 43,000) bound to it will enter agarose gels (58). However adenovirus DNA (35,000 base pairs) with two terminal proteins (MW = 55,000) covalently bound will enter agarose gels only in the presence of SDS (59). DNA-protein complexes stick to nitrocellulose filters (60). DNA-protein complexes can be extracted into phenol (59,61). Adenovirus DNA with two terminal proteins covalently linked is extracted into phenol (64).

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CHAPTER 2

METHODS

I. SYNTHESIS OF PDN

a) 8-Hydroxypsoralen (9): To an ice cooled solution of 10 g (0.046 moles) of 8-methoxypsoralen in 200 ml of dichloromethane was slowly added 10 g (0.11 moles) of boron tribromide in 50 ml of dichloromethane. A yellow precipitate formed. The reaction mixture was stirred at room temperature for 1 hour. Water (500 ml) was then added. A vigorous reaction occurred resulting in a white precipitate which was filtered, dried (P_2O_5) and recrystallized (CH_3CN) to yield 8.35 g (0.041 moles, 88%) of **9** as a light yellow crystalline solid: mp 184-185^oC, NMR (CD_3CN) 6.3 (d, 1, J=9 Hz, C3-H), 6.9 (d, 1, J=3 Hz, C4'-H), 7.4 (s, 1, C5-H), 7.8 (d, 1, J=3 Hz, C5'-H), 7.9 (d, 1, J=9 Hz, C4-H); UV max (CH_3OH) 306, 268, 260, 249, 242, 218 mu; lR (nujol) 1715 cm^{-1} (C=0). Anal. Calcd. for $C_{11}H_6O_4$: C, 65.35; H, 2.97. Found C, 65.10; H, 3.10.

b) 8(6-Bromo-1-hexoxy)psoralen (10): To 100 ml of absolute ethanol was added 2 g (0.01 moles) of **9**, 7.7 ml (0.05 moles) of 1,6-dibromohexane, and 1.12 g (0.01 moles) of potassium t-butoxide. This was heated at reflux for 1 week. The solvent and the unreacted 1,6-dibromohexane were removed and the product isolated by chromatography (Silica, CH_2Cl_2 1% CH_3CH) to yield 1.15 g (0.0032, moles 32%) of **11** as a light yellow oil: NMR ($CDCl_3$) 1.7 (m, 8, $-CH_2$), 3.4 (t, 2, J=6Hz, $-CH_2Br$), 4.5 (t, 2, J=6Hz, $-CH_2O-$), 6.35 (d, 1, J=9Hz, C3-H), 6.8 (d, 1, J=2Hz, C4'-H), 7.35 (s, 1, C5-H), 7.75 (m, 2, C5'H, C4-H); UV max (CH_3OH) 298, 248, 218 ; IR (thin film) 1740 cm^{-1}(C=0). Anal. Calcd. for $C_{11}H_{17}O_4Br$: C, 55.91; H, 4.66. Found: C, 56.19; H, 4.82.

c) Diethyl-1,6-hex-3-enedioate (2): To 30 ml of toluene was added 25g (0.17 moles) of trans- -dihydromuconic acid, 62 ml (1.04 moles) absolute ethanol and 0.14 ml concentrated H_2SO_4. This was heated at 115°. The acid dissolved and an ethanol water toluene azeotrope distilled over (74-76°C). The product was isolated by distillation to give 29.2 g (0.146 moles, 86%) of **2** as a clear liquid: bp 85-92° (0.5 mm) NMR ($CDCl_3$) 1.25 (t, 6, J=8Hz, $-CH_3$), 3.05 (m, 4, $\underline{CH_2}$-C=), 4.15 (Q, 4, J=8Hz, $-CH_2O-$) 5.66 (m, 2, =CH); IR (CH_2Cl_2) 1725 cm^{-1} (C=0).

d) 1,6-Hex-3-enediol (3) (Ref. 1): To a suspension of 3.79 g (0.1 moles) of lithium aluminum hydride in 100 ml of dry diethyl ether was added 10 g (0.05 moles) of diester **2** in 100 ml of diethyl ether at 0°C. The reaction was stirred for 1 hour. To this was added 3.8 ml water, 3.8 ml 15% NaOH and 11.4 ml water dropwise, sequentially. The reaction mixture was stirred for 15 minutes, filtered, and the precipitate was washed with diethyl ether. The washings were combined with the filtrate, dried (Na_2SO_4) and the ether was removed. The product was purified by flash distillation to give 4.8 g (0.042 moles, 84%) of **3** as a clear oil: bp 100-106° (0.6 mm); NMR ($CDCl_3$) 2.2 (Q, 4, J=6Hz, $-\underline{CH_2}$C=), 3.5 (Q, 4, J=6Hz, $-\underline{CH_2}$0), 4.15 (t, 2, J=6Hz, -O-H), 5.4 (m, 2, HC=).

e) 6(2-Methoxy-4-nitrophenoxy)hex-3-ene-1-ol (4): To 100 ml of DMF was added 64.26 g (0.35 moles) 4-nitroveratrole, 40 g (0.35 moles) of **3** and 18.9 g (0.35 moles) sodium methoxide. This was stirred at room temperature for 3 days. The reaction was poured into water (500 ml) and extracted with dichloromethane. The extracts were washed with 0.1 M NaOH three times, and with water three times, dried ($MgSO_4$) and decolorized (charcoal). The product was purified by chromatography on

silica (CH_2Cl_2 then CH_2Cl_2/CH_3OH 1:1) to give 12 g (0.045 moles, 12.8%) of **4** as a yellow crystalline solid: mp 89-91°; NMR ($CDCl_3$)δ 1.7 (s, 1, O-H), 2.3 (Q, 2, J=6Hz, -$\underline{C}H_2CH_2OH$), 2.55 (Q, 2, J=6Hz, -$\underline{C}H_2CH_2OPh$), 3.6 (t, 2, J=6Hz, -$\underline{C}H_2OH$); 3.85 (s, 3, -OCH_3), 4.05 (t, 2, J=6Hz, -CH_2OPh), 5.5 (m, 2, HC=), 6.8 (d, 1, J=8Hz, C6-H), 7.65 (d, 1, J=3Hz, C3-H), 7.8 (dd, 1, J=3 and 8Hz, C5-H); UV max (CH_3OH), 335, 300 (sh), 240, 210 mμ; Anal. Calcd. for $C_{13}H_{17}NO_5$: C, 58.43; H, 6.37; N, 5.24. Found: C, 58.52; H, 6.45; N, 5.19.

f) 1(2-Methoxy-4-nitrophenoxy)6-phthalimidohex-3-ene (5) (Ref. 2): To a solution of 11 g (0.041 moles) of alcohol **4**, 11.87 g (0.045 moles) of triphenylphosphine and 6.66 g (0.045 moles) of phthalimide in 150 ml dry THF was added 7.2 ml (0.045 moles) of ethylazodicarboxylate. The reaction was stirred overnight, the solvent was removed and the product was purified by chromatography on silica (CH_2Cl_2/hexane 1:1, then CH_2Cl_2) to give 16.1 g (0.04 moles 99%) of **5** as a light yellow crystalline solid: mp 75-77°; NMR ($CDCl_3$) δ 2.4 (m, 4, $\underline{CH_2}C$=), 3.7 (t, 2, J=7Hz, -CH_2N), 3.9 (s, 3, OCH_3) 3.95 (t, 2, J=7Hz, OCH_2-), 5.5 (m, 2, HC=), 6.75 (d, 1, J=9Hz, C6-H), 7.7 (m, 6, arom); UV max (CH_3OH) 338, 300, 218 mμ; IR (nujol) 1720 cm^{-1}(C=0). Anal. Calcd. for $C_{21}H_{20}N_6$: C, 63.64; H, 5.05; N, 7.07. Found: C, 63.76; H, 5.04; N, 7.02.

g) 1(2-Methoxy-4-nitrophenoxy)6-phthalimido-3,4-hexanediol (6) (Ref. 2): To a solution of 16.93 g (0.043 moles) of alkene 5 in 150 ml of dichloromethane was added 8.7 g (0.064 moles) of N-methylmorpholine-N-oxide in 40 ml of acetone water (1:1). This was stirred overnight. The product precipitate was filtered and recrystallized (CH_3OH) to give 14.72 g (0.034 moles 80%) of very light

yellow solid: mp 175-177°; UV max (CH_3OH), 335, 295, 220, mμ; IR (nujol), 1730 cm^{-1} (C=O). Anal. Calcd. for $C_{21}H_{22}N_2O_8$: C, 58.60; H, 5.12; N, 6.51. Found: C, 58.60; H, 5.24; N, 6.44.

h) 1(2-Methoxy-4-nitrophenoxy)-6-amino-3,4-hexanediol (7): To 500 ml of 95% ethanol was added 1.78 ml (0.039 moles) of hydrazine hydrate and 13.92 g (0.032 moles) of phthalimide **6**. This was heated at reflux overnight. The product was filtered and washed with ethanol to give 14.08 g (0.031 moles, 94%) of yellow solid. The ammonium salt (4.62 g 0.01 moles) was suspended in 50 ml 0.5 M NaOH and immediately extracted with dichloromethane. The extracts were washed with water, dried (Na_2SO_4) and the solvent removed to give 2.77 g (0.009 moles, 92%) of **7** as a yellow solid: mp 178-180° (as the hydrochloride); NMR (DMSO), δ 1.5 (m, 2, -$\underline{CH_2}CH_2NH_2$), 1.8 (m, 2, -$\underline{CH_2}CH_2OPh$), 2.65 (t, 2, J=6Hz, -$\underline{CH_2}$-NH_2), 3.4 (m, 8, O-H, N-H, $\underline{CH}OH$), 3.85 (s, 3, OCH_3), 4.2 (t, 2, J=6Hz, $\underline{CH_2}OPh$), 7.2 (d,1, J=9Hz, C6-H), 7.75 (d, 1, J-3Hz, C3-H), 7.9 (dd, 1, J=9Hz, J=3Hz, C5-H); UV max (CH_3OH) 330, 300 (sh), 235, 210 mμ. Anal. Calcd. for $C_{13}H_{20}N_2O_6$: C, 52.0; H, 6.67; N, 9.33. Found: C, 51.73; H, 6.70; N, 9.15.

i) Psoralen-diol-nitroveratrole (12): To 5 ml of dichloromethane was added 0.1 g ($3.3x10^{-4}$moles) of amine **7** and 0.26 ml ($1.3x10^{-3}$moles) of N-trimethylsilyldiethylamine. This was stirred for 3 hours. The solvent was removed and the reaction mixture was held under vacuum for 30 min. (0.5 mm, 50°). To this was added 25 ml of dry acetonitrile and 0.12 g ($3.3x10^{-4}$moles) of bromide **10**. The reaction mixture was heated under nitrogen at 80° for 36 hours, cooled, and 0.1 ml of conc. HCl was added. The mixture was stirred for 1 hour. The solvent was removed and the product was isolated from the resulting brown oil

by chromatography on silica (THF satd with aq. NH_3/CH_3OH 9:1) to give 0.044 g ($7.5x10^{-5}$ moles, 23%) of **12** as a yellow solid: mp 112-115°; NMR (DMSO) δ 1.92-1.36 (m, 12, aliphatic C-H), 2.63 (t, 2, J=5.8Hz, $N\underline{CH_2}CH_2CHOH$-), 3.3 (s, 6, NH, OH), 3.51 (t, 2, J=3.2Hz, $\underline{CH}OH$), 3.87 (s, 3, $-OCH_3$), 4.21 (t, 2, J=5.5Hz, $-CH_2OPh$), 4.39 (t, 2, J=6.5Hz, $-CH_2OPsor$), 6.43 (d, 1, J=9.6Hz, C4-H Psor), 7.09 (d, 1, J=2.2Hz C4'-H Psor), 7.17 (d, 1, J-9Hz, C6-H nitroveratrole), 7.68 (s, 1, C5-H Psor), 7.72 (d, 1, J=2.6Hz, C3-H nitroveratrole), 7.88 (dd, 1, J=9.0, 2.7Hz, C5-H nitroveratrole), 8.1 (d, 1, J=2.2Hz, C5'-H Psor), 8.14 (d, 1, J-9.6Hz, C4-H Psor). UV max (CH_3OH), 350 (sh), 300, 242, 210 mμ. $\epsilon^{300} = 1.49x10^4$L $mole^{-1}$ cm^{-1}. IR (thin film), 1725 cm^{-1} (C=0); Anal. Calcd. for $C_{30}H_{36}N_2O_{10}$: C, 61.64; H, 6.16; N, 4.79. Found: C, 61.51; H, 6.17; N, 4.74. The solubility in water is $2.27x10^{-5}$moles L^{-1}.

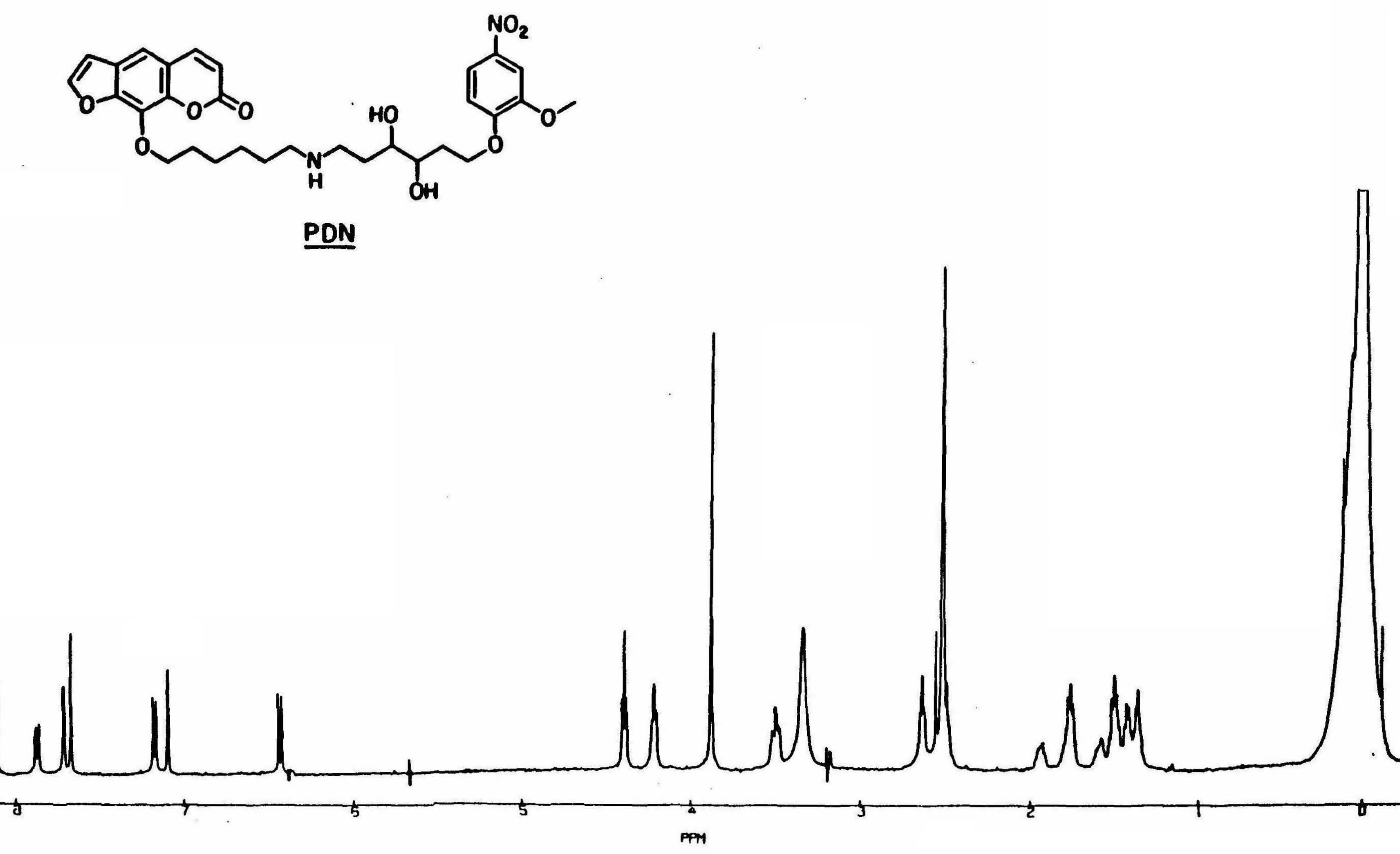

Figure 1. NMR (500 MHz) of PDN.

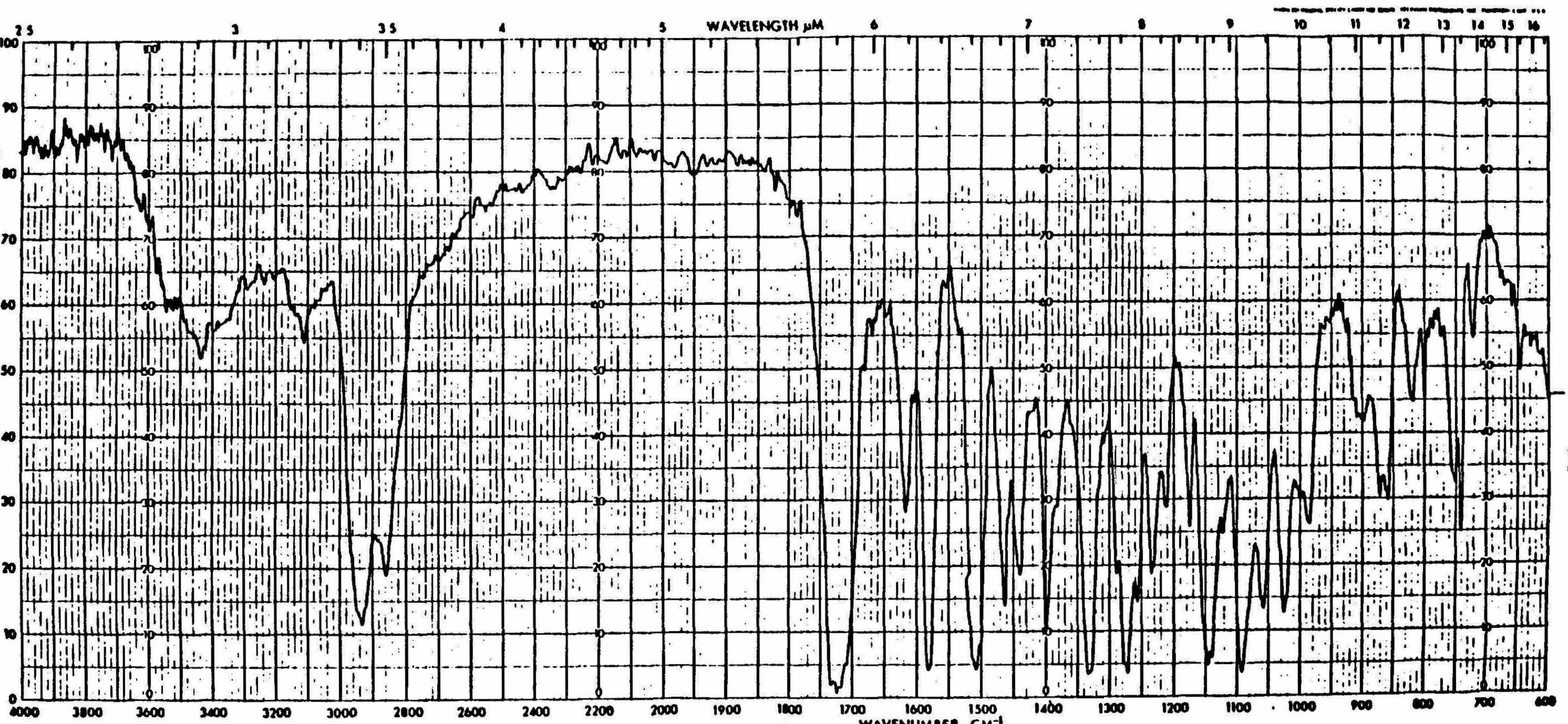

Figure 2. Infrared spectrum of PDN.

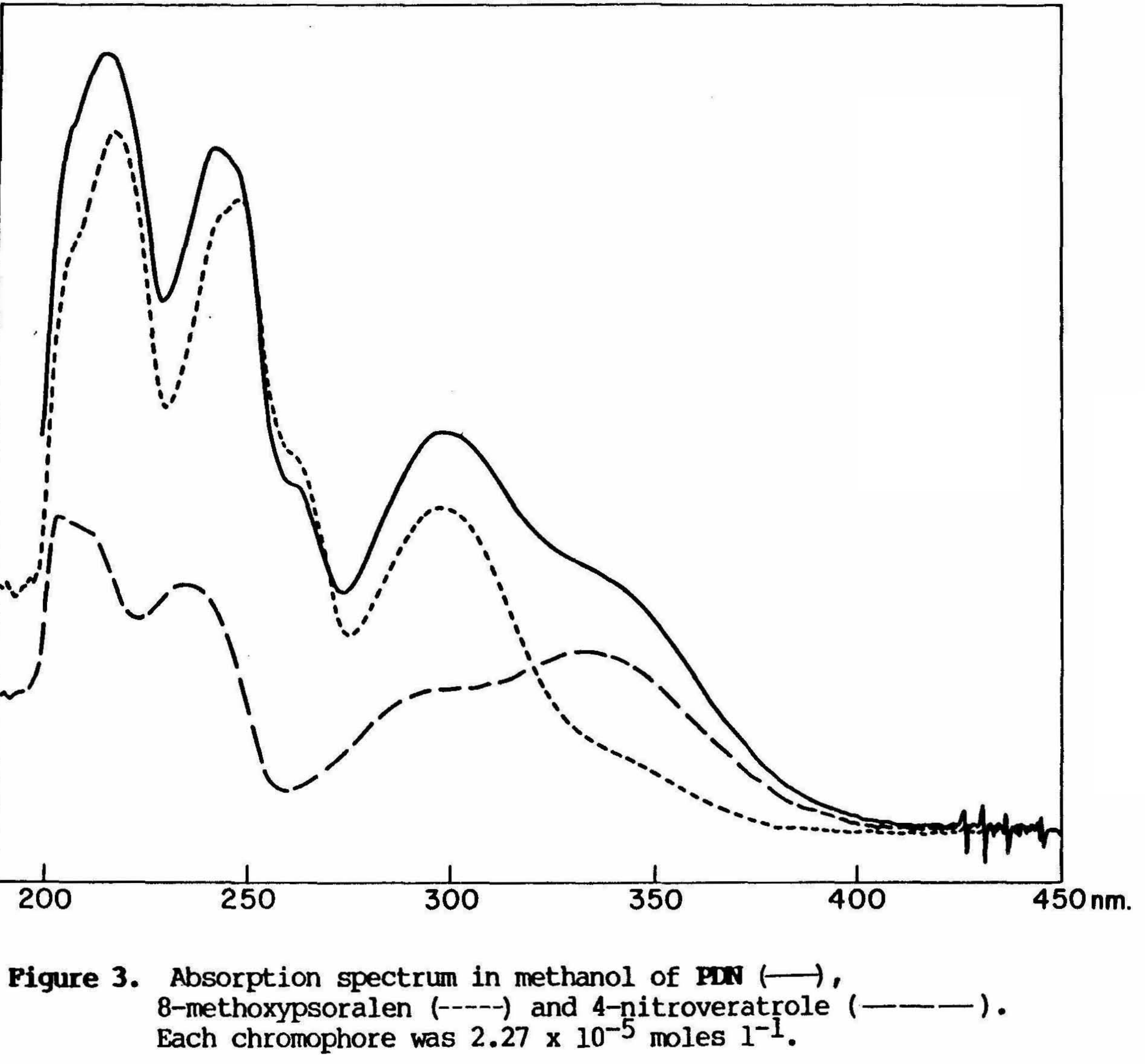

Figure 3. Absorption spectrum in methanol of **PDN** (——), 8-methoxypsoralen (-----) and 4-nitroveratrole (— — —). Each chromophore was 2.27 x 10^{-5} moles l^{-1}.

III. BIOCHEMICAL METHODS:

a) Materials: Sepharose was obtained from Pharmacia, agarose was from Sigma, acrylamide, bisacrylamide and TEMED were from Bio-Rad, grids and palladium platinum wire were from Pella, and restriction enzymes were from New England Biolabs. Bacteriophage λ and SV40 virus were obtained as a special order from Bethesda Research Laboratories. Bacteriophage T7 was a gift from Dr. Michael Reuben.

b) Buffers:

TAE: 0.04 M Tris base, 0.005 M sodium acetate, 0.001 M EDTA, pH 7.9 (acetic acid).

TAEN: 0.04 M Tris base, 0.005 M sodium acetate, 0.001 M EDTA, 0.1 M sodium chloride, pH 7.9 (acetic acid).

TAENE: 0.04 M Tris base, 0.005 M sodium acetate, 0.001 M EDTA, 0.1 M sodium chloride, 0.2 mg/L ethidium bromide, pH 7.9 (acetic acid).

PM: 0.1 M disodium hydrogen phosphate, 0.01 M magnesium chloride pH 6.0 (hydrochloric acid).

BglI: 0.066 M sodium chloride, 0.01 M magnesium chloride, 0.01 M Tris pH 7.4, 0.001 M 2-mercaptoethanol.

c) Crosslinking Procedure: 50 μl of the T7 phage sample (0.4 mg/ml) in PM buffer is treated with 5 μl of a saturated solution of **PDN** in CH_3OH:DMSO (10:1), placed in a Pyrex tube (2 mm internal diameter) and irradiated with a black lamp for 15 minutes (4) (See Figure 4). During the irradiation the sample is cooled with the fan from an electrophoresis apparatus.

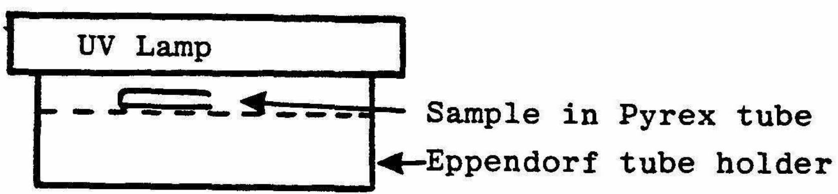

Figure 4. Apparatus used for crosslinking the phage.

d) Phenol Extraction: The phage was dissociated by heating with 1% SDS at 70° for 2 minutes. A saturated solution of phenol in TAE buffer was added (volume of phenol was one half sample volume) and the solution was stirred with a pipet-man tip until an emulsion formed. The sample was centrifuged on an Eppendorf centrifuge for 1 minute and the aqueous phase was removed, re-extracted with phenol and washed with ether (2x1 ml).

e) Lyophilization: The sample was made 1% in SDS and frozen in dry ice. Three holes were made in the bullet vial cap with a large syringe needle and the sample was placed in an erlenmeyer flask and maintained under vacuum (0.3 mm Hg) until dry.

f) Sepharose Chromatography: An aqueous suspension of Pharmacia sepharose 2BCL was degassed by maintaining under vacuum for 15 minutes. The sepharose suspension was added to a Bio-Rad column (0.8x10 cm) which was already two/thirds filled with water. This was allowed to settle up to 2 cm from the top of the column. Any excess

was removed. Water (10 ml) and then TAENE buffer (10 ml) were passed through the column before using to ensure that the column is uniformly packed and equilibrated with eluting buffer. The quality of the column was determined by chromatographing a solution containing 3 1 formamide 0.01% bromophenolblue in 50 1 TAENE buffer. For a good column the blue solution should enter the sepharose as a compact band and pass through the column without extensive broadening or streaking. The column was reused after washing with 5 ml of TAENE buffer.

The sample (containing up to 25 g of DNA and protein) to be deproteinized in 60 1 of TAENE buffer was carefully added to the sepharose column. It is important not to stir up the sepharose or the DNA and protein bands will be irregular and overlap. The DNA was eluted with TAENE buffer. The fractions showing enhanced ethidium fluorescence were collected and combined. For a crosslinked sample the unreacted reagent and protein with psoralen attached to it showed blue-green fluroescence. The chromatography can be readily followed by irradiating the column with a hand held long wavelength UV lamp (4).

g) Agarose Gel Electrophoresis:

Vertical agarose gels: Vertical agarose gels were prepared and run using the apparatus and procedure described by Studier (5). The gels were 0.7% agarose, 4 mm thick and each had 16 wells (5x11x2 mm^3). Electrophoresis was carried out in TAE buffer.

Horizontal agarose mini gels: All of the exploratory agarose gel electrophoresis was carried out on horizontal mini gels. These gels have the advantages of being easy to prepare in bulk quantities, of being stable to storage and of giving rapid separations of DNA. The

progress of the electrophoresis can be readily followed if the gel is developed in an ethidium containing buffer. The apparatus consists of a plexiglass box (16x6.2x2 cm^3). Platinum electrodes are cemented at each end and a plexiglass block (7x5.7x0.7 cm^3) is cemented in the center (Figure 5).

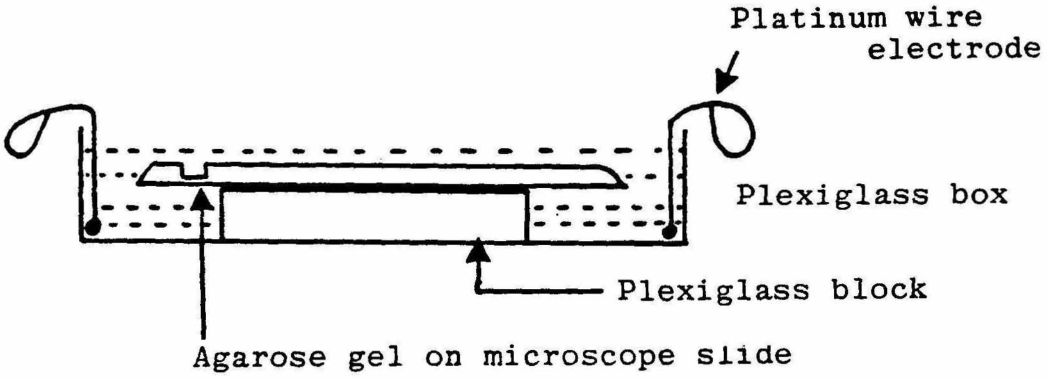

Figure 5. Apparatus for horizontal agarose mini gel.

<u>Preparation of gels</u>: A 16 tooth gel comb is supported above two microscope slides (5x7.5 cm^2) with two paper clips. A space of 1 mm is maintained between the slide and the comb. Agarose (0.7 g) is dissolved in 100 ml TAE buffer by heating on a water bath or in a microwave oven. The solution is cooled to 50° and poured over the slides. Surface tension is adequate to prevent the gel from overflowing. The gels are allowed to set for 1 hour before use. SDS agarose gels are similarly prepared. 5 ml of 10% SDS is added to the hot agarose solution before pouring. The gels were allowed to set overnight before use. Gels were prepared in batches of 8 and stored in the refrigerator in a large plexiglass box containing TAE buffer.

The gels are indefinately stable provided they are not allowed to dry out.

Electrophoresis procedure: The gel was immersed in 100 ml of TAE buffer containing 10 μl of ethidium bromide solution (10 mg/ml). The sample was made 2% in ficoll by the addition of ficoll-bromophenolblue (20%, 0.025%) solution and loaded onto the gel. 10-20 μl is a suitable sample volume. Typical electrophoresis voltage was 100V for 15 minutes for λ or T7 DNA and 60-80V for 30-60 minutes for SV40 DNA or for restriction fragments. The gel was destained in water, placed on a photodyne transilluminator and photographed with an Omega View 45F camera using Polaroid type 667 film and a 1 minute exposure. SDS agarose gels were extensively washed with water to remove the SDS before staining.

h) Polyacrylamide Gel Electrophoresis: SDS polyacrylamide gels were prepared and run using a modification of the procedure developed by Laemmli (6). The apparatus used was that described by Studier (5). The clear glass plates were separated by 1 mm plexiglass spacers and the comb had 16 teeth (5x12x1 mm^3).

Preparation of Gel: Stock solutions:

Solution A. Dissolve 30 g acrylamide and 0.8 g bis acrylamide in water. Dilute to 100 ml and store in a dark bottle in the refrigerator.

Solution B: 1.5 M Tris pH 8.8.

Solution C: 10% SDS in water.

Solution D: 0.5 M Tris pH 6.8.

Solution E: 10% ammonium persulfate in water.

Electrophoresis buffer: 0.05 M Tris base, 0.38 M glycine, 0.1% SDS.

The gel solution was made up by mixing the various solutions in the quantities shown in Table I. The TEMED was added immediately before pouring the gel. 5 ml of the separating gel solution was poured into the well on the gel stand. 50 μ l of TEMED was added and the gel plates were placed on the stand. After the solution had polymerized, 50 μ l of TEMED was added with stirring to the remainder of the separating gel solution and the gel was poured immediately up to 3 cm from the top of the plates. A 0.1% solution of SDS in water was then added to ensure a flat gel surface after polymerization. Gels were allowed to stand overnight before use. They can be stored for longer if they are sealed in plastic wrap. The SDS solution was then removed and the spacer gel solution was added. The gel comb was inserted and the gel was allowed to polymerize for 3 hours. The comb was then carefully removed using a syringe needle to separate the gel from the comb.

Electrophoresis: A mixture of the sample and formamide containing 0.01% bromophenol blue (10:1) was applied to the gel using a 50 μ l syringe (ficoll is not suitable for increasing the sample density). Electrophoresis was carried out at 60V until the sample had entered the separating gel and then at 120V for about 4 hours. Alternatively electrophoresis has been carried out at 50V overnight. During electrophoresis the buffer was circulated with a peristaltic pump.

Table I

	Separating Gel	Spacer Gel
Solution A	8.33 ml	1.6 ml
Solution B	6.25 ml	
Solution C	0.25 ml	0.08 ml
Solution D	—	2 ml
Solution E	0.125 ml	0.08 ml
Water	10 ml	4.24 ml
TEMED	50 μl	25 μl

i) Silver Staining of Polyacrylamide Gels: Silver staining of proteins in polyacrylamide gels is about 100 times more sensitive than coomassie blue staining. There are several procedures in the literature. The method used was described by Morrissey (11).

Procedure for silver staining:

1. The gel is washed in 50% methanol, 10% acetic acid for 30 minutes. The gel can be stored in this solution for several days.
2. The gel is then washed in 5% methanol, 7% acetic acid for 30 minutes.
3. The gel is soaked in 10% glutaraldehyde for 30 minutes. The glutaraldehyde solution can be reused at least 4 times. The solution should be filtered if any precipitate forms.
4. The gel is washed three times in 500 ml of distilled water for 1 hour. Repeat twice.
5. The gel is soaked for 30 minutes in 500 ml of water containing

1 mg of dithiothreitol.

6. The gel is soaked in 200 ml of 0.1% silver nitrate for 30 minutes.
7. The gel is rinsed once with distilled water and twice with 100 ml of the developing solution (50 μ1 of 37% formaldehyde in 100 ml of 3% Na_2CO_3), and soaked in 200 ml of fresh developing solution until the desired level of staining is attained. Staining is stopped by adding 10 ml of 2.3 M citric acid and shaking for 10 minutes.
8. The gel is stored in distilled water and photographed on a light box using an Omega View 45F camera and Polaroid film type 667.

If the gel does not stain satisfactorily it can be completely destained (6) and restained. During all the above steps the gel is agitated on a rotary shaker at 30 rpm (TekTator V Scientific Products). The staining process was carried out in a plexiglass box. The gel should be handled only with washed gloves. After staining, the silver nitrate solution is poured into a saturated solution of sodium chloride. The precipitated silver chloride is kept for eventual recovery of the silver. The sensitivity of the stain decreases with increasing acrylamide concentration and with increasing gel thickness. I have experimented with a number of different silver staining techniques. Considerable progress has been made since the publication of the seminal papers by Merril, Switzer, Van Keuren and Shifrin (7,8). The method described by Merril, Danau and Goldman (9) was satisfactory but it is more tedious and less reproducible than the

method described by Morrissey (11) which gives the same sensitivity. The photochemical silver stain (10) is rapid and easy to carry out. Unfortunately, this method stains DNA with high sensitivity and use of this method results in high background staining for samples containing large amounts of DNA. Useful discussions of the techniques are found in references 12, 17 and 18.

j) Electron Microscopy: The Kleinschmidt technique for spreading DNA was used (19-21).

Preparation of parloidion coated grids.

1. Add 2-3 drops of parloidion solution to water in a large petri dish.
2. After the amyl acetate has evaporated place several copper grids (Pelco 3H GC 200) dull side down on the unwrinkled parts of the film.
3. Remove the film from the water with a plastic wrap drum. (The drum is made by drawing a film of plastic wrap tightly over a glass petri dish).
4. Dry the film under a red lamp.
5. Remove the grids from the drum and store on a piece of filter paper in a petri dish. Good grids are covered with a reflective film and are stable for several weeks.

Sample spreading:

Preparation of hypophase: 1 ml TE buffer; 10 ml Formamide; and 89 ml distilled water.

Preparation of hyperphase: 5 1 cytochrome C solution; 5 1 TE buffer; 20 1 Formamide; X 1 DNA sample (0.025-0.005 g DNA); and (20-X) 1 distilled water.

1. Fill a petri dish (60x15 mm) to the point of overflowing with hypophase.
2. Slowly slide a teflon bar across two thirds of the surface.
3. Place a 3x1" microscope slide against the teflon bar (Figure 6). The slide should be cleaned in chromerge and washed with ammonia and water.
4. Rinse the slide with hypophase immediately before adding hyperphase.
5. Add the hyperphase slowly to the slide from a 50 1 screw pipette.
6. Wait 2 minutes for the DNA to spread.
7. Touch the surface with a grid, parloidion side down.
8. Immerse the grid for 30 seconds in $5x10^{-8}M$ uranyl acetate and for 10 seconds in 90% ethanol.
9. Place the grid on a filter paper to dry.
10. Shadow the grid using 1" of platinum palladium wire. (0.008 diameter). Grids were examined with a Phillips EM 300 electron microscope.

Figure 6 Apparatus for Kleinschmidt spreading of DNA. (a) Hypophase in a petri dish; (b) Microscope slide;: (c) Teflon bar; (d) Hyperphase in 50 L screw pipette; (e) Parloidion coated copper grid (20).

Solutions required for electron microscopy are:

1. 2.2% parloidion in amyl acetate. The solution is stored over 4A molecular seives.
2. 1 M Tris, 0.1 M EDTA, pH 8.5 (TE Buffer).
3. 90% ethanol.
4. Formamide (MCB formamide was used without purification).
5. Cytochrome C solution 0.5 mg/ml cytochrome C in 10 mM Tris, 1 mM EDTA pH 7.5. Solution is stored in the refrigerator.
6. Uranyl Acetate: 0.05 uranyl acetate in 0.05 M HCl. The solution is stored in the refrigerator.

To avoid dirty grids the following steps should be taken: All buffers used should be millipore filtered. The spreading should be carried out away from open doors and windows and in a box that shields from dust carrying air currents. The plastic tubes used should be

stored in a clean covered beaker to avoid dust sticking to them. The teflon bar should be cleaned with a wet tissue to avoid charging it. Plastic gloves should be washed to remove the powder from them. The microscope slide should be handled only with a clean forceps.

If the sample contains a large concentration of protein the DNA will not spread well. If the sample contains SDS the DNA will not stick to the grid. Both of these problems can be overcome by removing the protein or SDS before spreading.

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CHAPTER 3

RESULTS

Scheme 1. Synthesis of **PDN**.

SYNTHESIS

The crosslinking reagent was prepared from 8-methoxypsoralen and 4-nitroveratrole in seven steps (Scheme I). 8-Methoxypsoralen was demethylated with boron tribromide and alkylated with 1,6-dibromohexane to give the bromide **6** in 28% yield. Diol **3** prepared by a modification of the literature procedure was reacted with 4-nitroveratrole to give the alcohol **4** in 13% yield. This was converted to the phthalimide **5** (80%) which was oxidized with osmium tetroxide to the diol **6** (92%) and then treated with hydrazine to give the amine **7** in 87% yield. This was alkylated with psoralen-bromide **10** to give **PDN** in 23% yield.

PROTEIN-DNA CROSSLINKING

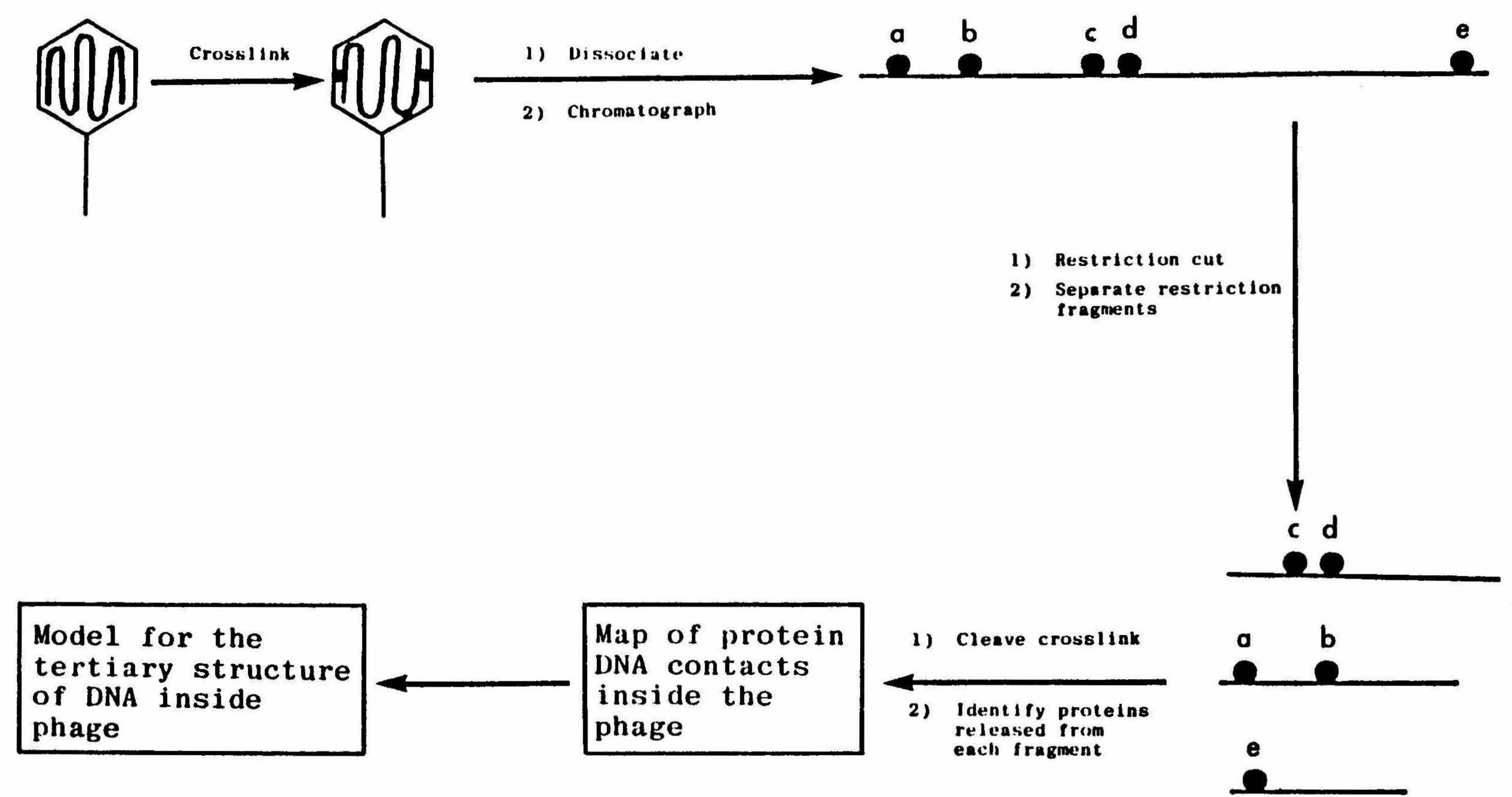
Crosslinking studies were carried out on bacteriophage λ , bacteriophage T7 and on SV40 virus. Bacteriophage λ presented technical difficulties which were not encountered with bacteriophage T7. For this reason T7 is the best studied system and will be described first. A brief survey of SV40 virus was undertaken.

I. BACTERIOPHAGE T7

a) Background:

T7 is an isometric bacteriophage. The phage has been well studied genetically and biochemically (for reviews see 1, 2, and 3). Its molecular weight is 50×10^6 daltons and the capsid diameter is 600A. The phage DNA (40,000 base pairs) has been partially sequenced (4). The restriction map for several enzymes has been published (5).

The phage is composed of 11 proteins. The location in the phage



Scheme 2.

Strategy for probing DNA packaging in phage T7 using protein DNA crosslinking.

of these proteins has been studied (1,6) Proteins Q, P9 and P10 are located in the head, proteins P8, P11, P12 and P17 are in the tail. Negatively stained electron micrographs of the phage indicate the presence of a protein core. One or possibly all of the proteins P14, P15 and P16 comprise the core (6).

The DNA in the phage is condensed 250 fold. Electron microscopy of disrupted T7 shows that the DNA is released from the phage head as a mass of concentric circles and as tightly wounds spools (7). The transient electric dichroism is consistent with a co-axial solenoid structure for the packaged DNA (8). X-ray diffraction studies indicate regular packaging of the DNA with an interplanar spacing of 27.5A (9). Recently Reuben (10) has shown that all the BglI restriction fragments of T7 can be crosslinked to each other by treating the phage with a bisazidomethidium interhelical DNA-DNA crosslinking reagent (11). This result contradicts the coaxial solenoid model. At this time the tertiary structure of the DNA in bacteriophage T7 is unknown.

We are interested in probing the tertiary structure of packaged T7 DNA using our protein-DNA crosslinking reagent. The approach that we attempted is outlined in Scheme 2. All of the steps in the scheme have literature precident. Carbodiimide crosslinked phage has been examined by electron microscopy (12), restriction fragments of adenovirus DNA with protein covalently attached to them have been separated on SDS agarose gels (13) and sodium periodate has been used to cleave diol containing protein-protein crosslinks (14).

Specifically we want to test the co-axial solenoid model and determine which proteins are involved in the proposed core. The

solenoid model predicts that 30% DNA on one end is in contact with the capsid proteins and that 13% of the DNA at the other end is in contact with the core proteins.

b) Results

1. Protein-DNA Crosslinking/Agarose Gel

5 1 of phage T7 (4 mg/ml) in =1.5 CsCl was diluted to 50 1 with =1.5 CsCl and dialyzed into PM buffer. The sample was divided into aliquots which were treated as follows:

1. 10 1 T7
2. 10 1 T7 irradiate for 15 minutes
3. 10 1 T7, 1 1 8-methoxypsoralen, 4-nitroveratrole (in MeOH DMSO 10:1, final concentration of each reagent = $3.2x10^{-5}$ moles/L) irradiate for 15 minutes.
4. 10 1 T7 + 1 1 **PDN** solution (in DMSO MeOH 1:10). Final concentration = $3.2x10^{-5}$ moles/L), irradiate for 15 minutes.

Each sample was dissociated with 1% SDS at 70° for 2 minutes and electrophoresed on an agarose mini gel.

Result: The DNA from the sample irradiated in the presence of the crosslinking reagent remains at the origin of the gel. Most of the DNA from the irradiated sample and from the sample treated with the separate chromophores enters the gel unaltered. (Figure 1)

2) Protein-DNA Crosslinking/Electron Microscopy

25 1 of crosslinked T7 phage was dissociated by heating with 1% SDS at 37°for 15 minutes and chromatographed on sepharose eluting with TAENE buffer. The DNA containing fractions were pooled (300 1) and a 1 1 sample was spread for electron microscopy. A non-crosslinked sample was similarly prepared.

Results: The DNA from the crosslinked sample formed rosettes (Figure 2). The DNA from the non-crosslinked sample was completely extended.

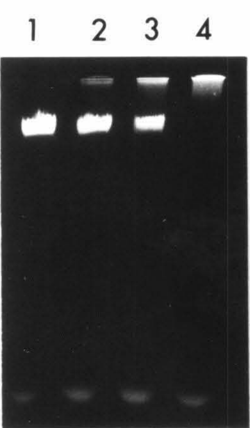


Figure 1. Agarose gel of crosslinked T7. (1) T7, (2) T7 + hν, (3) T7 + 8-methoxypsoralen + 4-nitroveratrole + hν, (4) T7+ PDN + hν.

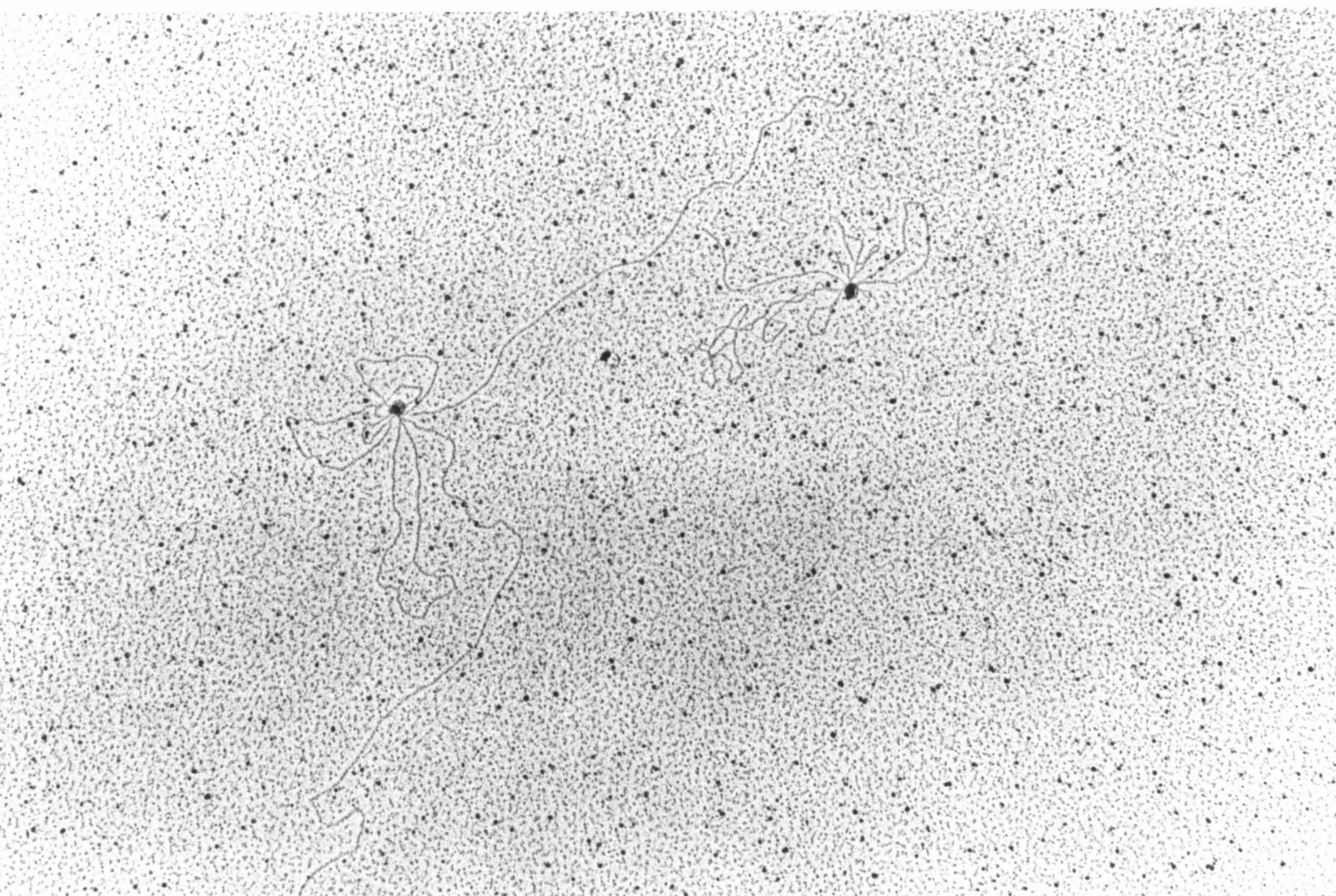


Figure 2. Electron micrographs of crosslinked bacteriophage T7.

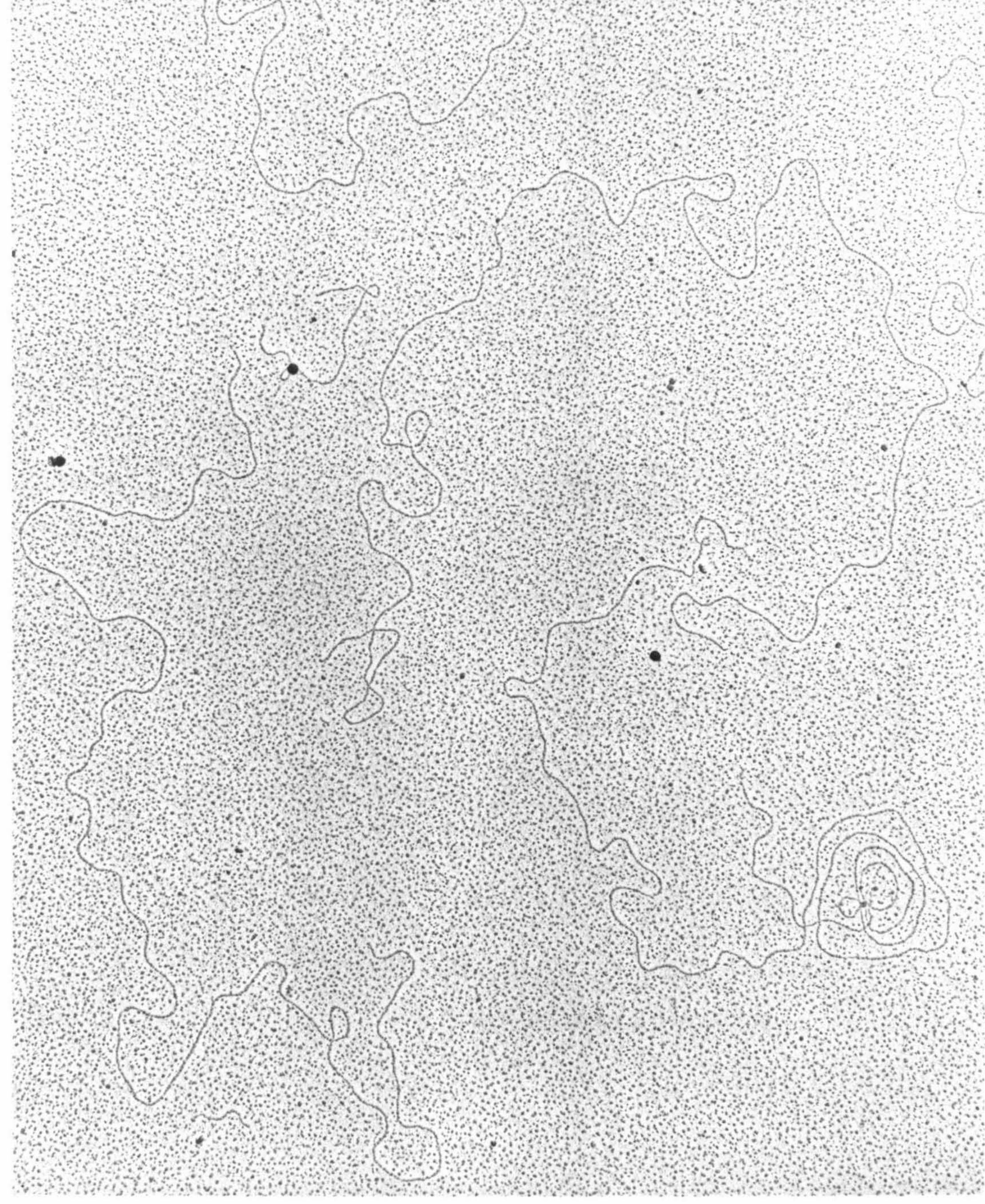


Figure 3. Electron micrograph of non-crosslinked bacteriophage T7.

(Figure 3). For a total of 300 pieces of DNA from each sample, 280 and 30 rosettes were counted for the crosslinked and the non-crosslinked samples, respectively.

3) Reversal of Protein-DNA Crosslink

50 μl of crosslinked T7 phage (0.4 mg/ml) was dissociated by heating with 1% SDS at 70° for 2 minutes. Samples for gel analysis were prepared as follows:

1) No treatment: 5 μl T7(CL) + 10 μl TAEN buffer

2) Sodium periodate cleavage of crosslink: 5 μl T7(CL) + 5 μl TAEN buffer + 5 μl 0.1 M $NaIO_4$. The reaction mixture was allowed to stand at room temperature for 1 hour.

3) Photoreversal of crosslink: 5 μL T7(CL) + 10 μl TAEN buffer. The sample was irradiated with short wavelength UV at room temperature for 15 minutes. The irradiation was carried out using the same apparatus used for the crosslinking experiment except the filter was removed from the UV lamp.

4) Phenol extraction: 10 μl T7(CL) + 20 μl TAEN buffer. The sample was phenol extracted (2x20 μl phenol) and washed with ether (2x1 ml). 15 μl was used for gel analysis.

5) Proteinase K treatment: 10 μl T7(CL) + 20 μl PM buffer + 1 μl proteinase K (10 mg/ml). The sample was heated at 55° for 30 minutes. 15 μl was used for gel analysis.

All samples were loaded onto a mini gel and electrophoresed at 80V for 1.5 hours.

Result: Treatment of the crosslinked phage with $NaIO_4$ cleaves the

crosslink. The crosslink cannot be reversed by short wavelength UV. The crosslinked phage was completely extracted into the phenol. Proteinase K treatment reverses the effect of crosslinking on the mobility of the phage DNA. (Figure 4)

4) Assay for Protein-Protein Crosslinking.

5 μl each of T7 and T7(CL) (0.4 mg/ml) were dissociated by heating with 1% SDS at 70° for 3 minutes. The samples were analyzed for protein-protein crosslinking on a silver stained polyacrylamide gel.

Result: There was no strong evidence for protein-protein crosslinking. Proteins P15 and P8 were absent, and P16, P9 and Q were depleted from the crosslinked sample. (Figure 5, lane 2) The protein band between P10 and P14 in the crosslinked sample is too intense to represent crosslinking of P13 and P14 (Figure 5).

5) Assay for DNA-DNA Crosslinking

50 μl of T7 (CL) (0.2 mg/ml) was dissociated by heating at 70°C for 2 minutes with 1% SDS and digested with proteinase K (1 mg/ml) at 55°C for 1 hour. The sample was then phenol extracted, dialyzed into BglI buffer and treated with 10 units of the restriction enzyme BglI at 37°C for 1 hour. A 0.1 μl sample was spread for electron microscopy and the number of crossed DNA fragments was counted. A non-crosslinked sample was similarly treated.

Result: From a total of 300 fragments counted for each sample 13 and 23 crosses were found for the non-crosslinked and the crosslinked samples, respectively.

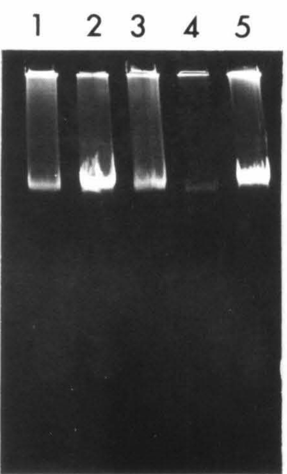


Figure 4. Gel analysis of crosslinked T7. (1) T7(CL), (2) T7(CL) + $NaIO_4$, (3) T7(CL) + $h\nu$, (4) T7(CL) phenol extracted, (5) T7(CL) proteinase K.

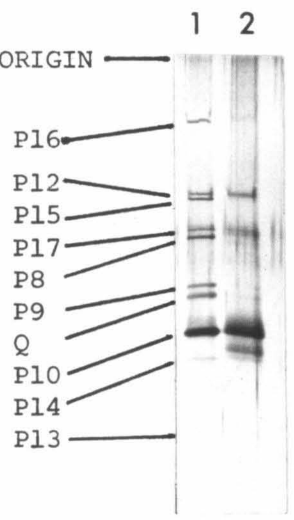


Figure 5. Comparison of the protein composition of crosslinked and non-crosslinked T7. (1) T7 (2 μg), (2) T7 (CL) 2 μg.

6) Crosslinked Protein DNA Complex/Gel Mobility.

Samples of crosslinked phage were treated as follows:

1) SDS dissociation: 5 μl T7(CL) + 1 μl 10% SDS + 5 μl TAEN buffer. The sample was heated at 70° for 5 minutes.

2) Urea SDS dissociation (5 M, 1%): 5 μl T7(CL) + 2 μl 10% SDS + 12 μl 8 M Urea in 0.05 M Tris 0.005 M EDTA 0.2 M NaCl pH 8.5 buffer. The sample was heated at 37° for 1 hour.

3) Guanidine hydrochloride sarkosyl dissociation (5 M 1%): 10 μl T7(CL) + 4 μl 10% sarkosyl + 26 μl 8 M guanidine hydrochloride in 0.01 M Tris 0.001 EDTA pH 8 buffer. The sample was heated at 37° for 1 hour, dialyzed into TAEN buffer. 20 μl of this sample was used for gel analysis.

4) Succinylation: 10 μl T7(CL) + 1 μl 10% SDS. The sample was heated at 70° for 2 minutes. 10 μl 0.2 M $NaHCO_3$ and 0.1 mg of succinic anhydride was added and the reaction mixture was allowed to stand at room temperature for 1 hour. The sample was then dialyzed into TAEN buffer and heated at 70° for 2 minutes. 20 μl of this sample was used for gel analysis.

The above samples were electrophoresed on a 0.7% agarose mini gel. A similarly prepared series of samples were electrophoresed on a 0.7% agarose 0.5% SDS mini gel.

Result: SDS dissociation and succinylation enabled most of the DNA to enter the SDS agarose gel. (Figures 6,7)

7) Protein Release and Detection/Silver Stained Polyacrylamide Gel.

100 μl of T7(CL) (0.4 mg/ml) was dissociated by heating with 1% SDS at 70° for 5 minutes and chromatographed on sepharose (2x55 μl aliquots) eluting with TAENE buffer. The DNA containing fractions

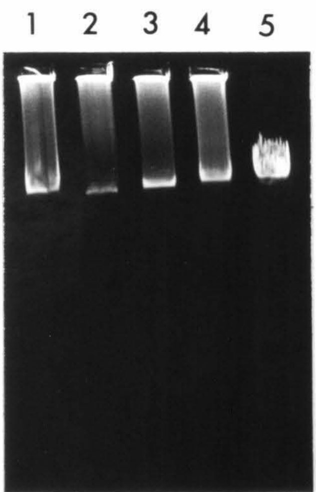


Figure 6. Agarose gel of crosslinked T7 dissociated in a variety of ways (1) T7(CL) SDS, (2) T7(CL) urea SDS, (3) T7(CL) guanidine hydrochloride sarkosyl, (4) T7(CL) SDS, succinic anhydride, (5) T7 non-crosslinked.

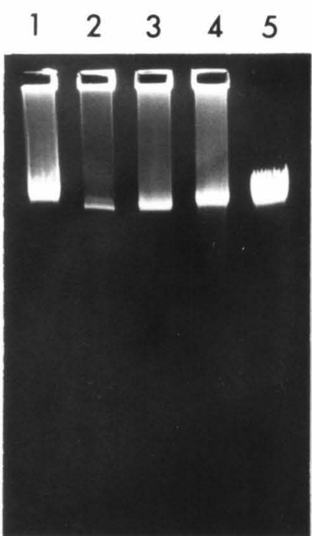


Figure 7. SDS agarose gel of crosslinked T7 dissociated in a variety of ways. (1) T7(CL) SDS, (2) T7(CL) urea SDS, (3) T7(CL) guanidine hydrochloride sarkosyl, (4) T7(CL) SDS succinic anhydride, (5) T7 non-crosslinked.

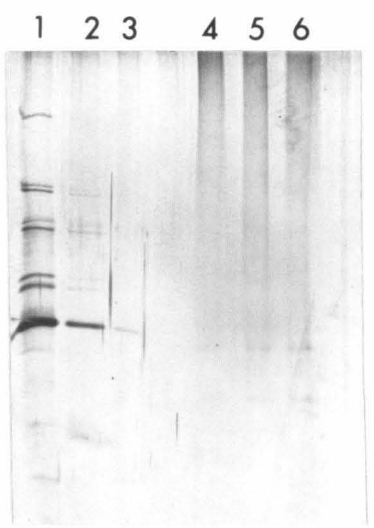


Figure 8. Silver stained polyacrylamide gel of periodate treated crosslinked T7 DNA protein complex. (1) 2 μg T7, (2) 0.2 μg T7, (3) 0.02 μ g T7, (4) T7 deproteinized, (5) T7(CL) deproteinized, (6) T7(CL) deproteinized + $NaIO_4$.

were combined made 0.1% in SDS, lyopholized and redissolved in 60 μl of water. 50 μl of non-crosslinked phage was similarly treated.

30 μl of the lyopholized crosslinked sample was treated with 10 μl of 0.1 M $NaIO_4$ at room temperature for 1 hour. The non-crosslinked, crosslinked and periodate treated crosslinked samples were electrophoresed on a polyacrylamide gel. Proteins were visualized by silver staining.

Result: Protein release by periodate treatment was not observed (Figure 8).

c) Discussion

Bacteriophage T7 can be crosslinked by treatment with PDN and irradiation at long wavelength. Because reagent reacts with thiols and amines these components must be absent from the crosslinking buffer. The crosslinking decreases with increasing pH (5-8) and is inefficient in the presence of borate. Very little crosslinking occurs in the absence of the reagent or in the presence of the separate chromophores.

The SDS dissociated crosslinked phage appears as a rosette under the electron microscope indicating that the DNA is crosslinked to the phage head. It was not possible to map the position of the phage head on the DNA for the crosslinked phage by electron microscopy because 40% of the phage heads in the non-crosslinked sample were still attached. DNA from the SDS dissociated crosslinked phage remains at the origin of an agarose gel. This band is almost completely absent from samples that have been phenol extracted before electrophoresis. Treatment of the crosslinked phage with periodate or proteinase K releases the DNA into the gel. These results indicate that the band

at the origin is a DNA-protein complex. Removal of the protein by proteinase K treatment or by cleavage of the crosslink restores the mobility of the DNA to that of the non-crosslinked sample. Psoralen mediated intrahelical RNA-RNA crosslinking can be reversed with short wavelength UV. The protein-DNA crosslink was not cleaved under these conditions indicating that photoreversal of the psoralen DNA monoadduct is much less efficient than photoreversal of the diadduct. Irradiation of the crosslinked sample in the presence of dithiothreitol also failed to reverse the crosslink by photochemical nucleophillic substitution on the nitroveratrole protein adduct.

DNA from crosslinked phage that has been dissociated with SDS enters SDS agarose gels as a band. This is consistent with the behavior of other DNA- protein complexes.

The reagent is bispecific. The protein composition of crosslinked and non-crosslinked phage was compared on a polyacrylamide gel. Proteins P15 (core, capsid) and P8 (tail, core) were absent, and P16, (core, capsid), P9 (capsid) and Q (capsid) were partially depleted from the crosslinked sample. This is indicative of selective crosslinking and we expected that these bands would be released from the crosslinked complex by periodate treatment. The protein band between P10 and P11 on the crosslinked sample is probably a contaminant as it is too intense to represent crosslinked P14 and P13 and migrates too fast to represent any other crosslinked complex. The gel analysis shows that the reagent does not form any protein-protein crosslinks. No evidence for DNA-DNA crosslinking was found when Bgl I restriction cut samples of crosslinked and non-crosslinked phage were compared by electron microscopy.

Attempts to identify which proteins are crosslinked to the DNA were unsuccessful. Treatment of the crosslinked complex with periodate failed to release detectable levels of protein. The silver stained polyacrylamide gel analysis would have detected less than 1% crosslinking to the DNA of the T7 proteins (except P13 and P11) and would have detected 0.1% of the major head protein. This result is clearly in contradiction to the protein depletion observed on polyacrylamide gels of crosslinked T7, to the electron microscopy of the crosslinked phage and to the fact that the crosslink can be readily cleaved with periodate. Proteins P15 and P8 are completely absent from a polyacrylamide gel of crosslinked T7. These proteins can be readily detected in a 0.2 g sample of T7 (Figure 8). The sample used to analyzed the crosslinked proteins contained 20 g of phage. This should give quantities of proteins P15 and P8 100 times greater than what can be minimally detected on the gel. One possibility is that the DNA-protein complex forms an aggregate which does not redissolve after lyophilization.

An alternative approach to identifying the crosslinked proteins would be to use P^{32} labeled phage. The crosslinked DNA-protein complex could be extensively digested with DNasae to give proteins radiolabeled with P^{32} oligonucleotides. The labeled proteins can be identified by autoradiography of a polyacryalmide gel. To construct a map of DNA protein contacts in the phage, the protein-DNA restriction fragments would be electroeluted from an agarose gel and similarly treated.

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III. BACTERIOPHAGE λ

a) Background

Bacteriophage λ is an isometric phage. Its biological properties have been extensively studied (1). The composition and structure of the phage are summarized in Tables I and II.

Table I

Composition of bacteriophage λ. (2,3)

Component	Molecular Weight
Intact phage	50×10^6
DNA (48000 base pairs)	31.8×10^3
Protein pD (440)[a]	11×10^3
pV (185)	31×10^3
pE (420)	38×10^3
pB*(12)	56×10^3
pB (3)	62×10^3
pH*(6)	90×10^3
pj (3)	140×10^3

(a) Estimated number of proteins per phage.

Table II

Structure of Bacteriophage λ. (4)	
Head diameter	580 A
DNA length	163 x 10^3 A
Tail length	1500 A

Several pieces of data relating to the structure of phage λ are in the literature. From biological studies it is known that the DNA is rapidly packaged into preformed phage heads (5-7) and that it is rapidly injected into bacteria during infection. Studies on the packaging of DNA from addition and deletion mutants show that the phage can package DNA in the range 75-108% of the wild type genome. Outside this range the infectivity of the phage decreases (8). This indicates that the phage head is filled almost to its maximum capacity with DNA. The major phage proteins have been localised by immune electron microscopy. Proteins pD and pE are in the phage head, pV is in the tail (3).

Phage λ DNA has a molecular weight of 31.8 x 10^6. Its ends are single stranded (12 bases) and are complementary. (9) The entire DNA has been sequenced (10) and its restriction map has been well characterized (11). The DNA undergoes a 300 fold condensation during packaging into the phage head. The right hand side of the DNA always protrudes into the tail (12,13). Electron micrographs of partially disrupted phage that has been crosslinked with nitrogen mustard show supercoiled regions (14,15). Electron micrographs of empty phage

heads indicate the presence of a protein core. (16,17) The phage gives an x-ray diffraction pattern that is consistent with the DNA being coiled as a co-axial solenoid (18). Linear circular dichroism studies are also consistent with this model. (19)

There are two serious problems with this model. Thomas found that λ phage head can be crosslinked to all regions of the DNA (12). The co-axial solenoid model would predict that the phage head should be crosslinked only to 22% of the DNA on the left end. Independently M. Mitchell (20) and B. Haas (21) have found that all the MST II and Bgl I restriction fragments of λ can be crosslinked to each other. The co-axial solenoid model would predict that only fragments within a layer or on adjacent layers can be crosslinked.

We set out to probe the tertiary structure of DNA inside the phage head using our protein DNA crosslinking reagent. Our approach was the same as that attempted for phage T7.

b) Results

1. Protein-DNA Crosslinking

5 μl of phage λ (4 mg/ml) in ρ=1.5 CsCl was diluted to 50 μl with ρ=1.50 CsCl and dialyzed into PM buffer. The sample was divided into 5 aliquots which were treated as follows:

1. 10 μl λ

2. 10 μl λ, irradiate for 15 minutes.

3. 10 μl λ, 1 μl 8-methoxypsoralen, 4-nitroveratrole (in MeOH DMSO 10:1 final concentration of each reagent=3.2×10^{-5} moles/l) irradiate for 15 minutes.

4. 10 μl λ + 1 μl PDN solution (in DMSO MeOH 1:10) (final concentration = 3.2×10^{-5} moles/l), irradiate for 15 minutes.

Each sample was dissociated with 1% SDS at 70° for 2 minutes and electrophoresed on an agarose mini gel.

Result: The DNA from the sample irradiated in the presence of the crosslinking reagent enters the gel as a streak. Most of the DNA from the irradiated sample and from the sample treated with the separate chromophores enters the gel unaltered. (Figure 1)

2. Protein-DNA Crosslinking/Electron Microscopy

25 l of crosslinked phage was dissociated by heating with 1% SDS at 70° for 2 minutes and chromatographed on sepharose eluting with TAENE buffer. The DNA containing fractions were pooled (300 l) and a 1 l sample was spread for electron microscopy. A non-crosslinked sample was similarly prepared.

Result: The DNA from the crosslinked sample formed tight rosettes (Figure 2). The DNA from the non-crosslinked sample was completely extended. From a total of 300 pieces of DNA from each sample, 290 and 25 rosettes were counted for the cross-linked and the non-crosslinked samples respectively.

3. Reversal of Protein-DNA Crosslink

50 l of crosslinked phage (CL) (0.4 mg/ml) was dissociated by heating with 1% SDS at 70° for 2 minutes. Samples for gel analysis were prepared as follows:

1. No treatment: 5 l (CL) + 10 l TAEN buffer.

2. Sodium periodate cleavage of crosslink: 5 l (CL) + 5 l TAEN buffer + 5 l 0.1 M $NaIO_4$. The reaction mixture was allowed to stand at room temperature for 1 hour.

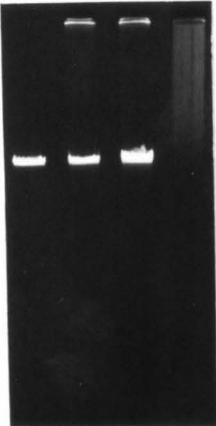

Figure 1. Crosslinking of phage λ with PDN. (1) λ , (2) $\lambda + h\nu$, (3) λ + 8-methoxypsoralen + 4-nitroveratrole + $h\nu$, (4) λ + PDN + $h\nu$.

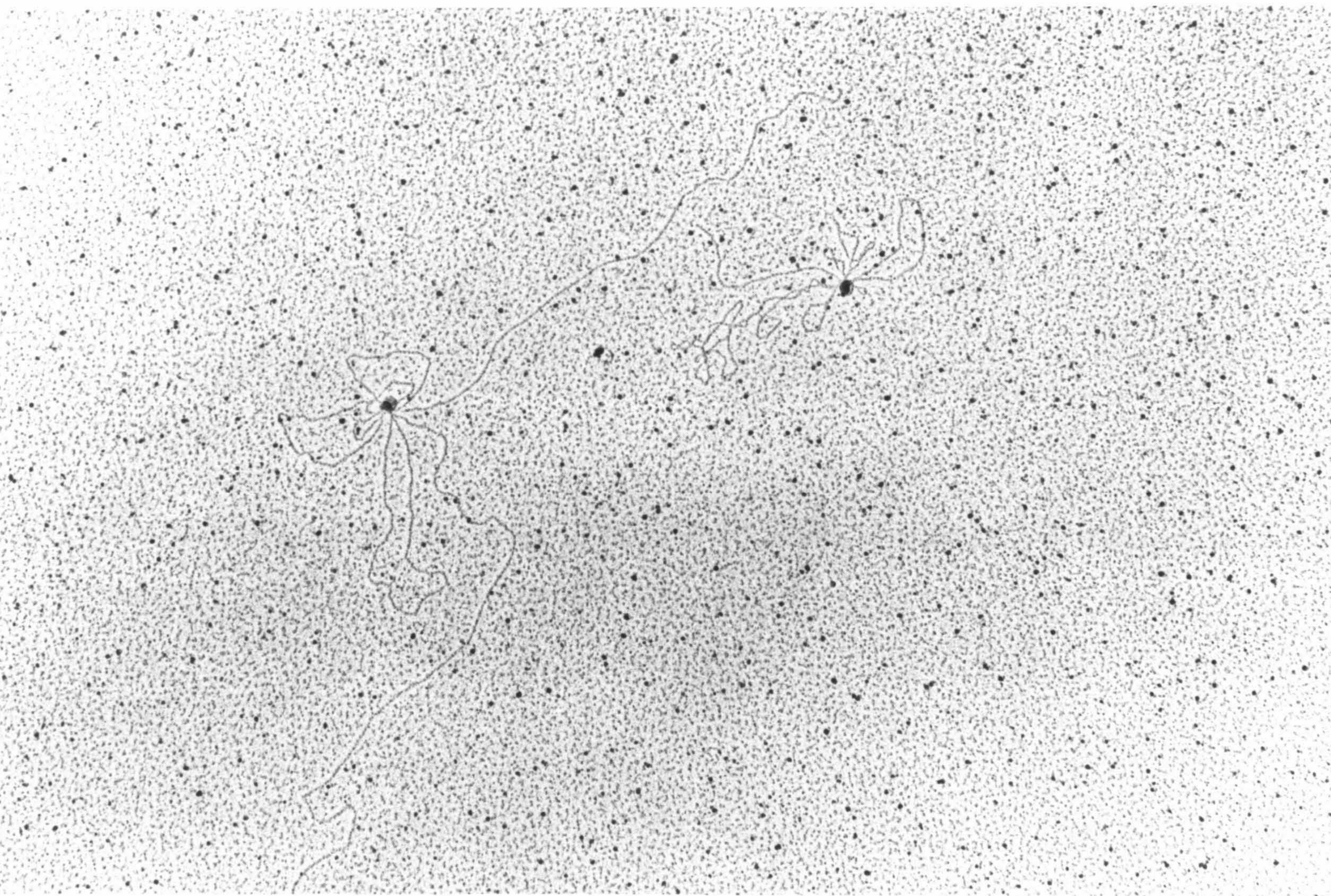

Figure 2. Electron micrographs of crosslinked bacteriophage T7.

3. Photoreversal of crosslink: 5 μl λ(CL) + 10 μl TAEN buffer. The sample was irradiated with short wavelength UV at room temperature for 15 minutes. The irradiation was carried out using the same apparatus as that used for the crosslinking experiment except the filter was removed from the UV lamp.

4. Phenol extraction: 10 μl λ(CL) + 20 μl TAEN buffer. The sample was phenol extracted (2x20 μl phenol) and washed with ether (2x1 ml). 15 μl was used for gel analysis.

5. Protenase K treatment: 10 μl λ(CL) + 20 μl PM buffer + 1 μl proteinase K (10 mg/ml). The sample was heated at 55° for 1 hour. 15 μl was used for gel analysis.

All samples were loaded onto a mini-gel and electrophoresed at 80V for 1.5 hours. (Figure 3)

Results: Treatment of the crosslinked phage with $NaIO_4$ cleaves the crosslink. The crosslink cannot be reversed by short wavelength UV. The crosslinked phage was completely extracted into the phenol. Unlike the T7 sample, proteinase K treatment does not reverse the effect of crosslinking on the mobility of the phage DNA.

4) Crosslinked Protein-DNA Complex/Gel Mobility

Samples of crosslinked phage are treated as follows.

1) SDS dissociation: 5 μl λ(CL) + 1 μl 10% SDS + 5 μl TAEN buffer. The sample was heated at 70° for 5 minutes.

2) Urea SDS dissociation (5 M, 1%): 5 μl λ(CL) + 2 μl 10% SDS + 13 μl 8 M Urea in 0.05 M Tris 0.005 M EDTA 0.2 M NaCl pH 8.5 buffer. The sample was heated at 37° for 1 hour.

3) Guanidine hydrochloride sarkosyl dissociation (5 M 1%):

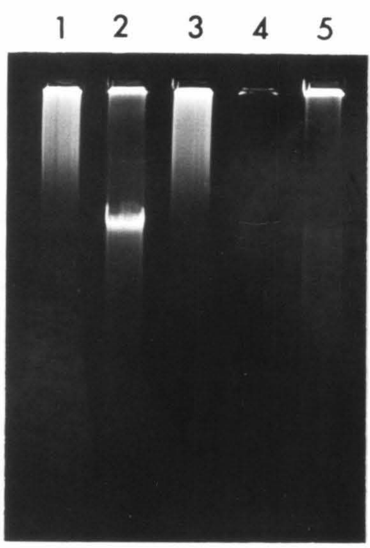

Figure 3. Gel analysis of the crosslinked phage. (1) λ (CL), (2) λ (CL) + $NaIO_4$, (3) λ (CL) + hν, (4) λ (CL) phenol extracted, (5) λ (CL) + proteinase K.

10 μl λ(CL) + 4 μl 10% sarkosyl + 26 μl 8 M guanidine hydrochloride in 0.02 M Tris 0.001 M EDTA, pH 8 buffer. The sample was heated at 37° for 1 hour, dialyzed into TAEN buffer. 20 μl of this sample was used for gel analysis.

4) Succinylation: 10 μl λ(CL) + 1 μl 10% SDS. The sample was heated at 70° for 2 minutes. 10 μl 0.2 M $NaHCO_3$ and 0.1 mg of succinic anhydride was added and the reaction mixture was allowed to stand at room temperature for 1 hour. The sample was then dialyzed into TAEN buffer and heated at 70° for 2 minutes. 20 μl of this sample was used for gel analysis.

The above samples were electrophoresed on a 0.7% agarose mini gel. (Figure 4) A similarly prepared series of samples were electrophoresed on a 0.7% agarose 0.5% SDS mini gel. (Figure 5)

Result: The DNA-protein complex did not enter the gel as a discrete band under any of the above conditions.

5) Electron microscopy of phage λ

Phage λ was dissociated with SDS (1%), urea (5 M) and guanidine hydrochloride (5 M). The samples were prepared as described above using 25 μl of λ for each sample. The denaturing agent was removed by chromatography on sepharose eluting with TAEN buffer and the samples were spread for electron microscopy.

Results: Empty phage heads were seen for the SDS and the urea dissociated samples. No phage heads were seen for the guanidine hydrochloride dissociated sample.

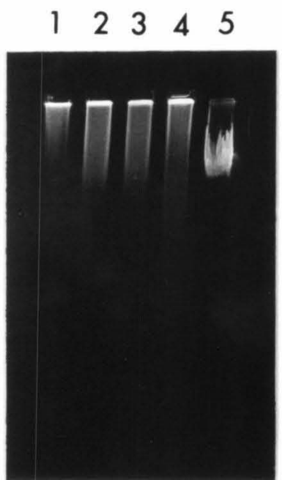

Figure 4. Agarose gel analysis of crosslinked λ dissociated in a variety of ways (1) λ (CL) SDS, (2) λ (CL) urea SDS, (3) λ (CL) guanidine hydrochloride sarkosyl, (4) λ (CL) SDS, succinic anhydride, (5) λ non-crosslinked.

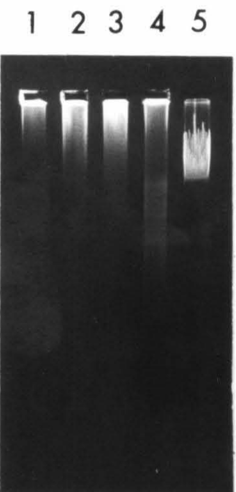

Figure 5. SDS agarose gel analysis of crosslinked λ dissociated in a variety of ways. (1) λ (CL) SDS, (2) λ (CL) urea SDS, (3) λ (CL) guanidine hydrochloride sarkosyl, (4) λ (CL) SDS succinic anhydride, (5) λ non-crosslinked.

<u>6) Protein Release and Detection/Silver Stained Polyacrylamide Gel</u>

100 1 of (CL) (0.4 mg/ml) was dissociated by heating with 1% SDS at 70° for 5 minutes and chromatographed on sepharose (2x55 1 aliquots) eluting with TAENE buffer. The DNA containing fractions were combined, made 0.1% in SDS, lyopholised and redissolved in 60 1 of water. 50 1 of non-crosslinked phage was similarly treated.

30 1 of the lyophilized crosslinked sample was treated with 10 1 of 0.1 M Na 10_4 at room temperature for 1 hour. The non-crosslinked, crosslinked and periodate treated crosslinked samples were electrophoresed on a polyacrylamide gel. Proteins were visualized by silver staining.

<u>Results</u>: All three samples were contaminated with non-crosslinked proteins. No difference was detected between the crosslinked periodate treated and non-periodate treated samples. (Figure 6)

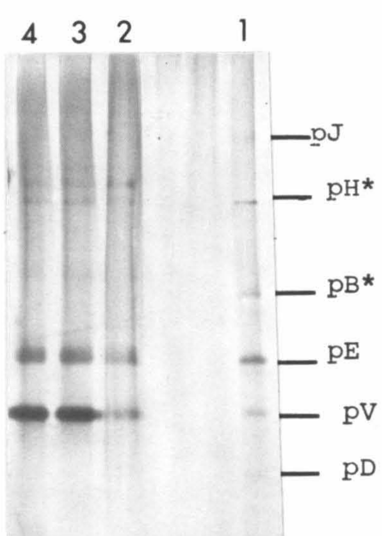

Figure 6. Silver stained polyacrylamide gel of periodate treated λ DNA protein complex. (1) λ proteins, (2) λ deproteinized, (3) λ (CL) deproteinized, (4) λ (CL) deproteinized + $NaIO_4$.

c) Discussion

Phage λ can be efficiently crosslinked by treatment with PDN and longwave length UV light. Very little crosslinking occurs in the presence of the separate chromophores or in the absence of the reagent. The SDS dissociated crosslinked phage appears as a dense rosette under the electron microscope indicating that the DNA is crosslinked to the phage head.

DNA from SDS dissociated crosslinked phage enters an agarose gel as a streak starting at the origin. The streak is removed by phenol extraction of the sample before electrophoresis. This shows that protein and not DNA-DNA crosslinking is causing the change in the mobility of the DNA. Treatment of the crosslinked phage with periodate cleaves the crosslink and the DNA now enters the gel with the same mobility as the non-crosslinked DNA. The crosslink was not cleaved by short wavelength UV.

Proteinase K treatment did not release the DNA from the protein. Dissociation of the crosslinked phage with SDS, Urea SDS or with guanidine hydrochloride sarkosyl did not give a DNA-protein complex that would enter an agarose or an SDS agarose gel as a band. Chemical modification of the SDS dissociated phage with succinic anhydride, a procedure that should decrease the electrostatic interaction between the proteins, also failed to give a DNA- protein complex that would enter agarose or SDS agarose gels as a band. When phage λ that has been dissociated with SDS, urea or guanidine hydrochloride is spread for electron microscopy intact phage heads are seen for the SDS and the urea dissociated samples. No phage heads were seen for the guanidine hydrochloride dissociated sample.

These observations are indicative of exceptionally strong protein-protein interactions in the λ phage head. Thus the head proteins are protected from proteolytic digestion, and DNA with intact phage head or large protein aggregates attached to it will not enter agarose gels.

It was not possible to determine which proteins were crosslinked to the DNA as sepharose chromatogrphy failed to give DNA samples free from contamination by non-crosslinked proteins. This is probably due to the fact that intact phage heads and tails and large protein aggregates will co-elute from sepharose in the void volume with the DNA. Due to this problem and because of our failure to get λ DNA-protein complexes to enter agarose gels as a band we were not able to complete the strategy for analysis of λ structure outlined in the introduction.

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III. SIMIAN VIRUS 40

a) Background

Simian Virus 40 (SV40) is a member of the papovairidae family. It is an icosahedral nonenveloped virus of molecular weight 27×10^6 and diameter 440Å. The virus induces tumors in monkeys and has been extensively studied (1). The viral DNA is superhelical and has a molecular weight of 3.4×10^6 (5200 base pairs). Its restriction map has been well characterized (2) and the entire genome has been sequenced (3).

When the virus is disrupted with triton X-100, the DNA is released as a closed circle containing between 20 to 22 nucleosome particles. This minichromosome has been used as a model for the second-order packaging of DNA in chromatin. The condensed form has been studied by electron microscopy, but no definite model for its structure has emerged (4-7). The relaxed and condensed forms of the minichromosome can be separated on low-salt agarose gels. (7) The distribution of the histones on the DNA has been analyzed by electron microscopy and by micrococcal nuclease digestion. (8) The internucleosomal distance varies between 15 and 250 base pairs. It is not known whether this distribution is an artifact. Crosslinking of the histones to the DNA should help to resolve this question.

Psoralen derivatives react readily with the intraviral DNA. Virus that has been crosslinked with trimethylpsoralen, denatured and examined under the electron microscope shows a preferential spacing of approximately 200 base pairs between crosslinks (9). Hydroxymethyl trimethylpsoralen preferentially binds around the origin of replication of intracellular SV40 chromatin. (10)

The proteins of SV40 have been separated on SDS polyacrylamide gels. The molecular weights and relative proportions of the capsid proteins are shown in Table I (11-13). VPI makes up 71% of the virus protein. The five histone proteins make up only 13% of the virus protein. Noncovalent complexes of SV40 DNA and VPI will enter agarose gels (14).

Table I Protein Composition of SV40 (11)

Protein	Molecular Weight	% of Virus Protein	Molecular Ratio	Number of Proteins per Virus
VP1	43,000	71	6	320
VP2	32,000	9	1	54
VP3	23,000	10	1.5	80
H3	14,000	6	1.5	80
H2a,H2b	12,500	4	1.1	60
H4	11,000	3	1.0	54

b) **Results**

1. Protein-DNA Crosslinking

25 μl of SV40 virus (5 mg/ml) in ρ=1.5 CsCl was diluted to 50 μl with ρ=1.5 CsCl, dialyzed against PM buffer and diluted to 250 μl with PM buffer. A 150 μl aliquot was treated with 15 μl **PDN** solution and irradiated for 15 mintues.

5 μl of crosslinked virus (0.5 mg/ml) was dissociated by heating

at 65° for 3 minutes with 1% SDS and 10 mg/ml DTT. A non-crosslinked sample was similarly dissociated and both samples were electrophoresed on a 0.7% agarose mini gel.

Results: The SV40 DNA from the crosslinked and the non-crosslinked virus samples enters the gel. (Figure 1)

2. Protein Release and Detection/Silver Stained Polyacrylamide Gel

100 μl of the crosslinked virus was dissociated by heating at 65° for 3 minutes with 1% SDS and 10 mg/ml DTT and chromatographed in two batches on sepharose, eluting with TAENE buffer. The samples were made 0.1% in SDS, combined, lyopholized and redissolved in 60 μl water by heating at 65° for 5 minutes. 50 μl of a non-crosslinked sample was similarly treated. 30 μl of the deproteinized crosslnked virus sample was treated with 10 μl of 0.1 M $NaIO_4$ at room temperature for 1 hour. The non-crosslinked, crosslinked, and the periodate treated crosslinked virus samples were electrophoresed on a polyacrylamide gel. The proteins were visualized by silver staining.

Result: The above treatment effectively deproteinizes the virus DNA. The sodium periodate treated sample did not show any protein. (Figure 2)

c) Discussion:

The crosslinked SV40 DNA-protein complex enters agarose gels as a broad streak. This mobility change may have resulted from DNA cleavage because the DNA also streaks on the sepharose column in the direction of decreasing size. No proteins were detected on a silver stained polyacrylamide gel of the periodate treated protein-DNA complex. Therefore I was unable to proceed with the analysis of the distribution of crosslinked proteins on the DNA.

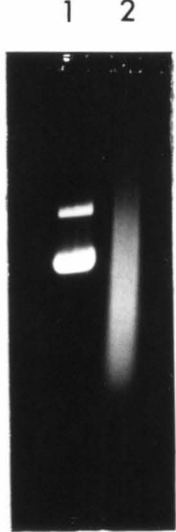

Figure 1. Agarose gel of crosslinked SV40. (1) SV40 (2) SV40(CL).

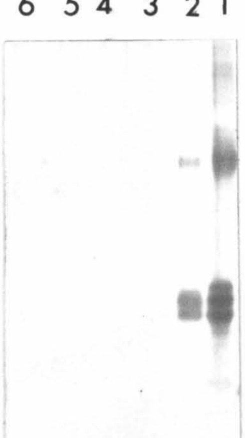

Figure 2. Silver stained polyacrylamide gel of periodate treated SV40 DNA protein complex. (1) 2.5 μg SV40, (2) 0.25 μg SV40 (3) 0.025 μg SV40, (4) 25 μg SV40 deproteinized, (5) 25 μg SV40(CL) deproteinized, (6) 25 μg SV40(CL) deproteinized + $NaIO_4$.

d) **References**

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V. SUMMARY

A new cleavable bispecific protein-DNA crosslinking reagent has been synthetized and characterized. Psoralen-diol-nitroveratrole (PDN) crosslinks protein to DNA in intact bacteriophage T7. No evidence for protein-protein or DNA-DNA crosslinking was obtained. The crosslink can be cleaved with sodium periodate. However, treatment of the crosslinked complex with sodium periodate did not release sufficient protein to be detected on a silver stained polyacrylamide gel. Technical difficulties with bacteriophage λ did not allow the crosslinking protocol to be fully tested.

PROPOSITION ABSTRACTS

Proposition 1: An approach to photoaffinity labeling of the active site of EcoRl with bromodeoxyuridine is proposed.

Proposition 2: Chemical studies on the oxidative decomposition of 1-aminocycloproylcarboxylic acid are proposed.

Proposition 3: Experiments are proposed to study the effect of micells on the polymerization of lipophillic derivatives of nucleotides and amino acids.

Proposition 4: RNA-RNA crosslinking with trimethylpsoralen is proposed as a probe of RNA tertiary structure in influenza virus.

Proposition 5: A suicide substrate for reverse transcriptase is proposed.