THE STRUCTURE OF THE BACTERIOPHAGE ALPHA DNA MOLECULE

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ABSTRACT

Selective precipitation with polyethylene glycol is an efficient method for concentrating and purifying bacteriophage α and other phages.

The phage α DNA molecule has a molecular weight of 33 million. When prepared by phenol extraction of crude phage suspensions, it contains many single-strand breaks. When prepared by phenol extraction of purified phage, it contains approximately one randomly-located single-strand break per molecule. The number of single-strand breaks can be further reduced by changing the conditions of the phenol extraction.

The complementary single strands of α DNA can be separated by MAK chromatography followed by self-annealing and hydroxylapatite chromatography, but this procedure results in extensive breakage of the strands. An alternative procedure has been developed using CsCl gradient centrifuging in the presence of polyguanylic acid (polyG) to give an efficient separation of the intact strands in 100 μg. quantities.

Both the L strand and the H strand of α DNA form complexes with polyG, although to different extents. The PolyG binding sites in the L strand appear to be confined to a small segment having a similar buoyant density to the H strand.

Sequences of consecutive pyrimidine nucleotides of all lengths up to 13 have been detected in diphenylamine-formic acid digests of α DNA. There is a slight general tendency towards clustering of the pyrimidine nucleotides, sequences of lengths 1-4 being present at

below random frequencies, and longer sequences being present at above random frequencies. These same general features are found in diphenylamine digests of the separated H and L strands. The distribution of pyrimidine nucleotide sequence lengths in α DNA does not appear to follow a rhythmic code of the type found in RNA phages.

Preliminary analyses have been made of the longest pyrimidine nucleotide sequences in α DNA, and of the distribution of various sequences between the two strands.

The dialysis of pyrimidine deoxyoligonucleotides was investigated, and found to be strongly influenced by cytosine content. This may reflect an unusual conformation of cytosine-rich oligonucleotides at low ionic strength. Gel filtration was found to provide a satisfactory method for the preliminary fractionation of diphenylamine digests on the basis of chain length.

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ABBREVIATIONS

α	alpha
p.f.u.	plaque-forming units
DNA, RNA	deoxyribonucleic acid, ribonucleic acid
L strand	purine-rich strand of alpha DNA
H strand	pyrimidine-rich strand of alpha DNA
DNase	deoxyribonuclease
A,G,C,T	adenine, guanine, cytosine, thymine
polyG	polyriboguanylic acid
polyIG, polyUG	copolymer of guanylic acid with inosinic acid, uridylic acid
PEG	polyethylene glycol
MAK	methylated albumin adsorbed on kieselguhr
SDDC	sodium diethyldithiocarbamate
EDTA	diaminoethanetetra-acetic acid
tris	tris(hydroxymethyl) aminomethane
SSC	0.15 M NaCl, 0.015 M sodium citrate (pH 7)
SP	saline phosphate buffer pH 6.8, containing 0.05 M phosphate
PB	phosphate buffer pH 7.0
DEAE-	N,N-diethylamino-ethyl-
TCA	trichloroacetic acid
PPO	2,5-diphenyloxazole
POPOP	1,4-bis-(5-phenyloxazolyl-2)-benzene
Py	pyrimidine nucleotide
p	esterified phosphate (5' to the left, 3' to the right)
d	deoxy-
A, n, ε	absorbance, refractive index, extinction
η, ρ	intrinsic viscosity, density
S^0	sedimentation coefficient at infinite dilution
I, M	ionic strength, molarity
P.S.F.	percentage sequence frequency
S.E.	standard error

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INTRODUCTION

A. The Structure of Phage DNA Molecules.

In 1959, Sinsheimer demonstrated that the entire DNA complement of bacteriophage ϕX-174 is contained in a single molecule[1]. A similar demonstration soon followed for bacteriophage T2[2]. The identity between the phage DNA molecule and the phage chromosome has now been universally accepted, and has provided the stimulus for detailed investigations of DNA structure.

The molecular weights of many phage and viral DNA molecules have been accurately measured, largely as a result of developments in the techniques of electron microscopy and zone sedimentation. Apart from an unusual group of small phages, including ϕX-174, in which the DNA molecule is a single-stranded circle of molecular weight approximately 2×10^6, all phages have been found to contain linear double-stranded DNA molecules ranging in molecular weight from 10×10^6 to 200×10^6. A useful summary of current knowledge is available in a review by Thomas and MacHattie[3].

The continued search for explanations for certain phage genetic phenomena in the physical structure of phage DNA has had several notable successes. The circularity of the genetic map of phage T2[4,5] led to experiments which demonstrated that the linear DNA molecules of this phage consist of a mixture of circularly permuted sequences[6,7]. A similar observation has been made for the DNA molecules of phage P22[8]. Other species, including phages T3, T5, T7, λ and SP82, contain a single non-permuted DNA sequence[7,9,10,11].

A hypothesis intended to explain the existence of two types of heterozygote in populations of phage T2[8,12] led to an ingenious demonstration that each molecule of T2 DNA is terminally redundant, carrying the same sequence of approximately 1-3% of the molecular length at each end[13].

Terminal redundancy has also been demonstrated in the DNA molecules of other phage species of both permuted and unique types[8,9].

A rather different form of terminal redundancy is found in the short single-stranded ends of the non-permuted DNA molecules of phage λ. These terminal sequences, only 20 nucleotides long[14], are complementary in base sequence[15]. They can be annealed together under appropriate conditions, causing the molecule to form a circle. Removal of the single-stranded ends destroys the infectivity of the DNA[16].

One of the most exciting discoveries in this field has been the demonstration that some phage DNA molecules have a highly organised structure at a level which can be described as molecular anatomy, where the units of structure are blocks containing tens of thousands of nucleotides. The most thoroughly studied case, the DNA molecule of phage λ, has been shown to contain at least six segments of different base composition, ranging in G + C content from 37 to 57 mole %[17]. Each segment appears to be reasonably homogeneous in G + C content, and the boundaries between segments are thought to be rather sharp: not more than a few % of the molecular length.

The internal heterogeneity of the λ DNA molecule has enabled the half fragments formed by shearing to be separated into two classes, corresponding to the left and right halves of the λ genetic map[10,16].

The DNA molecules of the temperate phages ϕ80, 434 and P22 can also be differentiated into several regions with distinctive G + C compositions[18]. The DNA molecules from the virulent phages of the T-series do not exhibit a marked segmental distribution of nucleotides when sheared to large fragments[19,20,21]. However, shearing these 'homogeneous' DNA molecules to a much smaller fragment size (molecular weight approx. 500,000) reveals a heterogeneity in base composition comparable with that of λ DNA[22].

Some phage DNA molecules exhibit a pronounced bias in base composition between the two complementary polynucleotide strands, allowing separation of the strands by chromatography or by centrifuging in density gradients[23,24]. A more universal bias between the two strands of phage DNA molecules is reflected in their preferential interaction with synthetic polyribonucleotides[25,26,27]. These differences appear to be correlated in some way with selection of the 'message' strand for the transcription of genetic information into messenger RNA[28,29,30].

B. Sequence Determination in DNA.

The problem of nucleotide sequence determination in DNA molecules has proved less tractable than the problems of DNA molecular structure. Many researchers appear to have been discouraged by the large size of even the smallest available homogeneous deoxyribonucleotide sequences[31]. Nevertheless, it may be confidently predicted that the next decade will see substantial progress in this field.

The determination of both DNA and RNA nucleotide sequences can be regarded as a problem essentially similar to the determination of protein

primary structure. The basic task in each case involves enzymic or chemical degradation of the polymer to a mixture of monomers and short oligomers, followed by separation and identification of these components by chromatography and electrophoresis. After complete elucidation of the short fragments, more limited degradation procedures must be used to obtain successively larger groups of oligomers. Eventually, sufficient overlap between known sequences should be obtained to define unambiguously the sequence of the intact polymer.

This approach, as briefly outlined recently by Holley[32], has resulted in the complete sequence elucidation of several transfer RNA[33,34] and 5s ribosomal RNA molecules[35,36]. Unfortunately, the complexity of the basic sequencing task increases very rapidly with increasing chain length. The lack of comparable progress in DNA sequencing can be directly attributed to the lack of availability of short (length 60-120 nucleotides) homogeneous species of DNA molecules, although the highly repetitious nucleotide sequences in the satellite band of Mouse DNA[37] may provide a solution to this problem.

A problem peculiar to DNA sequence determination is the lack of enzymes with a known sequence specificity. This situation has not changed markedly since the 1965 review by Burton[38], although a recent report suggests that the Streptococcal DNase A may have a useful specificity for moderately long tracts of adenine or thymine nucleotides[39], while the DNase K2 from *Aspergillus oryzae* has been used to prepare thymine-rich fragments of length 30-50 from the DNA of phage f1[40]. The known DNases with potential application to sequencing have been reviewed by Laskowski[41].

Fortunately, chemical methods of proven specificity are available for cleaving DNA. The most extensively used have been the reaction of DNA with diphenylamine and formic acid[42,43] which liberates sequences of consecutive pyrimidine nucleotides, and the reaction of DNA with hydrazine followed by alkaline hydrolysis[44,45], which liberates sequences of consecutive purine nucleotides. Other less widely used methods are available. Thymine nucleotides can be selectively destroyed by the reaction of DNA with an ammoniacal solution of osmium tetroxide[46], although some destruction of cytosine cannot be avoided. A method for the selective destruction of cytosine nucleotides and the release of sequences of consecutive thymidine nucleotides has been reported[47], and used to demonstrate sequences of general formula $T_n p_{n+1}$ of length up to n = 11 in the DNA of *Escherichia coli*[48]. Sequences of consecutive adenine nucleotides can be prepared by alkaline hydrolysis after oxidising the other bases with permanganate at pH 9[49]. Guanine can be selectively photo-oxidised[50], but no method for its selective removal has been reported.

Hall and Sinsheimer[51] have used the diphenylamine reaction to demonstrate sequences of up to 11 consecutive pyrimidine nucleotides in the DNA of phage ϕX-174. This DNA, which has a chain length of 5,500 nucleotides, was found to contain only four sequences of 9 consecutive pyrimidine nucleotides, two sequences of length 10, and one sequence of length 11. Petersen and Reeves[52] have recently reported a similar analysis of the DNA of phage f1, which was found to contain four pyrimidine nucleotide sequences of length 9, one of length 10, two of

length 11, one of length 13, and one of length 19-20. Sedat and Sinsheimer[53] examined the hydrazinolysis reaction products of ϕX-174 DNA, and were able to demonstrate sequences of consecutive purine nucleotides of lengths up to 13-14. However, some doubt was cast on the specificity of the hydrazinolysis and diphenylamine reactions by the lack of agreement between the total numbers of purine and pyrimidine nucleotide sequences in ϕX-174 DNA. A critical study[54] has suggested that hydrazinolysis may not be suitable for the quantitative determination of purine nucleotide sequences, while the low recoveries of long pyrimidine nucleotide sequences by Petersen et al[52]., together with the results of redigestion experiments[55], suggest that the diphenylamine reaction may result in limited non-specific cleavage.

Nevertheless, these methods should allow the sequencing of at least small portions of the largest DNA molecules.

Various techniques for the selective end-labelling of polynucleotides have become available in the last few years, and have been used to determine the terminal base sequences of several ribosomal RNA and phage RNA molecules[57,58] and two phage DNA molecules[14,59]. These methods, however, can find only limited application to high molecular weight DNA.

Further extension of the basic sequencing procedure will be dependent on methods for reproducibly splitting very large polynucleotides into smaller fragments. Brief enzymic degradation of the RNA molecules of phages Qβ and MS2 has been used to give two unequally-sized fragments[60,61], and separation of these by sucrose gradient sedimentation has enabled the

allocation of several long sequences of consecutive purine nucleotides to different regions of the MS2 RNA molecule[62]. Gel electrophoresis of partial digests of ribosomal RNA can give a range of clearly separated large fragments[63], and this method has been extended by Sanger et al. to the phage MS2 RNA molecule, with the isolation and complete sequencing of a 57-nucleotide segment which appears to code for part of the phage coat protein[64].

There would seem no reason why this approach should not be extended to the DNA molecules of phages ϕX-174 and f1. Although limited digestion with DNases has scarcely been explored, the folded secondary structure of single-stranded DNA[65] should allow at least partially selective cleavage by DNases with a higher affinity for native than for denatured DNA. The ϕX-174 DNA molecule appears to contain a block of nucleotides of unusual secondary structure, highly resistant to the action of exonucleases[66]. PolyG-binding sites are believed to be resistant to the action of pancreatic DNase[67]. It would seem that efforts to isolate short unusual DNA segments, followed by application of the known methods of partial and complete chemical degradation, are likely to be most fruitful in the near future.

In addition to the basic sequencing methods, an interesting although as yet largely unproven class of methods may be available through the use of the DNA polymerase of *E. coli*. The classic demonstration by Kornberg et al.[68] of the frequency of dinucleotide sequences in several DNA molecules has rather surprisingly never been taken to such logical extensions as the measurement of the purine neighbour frequencies for

sequences of consecutive pyrimidine nucleotides. Wu and Kaiser[14,69] have used the DNA polymerase to 'repair' the short single-stranded ends of phage λ DNA, and have partly deduced the nucleotide sequence. Khorana et al[70,71] have demonstrated that the DNA polymerase, in conjunction with the *E. coli* DNA ligase, can be used to synthesise an accurate deoxyribonucleotide replica of portions of a Yeast alanyl-transfer RNA. The demonstration that short oligonucleotides are needed for the initiation of DNA polymerase replication with the circular phage ϕX-174 DNA molecule as template[72] suggests a logical extension of this method to elucidate base sequences bordering on the longest sequences of consecutive purine or pyrimidine nucleotides. The ability of the DNA polymerase to incorporate ribonucleotides into a mixed co-polymer may also be of use[73].

An unproven technique of even greater long-term potential may be the direct visualisation of stained base-sequences under the electron microscope[74]. Several base-specific stains have been proposed for this purpose[75,76], although formidable practical difficulties remain.

C. The aims of the present work.

Although many aspects of the genetic code and control of genetic expression have been worked out from the genetics and biochemistry of RNA and protein synthesis, there remain important questions that can probably only be answered by DNA sequencing. The present work was undertaken as a preliminary attempt to correlate DNA sequence studies with the molecular anatomy of a phage DNA molecule, in an attempt to

cast some new light on (a) the initiation and termination of RNA message transcription from DNA templates (b) the Rhythmic Code in relation to DNA.

Selective Transcription of DNA.

It is now well established that in bacterial systems only one strand of the DNA double helix is transcribed into RNA at any one region of the chromosome. The mechanism of strand selection must depend on the recognition by some molecule, perhaps the DNA-dependent RNA polymerase itself, of unique structural features in the DNA. Direct evidence for this hypothesis has come mainly from the *in vitro* synthesis of RNA using purified RNA polymerase. It has been demonstrated that marked differences in the preference for the 5'-terminal nucleotide of the RNA product are determined by the source of the DNA template[77], presumably reflecting initiation of RNA synthesis at a restricted number of sites in each DNA molecule. RNA-DNA hybridisation has been used to show that the sequences transcribed from phage DNA molecules by purified RNA polymerase *in vitro* are identical to those transcribed during the early phases of phage infection[78,79,94], although this specificity is strongly dependent on a dissociable protein factor[79,80]. Purified RNA polymerase can bind to native DNA *in vitro* at only a limited number of sites[81]. In the case of several viral DNA molecules, the number of RNA polymerase-binding sites is in reasonable agreement with the number of genes expected to be transcribed at early stages of viral infection[81,82].

Indirect evidence that unique structural features in DNA may be associated with the strand selection mechanism has come from work on the

binding of polyribonucleotides to single-stranded DNA. Several bacteriophage DNA molecules have been shown to bind polyG and related polymers exclusively to the 'message' strand of the molecule[26,29,30]. In the case of phage λ, messenger RNA is transcribed from different regions of both complementary strands, but a close correlation is found with polyG-binding sites, which are also located in both strands of λ DNA.

On the assumption that the specificity of polyG-DNA complex formation depends on normal Watson-Crick base-pairing, it has been suggested that the polyG-binding sites may in fact be 'clusters' of short cytosine nucleotide sequences of combined length 14-20 nucleotides[28,31]. Since sequences of consecutive cytosine nucleotides occur rather rarely in DNA[32], it might be expected that the distribution of such sequences would show a very pronounced bias in distribution between the transcribing and non-transcribing regions of a phage DNA molecule. A direct attempt has been made in the present work to verify this hypothesis.

The Rhythmic Code.

Fiers et al. reported in 1965 that the distribution of purine nucleotide sequences of various chain lengths in pancreatic RNase digests of the phage MS2 RNA molecule showed small regular deviations from randomness, which they characterised as a 'rhythmic code'[33]. All purine nucleotide sequences of even length, i.e. lengths 2,4,6 etc., were present at less than the frequency expected for a randomly arranged polyribonucleotide of similar base composition. All purine nucleotide sequences of odd length i.e. lengths 1,3,5 etc., were present at above random frequency. Similar observations have been reported for the RNA

molecules of phages R17 and μ2[84,85]. It has been suggested that the rhythmic code may reflect some general restriction in the use of nucleotide sequences for the encoding of biological information[83,86].

Almost no comparable information has been available from DNA sequence studies. Sedat and Sinsheimer have reported the distribution of the purine nucleotide sequences released by hydrazinolysis from the DNA of phage ϕX-174[53] and their results show no sign of a rhythmic code. However, their procedure appears to result in significant losses of adenine[87,54]. Since the complementary structure of double-stranded DNA ensures that every sequence of consecutive purine nucleotides will be matched by a complementary sequence of consecutive pyrimidine nucleotides, relevant data can also be obtained from an analysis of pyrimidine nucleotide sequences. The only published analysis of the pyrimidine nucleotide sequences in a single DNA strand, that of Hall and Sinsheimer for the DNA of phage ϕX-174[51], shows no signs of a rhythmic code. However, these pyrimidine nucleotide sequences are not complementary to the purine nucleotide sequences in the messenger RNA of phage ϕX-174, since transcription takes place exclusively from the complementary ϕX-174 DNA strand, formed only after phage infection[88].

In the present work the distribution of pyrimidine nucleotide sequences was determined in both separated strands of a phage DNA molecule, in a direct attempt to demonstrate a DNA rhythmic code.

Phage Alpha.

The DNA molecule of phage α, a temperate bacteriophage of *Bacillus liberius*, was chosen to be suitable for these studies. α DNA was known

to be linear and double-stranded, with a molecular weight of 30-38 x 10^6, and to contain only the 4 normal bases: adenine, guanine, cytosine and thymine[89,90,91]. The two complementary strands of the a DNA molecule had been reported to be separable in CsCl density gradients and by MAK chromatography[90,92]. *In vitro* and *in vivo* studies had both shown that only the H strand is transcribed into messenger RNA[93,94]. It had been reported that only the H strand contains polyG-binding sites[26].

METHODS

Phage and Bacterial Strains

A clear mutant of bacteriophage α, α_{c3}^{186}, and its host *Bacillus tiberius*, were obtained from Dr. E.P. Geiduschek. Bacterial stocks were kept on nutrient agar slopes at 2-5°, and were sub-cultured at intervals of not less than 4 weeks. Occasionally fresh slopes were prepared from stab cultures maintained in the laboratory of Dr. R.J.T. Clarke. Phage T7 and *Escheriohia coli* strain W3110 were obtained from Dr. M.G. Smith. Phage f1, purified by the method of Marvin and Hoffman-Berling[95], was a gift from Dr. G.B. Petersen.

Bulk Growth of Phage Alpha

Phage α was grown in layers of soft agar by the confluent lysis method[96]. The following growth media were used:

Nutrient Broth	Soft Agar	Hard Agar
10 g. Difco Nutrient Broth	1 l. Nutrient Broth	1 l. Nutrient Broth
5 g. NaCl	7 g. Davis Agar	12 g. Davis Agar
1 l. distilled water		

These media were sterilised by autoclaving and stored at room temperature. Phage buffer contained 0.5 M NaCl, 0.01 M phosphate buffer pH 7.0, 0.01 M $MgSO_4$. For the large scale growth of phage, 250-500 ml. layers of hard agar were poured into heat-sterilised stainless steel trays (8 in. x 12 in.) fitted with lids, and allowed to set. These trays were dried briefly at 30° with the lids off. A mixture containing 50 ml. soft agar maintained at 45°, 5 ml. of an overnight culture or thick log phase culture of *B. tiberius*, and 0.1 ml. of α phage stock

containing about 10^6 p.f.u./ml. was poured onto each hard agar layer. The trays were then incubated upside down at 30° overnight.

To harvest the phage, 30 ml. of phage buffer was poured over each agar layer. After allowing the trays to stand at room temperature for 1 h., the soft agar layers were lifted off with the aid of a metal paint scraper. Phage particles were released from the combined soft agar layers and phage buffer by homogenising (5 minutes at the lowest speed setting in a Sorvall Omni-mixer) followed by low speed centrifuging (5 minutes at 7,000 r.p.m. in the GSA rotor of a Sorvall RC-2B centrifuge). The pellets of soft agar granules were rinsed by a second cycle of homogenising with phage buffer (20 ml./tray) and centrifuging.

The combined supernatants were made 1.5% (w/v) in polyethylene glycol (L. Light and Co. Ltd., Colnbrook, England, molecular weight 20,000) from a 30% (w/v) stock solution in 0.5 M NaCl, and centrifuged. This and subsequent centrifugings were for 5 minutes at 15,000 r.p.m. in the SS-34 rotor of a Sorvall RC-2B centrifuge. The supernatants were made 2.5% (w/v) in polyethylene glycol, and after 5 minutes the precipitate of phage was collected by centrifuging and the supernatants discarded. Each pellet of phage was resuspended in 1-2 ml. of phage buffer. The concentrated phage suspension was clarified by a final centrifuging and stored at $2\text{-}5^\circ$.

Routine phage yields ranged from 2 to 4 mg. of DNA per tray.

When required for electron microscopy, phage was freed of polyethylene glycol by two cycles of high-speed centrifuging (1 h. at 30,000 r.p.m. in the 30 rotor of a Spinco Model L ultracentrifuge) followed by resuspension in phage buffer.

Phage Assays.

α phage assays were carried out by the agar layer method[96], using bacteria from mid-log phase cultures. However, it was noticed that the titre of plaque-forming units (p.f.u.) was consistently 100-fold less than the titre of phage particles deduced from the yield of phage DNA after phenol extraction.

Growth of Radioactive Phages.

Phage α was labelled with ^{32}P or ^{33}P by adding 0.5-2.5 mC. of carrier-free ^{32}P-orthophosphate (Radiochemical Centre, Amersham, England), or ^{33}P-orthophosphate (New England Nuclear Corp., Boston, Mass.) to each soft agar layer. Greatly increased incorporations of label were obtained by reducing the volume of the hard agar layer to 50 ml.

^{3}H-labelled phage T7 was grown on *E. coli* strain W3110 in nutrient broth containing 2 μc/ml. ^{3}H-thymidine (methyl-T, Radiochemical Centre, Amersham, England) and concentrated by two cycles of differential centrifuging[139].

Phenol Extraction of Phage DNA.

Redistilled, water-saturated phenol was stored in the dark, and was buffered to a pH of 8-9 immediately prior to use by shaking with an equal volume of 0.1 M $Na_2B_4O_7$. Concentrated phage suspensions were dialysed overnight at 2-5° against 0.1 M tris/HCl buffer pH 8.0 containing 0.5 M NaCl and 0.001 M sodium diethyldithiocarbamate (SDDC), and the phage DNA released by shaking gently for 15 minutes with an equal volume of chilled, buffered phenol. The two phases were separated by brief centrifuging at 3,000 g., and the phenol layer removed by pasteur

pipette and discarded. The aqueous layer containing the DNA was then extracted with another equal volume of chilled buffered phenol, and finally dialysed in the cold against several changes of 0.01 M tris/HCl buffer pH 8.0 containing 0.1 M NaCl and 0.001 M EDTA. The A_{260}/A_{280} ratio of such preparations was 1.85-1.95, and the A_{260}/A_{230} ratio was 2.25-2.45. The DNA content was measured by taking the absorbance at 260 mμ in a 1 cm. cell to be 20.0 for a solution containing 1 mg. DNA/ml.

Electron Microscopy of Phages and Phage DNA.

This was carried out by Mr. Alan Craig, Plant Chemistry Division, D.S.I.R. Phage suspensions were diluted into distilled water, negatively stained with 2% potassium phosphotungstate pH 7.0, sprayed onto carbon films on copper supporting grids, and examined with a Philips EM 200 electron microscope.

Phage DNA, at 2 μg/ml. in 1.0 M ammonium acetate containing 0.5% formalin and 0.01% cytochrome C, was allowed to spread onto 0.3 M ammonium acetate containing 0.5% formalin. After 10 minutes the protein film was compressed to 0.9 dynes/cm^2. Samples were taken onto carbon films on copper supporting grids, and shadowed with platinum during a 360° rotation.

Boundary Sedimentation of DNA.

Boundary sedimentations of DNA were performed in a Spinco Model E analytical ultracentrifuge fitted with ultraviolet optics, in cells with a 30 mm. light path.

Native DNA was sedimented in buffers of pH 7-8 containing 0.15-1.0

M NaCl. Denatured DNA was sedimented in 0.1 M NaOH, 0.9 M NaCl. To eliminate any possibility of breakage by shear, concentrated DNA was pipetted into pre-assembled cells after removal of the upper window. The appropriate neutral or alkaline solvents, to a total volume of 1.8 ml., were introduced by syringe after re-assembly of each cell, and the contents gently mixed by rolling the cell for several minutes. The standard conditions used for assaying phage DNA intactness were: temperature, 18-22°; DNA concentration, 20 µg/ml; rotor speed, 37,020 r.p.m. Ultraviolet photographs of the cell were taken at 8 minute intervals, using Kodak Commercial or Kodak Professional film. Exposure times were chosen to give a silver grain density proportional to optical density in the cell. After developing, fixing and drying, the pictures were scanned in the boundary region with a Joyce-Loebl microdensitometer or with a Beckman Analytrol chromatogram scanner. The amount of breakage of the DNA was estimated as the percentage of ultraviolet-absorbing material trailing behind the symmetrical boundary (see figures 6 and 9).

In a few early experiments with highly fragmented DNA, heat-denatured DNA in 0.15 M NaCl, 0.015 M sodium citrate (SSC) was introduced into the ultracentrifuge cell by gravity flow through a No. 22 syringe needle. The mean sedimentation coefficient was calculated from measurement of the densitometer tracings and corrected to $S^0_{20,w}$ by the usual methods[97]. The mean molecular weight (M) of the DNA was deduced from the relationships $S^0_{20,w} = S_{20,w}(1 + 1.7\,[\eta]\,C)$, $[\eta] = 0.0049 \cdot M^{0.55}$, and $S^0_{20,w} = 0.022 \cdot M^{0.48}$ found by Eigner et al[98]. for denatured DNA in SSC.

Zone Sedimentation Through Sucrose Gradients.

Zone sedimentations of DNA were performed in the SW-39 rotor of a Spinco Model L preparative ultracentrifuge, using the procedures first developed by Burgi and Hershey[99], and extended by Thomas et al[100]. 4.8 ml. linear gradients of 5%-20% sucrose were prepared using a 2-chamber mixing device, and were stored at 2-5° for at least 1 h. prior to use. Neutral gradients contained 1-2 M NaCl and were buffered at pH 7-8 with dilute tris/HCl or phosphate buffers. Alkaline gradients contained 0.1 M NaOH, 0.9 M NaCl and were checked before use to ensure that the pH was greater than 11. Samples of DNA (usually less than 1 µg. per tube) were layered onto the gradients in 0.01 M tris/HCl buffer pH 8.0, loaded into a pre-cooled rotor, and centrifuged for 3-6 h. at approximately 5-10°. At the end of each run the rotor was braked to a halt, and the gradients sampled by puncturing the bottom of the tube and collecting 3-7 drop fractions, using negative pressure on the top of the gradient to control the flow.

Calculation of the distances moved by sedimenting zones assumed a constant drop size in all fractions. When 3-drop fractions were collected, the centre of a sedimenting zone could be located with a precision of about ± 2 drops.

Molecular weights were calculated from the relative distances sedimented[99,100,101], according to the relationships.

$$\frac{D_1}{D_2} = \frac{M_1}{M_2}^{0.35} \qquad \text{(neutral sucrose)}$$

and

$$\frac{D_1}{D_2} = \frac{M_1}{M_2}^{0.40} \qquad \text{(alkaline sucrose)}$$

Alkali Denaturation of DNA.

Phage DNA at concentrations of up to 200 µg/ml. in 0.01 M tris/HCl buffer pH 8.0 containing 0.1 M NaCl was denatured by adding a one-third volume of 0.4 M NaOH. After gentle mixing at room temperature for 2-5 minutes, the denatured DNA was chilled to 0^{o} in an ice-water bath and re-neutralised by adding a half volume of chilled potassium phosphate buffer containing 0.3 M KH_2PO_4 and 0.1 M K_2HPO_4.

To form polyG-DNA complexes, 0.25 µg. of polyguanylic acid (Miles Laboratories Inc., Elkart, Indiana, minimum molecular weight > 100,000) was added per µg. of DNA prior to alkali denaturation.

Handling of Single-stranded DNA.

High molecular weight single-stranded DNA is very shear-sensitive in the alkaline form (pH > 10.5) or at low ionic strengths (I < 0.1). The general precautions outlined by Davison and Freifelder[102] were used throughout the present study. Wherever practicable, native DNA was stored and handled at high concentrations (more than 100 µg/ml.) and denatured DNA was stored at pH 8.0 and at ionic strengths greater than 0.2. Vortexing or syringing of solutions containing DNA was avoided. Small volumes were transferred using wide-bore pipettes equipped with a screw-suction device for controlling low rates of flow (Clay Adams, N.Y., U.S.A.). Whenever practicable, single-stranded DNA was concentrated from dilute solutions by dialysis against solid sucrose, since lyophilisation or adsorption to hydroxylapatite resulted in breakage of most of the strands.

Shearing of DNA.

In several experiments, the alkaline form of α DNA was sheared by rapidly squirting the DNA solution 5-10 times through a No. 22 syringe needle. This reduced the mean size of the strands to that of quarter-length fragments (mean molecular weights of 4.6×10^6 and 5.2×10^6 were measured in two separate experiments by zone sedimentation through alkaline sucrose gradients).

Annealing of DNA.

Denatured α DNA at 5-100 µg./ml. was dialysed at 2-5° against twenty volumes of 30% (v/v) formamide containing 0.15 M NaCl, 0.015 M sodium citrate. After 12-40 h., formamide was removed by dialysing against 0.5 M NaCl, 0.01 M phosphate buffer pH 7.0.

MAK Chromatography.

MAK (methylated albumin adsorbed on Kieselguhr) was prepared by the method of Mandell and Hershey[103], except that methylation was allowed to proceed for 2 weeks instead of 3 days. Early experiments followed in every detail the procedure described by Cordes[91]. In later experiments, refrigerated columns (2-5°) and gradient elutions were employed[104]. The procedure was as follows: A freshly-prepared column of MAK (3 cm. x 1 cm. diameter) was rinsed (at 1 ml./min.) with 100 ml. of 0.1 M SP buffer (0.05 M phosphate buffer pH 6.8 containing 0.1 M NaCl) followed by 20 ml. of 0.6 M SP. Approximately 400 µg. of alkali-denatured, reneutralised α DNA was made 0.5 M in NaCl and pumped onto the column. The column was then rinsed with 20 ml. 0.6 M SP, followed by a 200 ml. linear gradient of 0.6-1.0 M SP, and 5 ml. fractions were

collected. DNA content was assayed by measuring the ultraviolet absorption at 260 mμ, and appropriate fractions were assayed for their content of L and H strands by CsCl gradient centrifuging. Fractions enriched for either strand were concentrated by adsorbing to hydroxylapatite, or by rotary film evaporation at 30° followed by dialysis against 0.1 M SP, and stored at 2-5°.

Hydroxylapatite Chromatography.

Crystalline hydroxylapatite $Ca_5(PO_4)OH$ was prepared from brushite by the method of Tiselius et al.[105], and stored at 2-5° in 0.01 M PB (phosphate buffer pH 7.0). Care was taken to avoid breaking the large, fragile crystals by pipetting or repeated resuspension. Of several independently prepared batches of crystals, no two had exactly the same properties for the chromatography of DNA. However, in every case phosphate gradient elution as described by Bernardi[106] readily separated single-stranded from native DNA. The most useful batch of hydroxylapatite gave approximately 75% recovery of high molecular weight single-stranded DNA, and 95-100% recovery of native α DNA, from columns eluted at room temperature. Up to 500 μg. of DNA in 0.5 M NaCl, 0.01 M PB could be loaded onto a short column (3 cm. x 1 cm. diameter) of hydroxylapatite, and eluted with a 50 ml. linear gradient starting with 0.15 M PB containing 0.3 M NaCl, and finishing with 0.3 M PB. Single-stranded DNA was eluted at 0.2-0.25 M PB. Complete elution of native DNA was accomplished by rinsing with a further 20 ml. of 0.3 M PB.

With an earlier batch of hydroxylapatite, good recoveries of high molecular weight single-stranded DNA could not be obtained by phosphate

gradient elution. This problem was overcome by the use of jacketed columns eluted at 70° [107]. DNA solutions were pre-heated to 70° and applied to 5 cm. x 1 cm. diameter columns of hydroxylapatite in 0.12 M PB containing 0.3 M NaCl. Under these conditions, single-stranded DNA passed straight through the columns, while native DNA was adsorbed. The irreversible adsorption of single-stranded DNA seemed to be dependent on molecular weight, since 90-95% recoveries were obtained of phage f1 DNA (molecular weight 1.6×10^6) or of α DNA which had been sheared to a molecular weight of approximately 500,000 by a single passage through a French pressure cell.

Density Gradient Centrifuging.

The theory and practice of equilibrium density gradient centrifuging has been adequately reviewed[108,109,110].

In the present work, analytical centrifugings were performed in a Spinco Model E analytical ultracentrifuge fitted with ultraviolet optics, using 12 mm. light-path cells and Kel-F centrepieces. When possible, a 4-place An-F rotor was used.

CsCl and Cs_2SO_4 mixtures were made up to approximately the desired initial density from concentrated stock solutions. Each sample, of total volume 0.8 ml., contained 0.5-2.0 μg. of DNA per density species. The initial density was determined by measuring the refractive index at 25°, using the relationships

$$\rho^{25^0} = 10.8601.\ n^{25^0} - 13.4974 \qquad (CsCl)$$

and $$\rho^{25^0} = 13.6986.\ n^{25^0} - 17.3233 \qquad (Cs_2SO_4)$$

CsCl mixtures contained 0.01 M tris/HCl buffer, pH 8.0. For accurate buoyant density measurements, the initial density was adjusted

to 1.710 ± .001 g/ml. (25°), and 0.5 µg. of DNA from *Pseudomonas aeruginosa* (ρ^0 = 1.727 g/ml.)[109] or *Tetrahymena pyriformis* strain W (ρ^0 = 1.685 g/ml.)[111] was included as a density reference. Exactly 0.8 ml. was syringed into each cell. Centrifuging was at 22-25° and 44,770 r.p.m. for 20-24 h., and the bands were located by ultraviolet photography and densitometry. Buoyant densities were calculated from the buoyant density (ρ^0) of the reference DNA using the relationship[108]

$$\rho = \rho^0 + 4.53.\ \omega^2\ (V^2 - V_0{}^2) \times 10^{-10}$$

where $\omega^2 = 22.02 \times 10^6$ at 44,770 r.p.m., and V and V_0 are the measured distances of the unknown and reference DNA bands from the axis of rotation.

For measuring the L and H strand content of samples of denatured α DNA, less care was taken with the initial density and sample volume, and centrifuge runs were often ended after 16-18 h. Native α DNA (ρ^0 = 1.705 g/ml.[90]) was included as a density reference. The relative content of the two strands was estimated from the relative heights of the H and L peaks in each densitometer tracing.

For mercury-binding experiments in Cs_2SO_4, the method of Nandi et al. was followed[112]. DNA samples were dialysed exhaustively against 0.1 M Na_2SO_4, 0.005 M borate buffer pH 9, to remove EDTA and NaCl, and were heat-denatured by heating to 100° for 5 minutes followed by rapid chilling in an ice-water bath. Cs_2SO_4 mixtures contained 0.005 M borate buffer pH 9.2. Mercuric ion was added from a solution containing 0.001 M $HgCl_2$ to give an r_f (moles Hg/moles DNA phosphate) of 0.3. Centrifuging was at 44,770 r.p.m. for 16-20 h.

Preparative CsCl gradient centrifugings were carried out in the 40 rotor of a Spinco Model L preparative ultracentrifuge, with the refrigeration set to maintain a temperature of roughly 5° during long runs. For separating polyG complexes of the H and L strands of α DNA, mixtures with an initial density (at 25°) of approximately 1.705 g/ml. were prepared by adding 0.875 ml. of a solution of α DNA, denatured and re-neutralised in the presence of polyG, to 3.125 ml. of a saturated solution of CsCl (ρ^{25^0} = 1.900 g/ml.) containing 0.01 M tris/HCl buffer pH 8.0. This initial density, substantially lower than the density at 25° of either of the polyG-αDNA complexes, was chosen to allow for the effects of temperature on the density of CsCl solutions and the degree of hydration of Cs-DNA[113]. The mixtures were poured carefully into polyallomer tubes and overlaid with paraffin oil to give a total weight of 14.8 g. per tube. In early experiments, centrifuging was for 35-45 h. at 36,000 r.p.m. In later experiments, the mixtures were made with an initial pre-formed step in CsCl concentration as described by Brunk and Leick[114], and the centrifuging time reduced to 12 h. At the end of each run, the rotor was braked to 5,000 r.p.m. and then allowed to coast to a halt. Tubes were removed with care to avoid disturbing the density gradient, and stored at 5° for up to 3 h. before sampling.

Diphenylamine Digests.

DNA solutions at concentrations of up to 4 mg/ml. were dialysed against distilled water and degraded with 2% (w/v) diphenylamine in 66% (v/v) formic acid for 18-25 h. at 30°[42]. Diphenylamine was removed by

ether extraction. Formic acid was removed by continuous liquid-liquid ether extraction or by several cycles of rotary film evaporation to dryness at 30°. Carrier diphenylamine digests were de-purinated after the preliminary ether extractions by passing through columns of Dowex50 x8 (H^+) and rinsing with distilled water until the absorbance at 260 mμ of the eluate was less than 0.05. This procedure was not used to de-purinate radioactive diphenylamine digests, since 2-3% of the radioactivity could only be recovered from the Dowex50 resin by eluting with 5 M aq. ammonia. In a single experiment outlined in Appendix II. the insoluble precipitate of guanine was removed from a combined digest of radioactive and non-radioactive bacteriophage DNA by filtering through a 1 cm. column of DEAE-cellulose in 2 M triethylammonium bicarbonate buffer pH 7.5.

Carrier Digests.

Calf thymus DNA was a gift from Dr. G.B. Petersen, and had been prepared by the method of Kay, Simmons and Dounce[115], after preliminary isolation of the nucleohistone[116]. Depurinated diphenylamine digests of calf thymus DNA were concentrated by rotary film evaporation at 30° to approximately 150 μMoles of phosphorus/ml., and stored at -20°.

In early experiments, preliminary fractionation of carrier digests was obtained by dialysing against distilled water with continuous stirring for 60-65 h., using Visking thin-wall dialysis tubing grade 20/32. Bacterial growth was prevented by adding a few drops of chloroform to the diffusate. This method was eventually found to be unsatisfactory (Appendix II.)[117].

In later experiments, preliminary fractionation of carrier digests was achieved by gel filtration (Appendix III.). Portions of digest corresponding to 50-200 mg. DNA were adjusted to pH 8.5 with aq. ammonia, concentrated by rotary film evaporation at 30°, and applied in 0.2-0.5 ml. layers to 150 cm. x 1 cm. diameter columns of Sephadex G50 gel (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M triethylammonium bicarbonate buffer pH 8.3. Best results were obtained by applying each sample to the surface of the gel as a dense layer, containing 5% (w/v) sucrose, beneath a layer of elution buffer. After elution into 5-10 ml. fractions, sequences of length approximately greater than 6 were pooled with 20% of the sequences of length approximately 3-5 and 5% of the sequences of length approximately 1-2. Occasionally, ^{32}P-labelled tetranucleotides were included in the carrier digest applied to the gel column, and all fractions eluted prior to the peak of radioactivity were pooled. Fractionated carrier sequences were dialysed briefly against distilled water (1-2 h.) and concentrated to dryness by rotary film evaporation at 30°.

Ion-Exchange Chromatography of Oligonucleotides.

The procedures used were those of Petersen and Reeves[118]. For separations on the basis of chain length, diphenylamine digests were chromatographed on DEAE-cellulose (Cellex D, Calbiochem. Los Angeles, Calif., U.S.A., small ion exchange capacity 0.93 meq./g.) using linear NaCl gradient elution in 0.1 M acetate buffer pH 5.4 containing 7 M urea. Urea, although not essential for these separations, was found to give higher recoveries of long sequences[119]. For separations on

the basis of base composition, oligonucleotides were chromatographed on the same resin using linear NaCl gradient elution in 0.1 M formic acid buffer (pH 3.5-4.3) also containing 7 M urea.

In later experiments, chain length separations employed a 'microgranular' form of DEAE-cellulose (Whatman DE-32, H. Reeve Angel, London, England, small ion exchange capacity 1.0 meq./g.). The resin was carefully pre-cycled with acid and alkali and equilibrated in batches with starting buffer after removal of fines. Columns (40-60 cm. x 1 cm. diameter) were packed by pumped buffer flow at 36 ml/h. as suggested by Thompson[120], and rinsed with starting buffer for several hours prior to use. Diphenylamine digests of radioactive DNA were supplemented with carrier (0.2-0.4 ml. of unfractionated carrier diphenylamine digest for separations of chain lengths up to 6, fractionated long sequences from 1-4 ml. of carrier diphenylamine digest for separations of chain lengths up to 13) and concentrated to dryness by rotary film evaporation at 30°. Each sample was taken up in 10 ml. of starting buffer, a 0.1 ml. aliquot set aside for radioactivity assay, and the remainder pumped onto the column and rinsed with several 5 ml. portions of starting buffer. Columns were eluted at 15-25 ml/h. with the following linear gradients: first gradient, 600 ml., 0-0.12 M NaCl; second gradient, 1200 ml., 0.12-0.18 M NaCl. The gradient was normally stopped after the heptanucleotide peak, and longer sequences eluted with 0.5 M NaCl. Longer sequences were alternatively eluted by continuing the second linear gradient.

Urea Solutions.

8-9 M solutions of urea in distilled water were decolorised by passing through 10-15 cm. x 5 cm. diameter charcoal columns containing 5 parts of Celite 545 (Koch-Light Laboratories Ltd., Colnbrook, England) per 1 part of activated charcoal (Darco G60, Darco Corp., N.Y., U.S.A.). Freshly prepared solutions had an absorbance of 0.015-0.005 in the wavelength range 260-300 mμ. Urea solutions were standardised by specific gravity measurements, taking the specific gravity of 7 M urea to be 1.113 g/ml. at 20°.

Desalting of Oligonucleotides.

(a) Dialysis. Brief dialysis (2-3 h.) against distilled water, or overnight dialysis against 0.1 M triethylammonium bicarbonate buffer pH 8.3, was used as a convenient method of desalting small volumes (up to 50 ml.) of nucleotides when quantitative recoveries were not regarded as essential. Dialysis against distilled water can result in selective loss of cytosine-rich sequences[117]. This is not a suitable procedure for terminally-dephosphorylated oligonucleotides, which pass very rapidly into the diffusate.

(b) Charcoal. Larger volumes of oligonucleotides were desalted on 5 cm. x 1 cm. diameter columns of the charcoal/celite mixture described above for the preparation of urea solutions[121]. Each column was washed with several column volumes of 1 M HCl followed by several column volumes of ethanol/water/ammonia 50:49:1 (v/v), and then rinsed with distilled water prior to use. Oligonucleotide mixtures containing 7 M urea were diluted with an equal volume of 0.5 M HCl and passed

through the columns at 2-5 ml/min. After rinsing with a few column volumes of water, the oligonucleotides were eluted with 50-100 ml. of ethanol/water/ammonia and concentrated to dryness by rotary film evaporation at 30°. This procedure gave recoveries of 90-100% for oligonucleotides of lengths 2-6, and is also suitable for terminally dephosphorylated oligonucleotides, but not for mononucleotide diphosphates.

(c) DEAE-Sephadex. The method of Rushizky and Sober[122] was preferred for the quantitative desalting of mononucleotides. Samples were diluted with water to less than 0.05 M NaCl, adjusted to pH 8.5 by dropwise addition of concentrated aq. ammonia and adsorbed onto 5 cm. x 1 cm. diameter columns of DEAE-Sephadex A25 (OH^-) (Pharmacia, Uppsala, Sweden). After rinsing with several column volumes of 0.01 M triethylammonium bicarbonate buffer pH 8.5, the nucleotides were eluted with 20-30 ml. 0.5 M triethylammonium bicarbonate pH 8.5 and the solvent removed by rotary film evaporation at 30°.

This method can also be used for quantitative desalting of oligonucleotides, however it was suspected during the course of this study that exposure to concentrated triethylamine can result in partial dephosphorylation of oligonucleotides. For this reason other methods were used when practicable.

Triethylammonium Bicarbonate Buffers.

2 M triethylammonium bicarbonate was prepared by bubbling CO_2 (generated by the deliquescence of dry ice) through a chilled saturated solution of redistilled triethylamine in water, with the relative rates

of input of CO_2 and triethylamine adjusted to maintain a pH of approximately 8.5. Concentrated solutions were stored at 2-5°.

Dephosphorylated Digests.

Human prostatic phosphomonoesterase was purified by salt fractionation[124] and ion-exchange chromatography[125] as described by Burton and Petersen[42]. Monoesterase activity was assayed by incubating a 0.2 ml. aliquot of 400-fold diluted enzyme at 37° with 2 ml. of 0.005 M disodium p-nitrophenylphosphate (NPP) and 1 ml. of 0.2 M acetate buffer pH 5.6. The reaction was stopped by adding 0.2 ml. of 2 M aq. ammonia, and the unit of activity defined as the amount of enzyme causing an increase of 1 in the absorbance at 400 mμ in 1 minute. Diesterase activity was assayed by replacing 0.005 M NPP with 0.005 M disodium p-nitrophenylthymidine-5'-phosphate. The preparation of phosphomonoesterase used for these experiments had a ratio of monoesterase/diesterase activities of 15,500.

Although a purified phosphomonoesterase with a comparable ratio of monoesterase/diesterase activities is available commercially (*E. coli* alkaline phosphatase, E.C.3.1.3.1, Worthington Biochemical Corp., Freehold, N.J., U.S.A., chromatographically-purified grade), the human prostate enzyme was preferred for two reasons:-

(1) It shows no product inhibition by inorganic phosphate.

(2) Very little buffering capacity is required to maintain the pH of optimum activity (5.6) during dephosphorylations. Hence there is no requirement for desalting prior to paper chromatography.

Ether-extracted diphenylamine digests were made 0.006 M in EDTA

and adjusted to pH 5.6 with a few drops of dilute NaOH. 15 units of phosphomonoesterase were added per μMole of total digest phosphorus, and the reaction mixture incubated at 37°. Aliquots (0.05 ml.) were taken at intervals for assay of inorganic phosphate release. When this appeared to reach a plateau, further enzyme (5 units/μMole phosphorus) was added and the incubation continued for several hours. The reaction was halted by shaking the digest with an equal volume of chloroform. After removal of the aqueous layer, the chloroform layer (and denatured protein interface) was rinsed twice with water. The combined aqueous layers were concentrated by rotary film evaporation at 30° to 0.5 ml., and any further precipitate centrifuged down and discarded. Finally, the digest was evaporated to dryness under a current of warm air in the tip of a conical glass centrifuge tube, and taken up in 0.1 ml. for spotting onto chromatography paper.

Paper Chromatography of Oligonucleotides.

Two-dimensional paper chromatography was carried out on acid-washed sheets of Whatman No. 1 chromatography paper (47 cm. x 57 cm.) as described by Petersen[49]. Ultraviolet absorbing spots were located by taking contact photographs in ultraviolet light[126]. ^{32}P-labelled spots were located by autoradiography, using Ilford Industrial B grade X-ray film. Spots initially containing 2,000 counts/min. (Cerenkov) gave detectable blackening after 2 week exposures. For quantitative measurements of ^{32}P activity the oligonucleotide spots were cut out, rinsed with ether (5 ml.), allowed to dry, and then eluted with water (10 ml.) and counted by Cerenkov emission. The radioactivity of the

wet paper residue (3-10% of the total) was included in the total for each spot, with no correction for counting efficiency. For measurements of $^{33}P/^{32}P$ ratio, the water eluate of each spot was evaporated to dryness at 95° and taken up in 1 ml. of water and 9 ml. of toluene/ tritonX-100 scintillator fluid.

Phosphate Estimations.

For assays of non-radioactive phosphate, duplicate estimations were made by the method of Chen, Toribara and Warner[127], slightly modified by the addition of 0.02 M EDTA to reagent A (1 part by volume of 6 N H_2SO_4, 1 part of 2.5% (w/v) ammonium molybdate, 2 parts by volume distilled water) just prior to use. This modification gives a lower and more consistent reagent blank (G.B. Petersen, personal communication).

For assays of total phosphorus, samples were first ashed with the ashing fluid of Hanes et al.[128,42]

For assays of the release of ^{32}P-labelled phosphate during phosphomonoesterase digestion of oligonucleotides, the method of Dreisbach[129] was slightly modified as follows. Each 0.05 ml. aliquot was taken into 0.5 ml. chilled 5% TCA in a conical glass centrifuge tube. After brief centrifuging at 3,000 g. to remove denatured protein, the supernatant was poured into a polythene-capped glass counting vial containing 2.5% ammonium molybdate (2.5 ml.), 1.5 M H_2SO_4 (2.5 ml.) and isobutanol/ xylene 35:65 (5 ml.). The vial contents were shaken vigorously by hand for 20 seconds, and the aqueous lower layer removed by Pasteur pipette. Cerenkov counts in the isobutanol/xylene layer were taken as a measure

of free ^{32}P phosphate. Two corrections were made:

(a) The efficiency of extraction of ^{32}P-labelled phosphomolybdate into the isobutanol/xylene layer was taken to be 97%.

(b) Corrections were made for counting efficiency by the Channels ratio method[180] using the following settings: channel A, 15-1000, 20% gain; channel B, 50-250, 2% gain. Cerenkov counting is more efficient in isobutanol/xylene than in water, but is strongly quenched by the yellow phosphomolybdate complex.

Success with this method requires immediate counting of each sample, since on standing a blue colour develops in the aqueous layer.

Radioactivity Estimations.

(1) For simple quantitative measurements of ^{32}P radioactivity, 0.1-0.2 ml. samples were dried onto disposable aluminium planchettes and counted with approximately 40% efficiency in a Beckman Low-Beta proportional counter. This method has the great advantage of not requiring time-consuming cleaning of glass vials.

(2) For double-label experiments involving the isotopes ^{3}H and ^{32}P, samples were dried onto filter paper squares (Whatman No. 1) in glass counting vials and counted in 5 ml. portions of a toluene scintillator solution containing (per litre) 10 g. of PPO and 0.25 g. of dimethyl-POPOP[181]. Spillover from each counting channel into the other was re-determined for every new batch of scintillator fluid, but no corrections were made for counting efficiency in individual vials.

(3) For quantitative measurements of ^{32}P radioactivity in experiments where complete recovery of the samples was desired, the Cerenkov method

was used[132,133]. Volumes of up to 20 ml. were counted in glass vials, with counting efficiencies of approximately 10% to 20% in different Packard liquid scintillation spectrometers, using a variety of window settings. This method is not susceptible to chemical quenching, nor is it affected by the usual range of pH changes and salt concentrations[132], but it gives characteristically different counting efficiencies in such solvents as 7 M urea, ethanol, and isobutanol/xylene 35:65[134]. Colour quenching, when suspected, was corrected for by the channels ratio method[130].

Semi-quantitative estimates of ^{32}P activity were sometimes made by dry Cerenkov counting of paper spots cut from chromatograms.

(4) Although Cerenkov counting was eventually realised to be the method of choice for measurements of ^{32}P radioactivity in 7 M urea buffers[133], two other methods were used in early experiments.

(i) 1 ml. samples were suspended as a thixotropic gel with 15 ml. of a scintillator mixture[135,136] containing (per litre) 150 g. naptha-lene, 10 g. PPO, 0.3 g. POPOP, 1 l. dioxane and 40 g. Cab-O-Sil (Trade-mark of Godfrey L. Cabot Inc.). No correction was made for counting efficiency.

(ii) 1 ml. samples were shaken with 9 ml. of the toluene/triton X-100 2:1 scintillator solution described by Patterson and Greene[137], containing (per litre) 1.5 g. p-terphenyl and 0.1 g. dimethyl-POPOP. An aqueous emulsion is formed which on standing gives a clear solution in which ^{32}P radioactivity can be counted with efficiencies of 58-61%. The counting efficiency was found to be about 1% less for samples

containing 7 M urea buffers than for samples containing water, with no effect of pH or ionic strength over the range used for ion-exchange chromatography. Chemical quenching is much less of a problem in this scintillator mixture[137] than in conventional scintillator mixtures such as Bray's fluid[138]. Colour quenching was corrected for using the Packard automatic external standardisation system, but corrections more than 1% were rarely necessary.

Counting of $^{33}P/^{32}P$ Ratios.

^{33}P is a β-emitter with a maximum energy of 0.25 Mev., and has a half-life of 25 days. For some experiments, samples were counted on paper, but for careful measurements samples were counted in the toluene/triton X-100 scintillator described above. Optimal gain settings and spillovers were re-determined for each experiment, and the counts in each channel were corrected using an Olivetti Programma 101 desk-top computer.

Vials in which significant quenching was detected by using the Packard automatic external standardisation system were not included in results.

All counting data were corrected for isotope decay.

Ultraviolet Absorption Measurements.

These were mostly carried out in a Beckman Model DU spectrophotometer, in quartz cells with a 1 cm. light-path, with an estimated reproducibility of ± 1%. Spectra were scanned using a Unicam SP800 recording spectrophotometer. DNA concentration measurements assumed that a 1 mg/ml. solution of native DNA has a 260 mμ absorbance of 20.0.

Oligonucleotide concentration measurements assumed an ϵ_M at 267 mμ and pH 1-2 of 9900/Mole of pyrimidine[42]. Other absorption data were measured relative to this basic value by diluting standard solutions of the deoxyribonucleoside-5'-phosphates dCp and dTp (Calbiochem., Los Angeles, Calif., U.S.A.) into various solvents. The results are tabulated below.

Solvent	Nucleotide	$\epsilon_{271} \times 10^3$/Mole	A_{280}/A_{260}	Isosbestic wavelength
0.05 M HCl pH 1-2	dCp	11.6	2.08	267 mμ
	dTp	9.6	0.71	
0.1 M formic acid 7 M urea pH 4.3	dCp	11.1	1.86	269.5 mμ
	dTp	9.4	0.77	
0.1 M acetate 7 M urea pH 5.4	dCp	11.0	1.23	268.5 mμ
	dTp	9.4	0.78	

Nucleotide Sequence Frequencies.

Percentage Sequence Frequency (P.S.F.) is defined as the number of sequences of a given length, base-composition, or sequence group per 100 nucleotide residues of DNA. Results from column chromatography were calculated by taking the total radioactivity recovered from the column, including the high-salt fraction, to be 100%. The actual recoveries of applied radioactivity, measured in several experiments, ranged from 96.8% to 99.7%. Random P.S.F. values were calculated assuming the pyrimidine content of double-stranded DNA to be 50%, and of the isolated H and L strands to be 54% and 46% respectively. I am indebted to Mr. Lloyd Thomas for discussing this problem with me and writing an appropriate computer program.

Estimation of Errors:

For results based on single experiments, such as the dialysis experiments in Appendix II., estimates are given of the cumulative errors of measurement, based on the 90% reliable error for radioactivity measurements, and a ± 1% error for absorbance measurements.

For results based on several experiments, ± S.E. represents the standard error for the mean.

RESULTS

The Growth and Purification of Bacteriophage Alpha

The growth methods used at the outset of this work were derived from those used by Cordes[91]. Since infective centres of phage α were found to die rapidly in the available liquid media, the phage was grown in layers of nutrient agar gel. After release from the gel by homogenisation with phage buffer, the phage particles were concentrated and freed from agar granules and intact bacteria by differential centrifuging[139].

The concentration of large amounts of phage α by this method, using the available high speed centrifuges, was found to be very time-consuming and to yield preparations contaminated with bacterial debris including bacterial DNA and a troublesome endonuclease. On the suggestion of Dr. Michael Clark, another method of phage concentration was adopted, based on selective precipitation with high molecular weight polyethylene glycol (PEG). This method had been used for the purification of plant viruses[140,141,142], and had been demonstrated to be applicable to bacteriophages[142], though not widely used for this purpose at the time of this study.

Phage α was readily precipitated by PEG at high salt concentrations. As shown in Table 1., the critical concentration with the batch of polymer used was 1.9% (w/v). Phage α was routinely precipitated from dilute suspensions with 2.5% PEG and could be concentrated by low speed centrifuging or by filtering suspensions through a thin layer of celite. When shaken with buffer containing less than 1.8% PEG the precipitates

Table 1. Precipitation of Phage Alpha by Low Concentrations of Polyethylene glycol.

A series of tubes containing phage α (10^{11} p.f.u./ml.), 0.5 M NaCl, 0.01 M phosphate buffer pH 7.0 and various concentrations of PEG were assayed visually for precipitation.

PEG Concentration (g/100 ml.)	Extent of Precipitation
6	+ +
4.5	+ +
3.0	+ +
2.5	+ +
2.1	+ +
2.0	+ +
1.9	+ -
1.7	-
1.5	-
1.0	-

of phage were rapidly dispersed. The appropriate concentrations for formation and dispersion of phage precipitates on a preparative scale were demonstrated by elution of precipitated phage from a celite column as shown in figure 1.

The small peak eluted between 2.5% and 1.0% PEG was shown by electron microscopy to contain nearly pure phage α. The recovery of phage α by this method was shown to be nearly quantitative as judged both by ultraviolet absorption measurements (Table 2) and infectivity assays (Table 3).

Many batches of phage α were prepared by this method. Concentrated suspensions had a very consistent colour: electric blue with a distinct tinge of brown. This was not altered on further purification by banding in a CsCl gradient. The A_{260}/A_{280} ratio for such preparations was 1.53 ± 0.02 (mean ± S.E. for 12 batches). The spectrum of a purified phage suspension is shown in figure 2.

When portions of such a suspension were rinsed by high-speed centrifuging, to remove traces of PEG, and examined by electron microscopy, almost no traces of bacterial debris were detected (figure 3), and the phage appeared morphologically intact with the same fraction of empty heads found in phage prepared by differential centrifuging.

Figure 1. PEG Gradient Elution of Phage Alpha from a Celite Column.

5 ml. of a soft-agar extract containing phage α was made 4% (w/v) in PEG and filtered through a 1 cm. x 1 cm. diameter column of celite. Phage was eluted with a linearly descending concentration gradient of PEG in 0.5 M NaCl, 0.01 M phosphate buffer pH 7.0. 5 ml. and 2 ml. fractions were collected and assayed for phage particles by ultraviolet absorption measurements at 260 mμ.

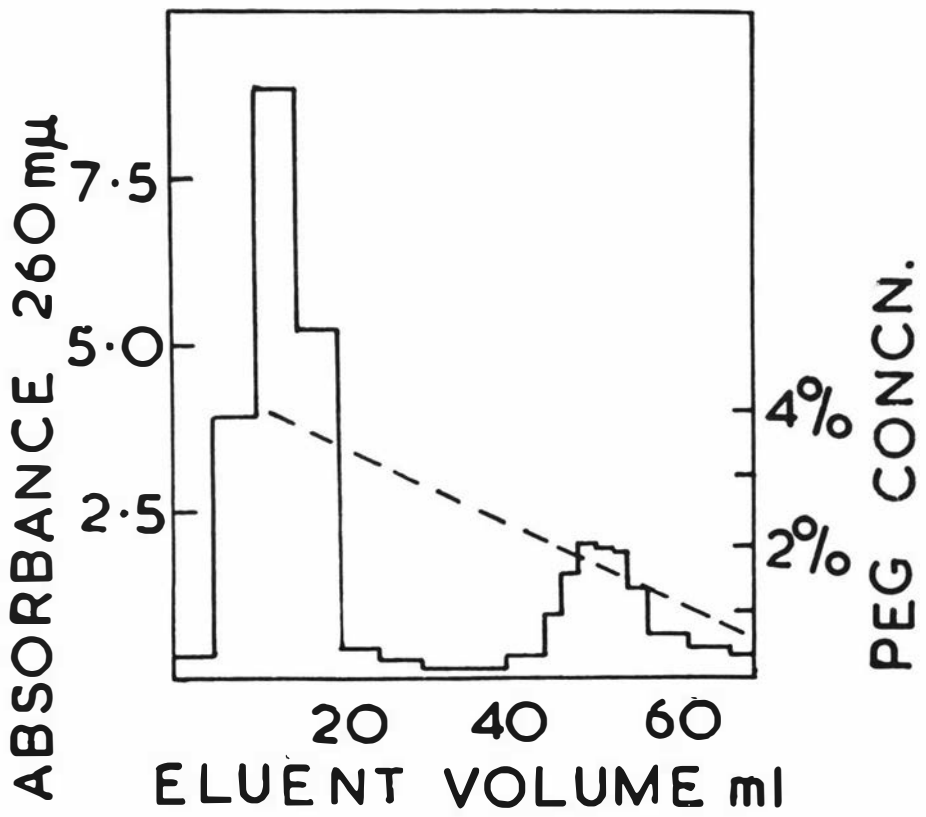


Table 2. Recovery of Ultraviolet Absorption after Precipitation of Phage Alpha by PEG.

A suspension of phage α prepared by differential centrifuging was made 0.5 M in NaCl and 1.5% (w/v) in PEG and centrifuged (5 min. at 15,000 r.p.m.). The supernatant was adjusted to 2.5% PEG and re-centrifuged. Ultraviolet absorbing material was assayed in all fractions after resuspension, by measurements at 260 mμ and 280 mμ.

Fraction	A_{260}/A_{280}	Units A_{260}	% of Total A_{260}
1.5% PEG ppt.	1.25	340	8.3
2.5% PEG ppt.	1.53	3,560	86.5
Final supt.	1.33	210	5.2

Table 3. Recovery of p.f.u. after Precipitation of Phage Alpha by PEG.

All fractions from two independent experiments carried out essentially as in the legend for table 2. were assayed for p.f.u. as described in methods. Results are expressed as mean p.f.u. ± standard error of the mean; number of estimates in parentheses.

Fraction	% Total p.f.u.	
	Experiment 1	Experiment 2
Crude phage	100 ± 6 (3)	100 ± 14 (5)
1.5% PEG ppt.	11.2 ± 3.2 (5)	7.0 ± 1.5 (5)
2.5% PEG ppt.	91 ± 9 (3)	109 ± 27 (4)
Final supt.	3.0 ± 1.6 (6)	3.4 ± 0.8 (5)

Figure 2. The Ultraviolet Absorption Spectrum of Purified Phage Alpha.

Phage α prepared by PEG precipitation was dialysed against 10^{-3} M EDTA for 6 hours, and the spectrum of an appropriate dilution scanned by double-beam spectrophotometer.

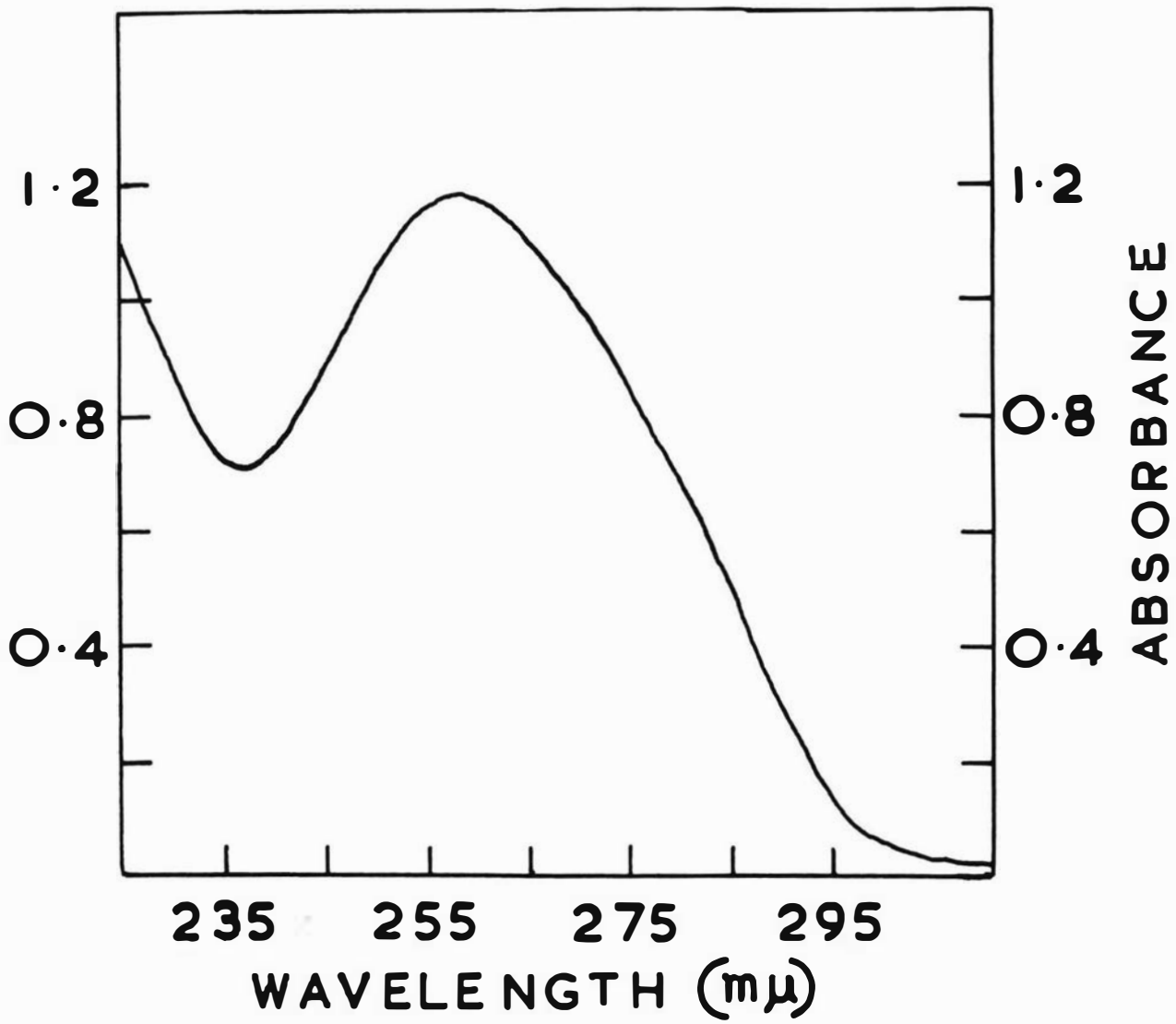
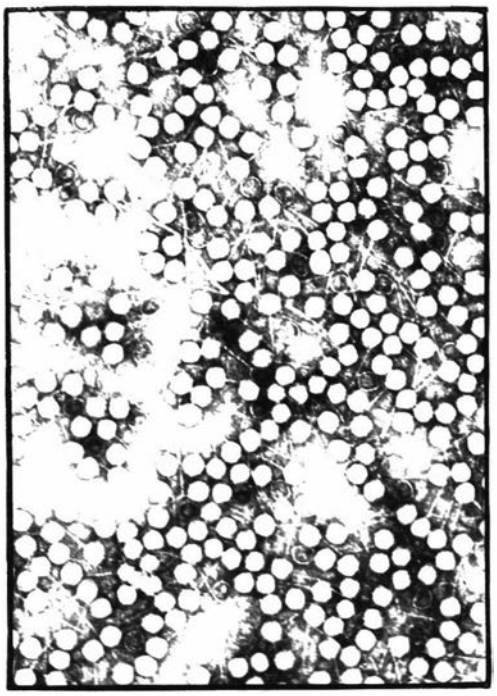


Figure 3. Electron Microscopy of Phage Alpha Prepared by PEG Precipitation.

Phage α prepared by PEG precipitation was rinsed twice with phage buffer by high-speed centrifuging. After dilution into distilled water, a portion was examined by electron microscopy.

Magnification 35,000 x



The Preparation of Strand Intact Alpha DNA

Phage α contains a single molecular species of double-stranded DNA, easily released from purified phage by shaking with buffered phenol[89,90]. Chemical studies have shown that it contains only the four normal bases, with a mean G+C content of 42.7%[89]. Cordes, using a method which can result in slight losses of thymine, found a mean G+C content of 44%[91]. Measurements of the melting temperature of α DNA in SSC, and of the buoyant density of α DNA in CsCl solutions, indicated G+C values of 42 ± 1% and 44 ± 1% respectively[91]. Thus there is a normal agreement between the physical properties of α DNA and the chemically-determined base composition.

Of these properties, only the buoyant density was re-investigated during the present study. A value of 1.705 ± .001 g/ml. was obtained, in agreement with the results of Cordes and others[89,91].

The sedimentation coefficient of the α DNA molecule was measured by Cordes using boundary sedimentation in an analytical ultracentrifuge[91]. She obtained an $S^0_{20,w}$ value of 35 from measurements of $S_{20,w}$ at a series of DNA concentrations in SSC. S^0 for a polymer can be related to the molecular weight M by relationships of the form $S^0 = K.M.^{\alpha}$. Using the values of K and α deduced for native DNA from a systematic survey of the literature by Eigner and Doty[143], $S^0_{20,w} = 35$ corresponds to a molecular weight of 29.7×10^6. Using the probably more reliable values for K and α deduced by Studier[144], a molecular weight of 32.4×10^6 is obtained.

This is roughly in agreement with the molecular weight of $34\text{-}40 \times 10^6$

deduced by Aurisicchio et al. from boundary sedimentation and autoradiography[89,145,146].

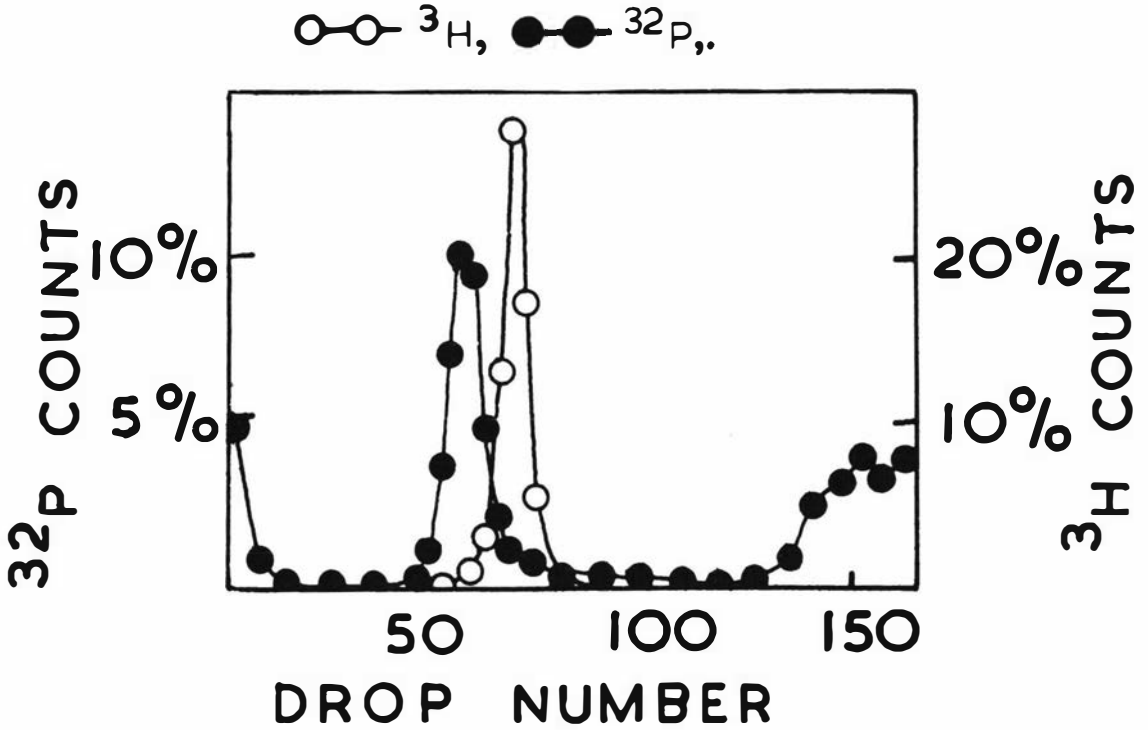
Cordes[91] also measured $S_{20,w}$ for a DNA under the conditions used by Rubinstein et al.[2], and deduced a molecular weight of 38 x 10^6. In contrast, $S_{20,w}$ measured for intact single strands of a DNA by Davison[147] led him to deduce a molecular weight of 30 x 10^6, although this estimate must have been subject to errors in measuring the concentration of intact strands.

Zone sedimentation[148,144] has considerable advantages over boundary sedimentation for the characterisation of high molecular weight DNA. The former method is less susceptible to artifacts caused by convective disturbances, DNA concentration and rotor speed[148,149,150]. The method developed by Burgi and Hershey[99], in which DNA molecules are sedimented through preformed sucrose gradients, was used in the present study. In 5-20% sucrose gradients at 5-10°C the distance D travelled by a sedimenting species is very nearly proportional to its sedimentation coefficient at infinite dilution[101]. The ratio of the distances two sedimenting species have travelled can be determined more reproducibly than the distance one has travelled, so two species labelled with different isotopes are usually sedimented in the same tube.

In my experiments, mixtures of ^{3}H-labelled T7 DNA and ^{32}P-labelled a DNA were sedimented together (figure 4) and the molecular weight and sedimentation coefficient of a DNA calculated from the ratio of the distances D travelled by the two species using the relationship $D_2/D_1 = S_2/S_1 = (M_2/M_2)^{\alpha}$ where the value of the exponent α was taken to

Figure 4. Measurement of the Molecular Weight of Native Alpha DNA by Zone Sedimentation.

A mixture of ^{32}P-labelled α DNA (0.5 μg.) and ^{3}H-labelled T7 DNA (0.3 μg.) was layered onto a neutral sucrose gradient containing 1 M NaCl, 0.02 M tris/HCl buffer pH 8.0, and sedimented for 6 h. at 35,000 r.p.m. The radioactivity in each 3-drop fraction was assayed by counting on paper in toluene scintillator fluid.



be 0.35 as found by Burgi and Hershey[99] and confirmed by Studier[144]. The values obtained for D_2/D_1 in two independent experiments were 1.098 ± .020 and 1.097 ± .020. Taking $S^0_{20,w}$ for T7 DNA to be 32 ± 0.8[144], $S^0_{20,w}$ for α DNA was calculated to be 35.1 ± 0.9 in close agreement with the result of Cordes[91]. Taking the molecular weight of T7 DNA to be 25 x 10^6, by collation of the results of Studier based on zone sedimentation of the native, alkaline, and neutral denatured forms of T7 DNA[144], the results of Abelson and others[151] based on zone sedimentation, electron microscopy, and autoradiography, and the results of Richardson[152] based on end-labelling, the molecular weight of α DNA was calculated to be 32.9 x 10^6.

There should be little difficulty in preparing DNA of this size completely unbroken. Thomas et al.[100,153] have reported that the DNA molecules of phages T2, T3, T7, λ, P1 and P22 can be isolated in highly strand-intact form as judged by zone sedimentation through alkaline sucrose gradients. Similar results for the DNA molecules of phages T2, T7 and λdg were obtained by Studier[144] and by Tomizawa and Anraku[154]. Davison, Freifelder and Holloway[155], using the criterion of boundary intactness in an analytical ultracentrifuge, found substantial amounts of strand breakage in the DNA molecules from several bacteriophages. However, in a later paper Davison and Freifelder[102] reported better than 90% strand intactness in the DNA molecules of phage λ, and 65-70% strand intactness in the DNA molecules of phage T2. Other workers[156,157] have reported finding essentially no strand breakage in the DNA molecules of phages T1 and SPP1.

A number of other phage DNA molecules do appear to contain single-strand breaks or nicks. These include the DNA molecules of phages SP50[158,159], PBS1[160], D3[147], and α[147,161]. Cordes[91] does not comment on the degree of strand intactness in her preparations of phage α DNA, but Davison[147] reported less than 50% intact strands. In the cases of phage T5 and the closely related phage P8 the DNA molecules have been shown to contain single-strand breaks at a number of fixed locations[151,162,163]. These breaks are under genetic control, and may serve some important function during the phage growth cycle.

During early experiments with phage α in the present investigation it was found that although the DNA molecules were intact in the double-stranded form, they contained many single-strand breaks as assayed by either zone or boundary sedimentation. In the experiments shown in figure 5, no intact strands were detected, and the mean molecular weight of the fragments was only 3.6×10^6. This DNA had been prepared by phenol extraction of crude phage concentrated from bacterial lysates by differential centrifuging, and the breakage was probably due to contamination with an endonuclease. The evidence for this is that:

(1) DNA showing a high degree of strand intactness was obtained if the phage was dialysed against EDTA (0.001 M) prior to phenol extraction. EDTA is an inhibitor of the many nucleases that require metal ions as co-factors.

(2) Highly intact DNA was obtained by phenol extraction of phage α that had been purified by selective precipitation with polyethylene glycol. This method routinely gave preparations of α DNA containing 45-55% intact strands.

Figure 5. Zone Sedimentation and Boundary Sedimentation of Nicked Alpha DNA.

(a) A solution of α DNA in 0.01 x SSC was heat-denatured (5 minutes at 100°, followed by rapid cooling) and adjusted to 1 x SSC and 20 μg/ml. A 1.8 ml. portion was transferred by gravity flow through a No. 22 syringe needle into a 30 mm. ultracentrifuge cell, and sedimented at a rotor speed of 37,020 r.p.m. Ultraviolet photographs were taken at 8 minute intervals and scanned with a Joyce-Loebl recording microdensitometer.

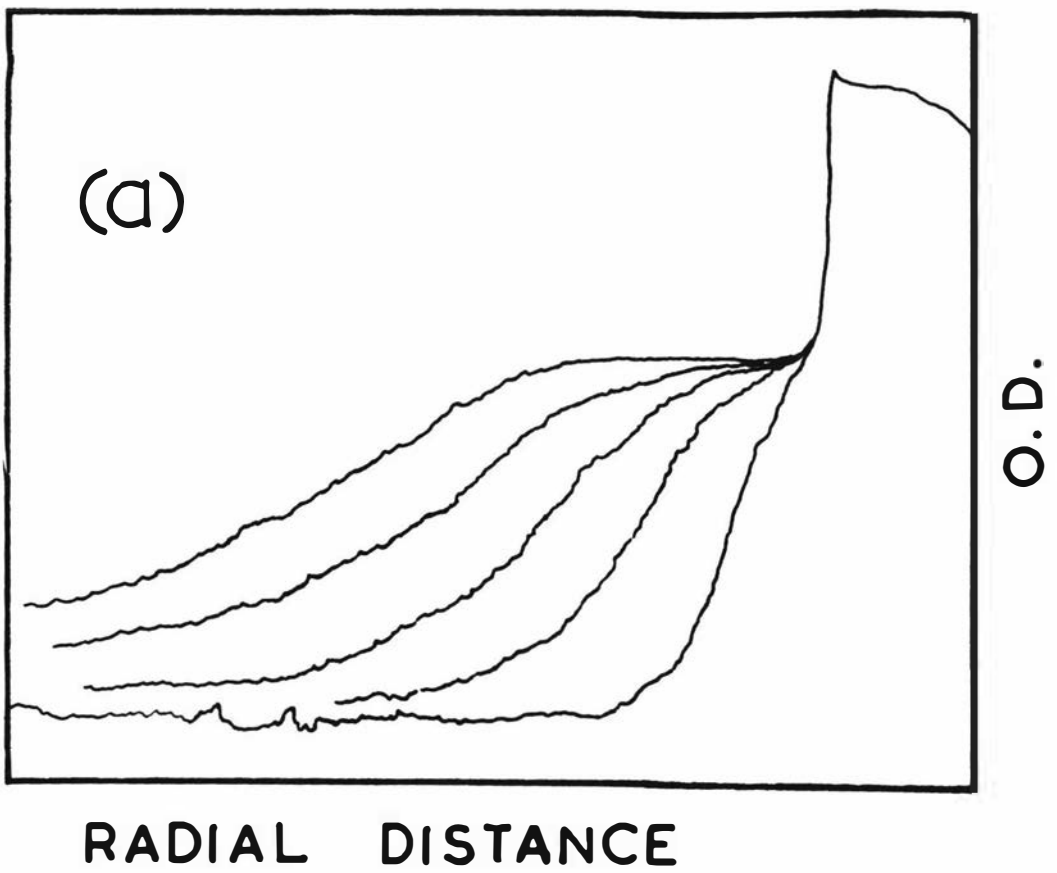
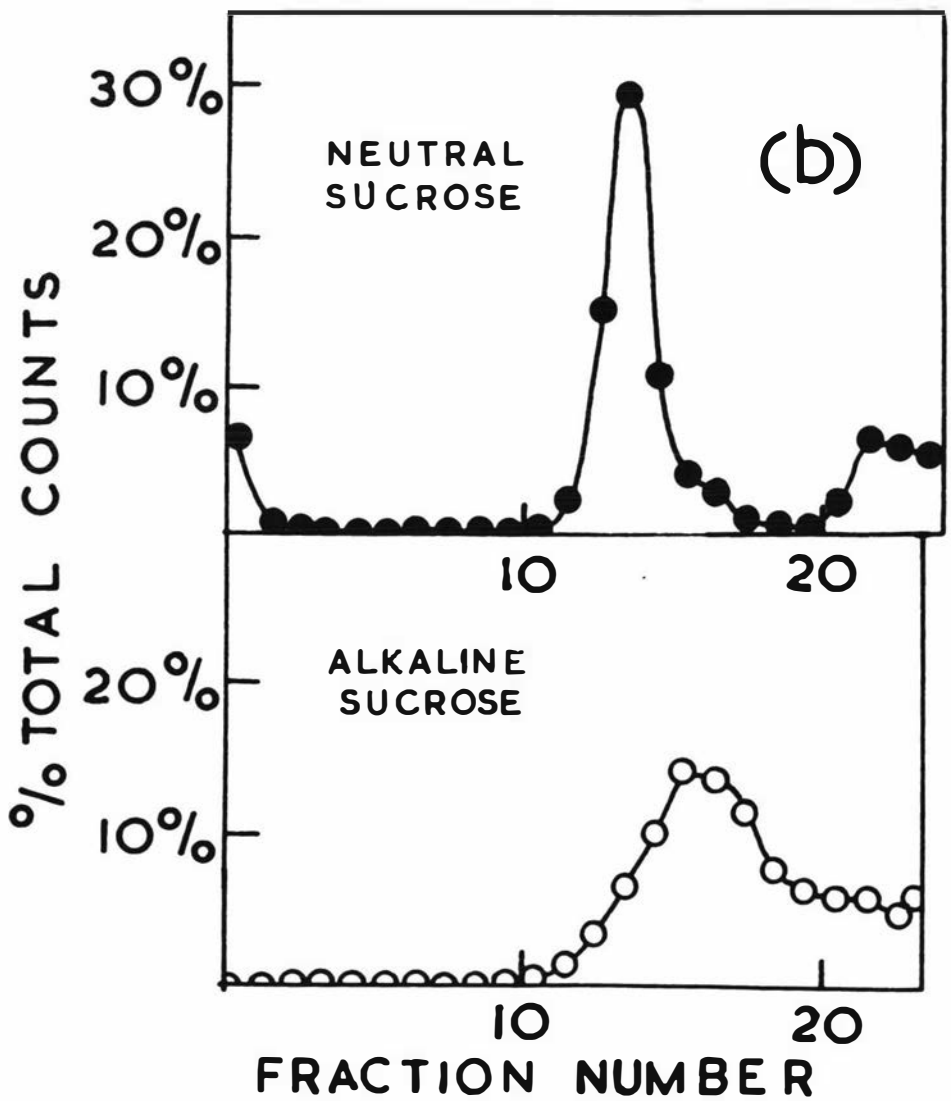


Figure 5. Zone Sedimentation and Boundary Sedimentation of Nicked Alpha DNA.

(b) 0.5 µg. portions of a preparation of ^{32}P-labelled α DNA (40,000 d.p.m./µg) were layered onto gradients of neutral sucrose (containing 2 M NaCl, 0.05 tris/HCl buffer pH 7.4) and alkaline sucrose (containing 0.1 M NaOH, 0.9 M NaCl), and sedimented for 4 h. at 35,000 r.p.m. 4-drop fractions were dried onto aluminium plachettes and assayed for radioactivity using a Beckman Low-Beta proportional counter.



Routine assays for DNA intactness by boundary sedimentation are shown in figure 6. Native α DNA gave very sharp boundaries with no detectable breakage, but on sedimentation in alkaline solution substantial amounts of trailing material were observed due to breakage of single strands. The amount of trailing material could be assayed with a reproducibility of within ± 5%, and was not increased by standing the DNA in alkaline solution for several hours prior to sedimentation.

These strand breaks were not found to occur at any fixed distance from the ends of the α DNA molecule, since no sharply-defined fragment sizes were found by zone sedimentation. Thus α DNA does not resemble the nicked DNA of phage T5, which gives four distinct sedimenting zones of strand fragments. Since the two complementary strands (L and H) of the α DNA molecule can be separated by centrifuging to equilibrium in density gradients of caesium chloride, it was possible to measure the distribution of strand breaks between the two strands. If breaks were mainly in one strand, the peak of intact strands isolated by zone sedimentation of denatured α DNA through neutral sucrose gradients should be enriched for the less-nicked strand. A double-label ratio experiment was carried out, in which ^{32}P-labelled intact strands isolated by preparative sucrose gradient sedimentation (figure 7(a)) were centrifuged in a caesium chloride density gradient with ^{33}P-labelled unfractionated strands. Preferential nicking of one strand should have been reflected in a higher than average $^{33}P/^{32}P$ ratio. As shown in figure 7(b), no such preference was detected. The actual distri-

Figure 6. Routine Assays for DNA Intactness by Boundary Sedimentation.

α DNA at 25 μg/ml. was assayed for intactness in (a) 1 M NaCl, 0.001 M tris/HCl buffer pH 8.0 (b) 0.1 M NaOH, 0.9 M NaCl, as described in Methods. Sedimentation was at 37,020 r.p.m. Photos were scanned with a Joyce-Loebl recording microdensitometer.

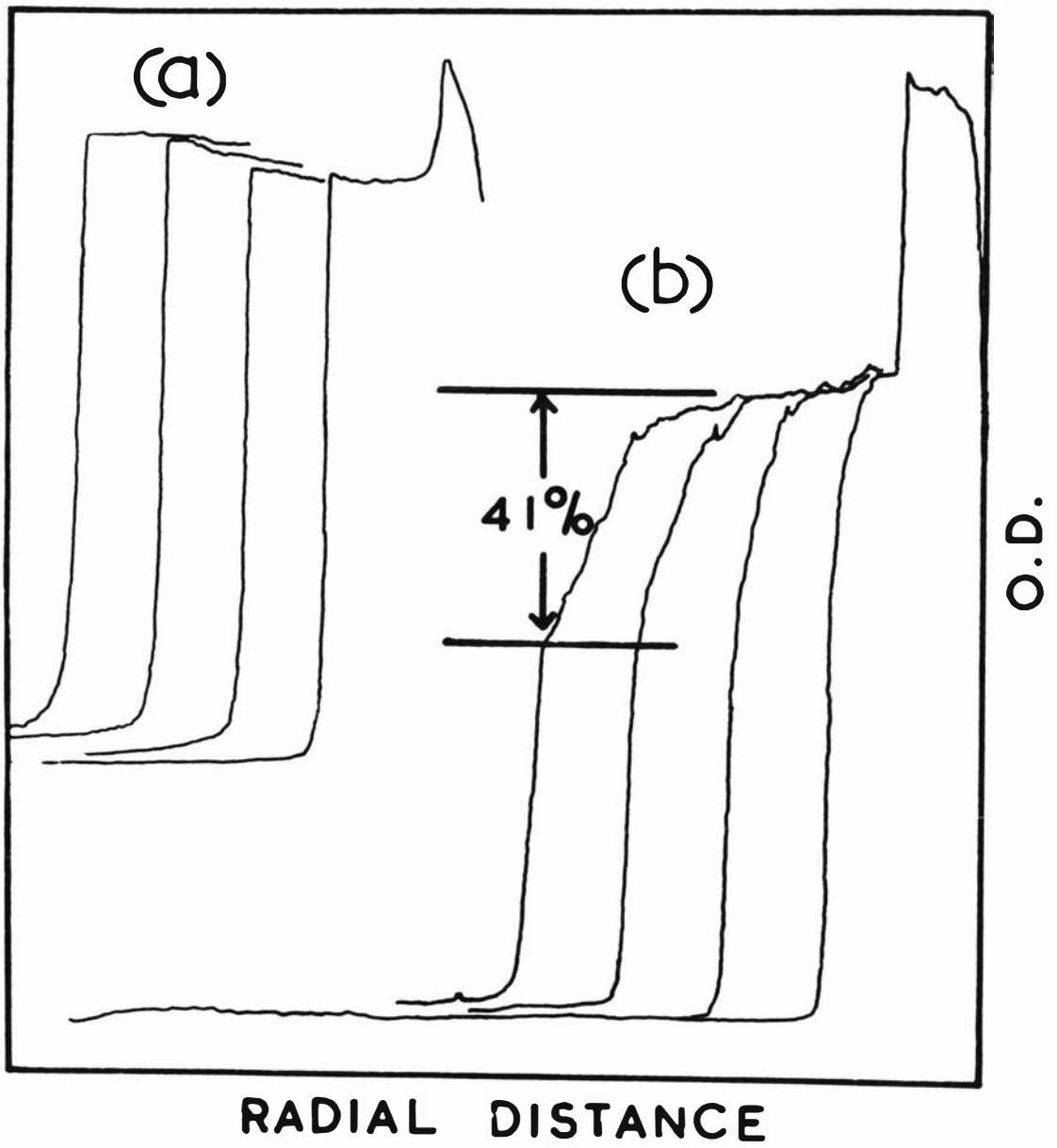


Figure 7. The Distribution of Strand Breakage Between the H and L Strands of Phage Alpha DNA.

(a) ^{32}P-labelled intact strands of α DNA were prepared by sone sedimentation of alkali-denatured DNA (3.5 μg.) through a neutral sucrose gradient (containing 2 M NaCl, 0.05 M tris/HCl buffer pH 7.4) for 3 h. at 38,000 r.p.m. Fractions 6-7 were taken as the peak of intact strands.

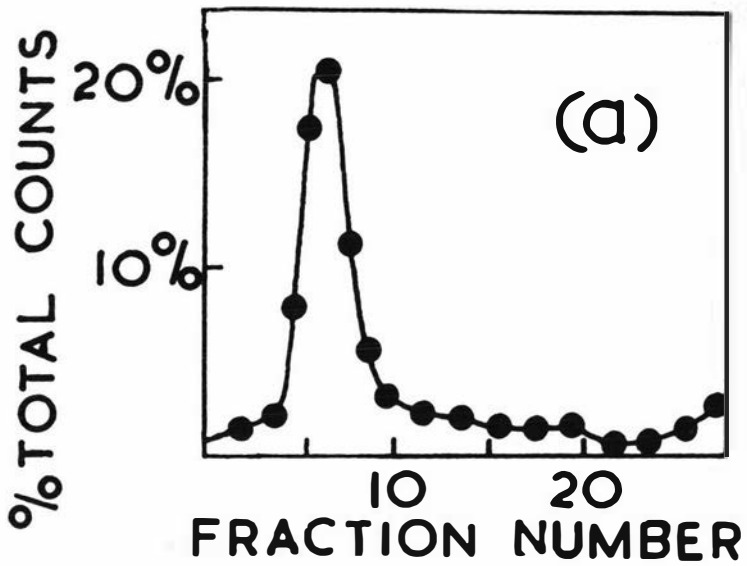
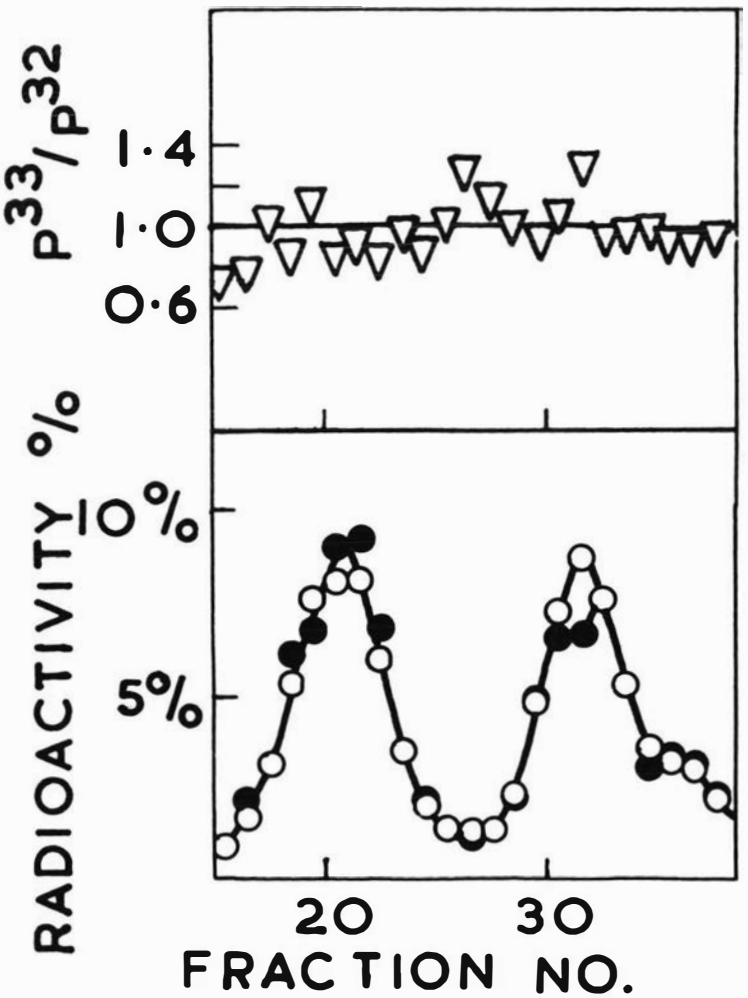


Figure 7. The Distribution of Strand Breakage Between the H and L Strands of Phage Alpha DNA.

(b) Dialysed ^{32}P-labelled intact strands from (a) were combined with un-fractionated ^{33}P-labelled α DNA (3.2 μg.) and the complementary strands separated by CsCl gradient centrifuging of the polyG complexes as described in Methods. The $^{33}P/^{32}P$ ratio for each 5-drop fraction was measured by counting on paper in toluene scintillator fluid. ○—○ ^{33}P, ●—● ^{32}P, ▽—▽ $^{33}P/^{32}P$ percentage ratio.



butions of radioactivity were: ^{33}P, 50.4% L strand, 49.6% H strand; ^{32}P, 47.5% L strand, 52.5% H strand. These are compatible with a slight (55:45) preference for nicking of the L strand, but are within range of the likely experimental error from a 50:50 distribution. It was concluded that the location of single-strand breaks in such preparations of a DNA is essentially random.

The origin of such random breakage is obscure. Two possibilities were considered: that either a DNA must be packaged in a nicked (or alkali-labile) state inside the phage particles, or that the breaks must be caused during phenol release of the DNA by endonuclease action. Phenol extraction must result in the eventual denaturation or removal of any endonucleases, since it was found that purified a DNA could be stored at 2-5° for several days without further strand breakage.

A direct attempt was made to detect endonuclease activity in a phage preparations by incubating small amounts of largely strand-intact ^{32}P-labelled DNA from phage λ b_2b_5 (a gift from Mr. Barrie J. Carter) with concentrated phage a prepared by selective precipitation with PEG followed by centrifuging in a caesium chloride density gradient. The result, shown in figure 8, was that most of the λ DNA remained strand-intact even after prolonged incubation with either intact phage a or osmotically-lysed phage a. This showed that such a phage preparations were not contaminated with an extraneous endonuclease active under the conditions of this experiment, but did not eliminate the possibility that an endonuclease might form an integral component of the phage particle, since such a component might not be released by the limited disruption resulting from osmotic lysis.

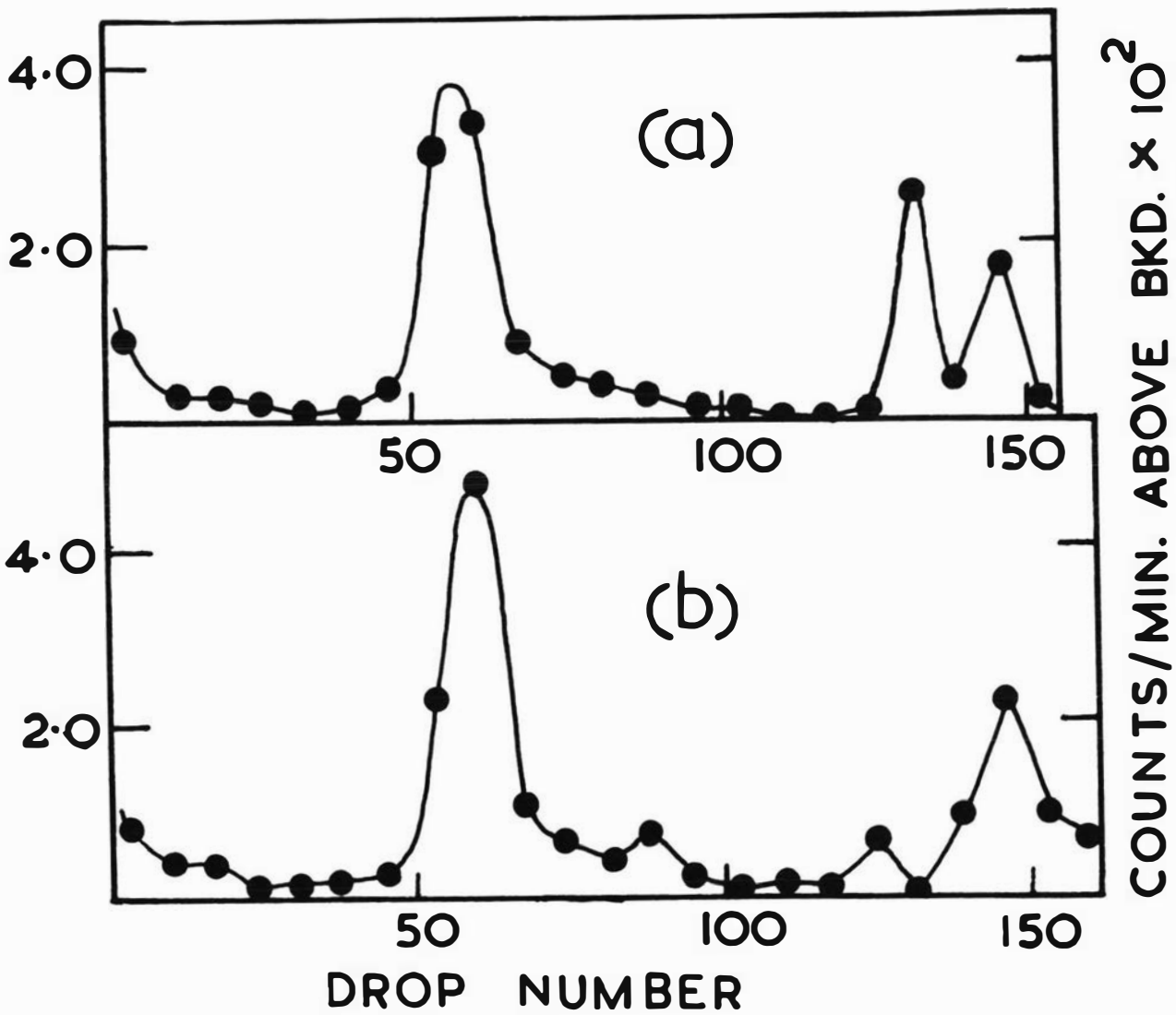
Figure 8. Zone Sedimentation of Phage Lambda DNA After Incubation With Intact and Osmotically-lysed Alpha phage.

Phage α prepared by selective precipitation with PEG was further purified by preparative CsCl gradient centrifuging (12 h. at 36,000 r.p.m., initial density of mixture 1.47 g/ml.). A portion was lysed by rapid 20-fold dilution into H_2O, and both lysed and intact phage were dialysed overnight at 2-5° against 0.5 M NaCl, 0.01 M phosphate buffer pH 7.0. After dilution to a concentration equivalent to 10^{11} p.f.u./ml., 0.2 ml. portions of both lysed and intact phage were incubated for 12 h. at 37° with 0.6 μMoles of $MgSO_4$ and 0.5 μg. of ^{32}P-labelled DNA prepared by phenol extraction of phage λ b_2b_5. After phenol extraction and dialysis against 0.1 M NaCl, 0.001 M EDTA, 0.01 M tris/HCl buffer pH 8.0, 0.05 ml. portions of each incubation mixture were sedimented through alkaline sucrose gradients for 4 h. at 38,000 r.p.m. 10-drop fractions were assayed for radioactivity by Cerenkov counting.

(a) Incubation mixture containing intact α phage.

(b) Incubation mixture containing lysed α phage.

Figure 8. Zone Sedimentation of Phage Lambda DNA After Incubation with Intact and Osmotically-lysed Alpha Phage.



One necessary consequence of the endonuclease hypothesis is that it should be possible to change the extent of strand breakage by changing the conditions during phenol release of the DNA. Two supporting observations were made:

(1) It was found repeatedly that by dialysing the phage suspensions against 0.01 M Tris/HCl buffer pH 8.0 containing 0.5 M NaCl and 0.001 M SDDC, and chilling to 2-5° prior to phenol extraction, the fraction of intact strands in the DNA obtained from portions of the same α phage preperation could be increased from 45-55% to 70-75%. EDTA was not an effective substitute for SDDC, and the high salt concentration was essential. However, no further increase in the strand-intactness of α DNA was achieved by increasing the SDDC concentration to 0.01 M, by including the powerful chelating agents 8-hydroxyquinoline and dibenzoylmethane at 1% concentration in the phenol layer, by carrying out the first phenol extraction with 50:50 phenol/$CHCl_3$, or by heat-shocking the dialysed phage (1 minute at 100°) immediately prior to phenol extraction. Sedimentation analysis of a preparation of α DNA containing 75% intact strands is shown in figure 9(a). The estimate of intact strands was not altered when the DNA was sedimented in the neutral denatured form after standing very briefly (1-2 minutes) in alkali (Figure 9(b).).

(2) A correlation was found between the temperature of the first phenol extraction and the strand-intactness of the resultant DNA. The results of two independent experiments are combined in figure 10. Although each experiment taken separately was inconclusive, there appeared to be an overall trend towards more strand breakage at higher

Figure 9. Boundary Sedimentation of Denatured α DNA Showing 75% Strand Intactness.

(a) Denatured α DNA in 0.1 M NaOH, 0.9 M NaCl. Rotor speed 37,020 r.p.m. Ultraviolet photographs taken at 8 minute intervals and scanned with a Joyce-Loebl recording microdensitometer.

(b) Denatured α DNA in 0.12 M phosphate buffer pH 7.4. Rotor speed 25,980 r.p.m. Ultraviolet photographs taken at 4 minute intervals.

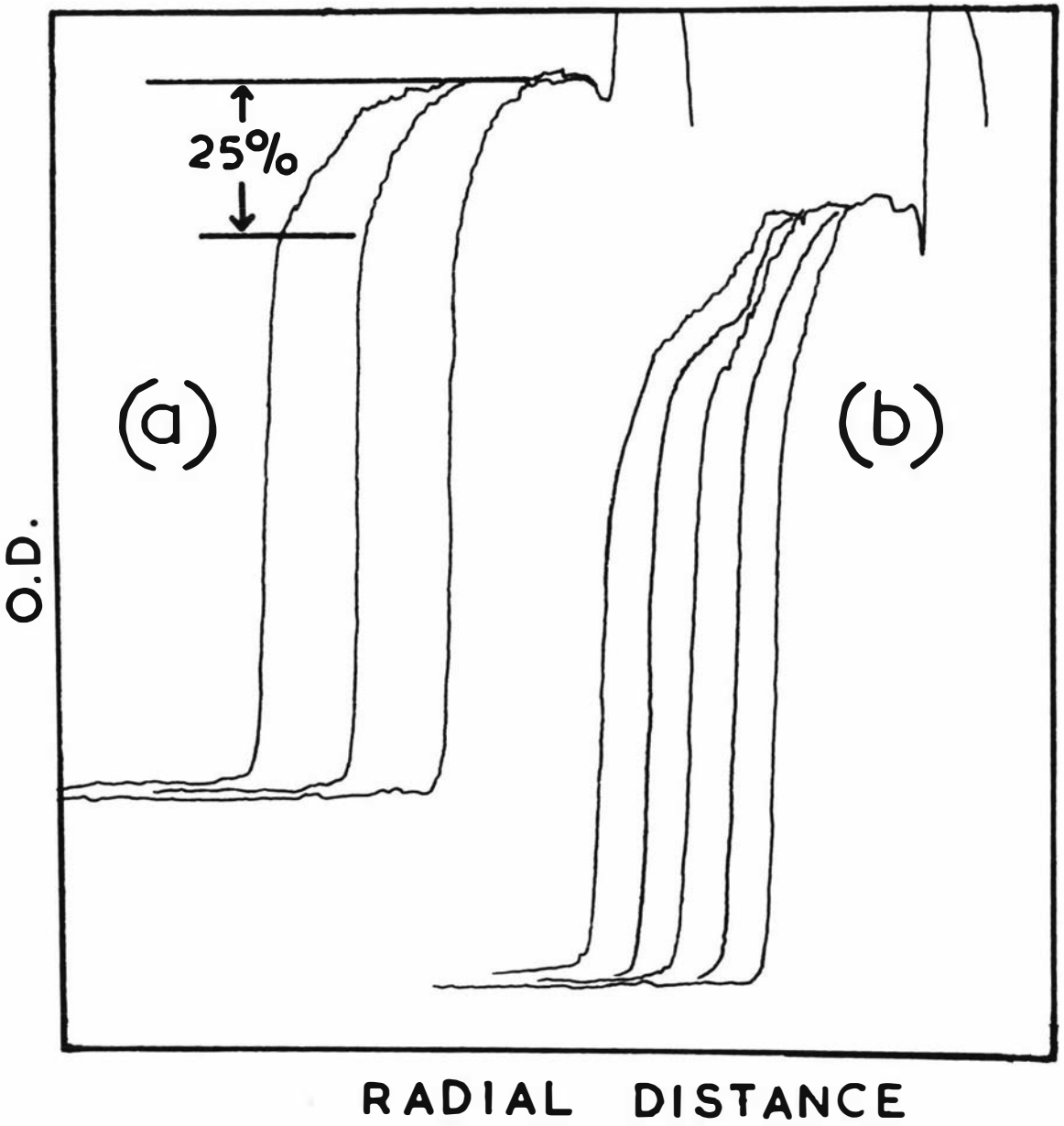
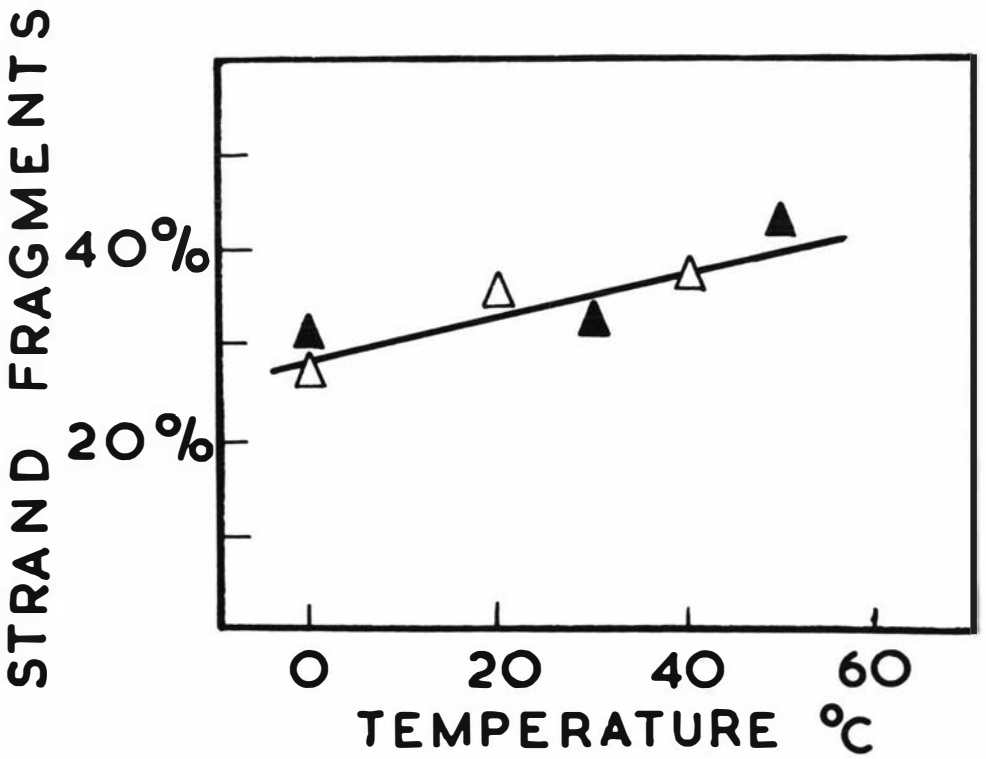


Figure 10. Strand-intactness and the Temperature of Phenol Extraction.

Phage α was prepared by two cycles of selective PEG precipitation followed by dialysis against 0.5 M NaCl, 0.001 M SDDC, 0.01 M tris/HCl buffer pH 8.0. Small portions of concentrated phage were allowed to equilibrate at the temperatures shown, then gently shaken with portions of warmed or cooled borate-buffered phenol and allowed to stand at the appropriate temperatures for 10 minutes. After separation of the layers by centrifuging, each aqueous layer was re-extracted once with buffered phenol at room temperature, dialysed against 0.1 M NaCl, 0.001 M EDTA, 0.01 M tris/HCl buffer pH 8.0 at 2-5°, and assayed for strand-intactness by boundary sedimentation in 0.1 M NaOH, 0.9 M NaCl.



temperatures. In a control experiment to test the possibility that breakage was caused by exposure to phenol, a preparation of purified α DNA containing 67-71% intact strands (two estimates gave the same range) was twice re-extracted with phenol at 30°. After dialysis, the DNA was found to contain the same fraction of intact strands (69-72%).

Separation of the Complementary Strands of Alpha DNA

Cordes[90,91] separated the complementary purine-rich (L) and pyrimidine-rich (H) strands of the α DNA molecule by MAK chromatography at pH 6.8, using a modification of the stepwise elution method of Sueoka and Cheng[163]. Heat-denatured α DNA was applied to the columns at a salt concentration (0.85 M SP) sufficient to completely elute the L strands. After rinsing with the same buffer, pure H strands were eluted with moderate yields (50%) by 1.0 M SP. The complementary nature of the L and H strand preparations was proven by measurement of their base-compositions (table 4.) and by their ability to re-associate in mixtures, but not separately, to give native α DNA as judged by both buoyant density and hyperchromicity measurements. The extent of contamination of each strand fraction with complementary strands was estimated to be not greater than 10%[91].

Aurisicchio et al. also fractionated heat-denatured α DNA by MAK chromatography at pH 7.9, but found their method suitable only for preparation of the L strands[92].

During the initial stages of this project, considerable difficulties were experienced with the method of Cordes, owing to an apparent lack of reproducibility in the salt concentrations required to elute the L and H strands. Similar problems with MAK chromatography have been encountered by other groups[164,104]. An interesting modification of the method was suggested by the results of Roger[104], who found that high recoveries and reproducible fractionations of denatured Pneumococcal DNA could be achieved by salt gradient elution of MAK columns at 5-6°.

Table 4. Base-compositions of the Separated H and L Strands of Alpha DNA.

Base-composition data recalculated from Cordes as mean ± standard error. Number of observations: L strand, 8; H strand 4.

	% G	% C	% A	% T	% G+C
L strand	23.3 ± 1.2	21.3 ± 0.3	30.0 ± 0.7	25.4 ± 1.2	44.6 ± 1.5
H strand	19.9 ± 1.0	24.2 ± 0.5	24.0 ± 1.4	32.1 ± 1.9	44.1 ± 1.5

During the present study, this observation was confirmed for the chromatography of denatured α DNA. One of several similar separations is shown in figure 11. It was found that early fractions from such columns were greatly enriched (90-100%) for L strands, as assayed by CsCl gradient centrifuging (figure 12.). Later fractions were enriched (60-80%) for H strands, but were always significantly contaminated with L strands. Total recoveries of denatured DNA ranged from 90-100%. However, attempts to scale up such columns for the fractionation of milligram quantities of denatured α DNA resulted in poor recoveries (30-50%) and mediocre separations, perhaps due to uneven flow through the columns.

It has been claimed that the problem of trailing of peaks during MAK chromatography of denatured DNA can be overcome by interrupted gradient elution[165], but this result was not confirmed in the present work. An interrupted gradient experiment is shown in figure 13. Although the first peak contained 90% L strands, the second peak, eluted by resuming the salt gradient, was only slightly enriched (60%) for H strands.

Enriched strand preparations were freed of complementary strands by self-annealing followed by chromatography on columns of hydroxylapatite. In order to avoid the hydrolytic strand breakage that can result from prolonged incubations at 60-70°, annealing was carried out at 0-5° in 30% (v/v) formamide using the salt concentrations suggested by Bonner et al.[166] Hydroxylapatite chromatography, a particularly useful method for the fractionation of polynucleotides[106,107] was used to remove double-stranded DNA after self-annealing. A

Figure 11. Gradient Elution MAK Chromatography of Denatured Alpha DNA.

Alkali-denatured α DNA (530 μg.) was fractionated by salt gradient elution from a 3 cm. x 1 cm. diameter MAK column at 2-5°, as described in Methods. 7-ml. fractions were assayed for DNA by ultraviolet absorption measurements at 260 mμ; and fractions 9, 11, 13 and 15 were assayed for H and L strand content by CsCl gradient centrifuging. (a) Elution profile of total denatured DNA. (——), 260 mμ absorbance; (— — —), NaCl concentration. (b) Deduced elution profiles for the complementary strands. (○○), L strands; (●●), H strands; (— — —), NaCl concentration.

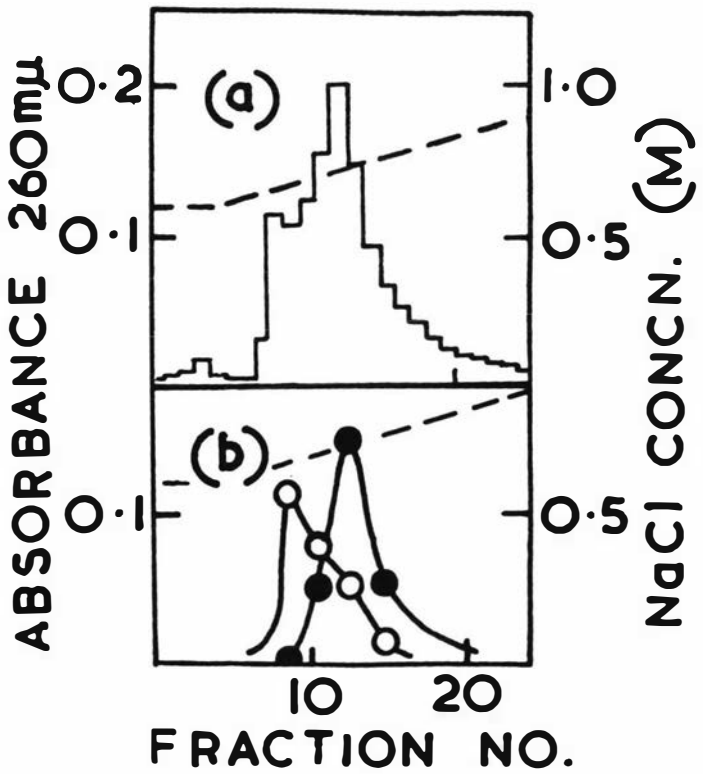


Figure 12. CsCl Gradient Centrifuging of Fractions from MAK Column Chromatography.

0.1-0.2 ml. aliquots from fractions 9, 11, 13 and 15 from figure 11. were centrifuged for 16-18 h. at 44,770 r.p.m. in CsCl density gradients, and ultraviolet photographs scanned with a Joyce-Loebl recording microdensitometer. Strand content was estimated from the relative height of the peaks (shoulders) of H and L. Density marker is native α DNA (ρ^0 = 1.705 g/ml.)

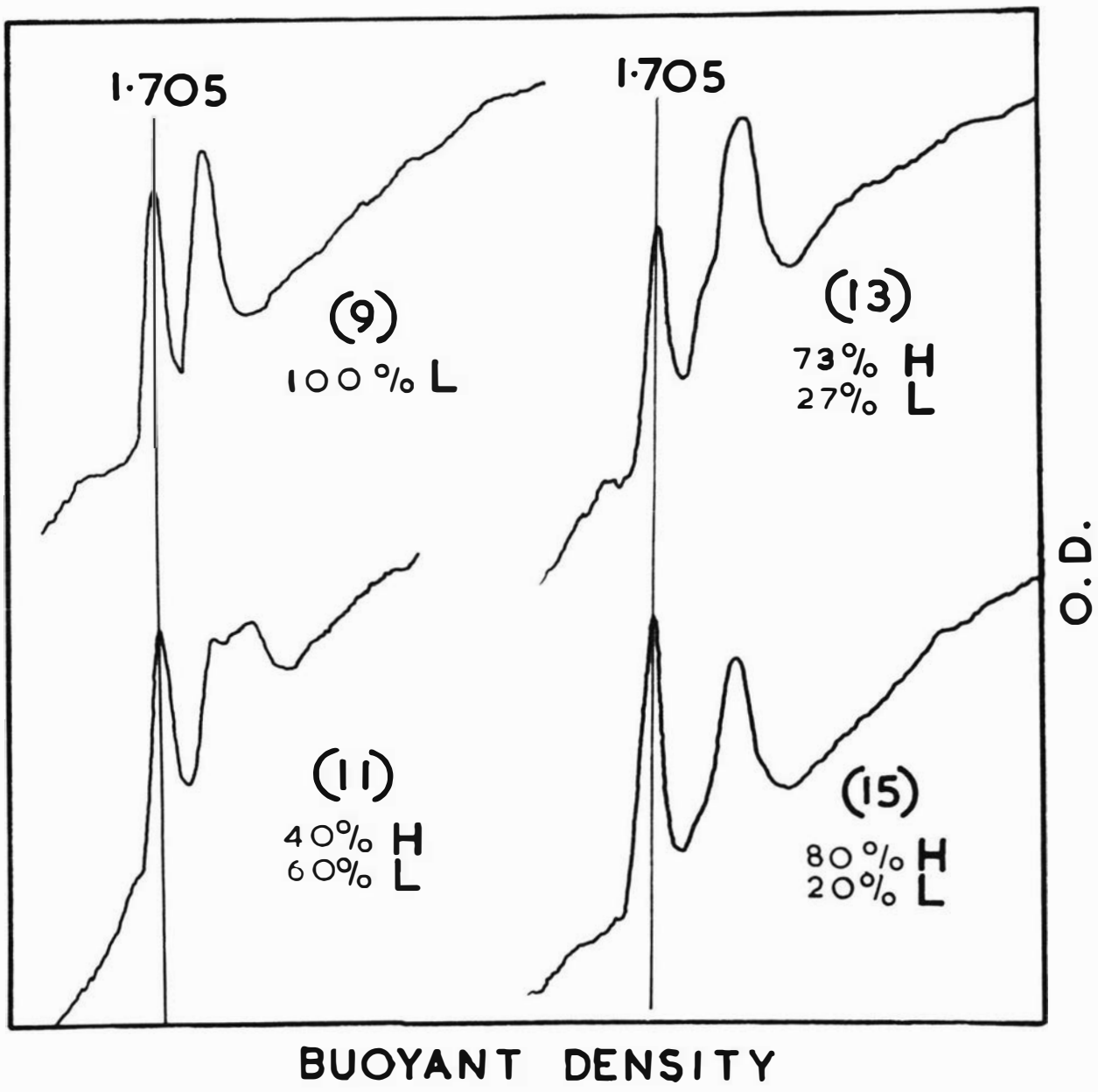


Figure 13. MAK Chromatography of Denatured Alpha DNA by Interrupted Gradient Elution.

^{32}P-labelled, alkali-denatured α DNA (1.5 mg.) in 0.6 M NaCl, 0.12 M phosphate buffer pH 7.4, was pumped onto a 3 cm. x 2 cm. diameter MAK column maintained at 2-5°. After rinsing with 0.6 M SP buffer pH 6.8, elution (at 3 ml/minute) commenced with an 800 ml. linear salt gradient (– – – –) formed from two flasks containing 0.6 M SP and 1.0 M SP. Ultraviolet absorption was monitored continuously using an ISCO eluent scanner. When the 260 mμ absorbance of the eluent had reached approximately 0.1, the salt gradient was interrupted, and elution continued out at a constant salt concentration until the absorbance of the eluent had returned to less than 0.03.

Salt Gradient elution was then resumed. Radioactivity was estimated in 20-ml. fractions by Cerenkov counting. Pooling of fractions for strand assay: L strand, 14-17; H strand, 23-30.

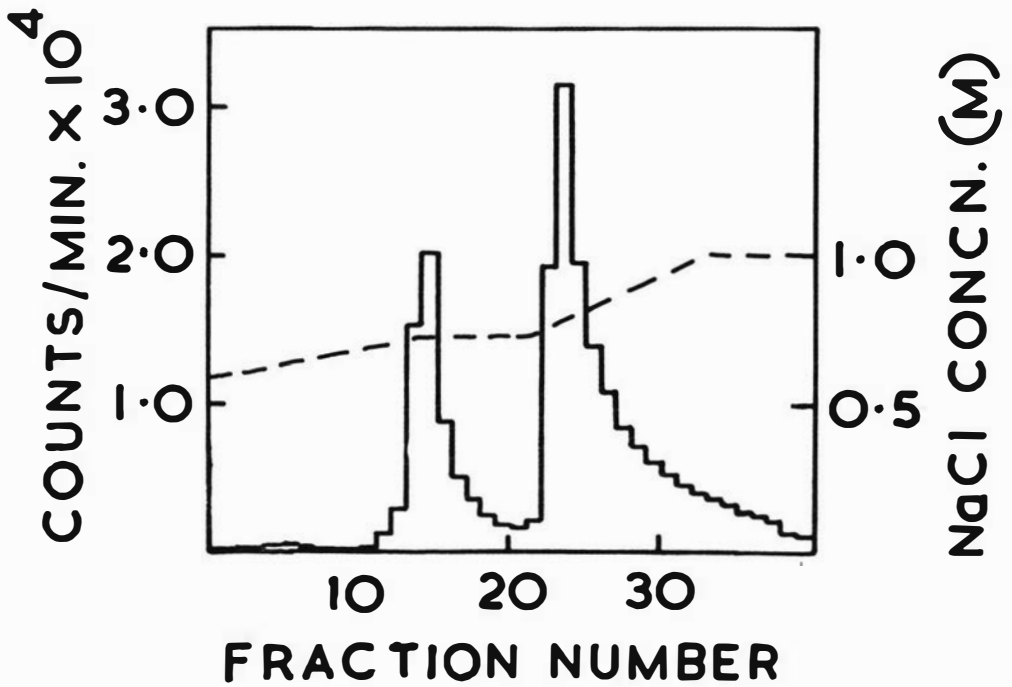


Figure 14. Hydroxylapatite Chromatography of a Self-annealed L Strand Fraction of Alpha DNA.

A 90 µg. sample of ^{32}P-labelled α DNA L strands, prepared by MAK chromatography, was self-annealed at 9 µg./ml. in 30% (v/v) formamide for 36 h., dialysed against 0.12 M phosphate buffer pH 7.0 containing 0.3 M NaCl, and loaded onto a 6 cm. x 1 cm. diameter column of hydroxylapatite. After rinsing with 10 ml. of the same buffer, the column was eluted (flow-rate 2 ml./minute) with a 30 ml. gradient of increasing phosphate buffer concentration (– – – –) followed by 20 ml. of finishing buffer. 2.5 ml. and 5 ml. fractions were assayed for radioactivity by Cerenkov counting. DS = double-stranded DNA, SS = single-stranded DNA. Total recovery of applied radioactivity was 92%.

Figure 14. Hydroxylapatite Chromatography of a Self-annealed L Strand Fraction of Alpha DNA.

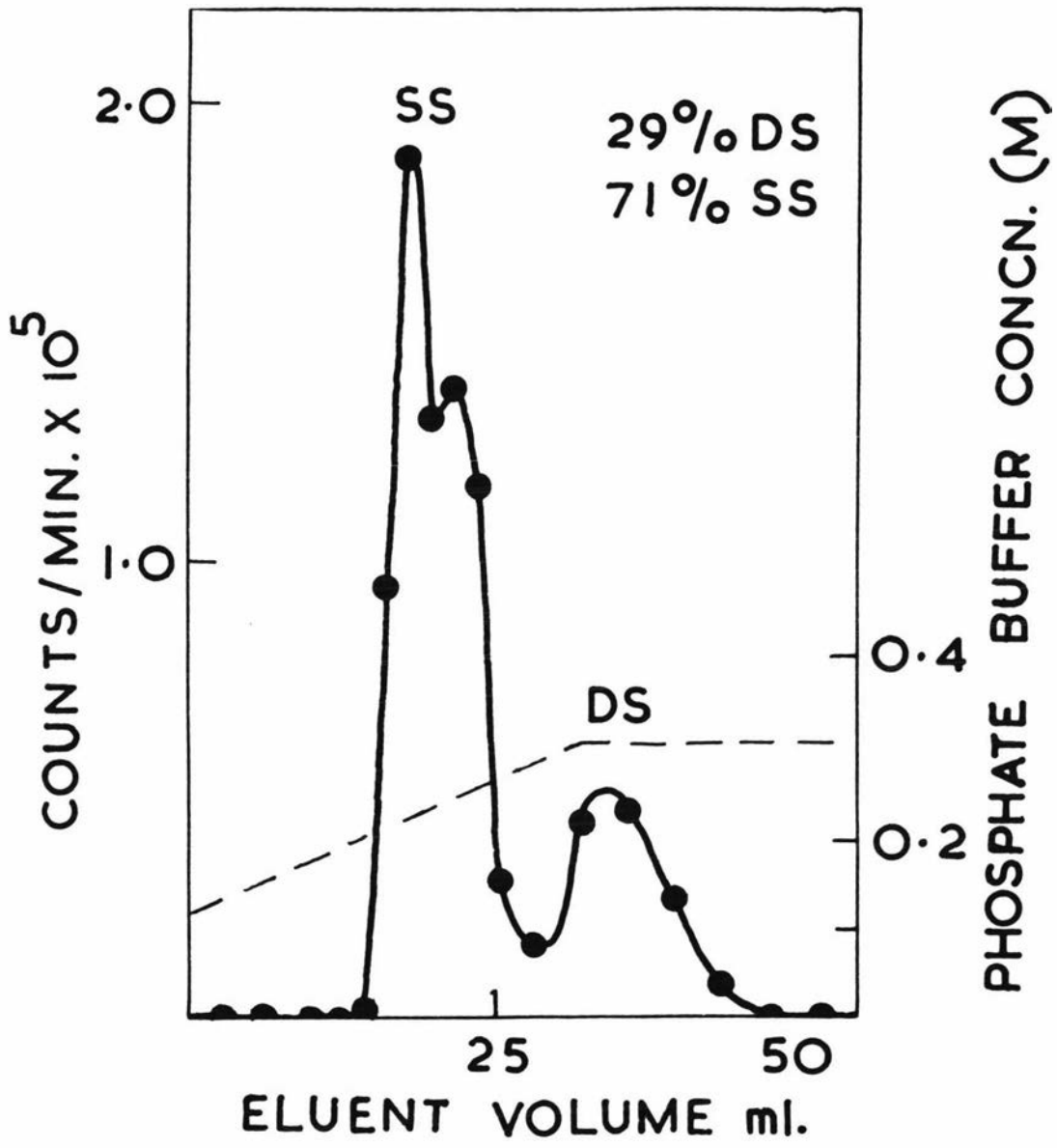
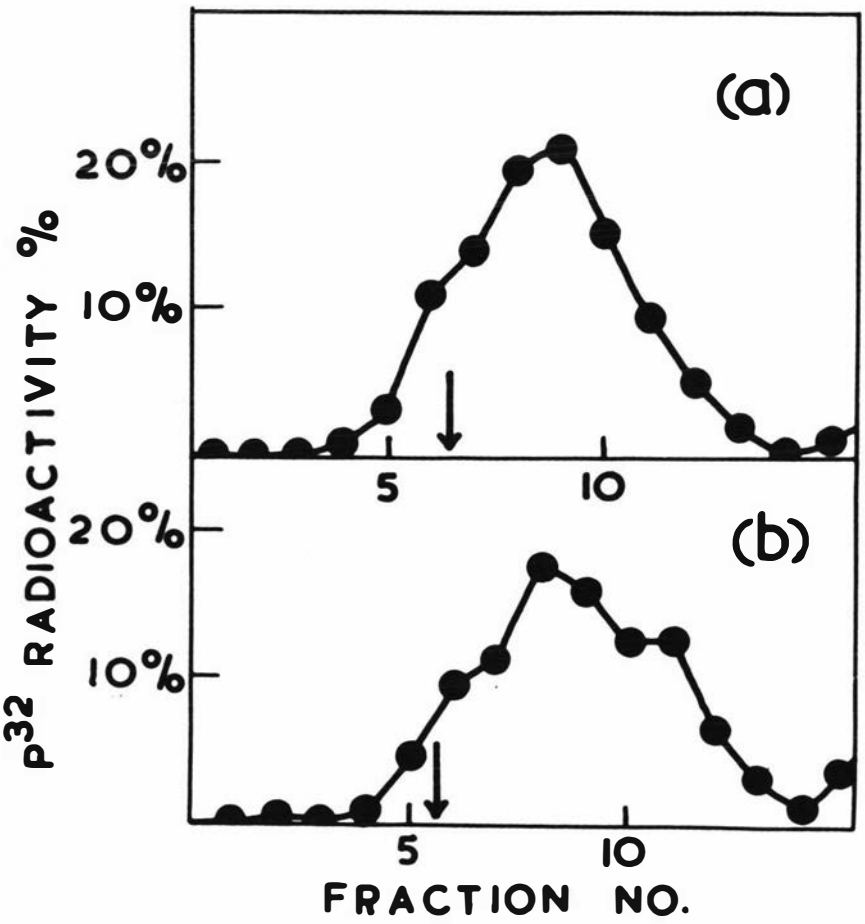


Figure 15. Zone Sedimentation of H and L Strands of Alpha DNA Prepared by Column Chromatography.

H and L strands of α DNA were prepared by MAK chromatography followed by self-annealing and hydroxylapatite chromatography as described in Methods. 2-3 μg. portions of (a), L strands; and (b), H strands; were layered onto alkaline sucrose gradients and sedimented for 4 h. at 35,000 r.p.m. 10-drop fractions were assayed for radioactivity by Cerenkov counting.

Arrow shows the position expected for a sedimenting zone of intact strands in each gradient.



typical separation is shown in figure 14. Alpha L strands prepared in this way contained no H strands detectable by CsCl gradient centrifuging in an analytical ultracentrifuge. A second cycle of self-annealing followed by hydroxylapatite chromatography gave less than 2% double-stranded DNA, showing that the concentration of complementary strands had been reduced to less than 1%.

This general strategy for the separation of the α DNA complementary strands was used in several preliminary experiments, but remained unsatisfactory in two respects:

(1) Preparation of H strands completely free from L strands required two successive cycles of self-annealing and hydroxylapatite chromatography and gave only low recoveries (5-10%).

(2) Both L strand and H strand preparations consisted mainly of half- and quarter-length fragments (figure 15). Strand breakage seemed to be a consequence of column chromatography of the high molecular weight separated strands. In view of the possibility, later confirmed, of intra-strand heterogeneity in base-composition, such preparations could not be assumed fully to represent the sequences found in the intact complementary strands of α DNA.

Density gradient centrifuging provided an obvious alternative strategy. The difference in buoyant density between the H and L strands in neutral CsCl, 0.008 g/ml.[89,90], does not allow an efficient separation by a single cycle of preparative ultracentrifuging. However, since the major difference in base composition between the two strands lies in the distribution of adenine and thymine (table 4.), a greatly enhanced buoyant density difference should be obtained by complexing

the strands with mercuric ions and centrifuging in neutral Cs_2SO_4. This procedure, developed by Davidson et al.[112], depends on the selective complexing of mercuric ions by thymine moities at pH 9. It was in fact found that under optimal conditions the buoyant density difference between the H and L strands of α DNA could be increased to at least 0.025 g/ml. (figure 16). However, the mercury-binding method was found to show an inconvenient susceptibility to interference by traces of EDTA and other anions capable of forming mercuric complexes and consequently was not further explored.

An increasingly widely-used method for fractionating denatured DNA depends on the ability to form specific complexes with a variety of synthetic polyribonucleotides. These complexes are stable in concentrated CsCl solutions, and can induce buoyant density differences between the complementary strands in a wide range of DNA molecules[25,26,27,28,29,30]. Sheldrick and Szybalski[26] have reported that the natural buoyant density differences between the complementary DNA strands found in several Bacillus phages, including phage α, can be enhanced by complexing the strands with polyriboguanylic acid (polyG) or the related co-polymer polyIG. PolyG-binding has been combined with CsCl gradient centrifuging in fixed angle rotors[167] for the separation of complementary strands from the DNA molecules of phages λ[27], T7[29] and SPP1[156]. A similar method using a different polyribonucleotide has been used to separate the complementary strands from the DNA molecules of phage T2[160].

In the present study, essentially the same method was used to separate the H and L strands of the DNA of phage α. The increased

Figure 16. Cs_2SO_4 Density Gradient Centrifuging of Mercuric Complexes of Denatured Alpha DNA.

2 µg. portions of α DNA were centrifuged to equilibrium in Cs_2SO_4 density gradients containing mercuric ions (Hg^{++}) at r_f = 0.3. (a) Native α DNA, initial density of Cs_2SO_4 (at 25^o) = 1.54 g/ml. (b) Heat-denatured α DNA, mean molecular weight 3.5×10^6, initial density = 1.59 g/ml. (c) Heat-denatured α DNA, 10-20% strand-intact, initial density = 1.59 g/ml. Ultraviolet photographs were scanned with a Joyce-Loebl recording microdensitometer.

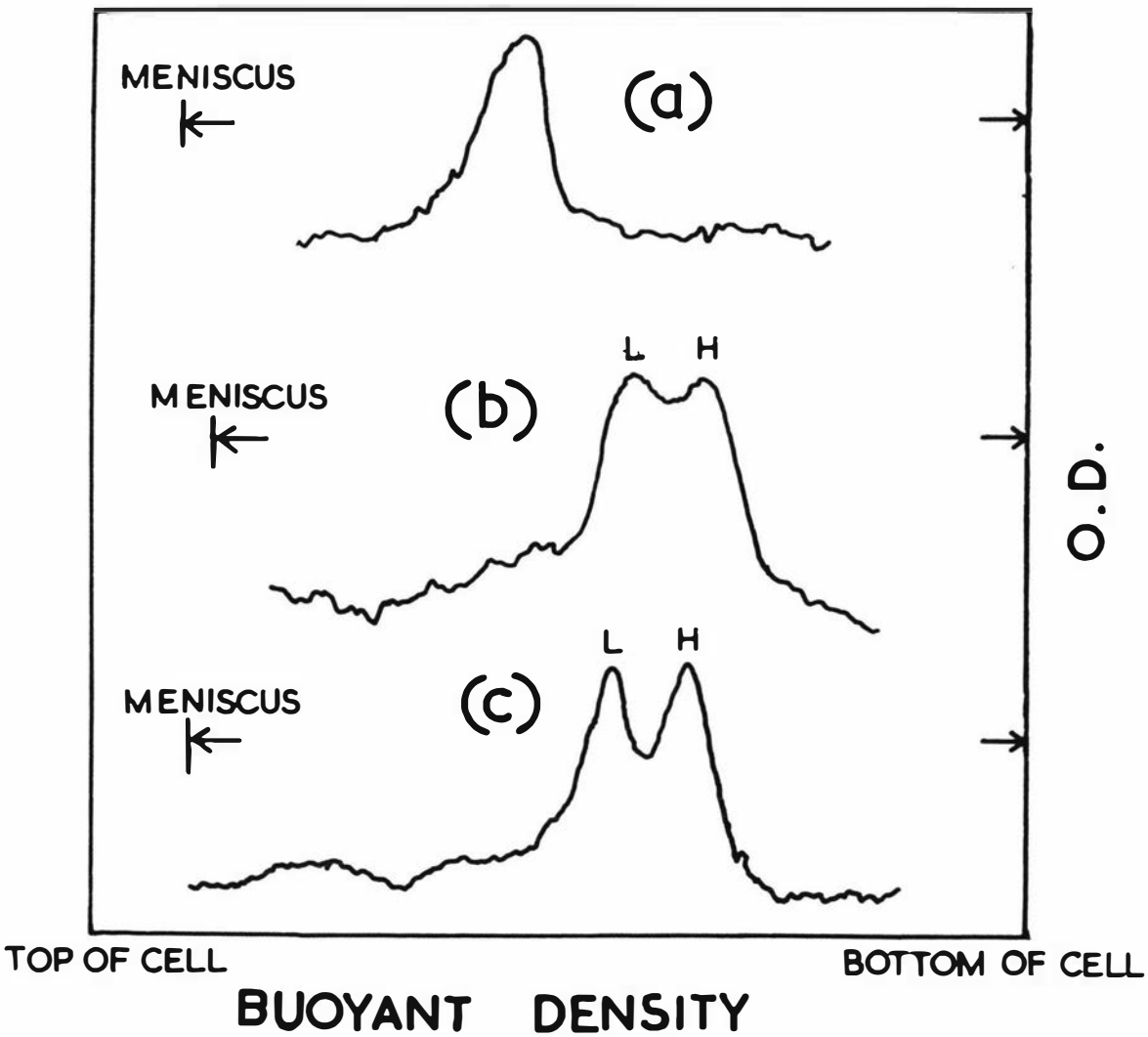
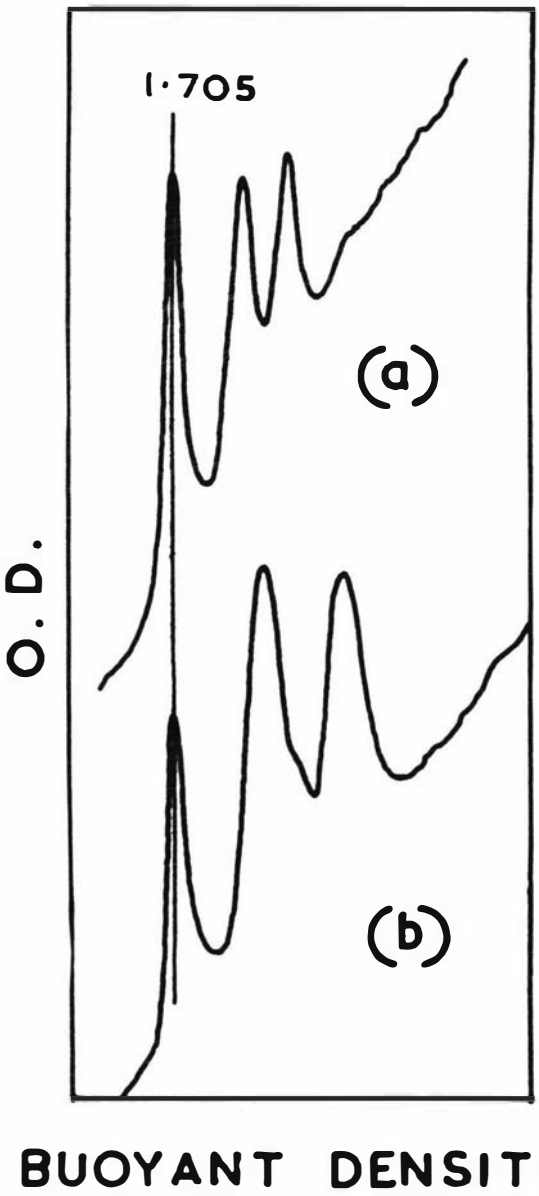


Figure 17. Effect of PolyG on the Buoyant Density of Denatured Alpha DNA.

1-4 μg. portions of denatured α DNA were centrifuged to equilibrium in CsCl density gradients. The density marker is native α DNA (ρ^{0} = 1.705 g/ml.). Ultraviolet photographs were scanned with a Joyce-Loebl recording microdensitometer. (a) No PolyG, (b) PolyG/DNA = 1/4.



density separation of strands resulting from polyG binding is shown in figure 17. When low DNA concentrations (less than 50 µg. DNA/ml.) were used during formation of the DNA-polyG complexes, the H and L strands were cleanly separated, with less than 5% cross-contamination. The use of higher DNA concentrations (up to 150 µg/ml.) resulted in the formation of a variable fraction of complexes containing both H and L strands, reducing the efficiency of the separation.

The separation obtained in preparative centrifugings is illustrated in figure 18(a). Radioactive strand preparations were assayed for complementary strands by re-centrifuging in the presence of added non-radioactive ø DNA and polyG, a procedure found to be more sensitive than analytical ultracentrifuging and less laborious than self-annealing followed by hydroxylapatite chromatography. One such assay is shown in figure 18(b). The accuracy of this method was established by an experiment (figure 18(c).) in which a 21% component of L strands was added to a preparation previously containing 95% H strands. Re-centrifuging gave estimates of 25.3% and 23.7% (two experiments) for the L strand content of the mixture.

When full precautions were taken to minimise shear damage during handling of the separated strands, 30-50% strand-intactness was maintained even after two cycles of CsCl gradient centrifuging (figure 19).

Figure 18. Separation and Assay of PolyG Complexes of the H and L strands of Alpha DNA by Preparative CsCl Gradient Centrifuging.

(a) 50 µg. of α DNA was complexed, by alkali-denaturation and re-neutralisation, with 12.5 µg. of PolyG. H and L strand polyG complexes were separated by centrifuging for 12 h. at 38,000 r.p.m. in a CsCl density gradient with a pre-formed concentration step. 5-drop fractions were assayed for ^{32}P radioactivity by Cerenkov counting.

(b) ^{32}P-labelled DNA pooled from the shaded L strand fractions of (a) was freed of CsCl by dialysis against 0.01 M tris/HCl pH 8.0, 0.001 M EDTA; and concentrated by dialysis against solid sucrose. A 20 µg. portion was combined with non-radioactive α DNA and polyG (polyG/DNA = 1/4), alkali-denatured and reneutralised, and centrifuged as in (a).

The H strand content was estimated from the ^{32}P radioactivity in fractions 5-9 as 3.8%.

(c) The assay method described in (b) was tested by adding a 21% (by radioactivity) component of ^{32}P-labelled L strands to a preparation of ^{32}P-labelled H strands whose purity had previously been estimated as 95%. (O-O), distribution of ^{32}P radioactivity; (▲–▲), estimated distribution of ^{32}P-labelled L strand. Result described in text.

Figure 18. Separation and Assay of PolyG Complexes of the H and L Strands of Alpha DNA by Preparative CsCl Gradient Centrifuging.

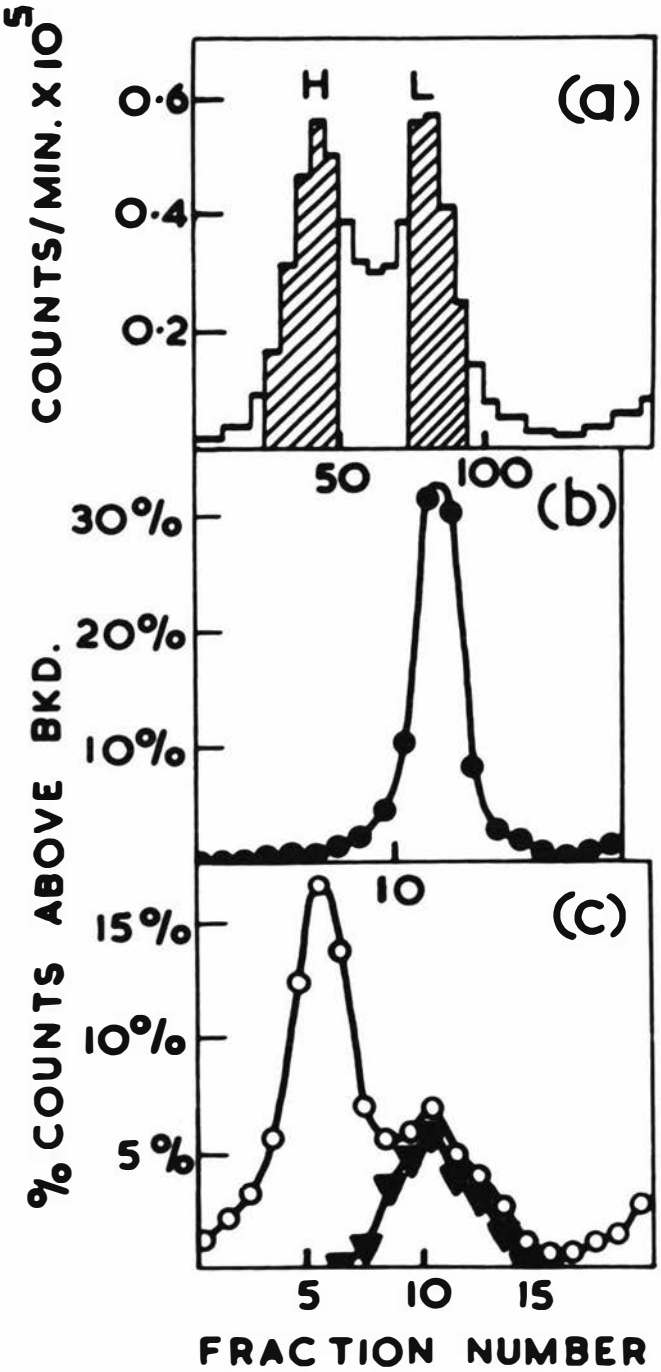
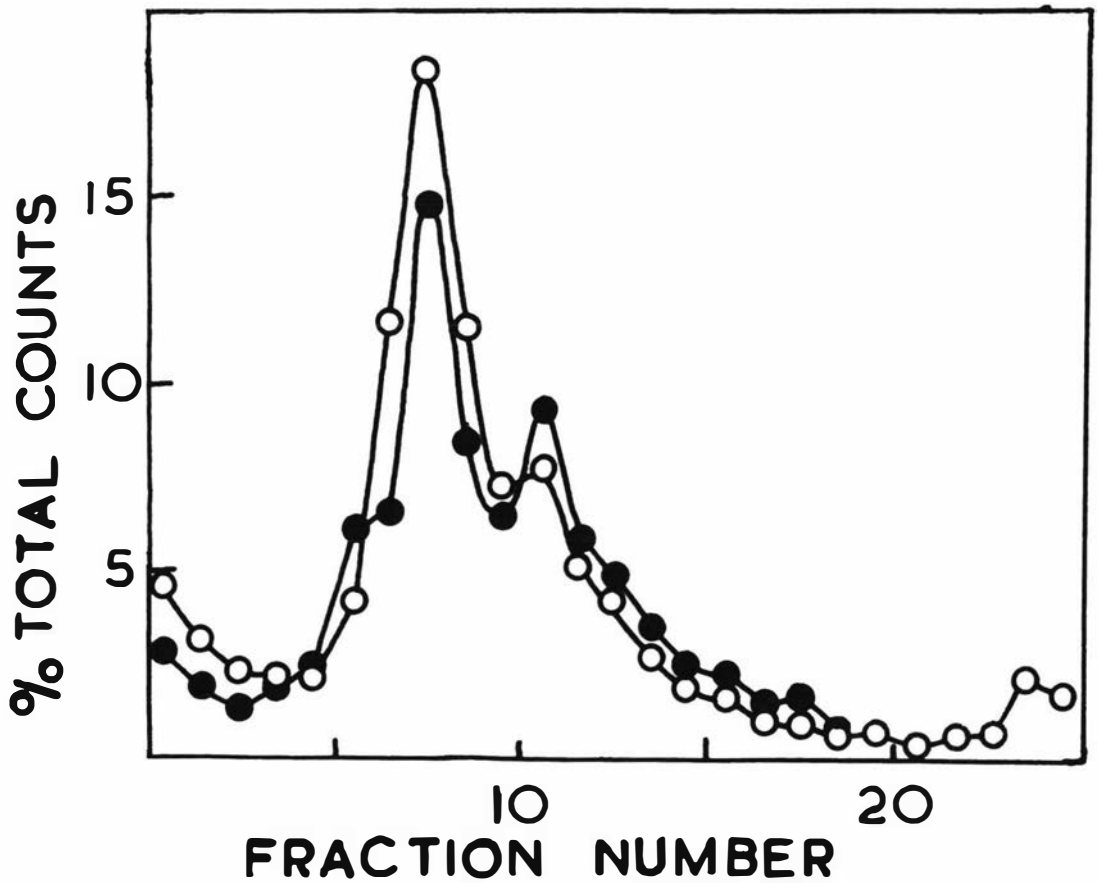


Figure 19. Zone Sedimentation of H and L Strands of Alpha DNA Prepared by CsCl Gradient Centrifuging.

^{33}P-labelled H strands and ^{32}P-labelled L strands of α DNA were prepared by two successive cycles of CsCl gradient centrifuging, as described in the legend of figure 25.

A mixture of the two species was dialysed against 0.01 M tris/HCl buffer pH 8.0 containing 0.001 M EDTA. A 1.0 µg. portion of this mixture was layered onto an alkaline sucrose gradient and sedimented for 3.5 h. at 38,000 r.p.m.; 5-drop fractions were assayed for ^{33}P and ^{32}P radioactivity by counting on paper in a toluene scintillator fluid. O—O ^{33}P, ●—● ^{32}P.



The Molecular Anatomy of Alpha DNA

The results of Cordes[91] suggested a rather homogeneous distribution of base composition within the α DNA molecule. No heterogeneity in buoyant density was found after shearing the native DNA to quarter-length fragments, and denaturation gave only the two bands, H and L, characteristic of the intact denatured DNA. Further shearing to eighths did not affect the buoyant density of the native DNA, which on denaturation gave a single broad band centred round a density intermediate between the heavy and light strand densities.

The results of Sheldrick et al.[26] also suggested that the differences between the two strands of α DNA might be fairly uniformly distributed along the length of the molecule. They concluded from a study of the Bacillus phages SP50, SP82, φμ-4 and α that in all these species only the H strands contained polyG-binding sites. Slight increases in the buoyant densities of the L strands at high polyG/DNA ratios were attributed to non-specific complexing. However, the strands of α DNA were found to be less separated at high polyG concentrations than the strands of the other Bacillus phages studied.

Observations made during the present work suggested a more complex molecular anatomy for α DNA. As shown in figure 20., the buoyant density of the L strand was found to be significantly increased in the presence of polyG. At suitably high polyG/DNA ratios, both L and H strands were carried completely outside the usual density range. Furthermore, when the polyG-DNA complexes were formed at high DNA concentrations (100-150 μg/ml.), a distinct third band was sometimes

Figure 20. Analytical CsCl Gradient Centrifuging of Denatured Alpha DNA - PolyG Complexes.

1-4 μg. portions of denatured α DNA (55% strand intact) were centrifuged to equilibrium in CsCl gradients. The density marker is native α DNA (ρ^0 = 1.705 g/ml.). Ultraviolet photographs were scanned with a Joyce-Loebl recording microdensitometer. (a) No polyG. (b) PolyG/DNA = 1/4, complexes formed at 20 μg. DNA/ml. (c) PolyG/DNA = 1/4, complexes formed at 120 μg. DNA/ml. (d) PolyG/DNA = 8/1.

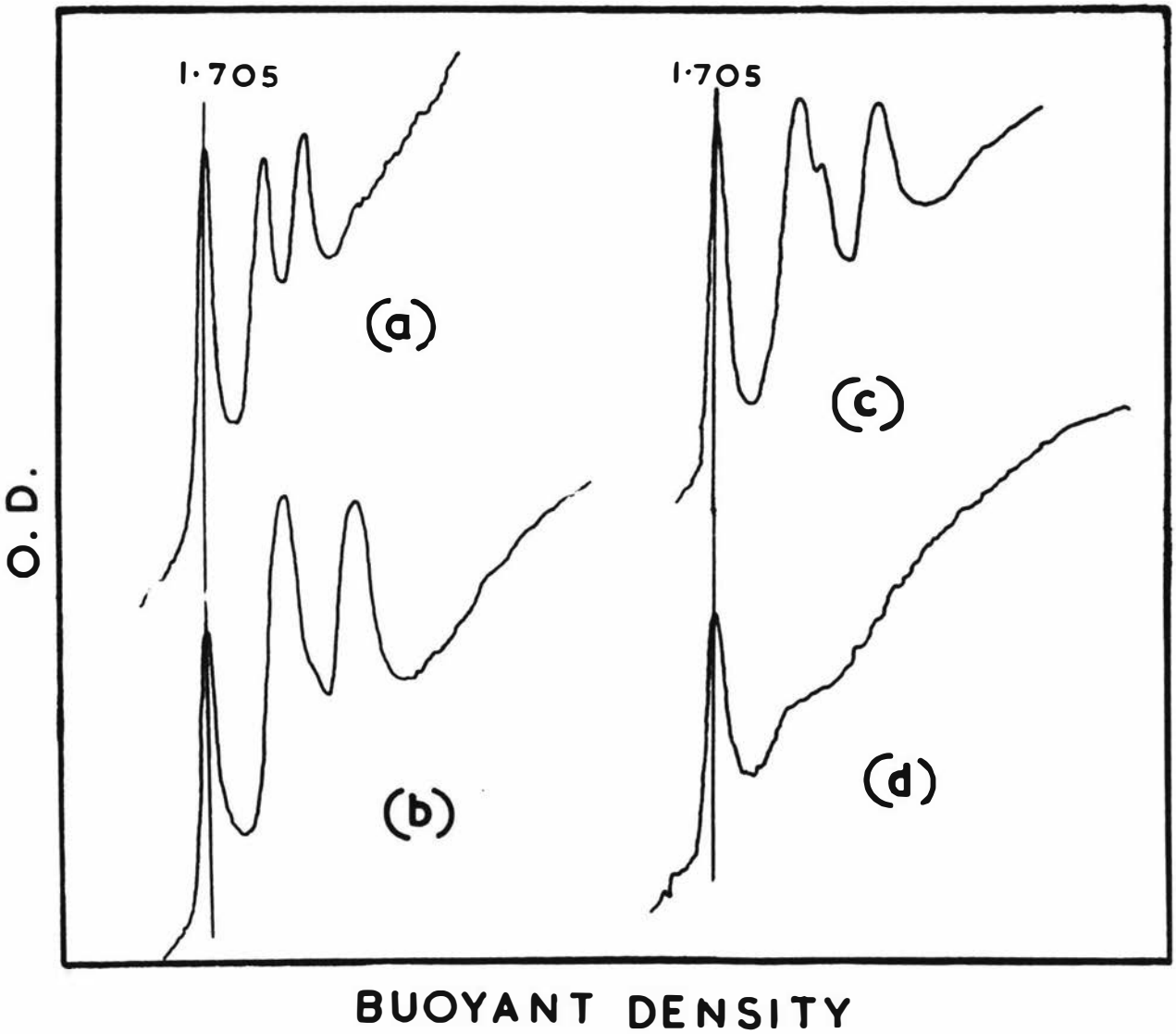
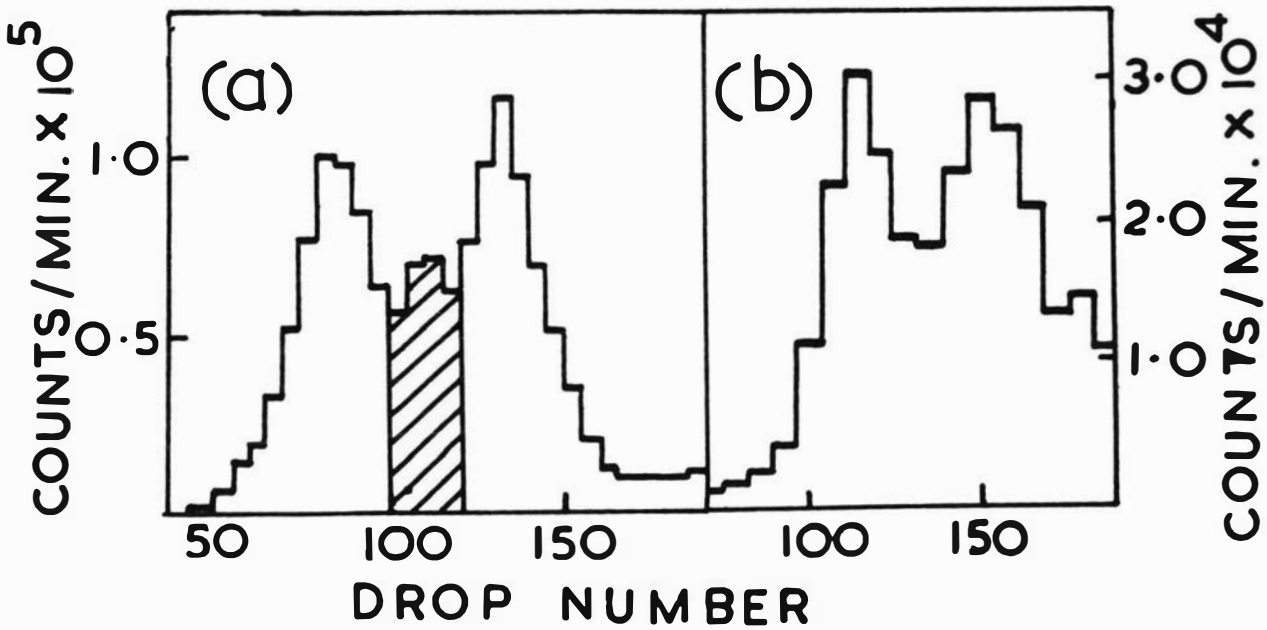


Figure 21. CsCl Gradient Centrifuging of the "Third Band" of Denatured Alpha DNA in the Presence of PolyG.

(a) ^{32}P-labelled α DNA was complexed with polyG (polyG/DNA = 1/4) at a DNA concentration of 120 μg/ml., and a portion centrifuged in a CsCl gradient (40 h. at 36,000 r.p.m.) to separate the two strands. 5-drop fractions were assayed for radioactivity by Cerenkov counting. (b) Central fractions from (a) were freed of CsCl by dialysis, re-complexed with polyG and non-radioactive α DNA at a DNA concentration of 30 μg/ml., and centrifuged in a CsCl



observed at a density intermediate between the H and L strands, suggesting the possibility of complexes containing both strands held together by polyG.

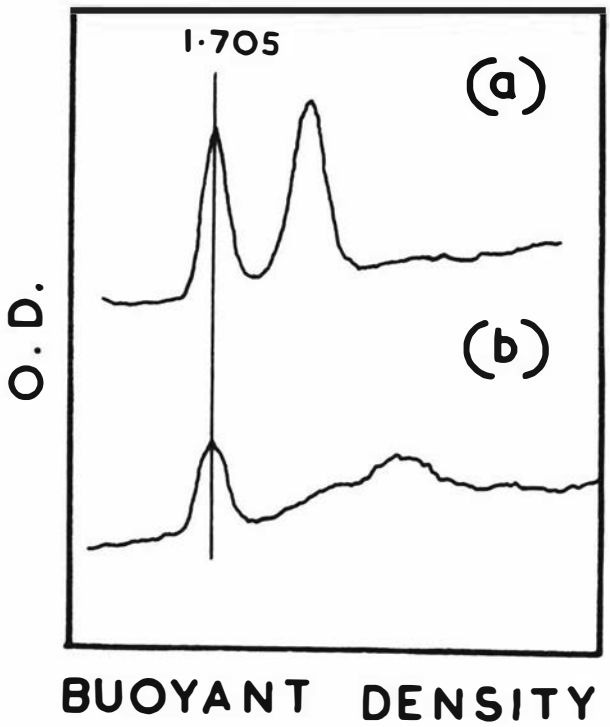
In support of this interpretation, when this middle band was isolated from a preparative CsCl gradient and re-centrifuged after dissociation and re-formation of the DNA-polyG complexes at a lower DNA concentration, two bands were obtained (figure 21).

These binding experiments were carried out under conditions in which the H and L strands cannot re-associate to form the double-stranded molecule. It was however necessary to demonstrate that the interaction of the L strands with polyG did not require the presence of H strands. In the experiment shown in figure 22 it was found that purified L strands, prepared in this case by MAK chromatography, mostly retained their polyG-binding capacity.

A later experiment suggested that the polyG binding sites might not be uniformly distributed through the L strand. ^{32}P-labelled L strands, freed of complementary strands by self-annealing followed by hydroxylapatite chromatography, were re-centrifuged in a caesium chloride density gradient with polyG and non-radioactive intact H and L strand markers. It was found that the ^{32}P-labelled L strands had lost ability to bind polyG (figure 23(a).), simultaneously giving rise to a small shoulder of heavy DNA. This heavy shoulder was also detected by re-centrifuging in the absence of polyG (figure 23(c).). ^{32}P-labelled H strand preparations, also freed of complementary strands by self-annealing followed by hydroxylapatite chromatography, were found to give rise on re-centrifuging to a shoulder of light DNA (figure 23(b).).

Figure 22. CsCl Gradient Centrifuging of the Isolated L Strands of Alpha DNA in the presence of PolyG.

L strands of α DNA, prepared by MAK column chromatography, were centrifuged to equilibrium in CsCl gradients. (a) in the absence of polyG. (b) after complexing with polyG (polyG/DNA = 1/4). Ultraviolet photographs were scanned with an Analytrol densitometer.



These unexpected results were taken to suggest that the purine-rich L strand might contain a small segment, perhaps 5-10,000 nucleotide residues, of pyrimidine-rich DNA. If this heavy segment were responsible for polyG binding by the L strand, then fragmentation of the L strands on storage at -20° and during hydroxylapatite chromatography might be expected to give rise to pieces of L strand containing no polyG-binding sites.

This interpretation was supported by an experiment in which ^{32}P-labelled whole α DNA was sheared to quarter-length fragments by repeated passages through a syringe needle, then denatured with intact non-radioactive α DNA and polyG and centrifuged in a CsCl gradient. It was found that most of the sheared L strand formed a sharp band at the density expected for intact L strands in the absence of polyG (figure 24). Most of the sheared H strand retained the heavy density, showing that polyG-binding sites are fairly evenly distributed in this strand.

The most convincing demonstration of a heavy segment in the L strand involved a double-label experiment in which purified ^{33}P-labelled H strands were mixed with purified ^{32}P-labelled L strands. This mixture was shown by alkaline sucrose gradient centrifuging (figure 19) to contain predominantly intact strands. In a CsCl density gradient the polyG complexes of the two strands gave sharp clearly-separated peaks (figure 2 (a).), although an incipient shoulder was found on the heavy side of the L strand peak. A mixture of the same strand preparations sheared to quarter-length fragments, centrifuged in a parallel CsCl gradient, showed a clear shoulder of light

Figure 23. CsCl gradient centrifuging of Purified ^{32}P-labelled H and L Strand Preparations of Alpha DNA.

^{32}P-labelled H strands and L strands of α DNA were prepared by CsCl gradient centrifuging of the polyG complexes, followed by self-annealing in 30% (v/v) formamide and hydroxylapatite chromatography, and stored at -20°. 0.2 μg portions were combined with 100 μg. portions of non-radioactive α DNA (70% strand intact), and alkali-denatured in the presence or absence of polyG prior to re-centrifuging.

5-drop fractions were assayed for radioactivity by Cerenkov counting, then diluted with 0.4 ml. portions of water and assayed for non-radioactive DNA by ultraviolet absorption measurements at 260 mμ.

(a) ^{32}P-labelled L strands, polyG/DNA = 1/4.

(b) ^{32}P-labelled H strands, no polyG.

(c) ^{32}P-labelled L strands, no polyG.

Figure 23. CsCl Gradient Centrifuging of Purified ^{32}P-labelled H and L Strand Preparations of Alpha DNA. (O–O), 260 mμ absorbance; (●–●), ^{32}P radioactivity.

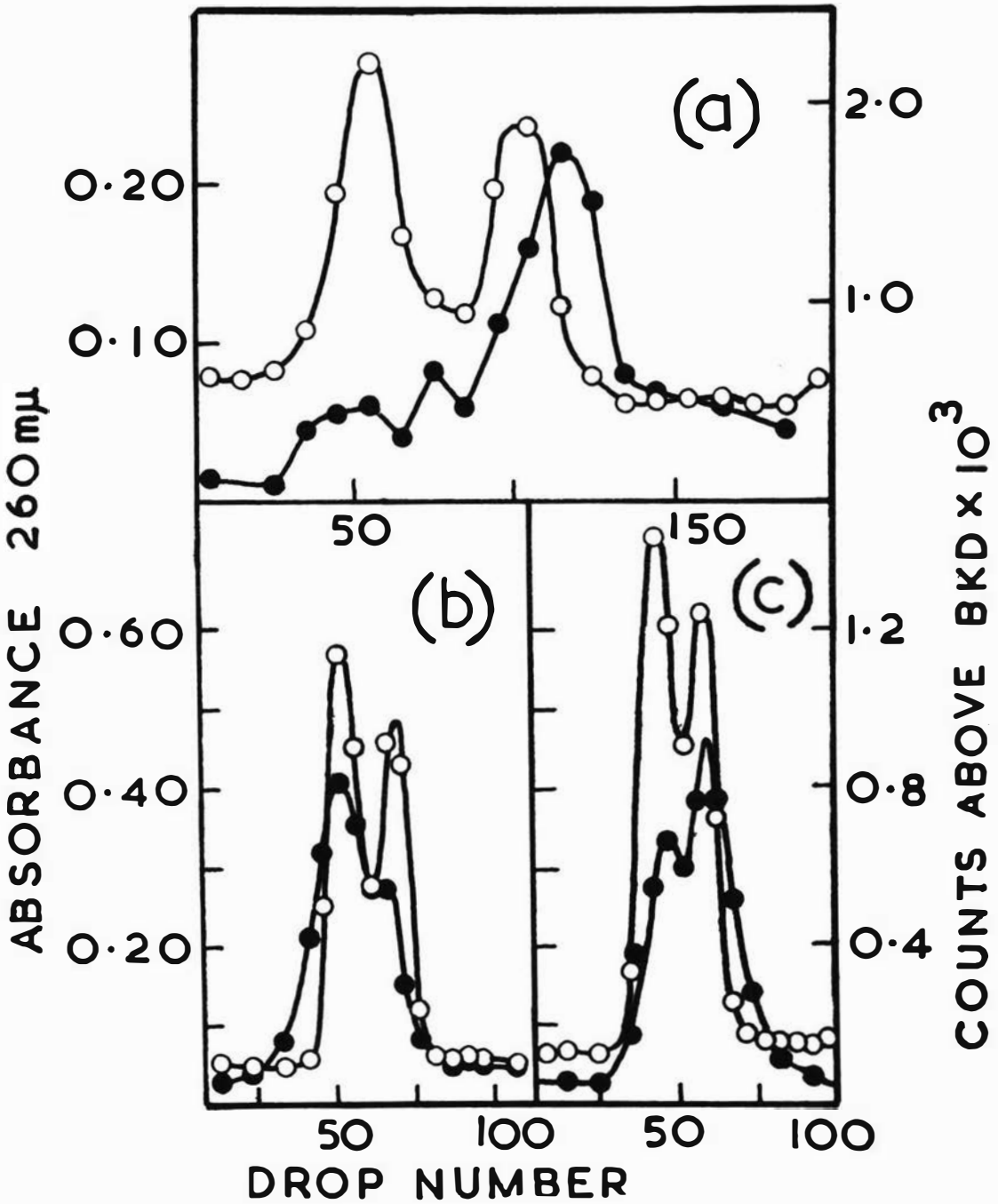


Figure 24. CsCl Gradient Centrifuging of Sheared and Unsheared Denatured Alpha DNA in the Presence of PolyG.

^{32}P-labelled α DNA (200 μg/ml.) in 0.1 M NaOH was sheared to approximately quarter-length fragments by ten passages through a No. 22 syringe needle. 4 μg. of this preparation was combined with 100 μg of non-radioactive α DNA (75% strand-intact), denatured and re-neutralised in the presence of polyG (polyG/DNA = 1/4), and centrifuged in a CsCl gradient (45 h. at 36,000 r.p.m.); 5-drop fractions were assayed for radioactivity and ultraviolet absorption as described for the previous figure.

○—○ 260 mμ absorbance, ●—● ^{32}P radioactivity.

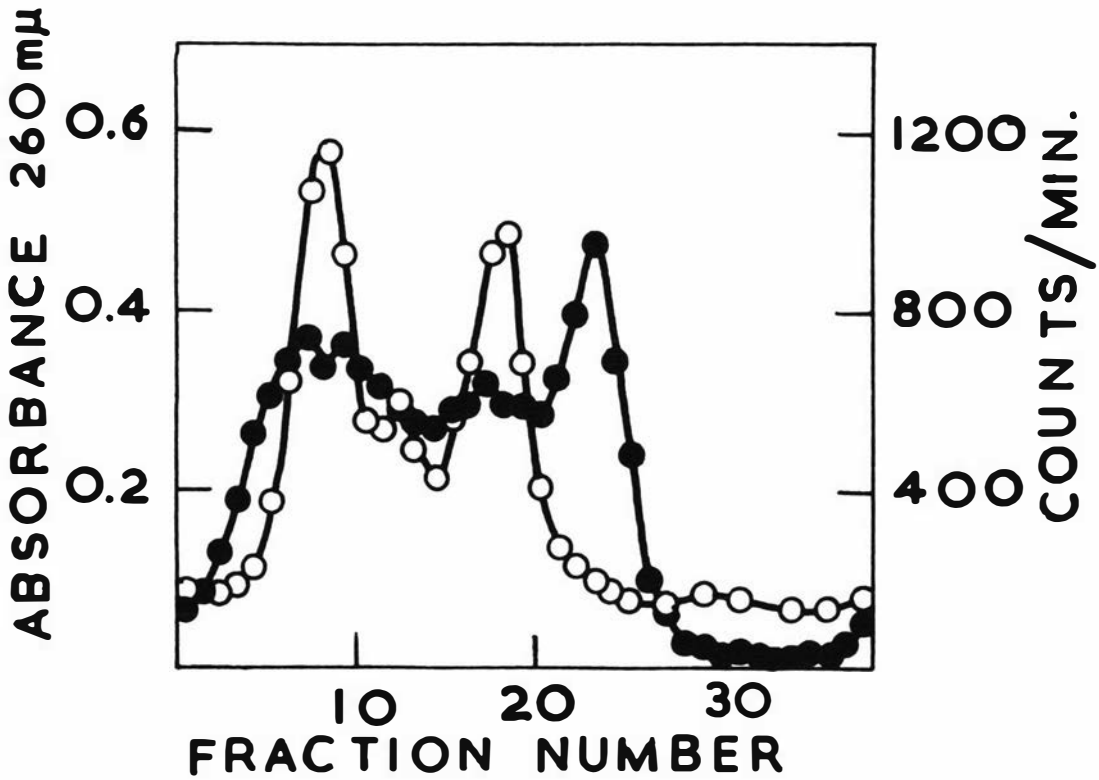


Figure 25. CsCl Gradient Centrifuging of Sheared and Unsheared Isolated H and L Strands of Alpha DNA in the Presence of PolyG.

Intact H strands were prepared from ^{32}P-labelled α DNA by CsCl gradient centrifuging of the polyG complexes. After concentrating the preparation by dialysis against solid sucrose, contaminating L strands were removed by a second cycle of CsCl gradient centrifuging.

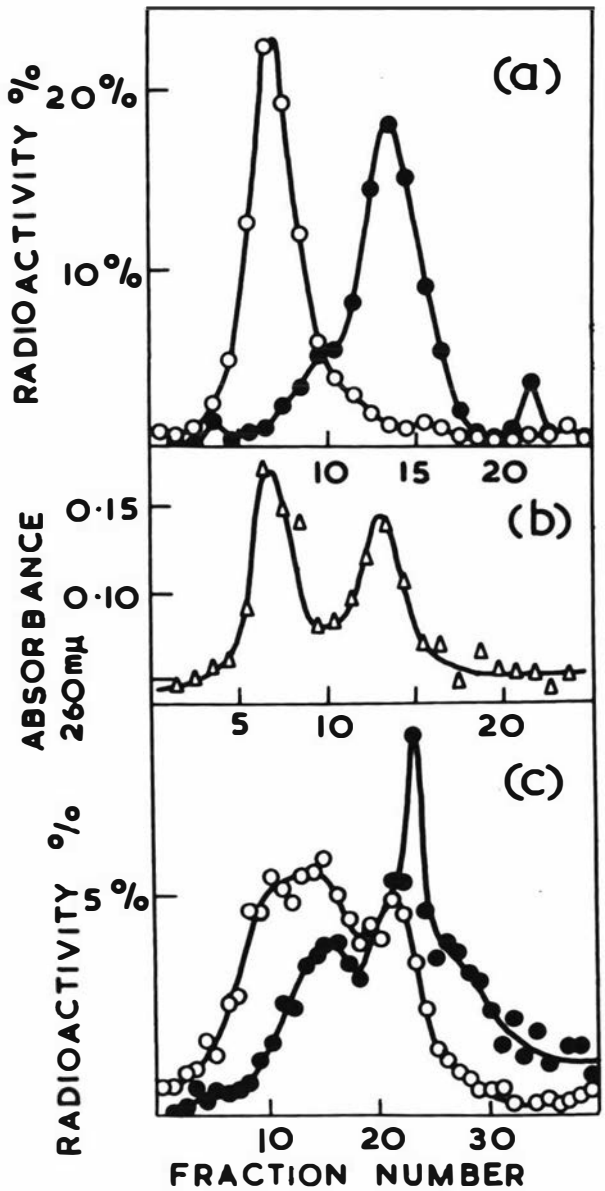
Intact L strands were prepared from ^{32}P-labelled α DNA by a similar procedure.

A mixture of these two strand preparations was combined with 50 µg. of non-radioactive α DNA (50% strand-intact) and denatured and re-neutralised in the presence of 15 µg. of polyG. This mixture was centrifuged for 12 h. at 36,000 r.p.m. in a CsCl gradient with a preformed concentration step. 7-drop fractions were made up to 0.5 ml. with water and assayed for (a) ^{32}P and ^{33}P radioactivity, by counting in toluene/triton X-100 scintillator fluid, and (b) Ultraviolet absorption at 260 mµ.

(c) An identical experiment after the labelled strand mixture had been sheared to approximately quarter-length fragments by ten passages through a No. 22 syringe needle.

Figure 25. CsCl Gradient Centrifuging of Sheared and Unsheared Isolated H and L Strands of Alpha DNA in the Presence of PolyG.

(●—●), % of total ^{32}P counts above background; (○—○), % of total ^{33}P counts above background; (Δ—Δ), 260 mμ absorbance in gradient (a); 7-drop fractions.



DNA formed from the H strand and a clear shoulder of heavy DNA formed from the L strand (figure 25(c)).

The formation of a shoulder of heavy DNA from purified intact L strands after shearing was also confirmed by analytical CsCl gradient centrifuging in the absence of polyG.

Pyrimidine Nucleotide Sequences in Alpha DNA

The logical first step in any attempt to correlate sequence studies with the molecular structure of a DNA molecule is to measure the frequency with which various separable groups of sequences occur in the intact DNA.

In the present study, sequences of consecutive pyrimidine nucleotides were released from α DNA by prolonged incubation at 30° with 66% (v/v) formic acid containing 2% (w/v) diphenylamine[42]. Diphenylamine and formic acid were removed by ether extraction[42], and the complex mixtures of oligonucleotides and inorganic phosphate were resolved by ion-exchange chromatography on columns of DEAE-cellulose[118].

A typical separation of the oligonucleotide chain length groups (isopliths) found in a diphenylamine digest of ^{32}P-labelled α DNA is shown in figure 26. Excellent separations of inorganic phosphate and isopliths of chain length 1-6 were routine. Rechromatography of each isoplith group to separate the oligonucleotide base composition groups (isomers) is illustrated by typical examples in figures 27, 28 and 29.

The distribution of isopliths was measured in several independent diphenylamine digests of ^{32}P-labelled α DNA. The results are presented in table 5. The variation between replicate digests was greater than the likely errors in ^{32}P counting and volume measurements, but the source of this variation was not established during the present work.

The distribution of isomers in each isoplith group was measured in at least two digests. The results are assembled in table 6.

Figure 26. Separation of Isopliths by Ion-exchange Chromatography.

Pyrimidine nucleotide sequences of increasing chain length from a depurinated diphenylamine digest of calf thymus DNA were resolved by ion-exchange chromatography on DE-32 resin, as described in methods. Flow-rate 15 ml/h., 10-ml. fractions. (– – – – –), NaCl concentration; (———), 269 mμ absorbance.

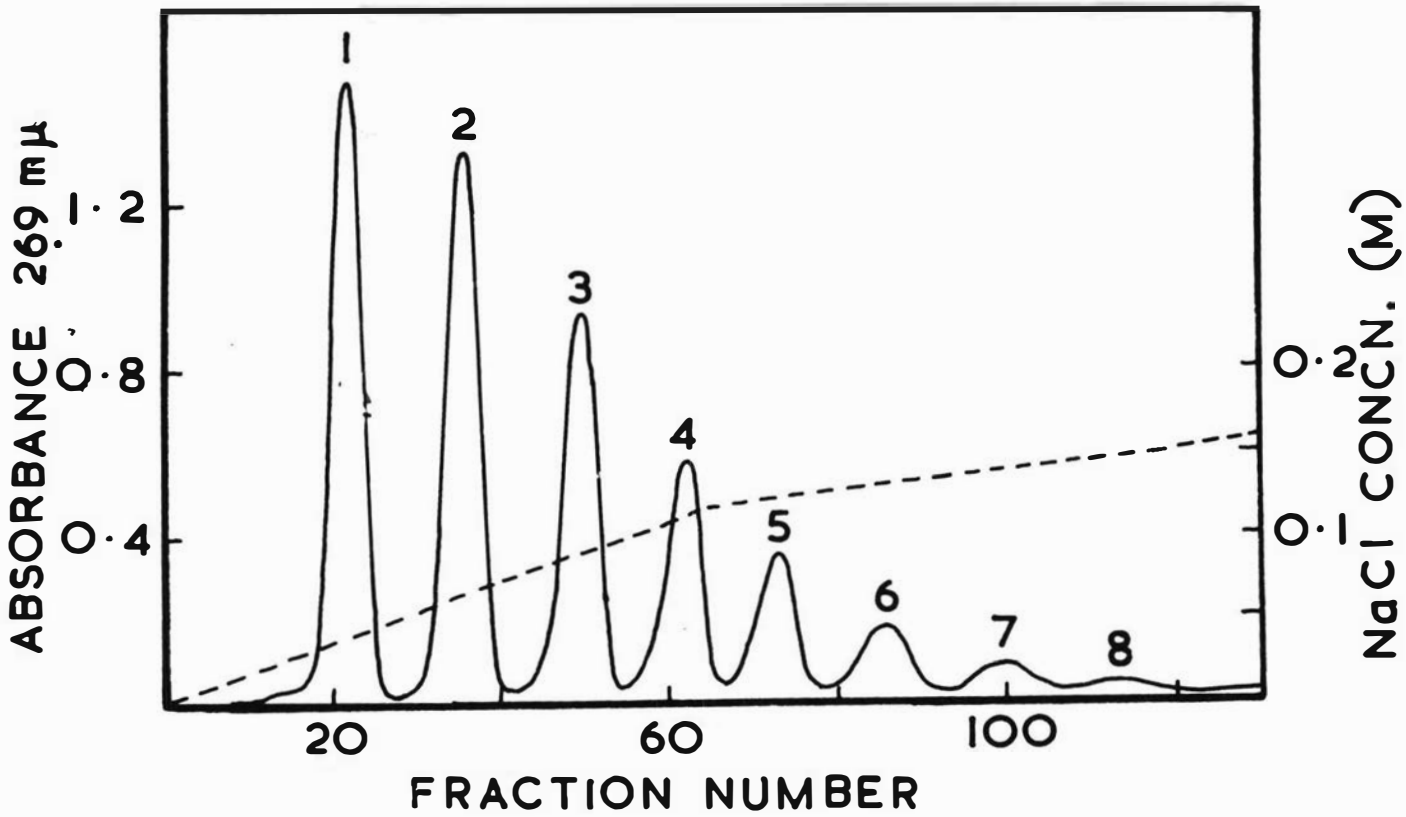


Figure 27. Separation of Isomers of Chain Length 1-2 by Ion-exchange Chromatography.

Isopliths of lengths (a) 1, and (b) 2, were desalted by brief dialysis against distilled water, and resolved into isomers by ion-exchange chromatography on 10 cm. x 1 cm. diameter columns of DEAE-cellulose (Cellex-D). (– – – –), NaCl concentration; (——), 254 mμ absorbance (ISCO scanner tracing); 5-ml. fractions.

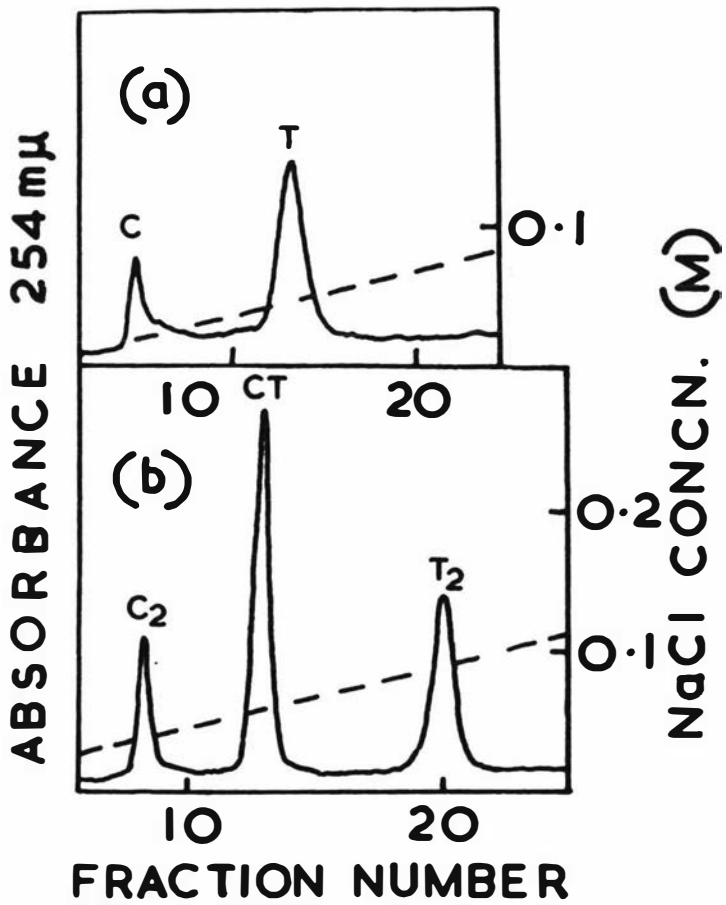


Figure 28. Separation of Isomers of Chain Length 3-4 by Ion-exchange Chromatography.

^{32}P-labelled isopliths of lengths (a) 3, and (b) 4, from a diphenylamine digest of phage α DNA, were desalted by overnight dialysis against 0.1 M triethylammonium bicarbonate buffer pH 8.5, and resolved into isomers by ion-exchange chromatography on 8 cm. x 1 cm. diameter columns of DEAE-cellulose; 7-ml. fractions were assayed for ^{32}P radioactivity by Cerenkov counting. (— — —), NaCl concentration; (——), ^{32}P radioactivity.

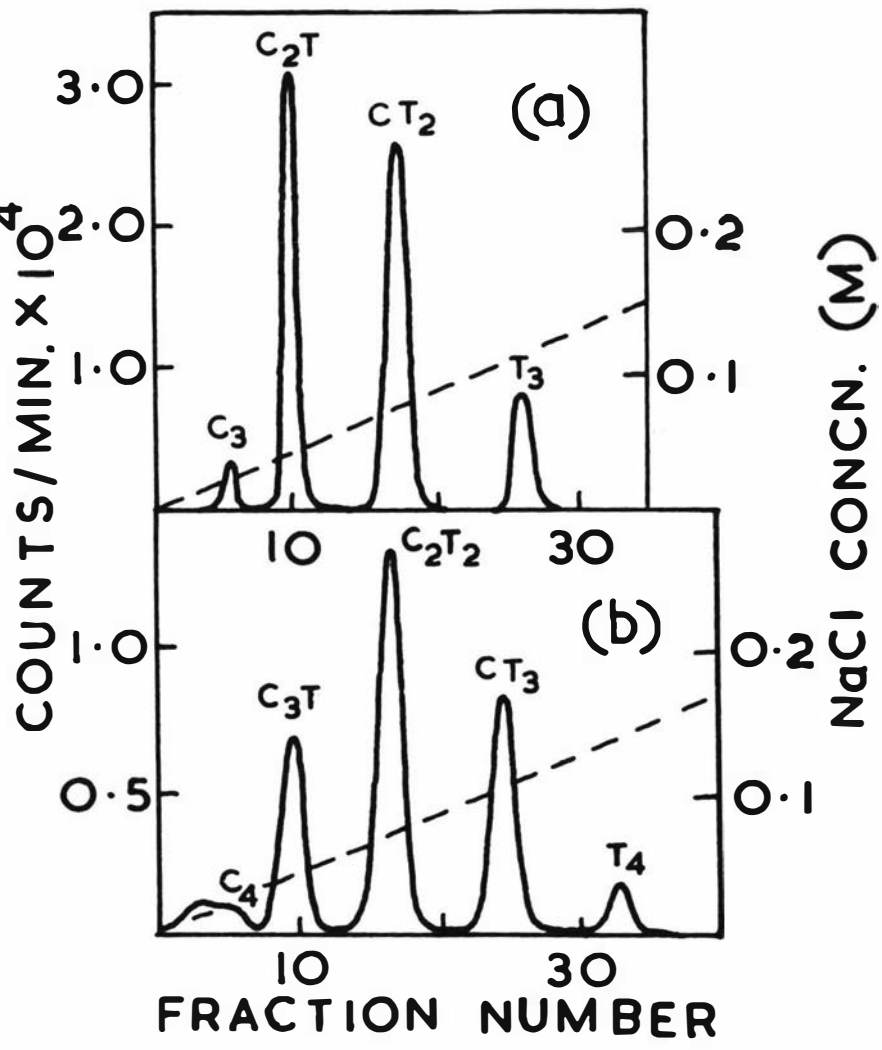


Figure 29. Separation of Isomers of Chain Length 5-6 by Ion-exchange Chromatography.

Isopliths of lengths (a) 5, and (b) 6, were desalted on short columns of DEAE-sephadex as described in Methods, and briefly dialysed against distilled water. Each sample was resolved into isomers by ion-exchange chromatography on an 18 cm. x 1 cm. diameter column of DEAE-cellulose (Cellex-D). (– – – –), NaCl concentration; (——), 270 mμ absorbance; 7-ml. fractions.

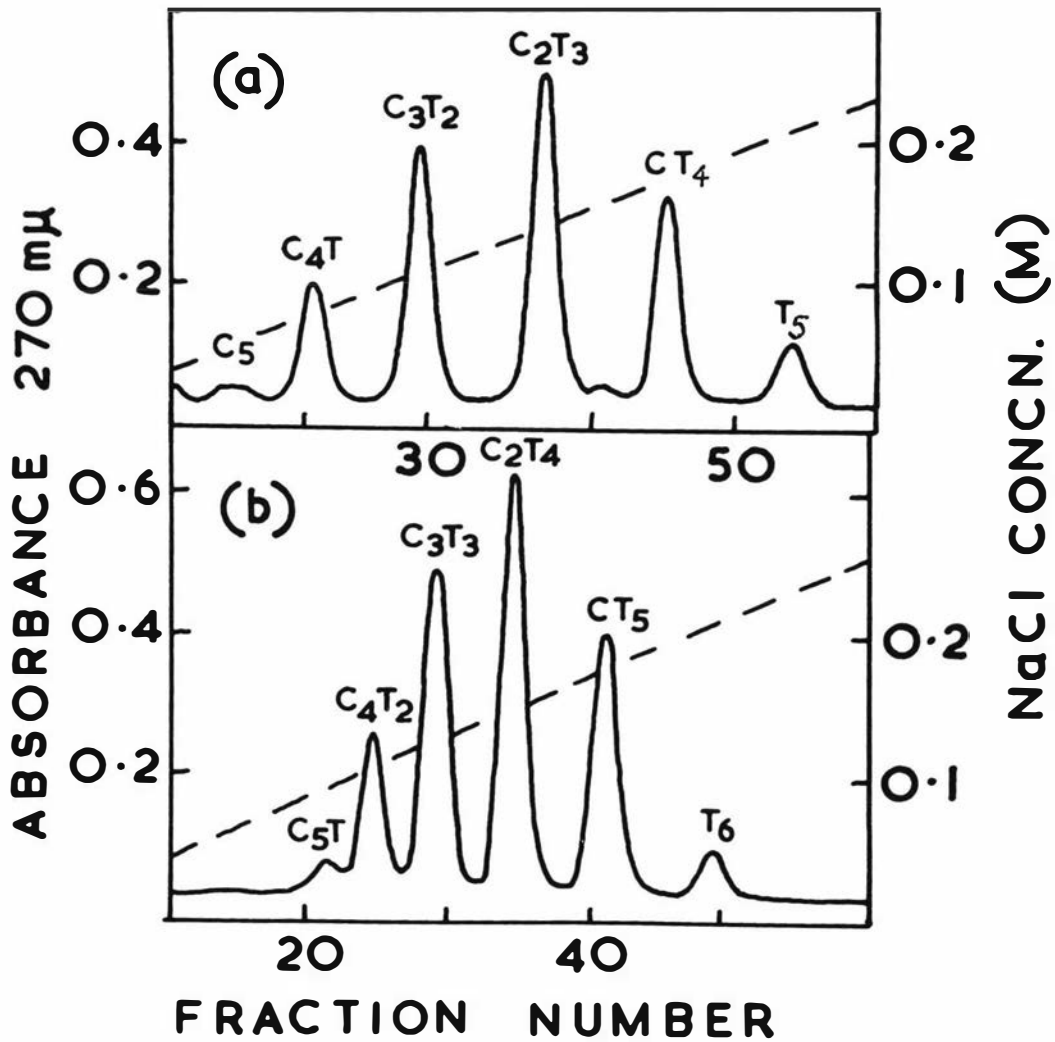


Table 5. Percentage Sequence Frequencies of Inorganic Phosphate and Isopliths of Chain Length 1-6 in Diphenylamine Digests of Alpha DNA.

Ether-extracted diphenylamine digests of ^{32}P-labelled a DNA were resolved on the basis of chain length by ion-exchange chromatography. ^{32}P content was estimated by counting in toluene/triton X-100 scintillator fluid (digests A-D) or by Cerenkov counting (digest E). Total recoveries of applied radioactivity were within the range of 97-100%.

Isoplith Group	Percentage Sequence Frequency						
digest	A	B	C	D	E	mean ± S.E.	Random
Inorganic Phosphate	27.2	27.2	27.5	26.5	27.5	27.2 ± 0.4	25.0
Chain Length 1	11.2	10.9	11.4	11.4	11.2	11.2 ± 0.2	12.5
2	5.77	5.57	5.87	5.95	5.77	5.79 ± 0.14	6.25
3	2.79	2.74	2.88	2.91	2.95	2.85 ± 0.09	3.13
4	1.39	1.27	1.35	1.47	1.30	1.36 ± 0.08	1.56
5	-	0.675	0.700	0.802	0.783	0.740 ± 0.062	0.781
6	-	0.490	0.432	0.474	0.498	0.474 ± 0.029	0.391
7	-	0.352	0.298	0.290	0.334	0.319 ± 0.029	0.195

Table 6. Percentage Distribution of the Isomers of Chain Length Groups 1-6 in Diphenylamine Digests of Alpha DNA.

Desalted isopliith groups were resolved on the basis of base composition by ion-exchange chromatography (digests A-D) or paper chromatography (digest F). ^{32}P content was estimated by counting in toluene/triton X-100 scintillator fluid (digests A-D) or by Cerenkov counting (digest F).

Isomer Group	Percentage Distribution					
	digest A	B	C	D	F	mean ± S.E.
Cp_2	-	39.2	38.9	-	-	39.1 ± 0.2
Tp_2	-	60.8	61.1	-	-	60.9 ± 0.2
C_2p_3	16.6	14.3	14.8	-	14.1*	14.9 ± 1.1
CTp_3	52.7	53.9	54.3	-	54.9*	54.0 ± 0.9
T_2p_3	30.8	31.8	30.9	-	30.9*	31.1 ± 0.5
C_3p_4	4.2	-	-	4.5	4.6*	4.4 ± 0.2
C_2Tp_4	38.8	-	-	37.8	37.0*	37.9 ± 0.9
CT_2p_4	42.6	-	-	42.6	43.0*	42.7 ± 0.2
T_3p_4	14.3	-	-	15.0	15.9*	15.1 ± 0.8
C_4p_5	2.0	-	-	-		(2.0)
C_3Tp_5	20.3	-	-	22.2		21.3 ± 1.3
$C_2T_2p_5$	42.7	-	-	40.0		41.4 ± 1.9
CT_3p_5	28.5	-	-	24.8		26.6 ± 2.5
T_4p_5	6.5	-	-	6.1		6.3 ± 0.3

Isomer Group	Percentage Distribution			
	digest A	B + C	D	mean ± S.E.
C_5p_6	-	(0.9)	0.8	0.8 ± 0.1
C_4Tp_6	11.0	10.5	8.7	10.1 ± 1.0
$C_3T_2p_6$	30.8	30.4	30.0	30.4 ± 0.4
$C_2T_3p_6$	36.9	38.4	38.1	37.8 ± 0.8
CT_4p_6	18.0	17.8	19.6	18.5 ± 1.0
T_5p_6	2.5	2.0	2.6	2.4 ± 0.3
C_6p_7	-	-	(0.2)	(0.2)
C_5Tp_7	-	4.2	3.8	4.0 ± 0.3
$C_4T_2p_7$	-	21.9	21.2	21.6 ± 0.5
$C_3T_3p_7$	-	35.5	35.3	35.4 ± 0.1
$C_2T_4p_7$	-	26.2	27.1	26.7 ± 0.6
CT_5p_7	-	11.6	11.8	11.7 ± 0.1
T_6p_7	-	(0.7)	0.6	0.6 ± 0.1

* measured by paper chromatography of digest F after treatment with prostatic phosphomonoesterase.

Table 7. Percentage Sequence Frequencies of the Pyrimidine Nucleotide Sequences of Length 1-6 in Diphenylamine Digests of Alpha DNA, as Compared With a Random Distribution.

These results have been collated from tables 5 and 6, assuming the standard errors to be cumulative. The ratio of the sequence isomers CpT and TpC was deduced from paper chromatography of digest F.

Isomer Group		Percentage Sequence Frequency			
		mean ± S.E.		random	
Cp_2		4.38 ± .10		5.38	
Tp_2		6.82 ± .14		7.13	
C_2p_3		0.86 ± .09		1.16	
CTp_3	CpT	3.13 ± .13	1.77	3.06	1.53
	TpC		1.43		1.53
T_2p_3		1.80 ± .07		2.03	
C_3p_4		0.13 ± .01		0.25	
C_2Tp_4		1.08 ± .06		0.99	
CT_2p_4		1.22 ± .04		1.31	
T_3p_4		0.43 ± .04		0.58	
C_4p_5		(0.027)		0.053	
C_3Tp_5		0.290 ± .030		0.283	
$C_2T_2p_5$		0.563 ± .065		0.562	
CT_3p_5		0.362 ± .054		0.497	
T_4p_5		0.086 ± .009		0.165	

Isomer Group	Percentage Sequence Frequency	
	mean ± S.E.	random
C_5p_6	(0.006 ± .001)	0.011
C_4Tp_6	0.075 ± .013	0.076
$C_3T_2p_6$	0.225 ± .020	0.202
$C_2T_3p_6$	0.280 ± .028	0.268
CT_4p_6	0.137 ± .018	0.177
T_5p_6	0.018 ± .004	0.047
C_6p_7	(0.0009)	0.0025
C_5Tp_7	0.019 ± .003	0.020
$C_4T_2p_7$	0.102 ± .008	0.065
$C_3T_3p_7$	0.168 ± .013	0.115
$C_2T_4p_7$	0.127 ± .009	0.114
CT_5p_7	0.056 ± .004	0.061
T_6p_7	(0.003 ± .001)	0.013

Frequencies in brackets are based on low counts or uncertain separations.

The mean frequencies for all 27 pyrimidine nucleotide sequences of length 1-6 found in diphenylamine digests of α DNA are collated in table 7, together with the frequencies expected for a DNA with a 43% G+C content but with a randomly arranged sequence of nucleotides.

Complete resolution of isopliths of chain length greater than six was found more difficult than had been anticipated. Petersen and Reeves[118] reported good separations of isopliths of chain length up to eighteen from diphenylamine digests of Calf thymus DNA, and a similar result was obtained early in the present work. However, these separations made use of a single batch of DEAE-cellulose with a particularly high resolving power. Several other batches of the same product were later found to be unsuitable.

It has been possible to completely resolve the nine oligonucleotides of length greater than 8 from diphenylamine digests of phage f1 DNA using Whatman DE-32, a more uniform grade of DEAE-cellulose (W.P. Tate, personal communication). However, complete resolution of the more complex mixture of long oligonucleotides found in diphenylamine digests of phage α DNA was not found to be as readily achieved.

The best separation of long α isopliths obtained in the present study is shown in figure 30. Carrier isopliths of lengths up to 16 were clearly resolved. Peaks of radioactivity were detected for lengths up to 13. The isoplith groups of length 8-13 were desalted by dialysing against distilled water, and resolved into groups of isomers by further column chromatography as shown in figures 31-36.

The distributions of radioactivity shown in figures 30, 35 and 36 suggest that the α DNA molecule contains only a few sequences of

Figure 30. Separation of the Longest Isopliths From a Diphenylamine Digest of Phage Alpha DNA.

An ether-extracted diphenylamine digest of 2.8 mg. of ^{32}P-labelled α DNA was combined with a dialysed, de-purinated diphenylamine digest representing 200 mg. of calf thymus DNA, and resolved into isopliths by ion-exchange chromatography on DEAE-cellulose (Cellex-D). 6.5-ml. fractions were assayed for carrier isopliths by ultraviolet absorption measurements at 271 mμ, and for ^{32}P radioactivity by counting portions in a toluene/Cab-O-Sil scintillator mixture.

Figure 30. Separation of the Longest Isopliths From a Diphenylamine Digest of Phage Alpha DNA.

(a) The separation of carrier isopliths of lengths 1-16. (——), 271 mμ. absorbance; (– – – –), NaCl concentration.

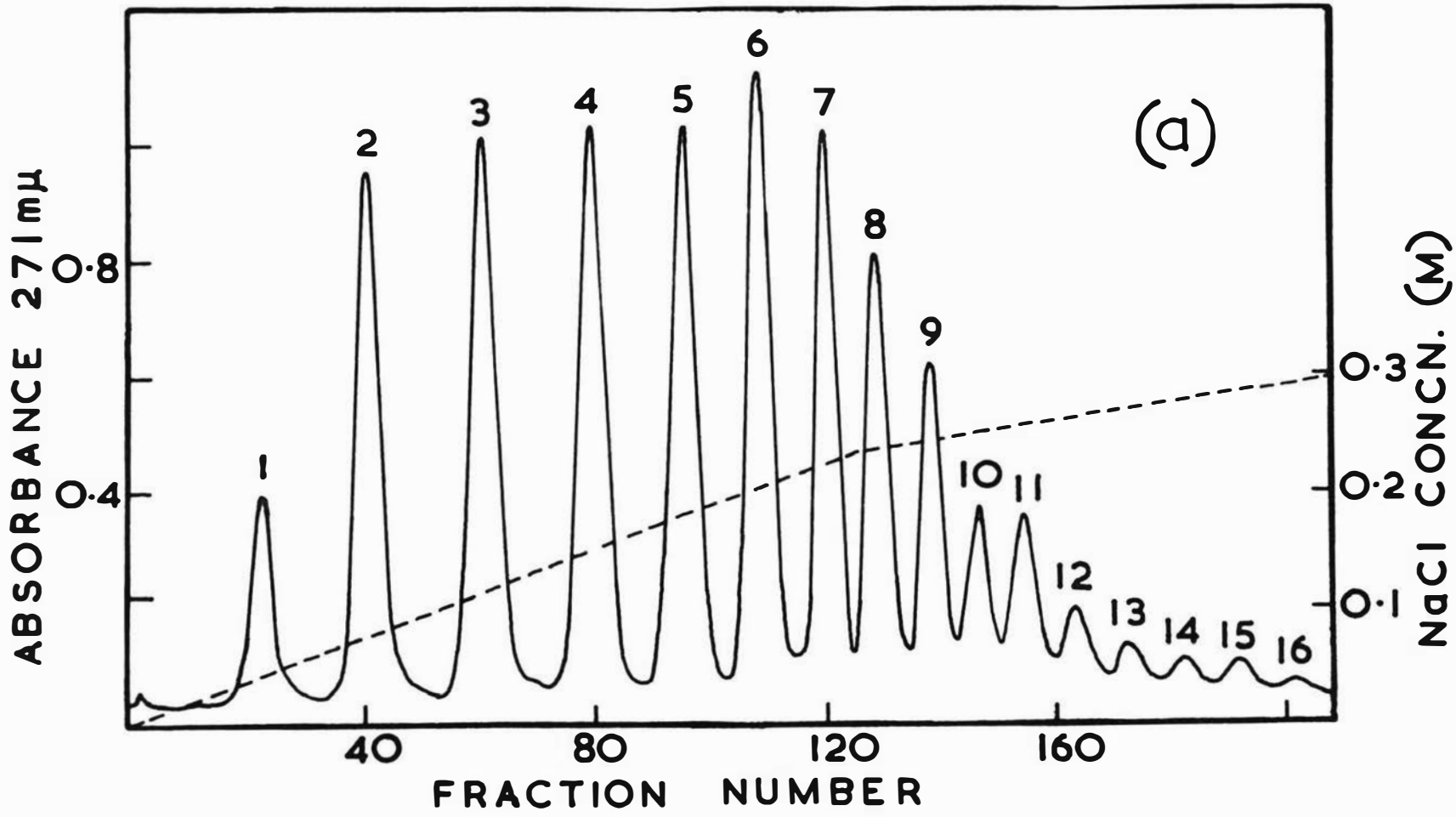


Figure 30. Separation of the Longest Isopliths From a Diphenylamine Digest of Phage Alpha DNA.

(b) The distribution of ^{32}P-radioactivity (●—●), and ultraviolet absorbance (○—○), in the longest isoplith peaks.

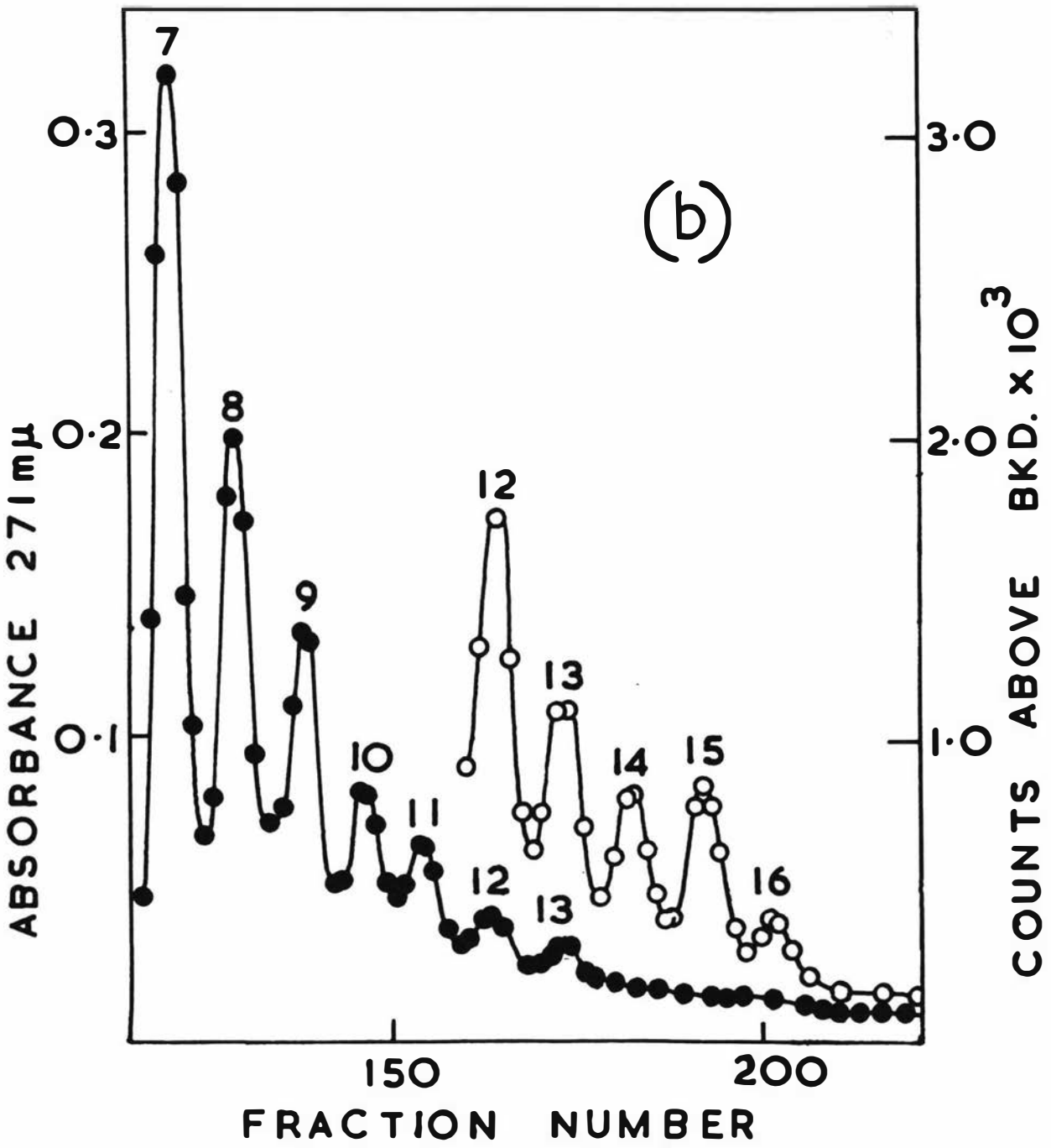


Figure 31. Separation of Isomers of Chain Length Eight From a Diphenylamine Digest of Phage Alpha DNA.

The isoplith peak 8 from figure 30. was desalted on a short column of DEAE-sephadex, and resolved into isomers by ion-exchange chromatography on a 12 cm. x 1 cm. diameter column of DEAE-cellulose. 5.5 ml. fractions were assayed for carrier isomers by ultraviolet absorption measurements at 271 mμ, and radioactivity was estimated in pooled peak and inter-peak regions by counting 1 ml. portions in a toluene/Cab-O-Sil scintillator mixture. (a) Distribution of ^{32}P radioactivity (100-minute counts above background). (b) Distribution of carrier isomers. (——), 271 mμ absorbance; (– – –), NaCl concentration.

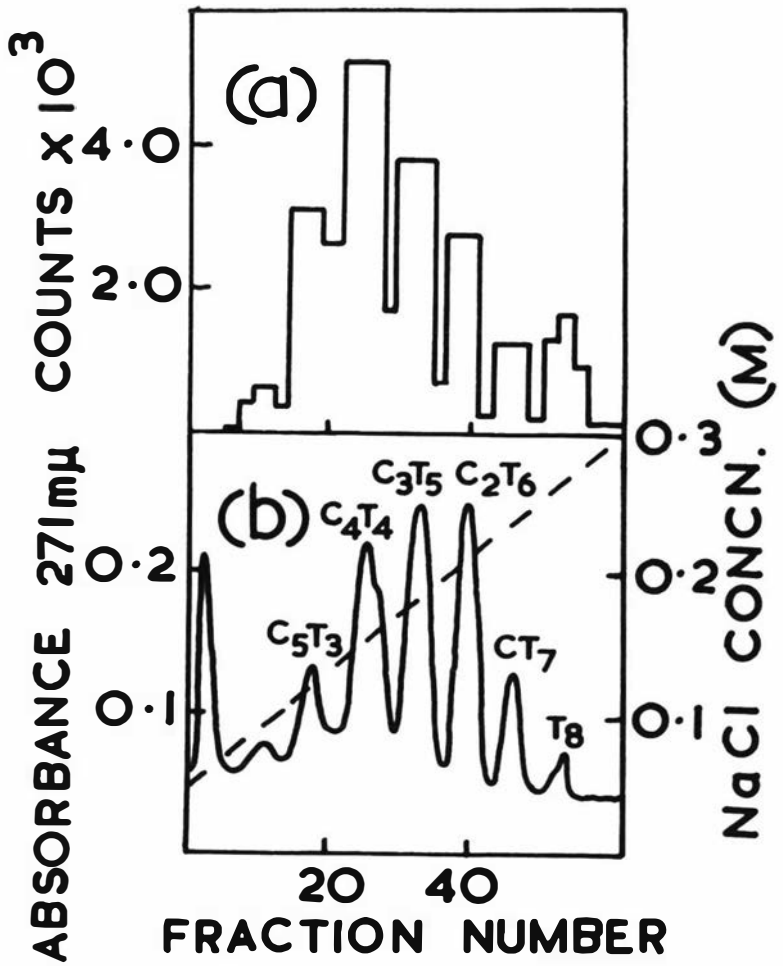


Figure 32. Separation of Isomers of Chain Length Nine.

The isoplith peak 9 from figure 30. was desalted by dialysing for 12 h. against distilled water, and resolved into isomers by ion-exchange chromatography as described in figure 31. (a) Distribution of ^{32}P radioactivity (100-minute counts above background). (b) Distribution of carrier isomers. (——), 271 mμ absorbance; (– – –), NaCl concentration; 3.1 ml. fractions.

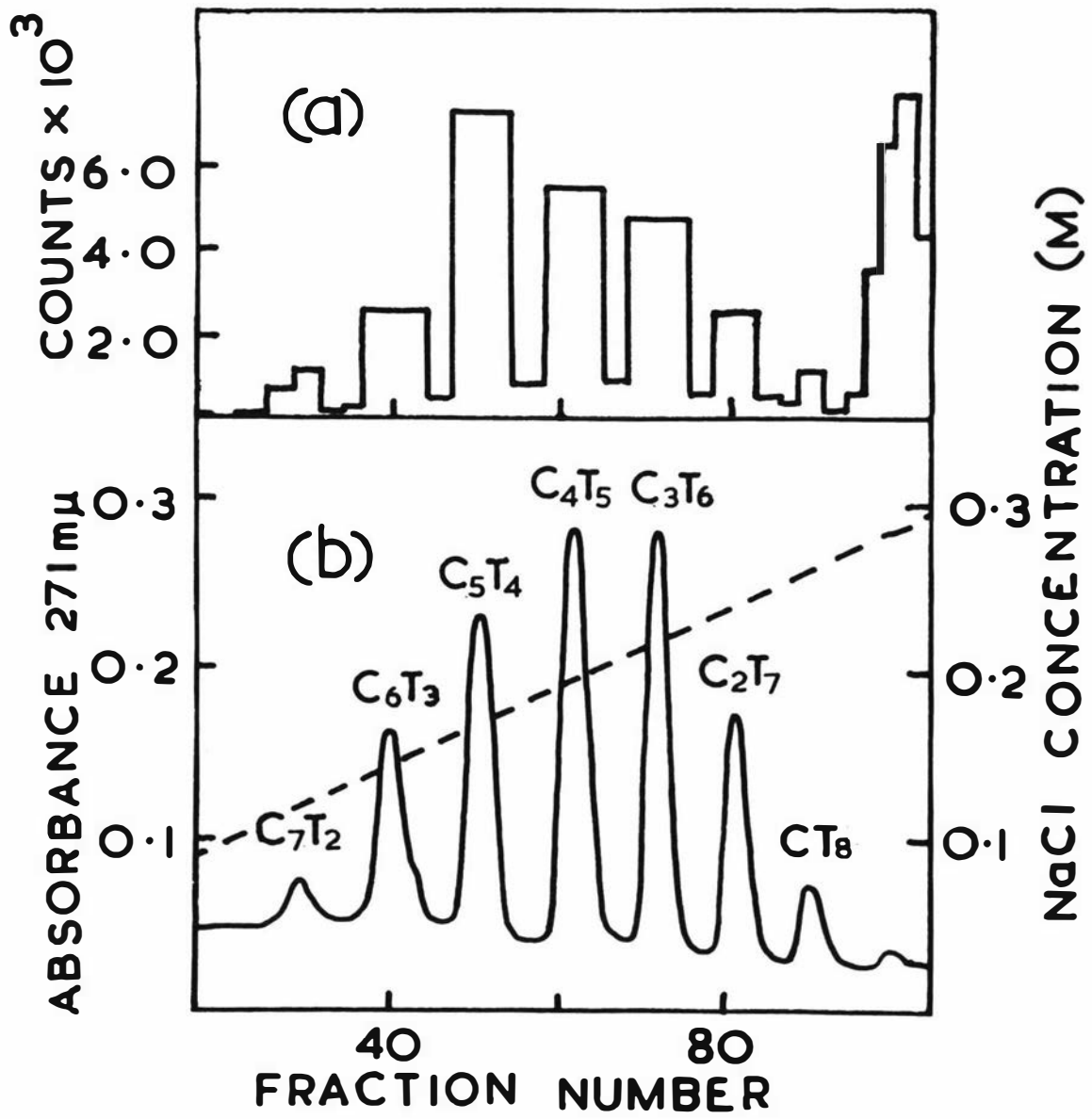


Figure 33. Separation of Isomers of Chain Length Ten.

The isoplith peak 10 from figure 30. was desalted by dialysing for 12 h. against distilled water, and resolved into isomers as described in figures 31-32. (a) Distribution of ^{32}P radioactivity (100-minute counts above background). (b) Distribution of carrier isomers. (——), 271 mμ absorbance; (– – –), NaCl concentration; 3.1-ml. fractions.

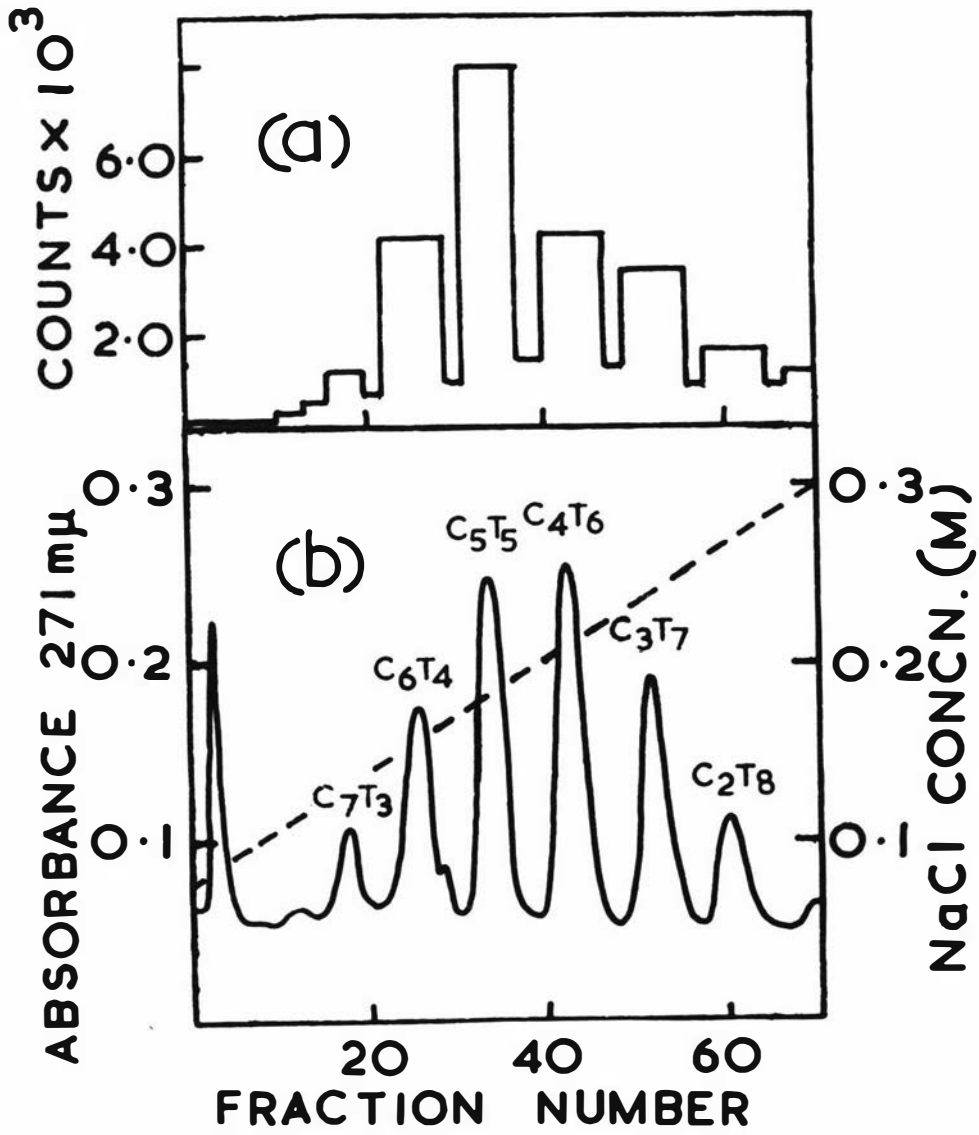


Figure 34. Separation of Isomers of Chain Length Eleven.

The isoplith peak 11 from figure 30. was desalted by a 12 h. dialysis against distilled water, and resolved into isomers by ion-exchange chromatography as described in figures 31-33. (a) Distribution of ^{32}P radioactivity (100-minute counts above background). (b) Distribution of carrier: (——), 271 mμ absorbance; (– – – –), NaCl concentration; 3.7-ml. fractions.

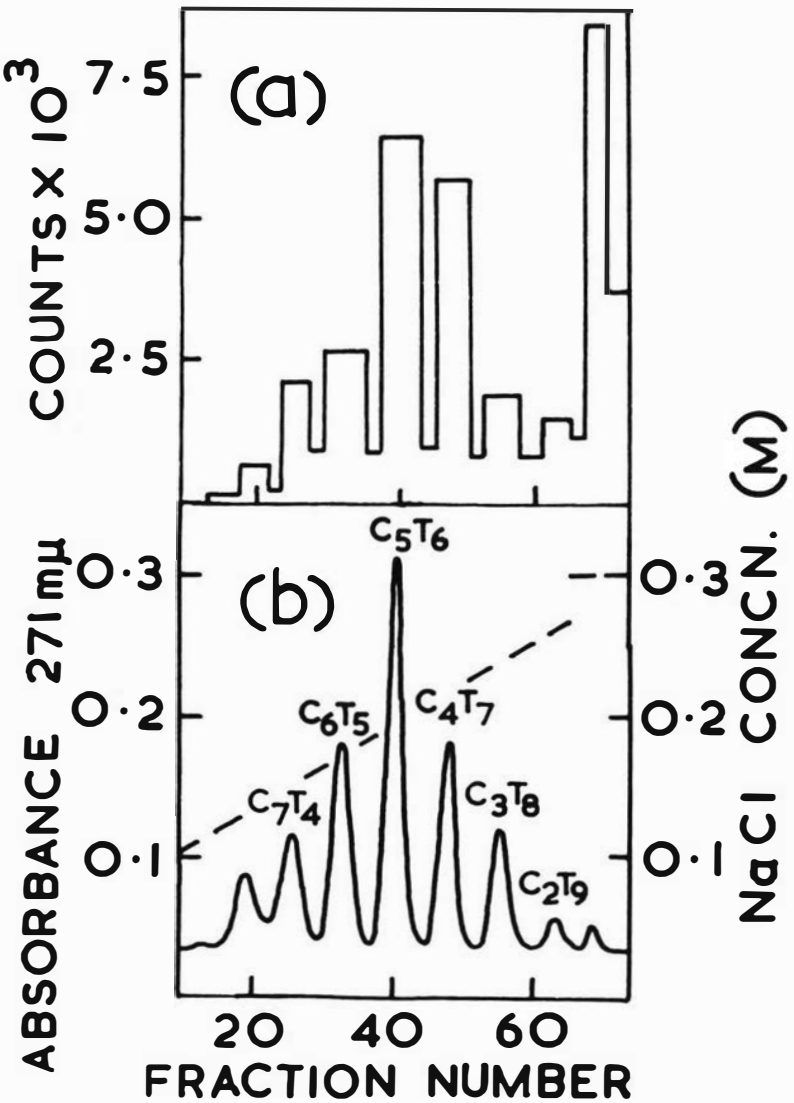


Figure 35. Separation of Isomers of Chain Length Twelve.

The isoplith peak 12 from figure 30. was desalted by a 12 h. dialysis against distilled water, and resolved into isomers by ion-exchange chromatography on a 13 cm. x 1 cm. diameter column of DEAE-cellulose. 2.6 ml. fractions were assayed for carrier isomers by ultraviolet absorption measurements at 271 mμ, and for radioactivity by counting 1 ml. portions in a toluene/ Cab-O-Sil scintillator mixture. (——), 271 mμ absorbance; (●—●), ^{32}P radioactivity, 50-minute counts above background; (– – – –), NaCl concentration gradient (200 ml. 0.05-0.30 M).

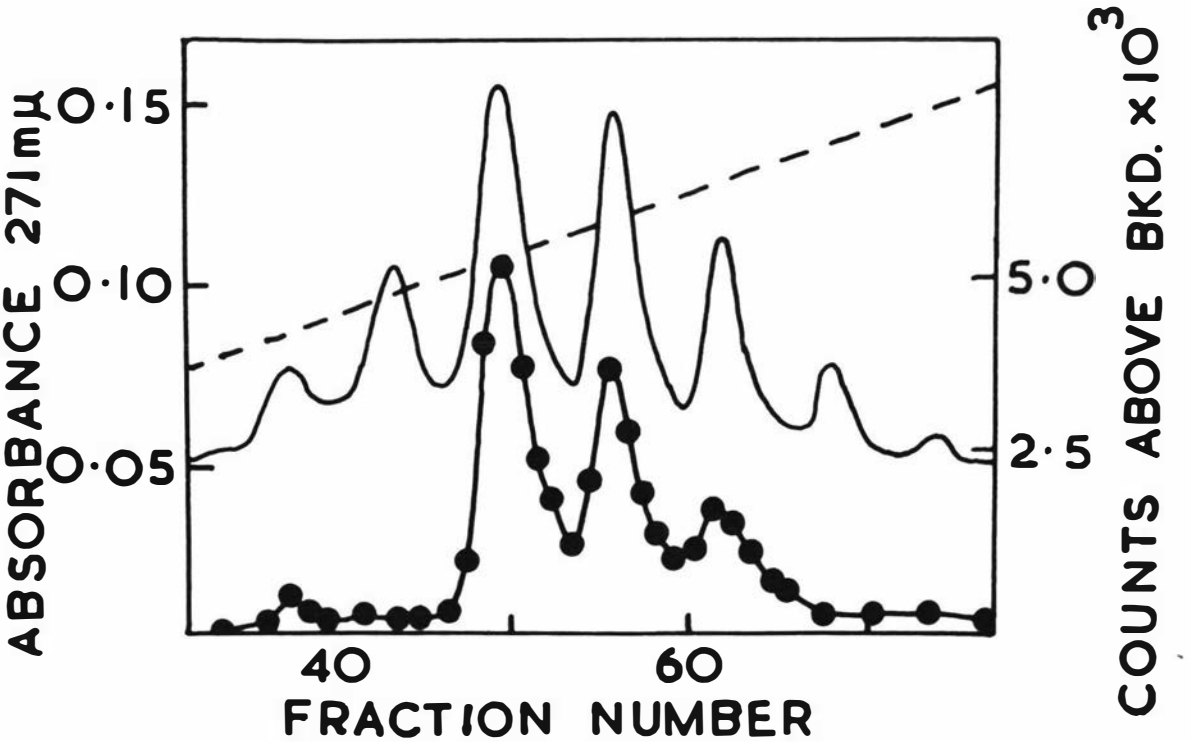
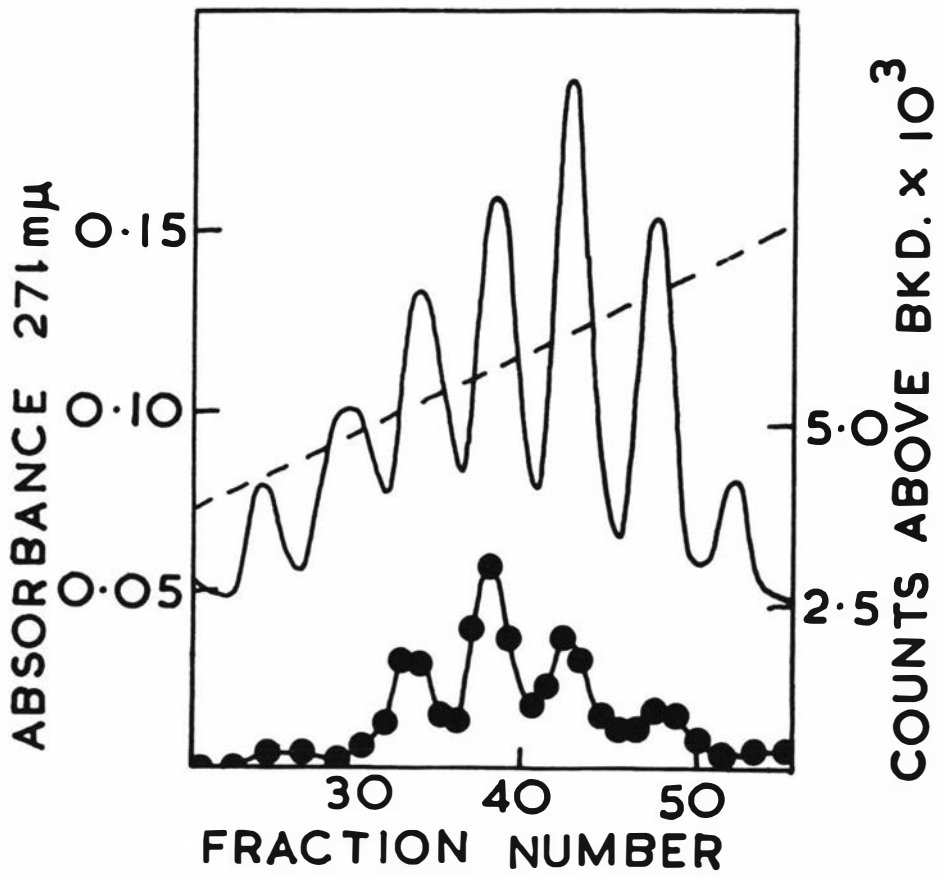


Figure 36. Separation of Isomers of Chain Length Thirteen.

The isoplith peak 13 from figure 30. was combined with carrier isopliths of similar length from another column, and desalted on a short column of DEAE-Sephadex. The sample was resolved into isomers as described in the previous figure. (——), 271 mμ absorbance; (●—●), ^{32}P radioactivity, 50-minute counts above background; (– – – –), NaCl concentration gradient (200 ml. 0.05-0.30 M); 3.6 ml. fractions.



consecutive pyrimidine nucleotides of length greater than 11. It was tentatively concluded that each α DNA molecule contains six pyrimidine nucleotide sequences of length 12, distributed between three isomer groups in a ratio of 3/2/1, and four pyrimidine nucleotide sequences of length 13, distributed between three isomer groups in a ratio of 1/2/1. Many more isomer groups were detected in the carrier, but did not contain significant radioactivity.

An interesting feature of figures 32 and 34 was the elution of relatively large amounts of radioactivity at the positions expected for the thymine oligonucleotides dT_9p_{10} and $dT_{11}p_{12}$. Such long sequences of consecutive thymine nucleotides would not be expected in a randomly arranged DNA molecule having the size and base composition of α DNA, and no trace of these sequences could be detected in the carrier digest. The '$dT_{11}p_{12}$' peak was re-chromatographed with fresh carrier isopliths of length 11, and eluted at the same position as before (figure 37). However, identification by methods other than ion-exchange chromatography was not practicable because of the low level of radioactivity in this experiment.

The distribution of long pyrimidine nucleotide sequences in α DNA deduced from these preliminary experiments is presented in table 8.

An alternative method of resolving the components of diphenylamine digests is two-dimensional paper chromatography after treatment with phosphomonoesterase[42,43]. The results of some preliminary experiments, included in table 6, show that this method gives isomer distributions in agreement with those obtained by ion-exchange chromatography. The

Figure 37. Rechromatography of the Thymine-rich Isomers of Length Eleven From a Diphenylamine Digest of Phage Alpha DNA.

Fractions 59-70 from figure 34. were combined with carrier isopliths of chain length 11 from a column similar to figure 30., and desalted by dialysing against distilled water for 12 h. The isomers were separated by ion-exchange chromatography as described for figures 31-34.(——), 271 mμ absorbance; (●—●), ^{32}P radioactivity (100-minute counts above background); (– – –), NaCl concentration gradient (300 ml. 0.1-0.35 M); 6-ml. fractions.

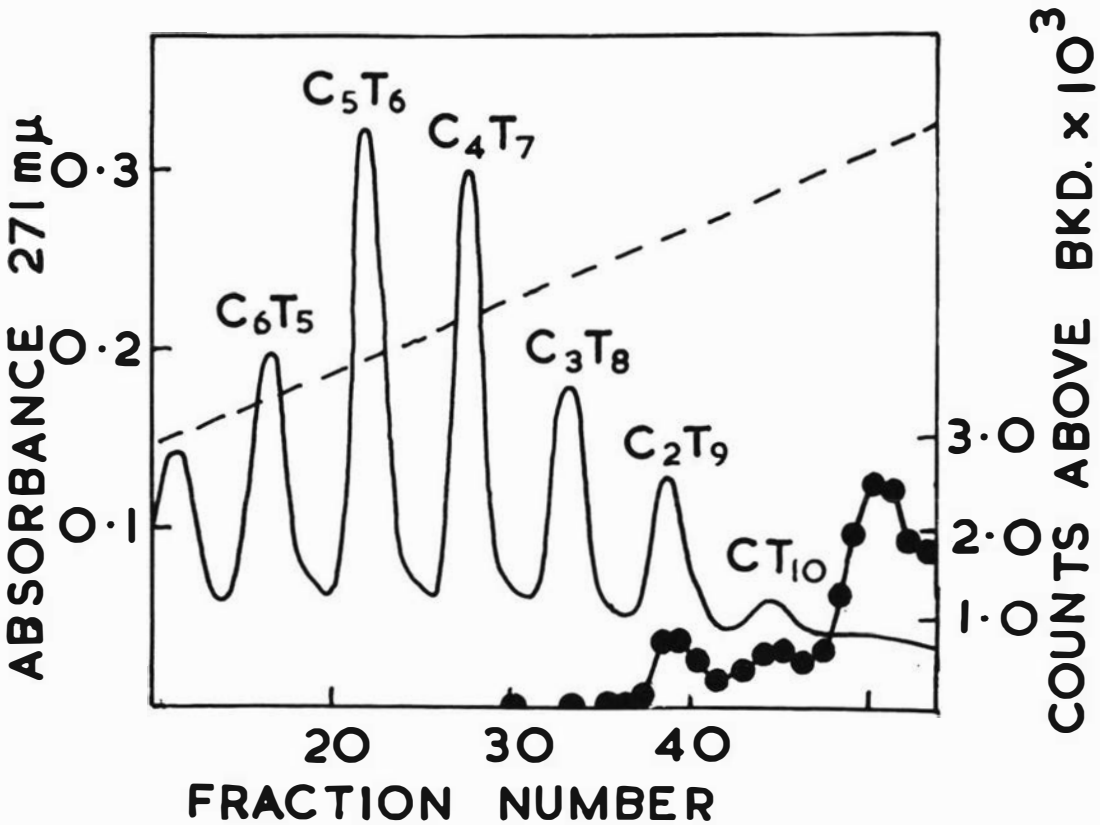


Table 8. Percentage Sequence Frequencies of the Longest Pyrimidine Nucleotide Sequences in Alpha DNA.

Data collected from figures 31-38. Isomer assignments are on the basis of elution position and A_{280}/A_{260} ratio measurements for the Calf Thymus carrier (J.M. Reeves, personal communication).

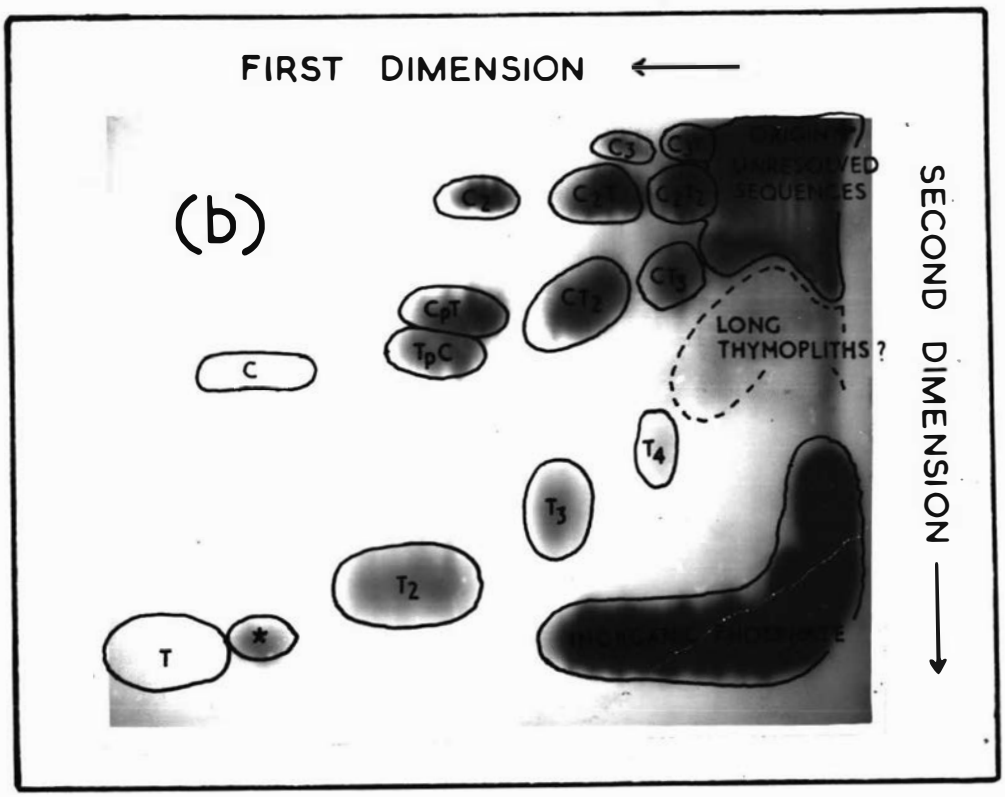
Isomer Group	P.S.F. found	P.S.F. random	Isomer Group	P.S.F. found	P.S.F. random	Isomer Group	P.S.F. found	P.S.F. random
$C_6T_2P_9$	0.002	0.006	$C_7T_3P_{11}$	0.001	0.001			
$C_5T_3P_9$	0.017	0.015	$C_6T_4P_{11}$	0.006	0.004			
$C_4T_4P_9$	0.022	0.025	$C_5T_5P_{11}$	0.010	0.006			
$C_3T_5P_9$	0.017	0.026	$C_4T_6P_{11}$	0.006	0.006			
$C_2T_6P_9$	0.009	0.017	$C_3T_7P_{11}$	0.005	0.005	$C_5T_7P_{13}$*	0.0030	0.0016
$C\ T_7P_9$	0.004	0.007	$C_2T_8P_{11}$	0.003	0.002	$C_4T_8P_{13}$*	0.0017	0.0013
T_8P_9	0.005	0.001	$C\ T_9P_{11}$	0.001	0.001	$C_3T_9P_{13}$*	0.0009	0.0007
$C_7T_2P_{10}$	0.001	0.002	$C_8T_3P_{12}$	0.001	0.000	$C_7T_6P_{14}$*	0.0008	0.0005
$C_6T_3P_{10}$	0.005	0.005	$C_7T_4P_{12}$	0.002	0.001	$C_6T_7P_{14}$*	0.0017	0.0007
$C_5T_4P_{10}$	0.013	0.010	$C_6T_5P_{12}$	0.003	0.002	$C_5T_8P_{14}$*	0.0008	0.0006
$C_4T_5P_{10}$	0.010	0.014	$C_5T_6P_{12}$	0.007	0.003	$C_4T_9P_{14}$*	(0.0003)	0.0004
$C_3T_6P_{10}$	0.008	0.011	$C_4T_7P_{12}$	0.005	0.003			
$C_2T_7P_{10}$	0.003	0.006	$C_3T_8P_{12}$	0.002	0.002			
$C\ T_8P_{10}$	0.001	0.002	$C_2T_9P_{12}$	0.001	0.001			
T_9P_{10}*	0.011	0.000	$C\ T_{10}P_{12}$	0.001	0.000			
			$T_{11}P_{12}$*	0.006	0.000			

* Isomer identification only tentative.

separation resulting from two-dimensional paper chromatography of a diphenylamine digest of a DNA is shown in figure 38.

Figure 38. Separation of Dephosphorylated Oligonucleotides From a Diphenylamine Digest of Phage Alpha DNA by 2-Dimensional Paper Chromatography.

A 100 µl. portion of a dephosphorylated diphenylamine digest of ^{32}P-labelled α DNA was subjected to 2-dimensional paper chromatography, and ^{32}P-labelled oligonucleotides were detected by autoradiography. (a) Autoradiograph (72 h. exposure). (b) Identity of spots. T and C were located by taking contact photographs in ultraviolet light.



* This spot was not identified in the present study. It does not coincide with any ultraviolet-absorbing spot on the chromatogram, and would therefore appear to be non-nucleotide in origin.

Pyrimidine Nucleotide Sequences in the Separated Complementary Strands of Alpha DNA.

The logical next step in the comparison of molecular structure and nucleotide sequence structure in the α DNA molecule was to determine the distribution of pyrimidine nucleotide sequences between the two complementary strands.

In an early experiment, the distribution of isopliths of chain-lengths 1-7 was measured in a diphenylamine digest of ^{32}P-labelled L strands prepared by MAK chromatography, self-annealing, and hydroxylapatite chromatography.

In a later experiment, the distribution of isopliths of chain-lengths 1-7 was measured in diphenylamine digests of two independent preparations of ^{32}P-labelled H strands, prepared in this case by preparative CsCl gradient centrifuging in the presence of PolyG, followed by self-annealing and hydroxylapatite chromatography.

The results from both these experiments are presented in table 9, together with calculated random distributions for each strand. For these calculations, the pyrimidine content of the α DNA L strand was taken to be 46 mole%, as measured by Cordes[91] (table 4). The pyrimidine content of the α DNA H strand was taken to be 54% (and hence complementary to the L strand) rather than 56% as measured on two preparations by Cordes[91], since the MAK chromatography procedure used for her preparations might be expected to result in 'H strands' depleted in the postulated light segment.

The distribution of isomers of chain lengths 1-5 was measured

Table 9. The Distribution of Isopliths of Chain Length 1-7 in the Separated Strands of Alpha DNA.

Data expressed as percentage sequence frequencies. H strand: two independent digests. L strand: one digest. Random distributions calculated from H = 54% Py, L = 46% Py.

Isoplith Group	H Strand		L Strand	
	found	random	found	random
Inorganic Phosphate	23.1, 26.9	21.2	33.2	29.2
Chain Length 1	10.3, 10.1	11.4	11.4	13.4
2	5.37, 5.34	6.17	5.95	6.17
3	2.77, 2.75	3.33	2.60	2.84
4	1.45, 1.37	1.80	1.15	1.31
5	0.881, 0.864	0.972	0.515	0.601
6	0.567, 0.516	0.525	0.282	0.276
7	0.466, 0.382	0.283	0.157	0.127

in both independent H strand digests. The observed percentage sequence frequencies are presented in table 10, together with the frequencies expected for a randomly-distributed polynucleotide containing 54% pyrimidines with C/C + T ratio of 0.43 (table 4).

Also presented in table 10 are the percentage sequence frequencies for isomers of chain lengths 1-2 found in diphenylamine digests of two independent L strand preparations. These were obtained by CsCl gradient centrifuging in the presence of polyG, followed by self-annealing and hydroxylapatite chromatography. Unfortunately, several fractions of each L strand preparation were lost during the latter step, and the nucleotide sequence results probably cannot be regarded as representative of the entire L strand. They are compared with the frequencies expected for a randomly-distributed polynucleotide containing 46% pyrimidines with a C/C + T ratio of 0.46 (table 4).

The distribution of each group of sequences within the a DNA molecule can be expressed as the ratio of the sequence frequencies in the H and L strands (H/L ratio). H/L ratios for inorganic phosphate and pyrimidine nucleotide sequences of lengths 1 and 2 can be calculated directly from the results in tables 9 and 10. They are presented in table 11, together with the values that would be expected in a quite randomly-arranged molecule, given the overall bias in base composition between the two strands. Also included are H/L ratios calculated indirectly by combining the results for the H strand in table 10 with the results for the whole DNA molecule in table 7.

The indirect approach can be extended to the isomers of lengths 3-5, but is open to rather large cumulative errors: not only because

Table 10. The Distribution of Isomers of Chain Length 1-5 in Diphenylamine Digests of the Separated Strands of Alpha DNA.

Data expressed as percentage sequence frequencies (two independent digests). Random distributions calculated from H = 54% Py, C/C + T = 0.43. L = 46% Py, C/C + T = 0.46

Isomer Group	H Strand		L Strand	
	found	random	found	random
C	3.33, 3.43	4.91	5.09, 5.37	6.17
T	6.97, 6.67	6.51	7.61, 7.93	7.24
C_2	(0.98, 0.84)	1.14	(0.102, 0.97)	1.31
(CT)	2.62, 2.72	3.03	2.99, 3.31	3.07
T_2	1.77, 1.78	2.00	1.49, 1.46	1.79
C_3	0.103, 0.102	0.265		
C_2T	1.03, 1.03	1.05		
CT_2	1.23, 1.21	1.39		
T_3	0.404, 0.396	0.617		
C_4	(0.048, 0.047)	0.061		
C_3T	0.292, 0.263	0.326		
C_2T_2	0.590, 0.551	0.648		
CT_3	0.371, 0.366	0.573		
T_4	0.109, 0.114	0.190		
C_4T	0.115, 0.130	0.094		
C_3T_2	0.263, 0.251	0.251		
C_2T_3	0.322, 0.294	0.333		
CT_4	0.150, 0.146	0.220		
T_5	0.032, 0.044	0.058		

Figures in brackets result from low counts or uncertain separations.

Table 11. Ratio of the Distribution of Some Sequences of Pyrimidine Nucleotides Between the Two Strands of Alpha DNA.

H/L ratios were calculated directly from the H and L strand results in table 10, or indirectly from the H strand results in table 10 and the whole DNA results in table 7, assuming cumulative errors no greater than the observed range. Random values are calculated from H = 54% Py, C/C + T = 0.43; L = 46% Py, C/C + T = 0.46.

Isomer Group	direct method	indirect method	random
Inorganic Phosphate	0.75 ± .06	0.85 ± .14	0.73
C	0.65 ± .03	0.63 ± .03	0.80
T	0.88 ± .04	1.00 ± .09	0.90
C_2	(0.91 ± .09)	(1.12 ± .35)	0.87
(CT)	0.85 ± .06	0.75 ± .08	0.99
T_2	1.20 ± .02	0.97 ± .09	1.12

of the limited number of sequence frequency measurements, but because of the rather large variation observed between replicate digests, particularly with respect to the distribution of isoplith frequencies (table 5).

In order to avoid this problem, a double-label ratio approach was devised, using another radioisotope of phosphorus, ^{33}P. Separated H and L strands were prepared from both ^{33}P-labelled a DNA and ^{32}P-labelled a DNA by CsCl gradient centrifuging in the presence of polyG. Each labelled strand preparation was assayed for contaminating complementary strands by re-centrifuging, then combined with complementary strands labelled with the other radioisotope. Diphenylamine digests of these mixtures were treated with prostatic phosphomonoesterase and resolved by 2-dimensional paper chromatography[43]. The H/L ratio for each separated group of isomers was measured directly by comparing the $^{33}P/^{32}P$ ratio with the $^{33}P/^{32}P$ ratio of the whole digest, after appropriate corrections for the degree of strand contamination in each preparation.

Some preliminary results obtained by this method are presented in table 12.

Table 12. Ratio of the Distribution of Some Sequences of Pyrimidine Nucleotides Between the Two Strands of Alpha DNA, as Measured by a Double Label Method.

The strand content of each mixture was estimated by recentrifuging in at least two CsCl gradients. Mixture A: ^{33}P, 18% L, 82% H; ^{32}P, 97% L, 3% H. Mixture B: ^{33}P, 88% L, 12% H; ^{32}P, 4% L, 96% H. Each digest was resolved on two separate paper chromatograms, and spots were located by ultraviolet photography. $^{33}P/^{32}P$ ratios were measured by counting the water eluate of each spot in a toluene/triton X-100 scintillator solution as described in Methods.

Isomer Group	H/L Distribution Ratio			
	digest A	digest B	mean ± S.E.	random
Inorganic Phosphate*	-	- , 0.90	(0.90)	0.90
C_2	0.94, 1.30	1.11, 1.02	1.09 ± .15	0.87
CpT	0.79, 0.62	0.77, 0.81	0.75 ± .09	0.99
TpC	0.96, 0.98	1.14, 1.10	1.05 ± .09	0.99
T_2	1.15, 1.16	1.14, 1.17	1.16 ± .01	1.12
C_2T	1.10, 1.11	1.18, 1.14	1.13 ± .04	1.08
CT_2	1.42, 1.24	1.45, 1.39	1.37 ± .09	1.22
T_3	1.54, 1.53	1.53, 1.48	1.52 ± .03	1.38
CT_3	1.63, 1.68	1.97, 2.08	1.84 ± .22	1.52

* after treatment with prostatic phosphomonoesterase.

DISCUSSION

A. The Molecular Structure of Alpha DNA.

The molecular weight estimated for phage α DNA in the present study, 32.9×10^6, is dependent on the estimated molecular weight of phage T7 DNA, as well as the reliability of the zone sedimentation method. While none of the available methods for measuring the molecular weight of large DNA molecules - sedimentation, electron microscopy, autoradiographic 'star-counting', and end-labelling - can as yet be regarded as having an absolute accuracy much better than ± 5%, they do give results that are consistent with each other when applied to DNA molecules from the 'standard collection' of *E. coli* phages[8]. By this criterion, in the absence of such unusual structural features as circularity or single-strandedness, zone sedimentation is a method of proven reliability. Electron microscopy of native α DNA, carried out by Mr. A. Craig using two different spreading procedures (those of Inman[169] and Beer[170]), has not revealed any unusual structure, so the above molecular weight is likely to be correct.

The result obtained in this project for the $S^0_{20,w}$ of α DNA, 35.1 ± 0.9, is in close agreement with the result obtained by Cordes using boundary sedimentation[91].

It is clear that phage α DNA, as isolated by phenol treatment, contains randomly located single-strand breaks. The origin of this breakage is obscure. Similar breakage has been noted by others in the DNA molecules of phages PBS1[160] and SP50[158,159], and in the latter case it has also been shown that at least some breaks occur in both

complementary strands[158]. It is not established whether this breakage is comparable with the lower level of breakage detected by Davison and Preifelder in several coli phage DNAs[102].

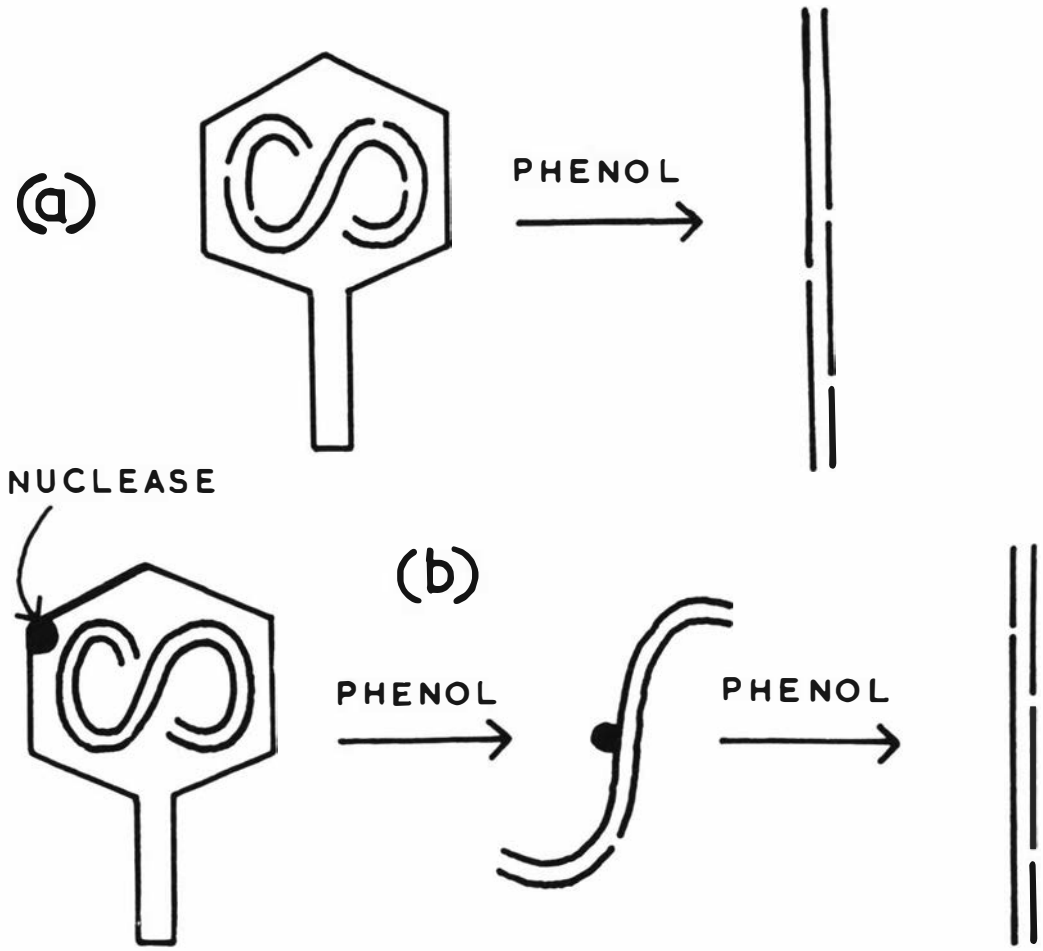
It seems to have been assumed by some previous workers that 'nicked' phage DNA molecules, other than those produced by radiation damage in DNA labelled at high specific activities[171], must be actually packaged in a nicked (or alkali-labile) state inside phage particles[155,160]. There is however another possibility: that strand breaks might be caused, during DNA release from the phage, by an endonuclease present either as an integral part of the phage particle or as a contaminant of even highly-purified phage. The alternatives are presented in figure 39.

It is clear from the results obtained early in this study with DNA extracted from crude phage α preparations (figure 5), that strand breakage can occur during phenol treatment. Such breakage was not induced by phenol treatment of purified DNA. The observation that most of the breakage could be prevented by dialysing the phage against EDTA suggests an analogy with the "unknown metal-activated degradation process" mentioned by Davison and Preifelder, which was apparently eliminated by the use of EDTA-containing buffers[102]. It is tempting to speculate that "metal-activated degradation" might represent the action of a metal-activated endonuclease. Clearly some such explanation must be invoked to explain the different extents of strand breakage noticed in phage SP50 DNA by Reznikoff and Thomas (who found 40-50% intact strands)[158] and Trautner et al. (who found no intact strands)[159]. However, since neither of these groups appears to have purified their

Figure 39. Alternative Explanations for the Origin of Single-strand Breaks in Purified Alpha DNA.

(a) DNA packaged in nicked form inside the α phage particle.

(b) α DNA packaged in strand-intact form inside the phage particle, but attacked during phenol release by an endonuclease present as an integral component of α phage.



phage exhaustively prior to phenol treatment, contamination of their preparations with extraneous endonucleases cannot be ruled out.

The experiment outlined in figure 8. of this study shows that purified α phage is not contaminated with an extraneous metal-activated endonuclease active under the conditions employed. Neither was any nuclease detected in osmotically-lysed α phage. However, this does not eliminate the possibility (figure 39 (b)) that such a nuclease might form an integral component of the phage particle, since such a component might not be released by the limited disruption resulting from osmotic lysis, or might not be active at high salt concentrations, or once released might be fairly rapidly inactivated.

An interesting parallel is provided by the RNA polymerase of Reovirus, which is found inside the Reovirus particle in intimate association with the ribonucleoprotein core[172]. This polymerase is enzymically active when isolated as part of the core, but appears to be immediately inactivated by treatment with reagents such as phenol that dissociate RNA from the core. It is known that at least some DNA-containing bacteriophages have a nucleoprotein core[173], and the occurrence of 'internal proteins' inside the head of phage T2 has long been recognised[174,175]. When phage α is heated to 60° in SSC buffer, it is known that the phage DNA is slowly released in association with a protein component of the phage[89,91].

It is tempting to speculate that a 'core' endonuclease may be responsible for the 25-30% breakage found in even the most intact

preparations of α DNA. In the absence of a direct demonstration of endonuclease activity, however, no conclusion can be reached, although the observed effects of temperature and ionic environment are suggestive. A useful further series of experiments might be to compare the effect of quite different methods of DNA release, such as lysing in alkali, heat-treatment under various ionic conditions, and pronase digestion, on the intactness of the DNA obtained.

Since some α DNA preparations were 75% strand intact, it can be stated unequivocally that at least half of the population of α DNA molecules are packaged inside phage particles in fully strand-intact form.

Strand-intact DNA is an essential prerequisite for the separation of intact complementary strands. Early experiments in this project, attempting MAK chromatography of highly-fragmented α DNA by the method of Cordes[91], were unsuccessful, probably because MAK chromatography recognises differences of size as well as differences of base-composition[103,163]. It is not clear why later MAK chromatography experiments (figures 11-13), which used highly strand-intact α DNA, did not give good separations. Possibly the variation between different batches of Keiselguhr and methylated albumin may be important for success with this rather empirical procedure.

It is interesting that Aurisicchio et al., who also fractionated denatured α DNA by MAK chromatography, reported that their isolated 'L strand' preparations had a mean sedimentation coefficient of only 13s as compared with 25s for heat-denatured unfractionated α DNA[92].

Even the latter value is much too low for intact strands of α DNA in SSC. It seems likely that their preparations of α DNA may have contained many single-strand breaks. If so, their 'L strand' preparations can hardly be regarded as representative of the entire L strand of α DNA.

The same criticism cannot be made of the results of Cordes[91], who demonstrated sedimentation coefficients (in SSC) of 50-58 s for both H and L strand preparations.

The difficulties with MAK chromatography in the present study were completely overcome by using the different technique of preparative CsCl gradient centrifuging of polyG-DNA complexes. This method allows an efficient separation of the H and L strands without extensive fragmentation, as shown in figures 18, 19 and 25. The upper limit of about 100 µg. of DNA per gradient makes the preparation of milligram quantities of separated strands laborious but by no means impossible.

The observed distribution of polyG-binding sites in the α DNA molecule is of considerable interest. Firstly, the observation that intact L strands bind polyG is at variance with the conclusion of Sheldrick and Szybalski[26]. Secondly, since polyG-binding sites are released from most of the L strand by shearing to quarter-length fragments, it is clearly necessary to study intact rather than fragmented DNA preparations if meaningful conclusions are to be made about the structure and function of α DNA. The conclusion that only the H strand of α DNA is transcribed into phage messenger RNA[93,94], which was reached from hybridisation experiments using 'L strands'

prepared by the method of Aurisicchio et al.[92], will certainly need to be re-examined. It would seem quite probable that at least the heavy segment of the L strand may also be transcribed, particularly in view of the correlation between polyG-binding sites and transcribing regions found in the DNA molecules of phages λ, T7 and T3[27,30,29].

It is clear that phage α, a temperate phage, does show signs of a segmental distribution of nucleotides in its DNA molecule. It differs from the temperate phages λ, φ80, 434 and P22, however, in that this segmental distribution cannot be demonstrated in the native DNA[91]. From CsCl density gradient experiments (figures 23 (b) and (c)) it would seem that the heavy segment in the L strand may have a base-composition similar to that of most of the H strand. α DNA can therefore be regarded as differing from an 'ideal' model, with a uniformly purine-rich L strand and a uniformly pyrimidine-rich H strand, by an inversion of 10-20% of the molecular length.

The distribution of G + C pairs in the (native) DNA molecules isolated from a large variety of bacteria is unimodal and approximately gaussian, as shown by buoyant density and thermal denaturation studies[109,176]. The DNA molecules from the virulent phages of the T series likewise do not have a marked segmental distribution of G + C pairs, although some heterogeneity between large fragments can be detected[19]. It has been suggested[18] that intramolecular heterogeneity may be a unique characteristic of the DNA of episomes: that category of the bacteria-afflicting parasites, normally taken to include the temperate phages, capable of a close association with the host bacterial chromosome. In agreement with this suggestion is the

observation that the DNA isolated from several bacterial sex factors can also be separated into regions of distinct base composition[177,178]. However, it would appear that phage α is an exception to this rule: it is not clear that the degree of structural heterogeneity in α DNA is greatly different to that which can be demonstrated in the separated strands of phage T2 (T4) DNA by polyUG binding[168].

Further logical developments in the study of the molecular structure of α DNA would be tests for cyclic permutation and terminal redundancy, and attempts to locate the heavy segment with respect to the two ends of the L strand. A useful technique might prove to be limited digestion with the phosphatase-exonuclease (exonuclease III) of *E. coli*.

B. Sequences of Consecutive Pyrimidine Nucleotides in Alpha DNA.

During the present study, sequences of consecutive pyrimidine nucleotides of all lengths up to 13 were resolved from diphenylamine digests of α DNA. While the existance of longer sequences (length 14-19) was not completely disproved, any such sequences must be very few in number.

The distribution of isopliths of length 1-7 was measured in several diphenylamine digests of whole α DNA (table 5). Despite the variation between replicate digests, there was a clearly non-random distribution of chain lengths. The main features can be summarised as follows.

(1) Inorganic phosphate, released by the diphenylamine reaction from the phosphodiester bridges between consecutive purine nucleotides[42],

was found at slightly more than the random frequency. This is indicative of a general tendency for the purine and pyrimidine nucleotides in α DNA to be bunched together in blocks of consecutive purines and pyrimidines[179], a tendency which α DNA shares with calf DNA but not with herring or salmon DNAs or the DNAs from *Escherichia coli*, *Pseudomonas aeruginosa*, or *Alcaligenes faecalis*[179].

(2) Isopliths of lengths 1-4 were found to occur less frequently than would be expected from a random distribution. The deficiency of mononucleotides was consistently greater than the deficiency of dinucleotides, and the most marked deficiency was usually found in the tetranucleotides.

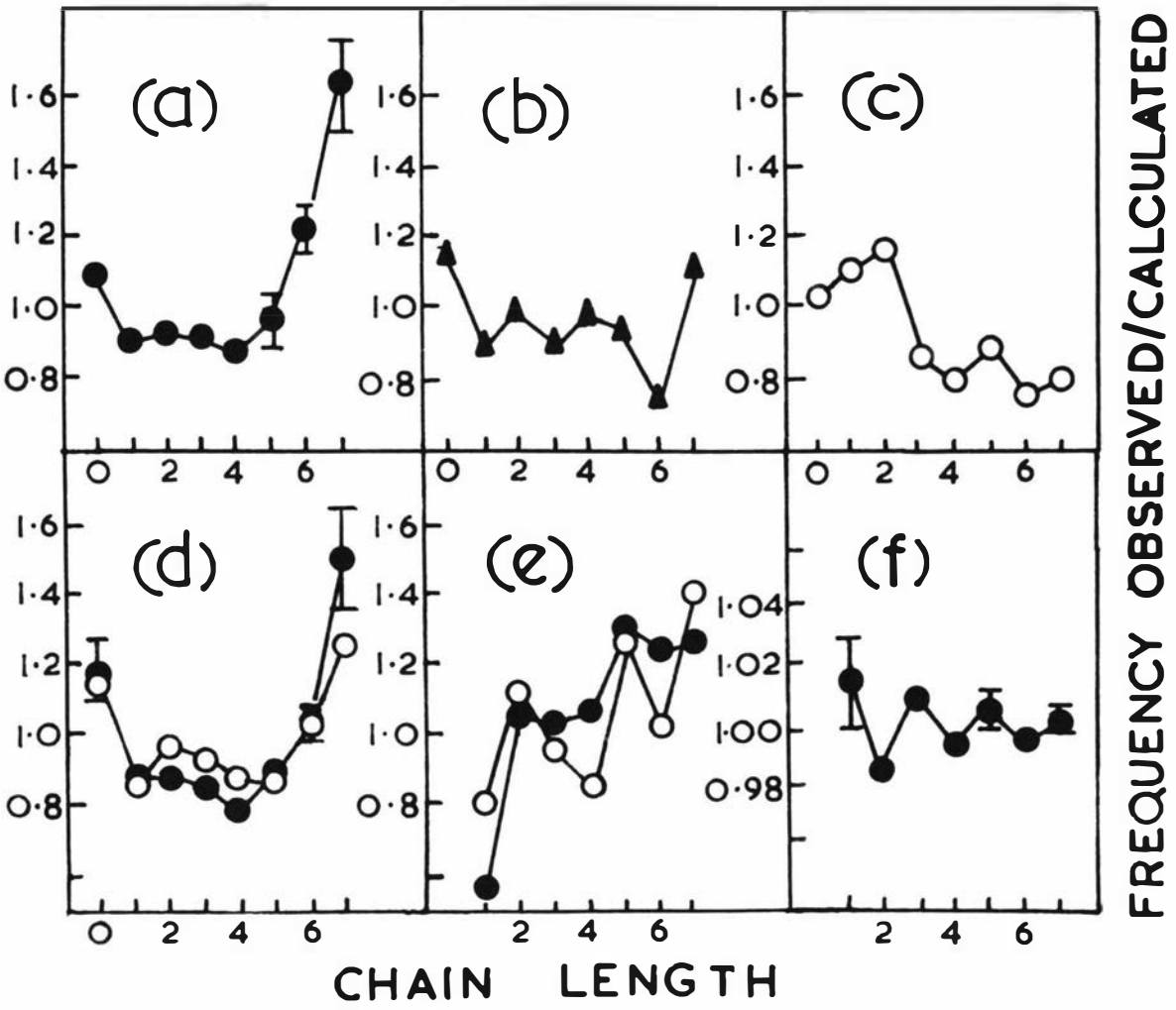
(3) Isopliths of lengths 6 and 7 were found to occur more frequently than expected, the excess of 7s being greater than the excess of 6s. An even greater excess of isopliths of lengths 8 and 9 was observed in several digests, although these results (not tabulated here) were based on very poor separations and showed large variations in frequency between replicate digests.

The excess of long isopliths and deficiency of short isopliths directly confirms the tendency, mentioned earlier, for bunching of the pyrimidine nucleotides in α DNA.

The ratio of observed to calculated frequency for the isoplith groups of length 1-7 in α DNA is presented graphically in figure 40(a). The distribution is quite different from that found in *E. coli* DNA by Cerny et al. (figure 40(c))[180], from that found in phage f1 DNA by Tate and Petersen (figure 40(b))[181], or from that found in phage ϕX-174 DNA by Hall and Sinsheimer (figure 40(e))[51]. None of

Figure 40. The Distribution of Pyrimidine and Purine Isopliths in Some Microbial Polynucleotides.

(a) Pyrimidine isopliths in native α DNA. Bars represent the standard error. (b) Pyrimidine isopliths in phage f1 DNA.[181] (c) Pyrimidine isopliths in DNA of *E. coli* K12.[180] (d) Pyrimidine isopliths in the separated strands of α DNA. (O—O), L strand, one measurement, (●—●), H strand, two measurements. Bars represent the observed range. (e) Pyrimidine isopliths (O—O),[51] and purine isopliths (●—●),[53] in phage φX-174 DNA. (f) Purine isopliths in the RNAs of phages MS2,[83] R17, M12[84] and μ2.[85] Bars represent the range between species.



these DNAs appears to have a distribution of isopliths very similar to that of any of the others. Nor is there any indication of the rhythmic code found in the purine isopliths of viral RNA by Fiers et al., Sinha et al., and Matthews (figure 40(f))[83,84,85,86].

As discussed on page 11, the pyrimidine isopliths in the transcribing strand of a DNA molecule are directly comparable with the purine isopliths in viral RNA. In the present study the distribution of isopliths of lengths 1-7 was measured for both separated strands of phage α DNA. Substantial differences were found between the H strand and the L strand (table 9). However, when the distribution found within each strand is compared with the distribution expected on a random basis, given the overall bias in base composition, the two strands show a remarkable similarity (figure 40(d)).

It must be concluded that the distribution of isoplith lengths in the α DNA molecule shows no signs of a rhythmic code. This is not necessarily to suggest that the evolutionary forces presumed to result in the rhythmic code[83,86] do not operate on DNA molecules. However, it would appear that other evolutionary forces are at work on the isoplith distribution in α DNA, apparently on both the transcribing strand and the non-transcribing strand of the molecule, which result in much greater deviations from randomness than those observed in viral RNA (figure 40(f)). It is interesting to note that the distribution of pyrimidine isopliths in the complementary strand of phage ϕX-174 DNA, as deduced from the distribution of purine isopliths in the viral strand (figure 40(e))[83], shows that in this case also there appear to be marked non-random features shared

by both strands of the DNA. One obvious feature is that in both strands isopliths of lengths 2 and 5 are greatly favoured over isopliths of lengths 1 and 4 respectively. The understanding of such phenomena will probably require a detailed understanding of the ways in which the DNA in various organisms functions as the genetic material.

The distribution of separable isomer groups in diphenylamine digests of α DNA (tables 6-8) shows several non-random features. This in itself is not surprising, considering the information-carrying role of DNA, and merely confirms the results of previous workers[42,43,182,183,82,184,180]. However, several features are worthy of comment.

(1) Sequences of consecutive cytosine nucleotides (cytopliths) were found at much less than the random frequency. As pointed out by earlier workers, a deficiency in cytopliths of length greater than 3 appears to be a general feature of many DNA molecules, including those from calf thymus, herring testis, *Micrococcus lysodeikticus* and *Escherichia coli*[82,184,185,183,180]. However, α DNA also appears to be deficient in cytopliths of lengths 1, 2 and 3 (table 7). A very marked deficiency of cytopliths of lengths 5 and 6 has been reported for some DNAs[85,82,180]. The α DNA molecule appears to contain 6-8 cytopliths of length 5, and may contain a single cytoplith of length 6.

(2) Sequences of consecutive thymine nucleotides (thymopliths) were also found at much less than the random frequency. This is rather an unusual feature, since Rudner et al., using sulphuric acid

hydrolysis, have reported that an *excess* of thymopliths appears to be a common feature of several bacterial DNAs[183]. The results of Cerny et al. confirm that there is a considerable excess of longer thymopliths in *E. coli* DNA, the excess over random increasing with chain length[180]. The thymopliths of lengths up to 7 in calf DNA have been found at frequencies rather close to the random[43,184].

If the late-eluting peaks of radioactivity in figures 32, 34 and 37 really represent thymopliths of lengths 9 and 11, then these sequences occur at dramatically high frequencies in the α DNA molecule. However, these sequences were not detected in other experiments, including experiments which used homologous carrier digests of α DNA. Since the identity of the 'long thymine sequences' was not established by methods other than ion-exchange chromatography, the possibility of some spurious explanation cannot be ruled out.

(3) There appears to be a non-random distribution of cytosine and thymine between the isoplith groups of α DNA. As shown in table 13, there is relatively less cytosine located in the short isopliths, and relatively more cytosine located in the long isopliths, than would be expected from the overall base composition of α DNA (42.6% G + C)[92]. In this respect also, α DNA differs from previously studied DNA molecules. Rudner et al.[183] have reported that several bacterial DNAs have a *more* than random amount of cytosine located in the short isopliths, and a *less* than random amount in the long isopliths, and this report is confirmed for *E. coli* DNA by the results of others[179,180].

As shown in table 13, the overall cytosine content for the

Table 13. Cytosine Content of the Isopliths of Length 1-6 in Diphenylamine Digests of Alpha DNA and *E. Coli* DNA.

Results are taken from tables 6 and 10, and from Cerny et al.[100]

Isoplith Group	C/C + T (Moles %)		
	whole molecule	H strand	*E. coli*
1	39.1	33.0	56.0
2	41.9	42.0	54.4
3	43.9	43.5	43.1
4	45.3	46.1	43.6
5	45.9	46.4	40.2
6	46.4	-	36.6
Total	42.6	41.1	50.3

isopliths of length 1-6 in a DNA is very close to the G + C content of a DNA, a result which can be taken as further evidence that the diphenylamine reaction does not result in selective destruction of either cytosine or thymine.

(4) a DNA contains many separable groups of isomers of lengths 9 and 10 (figures 32-33). There are also at least 6 separable groups of isomers of length 11 (figure 34), but the uneven distribution of radioactivity between these groups, recently confirmed in another experiment (G.B. Petersen, personal communication), suggests a rather restricted range of sequences. Only three separable groups of isomers of length 12, and three (possibly four) of length 13, were detected (figures 35-36). The quite non-random distribution of these sequences suggests very strongly that some of them occur only once in each a DNA molecule, and this conclusion is supported by the quantities of radioactivity found in each peak (table 8).

This probably represents the first isolation of 'unique' sequences from within a DNA molecule larger than the DNA of phage ϕX-174.

The distribution of separable isomer groups between the two strands of the a DNA molecule has so far received only a preliminary examination. However, on the basis of the results in tables 10-12 the following observations can be made.

(1) The H strand resembles the whole a DNA molecule in being deficient in cytopliths of length 1-3 and thymopliths of length 2-5, and in having cytosine concentrated in the longer isopliths (table 13).

(2) Cytosine monomers and CpT sequences appear to be concentrated non-randomly in the L strand, while thymine monomers and dimers and TpC sequences appear to be randomly distributed between the two strands.

Many interesting experiments remain to be carried out with α DNA. The distribution of cytopliths between the two strands, one of the direct objectives of this project, has not yet been accurately measured. No attempt has yet been made to locate the separable 'unique' sequences of consecutive pyrimidines with respect to the two strands of the DNA or the 'heavy-segment' region of the light strand. No very startling non-randomness in the distribution of pyrimidine sequences between the two strands has yet been detected, but even preliminary analyses have not yet been extended to isomer groups of length greater than 5, or isopliths of length greater than 7.

Dunedin, October 3, 1969.

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Appendix I. The Use of Polyethylene glycol for the Concentration and Purification of Bacteriophages f1 and T7.

The discovery that PEG precipitation provided an efficient method for concentrating and purifying large quantities of bacteriophage α suggested that it might also be useful for preparing the other bacteriophages being studied in this laboratory, f1 and T7. This proved to be the case.

Suitable concentrations for the precipitation and re-suspension of phage T7 were demonstrated by celite column chromatography as shown in figure 1. An electron micrograph of phage T7 purified by this method is shown in figure 2. Such preparations contained very little extraneous material. Figure 3 shows by way of contrast an electron micrograph of phage T7 prepared by salt precipitation followed by differential centrifuging. This preparation contained large amounts of bacterial debris.

Phage f1 was precipitated at rather lower PEG concentrations than phages T7 and α, as shown in table 1.

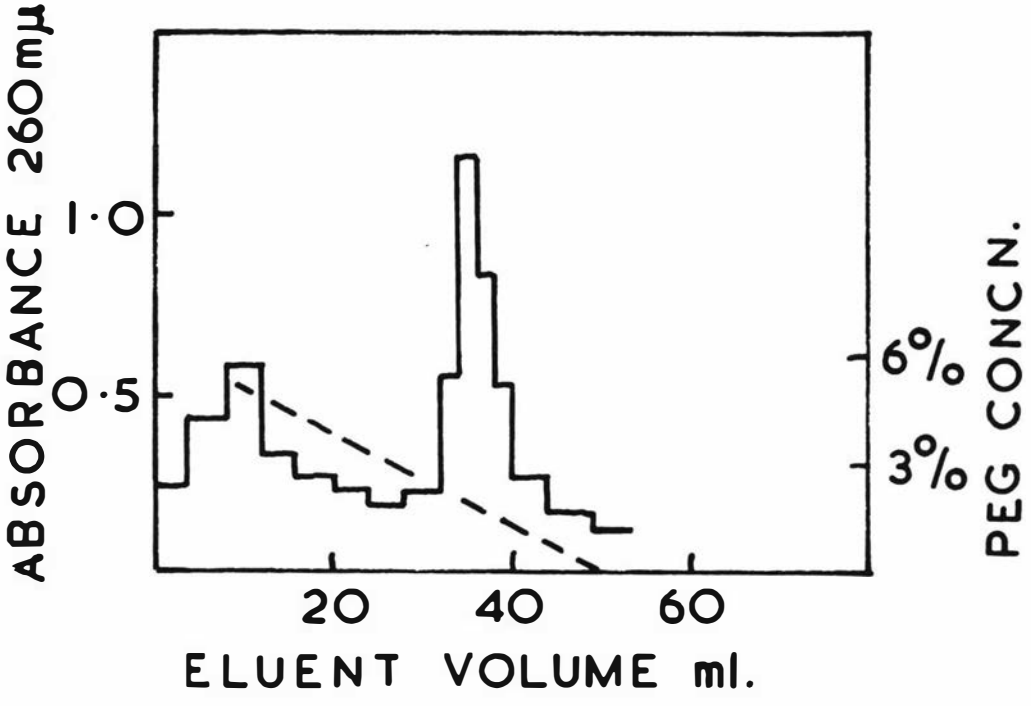
The recovery of phage f1 after PEG precipitation was quantitative (table 2), and f1 purified by this method showed the normal morphology when examined by electron microscopy (figure 4.).

Considerable difficulty was experienced in following the recovery of phage f1 by infectivity assays, and it may be that f1 is inactivated by PEG. Nevertheless, this selective precipitation method is now used for routine batch preparations of f1 in this laboratory (W.P. Tate, personal communication).

Appendix I.

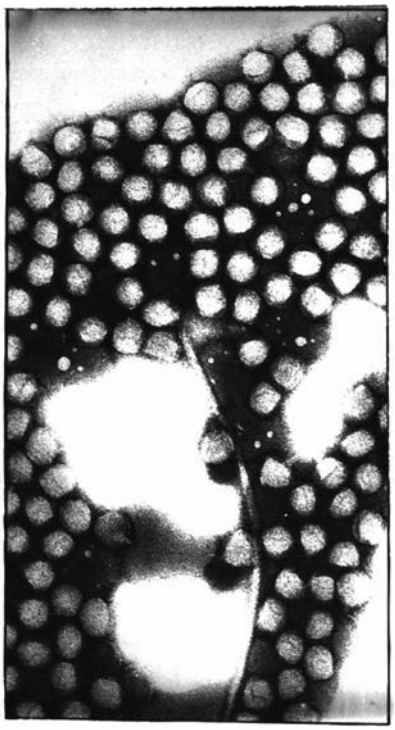
Figure 1. PEG Gradient Elution of Phage T7 from a Celite Column.

Phage T7 prepared by differential centrifuging was made 0.5 M in NaCl and 6% (w/v) in PEG and filtered through a 1 cm. x 1 cm. diameter column of celite. Phage was eluted with a linearly descending concentration gradient of PEG containing 0.5 M NaCl, 0.01 M phosphate buffer pH 7.0; 5 ml. and 2 ml. fractions were collected and assayed for phage particles by ultraviolet absorption measurements at 260 mμ.



Appendix I. Figure 2. Electron Microscopy of Phage T7 Prepared by PEG Precipitation.

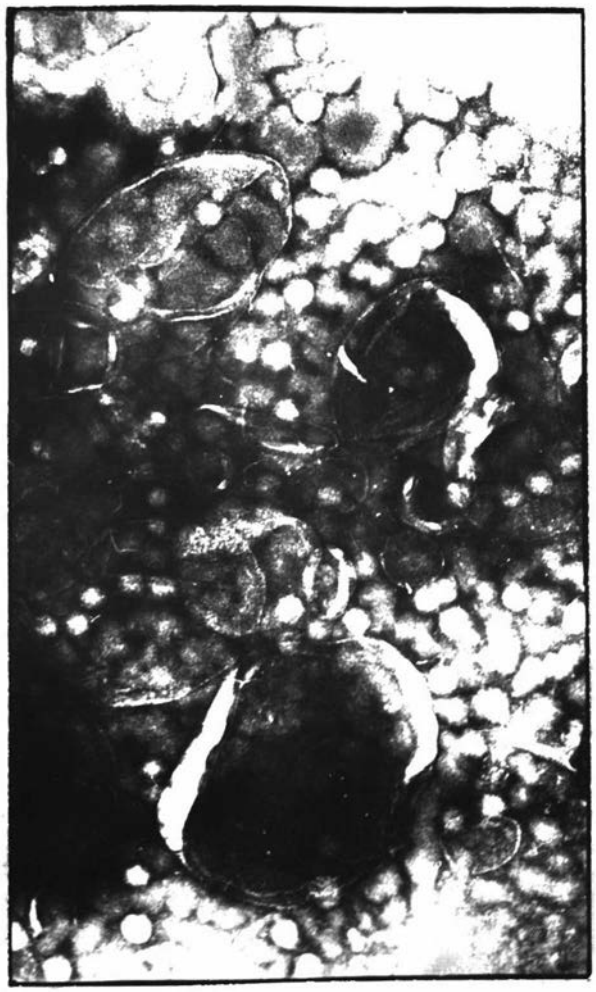
Phage T7 eluted in fractions 9-12 from the celite column described in figure 1, was rinsed twice with phosphate buffer by high speed centrifuging and examined by electron microscopy. Magnification 80,000 x.



Appendix I. Figure 3. Electron Microscopy of Phage T7 Prepared by Differential Centrifuging.

Phage T7 was concentrated from a bacterial lysate by ammonium sulphate precipitation followed by differential centrifuging. The concentrated phage suspension was clarified by a final low speed centrifuging (10 minutes at 10,000 r.p.m. in the SS-34 rotor of a Sorvall RC-2B centrifuge) and a portion of the supernatant examined by electron microscopy.

Magnification 64,400 x.



Appendix I. Table 1. Precipitation of Phage f1 by Low Concentrations of Polyethylene Glycol.

A series of tubes containing phage f1 (10^{12}/ml.), 0.5 M NaCl, 0.08 M borate buffer pH 8.0 and various concentrations of PEG were assayed visually for precipitation.

PEG Concentration (w/v)	Extent of Precipitation
3.0	+ +
2.5	+ +
2.0	+ +
1.75	+ +
1.50	+ +
1.25	+ -
1.0	-
0.5	-

Appendix I. Table 2. Recovery of Phage f1

After Precipitation by Polyethylene Glycol.

A suspension of phage f1 (10^{13}/ml.), prepared by differential centrifuging, was made 0.5 M in NaCl and 0.5% (w/v) in PEG and centrifuged (5 min. at 15,000 r.p.m.). The supernatant was made 1.75% in PEG and re-centrifuged. All fractions were rinsed twice in borate buffer by high speed centrifuging and assayed for phage by measuring the ultraviolet absorptions at 260 mμ and 280 mμ.

Fraction	A_{260}/A_{280}	Units A_{260}	% Total A_{260}
Initial phage suspension	1.10	460	100
0.5% ppt.	1.11	3.8	0.8
1.75% ppt.	1.10	469	102
Final supt.	1.10	0.9	0.2

Appendix I. Figure 4. Electron Microscopy of Phage f1 Prepared by Selective Precipitation with PEG.

A portion of a rinsed 1.75% PEG ppt. fraction of phage f1 (table 2.) was diluted into distilled water and examined by electron microscopy.

Magnification 35,000 x.

Appendix II. Dialysis of Pyrimidine Oligodeoxynucleotides.

The degradation of DNA with diphenylamine in formic acid results in a complex mixture of inorganic phosphate, free purine bases, and pyrimidine oligodeoxynucleotides of varying chain length and base composition with the general formula Py_np_{n+1}. Methods for the resolution of such mixtures by paper chromatography, paper electrophoresis and ion-exchange chromatography have been developed[1,2,3,4]. In all these methods, the total amount of material that can be conveniently resolved is limited to a few tens of µg. atoms of oligonucleotide phosphorus.

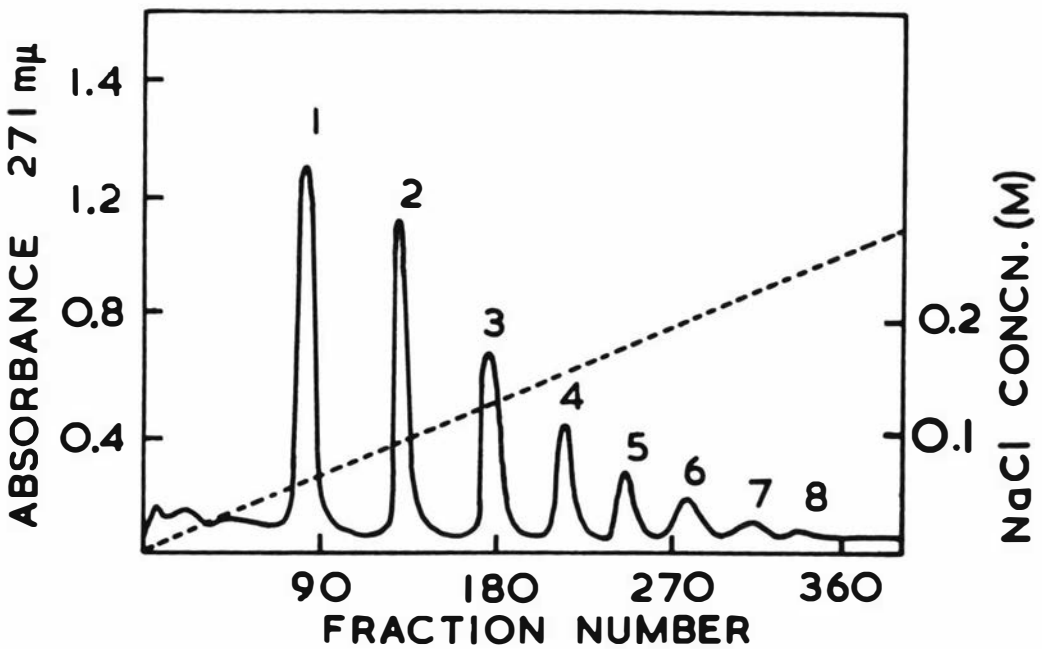
Since the long, rarely-occurring pyrimidine nucleotide sequences form only a very small fraction of a diphenylamine digest, their study is facilitated by prior removal of most of the shorter sequences. Prolonged dialysis against distilled water was at first used for this purpose, on the assumption that the losses of the longest oligonucleotides into the diffusate would be negligible, but it remained necessary to examine in detail the effect of chain length and base composition on the dialysis of pyrimidine oligodeoxynucleotides.

In a preliminary experiment, a 4-ml. portion of a diphenylamine digest of calf thymus DNA (representing 100 mg. of DNA) was dialysed against 800 ml. of distilled water for 60 h. at 4°. An aliquot (50 ml.) of the diffusate was evaporated to dryness at 30°, and the oligonucleotides separated on the basis of chain length by ion-exchange chromatography[4]. The elution pattern, illustrated in Figure 1, showed that appreciable amounts of sequences of chain length up to at least 7 had passed into the diffusate.

Appendix II.

Figure 1. Oligodeoxynucleotides in the Diffusate.

The products in the diffusate obtained after prolonged dialysis of a depurinated digest of calf thymus DNA were separated on the basis of chain length by ion-exchange chromatography on DEAE-cellulose; 2 ml. fractions were collected. ----, NaCl concentration.



In order to reduce the errors involved in measuring the diffusion rates of various oligonucleotides, another approach was adopted. A diphenylamine digest of non-radioactive bacteriophage α DNA, containing 425 ± 6 μg. atoms of phosphorus (25 ml.), was dialysed against 20 l. of distilled water at room temperature for 63 h. Phosphorus analyses showed a loss into the diffusate of 74 ± 3% of the nucleotide and oligonucleotide phosphorus.

The contents of the dialysis bag were combined with an undialysed digest of ^{32}P-labelled α DNA, containing 62 ± 1 μg. atoms of DNA phosphorus, and evaporated to dryness. After removal of the insoluble guanine residues, the oligodeoxynucleotides of the combined digest were separated on the basis of chain length by ion-exchange chromatography (Figure 2).

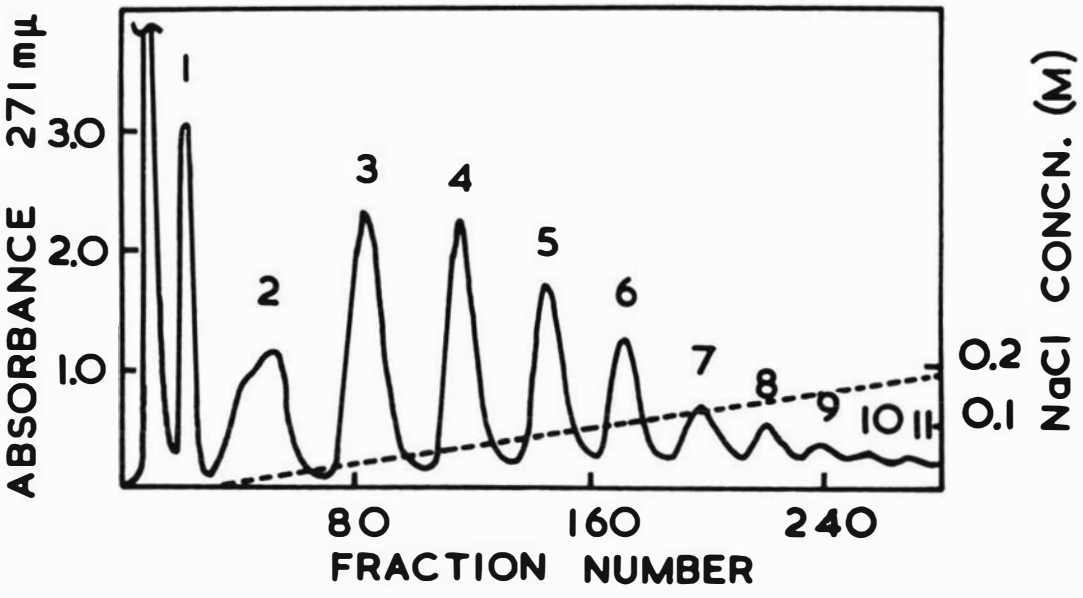
Peaks 2-6 were identified by rechromatography as pyrimidine oligonucleotides of length 1-5. The absorbance (271 mμ) and ^{32}P radioactivity of each peak was assayed. Assuming the base composition of each peak of oligodeoxynucleotides to be 43% cytosine, 57% thymine (from the cytosine to thymine ratio of phage α DNA)[5], the absorbance at 271 mμ gave a measure of the contribution to each peak from both the dialysed and undialysed digest, while the ^{32}P radioactivity gave the contribution from the undialysed digest alone. From these, the losses of unlabelled nucleotide material during dialysis were calculated. The percentage loss, plotted as a function of chain length, is given in Figure 3.

It can be seen that the removal of the mononucleotides was complete. The loss during dialysis declined with chain length in the range 1-6, but remained substantial for oligonucleotides as long as 11.

Appendix II.

Figure 2. Separation of Isopliths in a Mixed Diphenylamine Digest of Bacteriophage DNA.

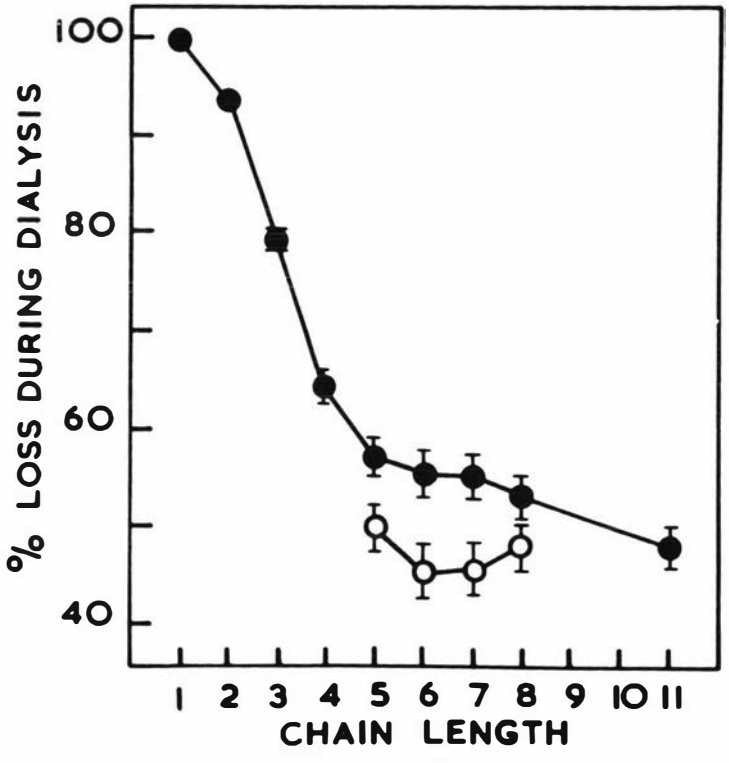
The first two peaks were eluted with 0.1 M acetate buffer (pH 5.4) containing 7 M urea. Sequences of greater chain length were eluted with a linear gradient of increasing NaCl concentration (-----) in the same buffer. Column length 44 cm., 5 ml. fractions.



Appendix II.

Figure 3. Chain Length and the Loss of Oligodeoxynucleotides During Dialysis.

●—●, mean percentage loss during a 63 h. dialysis for all sequences of a given chain length; O—O, mean percentage loss during the 63 h. dialysis for those sequences of each length remaining after a 45 h. re-dialysis. The bars represent the estimated reliable errors.



To determine whether base composition had any effect on the rate of dialysis of pyrimidine oligonucleotides, Peaks 1-5 of Figure 2 were desalted and separated into their components of differing base composition by ion-exchange chromatography[4]. Peak tubes were pooled and assayed for ^{32}P radioactivity and ultraviolet absorption. Calculations of percentage losses during dialysis of the non-radioactive digest were made as before, except that no assumptions were needed concerning the base composition of each peak.

A very marked effect of base composition, which became increasingly pronounced as the chain length increased in the range 2-5, was found. In every case, the loss during dialysis increased with increasing cytosine content. This was shown graphically when the percentage loss for each group was plotted relative to the percentage loss of the thymine oligomer of the same chain length (Figure 4).

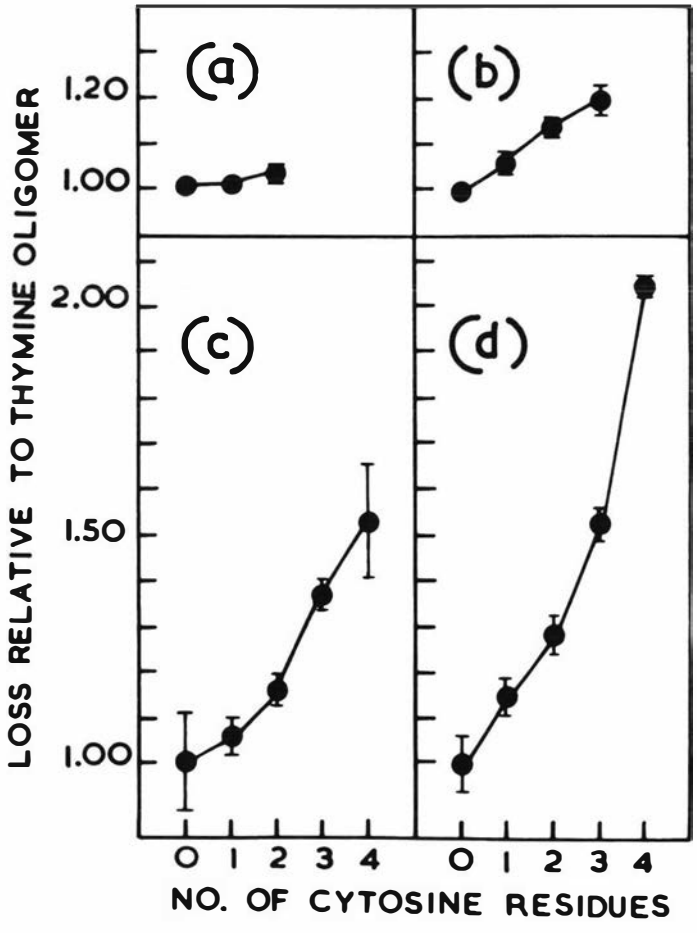
This base composition effect was found to strongly modify the effect of increasing chain length, as shown by plotting the percentage loss during dialysis against chain length for different compositional series (Figure 5).

It was clear that the losses shown in Figure 3 for sequences of chain length 3-5 must represent weighted mean values for mixtures of components of differing base composition which had dialysed at quite different rates. It was of interest to know whether this also held true for the longer sequence groups. Unfortunately the poor separation obtained on the basis of chain length for these sequences interfered with their further resolution on the basis of composition.

Appendix II.

Figure 4. Cytosine Content and the Loss of Oligodeoxynucleotides During Dialysis.

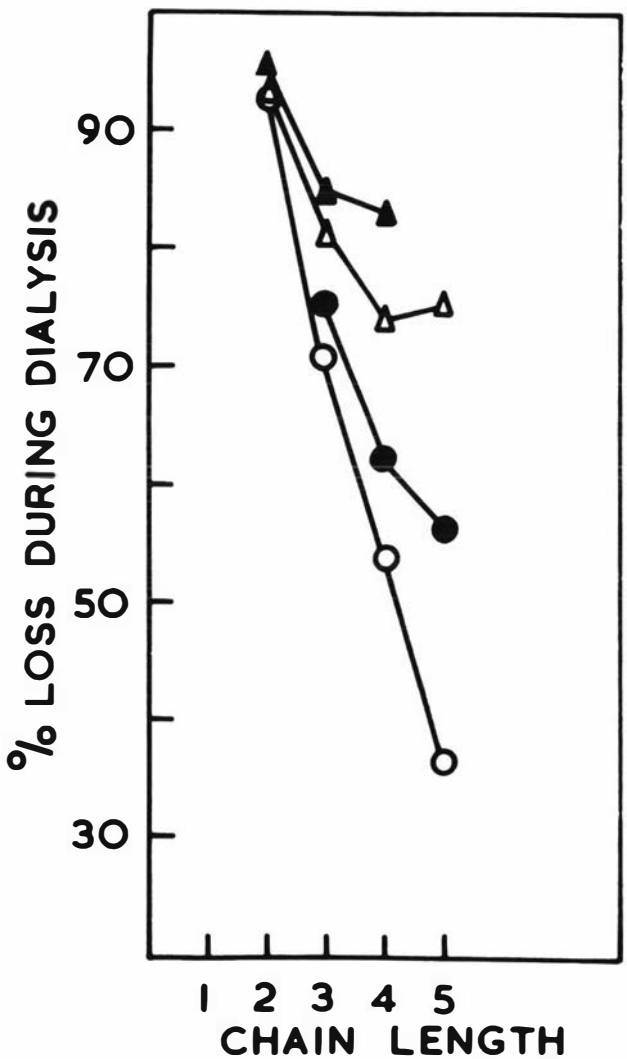
(a) dinucleotides; (b) trinucleotides; (c) tetranucleotides; (d) pentanucleotides. The bars represent the estimated reliable errors. The percentage losses for sequences of differing base content are expressed relative to the thymidine oligomer (T_nP_{n+1}) within each group.



Appendix II.

Figure 5. Base Composition and the Effect of Chain Length on the Dialysis of Oligodeoxynucleotides.

(▲—▲), C_np_{n+1}; (△—△), $C_{n-1}\ Tp_{n+1}$; (●—●), $C_{n-2}\ T_2p_{n+1}$; (○—○), T_np_{n+1}.



An alternative approach was designed in which 10-ml. portions of the sequence groups of chain length 5-8 were each re-dialysed for 45 h. against 2 l. of distilled water. The samples were acidified by the addition of 0.1 vol. of 0.5 M HCl and their ^{32}P radioactivity and ultraviolet absorption measured. The results are presented in Table I.

Table 1. Reduction of Specific Activity on Re-dialysis.

Chain Length	Counts/min. per m.mole pyrimidine before re-dialysis.	Counts/min. per m.mole pyrimidine after re-dialysis.
5	35.2 ± 0.9	31.3 ± 0.6
6	33.0 ± 0.8	29.0 ± 0.7
7	32.4 ± 0.8	28.4 ± 0.7
8	32.2 ± 0.8	28.4 ± 0.9

It was found that re-dialysis resulted in a reduction of the ^{32}P radioactivity relative to the ultraviolet absorption. This result was interpreted as showing that each of these sequence groups contained fractions (probably those oligodeoxynucleotides most rich in cytosine) that were dialysed out more rapidly than the remainder. These would have been depleted in the original non-radioactive digest, but not in the radioactive digest. The depletion of the cytosine-rich radioactive fractions during re-dialysis would have resulted in the observed reduction of specific activity. Conversely, it is readily shown that the sequences in each of these length groups that remained after re-dialysis represented a fraction that was dialysed out less rapidly than the mean (Figure 3).

At a late stage in this work it was realised that variation between replicate diphenylamine digests imposes an element of uncertainty on dialysis results obtained by an isotope dilution method. To confirm the main conclusions of the previous experiments, a single experiment was carried out by a more direct method. Purified tetranucleotides (10 ml., 0.61 μmoles pyrimidine/ml.) from a diphenylamine digest of calf thymus DNA were dialysed against 700 ml. distilled water at 4° for 65 h., and the bag contents and diffusate fractions separately concentrated to dryness at 30° and resolved into the components of different base composition by ion-exchange chromatography. The percentage loss for each group was assayed directly by ultraviolet absorption. A dramatic effect of cytosine content on the rate of dialysis was confirmed (figure 6). The mean loss of tetranucleotides into the diffusate was 21%: much less than in the previous series of experiments, which were however not carried out under comparable conditions of temperature and oligonucleotide concentration.

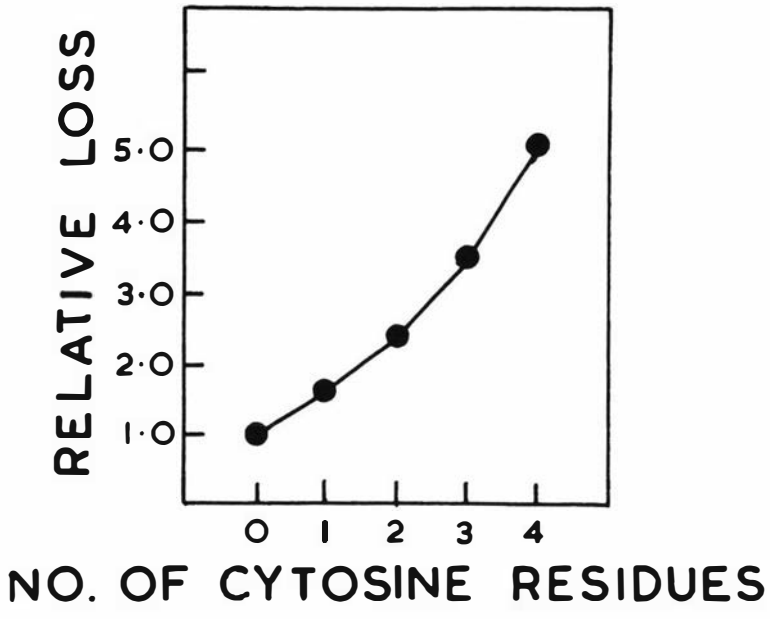
It is perhaps not surprising that appreciable losses occurred during the prolonged dialysis of quite long oligonucleotides. The largest oligonucleotides examined in this work have a molecular weight of less than 7,000, and the major reason for the slowness with which they penetrate dialysis membranes is probably the high negative charge they carry in neutral solution rather than size.* The pronounced effect of base composition could also be interpreted in terms of charge. Since

* It should be noted that no allowances were made for hypochromicity. This may mean that the losses of the longer oligonucleotides (figure 3) have been overestimated.

Appendix II.

Figure 6. Dialysis of Purified Tetranucleotides Against Distilled Water.

The % loss into the diffusate for each isomer group was assayed by ultraviolet absorption measurements at 270 mμ, and is expressed relative to the % loss of T_4p_5 (10.4%).



the pH during dialysis against distilled water was probably less than 7.0, an appreciable fraction of the cytosine residues may have been positively ionised, thus reducing the overall negative charge on cytosine-containing sequences. This interpretation does not however readily explain why the cytosine effect should have been very much greater for the tetra- and pentanucleotides than for the dinucleotides (Figure 4).

For this reason, it is preferred to interpret these results in terms of composition-dependent base interactions. These could involve stacking between adjacent bases, which recent studies with polyribonucleotides[6] have shown to have a profound influence on the conformation of polynucleotides in solution, or alternatively hydrogen-bonding between non-adjacent bases, which is believed to be the main force stabilising the conformation of transfer RNA. Hydrogen-bonding would seem a priori a more likely explanation, because although base-stacking interactions (in polyribonucleotides) are found in polycytidylic acid but not polyuridylic acid at low ionic strength[7,8], these interactions are non-cooperative, so that the difference in conformation between a stacked and an unstacked dinucleotide is unlikely to be very much less than the difference in conformation between a stacked and an unstacked pentanucleotide. In contrast to base-stacking, hydrogen-bonding between bases tends to be a strongly cooperative process, as shown by melting curves.

There are strong indications that the base interactions responsible for the cytosine effect on dialysis are cooperative in nature; not only from the re-inforcement by increasing chain length shown in figure 4, but from the upward curvature shown in figures 4 and 6. Furthermore,

the effect is strongly dependent on temperature (compare figure 4 with figure 6), a result not expected for base-stacking interactions.

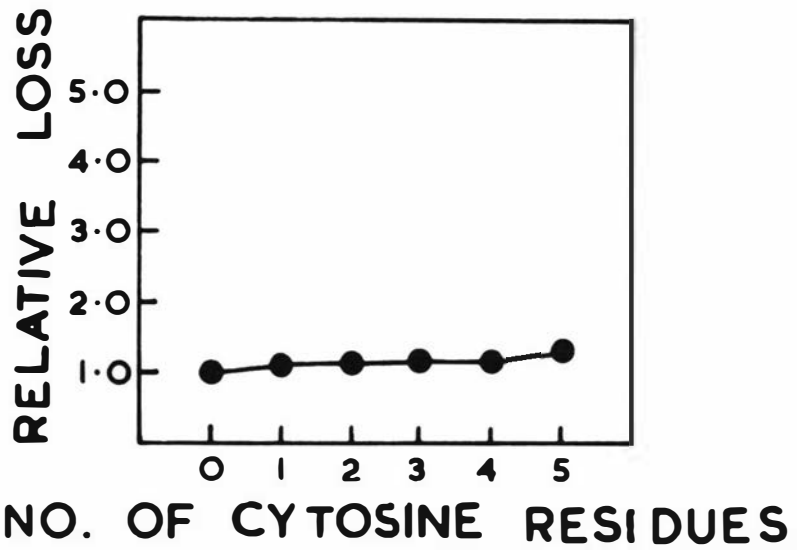
A single experiment showed a dramatic effect of ionic strength. Purified pentanucleotides (10 ml., 0.35 μmole pyrimidine/ml.) from a diphenylamine digest of calf thymus DNA were dialysed against 0.1 M triethylammonium bicarbonate buffer pH 8.3 at 4° for 84 h., and the loss of each isomer group measured directly, as in the previous experiment. The mean loss of pentanucleotides into the diffusate was 75%. As shown in figure 7, the cytosine effect was apparently completely eliminated.

This provided a useful means of desalting oligonucleotide mixtures without selective loss of the cytosine-rich sequences. However, prolonged dialysis remains clearly unsuitable for the preliminary fractionation of diphenylamine digests without substantial losses of even the longest pyrimidine nucleotides present, and dialysis against distilled water remains unsuitable for any purpose other than the rapid desalting of homogeneous sequence preparations.

Appendix II.

Figure 7. Dialysis of Purified Pentanucleotides Against 0.1 M Triethylammonium bicarbonate.

Losses of each isomer group into the diffusate are expressed relative to the % loss of T_5p. (65.4%).



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Appendix III. Gel Filtration of Pyrimidine Deoxyoligonucleotides

The discovery (appendix II.) that dialysis was an unsatisfactory method for the preliminary fractionation of diphenylamine digests made it desirable to develop an alternative.

It had been reported by Hohn and Schaller[1] that homologous series of deoxyoligonucleotides, of defined base composition and degree of phosphorylation, could be separated on the basis of chain length by chromatography on columns of Sephadex gel. Although they found a marked effect of base composition by comparing the two homologous series $d(pT)_n$ and $d(pA)_n$, the two pyrimidine series $d(pT)_n$ and $d(pC)_n$ showed closely similar elution patterns. These results were obtained using ammonium bicarbonate buffers of moderate concentration (0.01 M - 1.0 M) and pH 8.6, and are therefore compatible with the observation (appendix II.) of no base composition effect when pyrimidine pentanucleotides were dialysed against a similar buffer. Oligonucleotides were recovered quantitatively from the gel.

During the present study the absence of a strong base composition effect in gel filtration was confirmed using a wide range of buffers. The elution profiles in figure 1. show that in 0.1 M phosphate buffer pH 7.0 the cytosine-rich pentanucleotide dC_4Tp_6 is only slightly more retarded by sephadex G25 than the more thymidine-rich isomers making up the bulk of the pentanucleotide peak. The use of the less cross-linked gel G75 at low ionic strength (fig. 1b) or high pH (fig. 1c) did not result in any more marked effect of cytosine content. A cytosine-dependent fractionation was obtained by gel filtration on the

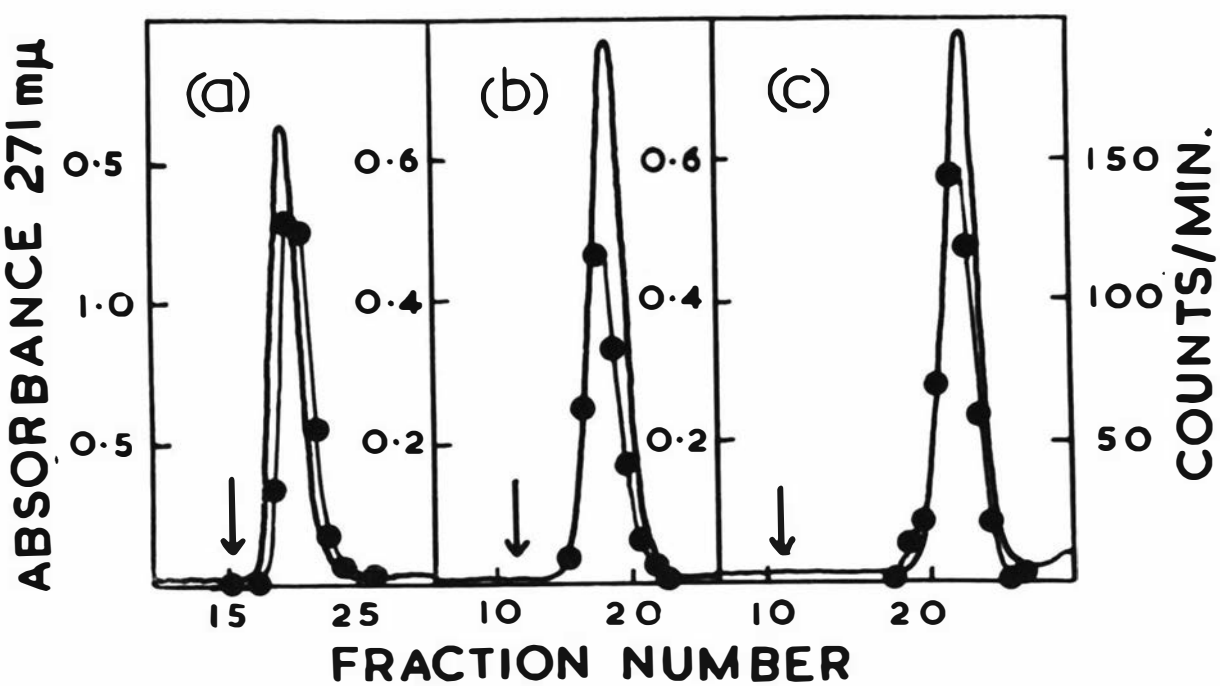
Appendix III.

Figure 1. Gel Filtration of Mixed Pentanucleotides.

^{32}P-labelled C_4Tp_6 isomers from a diphenylamine digest of phage α DNA were combined with a mixture of non-radioactive pentanucleotides from a diphenylamine digest of phage f1 DNA and desalted by dialysis. 0.5 ml. portions of the mixture were layered carefully onto 100 cm. x 1 cm. diameter columns of Sephadex gel and eluted at flow rates of 15-25 ml/h. 3-5 ml. fractions were assayed for ultraviolet absorption (271 mμ) and ^{32}P radioactivity (Beckman Low-Beta counter).

Gel grade and elution buffers:- (a) G25, 0.1 M phosphate buffer pH 7.0. (b) G75, 0.001 M phosphate buffer pH 7.0. (c) G75, 0.01 M borate buffer pH 10.0.

Arrows show void volume (DNA) for each column ——, 271 mμ absorbance; ●—●, ^{32}P.



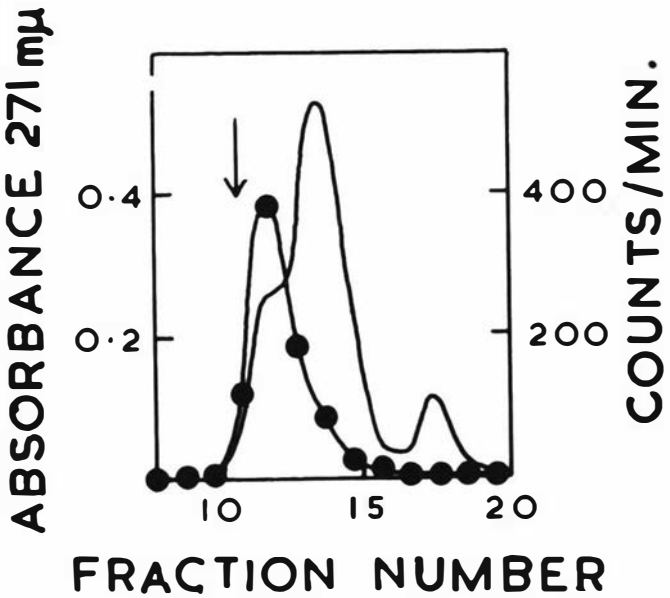
Appendix III.

Figure 2. A Base Composition Effect on Gel Filtration at pH 2.6.

Purified ^{32}P-labelled C_3Tp_5 and mixed non-radioactive tetranucleotides from a diphenylamine digest of phage f1 DNA were desalted by dialysis. A 0.5 ml. portion was layered carefully onto a 100 cm. x 1 cm. diameter column of Sephadex G15 and eluted with 0.1 M formic acid pH 2.6 at a flow rate of 18 ml/h. 4 ml. fractions were assayed for non-radioactive oligonucleotides by ultraviolet absorption measurements at 271 mμ, 260 mμ and 280 mμ. ^{32}P radioactivity was estimated by counting small portions with a Beckman Low-Beta Proportional counter.

Arrow shows void volume (Dextran blue).

——, 260 mμ absorbance; ●—●, ^{32}P.



tightly cross-linked gel G15 in 0.1 M formic acid pH 2.6, (Figure 2) but in this case dT_4p_5 (identified with the small trailing peak by its A_{280}/A_{260} ratio) was more retarded, and dC_3Tp_5 less retarded, than the main peak of tetranucleotides. This can probably be attributed to the weak ion-exchanging properties of sephadex gel that result from residual positively charged substituents in the polysaccharide matrix.

Sephadex gels proved to have excellent characteristics for preliminary fractionation of the oligonucleotides in diphenylamine digests on the basis of chain length. The most suitable buffers and gel grades, with typical results obtained in chromatography of depurinated diphenylamine digests, are shown in figure 3. Purine bases, when present, were eluted in a broad peak completely separated from the oligonucleotides. Inorganic phosphate was slightly more retarded than the peak of mononucleotides, but not completely separated. Routine fractionation of diphenylamine digests in this project employed sephadex G50 gel equilibrated with 0.1 M triethylammonium bicarbonate buffer pH 8.3. Similar fractionations were obtained using Sephadex G75 gel equilibrated with 0.01 M triethylammonium bicarbonate buffers. A joint experiment with Mr. W.P. Tate showed that such conditions gave quantitative recoveries (97.6% and 98.4% in two experiments) of ^{32}P-labelled oligonucleotides of chain length greater than six.

The partial resolution of a complex mixture of chain lengths suggested by the elution profile in figure 3 was confirmed by the excellent resolution of simple mixtures of chain lengths such as that shown in figure 4. No evidence was obtained from such elution profiles

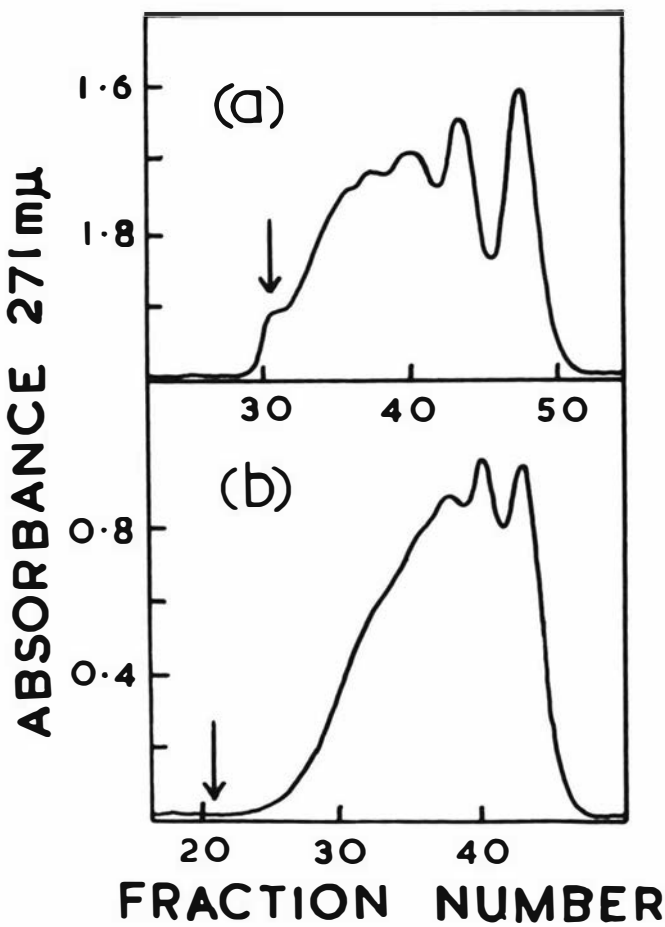
Appendix III.

Figure 3. Preliminary Fractionation of Diphenylamine Digests by Gel Filtration.

0.05 ml. portions of a depurinated diphenylamine digest of calf thymus DNA (50 mg. DNA/ml.) were chromatographed on 150 cm. x 1 cm. diameter columns of Sephadex.

Gel grades and elution details - column (a) G25, 0.1 M ammonium bicarbonate buffer pH 8.3, flow rate 20 ml/h., 2 ml. fractions. Column (b) G50, 0.1 M triethylammonium bicarbonate buffer pH 8.3, flow rate 15 ml/h., 3 ml. fractions.

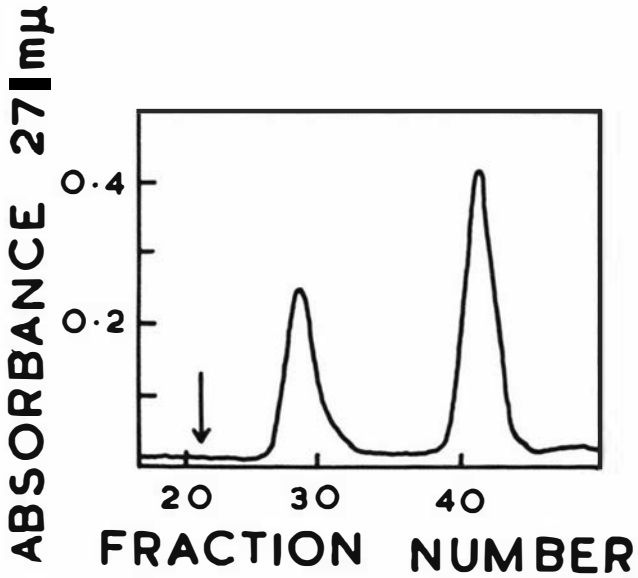
Arrows show void volume (Dextran blue) for each column.



Appendix III.

Figure 4. Gel Filtration of a Simple Mixture of Oligonucleotides on Sephadex G50.

Mononucleotides and nonanucleotides isolated by ion-exchange chromatography from a diphenylamine digest of calf thymus DNA were separated by gel filtration on a 150 cm. x 1 cm. diameter column of Sephadex G50 equilibrated with 0.1 M triethylammonium bicarbonate buffer pH 8.3. Flow rate 17 ml/h., 3.2 ml. fractions. Arrow shows void volume (Dextran Blue).



of any base composition dependent fractionation.

Another series of experiments in which balanced mixtures of cytosine mono- , di- , tri- and tetra- nucleotides were chromatographed on 150 cm. x 1 cm. diameter columns of Sephadex G25 gel showed that no unusually compact conformations for dC_3p_4 and dC_4p_5 could be demonstrated (at 25°C) in 0.1 M ammonium bicarbonate buffer pH 8.3, 0.1 M phosphate buffer pH 7.0, 0.1 M acetate buffer pH 5.5, or 0.1 M formate buffer pH 4.0.

Hohn and Schaller[1] concluded that the gel elution behaviour of an oligonucleotide was determined by the degree of cross-linking of the gel, the pH and ionic strength of the elution buffer, and the base composition and molecular charge of the oligonucleotide. During the present work it was found that the cations present in the elution buffer could also be of importance, as shown by comparison of the elution profiles in figures 3 and 5. In the presence of the bulky triethylammonium cation, the retention of oligonucleotides on either G50 or G25 gel was substantially reduced.

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Appendix III.

Figure 5. Effect of the Triethylammonium (Tea^+) Cation on the Gel Filtration of Oligonucleotides.

Further experiments on the type shown in figure 3. (a) Sephadex G50 equilibrated with 0.1 M tris/HCl pH 8.3, 0.1 M Tea^+ Cl^-. Flow rate 15 ml/h., 3 ml. fractions. (b) Sephadex G50 equilibrated with 0.1 M tris/HCl pH 8.3, 0.1 M NH_4^+ HCO_3^-. Flow rate 15 ml/h., 3 ml. fractions. (c) Sephadex G25 equilibrated with 0.1 M Tea^+ HCO_3^- pH 8.3. Flow rate 20 ml/h., 2 ml. fractions. Arrows show void volume (Dextran blue) for each column.