Interhelical DNA-DNA Crosslinking of Bacteriophage Lambda: Bis(monoazidomethidium)octaoxahexacosanediamine and Bis(psoralen)nonaethyleneoxy ether, Probes of Packaged Nucleic Acid

Thesis by

MARK ALLEN MITCHELL

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I give special thanks to my entire family for their constant support, encouragement and attempts to understand me and my work.

Generous financial support from Caltech, an Earle C. Anthony Fellowship, and the National Institutes of Health is also greatly appreciated.

Hermit hoar, in solemn cell,
Wearing out lifes evening gray;
Strike thy bosom, sage! and tell
What is bliss, and which the way?
Thus I spoke, and speaking sighed,
Scarce repressed the starting tear,
When the hoary sage replied,
'Come my lad, and drink some beer.

Anecdotes of Johnson

Mrs. Piozzi

To My Family

For Their Support

Abstract

A strategy for determining the structure of packaged bacteriophage DNA is developed. The synthesis and characterization of two new interhelical crosslinking reagents, bis(monoazidomethidium)-octaoxahexacosanediamine (BAMO) (13) and bis(psoralen)nonaethyleneoxy ether (BPNE) (7) are reported. Both BAMO and BPNE are capable of crosslinking neighboring DNA helices within intact bacteriophages λ and T7. BAMO crosslinks packaged bacteriophage λ DNA at low binding levels (r = 0.01 BAMO/bp) and can be photolyzed quickly (0.5-1 hr) with light of low energy (λ > 400 nm) to yield 17% crosslinked structures as observed under the electron microscope after isolation and restriction cleavage of the crosslinked DNA. BPNE yields 10% crosslinked structures when incubated for three hours with bacteriophage λ at a binding ratio of r = 0.28 BPNE/bp, photolysed for five hours (λ = 365 nm), followed by isolation and restriction digestion of the crosslinked DNA.

Methodology necessary to uniquely locate the site of crosslinking along the bacteriophage genome has also been developed. Directional measurements of restriction fragments have been carried out on photomicrographs of fragments labeled specifically at one end with electron dense avidin spheres. The avidin spheres are attached to the restriction fragments by incorporating a biotin containing nucleotide (Bio-dUTP) into the restriction fragment using T4 polymerase under standard labeling conditions. Subsequent restriction cleavage generates fragments labeled at one specific end. Incubation of avidin spheres with these biotinylated restriction fragments

results in avidin sphere binding specifically to the labeled end of the fragments.

We have begun to generate interhelical nearest neighbors maps from the measured crosslink positions for bacteriophages λ and T7. Models have been constructed for packaged T7 DNA according to the coaxial solenoid and coil of coils models discussed in the literature. Hypothetical interhelical nearest neighbors maps from these models have been prepared.

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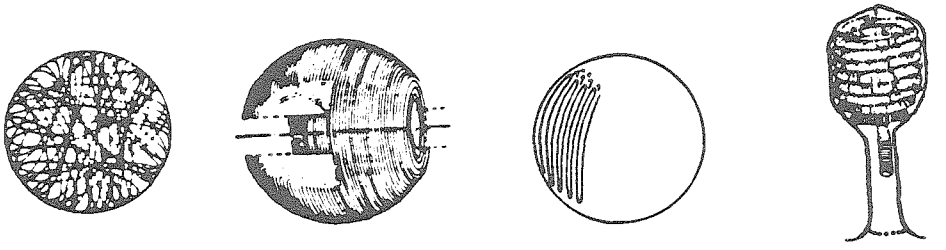
INTRODUCTION

Crosslinking agents have proven to be powerful tools for probing the higher order structure of biological macromolecules. Bifunctional protein-protein and protein-nucleic acid crosslinking reagents have been used to map protein-protein and protein-nucleic acid nearest neighbors in nucleosome and ribosome complexes.[1-3] This methodology has not been applied to mapping condensed nucleic acid structures such as in viruses, presumably because interhelical nucleic acid-nucleic acid crosslinkers have not been available. The design and synthesis of interhelical DNA-DNA crosslinkers and the development of the methodology to use them provides a new capability to probe the structure of packaged nucleic acids. We describe the synthesis of two new photoreactive bifunctional molecules containing the criteria necessary for an interhelical DNA-DNA crosslinker. We also describe the methodology used to evaluate their effectiveness as crosslinking reagents and to obtain maps of the nearest neighbor contact sites for the packaged DNA within intact bacteriophages λ and T7.

Determination of the structure of condensed nucleic acid within intact bacteriophages has been the subject of many physical and chemical studies. Physical techniques used to study bacteriophage structure have been: x-ray diffraction,[4,5] electron microscopy,[6,7] flow linear dichroism[8], optical rotary dispersion,[9] flow birefringence,[10]transient electric birefringence,[11,12] transient electric dichroism,[13] electron beam inactivation studies[14] and circular dichroism.[13,15] Data from these studies have resulted in the proposal of several models for the structure of such condensed nucleic acid complexes, yet insufficient data exist to make a definite

structural assignment (see Fig. 1).



A B C D

Fig. 1

Bacteriophage λ : Bacteriophage λ is a long tailed isometric bacteriophage, having an icosahedral head with a radius of 300 Å and a thickness of about 40 Å connected to a flexible tail 1500 Å long.[16] The empty capsid has a molecular weight of 21 x 10^6 daltons. The tail adds 6.1 x 10^6 and the DNA 31 x 10^6 daltons, yielding a total molecular weight of 58 x 10^6 daltons. The λ phage genome is 48,498 base pairs long and has been completely sequenced.[17] The λ DNA is packaged in its linear double helical form with its 12 base long, single stranded complementary projections at each end. The projection near the A gene is defined as the left cohesive end and is packaged first.[18] The right cohesive end of the packaged DNA is located near the head-tail junction and protrudes into the upper third of the tail.[19] Physical techniques used to study phage λ structure have been: x-ray diffraction,[4,5] electron microscopy,[6,7] flow linear dichroism,[8] and optical rotary dispersion.[9]

Richards et al obtained electron micrographs of several mildly disrupted bacteriophages, including phage λ , prepared by drying in

thin films of negative stain.[7] Phage treated in this manner appear gently disrupted and flattened, resulting in an essentially two-dimensional display of the nucleic acid. The DNA in their samples appeared in its simple, 25Å double helical form, not as a supercoil, and always as a set of concentric circles or as a tightly wound spiral. They interpreted their observations as being consistent with the DNA being packaged like a ball of string (A) or wound coaxially like a spool (B) and inconsistent with a chain folded (C) or coil of coils (D) structure.

North and Rich observed a 24Å x-ray reflection in x-ray photographs of unoriented bacteriophage λ and T7 gel specimens.[4] More recently Earnshaw and Harrison in their studies of phage λ , using x-ray cameras of greater resolution, not only observed the intense 24Å diffraction, but also detected a modulation of its intensity due to long range order within the phage head.[5] The intense 24Å diffraction results from adjacent segments of DNA packed in a locally parallel array. Assuming hexagonal packing of the helices, they calculated the interhelical spacing to be 27Å. The ripples that modulated the intensity of the 24Å diffraction had a wavelength of about 1/540Å or approximately the reciprocal of the internal head diameter, indicating coherence between the DNA on opposite sides of the phage particle. They were able to model this ripple effect by assuming the DNA exists in several concentric layers packed within the protein shell. Earnshaw and Harrison interpret their results as providing support for the spool (B) model for packaged bacteriophage λ DNA.

Hall and Schellman determined the flow linear dichroism of

bacteriophage λ both in the absence and presence of the intercalating dye, ethidium bromide.[8] In both experiments the magnitude of the observed dichroism did not correspond with that calculated for the simplest models, i.e., models in which the helix axis is wholly parallel or perpendicular to the phage axis. They discussed their results in terms of: inclined structures, in which the packaged structure is tilted away from the axial or transverse directions in the phage; orientational disorder, in which the orientation of the packaged structure varies from one phage particle to another; composite structures, in which the packaged structure is not uniform within the phage; and partial organization, in which the DNA within capsid phages does not have a definite structure and varies from one phage particle to the next. The dye binding studies indicated that the net orientation of intercalated ethidium bromide was not the same as the helix orientation. This suggests that the DNA within phage λ is inhomogenously ordered and that ethidium bromide does not penetrate to all DNA binding sites.

Maestre and Tinoco obtained optical rotary dispersion (ORD) spectra from a variety of bacteriophages including phage λ and T7.[9] They observed qualitative similarities between spectra from several phages and distinct differences between spectra from the intact bacteriophage and spectra from samples of osmotically shocked phage in which the DNA has been released from the capsid. Intraphage ORD data have been subject to a variety of inconclusive interpretations including: the condensed DNA exists in a reduced state of hydration; the condensed DNA exists at least partially in an altered secondary conformation; or the densely packed intraphage DNA may actually

possess anisotropic properties.

Bacteriophage T7: T7 is a short-tailed isometric bacteriophage with a capsid diameter of 600 Å and a tail length of about 140 Å.[20] The molecular weight of the entire phage is 50.6 x 10^6 daltons and the DNA, 6.5 x 10^6 daltons.[21] The T7 genome is 39,936 base pairs in length and has been completely sequenced.[21] Negatively stained electron micrographs indicate the presence of a protein core.[22] The left end of the genetic map is injected into E.coli first, indicating polarity of the packaging event.[23] Physical methods used to determine the structure of packaged T7 DNA include x-ray diffraction,[4] electron microscopy,[22] transient electric dichroism,[13] birefringence,[12] circular dichroism,[13] and optical rotary dispersion.[9]

The x-ray diffraction studies by North and Rich[4] and the optical rotary dispersion studies by Maestre and Tinoco[9] have already been discussed. The results for both T7 and phage λ are similar. Electron microscopy studies by Serwer indicate that T7 may have an internal core of protein.[22]

Kosturko, et al studied the transient electric dichroism of several phages including T7.[13] They found that all phages studied showed a large dipole moment, the reduced dichroism of each phage at perfect orientation was within the range +0.12 to +0.19, and that circular dichroism spectra suggest that the local DNA helicity is similar for each phage. Kosturko, et al. concluded that the measured dichroism suggested that the DNA is wrapped in a closely packed coaxial solenoid with the axis of the solenoid tipped $43.5^o \pm 2.5^o$ from an axis running from the apex of the phage through the tail. These dichroic results are similar to the flow linear dichroism

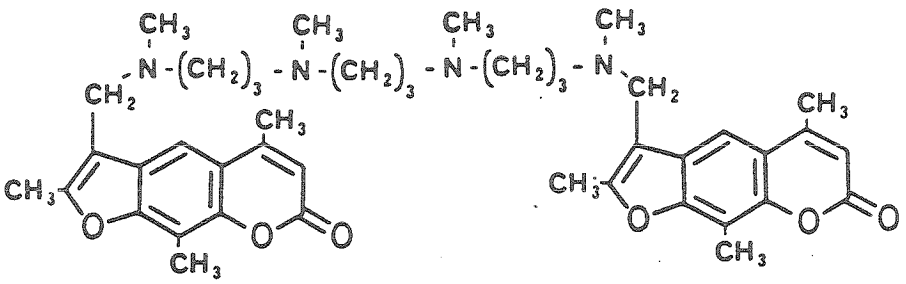
studies discussed for phage λ . Birefringence studies of T7 by deGroot, et.al. yielded dipole moment data comparable to the dichroism measurements.[12]

In addition to the physical studies discussed above, chemical modification studies have also provided information about the structure of intraphage DNA.[15] Chemical modification of functional groups normally protected by interplanary or complementary interactions is widely used as a technique to detect regions of altered helical structure. Intraphage modification experiments have been performed with O-methylhydroxylamine, glyoxal, bisulfite, nitrous acid and formaldehyde. In general, all studies conclude that approximately 20% of the intraphage DNA does not exist in the normal solution B conformation. If regions of altered helical structure are limited to certain regions of the DNA, the precise localization of the sites of chemical modification would be useful in determining the higher order packaged structure. These studies have not been done.

Crosslinking of the DNA to the tails of bacteriophage λ by a carbodiimide reagent has provided knowledge of the unique directionality of the DNA packing event.[15] Although not a direct probe of higher order structure, data such as this must also be accommodated by any structural model for intraphage DNA.

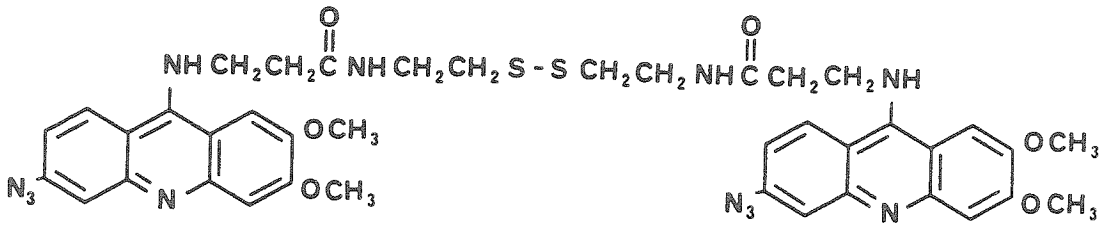
When the work reported in this thesis started in 1976, there were no interhelical crosslinking reagents available. Only recently have such reagents appeared in the literature. Haas, Murphy and Cantor (1982) have reported an interhelical crosslinking reagent, 1,15-bis(4'-trioxalen)-2,6,10,14-tetramethyl-2,6,10,14-tetraazapentadecane, BTPD (1) and its use as a probe of the arrangement of the DNA

inside bacteriophage λ .[24] Although their experiments did not uniquely localize the sites of crosslinking, they concluded that "hints of periodicity" and preferential crosslinking between certain regions of the genome "appear to rule out purely random packaging arrangements". Olomucki, et al. (1981) have reported the synthesis of a bis(azidoacridine) reagent , dithiobis[9-(2-ethylenecarbamoylethylamine)-2,3-dimethoxy-6-azidoacridine hydrochloride], $(AAS)_2$, (2), which they state could be used as an interhelical crosslinking reagent.[25] However, they reported no efforts demonstrating its usefulness as an interhelical crosslinker.



BTPD

1



$(AAS)_2$

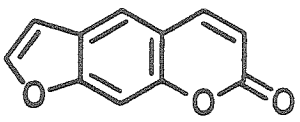
2

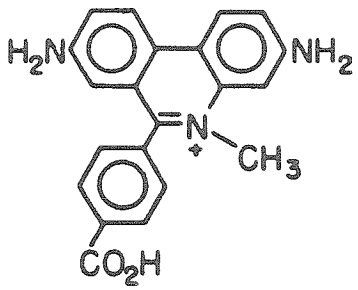
RESULTS AND DISCUSSION

I. Reagent Design and Synthesis

The design of our crosslinking reagents is partitioned into two aspects, the end group functionality used in the binding to the nucleic acids and the linkage which joins the two end groups together. Double helical nucleic acids are uniquely capable of undergoing an intercalative interaction with flat aromatic molecules. Such an interaction, in which the molecule slides between adjacent base pairs of the nucleic acid, is capable of delivering, specifically to the nucleic acid, a molecule which can be designed to undergo a desired chemical reaction at the intercalation site.

Psoralen (3)[26] and p-carboxymethidium (4)[27] represent two classes of molecules which intercalate into DNA (Fig. 2).





psoralen

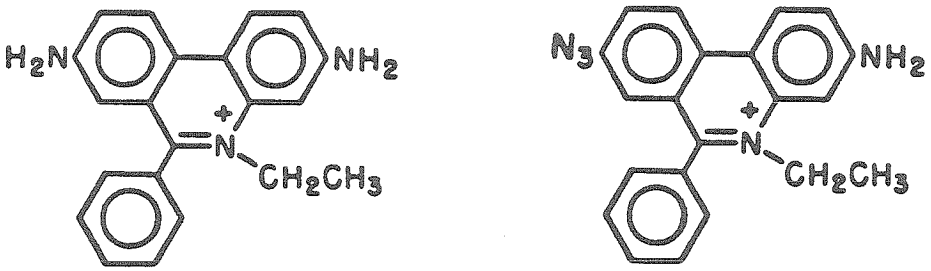
3

p-carboxymethidium
a phenanthridine

4

Figure 2

Psoralens themselves undergo a facile, photoinduced ($320 < \lambda < 380$ nm) ($2\pi + 2\pi$) cycloaddition to pyrimidine bases, effectively binding to both strands of the DNA.[28] An azide derivative (6) of a common phenanthridine, ethidium bromide (5), has been reported which undergoes a photoinduced ($\lambda \geq 400$ nm) covalent reaction with nucleic acids (Fig. 3).[29]



5 6

Figure 3

Both the psoralen and azidophenanthridine therefore would be suitable nucleic acid specific functionalities capable of covalent interaction with DNA.

The second critical aspect of the crosslinking reagent is the selection of an appropriate linker for the molecule. There are several properties that one would like to bestow upon the entire molecule which can be incorporated into the linker portion. Water solubility often becomes a problem when discussing psoralen derivatives and must be considered when designing a bispsoralen reagent.[30] The crosslinker, upon binding to one DNA helix, must reach a neighboring DNA helix. The linkage chosen to date for the crosslinker is a defined length oligoethyleneoxy oligoether.

Because we anticipated the synthesis and characterization of

bispsoralen molecules would be simpler, we initially undertook the synthesis of BPNE (7) shown in Figure 4. X-ray diffraction determinations of interhelix spacings of the packaged DNA in several bacteriophages all found spacings of 25-28Å.[4,5] Length measurements from a CPK model of BPNE suggested a maximum extension of nearly 40Å. Thus a nonaethyleneoxy linker seemed long enough to span contiguous DNA helices within bacteriophage capsids.

Demethylation of xanthotoxin (8) with boron tribromide in methylene chloride followed by deprotonation of the resulting hydroxypsoralen (9) with potassium hydroxide had been carried out by coworkers in an overall yield of 79%. It is reported here for completeness and continuity. Synthesis of nonaethylene glycol (11) was a modification of a previously developed procedure.[31] Condensation of one equivalent of triethylene glyol ditosylate with two equivalents of monosodium triethylene glycolate in excess triethylene glycol resulted in a 32% yield of nonaethylene glycol after distillation. Conversion of the nonaethylene glycol to the bistosylate 12 and condensation with two equivalents of the potassium salt of 8-hydroxypsoralen (10) resulted in a 97% overall yield of bispsoralen-nonaethyleneoxy ether (BPNE) after chromatography on silica gel 60 (methylene chloride:methanol; 80:20).

Initial efforts in our laboratories at Caltech to crosslink covalently closed circular DNA with BPNE proved unsuccessful. Collaborative efforts to crosslink intact bacteriophage λ with BPNE in the laboratory of Professor Charles Cantor (Columbia University) were also unsuccessful (1978). We therefore undertook the synthesis of a bis(monoazidophenanthridine) crosslinking reagent, a more serious

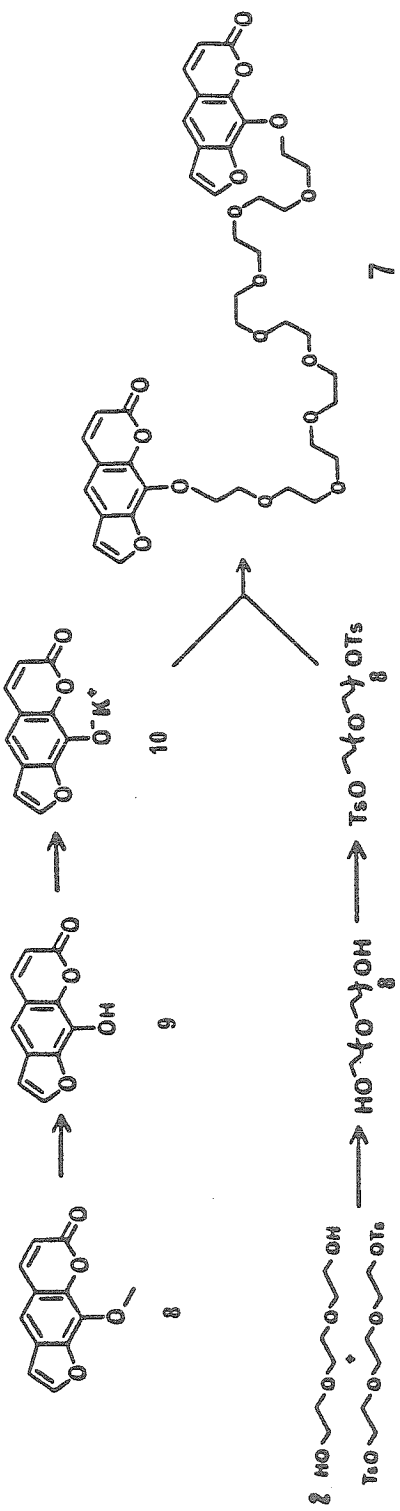


Figure 4

synthetic task. The azidophenanthridine chromophore is known to have a higher binding affinity (10^5 vs $10^3 M^{-1}$)[32] and a higher photobinding efficiency to double helical DNA than the psoralen chromophore.[33] The nonaethyleneoxy ether linker was retained in the new reagent. With such a reagent we have been successful in our interhelical crosslinking attempts.

The synthesis of bis(monoazidomethidium)octaoxahexacosanediamine (BAMO) (13), is shown in Figure 5.[34] This route required the prior synthesis of the monoazide derivative of p-carboxymethidium chloride.[35] All manipulations in the preparation and utilization of azidomethidium derivatives were performed under red light illumination. Diazotization of p-carboxymethidium chloride with isopentylnitrite in 0.2 M aqueous HCl at 0°C followed by reaction with sodium azide yielded the light-sensitive monoazido-p-carboxymethidium chloride (14) after chromatography on carboxymethyl cellulose (cellex CM, pH 2.8 aq HCl eluant). Literature ethidium azide syntheses were done in pH 1.6 aq HCl solutions (0.025M).[35] Para-carboxymethidium does not diazotize cleanly under these conditions and required a higher acid concentration to form the diazonium ion in solution. Although the monoazido-p-carboxymethidium chloride formed in this reaction has been characterized as the monoazide derivative through spectral and elemental analyses, it has recently been resolved into two spots on TLC plates (Cellulose, eluted with isopropanol: 0.025 M sodium phosphate pH 7, 50:50). We suspect our synthesis results in the formation of both monoazide isomers at the C3 and C8 positions on the chromophore (see Fig. 6). However, without further characterization

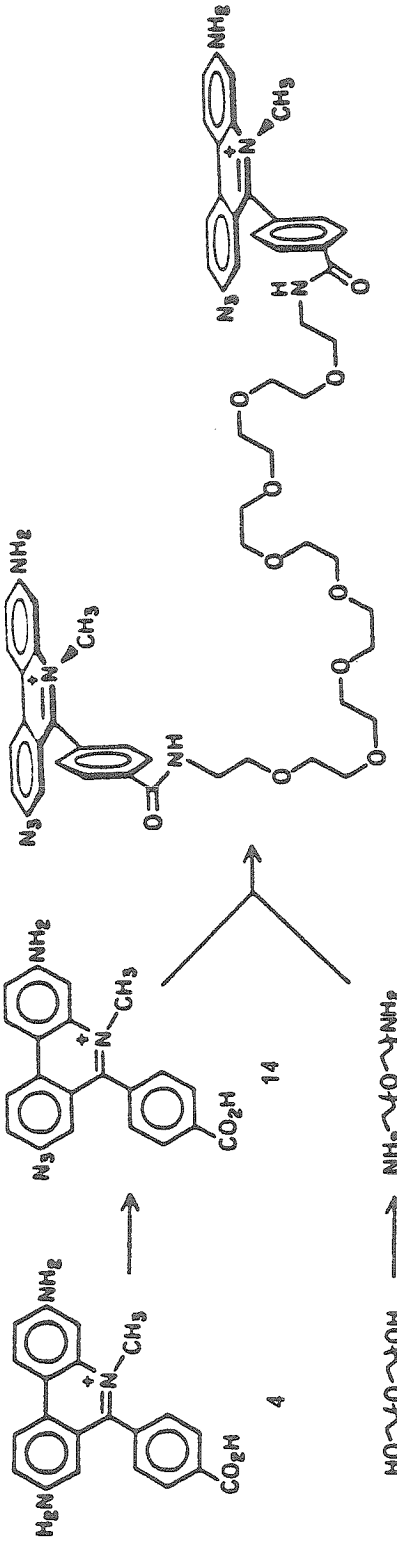


Figure 5

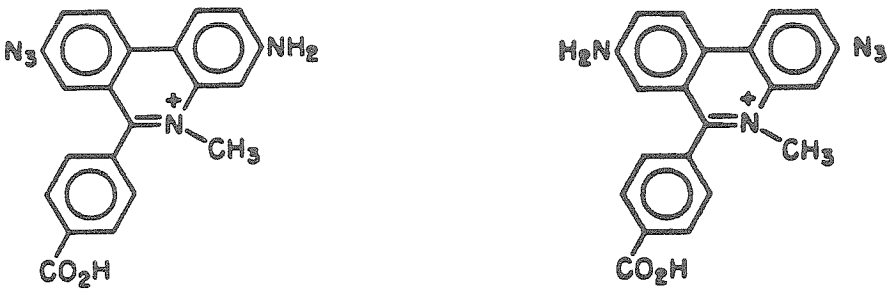


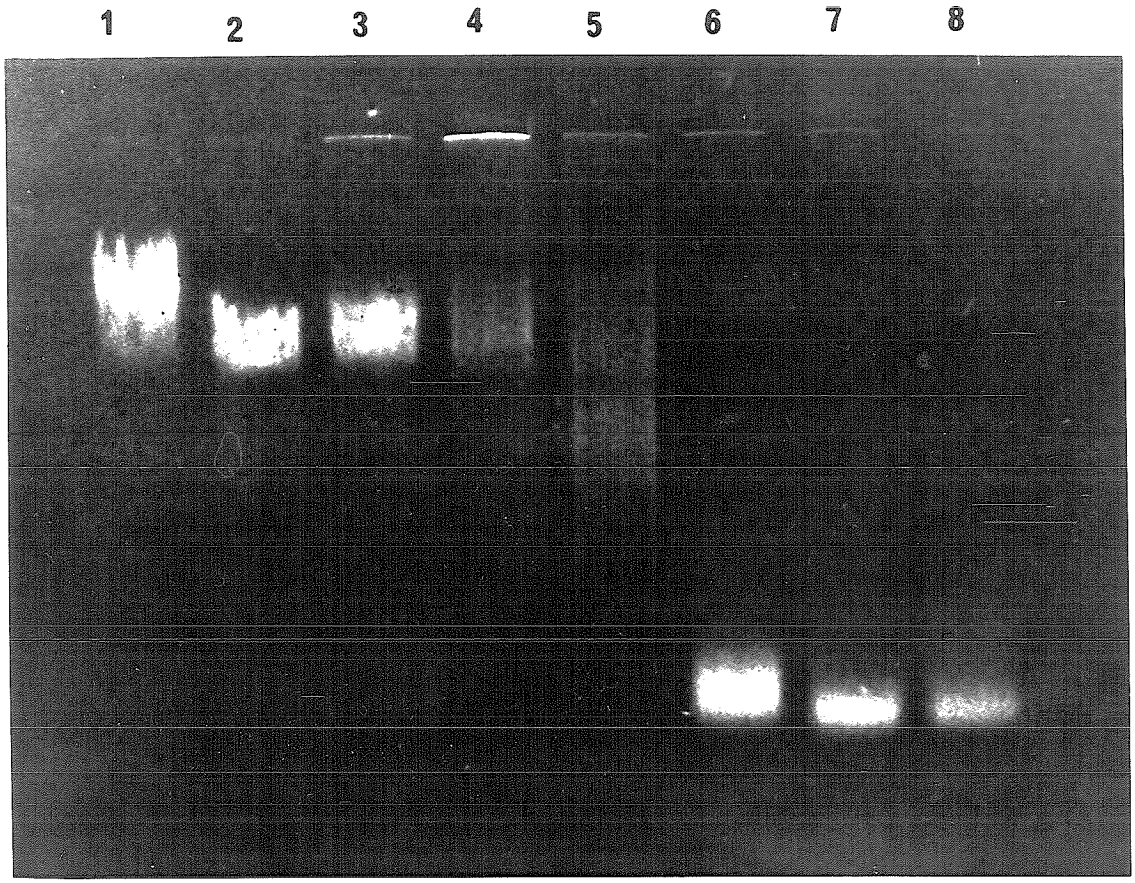
Figure 6

of these compounds such as X-ray data we continue to represent the product as the C8 derivative by analogy to the X-ray structure determination of ethidium monoazide.[37] The linker was synthesized by successive treatment of nonaethylene glycol (11) with p-toluene sulfonyl chloride, (pyridine, 0°C), potassium phthalimide (DMF, 90°C), and hydrazine hydrate (ethanol at reflux); yielding octaoxahexacosanediamine (15) after neutralization and workup. The reaction of 0.5 equivalents of diamine 15 with the acylimidazole ester of monoazido-p-carboxymethidium in dimethyl sulfoxide at 25°C for 24 hours yielded the light-sensitive deep red compound bis(monoazidomethidium)octaoxahexacosanediamine (BAMO) (13), which was purified by successive chromatography on silica gel (230-400 mesh ASTM) eluting with 0.006% methanolic HCl, then amberlite (XAD-2) first washing with water then eluting with methanol. Tritium labeled BAMO ([^{3}H]BAMO) (16) was synthesized similarly using tritium labeled diamine linker which was labeled by catalytic exchange at New England Nuclear Corp. (Boston, MA).

II. BAMO Crosslinking Assay (No Restriction Endonuclease Cleavage)

The ability of BAMO to effect interhelical crosslinking has been studied by gel electrophoretic and electron microscopic techniques. DNA isolated from BAMO crosslinked bacteriophage λ exhibits a faster mobility on 0.3% agarose gels than that of uncrosslinked λ DNA (Fig. 7). Compact forms of DNA have been shown to migrate faster than noncompact forms of DNA.[38] This higher mobility is interpreted to mean that the λ phage DNA, after crosslinking with BAMO, is unable to completely untangle upon bursting of the phage capsid and digestion of the capsid proteins. Presumably interhelical crosslinks are responsible for the maintenance of the compact form because photolysis of λ phage with ethidium azide does not result in DNA with altered electrophoretic mobility.[39] The correlation between increased crosslinking and increased binding ratio of BAMO is also evidenced on this gel (Fig. 7) by a trend of increased electrophoretic mobility with increased binding ratio.

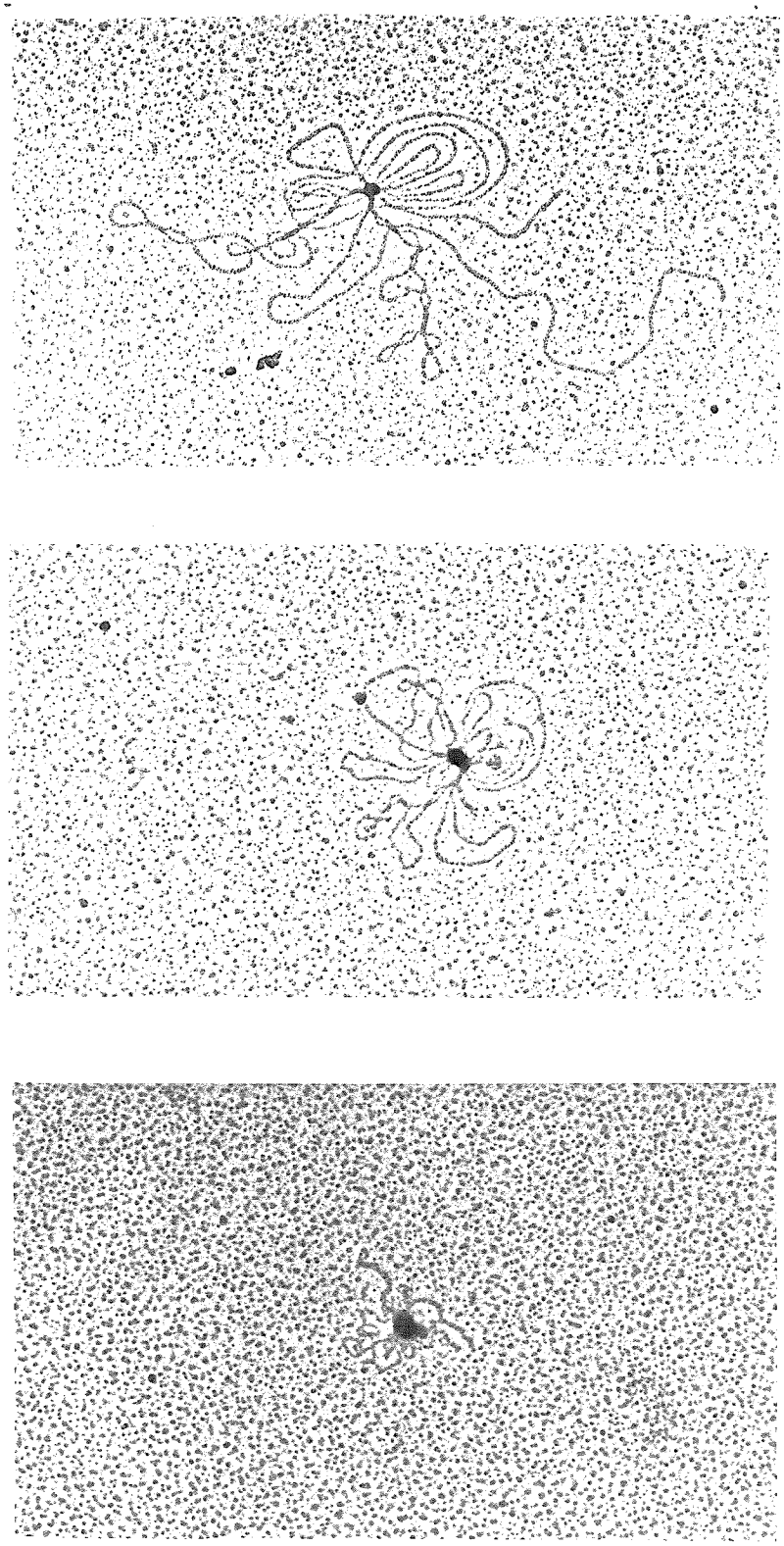
Electron microscopic analysis also provides evidence of the observation that a higher binding ratio results in a more compact form of λ phage DNA. Representative photomicrographs presented on the following page are from crosslinked samples in the binding ratio range of r=0.005 to r=0.2 BAMO/bp. These data provide supporting evidence that BAMO is capable of forming covalent interhelical crosslinks. However, such densely tangled structures are not helpful in providing data for a map of nearest neighbor interhelical contacts. Map data must be obtained from structures seen under the electron microscope which allow measurements defining the position of the crosslink.



0.3% agarose gel of BAMO crosslinked phage lambda at 3.0×10^{-4}M bp.

1) no BAMO
2) r = 0.005 BAMO/bp
3) r = 0.01
4) r = 0.02
5) r = 0.03
6) r = 0.05
7) r = 0.1
8) r = 0.2

Figure 7


INCREASING BAMO/bp

III. BAMO Crosslinking Assay (Restriction Endonuclease Cleavage)

Using low binding ratios, $r \leq 0.01$, DNA isolated from BAMO crosslinked λ phage exhibits a gel mobility identical to that of uncrosslinked λ phage DNA. λ DNA crosslinked at these low binding ratios also is a functional substrate for a variety of restriction endonucleases. Restriction maps for the enzymes used in these studies are shown in Figure 8.[40,41] If two regions of the λ phage genome which become crosslinked, lie on different restriction fragments, the resulting electron microscopic observation should be two overlapping restriction fragments. Representative photomicrographs of such structures are shown on the following pages. Observed crosslink frequencies for several restriction enzymes are presented in Table 1. An observed DNA species was scored as a "linear restriction fragment" when it exhibited only two ends, as a "crosslinked structure" when 2M distinct ends (where $M > 1$) were observed continuously connected or as a "rosette" when the DNA species was tangled to such an extent that the path of the DNA could not be distinctly followed. BAMO crosslinked phage yielded 16-17% crosslinked structures and 10% rosettes in studies with three different restriction endonucleases. Control experiments either in the absence of BAMO or in the presence of ethidium monoazide (EN_3) yield only 2-3% crosslinked structures and no rosettes. These results are presented in Table 1.

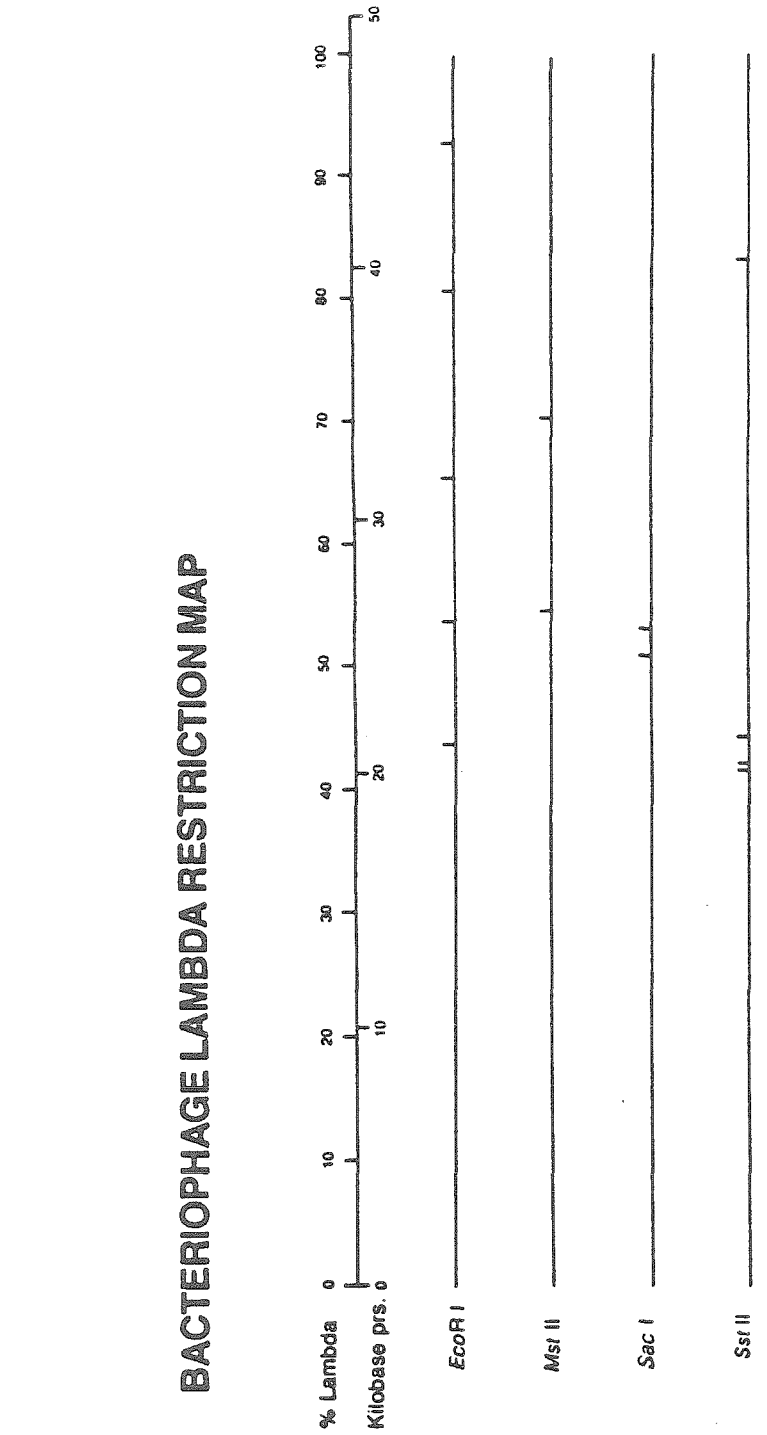
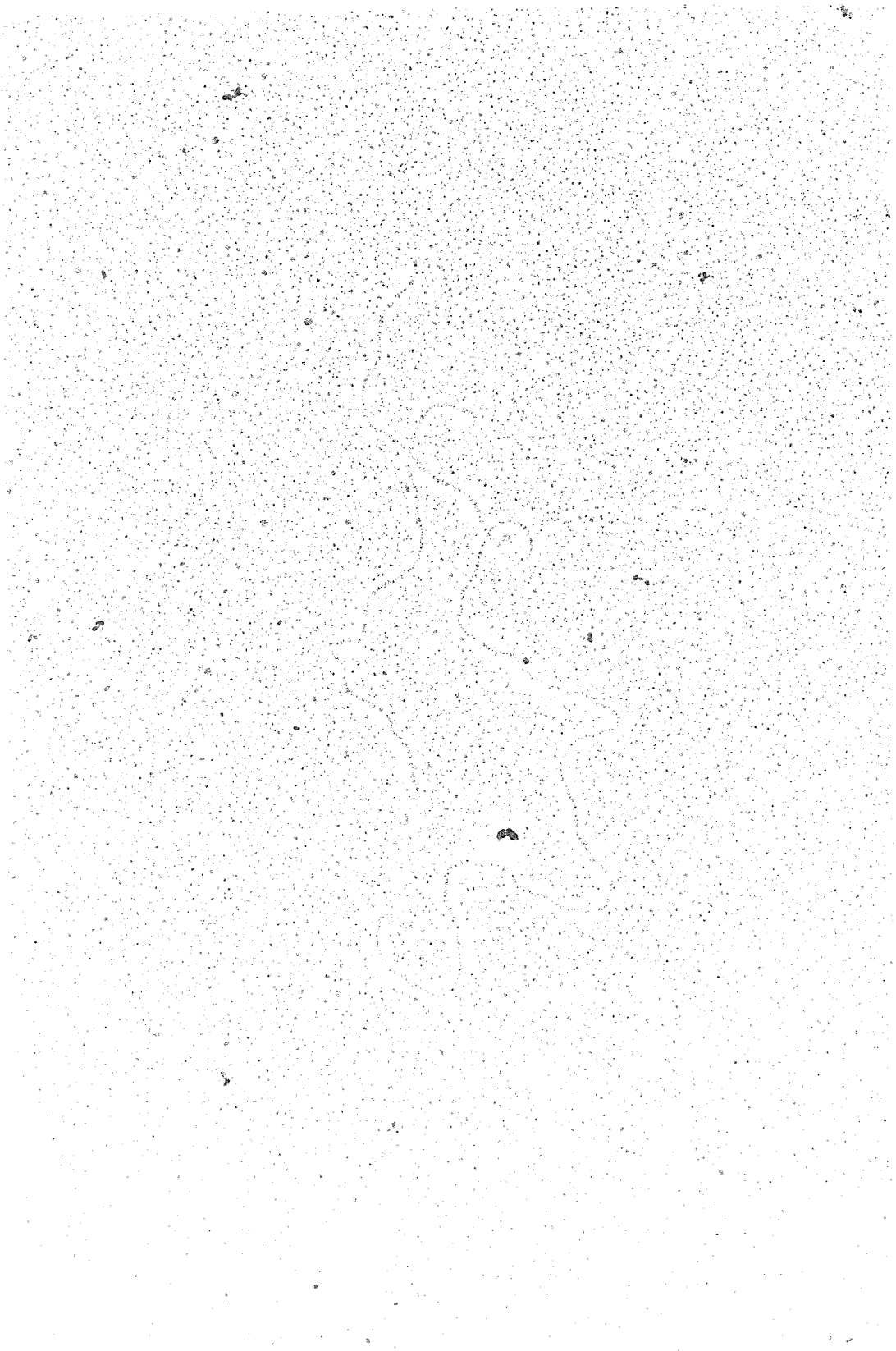
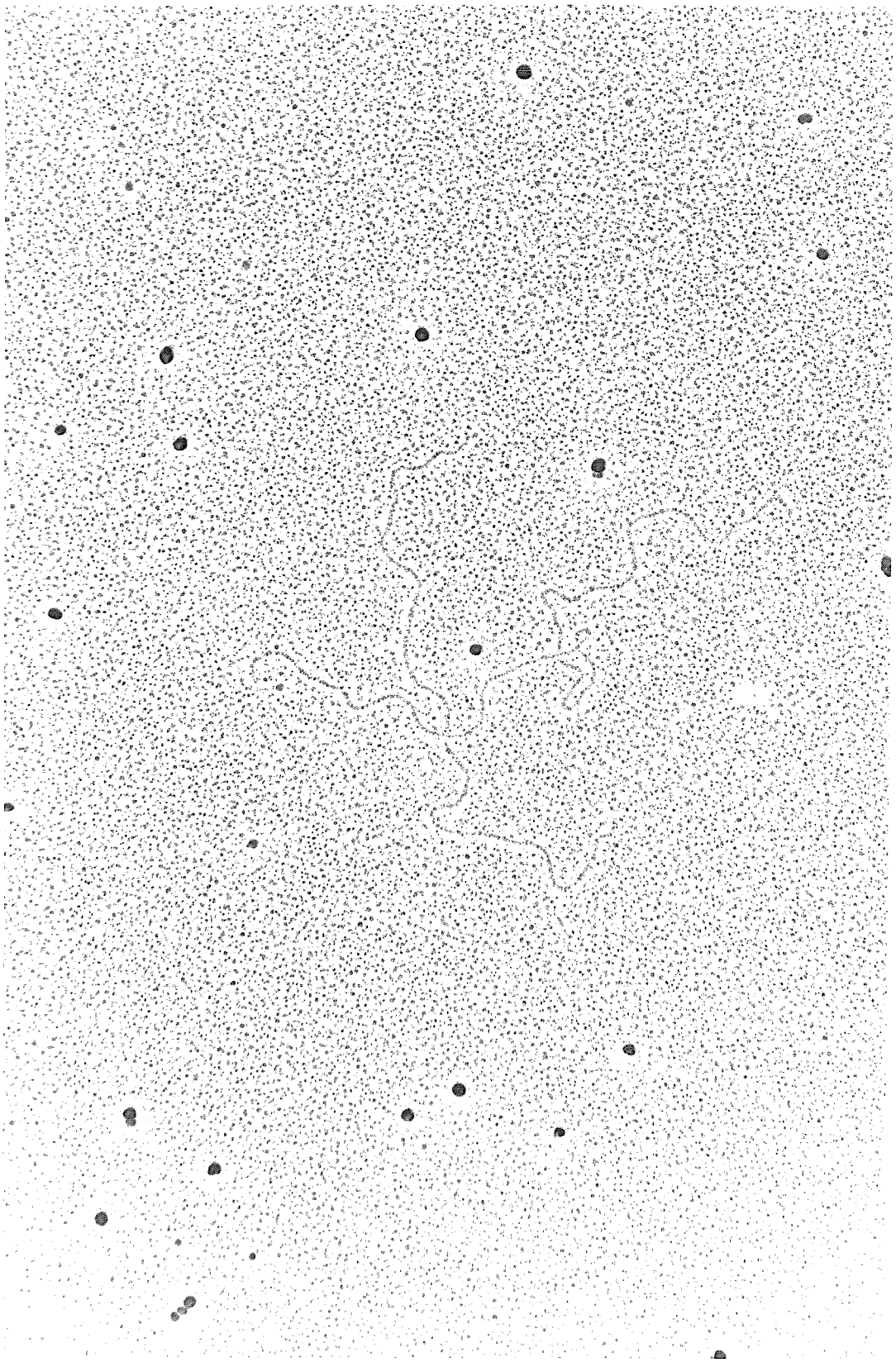
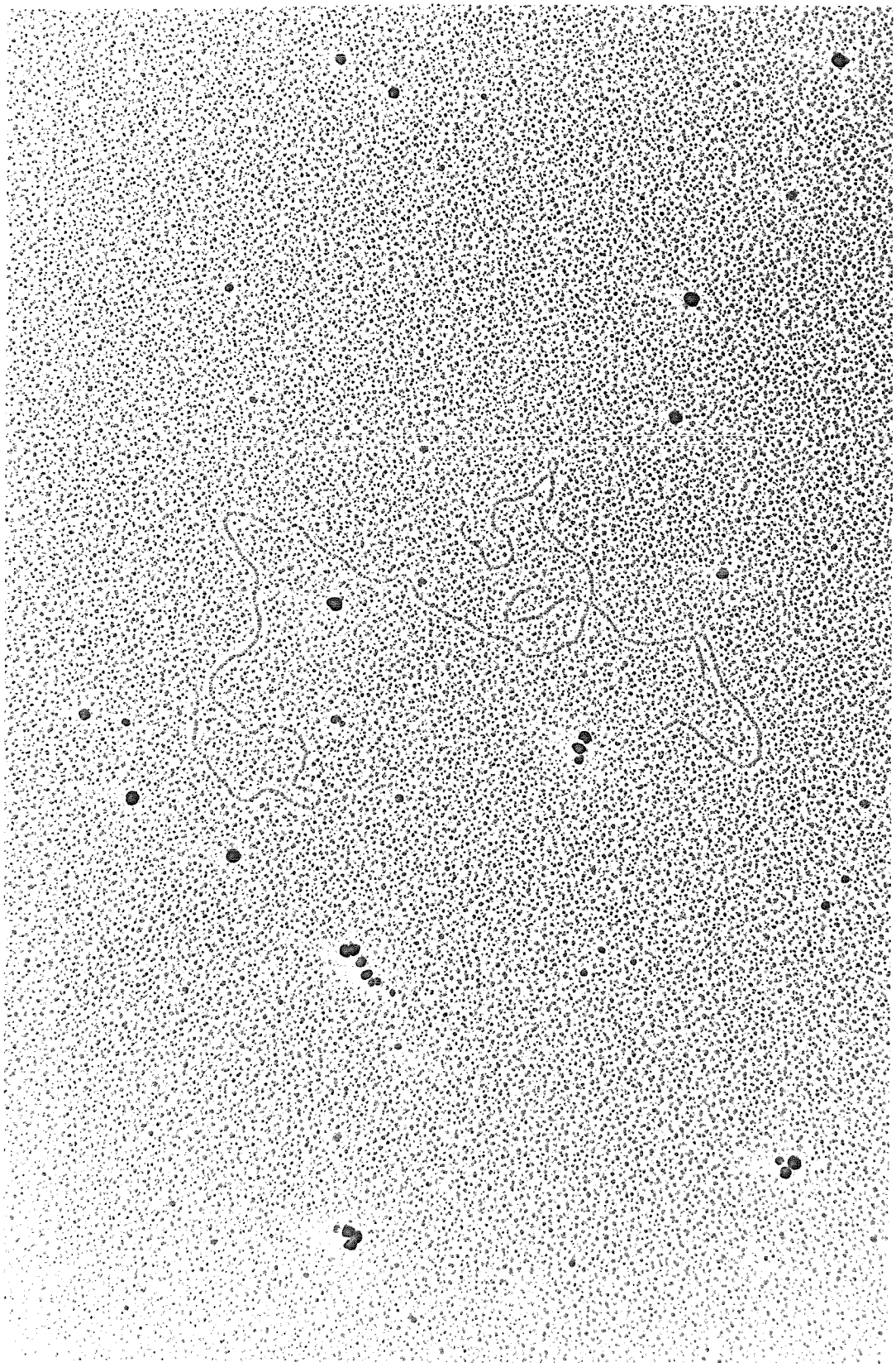

BACTERIOPHAGE LAMBDA RESTRICTION MAP
% Lambda
Kilobase prs.
EcoR I
Msl II
Sac I
Sst II

Figure 8







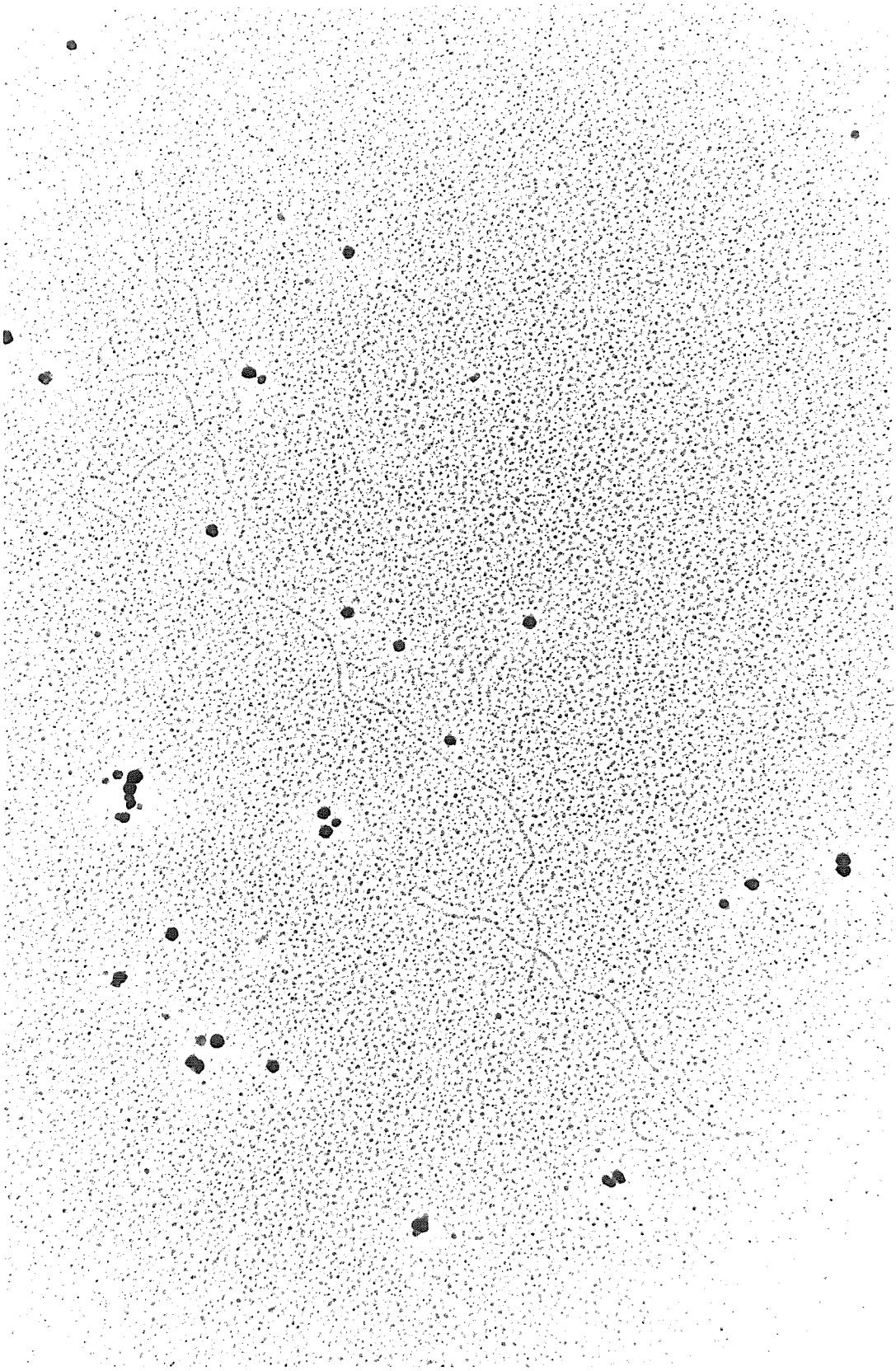


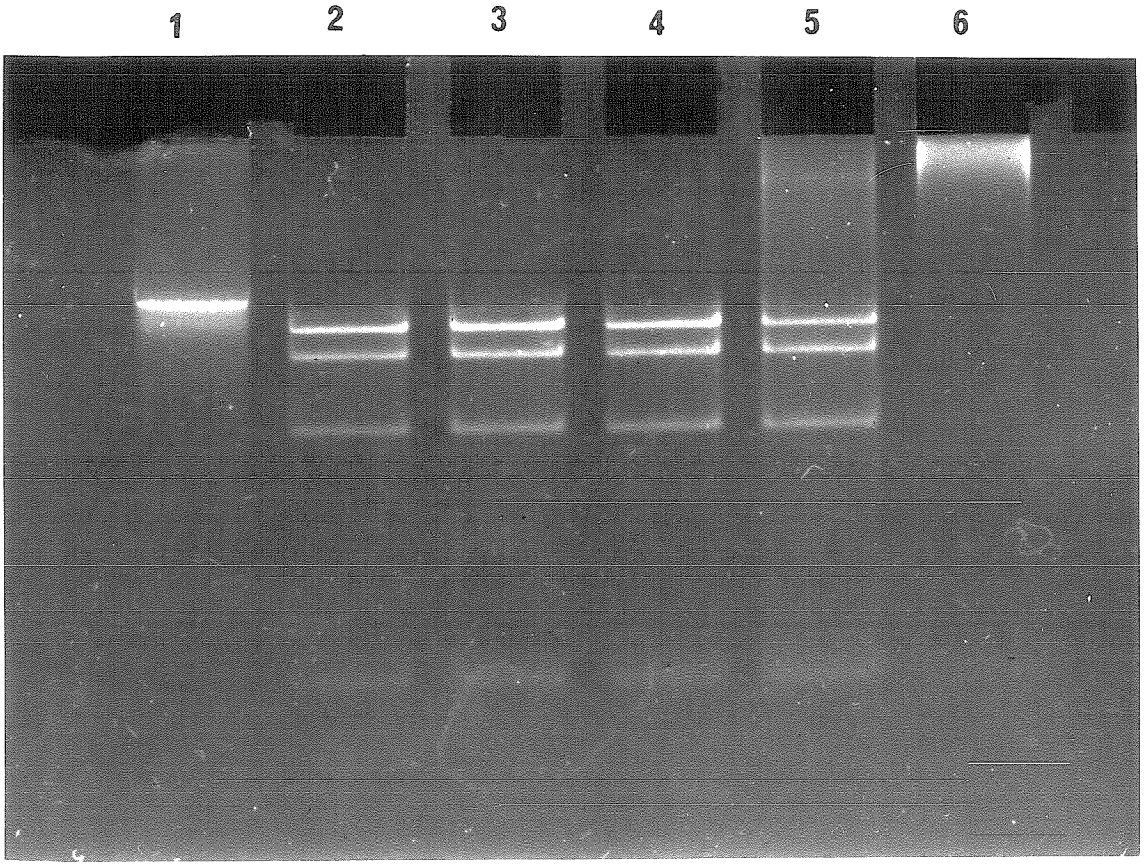
TABLE 1

Crosslinking Assay with Restriction Endonuclease Cleavage

Restriction Endonuclease	N[a]	Reagent	% Linear Fragments	% Crossed Structures	% Rosettes Remaining
Mst II	3	BAMO[c]	73	17	10
		—[b,c]	97	3	0
Sac I/Sst II	4	BAMO[d]	74	16	10
		—[b,d]	98	2	0
Eco Rl	6	BAMO[c]	83	17	0
		EN_3[c]	98	2	0
		—[b,c]	98	2	0

(a) N is the number of restriction fragments obtained by digestion of phage DNA with the listed restriction enzyme system.
(b) a — indicates a control in the absence of any reagent.
(c) 500 DNA species counted.
(d) 200 DNA species counted.

Agarose gel electrophoresis (0.8%) of DNA restriction fragments from crosslinked intact phage at this low binding level (r=0.01) yields predominantly the standard restriction fragment pattern. However, the presence of a faint smear migrating slower than full length λ DNA is visible. No distinct banding within this smear is discerned for phage λ DNA (see Figure 9). Electron microscopy identifies this slower moving DNA as crossed restriction fragments.



0.8% agarose gel of BAMO crosslinked phage lambda at 3.0×10^{-4}Mbp restriction cleaved with SacI/SstII.

1) noBAMO and no SacI/SstII
2) r = 0.005 BAMO/bp
3) r = 0.01
4) r = 0.025
5) r = 0.05
6) r = 0.1

Figure 9

IV. Radiolabeled BAMO Photobinding Experiments

Evaluation of the quantity of BAMO covalently bound to DNA in intact bacteriophage λ was carried out using tritiated BAMO synthesized as described above. The three experimental variables studied were: length of incubation time in the dark, length of photolysis time, and binding ratio (BAMO/bp). The results of these studies are plotted in figures 10, 11, and 12. It is seen from Fig. 10 that BAMO equilibrates with the packaged DNA in less than 1 hour. This observation is consistent with the high degree of ionic permeability bacteriophage λ exhibits with respect to osmotic shock[42] and loss of polyamine content when dialyzed against divalent cations.[43] Although Figure 11 shows a slight positive slope with respect to photolysis time, BAMO is effectively photolyzed within 30 minutes under the low intensity irradiation conditions used. This result demonstrates the high photolability of the methidiumazide moiety. Binding ratio experiments were performed under the standard experimental conditions of 2 hour incubation in the dark followed by 1 hour photolysis. As shown in Fig. 12, the covalent attachment of BAMO to intact λ phage DNA follows a linear dependence with respect to binding ratio. The slope of the least squares line in Fig. 12 is 0.063 indicating that in the range of 0.005 to 0.05 BAMO/bp, a constant 6.3% of the total BAMO initially in solution becomes covalently attached to the bacteriophage DNA.

These results can be used to give a qualitative understanding of the efficiency of BAMO as a crosslinking reagent. Although covalent binding of BAMO to DNA does not necessarily result in interhelical crosslinking, the level of covalent binding does yield an upper limit

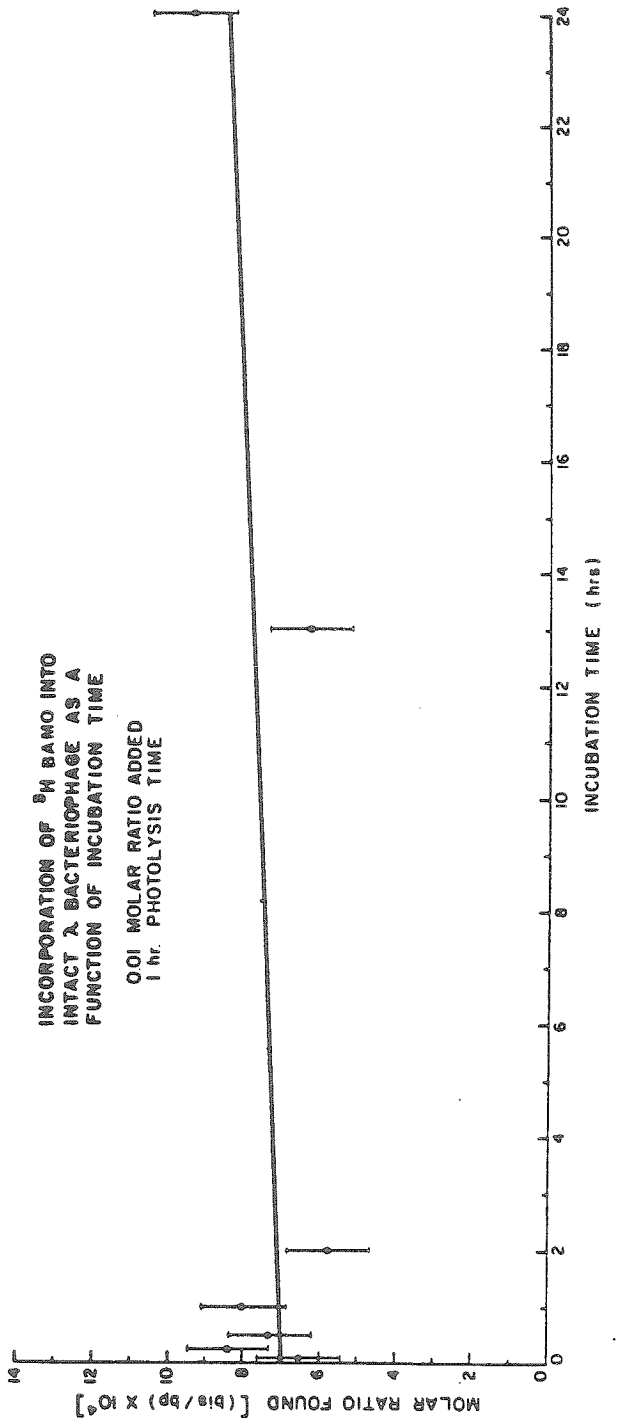

INCORPORATION OF ^{3}H BAMO INTO INTACT λ BACTERIOPHAGE AS A FUNCTION OF INCUBATION TIME
0.01 MOLAR RATIO ADDED
1 hr. PHOTOLYSIS TIME
MOLAR RATIO FOUND [(bis/bp) x 10^{4}]
INCUBATION TIME (hrs)
0
2
4
6
8
10
12
14
16
18
20
22
24

Figure 10

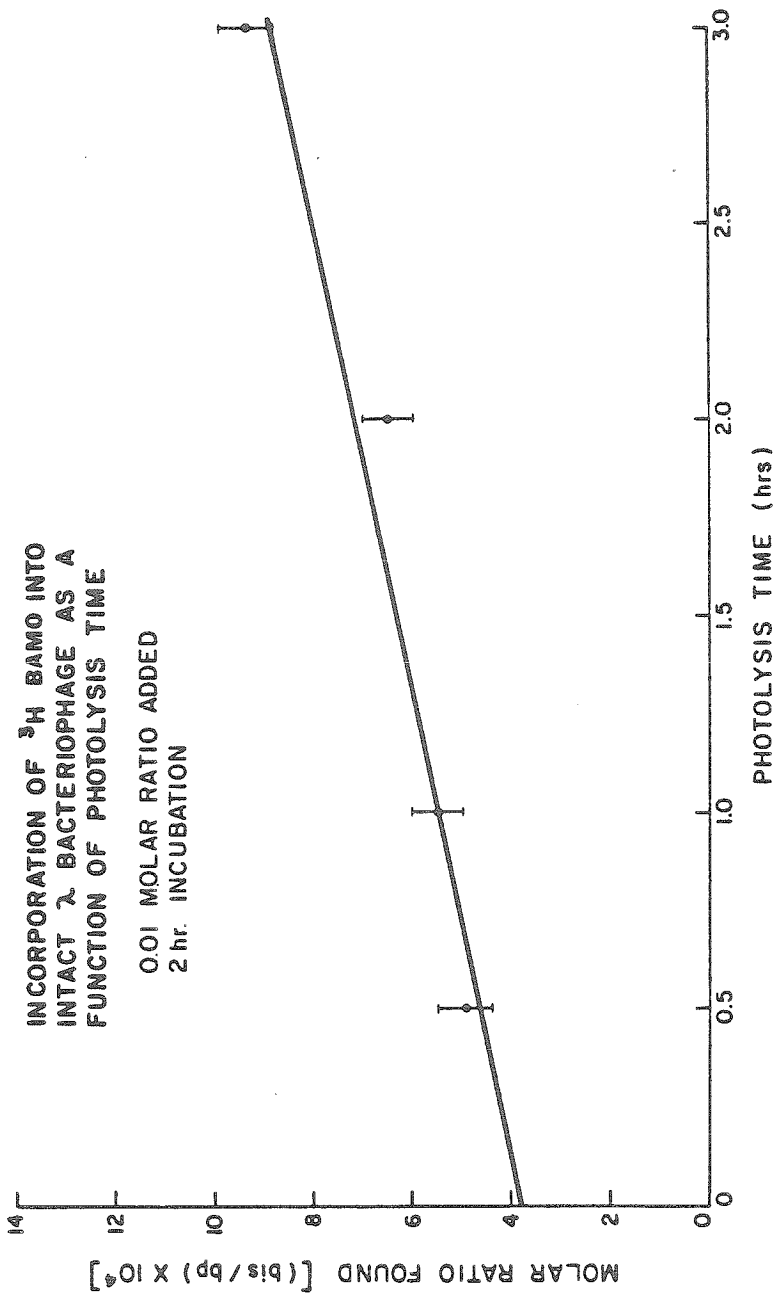

INCORPORATION OF ³H BAMO INTO INTACT λ BACTERIOPHAGE AS A FUNCTION OF PHOTOLYSIS TIME
0.01 MOLAR RATIO ADDED
2 hr. INCUBATION
MOLAR RATIO FOUND [(bis/bp) x 10⁴]
PHOTOLYSIS TIME (hrs)
0
2
4
6
8
10
12
14
0
0.5
1.0
1.5
2.0
2.5
3.0

Figure 11

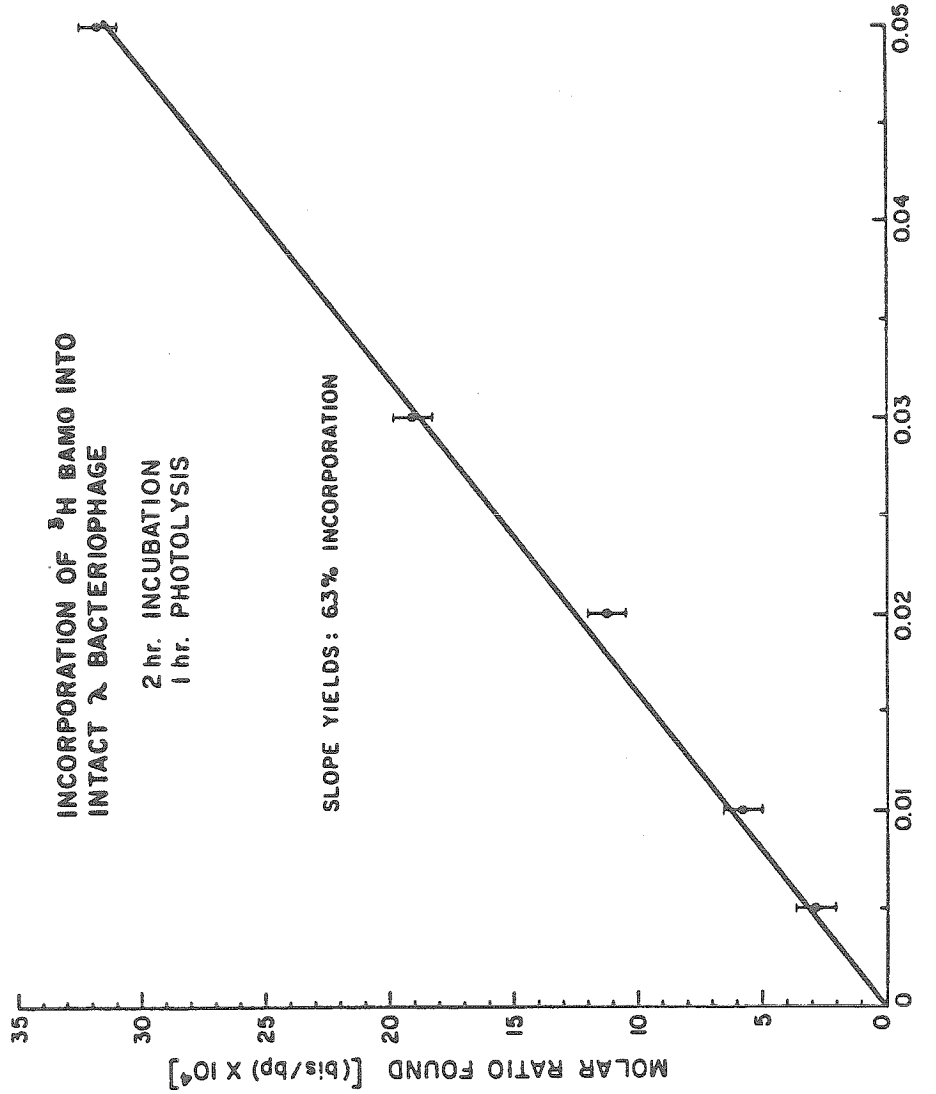

INCORPORATION OF ^{3}H BAMO INTO
INTACT λ BACTERIOPHAGE
2 hr. INCUBATION
1 hr. PHOTOLYSIS
SLOPE YIELDS: 63% INCORPORATION
MOLAR RATIO FOUND [(bis/bp) X 10⁴]
MOLAR RATIO ADDED (bis / bp)
0
5
10
15
20
25
30
35
0.01
0.02
0.03
0.04
0.05

Figure 12

for the number of molecules required for a given observed crosslinking level. For example, a binding ratio of r=0.01 BAMO/bp results in a covalent binding level of 0.00063 BAMO/bp. Since bacteriophage λ consists of approximately 48,000 bp an upper limit of (0.00063x48,000 = 30) thirty molecules of BAMO/genome gives rise to the crosslinking level observed at that binding ratio. As previously discussed, an incubation binding ratio of r=0.01 affords 15-20% crosslinked species seen under the electron microscope for restriction enzyme systems that cleave at 2,3 and 5 sites.

The system generating 6 fragments affords one crosslinked

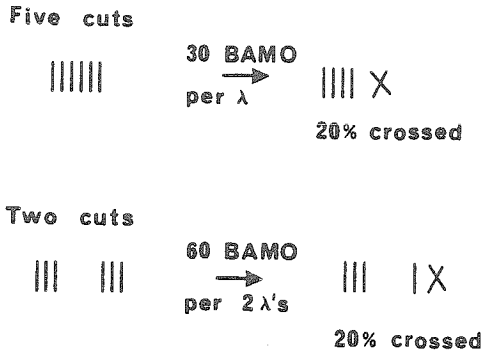


Figure 13

structure per genome or one crosslink per 30 BAMO molecules. The system generating 3 fragments yields one crosslinked structure for every two genomes or one structure per 60 BAMO molecules. It's not unreasonable to assume that a portion of the remaining 29/30 or 59/60 molecules also result in interhelical crosslinks which, however, are not assayed by the particular restriction enzyme employed. Experiments cleaving λ DNA into many more than 6 fragments would probably result in an even higher apparent crosslinking efficiency

than one per thirty.

Control experiments were performed on nonirradiated samples of tritiated BAMO incubated with bacteriophage λ. Results indicated that the experimental protocol resulted in efficient removal of noncovalently bound BAMO from the DNA.

V. End Labeling of Restriction Fragments

Determination of the unique position of each interhelical crosslink on the λ phage genome requires the ability to assign the directionality or polarity of each restriction fragment on electron photomicrographs. Without directionality a given observed cross represents a set of four potential unique crosses as shown by the schematic drawings in Figure 13. Only one of

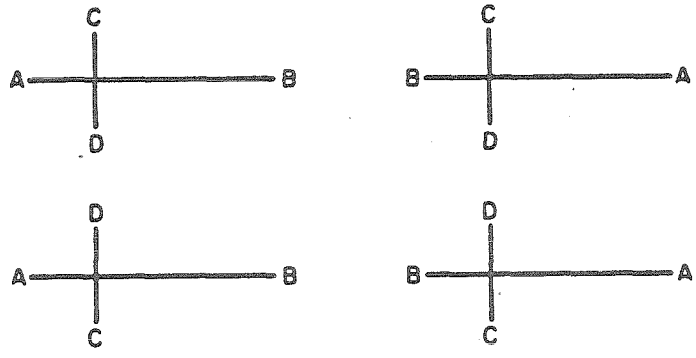


Figure 14

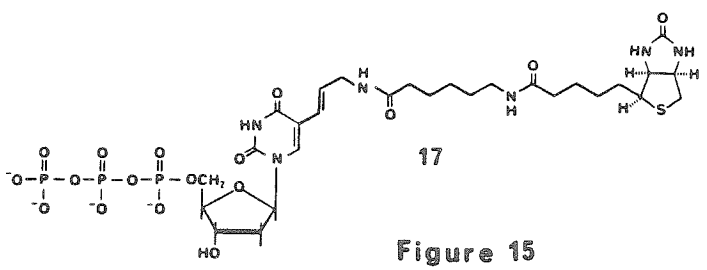
the restriction fragment alignments within the set of four uniquely defines an interhelical crosslink position. There are currently no generally useful methods available for solving this directionality or alignment problem for DNA restriction fragments in the literature. In fact, the interhelical crosslinking data from the laboratory of Professor Charles Cantor suffer from this lack of directionality, making interpretation of their data difficult at best.[24] Recently, Wollenzien and Cantor have developed a tedious method for determining the directionality of E.coli 16S rRNA.[44] Their method involved the generation of a hybridization probe labeled with psoralen monoadducts. Photolysis of samples after hybridization covalently links the probe to the complementary RNA, thus labeling it at a unique position. The unique position of the label along the RNA sequence defines the

directionality of the entire RNA. Unfortunately, this method suffers from low labeling efficiency.

We have developed a protocol which solves this alignment problem by generating restriction fragments uniquely labeled at one specific end with an electron dense label. By knowing which end is labeled, from knowledge of the genomic restriction map, the directionality of each restriction fragment is defined.

It has been shown that polymethacrylate spheres prepared by emulsion polymerization and covalently labeled with avidin can serve as useful electron dense labels for biological macromolecules containing biotin.[44] Our labeling procedure takes advantage of the high binding affinity ($K=10^{16}-10^{17}$) between the biotin moiety and the protein avidin.[43] The resulting biotin-avidin complex is stable to normal denaturing conditions, such as high salt concentration, 99% formamide, 8 M urea and 6M guanidine·HCl.[45,46]

Recently, the ability of several nucleic acid polymerases to utilize as substrate a modified deoxyuridine triphosphate containing the biotin functionality has been reported.[46] The biotinylated dUTP(Bio-dUTP) (17) derivative used in our studies is shown in Fig. 15. Although Bio-dUTP is currently available commercially (Enzo Biochem., Inc., New York, NY) its synthesis, developed prior to its availability, is reported in the experimental section.



17

Figure 15

The ends of DNA restriction fragments can be efficiently labelled with Bio-dUTP using bacteriophage T4 DNA polymerase under standard enzymatic conditions.[47,48] Figure 16 details the protocol developed for generating restriction fragments labeled specifically at one end suitable for directional measurement on electron photomicrographs. After incubating the biotinylated restriction fragments with avidin spheres and spreading for electron microscopy using the modified Kleinschmidt technique; up to 50% of the restriction fragments appear labelled at one end under the electron microscope. Applying this procedure to BAMO crosslinked λ phage samples generates a population of crosslinked DNA restriction fragments, some of which have two of the four ends of the cross labelled. Representative photomicrographs of doubly end labeled crosses are presented on the following pages. A two dimensional representation of the unique interhelical crosslink locations, a nearest neighbors map, is shown for bacteriophage λ in Figure 17.

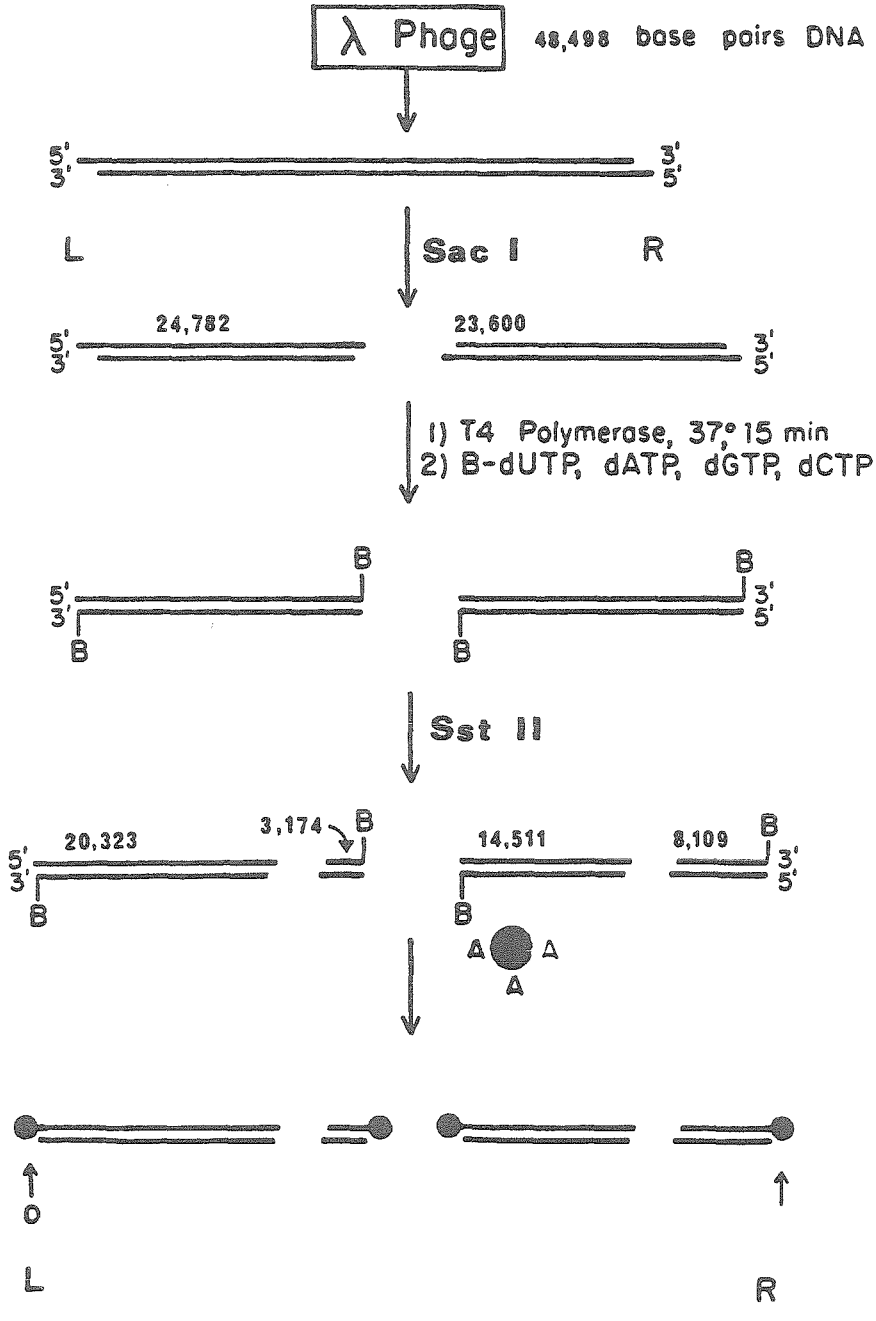
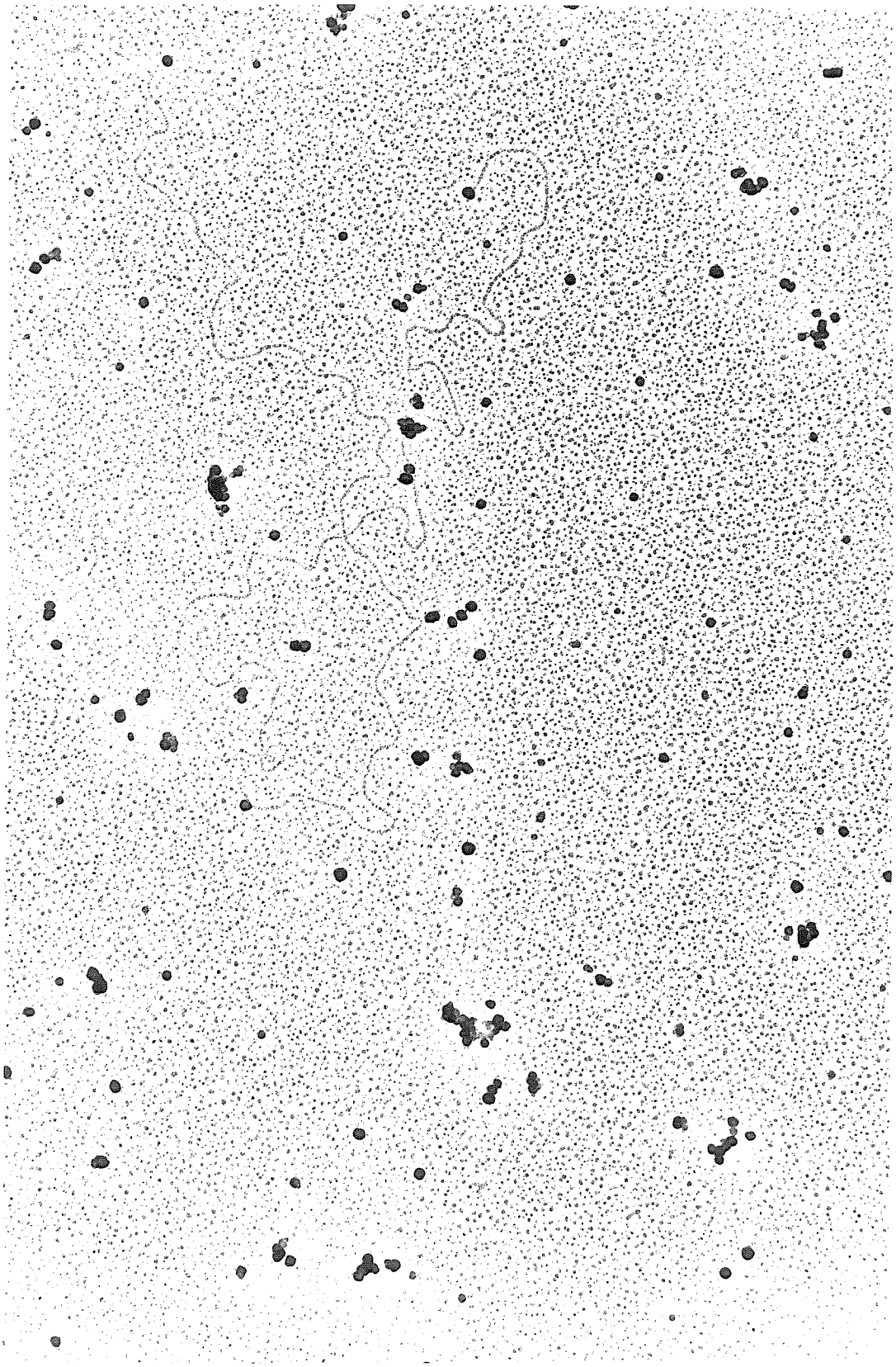
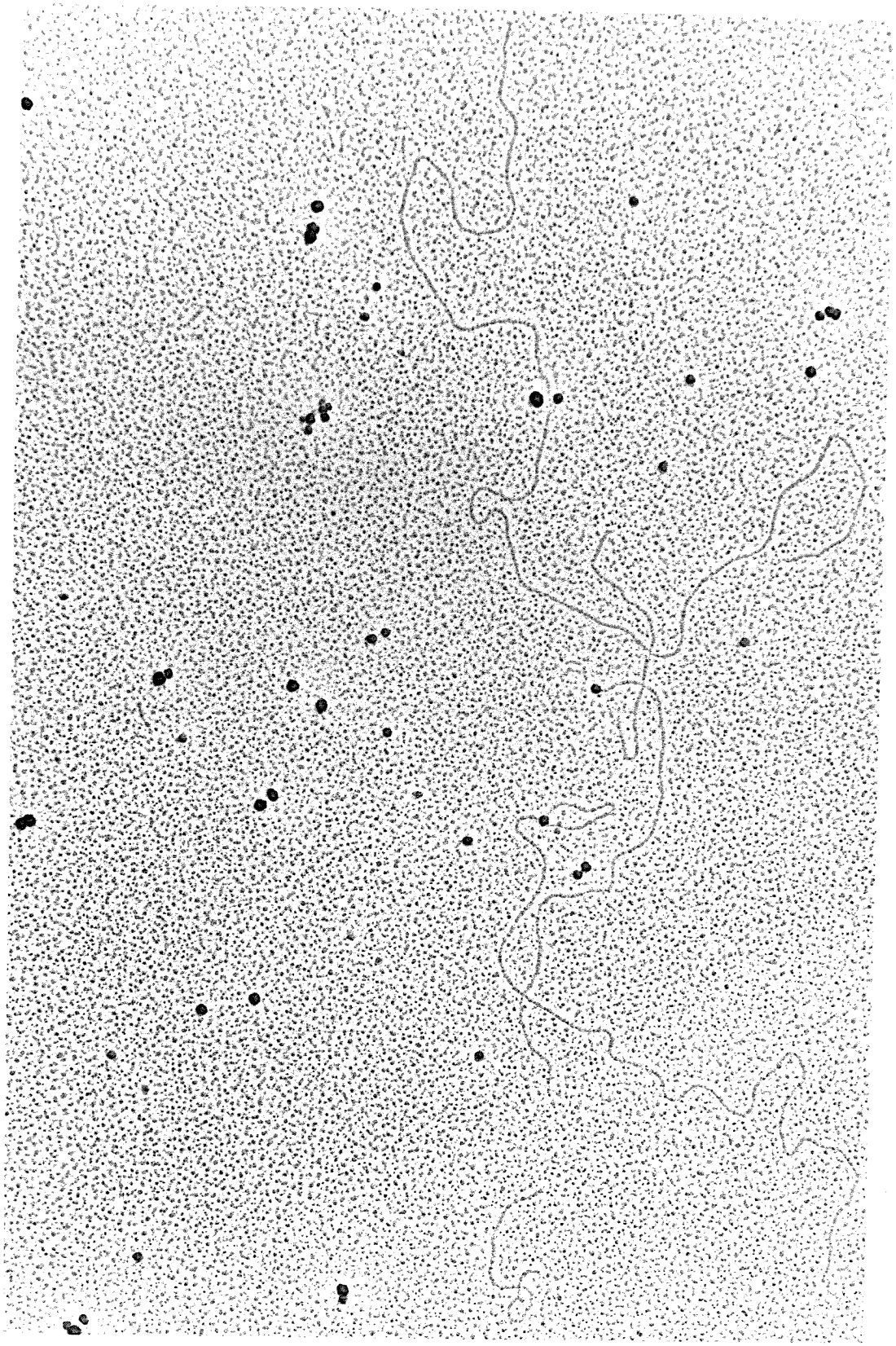
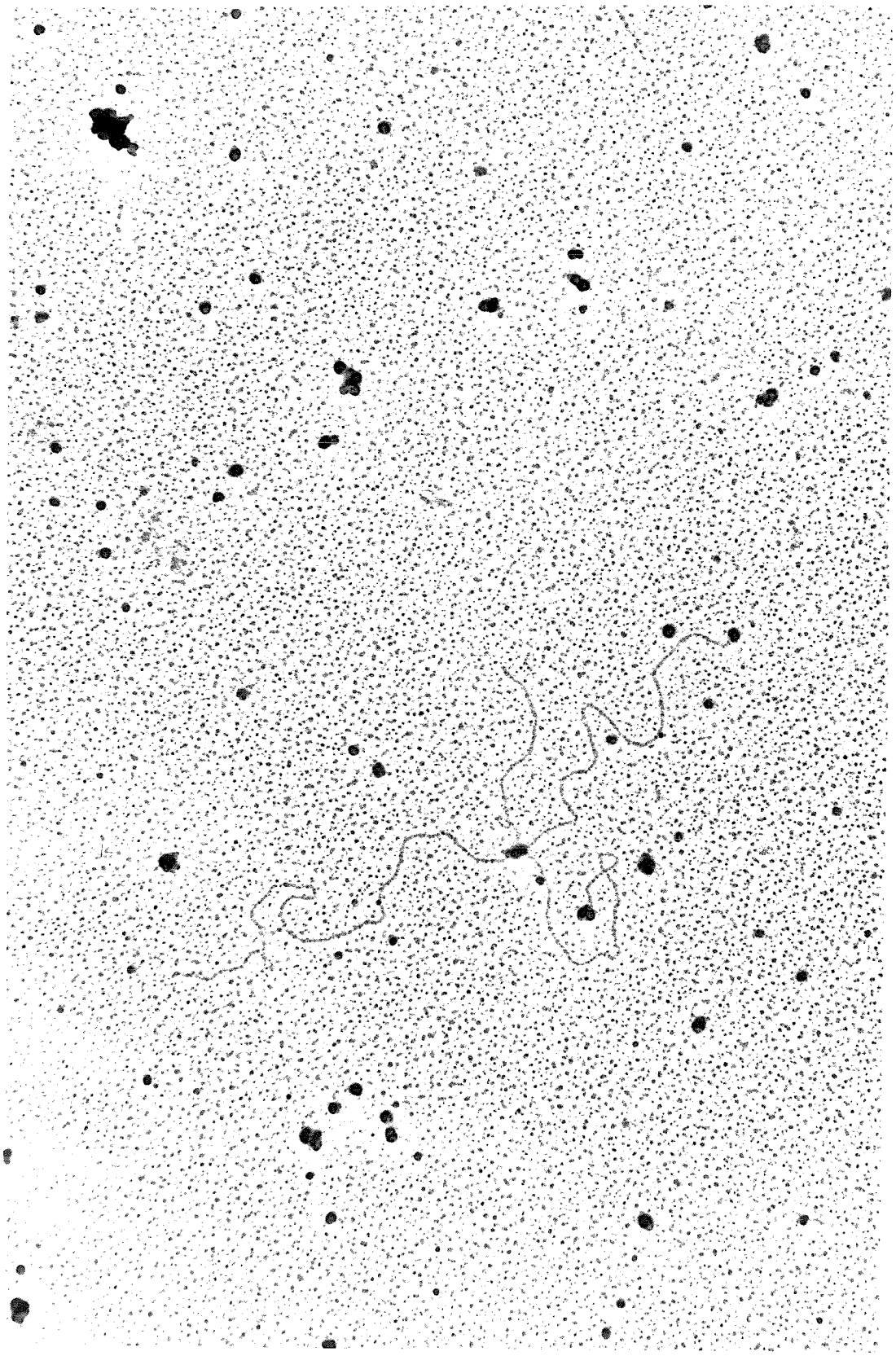
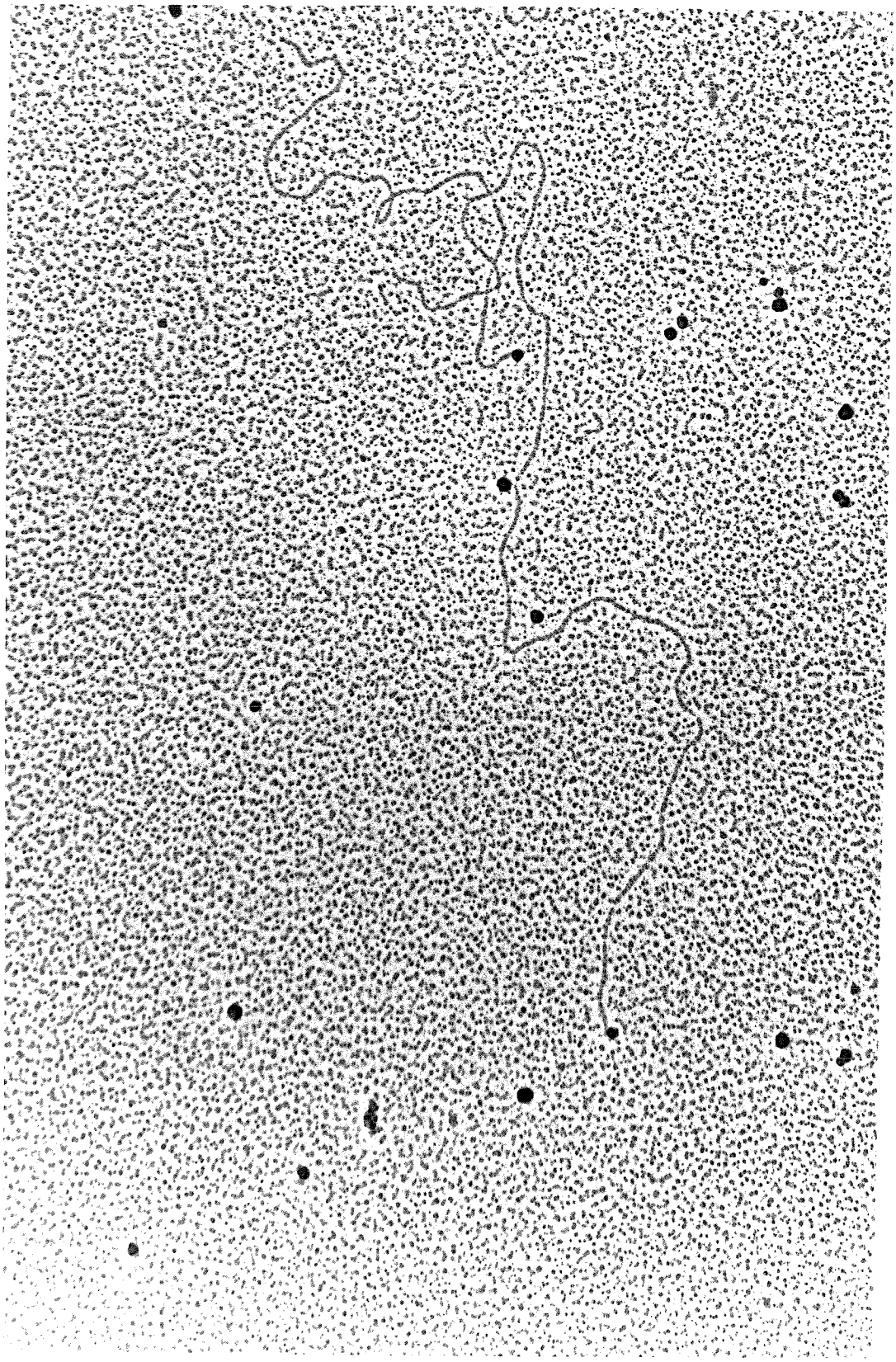

λ Phage
48,498 base pairs DNA
5'
3'
3'
5'
L
Sac I
R
24,782
23,600
1) T4 Polymerase, 37°, 15 min
2) B-dUTP, dATP, dGTP, dCTP
B
Sst II
20,323
3,174
14,511
8,109
A
O
L
R

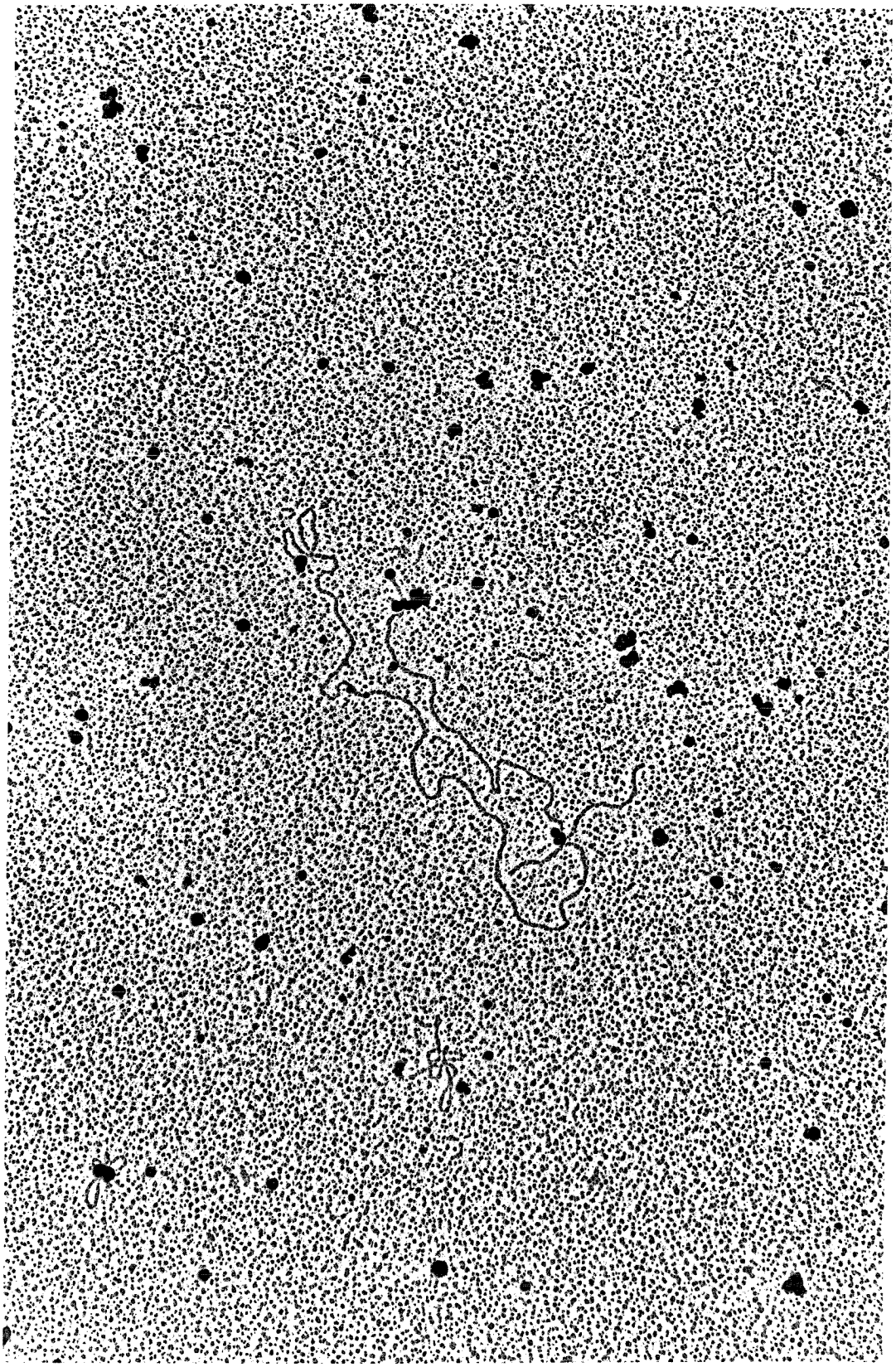
Figure 16

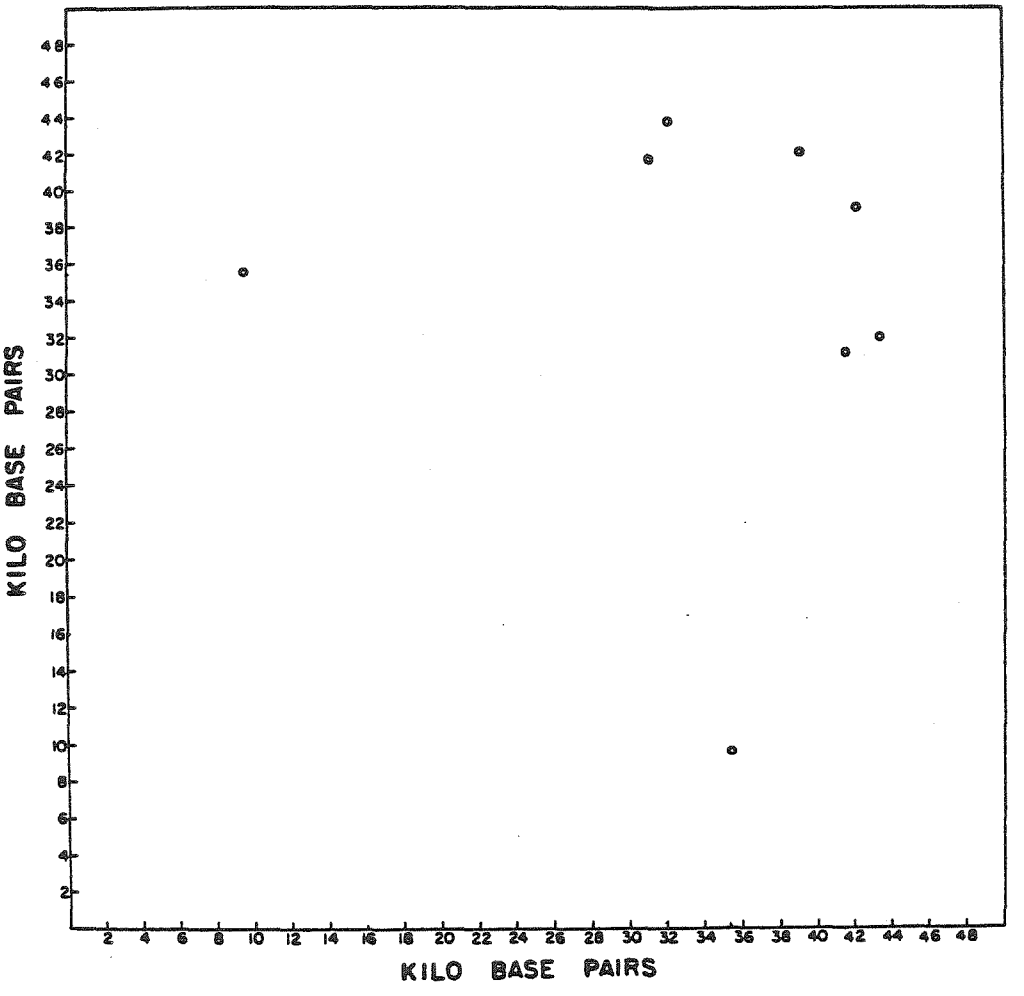












PHAGE LAMBDA NEAREST NEIGHBORS MAP

Figure 17

VI. BPNE Crosslinking Assay (Restriction Endonuclease Cleavage)

Having had success with BAMO as a crosslinking reagent for bacteriophage λ, a reevaluation of the effectiveness of the bispsoralen reagent (BPNE), in our own hands, was called for. Irradiation was performed with light from two GE F15T8BLB bulbs fitted into the same desk lamp apparatus used for BAMO irradiation. The light was filtered through 1/4" plexiglass. Samples were prepared in 1.5 ml Eppendorf centrifuge tubes and irradiated from above by placing the plexiglass directly on top of the open sample tubes and the light source directly on top of the plexiglass. After irradiation the samples were prepared for electron microscopic analysis by the protocol previously described for BAMO crosslinking experiments. Results from these studies are shown in Table 2 along with the data from the analogous BAMO experiment for comparison.

BPNE crosslinking of bacteriophage λ at a binding ratio of 0.28 BPNE/bp, incubated for 3 hours and photolyzed for 5 hours yielded 10% crosslinked structures. This is similar to the 17% crosslinked structures for BAMO at a binding ratio of 0.01 BAMO/bp, incubated for 2 hours and photolyzed for 1 hour. The BPNE crosslinking results indicated that BPNE is indeed a useful interhelical crosslinking reagent at least within the limits of the experimental variables studied; namely: 3-7 hour incubation, > 2 hour photolysis at a binding ratio of 0.28 and a λ phage concentration of 3×10^{-4} Mbp. Apparently the experiments performed for us by the Columbia group which indicated the ineffectiveness of BPNE as an interhelical crosslinker were not carried out within these limits and therefore were misleading.

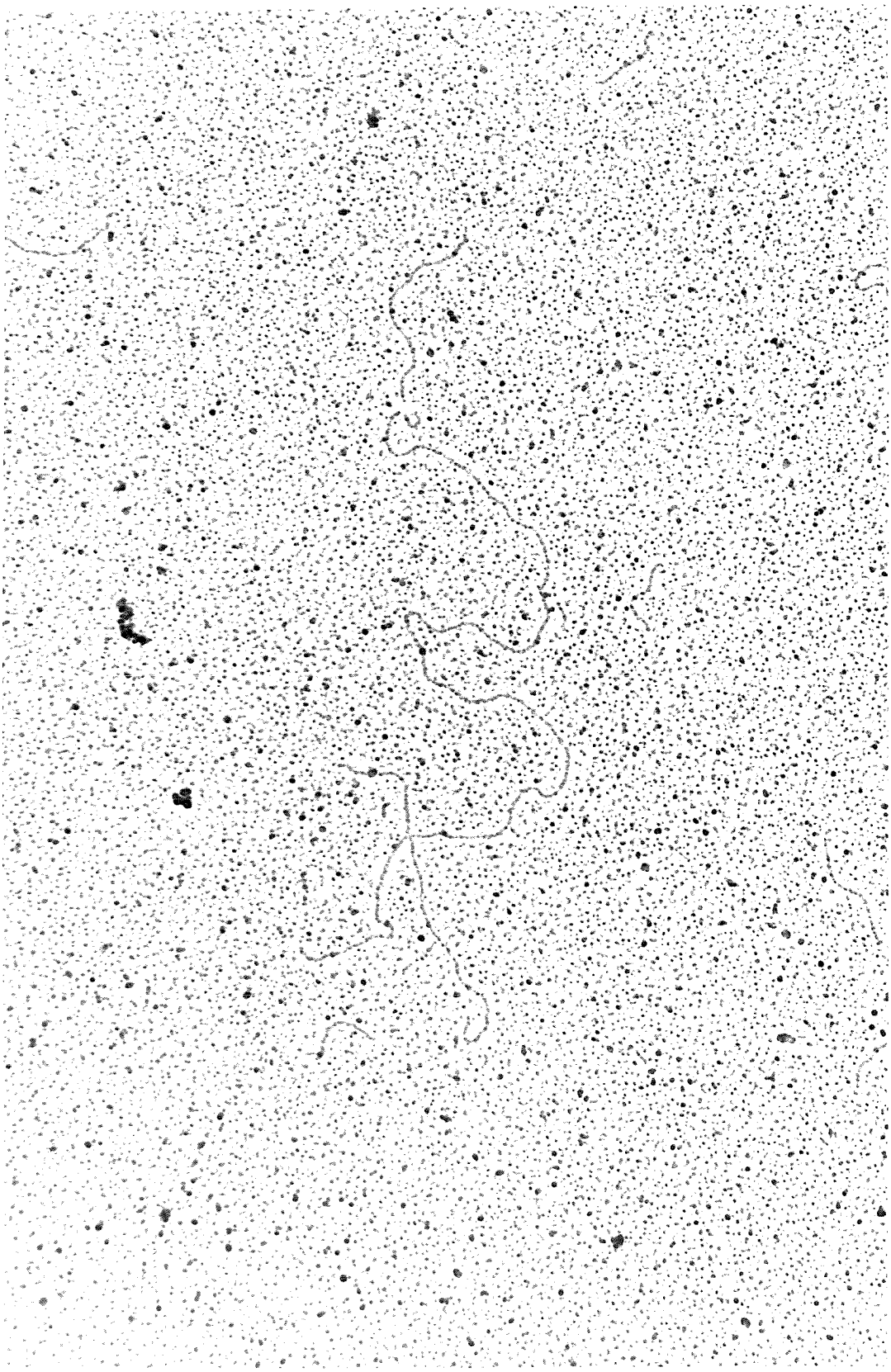
Table 2

BPNE Crosslinking Assay with SacI/SstII

Restriction Endonuclease Cleavage[a]

Reagent	Binding Ratio (drug/bp)	Incubation Time	Incubation Time	% Linear Fragments	% Crosslinked Structures	% Rosettes Remaining
BPNE	0.28	7	10	73	20	7
---	--	7	10	95	2.5	2.5
BPNE	0.28	3	5	90	10	0
BPNE	0.28	3	2	100	0	0
BAMO	0.01	2	1	73	17	10

a) Sample contained bacteriophage λ at a concentration of 3×10^{-4} Mbp.



Both BAMO and BPNE may prove to be useful reagents for other systems depending upon the molecular or photochemical properties desired by the investigator. Although BAMO requires less energetic light for photolysis than BPNE, it is a charged molecule whereas BPNE is uncharged. This study was not intended to be a thorough comparison of the two interhelical crosslinking reagents. A brief summary of the molecular properties and crosslinking results obtained to date along with the results for BTPD, the reagent from the Columbia group are presented in Table 3.

TABLE 3

Reagent	Tether Length (# atoms)	Ionic Characteristic	Effective Binding/Ratio Reagent/bp	Effective Incubation Time	Effective Irradiation Time	Lamp Source	Wavelength Required for Photolysis	% Crosslinked Structures
BAMO (13)	30	charged	0.01	1 hr	30 min	2 x F15T8 CW	> 400 nm	17
BPNE (7)	28	neutral	0.28	> 3 hr	> 3 hr	2 x F15T8 BLB	365 nm	10
BTPD[a] (1)	15	charged	0.5	15 min	5 min	2 x H400A33-1/T16	365 nm	31
$(AAS)_2$ (2)	16	neutral	---	---	5 min	1 x F8T5 BLB	365 nm	--

a) data extracted from ref. 24

b) Ref. 25, no crosslinking data is available for $(AAS)_2$. Irradiation parameters listed are for the monomeric reduced thiol derivative.

VII. BAMO Crosslinking of Bacteriophage T7

Collaborative efforts were undertaken with Dr. Michael Reuben in our laboratory to determine the packaged DNA structure of bacteriophage T7. Results for T7 are qualitatively similar to those for bacteriophage λ . DNA isolated from T7 after BAMO crosslinking exhibits a faster agarose gel mobility and appears as rosettes under the electron microscope. Restriction endonuclease cleaved crosslinked T7 DNA exhibits a slower gel mobility than the uncrosslinked fragments and appears as crosses under the electron microscope. In fact, the slow moving crosslinked T7 fragments are resolved into distinct bands whereas phage λ fragments appeared only as an unresolved smear. The cause of this enhanced resolution for crosslinked T7 fragments is not known. Figure 18 details the protocol developed for generating T7 restriction fragments labeled with avidin spheres specifically at one end suitable for directional measurement on electron micrographs. A nearest neighbors map of the measured locations of the crosslink positions obtained to date for T7 restriction fragments generated as described in Figure 18 is shown in Figure 19.

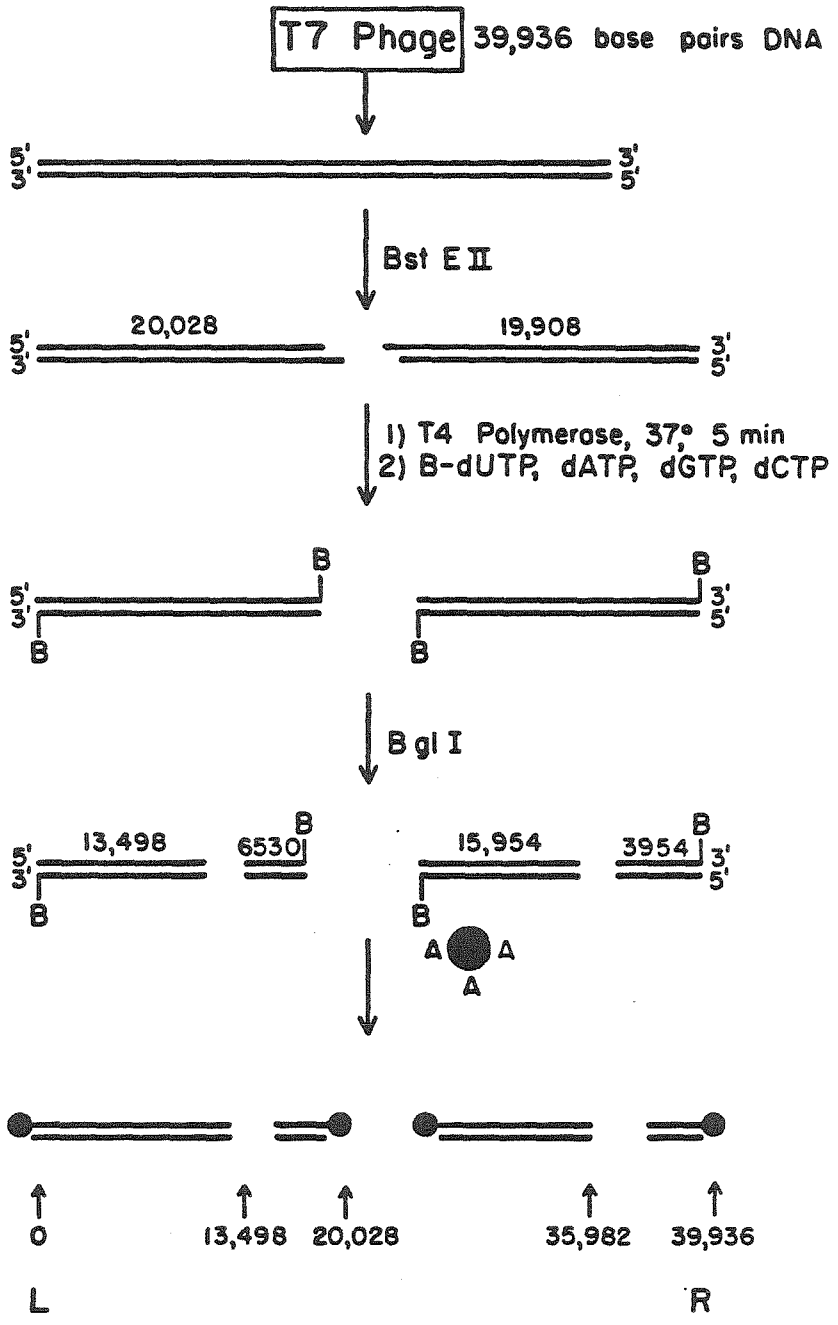
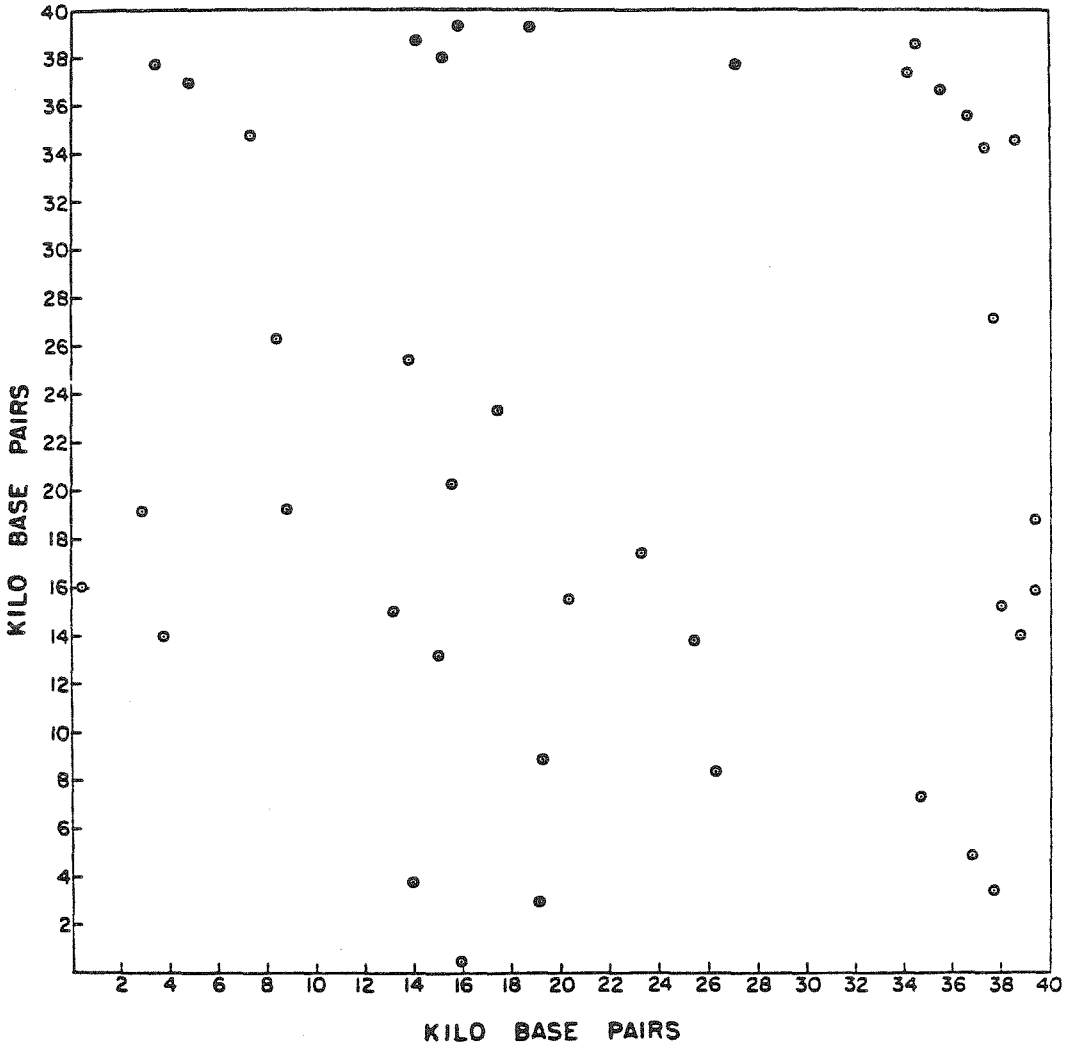

T7 Phage
39,936 base pairs DNA
5'
3'
3'
5'
Bst E II
20,028
19,908
1) T4 Polymerase, 37° 5 min
2) B-dUTP, dATP, dGTP, dCTP
B
Bgl I
13,498
6530
15,954
3954
A
0
13,498
20,028
35,982
39,936
L
R

Figure 18

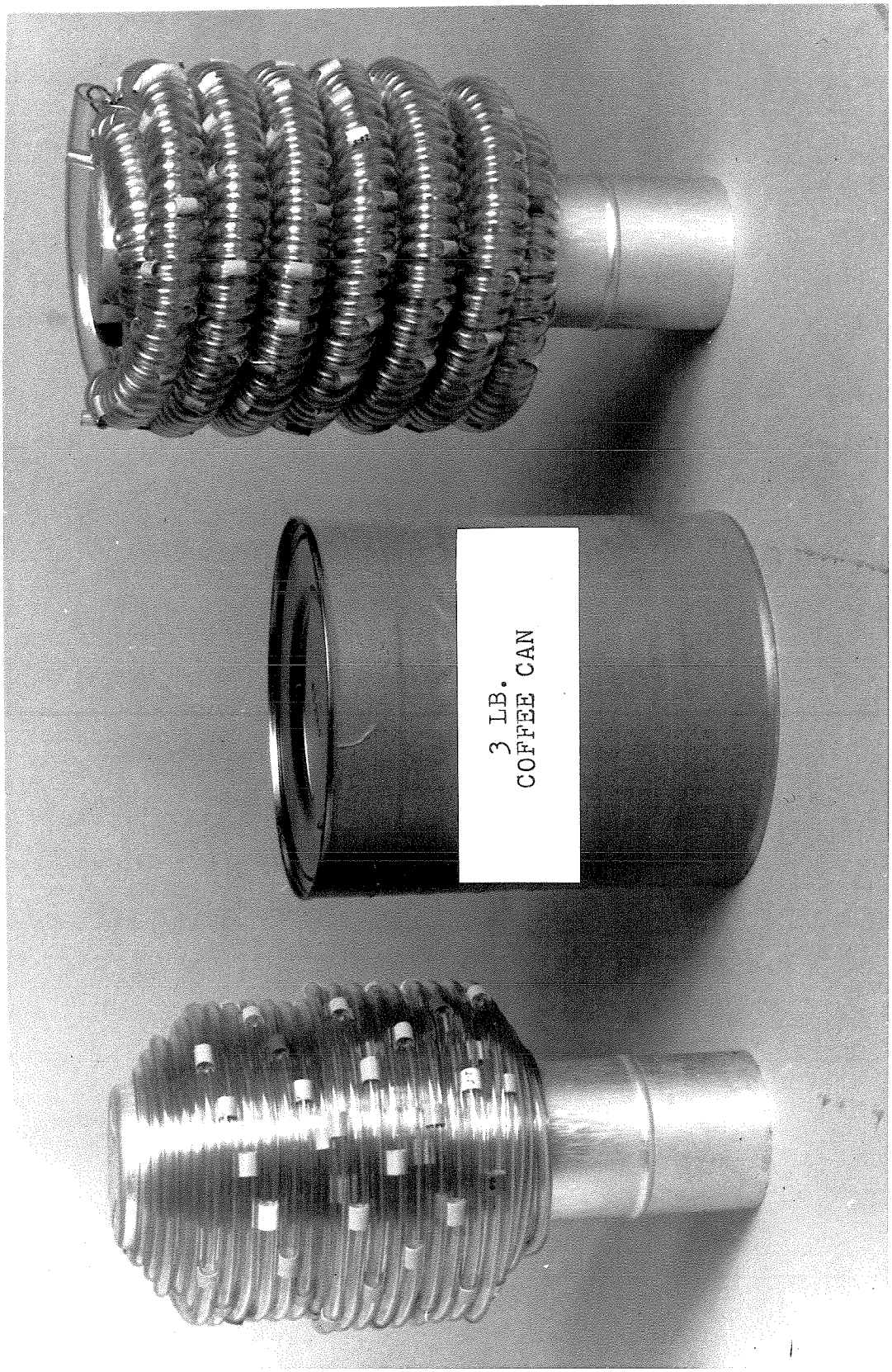


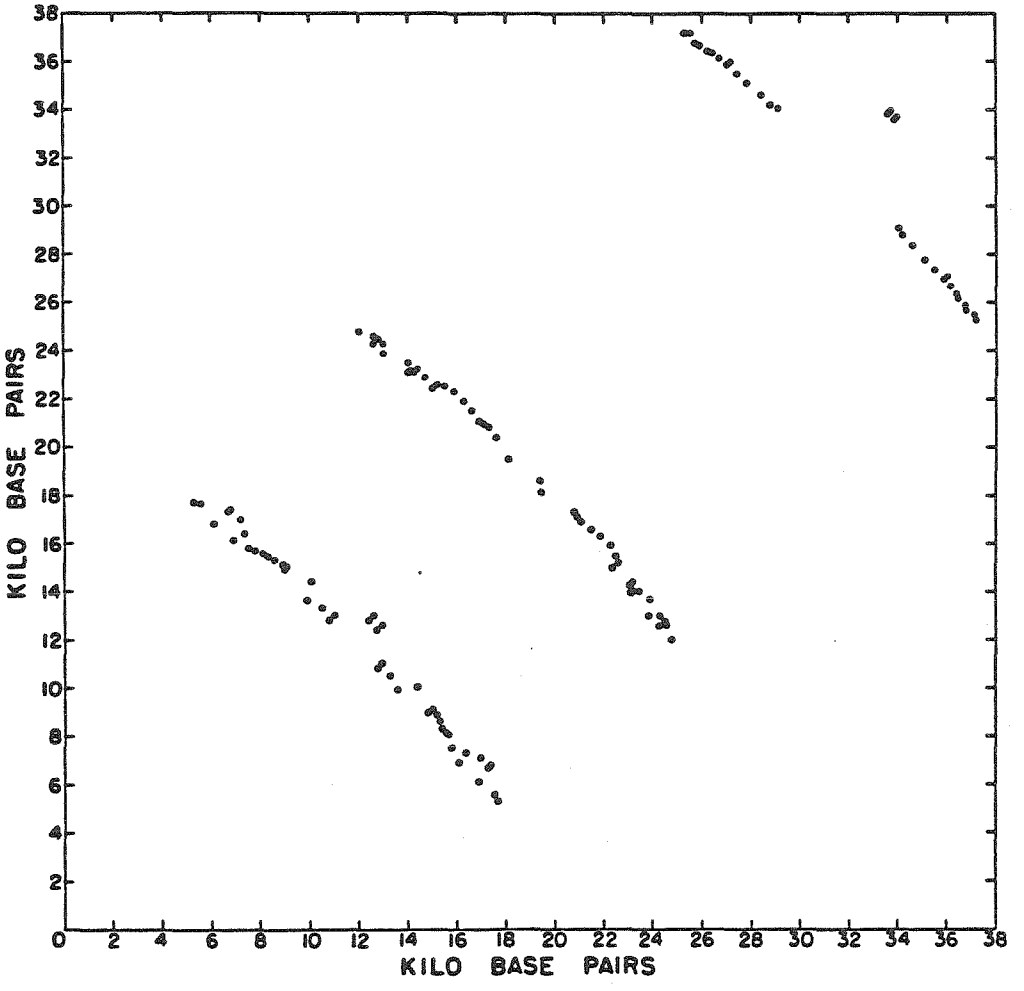
T7 NEAREST NEIGHBORS MAP

Figure 19

VIII. Scale Model Building of Coaxial Solenoid and Coil of Coils for Packaged T7 Genome

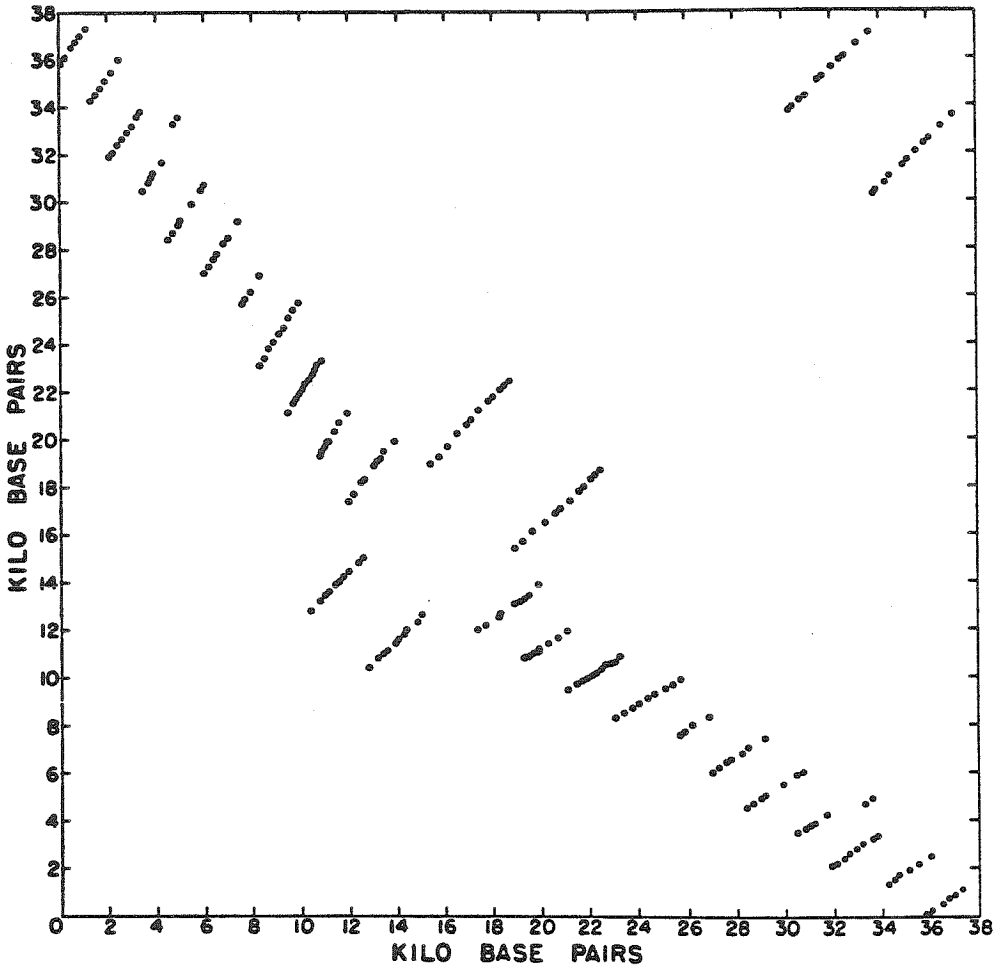
In order to help interpret the results obtained from the nearest neighbors map, scale models of a coxial solenoid and a coil of coils were made out of 1/4 inch tygon tubing. The scale was one inch equals 100 Å, which allowed the 1/4 inch diameter tubing to approximate a 25 Å wide helix of double stranded DNA. Assuming packaged DNA to be in a B-conformation with each base pair contributing 3.4 Å of length, our models representing 39,936 base pairs of T7 DNA, were 113 feet long. This entire length was then condensed into a cylinder with a radius of 3 inches and a height of 6 inches based on the T7 dimensions of Stroud, et.al.[20] A small photograph of the scale models is shown on the following page. Idealized data taken from these models based on the same restriction sites used to acquire the experimental data provide a basis for comparison of possible packaging models for the T7 bacteriophage with its actual mode of packaging. These idealized data are graphically presented in Figures 20 and 21 as hypothetical nearest neighbors maps. These models have been built to show that the different packaging models discussed in the literature can be distinguished with data generated from studies detailed in this thesis.


3 LB.
COFFEE CAN



HYPOTHETICAL COAXIAL SOLENOID NEAREST NEIGHBORS MAP

Figure 20



HYPOTHETICAL COIL OF COILS NEAREST NEIGHBORS MAP

Figure 21

IX. Summary

We have developed a strategy for determining the higher order structure of packaged bacteriophage DNA. To this end we have synthesized and characterized two interhelical crosslinking reagents; a reagent type previously unavailable from the literature. We have also developed the methodology necessary for directional measurement of restriction fragments on electron photomicrographs. By crosslinking the DNA within the intact bacteriophage followed by restriction digestion and end labeling of the isolated DNA we can uniquely locate the site of crosslinking along the bacteriophage genome. These data produce an interhelical nearest neighbors map of interhelix contact sites within the packaged structure. By comparing maps generated from our data to hypothetical maps generated from scale models we have made of packaged DNA we hope to determine the structure of packaged bacteriophage DNA.

We have synthesized and characterized the two new interhelical crosslinking reagents, bis(monoazidomethium)octaoxahexacosanediamine (BAMO) and bispsoralen-nonaethyleneoxy ether (BPNE); and have demonstrated their ability to crosslink neighboring DNA helices within intact bacteriophages λ and T7. BAMO efficiently crosslinks packaged bacteriophage DNA at low binding levels (r = 0.01 BAMO/bp) and can be photolyzed quickly (0.5-1 hr) with light of low energy (λ > 400 nm). Incubation of bacteriophage λ (3×10^{-4} Mbp) with BAMO (r = 0.01 BAMO/bp) for 2 hrs in the dark followed by irradiation (λ > 400 nm) for 1 hr routinely yields 16-17% crosslinked structures under the electron microscope after restriction endonuclease digestion. BPNE also efficiently crosslinks packaged bacteriophage DNA as indicated by

our preliminary studies. Incubation of bacteriophage λ (3×10^{-4} Mbp) with BPNE (r = 0.28 BPNE/bp) for 3 hrs in the dark followed by irradiation (λ = 365 nm) for 5 hours yields 10% crosslinked structures under the electron microscope after restriction endonuclease digestion.

Directional measurements of restriction fragments have been carried out on photomicrographs of fragments labeled specifically at one end with electron dense avidin spheres. The avidin spheres are attached to the restriction fragments by incorporating a biotin containing nucleotide (Bio-dUTP) into the restriction fragment using T4 polymerase under standard enzymatic conditions. Incubation of avidin spheres with biotinylated restriction fragments results in avidin sphere binding specifically to the labeled end of the restriction fragment.

We have begun to generate interhelical nearest neighbors maps from the measured crosslink positions. Models have been constructed for packaged bacteriophage T7 DNA according to the coaxial solenoid and coil of coils models discussed in the literature. Hypothetical nearest neighbors maps from these models have been prepared. These maps readily distinguish the two models and provide hope that our crosslinking approach will allow us to propose a structure for packaged bacteriophage DNA when enough data points have been measured. We anticipate that the reagents synthesized and the technology developed will be useful in probing the higher order structure of other packaged nucleic acid systems.

EXPERIMENTAL

Materials and Methods

Melting points were obtained using a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Caltech Analytical Facility, Pasadena, Ca.; and Galbraith Laboratories, Inc.; Knoxville, Tennessee. Infrared spectra (ir) were recorded on a Perkin-Elmer Model 257, Grating Infrared Spectrophotometer or a Beckman IR-4240 spectrophotometer. Ultraviolet-visible spectra (UV-vis) were recorded on a Beckman Model 25 spectrophotometer. Nuclear magnetic resonance (nmr) spectra were obtained on a Varian Associates A-60 or EM 390, Joelco FX90, or Bruker WM500 Spectrometer and are uncalibrated. Chemical shifts are given as parts per million (ppm) downfield from tetramethyl silane (TMS) in units and coupling constants in cycles per second (Hz). Nuclear magnetic resonance data are reported in the order: chemical shift; multiplicity, s-singlet, d=doublet, t=triplet, m=multiplet; number of protons; coupling constants; assignment.

Most reagent grade chemicals were used without further purification. Dimethyl sulfoxide, N,N -dimethylformamide and pyridine were dried over 4A molecular sieves. Triethylamine was dried over potassium hydroxide pellets. Restriction endonuclease SacI, MstII, and EcoRl were purchased from New England Biolabs, Inc. (Beverly, MA). Restriction endonucleases SstII and T4 polymerase were purchased from Bethesda Research Laboratories, Inc. (Rockville, MD). Proteinase K was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Biotinylated-dUTP was purchased from Enzo Biochem., Inc. (New

York, NY). Carboxymethyl cellulose (Cellex CM) was purchased from Bio-Rad Laboratories (Richmond, CA). Sepharose 2B-Cl was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Bovine Serum Albumin was purchased from Miles Laboratories (Elkhart, IN) and was their Pentex Bovine Albumin Crystallized grade. Supplies for electron microscopy can be purchased from Ted Pella, Inc. (Tustin, CA).

Agarose gels were poured and run on a horizontal gel apparatus similar to McDonnell et al.[50] Typical gels were prepared with a buffer containing 40 mM Tris acetate, 5 mM sodium acetate, 1 mM Na_2EDTA, pH 7.8 and 0.3-1.0% (W/v) agarose. Gels were stained with ethidium bromide (2 μg/ml), visualized with a long wavelength ultraviolet transilluminator (Fotodyne, New Berlin, Wisconsin) and photographed from above using Polaroid type 55 land film.

DNA samples were spread for electron microscopy by the standard formamide-cytochrome C method of Davis et al.[51] The spreading solution typically contained 100 mM Tris-HCl, 10 mM Na_2EDTA (pH 8.5), 0.1 mg/ml cytochrome C, 50% formamide, and 0.01 μg DNA. The samples were spread onto a hypophase consisting of 10 mM Tris-HCl, 1.0 mM Na_2EDTA (pH 8.5), and 20% formamide. DNA samples were mounted onto parlodion coated 200 mesh copper grids, stained with uranyl acetate and rotary shadowed with platinum-palladium (80/20). DNA was visualized on a Phillips EM301 Transmission Electron Microscope. Length measurements were made from enlarged 35 mm negatives using an X-Y digitizer and length measuring device attached to a Hewlett-Packard calculator.

3,6,9,12,15,18,21,24 octaoxahexacosane 1,26 diol (nonaethylene glycol) (11)

Nonaethylene glycol was synthesized by an adaptation of a procedure by Bomes et al.[31] In a 500 ml 3-necked roundbottom flask fitted with stopper, septum inlet and an argon inlet tube was placed 165 g (1.1 mol) of triethylene glycol, previously dried over 2 changes of 4A molecular sieves. An ether suspension of ether washed 50% sodium hydride dispersion 10 g (0.2 mol) was slowly added to the stirred triethylene glycol. The mixture was stirred at room temperature for two hours. To the mixture was added all at once 38.0 g (0.082 mol) of triethylene glycol ditosylate (mp 79-81°C, lit 81-82°C). The mixture was stirred and heated at 90°C for two days. The reaction was treated with 200 ml of water and heated at reflux for two hours. After cooling to room temperature the reaction mixture was treated successively with two 30 g batches of DEAE-A50-120 anion exchange resin. The solution was filtered and then distilled in vacuo to remove water and excess triethylene glycol. The remaining residue was bulb to bulb distilled. Low boiling material was removed at 180°C/7 microns Hg with the product collected at 240°C/7 microns Hg. Redistillation yielded analytically pure nonaethylene glycol (10.8 g, .026 mol, 32%); ir (neat) 3500 (alcohol), 2780 (alkane), 1350, 1260, 1170, 1110, 1035, 940 and 755 cm^{-1}; nmr ($CDCl_3$) δ 3.67 (s, 36H, $O\underline{CH_2CH_2}$), and 3.20 (s, 2H, $O\underline{H}$).

Anal. Calcd. for $C_{18}H_{38}O_{10}$: C, 52.16; H, 9.24. Found: C, 52.39; H, 9.17.

Nonaethylene glycol ditosylate (12)

To a solution of 3.0 g (7.2 mmol) nonaethylene glycol in 25 ml

dry pyridine, cooled to 0°C in an ice bath, was added 55 g (29 mmol) of p-toulenesulfonyl chloride. The solution was stoppered and stored at 4°C overnight. The pyridine was removed in vacuo. The remaining residue was washed thoroughly with hexane, dissolved in methylene chloride and extracted with cold 5% HCl. The methylene chloride solution was evaporated to dryness in vacuo. The remaining residue was again washed thoroughly with hexane, dried in vacuo and subjected to flash chromatography on silica (THF eluant) to yield analytically pure nonaethylene glycol ditosylate (5.2 g, 100%); ir (neat) 2860 (alkane), 1595, 1190 and 1170 (tosyl), 1440, 1356, 1100, 1010, 920, and 815 cm^{-1}; nmr ($CDCl_3$) δ 2.45 (s, 6H, $\underline{CH_3}$) 3.64, 3.61, 3.58 (m, 32H, $O\underline{CH_2CH_2}O$) 4.19 (m, 4H, $S-O-\underline{CH_2}-CH_2$) 7.38 (d, 4H, $\underline{J}$=8Hz, arom.) and 7.82 (d, 4H, $\underline{J}$=8Hz, arom.).

Anal. Calcd. for $C_{32}H_{50}O_{14}S_2$: C, 53.17; H, 6.97. Found: C, 53.02; H, 6.94.

3,6,9,12,15,18,21,24 octaoxahexacosane 1,26 diamine (15)

In a flame dried 250 ml 3 necked roundbottom flask fitted with an argon inlet and septum inlets was placed 5.0 g (6.9 mmol) of nonaethylene glycol ditosylate and 100 ml of dry N,N -dimethylformamide. Potassium phthalimide (10.3 g, 55.3 mmol) was added and the mixture stirred at 90°C overnight. The N,N -dimethylformamide was removed in vacuo. The remaining residue was taken up in methylene chloride, washed three times with water and dried (K_2CO_3). Evaporation of the methylene chloride in vacuo yielded crude nonaethylene glycol bisphthalimide. The crude bisphthalimide was dissolved in 80 ml of ethanol and treated with (2.68 ml/55.3 mmol) of hydrazine hydrate. The solution was stirred overnight at

reflux. The ethanol was removed in vacuo. To this was added 50 ml of 5% HCl and the reaction mixture was heated at reflux for 4 hours. After evaporating the reaction mixture to dryness excess potassium hydroxide solution was added and stirred until all solids dissolved. The mixture was evaporated to dryness in vacuo. The remaining residue was triturated three times with 100 ml portions of dry triethylamine (dried over KOH pellets). Evaporation of the triethylamine in vacuo yielded crude diamine as a yellow oil. The yellow oil was distilled (bulb to bulb). Low boiling material was removed at 150°C/7 microns Hg and the product collected at 200°C/7 microns Hg. Redistillation yielded 1.0 g (35%) of analytically pure 3,6,9,12,15,18,21,24 octaoxahexacosane 1,26 diamine; ir (neat) 3380 (amine), 2880 (alkane), 1600, 1460, 1355, 1300, 1250, 1120, 950 and 850 cm^{-1}; nmr ($CDCl_3$) δ 1.50 (s, 4H, $\underline{NH_2}$), 2.84 (t, 4H, $\underline{J}$=4Hz, $N\underline{CH_2}$) 3.49 (t, 4H, $\underline{J}$=4Hz, $O\underline{CH_2}CH_2NH_2$) and 3.63 (s, 28H, $O\underline{CH_2}\underline{CH_2}O$).

Anal. Calcd. for $C_{18}H_{40}N_2O_8$: C, 52.41; H, 9.77; N, 6.79. Found: C, 52.58; H, 9.64; N, 6.79.

Para-carboxy(monoazidomethidium)chloride monohydrate (14)

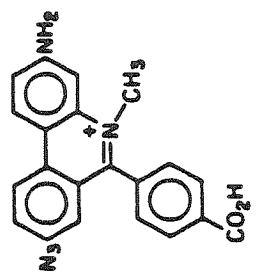
This synthesis was carried out entirely under red light illumination. To a solution of p-carboxymethidium chloride[35] (100 mg, 0.23 mmol) in 40 ml of 0.2 M aqueous HCl stirred at 0°C was added dropwise isopentylnitrite (35 μl, 0.24 mmol) dissolved in 2 ml of THF. After stirring at 0°C for 15 minutes, sodium azide (2.1 ml of a 0.115 M solution, 0.24 mmol) was added dropwise. Stirring was continued for thirty minutes. After neutralization with 1 M NaOH the solution was evaporated to dryness. The residue was redissolved in a minimal volume of hot aqueous HCl (pH 2.8) and applied to a 40 cm x 2.5 cm

carboxymethyl cellulose column. The column was developed with aqueous HCl (pH 2.8) and separated the mixture into three colored bands. The first fraction, yellow in color, p-carboxy(bisazidomethidium) by analogy to the synthesis of ethidium azide derivatives was not collected. The second fraction, orange in color, was collected, combined with the orange fraction from a second identical preparation and evaporated to dryness in vacuo with minimal heating. After chromatography on a clean carboxymethyl cellulose column, and evaporation of the solvent, the product was recrystallized from 1M HCl. The combined preparations yielded 66 mg 34% of analytically pure p-carboxy(monoazidomethidium) as the chloride monohydrate; ir (KBr) 3440, 3200, 2118 (azide), 1720, 1630, 1620, 1490, 1315, 1265, 1230, 1180, 1165, 1110, 1020, and 830 cm^{-1}; UV-vis λ_{max} 464 nm, $\epsilon = 5.39 \times 10^3$/mol cm; nmr ($d_6$·dimethyl sulfoxide) δ 4.06 (s, N-$\underline{CH_3}$), 4.18 (s, N-$\underline{CH_3}$), 6.33 (s), 6.47 (s), 6.79 (s), 6.87 (s), 7.41 (s), 7.46 (d, J=10Hz), 7.64 (d, J=10Hz), 7.84 (m), 7.96 (d, J=10Hz), 8.09 (s), 8.28 (s), 8.30 (s), 8.31 (s), 8.86 (m), 8.95 (d, J=10Hz) and 9.00 (d, J=10Hz). The two resonances at δ 4.06 and δ 4.18 assigned to the phenanthridinium methyl group, indicate the possibility of two positional azide isomers being present (see attached spectra).

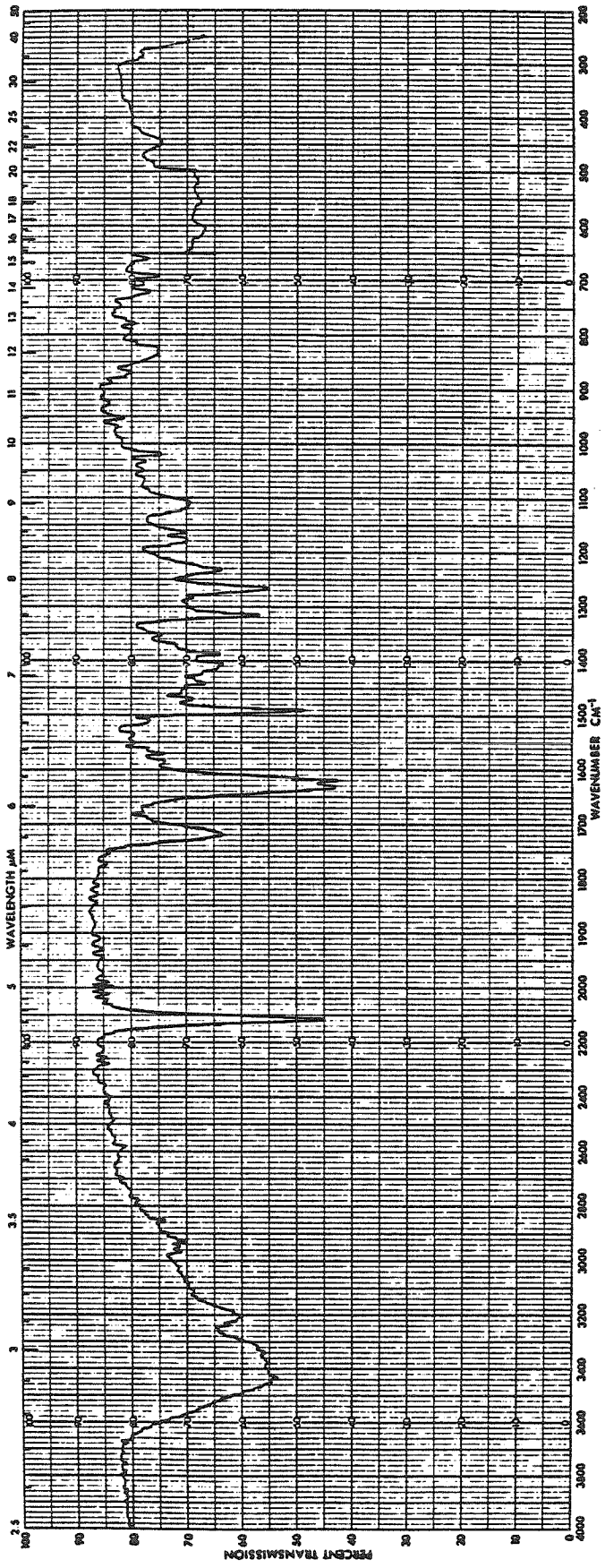
Anal. Calcd. for $C_{21}H_{18}N_5O_3Cl$: C, 59.51; H, 4.28; N, 16.52. Found: C, 59.41; H, 4.36; N, 16.46.

Bis(monoazidomethidium)octaoxahexacosanediamine (BAMO) (13)

This synthesis was carried out entirely under red light illumination. To a 50 ml roundbottom flask equipped with a N_2 inlet tube and two septum inlets, flame dried and cooled under a nitrogen flow was added p-carboxy(monoazidomethidium) chloride (170 mg, 0.4


NH2
CH3
N
+
N3
CO2H

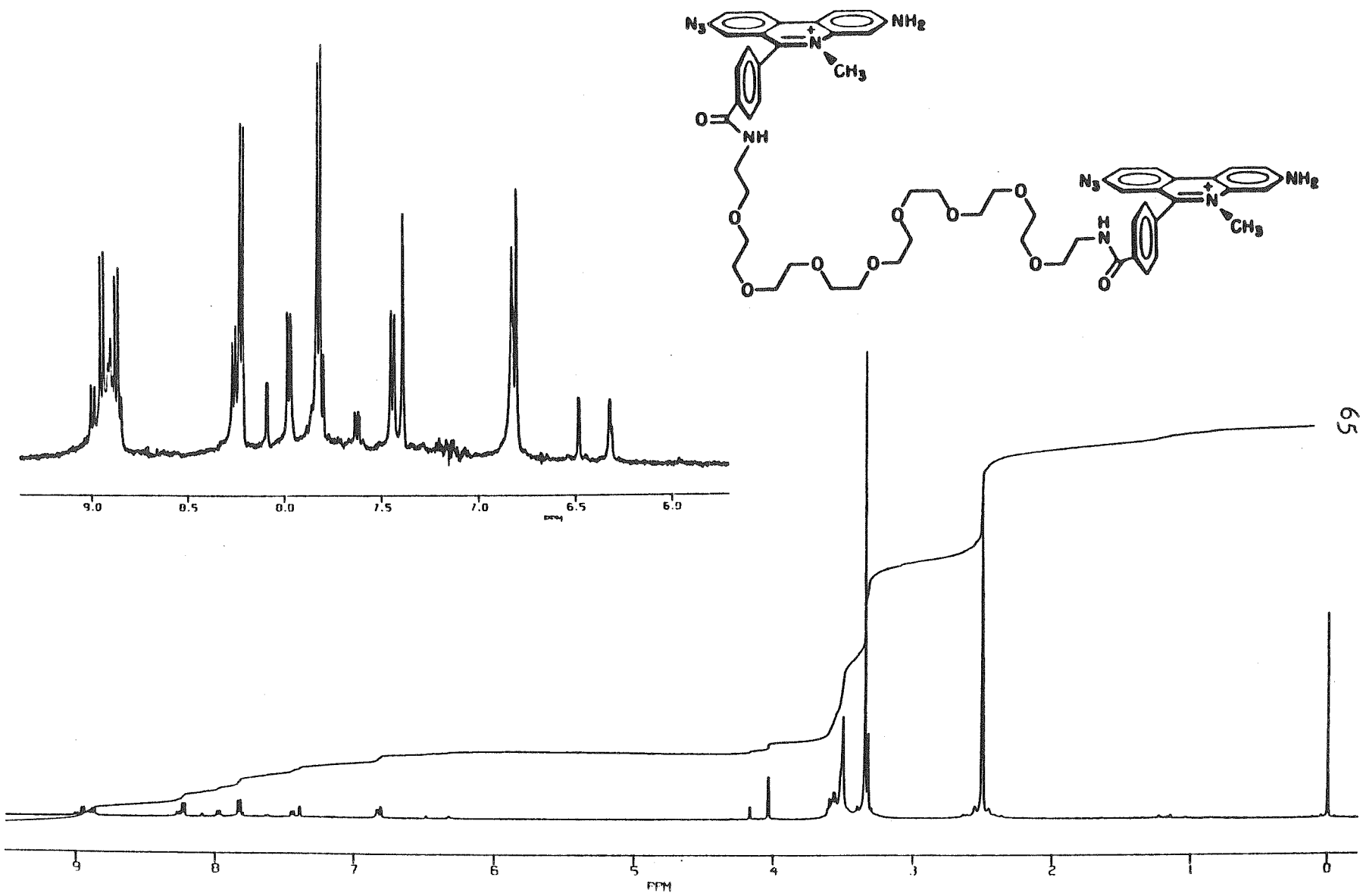
9
8
7
6
5
4
3
2
1
0
PPM



INFRARED SPECTRUM OF PARA-CARBOXY(MONOAZIDOMETHIDIUM)CHLORIDE

mmol), dry N-ethylmorpholine (76 μl, 0.6 mmol) and 20 ml dry dimethyl sulfoxide (dried over 4Å sieves). To the stirred solution was added carbonyl diimidazole (71 mg, 0.44 mmol, in 1 ml dry dimethyl sulfoxide). After stirring the solution for two hours, octaoxahexacosanediamine (82.5 mg, 0.2 mmol in 3 ml dimethyl sulfoxide) was added to the reaction mixture. The reaction was stirred 48 hours and then evaporated to dryness in vacuo. The residue was repeatedly washed with hexane and ethyl ether to remove residual dimethyl sulfoxide. The product was purified by silica gel chromatography (230-400 mesh ASTM) using 0.006% methanolic HCl as the eluant. Final purification was carried out by loading the product dissolved in water onto an amberlite XAD-2 column and washing extensively with more water. Elution with methanol yielded analytically pure bis(monoazidomethidium)octaoxahexacosanediamine dichloride dihydrate; ir (KBr) 3420, 2915, 2118 (azide), 1620, 1400, 1388, 1260, and 1100 cm^{-1}; UV-vis λ_{max} 481 nm, ϵ = 8730/mol cm; nmr (d_6-dimethyl sulfoxide) δ 3.49 (OCH_2CH_2O), 3.58 (m, CH_2CH_2), 4.03 (s, $N-CH_3$), 4.17 (s, $N-CH_3$), 6.33 (s), 6.49 (s) 6.82 (m), 7.44 (d, J=10Hz), 7.63 (d, J=10Hz), 7.80 (s), 7.82 (s), 7.84(s), 7.99 (d, J=10Hz), 8.09 (s), 8.23 (d, J=10Hz), 8.27 (d, J=10Hz) and 8.93 (m). The two resonances at δ 4.03 and δ 4.17, assigned to the phenanthridinium methyl group, indicate the possibility of two positional azide isomers being present (see attached spectra). Integration confirms the ratio of chromophore/linker = 2/1.

Anal. Calcd. for $C_{60}H_{70}N_{12}O_{12}Cl_2$: C, 58.96; H, 5.77; N, 13.75. Found: C, 58.78; H, 5.92; N, 13.53.


N3
NH2
CH3
O
NH
O
O
O
O
O
O
O
O
O
O
O
O
N3
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H
N
O
9.0
8.5
8.0
7.5
7.0
6.5
6.0
PPM
9
8
7
6
5
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3
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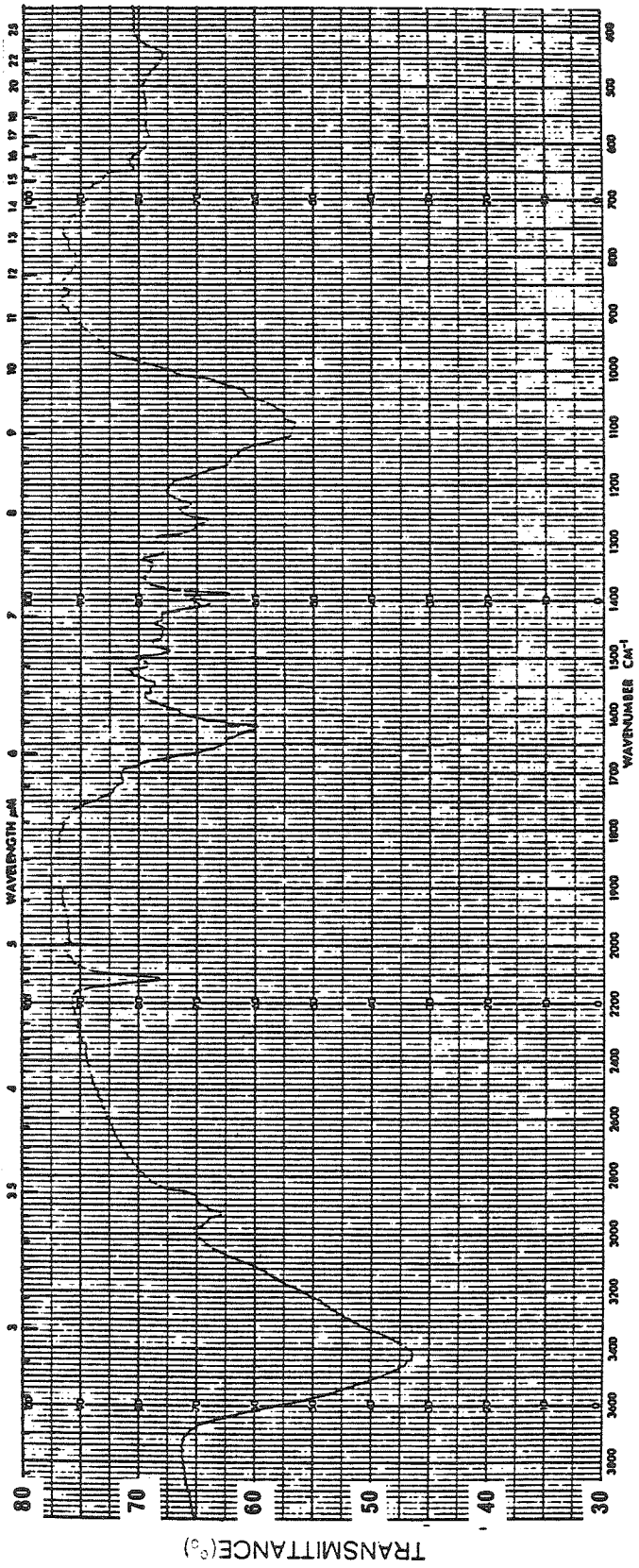
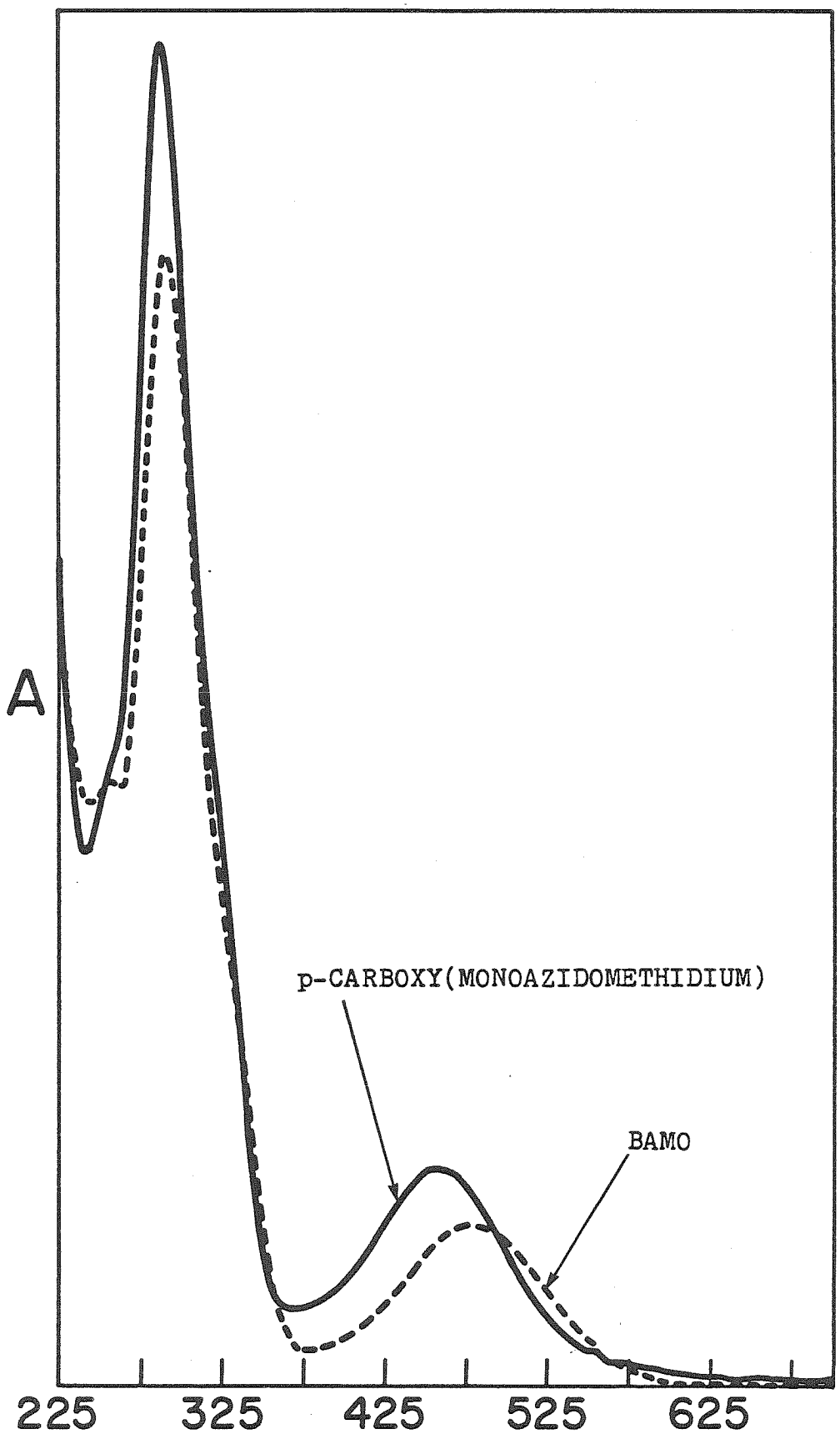
1
0
PPM



INFRARED SPECTRUM OF BAMO


A
p-CARBOXY(MONOAZIDOMETHIDIUM)
BAMO
225
325
425
525
625

[^{3}H]BAMO (16)

Tritiated BAMO was synthesized by the procedure described above using octaoxahexacosanediamine tritiated by New England Nuclear Corp. (lot 1365-237A) according to the procedure below and repurified in our laboratory.

Octaoxahexacosanediamine (80 mg) was dissolved in 2 ml of N,N - dimethylformamide. To this was added 5% Rh/Al_2O_3 catalyst (50 mg) and 25 Curies of tritium gas. The reaction mixture was stirred overnight at room temperature. Labile tritium was removed in vacuo, using water as solvent. After filtration from the catalyst the product was again taken to dryness in vacuo and then dissolved in 25 ml of chloroform/methanol (2:3). In our laboratory, the vial was opened and the solvent evaporated under a nitrogen gas flow with gentle warming. Aqueous potassium hydroxide was added to the vial to fully neutralize the diamine. After evaporation to dryness in vacuo, the remaining residue was triturated three times with dry triethylamine (25 ml). Evaporation of the triethylamine under a nitrogen flow afforded a yellow oil which was distilled (bulb to bulb) 220-240°C; 0.2-0.3 mm to yield octaoxahexacosanediamine (33 mg). The following synthesis was carried out entirely under red light illumination. A solution of p-carboxy(monoazidomethidium) (34.0 mg, 80 μmol) in 5 ml of dry dimethyl sulfoxide and N-ethylmorpholine (100 μl) was stirred for 15 minutes. To this was added carbonyl diimidazole (212 μl of a solution 0.122 g in 2.0 ml dimethyl sulfoxide). After stirring two hours ^{3}H diamine (0.45 ml of a solution containing 33 mg in 1.0 ml dry dimethyl sulfoxide) was added via syringe. After stirring overnight, the reaction was evaporated to dryness in vacuo and washed with petroleum

ether and ethyl ether to remove residual dimethyl sulfoxide. The remaining solid was twice chromatographed on a 3/8"x2" silica gel 60 (230-400 mesh) column using 0.006% methanolic HCl as eluant. The slow mobility fraction collected showed the same TLC mobility (silica gel, 20 ml methanol with 5 drops conc. HCl) as BAMO synthesized with nontritiated diamine. Specific activity by UV calibration of sample concentration was 1.1 Ci/mmol.

8-Hydroxypsoralen (9) and the corresponding potassium salt (10)

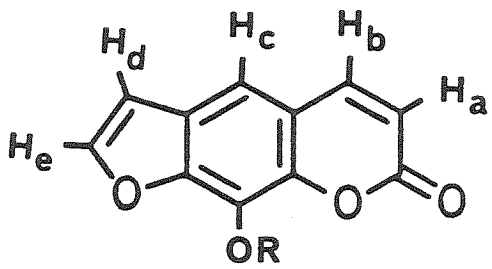
The potassium salt of 8-hydroxypsoralen was a gift from M.M. Becker, which can be synthesized from 8-methoxypsoralen by the following experimental procedures.

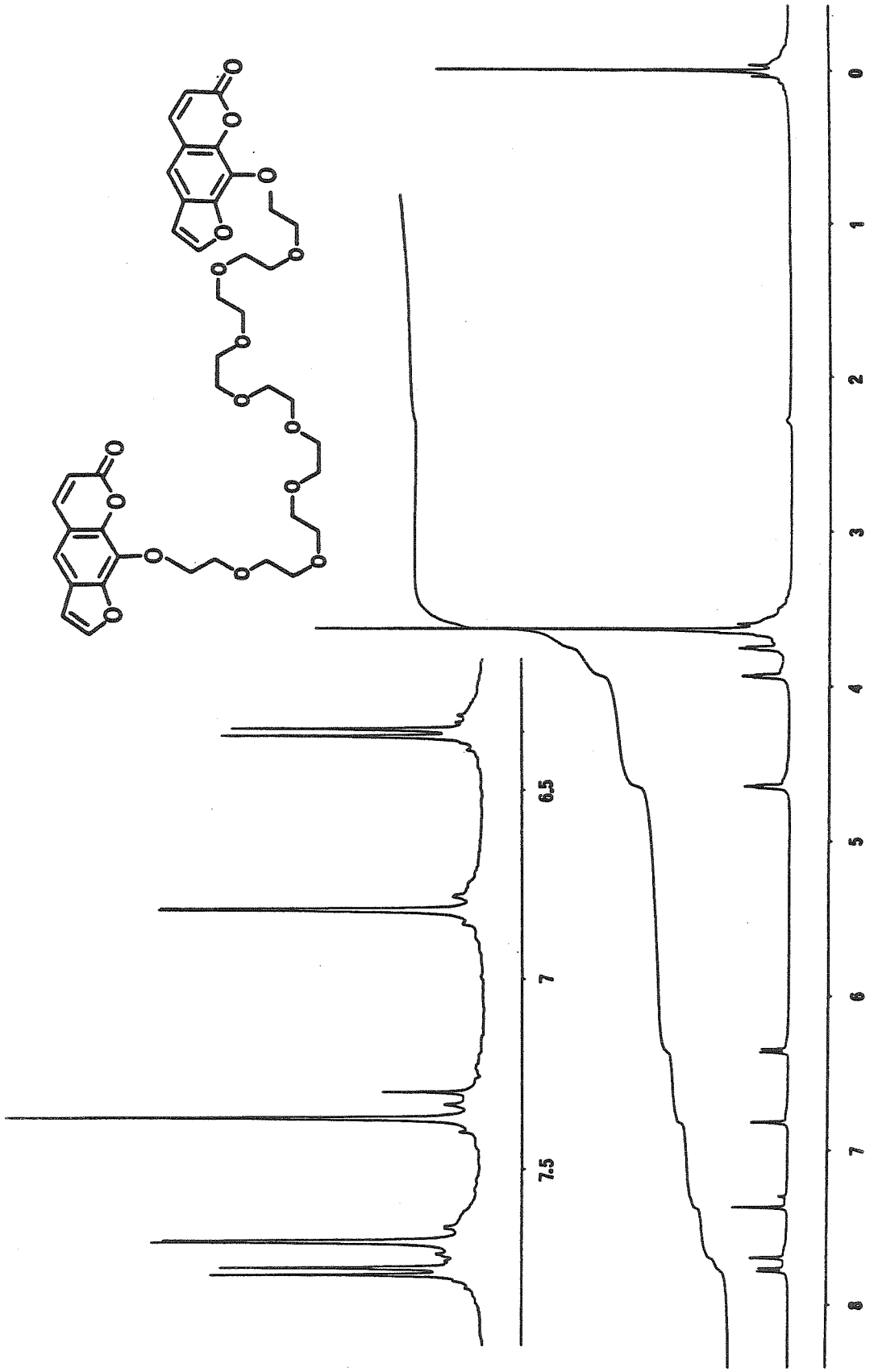
To a solution of 8-methoxypsoralen (10 g, 46 mmol), in 200 ml of dry methylene chloride is added a solution of boron tribromide (10 ml, 106 mmol) in 50 ml of methylene chloride. After stirring for 5 hours the reaction is transferred to a 1 liter beaker. Water (450 ml) is added with stirring, resulting in the precipitation of a white solid. The solid is filtered, dried in vacuo and recrystallized from acetonitrile, yielding 8-hydroxypsoralen (8.2 g, 41 mmol, 87%). Sublimation at 0.1-0.3 mm (230°C oil bath) affords light yellow crystals m.p. 239-240°C. To a solution of 8-hydroxypsoralen (8.0 g, 39 mmol) in 500 ml of dry methanol at 0°C is added with stirring a solution of potassium hydroxide (2.2 g, 33 mmol) in dry methanol over a fifteen minute period. Sufficient methanol is added to dissolve remaining solids. The solution is filtered and stored over 3Å molecular sieves overnight protected from moisture with a calcium chloride drying tube. Evaporation of the methanol yields a bright yellow solid, the potassium salt of 8-hydroxypsoralen, (8.6 g, 36 mmol

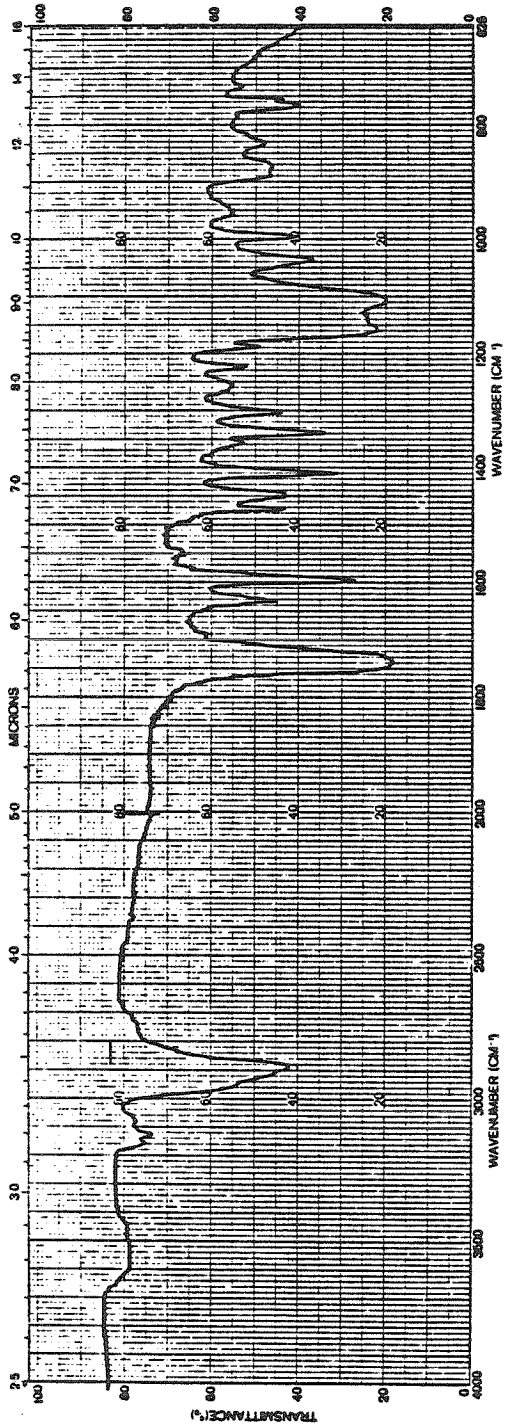
91%).

Bis(psoralen)nonaethyleneoxy ether (BPNE) (7)

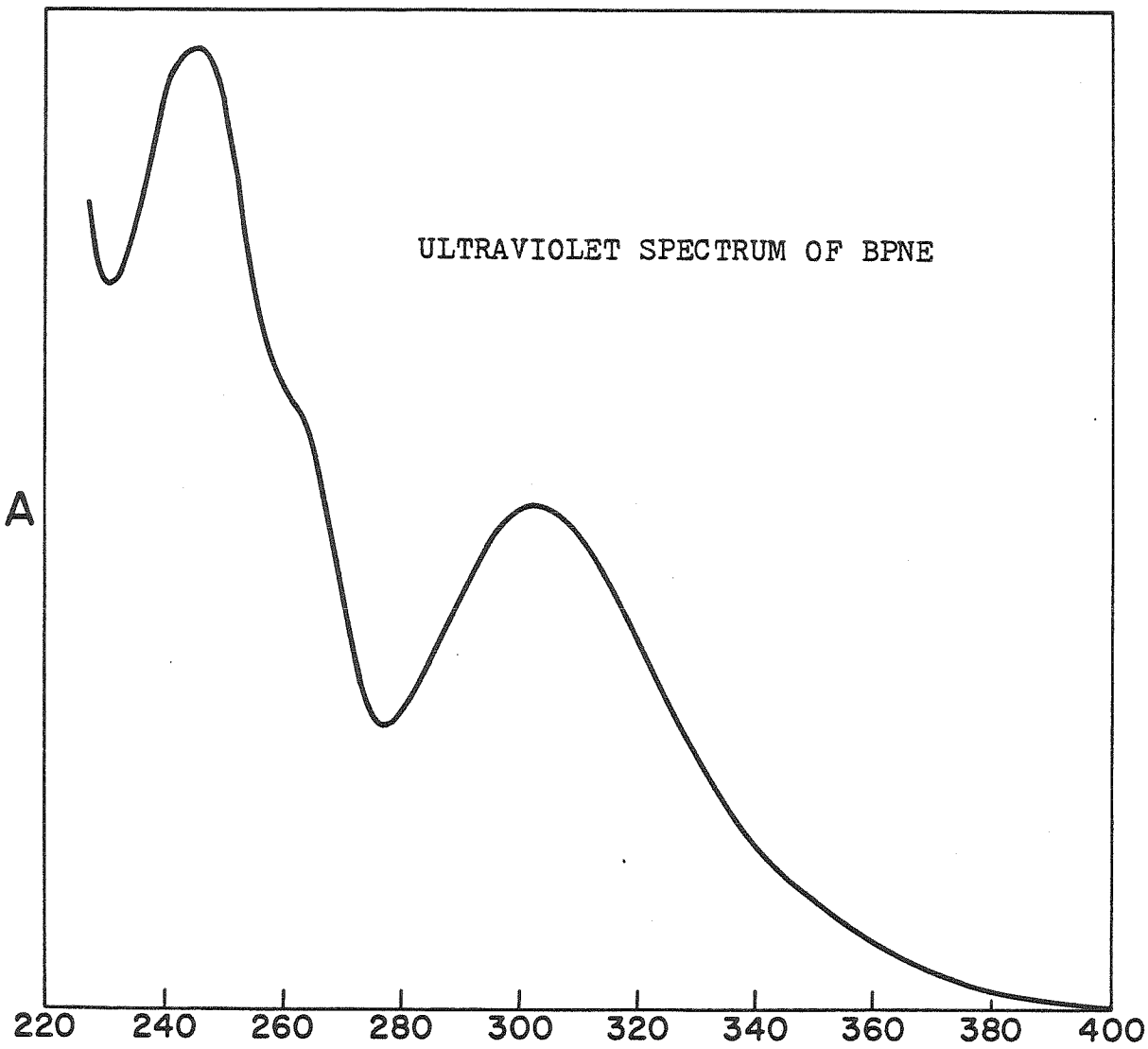
To a well dried 250 ml roundbottom flask equipped with a reflux condenser, nitrogen inlet tube and two septum inlets was added one stirbar, nonaethylene glycol ditosylate (0.92 g, 1.3 mmol), the potassium salt of 8-hydroxypsoralen (3.1 g 13 mmol) and dry N,N -dimethylformamide (150 ml, dried over 4Å sieves). The mixture was heated to 90°C while stirring under a nitrogen atmosphere. After stirring overnight at 90°C, 50 ml of water was added to the mixture and heating was continued for two hours. After evaporation of the reaction mixture to dryness in vacuo, the product was chromatographed in 100 mg portions on 500 g silica gel 60 (70-230 mesh ASTM) eluted with a 70:30 mixture by volume of acetonitrile:chloroform. Elution was followed by TLC on silica gel plates developed with 90:10 chloroform; acetonitrile. The fractions containing the major product were combined and evaporated to yield analytically pure bis(psoralen)nonaethyleneoxy ether (0.97 g, 97%) as a light yellow oil; ir (neat) 3120, 2880, 1735, 1630, 1595, 1470, 1450, 1410, 1340, 1300, 1255, 1220, 1155, 1100, 1035, 990, 950, 880, 830 and 765 cm^{-1}; nmr ($CDCl_3$) 3.5-4.8 (m, 36H, $O\underline{CH_2CH_2}O$), 6.31 (d, 2H, $\underline{J}$=9.5 Hz, Ha) 6.81 (d, 2H, $\underline{J}$=2Hz, Hd), 7.39 (s, 2H, Hc), 7.71 (d, 2H, $\underline{J}$=2, He) and 7.79 (d, 2H, $\underline{J}$-9.5 Hz, Hb).[52]




6.5
7
7.5
0
1
2
3
4
5
6
7
8



INFRARED SPECTRUM OF BPNE


ULTRAVIOLET SPECTRUM OF BPNE
A
220
240
260
280
300
320
340
360
380
400

Anal. Calcd. for $C_{40}H_{46}O_{16}$: C, 61.37; H, 5.92. Found: C, 61.15; H, 5.78.

Buffers

All manipulations of bacteriophage λ and isolated λ DNA were carried out in a standard incubation buffer of: 10 mM Tris, 10 mM $MgCl_2$, 150 mM NaCl, pH 7.5. Reactions utilizing T4 polymerase or restriction endonucleases SstII and SacI were carried out in a buffer designated as TA buffer which consisted of: 33 mM Tris acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 100 μg/ml nuclease free Bovine Serum Albumin (BSA) and 0.5 mM dithiothreitol. Reactions utilizing the restriction endonuclease MstII were carried out in: 6 mM Tris, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 150 mM NaCl, and 100 g/ml BSA. EcoRI reactions were carried out in 100 mM Tris, 50 mM NaCl, 5 mM $MgCl_2$ and 100 μg/ml BSA.

Avidin Spheres

The preparation of polymethacrylate spheres followed exactly the procedure described by Manning et al.[45] The procedure for covalently binding avidin to the spheres was also exactly as done by Manning et al. except reactions were often run on a smaller scale (0.1 ml total volume). Purification was done by electrophoresis on a short (≅ 2") column of 0.7% agarose with a dialysis bag attached to the bottom of the tube. This procedure eliminated most of the oligomerized spheres as well as uncoupled avidin. The main band of spheres could be visualized by turbidity in the gel.

Bacteriophage λ

Bacteriophage λ was produced by thermal induction of the E. coli. lysogen W3110 bearing λ cI 857S7. Three liters of media divided between four, two liter flasks containing the E. coli lysogen was incubated at 37°C in rich media to a density of $4x10^8$ cells/ml. The lytic cycle was induced by immersing the flasks in 45°C water for 15 minutes with occasional shaking. After shaking an additional 5 hrs. the infected cells were lysed in the media by adding 0.5 ml chloroform to each flask. The flasks were shaken an additional 15 minutes at 37°C. Bacterial debris was spun down in a J6 Centrifuge, 4.2 rotor at 4200 rpm for 30 minutes (2000 g at inner radius, 5000 g at outer radius). Solid sodium chloride was added to $\geq$ 0.5 M (30 g/l), and PEG-6000 (Carbowax 6000) added to 10%. Phage were allowed to precipitate overnight. The pellet was harvested as above (4200 rpm, 30 minutes). The pellet was resuspended in 10 mM Tris, 10 mM mg SO_4, pH 7.5 (30 ml). Resuspended phage were given a gentle spin to remove debris (2000 rpm, SS34 rotor, 10 minutes). Solid CsCl was added to give a density of 1.5 g/ml (0.8 g CsCl per ml resuspended phage). The phage suspension was poured into polycarbonate 5/8" x 3" tubes, balanced and spun at 35000 rpm for 30 hours in a Type 40 rotor. The blue opalescent phage band was removed by inserting a micropipet from above. Bacteriophage λ was further purified by a second CsCl density gradient centrifugation as described above. Bacteriophage λ was stored at 4°C in the density gradient solution and dialyzed into the standard incubation buffer of 10 mM Tris, 10 mM $MgCl_2$, 150 mM NaCl pH 7.5 only just prior to use. The bacteriophage λ stock solution was $3.23x10^{-3}$ M in base pairs as determined by UV absorption. ϵ_{260}

37000/Mbp cm).[19,51]

Stock Solutions of BAMO and [^{3}H] BAMO

Samples of BAMO and [^{3}H] BAMO were dissolved in 10 mM potassium cacodylate pH 7.5 and diluted to a standard stock concentration of $3x10^{-5}$ M (ϵ_{481}, 8730/M cm).

Photolysis of Samples

Samples prepared in 1.5 ml Eppendorf centrifuge tubes were irradiated from above through a pyrex crystallizing dish filled to a level of 5 cm with 2.9 M sodium nitrite solution placed directly over the open sample tubes. The illumination source was a desk lamp fitted with two GE F15 T8 CW light bulbs, placed directly above the sodium nitrite filter.

Incorporation of [^{3}H] BAMO into Bacteriophage λ

The covalent incorporation of [^{3}H] BAMO onto the DNA of intact Bacteriophage λ was studied as a function of three experimental variables: incubation time in the dark, time of irradiation and binding ratio (BAMO/bp).

A. Incubation Time in the Dark. Samples were prepared by diluting Bacteriophage λ (3.72 μl of stock solution), 5x concentrated incubation buffer (7.26 μl) and [^{3}H] BAMO (4.0 μl of stock solution) to a total volume of 40 μl with water. This results in final concentrations of $3x10^{-6}$ M BAMO and $3x10^{-4}$ M base pairs. Samples were incubated in the dark for times ranging from one minute to 24 hours. After irradiating samples for 1 hour as described above, sodium docecyl sulfate (6 μl of 10% solution) and Proteinase K (6 μl of 10 mg/ml of stock solution) and water to 60 μl total volume were added to each sample to burst the bacteriophage heads allowing isolation of

the DNA. These samples were incubated at 55°C for 1 hour then at 37°C for 4 hours. Each sample was applied to a 1.5 ml Sepharose 2B-CL column equilibrated with incubation buffer. DNA containing fractions were combined, concentrated to a volume of approximately 100 1 by extraction with 2-butanol then ether, and dialyzed into incubation buffer. The quantities of DNA in each sample was determined by UV absorption (ϵ_{260}, 13,200/Mbp cm)) and the quantity of [^{3}H] BAMO (3.3×10^9 cpm/μmol) covalently bound to the DNA by scintillation counting of a measured volume of the UV sample diluted into 10 ml of Aquasol 2. incubation time in the dark, time of irradiation and binding ratio (BAMO/bp).

B. Time of Irradiation. Samples were prepared as for "incubation time in the dark" experiments described above and incubated in the dark for a standard time of 2 hours. Samples were then irradiated for times ranging from 30 minutes to 3 hours, and analyzed as described above.

C. Binding Ratio. Samples were prepared by diluting bacteriophage λ (3.72 μl of stock solution), five times concentrated incubation buffer (7.26 μl) and ^{3}H BAMO (aliquots ranging from 2.0 μl to 20 μl) to a total volume of 40 μl with water. This results in binding ratios ranging from 0.005 to 0.05 BAMO/bp at a bacteriophage λ concentration of 3×10^{-4} M in base pairs. After incubating the samples in the dark for 2 hours and irradiating them for 1 hour as described above, sodium dodecyl sulfate (6 μl of 10% solution), proteinase K (6 μl of 10 mg/ml stock solution) and water to 60 μl total volume were added to each sample to burst the bacteriophage heads allowing isolation of the DNA. These samples were incubated at 55°C for 1 hour

then at 37°C for 4 hours. Each sample was applied to a 1.5 ml Sepharose 2B-CL column equilibrated and developed with incubation buffer. DNA containing fractions were combined, concentrated to a volume of approximately 100 μl by extraction with 2-butanol, then ether, and dialyzed into incubation buffer. The quantity of DNA in each sample was determined by UV absorption (ϵ_{260}, 13200/Mbp cm); and the quantity of [^{3}H] BAMO (3.3×10^9 cpm/μmol) covalently bound to the DNA by scintillation counting of a measured volume of the UV sample diluted into 10 ml of Aquasol 2.

Control experiments were performed with samples prepared as above except without irradiation to determine the efficiency with which the workup procedure removed noncovalently bound [^{3}H] BAMO from the DNA.

Gel Electrophoresis and Electron Microscopy of Unrestricted DNA from BAMO Crosslinked Bacteriophage λ

Samples were prepared by diluting bacteriophage λ (3.72 μl of stock solution), five times concentrated incubation buffer (7.26 μl) and BAMO (aliquots ranging from 2 μl to 12 μl of $3x10^{-5}$ M stock or 2 μl to 8 μl of $3x10^{-4}$ M stock) to a total volume of 40 μl with water. This results in binding ratios ranging from 0.005 to 0.2 BAMO/bp at a bacteriophage λ concentration of $3x10^{-4}$ M in base pairs. After incubating the samples in the dark for 1 hour and irradiating them for 1 hour as described above, sodium docecyl sulfate (6 μl of 10% solution), Proteinase K (6 μl of 10 mg/ml stock solution) and water to 60 μl total volume were added to each sample. These samples were incubated at 55°C for 1 hour then 37°C for 10 hours. Each sample was applied to a 1.5 ml Sepharose 2B-CL column equilibrated and developed with incubation buffer. DNA containing fractions were combined, concentrated to a volume of 80 μl by extraction with 2-butanol, then ether, and dialyzed into incubation buffer. To an aliquot (12 μl) of each solution was added 5 μl of gel loading solution (25% Ficoll, 0.0025% Bromophenol Blue in water). Samples were loaded onto a 0.3% horizontal agarose slab gel. The gel was run at 30 volts (2.0 v/cm) for 12 hours, stained with ethidium bromide and photographed. Another aliquot from each sample was diluted to 0.01 g DNA/ml and spread for electron microscopy from a hyperphase of 50% formamide onto a hypophase of 20% formamide.

Gel Electrophoresis and Electron Microscopy of Restricted DNA From BAMO Crosslinked Bacteriophage λ

Samples prepared, crosslinked, burst and Sepharose purified as described above were dialyzed into the appropriate buffer for various restriction enzymes. Samples were incubated at 37°C for the appropriate time. Aliquots were analyzed on a 0.8% agarose horizontal slab gel. Gels were stained with ethidium bromide and photographed. Another aliquot from each sample was diluted to 0.005 μg DNA/ml and spread for electron microscopy from a hyperphase of 50% formamide onto a hypophase of 20% formamide.

End Labeling of Sac I/SsT II Restriction Fragments

The labeling of restriction fragments using T4 polymerase and biotinylated dUTP (Bio-dUTP) was carried out according to the procedure described by P.H. O'Farrell in a technical report on T4 polymerase published by Bethesda Research Laboratories, Inc.[48] The reaction buffer designated TA buffer has a composition of: 33 mM Tris acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 100 μg/ml nuclease free Bovine Serum Albumin (BSA) and 0.5 mM dithiothreitol; and was stored as frozen aliquots of a 10 times concentrated buffer. Purified crosslinked bacteriophage λ DNA (2 μg), 10 times concentrated TA buffer (2 μl) and water to give a total volume of 20 μl were combined and treated with 20 units Sac I restriction endonuclease for 1 hour at 37°C. T4 polymerase (2.5 units) was added directly to the restriction mixture and incubated for 15 minutes at 37°C. One microliter of a solution containing 2 mM each of dATP, dGTP and dCTP was added to the reaction mixture along with 7 μl of a 0.3 mM Bio-dUTP solution. After incubation at 37°C for 45

minutes, 1 μl of a 2 mM dUTP solution was added and incubation continued for an additional 30 minutes. T4 polymerase was then inactivated by heating to 70°C for 5 minutes. After cooling the reaction mixture to 37°C, restriction endonuclease Sst II (14 units, 2 μl) was added and the solution incubated an additional 1 hour at 37°C. After the addition of 5 M NaCl (9 μl) and 0.2 M Na_2EDTA (3 μl) the solution was loaded onto a 1.5 ml Sepharose 2B-Cl column and eluted with incubation buffer. DNA containing fractions were collected and combined. Incubation of aliquots of this solution containing 0.01 μg DNA with avidin spheres (2 μl) provided end labeled DNA suitable for electron microscopy.

Electron Microscopy of Restricted DNA from BPNE Crosslinked Bacteriophage λ

Samples were prepared by diluting bacteriophage λ (7.44 μl of stock solution), ten times concentrated incubation buffer (7.26 μl) and BPNE (65.3 μl of 1.0×10^{-4} M stock solution). This results in a binding ratio of 0.28 BPNE/bp at a bacteriophage λ concentration of 3×10^{-4} M in base pairs. After incubating the samples in the dark for either 3 or 7 hours, they were irradiated from above by placing 1/4" plexiglass directly on top of the open sample tubes and the light source (two GE F15T8BLB bulbs) directly on top of the plexiglass. The samples were irradiated for times ranging from 2 to 10 hours. Samples were burst with sodium dodecyl sulfate/proteinase K and sepharose purified as described above for BAMO crosslinking experiments. A 2 μg aliquot of the purified DNA was concentrated to a volume of 45 μl by extraction with 2-butanol, then ether and dialyzed into 5 mM Tris, 0.5

mM Na_2EDTA pH 7.5. To this sample was added 5 μl of 10 times concentrated TA buffer, 20 units of Sac I (2 μl) and 14 units of Sst II (1 μl) restriction endonucleases. After incubation for 30 minutes at 37°C, a 5 μl aliquot of this restriction sample was diluted into 10 mM Tris, 1 mM Na_2EDTA, pH 7.5 (120 μl). Two microliters of this diluted sample was spread for electron microscopy from a hyperphase of 50% formamide onto a hypophase of 20% formamide.

Preparation of Biotinylated deoxyuridine 5'-triphosphate (Bio-dUTP) (17)

The synthesis and purification of 5-mercurideoxyuridine 5'-triphosphate (5-Hg dUTP) and 5-(3-amino)allyldeoxyuridine 5'-triphosphate (AA-dUTP) were carried out exactly as prescribed by Langer et al.[47] Final purification of AA-dUTP was achieved by high pressure liquid chromatography on a 25 cm Whatman Partisil-10 ODS-2 column using 0.5 M acetic acid adjusted to pH 4.3 with triethylamine as the eluant. This chromotography is crucial and other types of reverse phase columns cannot be substituted.

Biotin-N-hydroxysuccinimide ester (NHS-Biotin) was prepared as prescribed by Bayer et al.[53] To a solution of biotin (Sigma, 1.0g, 4.09 mmol) and N-hydroxysuccinimide (0.6g, 5.2 mmol) in dry N,N -dimethylformamide (12 ml) at room temperature was added dicyclohexylcarbodiimide (0.8g 3.9 mmol). The reaction mixture was stirred overnight and then filtered. The filtrate was evaporated to dryness. The remaining residue was washed with ether and recrystallized from isopropanol (≅ 250ml) to yield NHS-Biotin (1.06g, 76%). NHS-Biotin was coupled to 6-aminihexanoic acid according to the following procedure. NHS-Biotin (0.341g, 1 mmol) and 6-aminohexanoic acid (0.157g, 1.2 mmol) were suspended in 20 ml of N,N -dimethylformamide/water (3:1) and stirred for 20 hours at room temperature. The reaction mixture was filtered and the solid recovered. The solid was washed with 1M HCl then with water, dried in vacuo at room temperature and used without further purification. The resulting product had been synthesized previously by another route.[54] This biotinylated hexanoic acid was converted to its

N-hydroxysuccinimide ester as follows. To a solution of biotinylated hexanoic acid (0.2g, 0.559 mmol) and N-hydroxysuccinimide (77 mg, 0.67 mmol) in 5 ml dry N,N -dimethylformamide was added dicyclohexylcarbodiimide (0.127g, 0.62 mmol). The reaction mixture was stirred for 24 hours at room temperature and filtered. The filtrate was evaporated to dryness. The remaining residue was washed with ether and recrystallized from isopropanol. The resulting solid was analyzed by thin layer chromatography on silica using methylene chloride/methanol (90:10) or methylene chloride/methanol/acetic acid (9:1:1) as eluant. This analysis indicated the presence of approximately 20% contaminating unconverted biotinylated hexanoic acid. The product was used without further purification. AA-dUTP was converted to Bio-dUTP using biotinylated hexanoic NHS-ester according to the procedure described by Langer et.al.[47] AA-dUTP (3.2 mg, 0.005 mmol) dissolved in 1.0 ml of 0.1M sodium borate, pH 8.5, was treated with biotinylated hexanoic NHS-ester (3.8 mg in 0.1 ml) of N,N -dimethylformamide). The reaction mixture was left at room temperature for 4 hours and then loaded directly onto a 30 ml column of DEAE-Sephadex A-25 previously equilibrated with 0.1M Et_3NHCO_3, pH 7.5. This column was prepared, loaded and eluted at 4°C. The column was eluted with a 400 ml linear gradient (0.1-0.9 M) of Et_3NHCO_3. Fractions containing Bio-dUTP which eluted at 0.55-0.65 M Et_3NHCO_3, were desalted by rotary evaporation in the presence of methanol and then dissolved in water. The solution of Bio-dUTP was briefly stirred in the presence of Dowex 50 (Na^+), filtered and precipitated by the addition of 3 volumes of cold acetone, washed with diethyl ether, dried under reduced pressure and stored at -20°C. Solutions of

Bio-dUTP were made up in 20 mM Tris·HCl, pH 7.5 and filtered through a 0.45 μm filter. λ_{max} 289 nm, λ_{max} 240 nm, λ_{min} 267 nm. The λ_{max} at 289 nm indicates the presence of an exocyclic double bond conjugated with the pyrimidine ring. And in contrast to AA-dUTP, Bio-dUTP does not give a positive ninhydrin reaction. This Bio-dUTP is currently available commercially from Enzo Biochem (New York, NY) under the trade name of Bio-Probe™ Biotinylated Probe. The commercial product is supplied as the triethyl- ammonium salt dissolved in 50 mM Tris·HCl, pH 7.4. The reported spectral data for the commercial product are: λ_{max} 290 nm (ϵ = 7000), λ_{min} 262 nm (ϵ = 4400). The purity of Bio-dUTP reported can be assayed by high pressure liquid chromatography using an Ultrasphere ODS-18 column. The Bio-dUTP is eluted with a solvent consisting of 30% Methanol and 70% 0.5M ammonium phosphate buffer, pH 3.5. At a flow rate of 1.0 ml/min, Bio-dUTP has a retention time of 7.3 minutes.

References

1) Brimacombe, R.; Stoffler, G.; Whittman, H.G.; Ann. Rev. Biochem. (1978), **92**, 217-249.

2) Kornberg, R.D.; Ann. Rev. Biochem. (1977), **46**, 931-954.

3) Shick, V.V.; Belyavsky, A.V.; Barykin, S.G.; Mirzabekov, A.D.; J. Mol. Biol. (1980), **139**, 491-517.

4) North, A.C.T.; Rich, A.; Nature (1961), **191**, 1242-1245.

5) Earnshaw, W.C.; Harrison, S.C.; Nature, (1977), **268**, 598-602.

6) Cummings, D.J.; Chapman, V.A.; DeLong, S.S.; J. Mol. Biol. (1965), **14**, 418-422.

7) Richards, K.E.; Williams, R.C.; Calendar, R.; J. Mol. Biol. (1973), **78**, 255-259.

8) Hall, S.B.; Schellman, J.A.; Biopolymers, submitted.

9) Maestre, M.F.; Tinoco, I.; J. Mol. Biol. (1967), **23**, 323-335.

10) Gellert, M.; Davies, D.R.; J. Mol. Biol. (1964), **8**, 341-347.

11) Maestre, M.F.; Biopolymers (1968), **6**, 415-430.

12) deGroot, G.; Greve, J.; Blok, J.; Biopolymers (1977), **16**, 639-654.

13) Kosturko, L.D.; Hogan, M.; Dattagupta, N.; Cell (1979), **16**, 515-522.

14) Cole, A.; Langley, R.; Biophys. J. (1963) 3, 189-197.

15) in Tikchonenko, T.I.; "Comprehensive Virology" (Fraenkel-Conrat, H. and Wagner, R.R., eds.), Vol. 5, pp. 1-117,

Plenum Press, New York.

16) Hohn, T.; Katsura, I.; Current Topics in Microbiol. Immunol. (1977), **78**, 69-110.

17) Sanger, F.; Coulson, A.R.; Hong, G-F.; Hill, D.F.; Petersen, G.B.; J. Mol. Biol. (1982), in press.

18) Emmons, S.; J. Mol. Biol. **83**, 511-525.

19) Thomas, J.O.; J. Mol. Biol. (1974) **87**, 1-9.

20) Stroud, R.M.; Serwer, P.; Ross, M.J.; Biophys. J. (1981), **36**, 743-757.

21) Studier, F.W.; Virology (1979), **95**, 70-84.

22) Serwer, P.; J. Mol. Biol. (1976), **107**, 271-291.

23) Pao, C-C.; Speyer, J.F.; J. Virol. (1973), **11**, 1024-1026.

24) Haas, R.; Murphy, R.F.; Cantor, C.R.; J. Mol. Biol. (1982), **159**, 71-92.

25) Olomucki, M.; Jerram, M.; Parfait, R.; Bollen, A.; Gros, F.; Bioorg. Chem. (1981), **10**, 455-469.

26) Scott, B.R.; Pathak, M.A.; Mohn, G.R.; Mutation Res. (1976) **39**, 29-74.

27) a) Wakelin, L.P.G.; Waring, M.J.; Mol. Pharm. (1974) **10**, 544-561; b) Tsai, C.C.; Jain, S.C.; Sobell, H.M.; Proc. Natl. Acad. Sci. USA (1975), **72**, 628-632.

28) Hearst, J.E.; Ann. Rev. Biophys. Bioeng. (1981) **10**, 69-86.

29) Graves, D.E.; Watkins, C.L.; Yielding, L.W. Biochemistry (1981) **20**, 1887-1892.

30) Isaacs, S., Shen, C-K.J.; Hearst, J.E.; Rapoport, H.; Biochemistry (1977) **16**, 1058-1064.

31) Bomes, B.; Heitz, W.; Kern, W.; J. Chromatogr. (1970) **53**, 51-54.

32) Garland, F.; Graves, D.E.; Yielding, L.W., Cheung, H.C.; Biochemistry (1980) **19**, 3221-3226.

33) Bolton, P.H.; Kearns, D.R.; Nucl. Acids Res. (1978) **5**, 4891-4903.

34) Mitchell, M.A.; Dervan, P.B.; J. Am. Chem. Soc. (1982), **104**, 4265-4266.

35) Para-carboxymethidium chloride was obtained from previous work. Dervan, P.B.; Becker, M.M.; J. Am. Chem. Soc. (1978) **100**, 1968-1970.

36) Graves, D.E.; Yielding, L.W.; Watkins, C.L.; Yielding, K.L.; Biochim. Biophys. Acta (1977), **479**, 98-104.

37) Sternglanz, H.; Graves, D.E.; Yielding, L.W.; Bugg, C.E.; J. Cryst. Mol. Struct. (1978) **8**, 93-103.

38) Liu, L.F.; Davis, J.L.; Nucl. Acids Res. (1981) **9**, 3979-3989.

39) Data not shown.

40) The restriction map for Mst II was obtained from New England Biolabs (Beverly, MA).

41) Other restriction maps were obtained from: Daniels, D.L.; Blattner, F.R.; Virology (1982), **117**, 81-92.

42) Ames, B.N.; Dubin, D.T.; J. Biol. Chem. (1960) **235**, 769-775.

43) Personal observation.

44) Wollenzien, P.L.; Cantor, C.R.; Proc. Natl. Acad. Sci. USA, (1982), 79, 3940-3944.

45) Manning, J.E.; Hershey, N.D.; Broker, T.R.; Pellegrini, M.; Mitchell, H.K.; Davidson, N.; Chromosoma (1975) 53, 107-117.

46) Bayer, E.A., Wilchek, M.; Methods of Biochem. Analysis (1980) 26, 1-45.

47) Langer, P.R.; Waldrop, A.A.; Ward, D.C.; Proc. Natl. Acad. Sci. USA (1981) 78, 6633-6637.

48) O'Farrell, P.H.; Focus (1981), 3, 1-3. This publication is a technical report on T4 polymerase published by Bethesda Research Laboratories, Inc. A preliminary report of this work has been published.[49]

49) O'Farrell, P.H.; Kutter, E.; Nakanishi, M.; Mol. Gen. Genet. (1980), 179, 421-435.

50) McDonnell, M.W.; Simon, M.N.; Studier, F.W.; J. Mol. Biol. (1977) 110, 119-146.

51) Davis, R.W.; Simon, M.N.; Davidson, N.; Meth. Enzymol. (1971) 21, 413-428.

52) Jarvis, M.W.; Moritz, A.G.; Aust. J. Chem. (1968) 21, 2445-2449.

53) Bayer, E.A.; Skutelsky, E.; Wilchek, M.; Meth. Enzymol. (1979), 62, 308-315.

54) Green, N.M.; Biochem. J. (1963), 89, 599-609.