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INTRASTRAND SECONDARY STRUCTURE IN CHROMATOGRAPHICALLY FRACTIONATED STRANDS OF *BACILLUS SUBTILIS* DNA

by

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Abstract

INTRASTRAND SECONDARY STRUCTURE IN CHROMATOGRAPHICALLY FRACTIONATED STRANDS OF BACILLUS SUBTILIS DNA

by

DENISE ALLENE GALLOWAY

Adviser: Professor Rivka Rudner

A population of molecules from fractionated B.subtilis DNA has been isolated which is rich in non-transcribed, non-transforming, presumably regulatory sequences. These structures have been shown to be scattered throughout the chromosome and to comprise 5-10% of the genome. These structures were isolated by hydroxyapatite (HA) fractionation of the self-annealed separated strands. Bacillus subtilis DNA was strand separated methylated albumin kieselguhr (MAK) chromatography. The isolated strands were self-annealed at 68^0 and the material which had renatured was separated from the rest of the DNA by hydroxyapatite.

Both strands of DNA (designated L and H) showed the same amount of self-annealing material as judged by physical parameters, e.g. elution from hydroxyapatite, melting profiles. The biological activity of the self-annealing structures from the two strands was not equivalent. Only the structures derived from molecules of the late eluting H strand showed increased levels of transforming activity (6-10% that of native DNA) following self-annealing. The late eluting DNA of the MAK H

strand has been shown to be of biological importance because of its high levels of hybridizability to both pulse-labeled and ribosomal RNA.

The base composition of the self-annealed DNA showed no unusual characteristics as determined by melting profiles and by direct analysis. The buoyant density of the self-annealed DNA is consistent with that of renatured B. subtilis DNA. The extent of base pairing in the self-annealed molecules was investigated by treatment with S_1 nuclease and temperature absorbance measurements. Twenty five percent of the self-annealed molecule from the HA purified fraction was base paired.

The chromosomal location of the self-annealing DNA was determined by the ability to transform for widely scattered markers, e.g. adenine, leucine, methionine, threonine, tryptophan, and histidine. The low levels of transforming activity associated with these structures indicated that there were many non-transforming sequences present in the population. The biological information carried by these structures was further investigated by hybridization to a pulse-labeled log-phase RNA. This showed that the self-annealed structures of the H strand contained about 20% of the transcribed sequences present in the total MAK H strand, indicating the presence of many non-transcribed sequences. Hybridization to the isolated components of stable RNA indicated that these genes were present in the self-annealing fraction but with different frequencies, with the 16S and 5S genes having a greater likelihood of fractionating with the self-annealed molecules.

A model has been proposed to explain the existence and function of intrastrand secondary structures. A wide variety of regulatory proteins have been shown to recognize sequences of DNA which contain

self-complementary sequences, i.e. ATGCTAGCAT . These sequences could serve as nuclei for reassociation when isolated strands are self-annealed. Extensive secondary structures around the initiator codon of messenger RNAs may be necessary for either ribosome binding or for protection from nucleases. The secondary structure seen in the RNA could be reflected in the DNA which codes for it. While these structures have only been isolated *in vitro*, they may also serve as regulatory sites *in vivo*.

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INTRODUCTION

The ability of isolated strands of Bacillus subtilis DNA to form regions of secondary structure was first described by Rudner and Remeza (1973). This observation implied that a single strand of DNA contained self complementary sequences which could form secondary structure upon annealing. It is the purpose of the study presented here, to investigate the biological implications of such structures, as well as to characterize their physical and chemical properties.

The intrastrand structures were analysed with regard to the following parameters: molecular size, buoyant density, base composition, thermal denaturation profile, retention by hydroxyapatite, and resistance to S_1 nuclease. The biological nature of these structures was studied by investigating their transforming activity for a wide variety of markers, and their hybridizability to both pulse-labeled and stable RNA. The experiments described were performed with two goals in mind: first, to see if the DNA capable of forming these structures was, in any way, dissimilar from the rest of B. subtilis DNA, and second, to determine what types of sequences (regulatory, structural genes, ribosomal genes) were involved in secondary structures.

In this introduction, an attempt will be made to provide a basis for understanding why intrastrand secondary structure exists and also for understanding the relevance of the results obtained. To that end a discussion of factors affecting the stability of both double stranded and single stranded polynucleotides is presented. In addition, evidence of this type of structure in other systems, eukaryotes, plasmids and viruses

is also given, as well as evidence that such sequences or structures may be recognized by regulatory proteins.

1. Stability of the helix

In 1953 Watson and Crick deduced the structure of deoxyribonucleic acid (DNA) from its X-ray diffraction pattern. DNA is a double strand, right-handed helix. The two strands are held together by the hydrogen bonding of the interstrand pairs of complementary purines and pyrimidines. The helical structure is further stabilized by the bonds between water molecules and virtually all the surface atoms in the sugar and phosphate groups. Further stabilization occurs through stacking interactions: intrastrand attraction of the adjacent bases.

The problems in, and methods of calculation the contributions of the bases to helix stability have been described by DeVoe and Tinoco (1962). They avoided the involvement of the solvent and calculated the free energy of the configuration in a vacuum. Bradley et al (1964) have made corrections in their calculations which automatically included the effects of hydrogen bonding. The value calculated by Nash and Bradley (1965) for the Watson-Crick AT pair was about -5.3 kcal per base pair while the energy of the Watson-Crick GC pair was about -17 to -19 kcal per base pair. The greater energy of the interaction of the GC pair was entirely attributable to the additional hydrogen bond.

The contribution of "stacking interactions" to the stability of the helix has been investigated by Crothers and Zimm (1964). They determined that the free energy change in stacking a hydrogen vonded base pair on an already existing hydrogen bonded base pair was on the order of -7kcal. The contributions to the free energy of formation must also include the interactions of the negatively charged phosphate groups of the backbone.

This included both the electrostatic interactions between the phosphate groups themselves, and their interaction with the ions in the solvent. Most of these calculations assumed that the intrachain phosphate repulsions were the same in the helix and the coil, since the distance between phosphates in the helix is almost the maximum possible for the fully extended chain, and that these repulsions would tend to maintain extended configurations in the random coil.

The stability of the double strand helix (Levine et al, 1963) as well as the single strand polynucleotides (Hamaguchi and Geiduschek, 1962) is very dependent on the nature of the solvent. Sinanoglu and Abdulmer (1964) examined the effects of various solvents on the DNA helix-coil transition. They found that the largest energy term comes from enlarging the cavity in the solvent to accomodate the bases of the coil form. This varied from -38 kcal/mole of base pairs in water to -12 kcal in mathanol. They showed that the change was mainly due to enthalpy rather than entropy. The conclusion of these studies was that a) the stability of the helix is favored in water because of the unfavorable enthalpy changes due to the interactions with the solvent in a helix-coil transition, and b) other solvents, such as methanol or formamide, have a destabilizing effect on the helix by reducing the energy required for helix-coil transitions.

At physiological temperatures the stability of a double strand helical molecule is favored. The disruption of the helix breaks hydrogen bonds and brings the hydrophobic bases into contact with water. Since the DNA molecule has a great many weak bonds arranted so that one bond break involves the simultaneous breaking of other bonds, the reformation of the hydrogen bond is highly favored.

At one time, tautomeric shifts involving the movement of a hydrogen atom from one ring nitrogen to another oxygen atom, were thought to occur fre-

quently, This is now known not to be true (Watson, 1970). The ring nitrogen usually exists in the amino form (NH_2) and only rarely in the imino form (NH). Likewise, oxygen atoms attached to the C_6 atoms of guanine and thymine are usually in the keto form (C=O)rather than in the enol form (C-OH). These relatively fixed locations are essential for the correct pairing of adenine with thymine and guanine with cytosine.

The helical structure of the polynucleotide is not entirely static however, but is constantly undergoing some disruption and reformation of its structure. The studies measuring the rates of tritium exchange have shown that base bound protons are able to exchange with the solvent (Englander and Englander, 1965; Printz and Von Hippel, 1965).

2. Stability of RNA

In contrast to the structure of DNA, ribonucleic acid (RNA) generally exists as a single stranded structure. This is true of all of the species of RNA transcribed from cellular DNA as well as for RNA from a variety of RNA viruses. Single strand RNA molecules showed a temperature-dependent effect on optical activity and hyperchroism in solution. Doty et al (1959) and Spirin (1960) explain this behavior as due to extensive base pairing to form double stranded regions, rather than being due to single strand base stacking. Cantor et al (1966) substantiated this conclusion by optical rotatory dispersion analysis of alanine tRNA.

Looking at the rate at which tRNAs exchanges tritium with the solvent, Englander and Englander (1965) established an upper limit of 82% of the bases involved in base pairing. The base pairing did follow the typical Watson-Crick pairing as shown with homopolymers. Poly rA, although a random coil, showed temperature dependent hyperchroism and optical rotatory dispersion patters which were interpreted to indicate base pairing (Fresco

and Klemperer, 1959). Using the homoribopolymers N_6-hydroxyethyl adenylic acid (Van holde et al 1965) and N_6, N_6-dimethyl adenylic acid (Griffen et al 1964), both of which block the normal hydrogen bonding of adenine, it was shown that their optical rotatory patterns and the temperature dependent absorbance was not much different than with poly rA, indicating that base pairing was not responsible for these phenomena. The patters were, however, quite different that those obtained with polymers which were capable of true Watson-Crick pairing.

Although the ordered secondary structure of many RNAs has been confirmed, the energy source for the stabilization of these structures in unknown. It has been calculated (McDonald et al, 1965) that the stabilization will be favored by the stacking of bases, which will reduce their interactions with the solvent, though the contributions will be smaller than in the double strand structures. There was evidence from nuclear magnetic resonance studies that the bibose ring was held in a mroe rigid structure than the deoxyribose ring perhaps due to the hydrogen bonding of the 2'OH groups (McDonald et al, 1965). Studies comparing the synthetic single stranded polydeoxyribopolymers formed a less stable single strand structure based on denaturation data (T'so et al, 1963).

At first it was thought that DNA only existed as a double strand structure, except as a transient state during replication and repair of the double strand molecule. In 1959, Sinsheimer showed that during the life cycle of the bacteriophage ØX 174, the DNA was entirely single stranded except for replication. Other single stranded phages, e.g. F1, M13, etc. have also been described. Although the physical chemistry of nucleic acids clearly indicates that a double helical structure is more energetically favored, biological organisms have been able to maintain single stranded DHA molecules.

3. Self complementary sequences in DNA and their interactions with proteins

Grier (1966) has proposed that DNA can exist in structures other than the Watson-Crick double helix. A sequence of symmetrically arranged bases could rearrange their hydrogen bonds to form a cloverleaf structure. Gilbert and Muller-Hill (1967) and Sobel et al (1971) have suggested that specific structural proteins, such as repressors, polymerases, melting proteins, or recombinational proteins can induce this change.

Sobel has extended this model to suggest a general mechanism for protein recognition of DNA sequences. It involves recognition of two-fold or four-fold symmetry. By postulating a tandem genetic duplication of a symmetrical sequence he showed how tetrameric proteins could recognize these structures. If the DNA is capable of forming a cloverleaf structure with the repeated sequences, the loops could serve as a recognition site. A model for this type of structure is given in Figure 1.

There is growing evidence that many proteins and other smaller molecules are able to react specifically with certain sequences of DNA by recognizing regions of symmetry. Sobel et al (1968) have proposed a stereochemical model for actinomycin-DNA binding from X-ray crystallographic data which involved intercalation of the atibiotic and recognition of the symmetrical sequence:

G C
C G

The acid DNase from spleen causes double strand scissions in DNA and is strongly inhibited by actinomycin D. Since it is a dimer of two identical subunits Bernardi (1968) has postulated that the enzyme can recognize a dyad axis on DNA and that each subunit has a catalytic site with which it can simultaneously cleave a phosphodiester bond on each strand.

A variety of restriction enzymes have been shown to recognize sym-

metrical sequences. Kelly and Smith (1970) first characterized a hexanucleotide sequence recognized by a restriction enzyme from Hemophilus influenza:

G T Py Pu A C

C A Pu Py T G

Evidence that two-fold symmetry was involved at the recognition site was provided by Meselson and Yuan (1968) who made heteroduplixes of DNA in which one strand was modified and showed that the other strand was protected from cleavage also.

The repression of the lactose operon occurs by the interaction of a 150,000 dalton tetramer of identical subunits with a region of DNA about 27 base pairs long. Gilbert and Maxam (1973) were able to obtain the fragment of DNA which was protected from pancreatic DNase digestion by the repressor. The sequence they obtained showed very good symmetry:

T G G A A T T G T G A G C G G A T A A C A A TT

A C C T T A A C A C T C G C C T A T T G T T AA

This would allow the repressor to interact with the DNA on a two-fold axis of symmetry. Steitz et al (1974) has suggested from X-ray crystallographic studies of the repressor-operator complex that two subunits interact with the left hand region and the other two subunits interact with the right hand region related by a two-fold axis of symmetry, although the overall structure of the tetrameric repressor is not exactly symmetrical.

Similar studies protecting the λ operators with repressor have led to the sequencing of these regions by Maniatis and Ptashne (1973). They found a series of concatenated binding sites of 35 - 100 base pairs in length. A dimer binds to the original site on the DNA and is subsequently filled with more dimer repressor molecules. The two λ operators are not identical in their sequence. The λ repressor does not act as a melting protein but binds to, and stabilizesthe helical structure.

Another approach has also been used to isolate promoter regions. The genes for a tRNA molecule have been chemically synthisized by Khorana et al (1972). These genes can be annealed to the complementary strands of ϕ 80 p Su III which contains the gene for $tRNA_{tyr}$. Elongation in one direction by DNA polymerase I gives the sequence of the promoter, and elongation in the other direction gives the sequence of the terminator. The sequence adjoining the 3' terminus of the E. coli $tRNA_{tyr}$ determined by Loewen et al (1974) is given below:

3' G G T A G T G A A A G T T T T C A G G G A C T T G A

C C A T C A C T T T C A A A A G T C C C T G A A C T

The terminal sequence corresponding to the CCA of the t RNA is shown in this sequence. The possible terminator site contains a symmetrical sequence of 6 bases which may be used as a recognition site for rho, a terminator protein which is composed of identical subunits (Oda and Takanami, 1972).

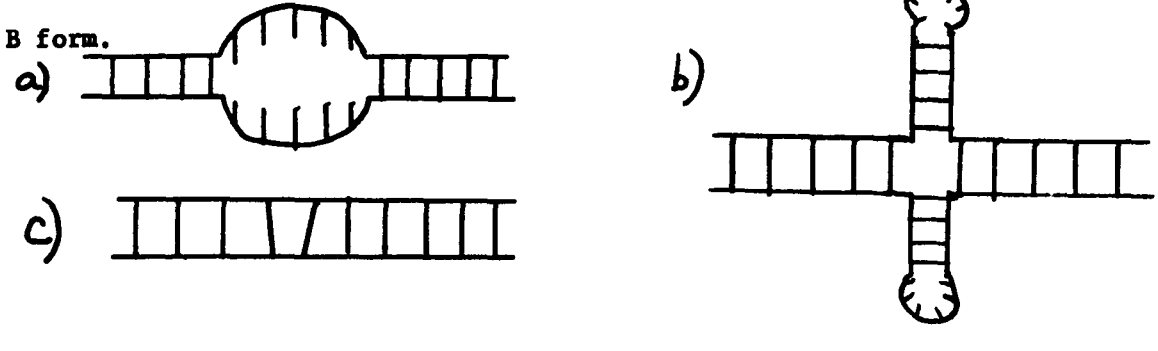
Robertson et al (1973) have isolated a fragment of the + strand of ϕX 174 DNA which was protected from the action of pancreatic DNase by its binding to the ribosomes. This strand contains the same sequences as the mRNA and should probably contain the information for initiation of protein synthesis. The sequence they found contained an ATG initiator codon at the top of the hairpin loop, suggesting that the secondary structure can run into the beginning of a structural gene.

Bronson et al (1973) have obtained a transcription product from the tryptophan operon containing a nucleotide sequence for the first eleven amino acids of the anthranilate synthetase enzyme and a "leader" sequence of about 150 nucleotides. Unlike the ϕX 174 fragment, the initiation codon for the trp E gene could not be drawn as part of a hairpin loop. Preliminary evidence, however, suggested that there is extensive secondary structure in the leader sequence.

As more operator, promoter, and terminator sequences are determined, a clearer understanding of protein - DNA interactions will come forth. It is possible that a common feature is these types of regulatory sites will be the requirement for symmetrical sequences. Heyden et al (1972) reported that the presumptive promoter regions of several bacteriophage, isolated by virtue of their protection from DNase digestion by RNA polymerase were AT rich (60-65%) but would not anneal to each other. By the direct sequencing of promotor sites, Pribnow (1975) has shown a common sequence for the RNA polymerase protected fragments of T_7A_3, fd, SV40, lambda, tyrosine t RNA, and lac wild type. The sequence contains a self complementary arrangement:

T A T Pu A T G 3'
A T A Py T A C 5'

Based on thermodynamic considerations, Sobell (personal communication) suggested that the recognition of these sequences occured in the intact duplex rather than by a mechanism which involved strand separation or the formation of Grier structures. The work of Chan and Wells (1974) however, suggested that the lactose operator may exist in some form other than a double helix prior to the binding of repressor. They have shown the repressor binding capacity to be exquisitively sensitive to the action of two single strand specific nucleases but not to nonspecific nicking agents. They proposed three possible models to explain its tructural uniqueness: a) a region of low thermostability due to an AT rich region, b) a cruciform structure, or c) a helical configuration in a different, non-DNA B form.



The operator sequence data would allow either a or b, but the insensitivity of the nuclease to salt and to temperature favors c. Such helical variances have been recognized in DNA polymers (Wells et al, 1970) and Bram (1971) has suggested that regulatory proteins may be able to recognize unique helical forms.

The normal in vivo structure of cellular DNA, is predominantly in the double helix with only a transient formation of single stranded structures for replication, recombination, or repair. RNA on the other hand, usually exists as a single strand polynucleotide chain, which stabilizes its structure by forming extensive intrastrand secondary structure. When DNA strands are denatured and separated, the potential for each of these strands to form secondary structure exists. Given the prevalence of symmetrical sequences for various regulatory functions, these self-complementary sequences could serve as nuclei for the formation of intrastrand secondary structure in isolated strands of DNA.

4. Self complementary sequences in eukaryotic DNA

Self-complementary sequences resulting from an inverted repetition have been shown to be a general feature of DNAs from higher eukaryotes. These inverted repeats, e.g. $\begin{matrix}\text{ACCTATAGGT}\\ \text{TGGATATCCA}\end{matrix}$ were termed palindromes by Wilson and Thomas (1973) which is not in agreement with the usual usage of the word to refer to a sequence that reads the same in both directions, e.g. ATCGCTA. The structures described by Wilson and Thomas contained between 300 and 1200 nucleotides in clusters of two to four. Based on these calculations, eukaryotic DNA could contain thousands to hundreds of thousands of these clusters. They were isolated by denaturation with strong alkali and reneutralized at high dilution, followed by absorbtion to hydroxyapatite. Under these conditions only inverted repetitions that are adjacent to each other were able to reassociate to form a hairpin loop with very few

nucleotides in the "turnaround" region. Electron microscope studies have shown (Wilson and Thomas, 1973) that these structures are due to intrachain reassociation rather than to a cross-link between the two strands. The thermal stability of the hairpins was the same as that of the bulk DNA of comparable size. This was unlike the rapidly reassociating DNA described by Britten and Smith (1970) which had a low thermal stability, but it was similar to the thermal stability reported for isolated chains of mouse satellite DNA.

The functinn of these palindromes is still a matter of speculation. As early as 1958 Schwartz argued that the formation of these cruciform structures could aid in the segregation of chromatids. Sobel (1972) has formulated a model for recombination in eukaryotes involving these structures. Grier (1966) has suggested that they serve as recognition sites for proteins. Support for this idea comes from the nature of the sites recognized by restriction enzymes, the lac repressor and RNA polymerase. The palindromes reported by Wilson and Thomas, however, are at least 10 times longer than those reported in prokaryotes or in viral RNAs.

Another problem in determining the function of palindromes is that these structures have not been detected in purified native DNA. There is no evidence the DNA as it exists in cells, changes from a lineform to a cruciform structure. If the palindromes exist as hairpin loops, it must be a rare event at any given time.

5. Self complementary sequences in bacteriophage and plasmid DNA

Under the isolation conditions described by Wilson and Thomas, neither T_7 nor E. coli showed any detectable inverted repetitions. Self associating single chains have been described in the DNA of several bacteriophages and prokaryotes. Forsheit and Ray (1970) have found two conform-

ations of the single strand DNA phage, M13, measuring differences in sedimentation coefficient and by electron microscopy. The slower sedimenting form, present at high ionic strength, was though to be a more extended form having single stranded "bushes" separated by more rigid double strand regions. The faster sedimenting form, present in low ionic strength, formed a compact structure with many randon hydrogen bonds.

Schaller et al (1969) have found that about 2% of fd DNA remained resistant to E. coli exonuclease I, following a partial digestion with a single strand specific endonuclease from Neurospora crassa. The fd core contained a few fragments of chain length 35 to 45 nucleotides long, the fragments were characterized by a high G=C content, a pyrimidine isostich pattern that was less complicated than that of the total phage, and the retention of secondary structure following denaturation and renaturation, indicating a hairpin loop structure. A similar study on the digestion of bacteriophage f 1 DNA with DNase K_2, an exonuclease from Aspergillus oryzae, was reported by Shishido et al (1969). they found a T rich fragment, 30-50 nucleotides long, which showed hypochromicity on annealing and a high degree of renaturability.

Inverted repeats have also been described in E. coli plasmid DNA by Sharp et al (1973). In isolated strands of plasmid DNA, which were to be used in heteroduplex studies, loops containing duplicated inverted sequences reported by Wilson and Thomas, the inverted repeats were not tandemly arranged, giving rise to various size loops. The size of the unpaired loops varied between 198 to 7.9 kilobases (kb), and the number of bases in one arm of the stalk varied between .7 and 1.4 kb. The intramolecular recombinational events causing these inverted repeats seem to occur much less frequently in plasmids or in prokaryotes than they do in eukaryotes.

6. Self complementary sequences in prokaryote DNA

The ability of prokaryotic DNAs to form intrastrand secondary structure has been less well characterized. Stoller and Grossman (1962) reported that denatured E. coli DNA seemed to form intrachain hydrogen bonds based on reactivity with formaldehyde and resistance to E. coli exonuclease I. Self-complementary sequences have been reported in E. coli by Kato et al (1974). They took denatured DNA and renatured it at 60° at a cot of 0.5. They removed the single strand DNA with an exonuclease from N. crassa and purified the remaining DNA on MAK columns following the procedure of Brahic and Fraser (1973). They repeated this procedure twice more with the remaining material, each time renaturing at a cot of 0.5. This method enriched for the reassociated single strand material, which can not be detected by the normal reassociation kinetics of Britten and Kohne (1968).

The DNA they isolated by this method represented about 8% of the chromosome, of which they estimated 1/4, or 2% of the genome, was repetitive sequences. The melting profile of the fraction suggested it had the same overall base composition as total E. coli DNA. In neutral sucrose gradients the size of the original native fragment was 3.8×10^5 daltons, and the self-complementary fraction was 1×10^5. In alkaline gradients the respective sizes were 1.9×10^5 and 8.1×10^4. There was the expected two-fold difference for the native sample, but only a 1.3 fold difference for the self-complementary DNA, suggesting it contained a hairpin loop structure with unpaired bases in it.

Electron microscope studies by Chow and Davidson (1973) were unable to find any inverted repeats in B. subtilis DNA. They denatures B. subtilis 168 DNA in alkali, reneutralized it, and dialysed it against 60% formamide, 0.1 M Tris, 0.01 M EDTA, pH 8.5 at 4°C, and then against 0.01 M Tris, 0.001 M EDTA, ph 8.0 at 4°C. These conditions in other instances have

have been suitable for detecting inverted repeat sequences. Their studies were only able to detect loop formation in the regions of the rRNA cistrons where multiple copies of the genes can reassociate out of register. The average length of the DNA fragment was reported to be 130 kb, which corresponds to a molecular weight of 1.8×10^7 daltons.

Using isolated strands of B. subtilis DNA, the data presented here, and that of Rudner and Remeza (1973) and Galloway and Rudner (1974) have shown the presence of self complementary sequences in B. subtilis DNA. A population of molecules from MAK fractionated strands of B. subtilis DNA which are able to be fractionated on hydroxyapatite following self-annealing at 68° have been isolated. The kinetics of reassociation of these fragments followed first order kinetics, indicating that it is an intrastrand structure, rather than a bimolecular duplex.

It seems possible that eukaryotic, viral, and prokaryotic DNAs are all capable of forming intrastrand secondary structure, but to varying extents. These structures may be present in vivo or in purified native DNA although evidence for their presence is lacking. These sequences can be detected in the isolated strands of DNA following renaturation. The following biological roles for these sequences are possible: 1) they serve as recognition sites for repressors, polymerases, nucleases required for recombination or repair, and other regulatory proteins by offering two-fold axes of symmetry, 2) they may be part of structural genes which show the same ability to fold on themselves that structural genes of viral RNA molecules show, 3) they may be part of genes which code for stable RNA components, known to have a well defined secondary structure, 4) they may represent regions of DNA with unusual base composition, either AT or GC rich sequences, which could serve either as regulatory sites, spacers, or be a random occurence, 5) They could be intramolecular recombinations which

which occur randomly but are favored by offering additional sites of homology for genetic recombination, 6) the self-complementary sequences could be used to align the single strand ends of a viral DNA for ring closure or for integration into the host chromosome. Another possibility is that the intrastrand secondary structure serves no biological function and exists merely as a statistical probability for self-complementary sequences to occur which seems unlikely based on mathematical calculations of probability.

The purpose of the studies presented here is to characterize the self-annealing structures in MAK fractionated strands of B. subtilis DNA. The molecules were isolated from the bulk of the DNA by hydroxyapatite chromatography following renaturation at 68°. The molecules containing secondary structure eluted at 0.2 - 0.22 M sodium phosphate buffer. The molecules contained both the reassociated regions and the single stranded sequences present on the modecule. The extent of secondary structure present in the fraction was determined by temperature absorbance measurements and by digestion with the single strand nuclease S_1, from Aspergillus oryzae. Further characterizations of this material including its base composition, molecular weight, and buoyant density were carried out.

Although both strands of DNA contained self-annealing DNA, the biological activity of the two fractions was different. The biological nature of the self-annealing DNA was investigated by assaying the transforming activity of the DNA for a variety of markers, thereby ascertaining its location on the B. subtilis chromosome. The presence of transcribed sequences in this material was assayed by its hybridizability to both pulse labeled log phage RNA and to the isolated components of stable RNA. These studies are important in determining the nature and significance of intrastrand secondary structure in prokaryotic DNA.

MATERIALS AND METHODS

BACTERIAL STRAINS

B. subtilis A26u$^-$, a uracil auxotroph of strain 168 provided the source of most of the DNA used. It was also used in the preparation of the ^{3}H-uridine labeled RNA. The strain was provided by D. Dubnau. Preliminary experiments also used the prototrophic strain W23S^{r}E^{r}M^r as a source for DNA. The mutant derivatives of the *B. subtilis* strain 168, BD-204 (*hisB*$_2$, *thy A*, *thy B*) and Mu8u5u16 (*leu8*, *met5*, purA16) were used in the preparation of ^{3}H-thymidine and ^{3}H-adenine labeled cells, respectively. The following mutant derivatives of the 168 strain were used as the recipients in transformation assays: Mu8u5u16 (*leu8*, *met5*, *purA16*) BD-170 (*thr-5*, *trp-2*), and SB-25 (*hisB*$_2$*trp-2*). The latter two were kindly supplied by D. Dubnau.

GROWTH OF UNLABELED CELLS

Cells were inoculated into Pennassay (Difco Antibiotic Selection Media No. 3) and grown until stationary phase. A dilution of this culture, 0.2 ml of a 10^{-3}, was inoculated into a 500 ml of medium containing veal infusion broth (Difco) and 0.50% yeast extract. The cells were grown overnight at 37^o with shaking. The cells were then harvested by centrifugation.

GROWTH OF ^{3}H-THYMIDINE LABELED CELLS

A thymine requiring strain of *B. subtilis* was grown in Spizizen salts (see appendix A) supplemented with 0.50% glucose, 1.0% glutamate, 0.5% vitamin-free casamino acids, 100 ug/ml l-histidine, 50 ug/ml l-tryptophan, and 2 ug/ml unlabeled thymidine. After one doubling, 1 uCi/ml of thymidine methyl ^{3}H (specific activity 54 Ci/mM, Schwarz-Mann) was added and the cells were allowed to grow until the beginning of stationary

phase. Bacterial growth in all cases was determined turbidimetrically with a Klett, using a red filter (660 nm). The cells were harvested by centrifugation.

GROWTH OF ^{3}H-ADENINE LABELED CELLS

An adenine (leucine and methionine) requiring strain of B. subtilis 168, Mu8u5u16, was grown in Spizizen salts supplemented with 0.50% glucose, 1.0% glutamate, 0.1% vitamin-free casamino acids, 50 ug/ml leucine, 50 ug/ml l-methionine and 10 ug/ml unlabeled adenine. After one doubling 4 uCi/ml of ^{3}H-adenine were added and the cells were grown to stationary phase. The cells were harvested by centrifugation.

GROWTH OF ^{32}P-labeled cells

Two separate preparations of ^{32}P-labeled cells were made using a B. subtilis 168 strain, A26u^{-}. In both cases the radioactive phosphate was in a medium that had been previously used for pulse labeling and was re-adjusted to support growth. The cells were adapted over two days to a low phosphate medium, 5 x 10^{-4}M PO_4, in Spizizen salts (minus phosphates), 0.50% glucose, 1.0% glutamate, 0.1% casamino acids, and 100 ug/ml uridine. Cells were transferred from this medium to Sterlini and Mandelstam medium (see appendix A) and allowed to grow for one doubling. A26u^{-} cells in Resuspension medium (see appendix A) were pulse labeled with 33 uCi/ml of $H_3{}^{32}PO_4$ (Schwartz and Mann) in both experiments. The labeling was stopped by adding frozen 2x Adjustment medium (see appendix A) to supply nutrients which may have become depleted. The cells were then spun down and then the remaining media was diluted with the A26u^{-} cells in Sterlini and Mandelstam medium to give a final concentration of approximately 7u Ci/ml of phosphate. The cells were grown to stationary phase and harvested by centrifugation.

GROWTH OF COMPETENT CELLS

Competent cells were prepared by a modification of the two-steop growth procedure of Anagnostopoulos and Spizizen (1961). Cells grown overnight in Pennassay were resuspended in medium I which contained Spizizen salts, 0.5% glucose, 0.02% vitamin free casamino acids, 0.1% yeast extract, 0.8% l-arginine, and 100 ug/ml of each required amino acid or nucleoside except for 50 ug/ml of tryptophan and histidine. The cells were grown from 4 to 4 1/2 hours, until stationary phase was reached. The cells were then diluted 1:10 into medium II which contained Spizizen salts, 0.5% glucose, 0.01% vitamin-free casamino acids 0.05% yeast extract, 2.5×10^{-3}M $MgCl_2$, 10^{-3}M $CaCl_2$, 5×10^{-4}M spermine tetrahydrochloride, and 50 ug/ml of each required amino acid or nucleoside except for 5 ug/ml of tryptophan and histidine.

The cells were grown in this medium for 90 minutes at which time they were at maximum competency. The cells were either used immediately for transformation or concentrated 10 fold by centrifugation, adjusted to 10% glycerol, and frozen.

EXTRACTION OF DNA

The DNA was extracted by a modified Marmur (1960) procedure. The harvested pellet was washed three times with saline-EDTA (0.15M NaCl 0.1 M EDTA, pH 8.0) and resuspended in 5ml saline-EDTA per gram of cells. Lysozyme (salt free, Worthington) was added to a concentration of 2 mg/ml. Lysis was allowed to continue at 37^{o} until the solution turned caramel in color and became very viscous. Sodium lauryl sulphate (SLS) was added to a concentration of 2% and the solution was heated at 60^{o} for 10 minutes until complete lysis had occured. The solution was then cooled and adjusted to 1M sodium perchlorate. An equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added and the mixture was shaken vigorously for 30

minutes followed by centrifugation at 10,000 g for 5 minutes. Two volumes of cold ethanol were added to the supernatent to precipitate the DNA. The highly polymerized DNA was spooled and dissolved in 0.1 x SSC (1 x SSC - 0.15 N NaCl, 0.015 M Na citrate). The solution was treated with pancreatic RNase (50 ug/ml, Worthington(and RNase T_1 (50 ug/ml, Worthington) for 90 minutes at 37°. The solution was further deproteinized by the action of phenol, ether (to remove traces of the phenol) and chloroform-isoamyl alcohol, and finally reprecipitated with ethanol. The DNA was redissolved in 0.1 x SSC. A second round of purification was done starting with the RNase treatment and including the deproteinization steps. After precipitation in ehhanol for the third time, the DNA was dissolved in 0.1 x SSC and dialyzed against 1 x SSC overnight at 4° (see table 1 for the specific activities of the radioactively labeled preparations).

DETERMINATION OF THE CONCENTRATION OF DNA

The UV absorbance of the sample was measured with a Beckman DU Model spectrophotometer. The samples were placed in quartz cuvettes and the obsorbancy at the maximu, 260 nm, was measured. The concentration was determined using E_{260} for native DNA of 20. The contamination with protein was determined by taking the ratio of the absorbance at 280 nm to that at 260 nm. The expected ratio for pure DNA is 0.5.

PREPARATION OF RNA

The RNA preparations used were the generous gift of Dr. Yoshiko Setoguchi. The RNA was extracted from ^{3}H-uridine labeled cells of the B. subtilis 168 strain A26u$^-$ following the procedure of Oishi and Sueoka (1965) and as described by Margulies et al 1970). The ribosomal RNA preparations were extracted from cells that had been grown to almost station-

ary phase in radioactive uridine, then resuspended in high concentrations of non-radioactive uridine for 40 minutes. Actinomycin D (5 ug/ml, Schwartz-Bio Research) was then added for 10 minutes to exhaust mRNA (Levanthal et al, 1972). A pulse-labeled log phase RNA was extracted from B. subtilis A26u$^-$ cells grown in a rich medium then shifted down to a low-uracil containing medium (Margulies et al, 1970). After a lag period ^{3}H-uridine was added for 90 seconds and the pulse was rapidly stopped by the addition of ice. The ribosomal content of this preparation was shown to be 33% by hybridization competition experiments.

A second preparation of ^{3}H-uridine labeled RNA was obtained from a graduate Molecular Biology course, Fall 1971. The 16S and 23S species of the MAK fractionated RNA were pooled and used in hybridization experiments. See Table 1 for the specific activities of the labeled RNA preparations.

DENATURATION OF DNA

Thermal method

DNA was diluted to concentrations of less than 30 ug/ml in a solvent containing less than 0.1 NaCl and placed in a water bath at 95^o for 10 minutes. The sample was removed and immediately placed in ice.

Alkali Method

DNA at the concentration and ionic strength stated above were brough to a pH of 12.7 with 3N NaOH (as monitored with a pH meter). The solution was stirred for 15 minutes and then neutralized with 3N HCl to a pH of 6.5 to 7.0.

RENATURATION OF DNA

In the standard conditions DNA was renatured at 68^o for 2 hours in at least 0.33 M Na^+. The conditions of renaturation were investigated by

varying the time, temperature, and ionic strength required. When a duplex between two strands of DNA was being reassociated, the concentration was at least 20 ug/ml of DNA. There was no adjustment of concentration in the reassociation of resolved single strands to form intrastrand secondary structure.

METHYLATED ALBUMIN KIESELGUHR (MAK) CHROMATOGRAPHY

1. Preparation of Methylated Albumin

The procedure used for the esterification of the albumin was that given by Mandell and Hershey (1960). Bovine serum albumin powder, "Fraction V" (Schwartz - Mann) was used. The albumin (5g) was suspended in 500 ml absolute (redistilled) methyl alcohol to which 4.2 ml of 12N HCl was added. The mixture was kept in the dark for 3 days and shaken occasionally. The precipitate was collected by centrifugation and washed twice with methyl alcohol and twice with anhydrous ether. Ether was removed by evaporation and the material reduced to a powder. It was generally stored in the dry form, however it could be kept as a 1% solution in H_2Q also.

2. Preparation of Coated MAK

Twenty grams of kieselguhr were boiled with 100 ml of 0.05 M $NaPO_4$ buffer pH 6.8 - 0.1 M NaCl. After cooling 5 ml of 1% methylated albumin were added. This was poured into a column and washed with 250 ml of the buffer, followed by 250 ml of 0.05 M $NaPO_4$ buffer - 0.4 M NaCl. The remaining kieselguhr coated with methylated albumin, was resuspended in 125 ml 0.05 M $NaPO_4$ buffer - 0.4 NaCl and used subsequently as the coated MAK.

3. Preparation of MAK Columns

The columns were filled with methylated serum albumin absorbed on kieselguhr as described by Mandell and Hershey (1960). The amount of the

ingredients could be altered depending on the quantity of nucleic acids to be fractionated. A recipe for what was denoted as a full MAK was used for a column having the dimensions 1.9 x 15.5 cm and was able to fractionate 5-6 mgs of DNA. The column consisted of four layers, the bottom and top were a thin layer of cellulose (1g) Whatman Chromedia) in 0.05 M sodium phosphate buffer, pH 6.8-0.3M NaCl. The same buffer was used throughout for packing the column. The second layer contained 8 grams of kieselguhr (Hyflosupercel - Fisher) boiled in 40 ml of buffer to which 2 ml of a 1% solution of methyated albumin were added after cooling. The third layer contained 6 grams of kieselguhr, boiled in 40 ml of buffer to which 10 ml of coated MAK were added after cooling. The two layers were added to the column in 5 ml aliquots. The column was washed with 100 ml of the buffer to remove any non-absorbed methylated albumin. The column was then equilibrated with the starting buffer which was between 0.3 and 0.7 M NaCl in 0.05 M sodium phosphate pH 6.8. The starting salt concentration varied with the nature of the necleic acid to be fractionated and the number of times the column had been used. All phosphate buffers for both MAK and HA chromatography were adjusted to pH 6.8 unless otherwise specified.

4. Fractionation of DNA on MAK Columns.

Denatured DNA can be fractionated into complementary strands by the intermittent gradient elution method described by Rudner et al (1968). DNA was dialysed against a starting buffer of 0.6 or 0.7 M NaCl in $NaPO_4$ pH 6.8 and absorbed onto the column. The packing of the column, absorbtion of the DNA and its elution were all done at a constant flow rate of ca 65 ml/hr. Low molecular weight DNA, low molecular weitht RNA and native DNA are not retained at this molarity. A linear gradient of 0.6 or 0.7 to 1.5 M NaCl in $NaPO_4$ buffer was applied (total volume 500 ml

for a full MAK). When the first fraction began to elute (around 0.8 - 0.9M) the gradient was discontinued and all of that fraction was allowed to elute at the same molarity. When the maximum absorbance of the first fraction was reached, the gradient was re-established and the second fraction began to elute. The elution of the DNA was monitored with an Isco continuous flow ultra-violet monitor. The eluent was collected in five ml fractions. The columns were reused several times after equilibration with an initial buffer of a lower molarity.

5. Purification and Concentration of DNA on MAK Columns.

DNA was freed of both protein and polysaccaride by passage through a MAK column. It was especially useful in removing teichoic acid from p^{32}-labeled DNAs. Native DNA was absorbed to the column at 0.3 M NaCl and washed with 0.5 M NaCl to remove the low molecular weight DNA and other impurities. The DNA was then repidly eluted with 2M NaCl and this fraction was collected. This was also useful in concentrating dilute nucleic acids. The dilute DNA or RNA was absorbed onto the column and rapidly eluted with high salt (2M NaCl) in a small volume.

6. Fractionation of RNA on MAK Columns

The amount of RNA which can be fractionated by a MAK column is half that of DNA for a given size column. The RNA was adsorbed to the column in 0.05 M $NaPO_4$ buffer pH 6.8 - 0.3 NaCl and eluted with a linear gradient of 0.3 to 1.4 M NaCl in buffer. The RNAs were eluted according to size, with the smallest species eluting first. Any DNA present in the sample eluted between the 4-5S fraction and the 16S fraction. Greater resolution between the 16 and 23 S species was obtained by interrupting the gradient as the 16S fraction began to elute and re-establishing the gradient once

the 16 S peak had reached maximum absorbance (Margulies et al, 1970).

HYDROXYAPATITE (HA) CHROMATOGRAPHY

Preparation of Hyroxyapatite

The procedure of Miyazawa and Thomas (1965) was followed for the preparation of hydroxyapatite. Two liters of 0.5 M $Na_2HPO_4 \cdot 7H_2O$ (Fisher) and two liters of 0.5 M $CaCl_2$ (Fisher) were mixed at a rate of 264 ml/hr with constant stirring. The crystalline precipitate was washed 4 times with 4 l of H_2O then resuspended in 4 l of H_2O. The suspension was boiled with 100 ml of 40% NaOH (w/w) for 1 hr. Following this, four successive washes were done with 4 l of H_2O with particular care being taken to decant the fine material. The precipitate was then resuspended in 4 l of 0.01 M sodium phosphate buffer, boiled for 5 minutes, resuspended, decanted, and boiled again for another 15 minutes. It was again decanted and resuspenced in buffer. The precipitate was washed 4 times in 0.01 M $NaPO_4$ buffer and after the last decantation, the remaining precipitate was resuspenced in 0.001 M $NaPO_4$ buffer and stored with a few drops of chloroform at 4^oC.

Fractionation of DNA on Hydroxyapatite

Fractionation of DNA by HA followed the procedures recommended by Bernardi (1971). The columns used were 1cm in diameter and were packed to a height of 1cm per 10D (260 nm) unit of DNA to be fractionated. The columns were prepared by adding enough HA to achieve the desired height. The columns were then washed with 0.05 M $NaPO_4$ buffer. The DNA was adsorbed to the column at the initial molarity of 0.05M and eluted with a linear gradient (usually 0.05M - 0.5 M $NaPO_4$buffer pH 6.8). The gradient might vary with the preparation of hydroxyapatite as each batch varies. Packing of the column the adsorption of the DNA, and its elution were all carried out at a constant flow rate of less than 40 ml/hr. The volumn of the grad-

ient used varied with the amount of DNA fractionated: less than 3 O.D., 200 ml; 3 - 5 O.D., 250 ml; 5 - 8 O.D., 300 ml; over 8 O.D., 350 ml. The elution of the DNA was monitored with an ISCO continuous flow untraviolet monitor. The eluent was collected in 4 ml aliquots. Single stranded DNA eluted at around 0.18 M $NaPO_4$ and double stranded or native-like NDA eluted between 0.20 and 0.22 M $NaPO_4$. Fractionation of denatured and self-annealed DNA led to incomplete recoveries (50-90%).

POLY LYSINE KIESELGUHR CHROMATOGRAPHY (PLK)

The fractionation of nucleic acids on PLK columns was performed as described by Ayad and Blamire (1968). This method was used for the purification of the ^3H-adenine labeled B. subtilis DNA that was used as a marker for the buoyant density determinations. Ten grams of kieselguhr were boiled with about 40 ml of 0.4 M NaCl 0.02 M KH_2PO_4 to remove trapped air. After cooling this was mixed with 15 - 25 mg of poly-lysine (Sigma). A 50 ml lysate, prepared by incubation with lysozyme (1 hr., 37°, 2 ug/ml) SLS (10 min., 60°, 2%) and RNase A (30 min., 37°, 50 ug/ml) was then added to the poly lysine kieselguhr. The proportion of the polylysine added can be varied with the amount of DNA that is thought to be present in a lysate in a ratio of 10:1, polylysine to DNA. This mixture was poured into a column on top of a cellulose layer and washed with the starting buffer to remove low molecular weight material. When no UV absorbing material was eluting, a linear gradient of 0.4 to 4 M NaCl 0.02 M KH_2PO_4 (total vol. 300 ml) was started. A peak of RNA resistant to RNase eluted just before the major peak of DNA. The elution of the nucleic acids was monitored with an ISCO continuous flow UV monitor. The DNA fraction was pooled and used as a radioactive marker (see Figure 2).

TEMPERATURE ABSORBANCE MEASUREMENTS

Melting profiles were obtained by measuring the increase in absorbancy at 260 nm recorded on a Gilford automatic recording spectrophotometer. The DNA solutions were at concentrations between 10 and 25 ug/ml in 1 x SSC. The initial absorbancy at 25°C was determined, then the DNA, in stoppered quartz cuvettes, was slowly heated to 95°C by circulating water. The spectrophotometer measured the absorbancy at the increasing temperatures and plotted out both the $O.D._{260}$ and the temperature on an x-y recorder. The hyperchromic increment (80-95°) represented the percentage of the hyperchromic rise that was observed during heating, in the range of 80 - 95°. Estimation of the percent helical structure was calculated from the hyperchromic rise from 80 - 95°C by using the following expression:

$$\frac{100\ (A^{95} - A^{80})}{A^{95} - (A^{95}/1.38)}$$

where A^{80} and A^{95} are the absorbances recorded at 80 and 95°C respectively, and 1.38 represents the maximal increase in relative absorbance recorded for native DNA at 95°C (Rudner et al, 1967). An example of the calculations is given in the appendix B.

BASE COMPOSITION ANALYSIS

Enzymatic Digestion of the DNA

A ^{32}P-labeled DNA was enzymatically digested to 5' mononucleotides following a modification of the procedures of Rudner (1961) and Mushynski and Spencer (1970). One ug of radioactive DNA (Spec. act. ca. 10^5 CPM/ug) and 500 ug of unlabeled calf thymus DNA were mixed and digested with 10 ug/ml pancreatic DNase (electrophoretically pure - Worthington) in 0.02 M Tris-HCl, pH 7.2, 0.05 M $MgCl_2$ for one hour at room temperature. To this, 250 ug.ml of snake venon phosphodiesterase (Worthington) in 0.02 M Tris-HCl pH 7.2, 0.05 M $MgCl_2$ was added and incubated at 37° for 18 hours. The

digestions were carried out in screw-top conical tubes.

Chromatography of the nucleotides

A 50 ul aliquot of the hydrolysate was spotted 10 cm from the end of a piece of Whatman filter paper NO. 1 and placed in a tank designed for descending chromatography. The solvent used for separation of the nucleotides was 5 parts redistilled isobutyric acid (Fisher) and 3 parts 0.5 N NH_4OH (Magasanik et al, 1950). After 18 - 24 hours the paper was removed from the solvent and dried. The four nucleotide spots were visualized by ultra-violet light and eluted for 1 hr. at 37^{o} in 3 ml of 0.05 M HCl, except for the spot which was known to be guanosine 5' phosphate, which was eluted in 0.1 M HCl. A 0.5 ml aliquot was removed for determination of radioactivity and the remainder of the sample was used for a spectral analysis and indentification of the spots. The order of migration of the spots was AMP, CMP, TMP, and GMP, with AMP being the fastest. The base composition of the DNA fractions was determined by the percent radioactivity in each of the spots.

EQUILIBRIUM DENSITY GRADIENT CENTRIFUGATION

The buoyant density of the DNA specimens in a CsCl solution was determined by means of sensity gradient centrifugation in a preparative Beckman Model L65B centrifuge. The density of a ^{3}H-labeled B. subtilis DNA was determined to be 1.703 g/cm^3 when it was run against E. coli Hfr H DNA and against Serratia marcessans DNA of buoyant densities of 1.710 and 1.760 g/cm^3, respectively. The H^3-B. subtilis DNA was then used as an internal reference with the unlabeled fractions of B. subtilis DNA. Unlabeled DNA (50 - 100 ug) and 50,000 CPM of ^{3}H-marker DNA were mixed with CsCl (optical grade - Harshaw Chemical Corp.) and adjusted to an initial density of 1.705. The density of the sample was inferred from

the refractometric measurements made with a Bausch and Lomb refractometer. The samples were placed in nitrocellulose tubes which had been coated with a 1% solution of bovine serum albumin to prevent denatured DNA from adhering to the tubes. The samples were immediately overlaid with mineral oil to prevent evaporation. Centrifugation was carried out in either the 40 or 50 Tk rotor, at 39,000 RPM for 65 hours at 19°. The tubes were then removed, pierced at the bottom with a syringe needle and 20 drop fractions (about 0.2 ml) were collected. The refractive index of every fifth sample was determined. A 20 ul sample was removed, spotted on GF/C filters, dried and placed in vials for determination of radioactivity. The remainder of the fraction was diluted with 0.3 ml 0.1 x SSC and the absorbance at 260 nm was measured. The fractions were then transferred to conical tubes and stored over chloroform for transcription assays.

The refractiometric measurements of the samples after fractionation did not prove to be a reliable method for determining the density, as the time between collecting the gradients and determining the refractive index varied enough to allow differing amounts of evaporation. The density of the fractions was determined following the relationship

$$P = P_o + 4.2\, w^2 \,(\, r^2 - r_o^{\,2}\,) \times 10^{-10}\ g/cm^3$$

where p and P_0 are the buoyant densities of the unknown DNA and the marker respectively, and r and r_o are the radii from the center of rotation of the unknown and marker DNA bands (Meselson, 1957).

A method had to be devised to determine the radius from the band to the center of rotation. The fixed angle of the rotor is 26° and the radius to the end of the tube is 8.1 cm as given by the Beckman manual. A triangle can be constructed having a hypotenuse that is 8.1 cm/sine 26° or 18.476 cm. Since the entire sample takes up 3.0 cm, one can divide this by the number of fractions and determine the distance in cm of the DNA

band from the bottom of the tube by subtraction from the hypotenuse. The radius to the band then, is equal to the new hypotenuse x sine 26^{o}. A sample of these calculations is given in appendix C.

SEDIMENTATION VELOCITY CENTRIFUGATION

Sedimentation velocity studies were performed using a Beckman Model E Analytical Ultracentrifuge equipped with ultraviolet absorbtion optics. A 30 mm single sector cell was used to provide a longer light path which was useful with low solute concentrations. A Kel-F 4^{o} centerpiece and quartz windows were used. The camera required a 21/2 x 31/2 inch film holder and Kodak Commercial film, allowing 12 exposures per film. The film was developed with Kodak developer D-11. A Beckman Model RB Analytrol with a film densitometer attachment was used to obtain tracings of the photographic films.

Centrifugation was performed at 24,000 RPM. A constant temperature of 20^{o} was maintained during the run by the Rotor Temperature and Control Unit (RTIC). The DNA concentrations used varied between 8 and 30 ub/ml. All of the samples, except for the native DNA were kept in an alkaline environment; 1 x SSC, 0.2N NaOH, to minimize the conformational differences of the various denatured fractions. The samples were loaded into the cell with a syringe and a 22 guage needle which had been cut to 4 mm to minimize shear of the DNA molecules. Centrifugation was carried out until the DNA had sedimented more than half the length of the cell (around 60 minutes). Pictures of the sedimentation were taken every 4 minutes, automatically, with an exposure time of 25 to 60 seconds, depending on the DNA concentration. The developed film plates were then traced with the Analytrol for determination of the sedimentation coefficient. The distance from the meniscus to the plateau was measured to determine

the midpoint of the boundary. This value was corrected for magnification and subtracted from a known value for the distance to the outer reference hole (7.30cm). This then represents the distance from the center of rotation to the boundary (x). A plot of the natural log of x against the time of sedimentation was obtained. The sedimentation coefficient was obtained from the relation

$$s = 1/w^2 \left(d \ln x / d t \right)$$

where d ln x / d t is the slope of the line (Svedberg and Pederson, 1940). At the low DNA concentrations used the sedimentation coefficients did not need to be extrapolated to zero concentration. Estimations of the molecular weights of the DNA based on the sedimentation coefficient were given by Studier (1965).

$$\text{native DNA } s^0_{20,w} = 0.0882M^{0.346}$$
$$\text{alkaline DNA } s^o_{20,w} = 0.0528M^{0.400}$$

An example of the calculations is shown in Appendix D.

S_1 NUCLEASE

Preparation of S_1 Nuclease

Two batches of S_1 nuclease, a single strand specific nuclease from Aspergillus oryzae, were used in these studies. One was purchased from Miles Laboratories and the other was made in this laboratory in association with Ms. Marie LeDoux, using in combination and modifications of the procedures of Ando (1966) and Vogt (1973) as outlined below. Twenty grams of commercial Takadiastase (Sigma), a mexture of enzymes produced by Aspergillus oryzae, were dissolved in 133 ml of water and 70.35 g of ammonium sulphate were then added. The mixture was centrifuged and 20 g of ammonium sulphate were added to the supernatent. The precipitate from the second fractionation was dissolved in 33.3 ml of water and dialysed against 2 l of water with several changes. Ammonium sumphate was added to 70% (w/v),

stirred for 3 hours, centrifuged, and the precipitate was dissolved in 12 ml of buffer B; containing: 0.02 M Tris-HCl pH 7.5, 0.05 M NaCl, 0.1 mM $ZnSO_4$, 5% glycerol, and then diluted with 50 ml buffer B minus the NaCl. This solution was adsorbed at a flow rate of 20 ml/hr onto a DEAE - celulose DE-23 column (2 x 18 cm) equilibrated and then washed with 200 ml of buffer B. The enzyme was eluted with a linear gradient of 0.05 to 0.35 M NaCl in buffer B. The fractions were assayed for nuclease activity as described below. The fractions with activity were pooled and reapplied to a DEAE-cellulose column and eluted with 0.5 M NaCl. The active fractions were stored in 50% glycerol.

S_1 Nuclease Treatment

A variety of conditions were tried to optimize the degradation of denatured DNA and minimize its activity with a native DNA substrate. This included varying the pH, temperature, and ionic strength of the reaction. The optimal reaction mixture for the enzyme prepared in this laboratory was: 0.03 M sodium acetate pH 4.5, 3×10^{-5} M $ZnSO_4$, 0.15M NaCl, 20 us/ml sheared, denatured calf thymus DNA, and appropriate quantity of radioactive DNA, and 95 U of enzyme (one unit was defined as the amount of enzyme necessary to render acid soluble 1 ug of denatured DNA in one hour (Ando, 1966). The mixture was incubated in a sealed conical tube at 50^{o} for 1 hr. or aliquots were removed at various time intervals, when determining the kinetics of the reaction. The reaction was stopped by adding 10% TCA to a final concentration of 5% TCA. This was kept on ice for 2 hrs. The TCA precipitate was collected on GS/A filters and placed in vials for determination of radioactivity. The reaction mexture for the commercially prepared enzyme was: 0.3 mM Na acetate pH 4.5, 1×10^{-6} M Zn Cl_2, 0.1 M NaCl. 20 ug/ml sheared, denatured calf thymus DNA, radioactive DNA and 500 U

enzyme as determined by Miles Laboratory. The mixture was incubated at 37^{o} for 1 hr. and the TCA precipitable counts were measured as above.

GENETIC TRANSFORMATION OF BACILLUS SUBTILIS

Transformation Assay

The growth of recipient cells to competency has been described earlier. The concentration and ionic strength of the solvent of the DNA aften varied. In instances where different DNA samples were to be compared, all the DNAs were adjusted to the same concentration and to 1 x SSC. If the fractions of a column were to be estimated qualitatively for the presence of transforming activity, the DNA and salt concentrations were not always adjusted. Transforming ability was measured as follows: to 0.9 ml of competent cells, 0.1 ml of DNA was added and the mixture was incubated at 37^{o} for 30 minutes. The reaction was terminated by the addition of 10 ug/ml of pancreatic DNase (Worthington) in 0.2 M $MgSO_4$. The cultures were then appropriately diluted in saline and plated on selective media. The number of viable cells in the tube was determined by plating on TBAB plates (see Appendix A). The ratio of transformants to recipient cells was determined.

Selective Media

The minimal agar plates (see Appendix A) were supplemented with 1% sodium glutamate, 50 ug/ml L-tryptophan, 50 ug/ml L-histidine (Cal-Biochem). Plates containing the following additives were used for the recipient cells indicated: BD - 170, 0.02% threonine assay medium (Difco), Mu8u5u16, 3 types of plates were used a) 100 ug/ml l-leucine, 100 ug/ml l-methionine (to assay adenine) b) 100 ug/ml adenine, 100 ug/ml l-methionine and 0.02% methionine assay medium.

HYBRIDIZATION TO RNA

The Liquid Method

Each reaction contained not less than 0.1 ug of non-radioactive DNA (2 ug/ml, in a range of 2 - 10 ug/ml) and a H^3-uridine labeled RNA in a total volume of 0.05 - 0.1 ml. The ratio DNA:RNA varied depending on what was being assayed, and the absolute amounts of the necleic acids used depended on the specific activity of the RNA sample and the efficiency of hybridization. The reaction was carried out in 6 x SSC at 68° for 18 hours. The reaction was stopped by chilling on ice and diluting with 10 ml of 2 x SSC. All hybridizations took place in screw-top conical tubes. The mixture was filtered onto Schleicher and Schuell (S + S) nitrocellulose filters which had been pre-soaked in 2 x SSC. The filters were washed with 100 ml 2 x SSC and treated for 1 hr. with 20 ug.ml RNaseA (Worthington) and 20 U/ml RNase T_1 (Worthington) in 0.2 M Na acetate buffer pH 5.5. Both enzymes had been treated at 80° for 10 minutes to destroy DNase activity. The filters were then washed with 50 ml 2 x SSC on each side, dried, and prepared for radioactive counting.

The Filter Method

Nitrocullulose filters (S + S) were soaked in 0.1 N KOH for 30 minutes to reduce the binding of native DNA to the filters. They were then soaked in 2 x SSC for 30 minutes. To each filter, 10 ug of a ^{32}P-labeled DNA were slowly added using a Membrane filter Apparatus (New Brunswick). Following the method of Gillespie and Spiegelman, (1965) the filters were washed with 100 ml 2 x SSC, dried for 2 hrs. at 80°, and 2 hrs. at room temperature. Once dry, the filters were stored in a desicator. The hybridization reaction took place in scintillation vials with one or more filters, depending on the amount of DNA required. A ^3H-uridine labeled RNA in 2x SSC was

added in a sufficient volume to just cover the filter (1.0 ml when a single filter is added). The mixture was incubated at 66° for 16 hrs. The reaction was stopped by chilling the vials on ice. The filters were washed and treated with RNase, as described for the liquid method, and prepared for radioactive counting as described below.

DETERMINATION OF RADIOACTIVITY

A Packard Tricarb Liquid Scintillation Counter equipped for beta and gamma counting was used. For counting tritium the setting was 70 - 1,000 with a 50% amplification. For counting ^{32}P the setting was 70 - 1,000 and a 2.4% amplification. When both isotopes were used in a double label experiment, the settings were 70 - 750, 50%, and 130 - 850, 1.1%. The spill of ^{32}P into the ^{3}H channel was determined to be 1.34%.

Aqueous samples were counted in Bray's scintillation fluid, prepared as follows: Solution A contained 120 g napthalene (Fisher), 4 g PPO (New England Nuclear) 200 ml methanol (Fisher) and 40 ml ethylene glycol (Fisher) added to 1 l of p-dioxane (Fisher). Solution B contained 4 g POPOP (NEN) dissolved in a liter of p-dioxane. The two solutions were then mixed. The proportion of aqueous sample to scintillation fluid was 1:5.

For counting all other samples a toluene based scintillant was used. This was prepared by adding 6 g PPO and 0.5 g POPOP to 1 l of toluene.

RESULTS

I. Preparation of Chromatographically Fractionated Strands of B. subtillis DNA

A. Methylated albumin kieselguhr chromatography (MAK strands

MAK fractionation was used as a means of strand separating B. subtilis DNA. This method has been shown to result in the separation of denatured DNA into tro fractions of strand fragments which are thought to be complementary (Rudner et al, 1968; Karkas et al,1968; Rudner et al, 1968a; Rudner et al, 1969). The two fractions were designated light (L) and heavy (H) on the basis of their buoyant densities. The bulk of the work presented here required the fractionation of DNA into complementary strands as a first step. A great many fractionations were performed to provide all the necessary material. One typical fractionation is shown in Figure 3. The material which eluted during the application was low molecular wright DNA. A minor peak of DNA eluted ahead of the major fractions. This DNA has been shown to contain native-like melecules (Rudner and Remez, 1973). For subsequent chromatography on hydroxyapatite, fractions were pooled and an aliquot removed to be used as the MAK strand fraction; the remainder of the fraction was used for self-annealing. Usually only the early eluting L and late eluting H fractions were used to prevent the possibility of cross contamination of the two fractions.

HYDROXYAPATITE (HA) fractionation

In general, HA was used to fractionate those DNAs which contained secondary structure (Bernardi, 1969). Fractionation of the self-annealed material was accomplished after incubating the MAK fraction at 68^{o} for 2 - 3 hours. A typical elution pattern of the fractionation of the self-annealed MAK H strand is shown in Figure 4. As in the case of the MAK

fractionation, this procedure was used as a common preparative method to get either the self-annealed fraction or the purified strand fraction to use for further studies, therefore the figure shown is merely representative of this type of fractionation and is not the exact fractionation used in all the experiments.

The first fraction of DNA eluted at 0.18 M $NaPO_4$ and was designated HI. It corresponds to single stranded DNA. The second fraction eluted at 0.22 M and eas designated HII. It corresponds to native-like DNA. Bernardi (1969) has shown that there are preferential losses of the single strand fractions, leading to poor recoveries. The amount of DNA which eluted in fraction II was used to estimate the amount of self-annealing DNA present in a strand. The molecules which were isolated in the second fraction were not entirely double stranded, but contained both helical and single strand regions, as presented later in the data.

When the MAK L fraction was self-annealed in the same manner as that described for the MAK H fraction and chromatographed on HA, a profile was obtained that was indistinguishable from that of the MAK H strand. The relative amounts of peaks I and II, the total recoveries, and the elution molarities were the same for both strands. A typical profile for the fractionation of the self-annealed MAK L strand by HA chromatography is shown in Figure 5.

HA chromatography was also used to separate L + H duplexes from the single strands which did not reaccoaiate. This was done to investigate the biological activity of duplexes before and after hydroxyapatite, the results will be discussed in the transformation section. The L and H strands were obtained either from MAK fractionated DNA or from the first fractions of HA fractionated self-annealed strands. The sample was chromatographed as described above, with the difference being that the major peak eluted

as native-like DNA.

FRACTIONATION OF RNA BY MAK CHROMATOGRAPHY

The actinomycin D treated ^{3}H uridine labeled RNA prepared by Dr. Y. Setoguchi was fractionated into its constituent species to test the hybridizability of the DNA to various species of stable RNA. A MAK column was prepared using 1/2 the recipe, as described in Materials and Methods, and equilibrated with 0.3 M NaCl in 0.05 M sodium phosphate buffer. The elution profile of the MAK fractionated RNA is shown in Figure 6. Figure 6 shows both the UV absorbance profile and the pattern of radioactivity. No UV detectable DNA was present in this preparation as shown by its absence in the region between 5S and 16S.

TRANSFORMING ACTIVITY OF THE CHROMATOGRAPHICALLY FRACTIONATED STRANDS OF B. SUBTILIS

The transforming activity of B. subtilis DNA was used as an assay for both the presence of secondary structure in a particular fraction, and also to determine what regions of the chromosome were present in any given fraction. The transforming activity of fractions along the MAK elution profile and the HA elution profiles of L and H self-annealed DNA were assayed. Each fraction was adjusted to 10 ug/ml and dialysed against 1 x SSC. The transforming activity of DNA along the MAK elution profile is given in Figure 7, assayed with the thr - 5 marker using a BD 170 recipient. There was a residual transforming activity which was associated with the early eluting minor component and trailing into the L fraction. A slightly higher transforming activity was seen in the region of cross contamination between the L and H fractions.

The same MAK elution profile was assayed with the threonine marker after the fractions were self-annealed. A portion of the MAK fraction

was annealed in the MAK salt at 68° for 2 hrs. and then the DNA concentration was adjusted. The results are shown in Figure 8. Neither the minor peak of DNA nor the L fraction showed any increase in transforming activity. Only the late eluting region of the H fraction showed an increase. The transforming activity along the elution profile of HA fractionated self-annealed H strand is shown in Figure 9. Fraction I was entirely devoid of transforming activity. This was true when other markers were tested and was also the case for the first fraction of the HA fractionated self-annealed L strand. The H II fraction showed an transforming activity that was 6-10% of the initial activity. The LII fraction showed a transforming activity which was 0.5 - 3% of the initial activity. The restoration of transforming activity after self-annealing was unique to the H strand. The transforming activities of the duplexes formed from the fractionated strands was also assayed. Table 2 shows these results. The markers assayed were thr-5, purA-16, leu-5, and met-8. For all markers assayed, the MAK L+H duplex showed a higher transforming activity than the HA fractionated LI+HI duplex.

The possibility was considered that hydroxyapatite itself, might in some way decrease the transforming activity of DNA, and thereby explain the low levels of transforming activity associated with the HA LI+HI duplex. The effect of hydroxyapatite on transforming activity was measured in two ways: 1) a MAK L+H duplex was assayed before and after passage through an HA column, and 2) a duplex was formed by adding LI, LII, HI, and HII together and then assayed. The former experiment would show whether passage through hydroxyapatite caused any sort of inactivation of the DNA; and the latter experiment would show whether the decreased transforming activity after HA fractionation was reversible. The DNA concentration of all the duplexes was adjusted to 5 ug/ml prior to transforming.

The data in Table 2 show the same activity for the reconstituted duplex as for the MAK duplex for the threonine and adenine markers, and a 10% lower activity for the reconstituted duplex with the methionine and leucine markers. The MAK duplex, after passage through HA also showed some reduction in transforming efficiency, about a 10% decrease. These results suggested that the HA chromatography was not responsible for the observed decrease in transforming activity of the hydroxyapatite duplexes. The ability of the HII fraction to transform for markers which were located all over the chromosome showed that the self-annealing fraction was not restricted to any particular region of the chromosome.

TEMPERATURE ABSORBANCE MEASUREMENTS OF THE FRACTIONATED STRANDS OF DNA

The various fractions of DNA were melted out to characterize the nature and extent of secondary structure in these fractions. Melting curves of the self-annealed MAK L, MAK H and MAK L+H duplex are shown in Figure 10. Estimations of the amount of helicity present in these fractions was based on the hyperchromic rise between 80 and 95°C as described in the Materials and Methods section. Both the MAK L and H strands showed some secondary structure, around 5% that of native DNA. The renaturation of the L+H duplex showed a restoration of helicity that was 77% that of native DNA. This was more than was present in the individual strands and indicated that the L _ H strands were complementary and able to form duplexes upon renaturation.

Melting curves of Fraction I of the H and L self-annealed DNAs, and the duplex formed from the renaturation of LI and HI are shown in Figure 11. Boththe H and L fractions were entirely single stranded, showing no hyperchromic rise between 80 and 95°. The duplex formed by the renaturation of LI and HI showed a helical structure that was 34% that of native DNA. The

decreased ability to form duplexes suggested that these fractions did not contain as many complementary sequences as did the MAK fractions. Melting curves of the second fraction of HA eluted DNA are shown in Figure 12. Both the L and the H strands showed the same extent of secondary structure. They had a hyperchromic rise that was about 22% that of native DNA.

To determine if the difference in duplex formation between the MAK and HA fractionated strands was due to HA chromatography, a reconstitution experiment was done. Fractions I and II were added together in the same proportions as they eluted from the HA column. Equal amounts of the total L and total H mixtures were added together in 2 x SSC, to give a final concentration of 20 ug/ml. The mixture was annealed at 68° for 2 hours, diluted to 1 x SSC, cooled, and then melted out. A comparison between the melting curves of the MAK L+H duplex, the HA LI+HI duplex, and the reconstituted duplex is seen in Figure 13. The reconstituted duplex showed the same hyperchromic rise as the MAK duplex, whereas the HA LI+HI duplex was only 60% as efficient as the MAK duplex. In all of the fractions shown and in the resulting duplexes, the Tm of the DNA in 1 x SSC was closest to 88°. This value was the same as that of native B. subtilis DNA. Schildkraut et al (1962) have shown that the Tm of a DNA in a given salt is directly related to the %GC content of the DNA. This suggested that the overall base composition of the DNA which was capable of forming secondary structure was the same as that of native B. subtilis DNA.

MOLECULAR SIZE OF THE CHROMATOGRAPHICALLY FRACTIONATED STRANDS OF DNA

The size of the fractions was determined to see if there was anything unique about the fraction which could self-anneal, and to see if there were any differences between the MAK and HA strand fragments which could explain their differential ability to form duplexes. The distribution of sizes along the HA elution profile was also investigated to determine

if the basis of elution was due solely to secondary structure. The sedimentation coefficients of the different fractions were determined by sedimentation velocity centrifugation. The results of these determinations are shown in Table 3. Extraction of the B. subtilis chromosome by the described Methods and further manipulations give a sheared population of molecules with an average molecular weight of 12×10^6. The MAK H strand fragments are larger than the MAK L fragment which would be expected due to the greater lability of the purine rich L strand (Rudner and Remeza, 1973). Both fractions I and II of HA eluted DNA showed the same sedimentation coefficient for a given strand. The values presented for the H strand showed that the HA fractions were about 10% smaller than the MAK fractionated strand. This difference is within experimental error. It was concluded that the size of the fragments did not influence the observed differences in the transforming activity and hypochromicity after annealing of the MAK v. HA duplexes.

Bernardi had shown that hydroxyapatite fractionated cucleic acids on the basis of their secondary structure, irrespective of molecular weight. The sedimentation coefficient of fractions along the HA elution profile was determined to verify these results. The data for this experiment is given in Figure 14. The fractions were not adjusted to a common DNA concentration because at the low concentrations used there is no effect on the sedimentation coefficient. The data was in agreement with that of Bernardi, in that fractionation was not dependent on molecular size.

BASE COMPOSITION OF THE CHROMATOGRAPHICALLY FRACTIONATED STRANDS OF DNA

The base composition of the fractions was determined to see if the self-annealing activity was due to any particular richness in either AT or GC rich sequences. A ^{32}P-labeled DNA was digested to 5' mononucleotides and chromatographed as described in the Materials and Methods section.

The results are given in Table 4. Native B. subtilis 168 DNA, as described by Rudner et al (1968a) is an AT rich DNA with a molar ratio of A+T/G+C = 1.35. The MAK fractionated strands showed an asymmetry in their purine and pyrimidine distribution, with the H strand having a Pu/Py ratio of 0.92. The HI fraction showed a Pu/Py ratio of 0.89, and that of the HII was 1.07. There was no AT or GC richness associated with the DNA which is capable of self-annealing. Although the HII fraction showed an increase in purine/pyrimidine ratio Pu/Py = 1.07 the AT and GC pairing did not improve.

It was thought that the single stranded regions in the HII molecules might obscure the AT or GC rich sequences involved in base pairing when the total modecule was analysed. Therefore the HII fraction was treated with a single strand nuclease from Aspergillus oryzae, S_1, and then the base composition was determined. This data showed the greatest shift in dissymmetry between purines and pyrimidines (Pu/Py = 1.18) and a large difference between A+T and G+C (A+T/G+C = 1.32). This observation was not expected since treatment with S_1 neclease should remove single strand DNA and leave a more paired structure with a Pu/Py closer to 1. In view of other data presented, it is clear that the treatment with S_1 neclease did not remove all the single strand regions, presumably leaving the loops and regions joining the two helical pieces intact. This data suggested that the DNA associated with the secondary structure, either in the loops or adjacent sequences, is extremely purine rich, especially adenine rich. A second possibility to explain these rather puzzling results is that S_1 nuclease has a greater preference for unpaired pyrimidines than for purines. This specificity has not been described for the enzyme but deserves investigation.

EQUILIBRIUM DENSITY GRADIENT CENTRIFUGATION

Another technique which was useful in the isolation of the self-annealing fraction was preparative CsCl gradients. This had the advantage of allowing resolution of the renatured parts of the molecule from the single stranded regions. A ^{3}H-adenine labeled native B. subtilis DNA was found to have a buoyant density of 1.703 g/cm^{3} by calculating its density relative to an E. coli Hfr H DNA and a Serratia marcessans DNA. This radioactive DNA was then used as the marker in subsequent determinations. Table 5 gives the buoyant densities determined for the fractionated strands. The difference in the buoyant densities of the MAK fractionated L and H strands is consistent with the difference in purine distribution. Fraction II of HA eluted DNA had both a main peak which banded at a density expected for renatured DNA and a shoulder that trailed into the denatured region. Figures 15-18 show the profiles obtained for the fractionated samples of H strand DNA. The MAK H strand (Figure 15) showed a sharp band with a buoyant density of 1.720 g/cm^{3}. When this strand was self-annealed (Figure 16) the DNA showed a heterogenous density profile with renatured and single strand positions. The first fraction of HA eluted DNA, HI (Figure 17) showed a sharp peak of denatured DNA with a buoyant density of 1.723 g/cm^{3}. The second fraction of HA eluted DNA, HII (Figure 18) showed a peak in the renatured region with a density of 1.707 g/cm^{3}.

Experiments were performed to determine whether the helical or single stranded DNA was responsible for the transforming activity. Gradients were run which contained only the sample DNA, without a native DNA marker. Each fraction from the gradient was diluted to a final volume of 0.5 ml with 1 x SSC and assayed for transforming activity. Figure 19 shows an assay of the transforming activity along the Cs Cl gradient for the HII DNA using the leucine marker.

For all the markers assayed, thr-5, purA-16, leu-5, and met-8, the transforming activity was associated with the DNA which banded in the renatured density position. The transforming activity of all the fractions was low. The transforming activity of the peak for the H II fraction was from 1 to 4 x 10^2 transformants/ml of recipient cells. The MAK H strand showed around 1.5 x 10^2 transformants, and the HI fraction, as expected from its behavior after HA chromatography, showed no transformants at all. The low levels of transforming activity seen were, in part, due to the high concentrations of CsCl present and to the low DNA concentrations used. The control value for native DNA in CsCl was only 2.3 x 10^4 transformants (for the leucine marker). The association of the transforming activity with the DNA which bands like native DNA reflected the requirement for double stranded DNA. The fact that transformation of markers all over the chromosome took place, supports the earlier finding that the self-annealing fraction was not restricted to any area of the chromosome.

DIGESTION OF FRACTIONATED STRANDS OF DNA WITH S_1 NUCLEASE

S_1 nuclease, a single strand specific nuclease isolated by Ando (1966) from Aspergillus oryzae was used to determine the extent of secondary structure in the various chromatographic fractions. The self-annealed fractions were shown to have DNA that was resistant to the action of S_1 nuclease. The S_1 resistant core of HII was analysed with respect to its base composition, transforming activity and hybridizability. It was thought that the removal of the single stranded ends of the molecule would give a clearer understanding of the nature of the self-annealing material.

Before this could be done, the optimum conditions for degradation of the structure had to be determined. The extent of degradation varied, and was strongly dependent on the NaCl concentration used (Sutton, 1971; Vogt,

1973; Crosa et al, 1973). Low salt concentrations favor the destabilization of the helix offering more single strand sites as substrate. This is particularly important for the endonucleolytic activity of the enzyme. High salt stabilized the helix presenting fewer sites for digestion. Maximum degradation of single stranded DNA was achieved in 0.05 M NaCl but at that ionic strength, only 76% of the native DNA remained after 1 hour of incubation. In 0.2 M NaCl 92% of the native DNA remained TCA precipitable after 1 hour but 24% of the denatured DNA was not degraded. The data in Table 6 show the effect of salt and temperature on the commercial enzyme. All of the subsequent assays using this enzyme were carried out at 0.1 M NaCl, 37°. Although these conditions do not allow maximum degradation of single stranded DNA (14% remained acid preciptable) the helical structure was more efficiently protected.

Figure 20 shows the kinetics of the reaciton with the fractionated strands. The results were consistent with the expected behavior of the strands. The HII fraction showed the greatest resistance to the action of S_1 nuclease, 56% whereas the HI fraction which is totally devoid of secondary structure shows only 17% resistance to S_1 after 1 hour. The non-self-annealed MAK fraction was only slightly more resistant than the HI fraction (19%).

The base composition of the S_1 resistant core of HII DNA was determined by digesting a sample containing ^{32}P labeled HII DNA and a sheared, denatured calf thymus DNA carrier with our enzyme preparation. The sample was dialysed overnight against 0.02 M Tris-HCl, 0.05 M $MgCl_2$pH 7.2, to remove free nucleotides. This sample was then digested to 5' mononucleotides and chromatographed as described earlier. The results are shown in Table 4.

The transforming activity of the HII fraction was tested with three markers, purA16, leu-8, met-5 with DNA that had been digested with S_1 nuclease for 1 hour. Since this was not a radioactive DNA, the activity of the enzyme was not measured. The results are given in Table 7. The results show a 2 to 3 fold decrease in transforming activity of the HII resistanct core except for the leucine marker. It is possible that the genes coding for the leucine marker are either in some unique structural configuration that make them sensitive to S_1 nuclease or are close to a site where the DNA is preferentially sheared during extraction, always leaving it at the end of a molecule and therefore more susceptible to nucleases.

The retention of transforming activity by the HII resistant core suggested two possibilities; either the self-complementary sequences carried structural gene material or, the S_1 treatment did not remove all the single strand regions and it was this DNA which was involved in transformation. The second possibility had some support because of the discrepancy between the amount of DNA which remained S_1 resistant, 56% and the % helicity, 22% in the fraction found by temperature absorbance measurements. Even taking into account the inefficiency of the enzyme in degrading purely single stranded DNA (15% of HI is not degraded) the difference must have been due either to the single stranded DNA of the loop, or to single strand stretches between the loops. The former possibility could not be ruled out though, especially in light of the association of the self-annealed material on CsCl gradients with the transforming activity.

The S_1 resistant core of HII DNA was hybridized to pulse-labeled RNA to see if the removal of the single strand regions decreased the hybridizability to this messenger rich fraction. The details of the experiment will be presented in the following section. It was found that

when the DNA was mildly digested to give 79% TCA precipitable, the hybridization officiency dropped to 72% of its original value. These results suggested that the S_1 resistant core of HII DNA contained transcribed sequences, probably including mRNA sequences.

Hybridizability of the fractionated strands of DNA to ^3H Uriding - pulse Labeled log phase RNA

The hybridizability of the fractionated strands to pulse labeled log phase RNA was investigated to determine the extent of transcribed sequences present in these fractions. A pulse labeled RNA was used to enrich for structural genes and their related regulatory sequences, rather than stable RNA components. Hybridization competition experiments using a DNA/RNA ratio of 125:1, and a ratio of labeled RNA to unlabeled rRNA competitor of 1:100, showed that the rRNA content of this preparation was 33% (Y. Setoguchi unpublished data). Margulies et al (1970a) have shown that of the hybridizable RNA, 70-80% hybridized to the H strand of MAK fractionated B. subtilis DNA. Of particular interest was Margulies' observation that the majority of mRNA species hybridized to the late eluting region of MAK H strand DNA. It was from this region that biologically active self-annealed molecules were derived.

The hybridizability of the pulse labeled RNA to the MAK and HA fractionated DNA at a DNA:RNA ratio of 1:50 is shown in Table 8. The characteristic asymmetry of the hybridizability to the MAK H and L strands was seen, with the ratio being 3.62. The late eluting H fraction was shown to be particularly hybridizable, with 2.44% of the RNA hybridizing to late H and only 0.92% hybridizing to early H. The hybridizability of the HII and LII fractions was not measured in liquid hybridizations because of their propensity for self-annealing. Both LI and HI hybridized poorly in com-

parison with the MAK fraction. The HI fractions from the early and late eluting MAK H strands hybridized only 28 and 37% as efficiently as the corresponding MAK fraction. When a high DNA/RNA ratio (120:1) was used, the hybridizability of the HI fraction improved to 77% that of the MAK H fraction (Figure 21). The hybridizability of the HII DNA could not be directly determined in the above experiment because of the rapid reassociation of the self-annealing DNA with itself rather than with RNA. This problem was overcome be denaturing the DNA, binding it to nitrocellulose filters, and then hybridizing it to RNA. The results of these experiments are given in Table 9. The data show that HI and HII hybridize 56.7 and 17.7% respectively, as well as the MAK H strand.

The differential hybridizability of the H strand fractions to pulse labeled RNA as a function of increasing DNA concentrations is shown in Figure 21. The results indicated that there really was a difference in the MAK H and HA HI populations. HI did not contain all of the sequences present in the MAK H fraction. At a saturation ratio of 120:1 (DNA/RNA) the MAK H and HI fractions gave values of 96 and 69% of the RNA hybridized, respectively. At the same ratio, only 31% of the RNA was hybridized to the HII DNA. This indicated that although the HII fraction contained some transcribed sequences it was rich in sequences which were either not transcribed or not present in a population of pulse labeled log phase RNA transcripts.

The hybridization of the HII DNA to a pulse labeled RNA population might have been due to the single strand part of the molecules rather than to the self-annealing portion. To investigate this possibility, the HII DNA was digested with S_1 nuclease, denatured, bound to a nitrocellulose filter, and hybridized to RNA. The last line in Table 9 shows that the digested core hybridized 12.9% of the RNA, which meant that it retained 73% of the hybridizability of the HII DNA. The extent of digestion of the

HII DNA was measured by TCA precipitation and was found to be 79% remaining. This data strongly indicated that the helical structures contained transcribable sequences.

HYBRIDIZABILITY OF FRACTIONATED STRANDS OF DNA TO ^{3}H-URIDINE LABELED RIBOSOMAL RNA

The fractionated strands were hybridized to ribosomal RNA to determine the extent of stable RNA cistrons in these fractions, particularly with self-annealing material. Margulies et al (1970) have shown that transcription of the stable RNA components occured entirely off the H strand of B. subtilis DNA. Therefore the experiments were only performed with the H strand fractions. Table 10 shows the results of these hybridizations. The data showed that .75% of DNA H hybridized to ribosomal RNA which corresponded well to the amount of DNA coding for the ribosomal genes (Oishi and Sueoka, 1969; Margulies et al, 1970). The HI fraction only showed 0.53% hybridized. This suggested that there were some ribosomal sequences which were not present in HI and were capable of fractionating with HII. The hybridizability of the HII fraction was not determined by this method.

The hybridizability of the isolated RNA components to DNA fractions which had been denatured and bound to filters was determined and the results are given in Table 11. The MAK H strand hybridized twice as well to the 23S fraction as to the 16S fraction, as would be expected from the size of the RNAs. Both HI and HII were able to hybridize to all of the components but with different efficiencies. The 16S and 5S species were much more likely to hybridize to the self annealing H strand DNA than were the 23S and 4S RNAs. The 16S RNA hybridized equal as well to the MAK H, HA HI and HII 85% and 89% as well as to the MAK H strand, respectively.

The ability of the HI fraction to hybridize to the stable RNA indicated that all of these cistrons must be able to exist without secondary structure. But since HII could also hybridize to the ribosomal RNA with different efficiencies, there must be differences in the secondary structure within or adjacent to these cistrons.

DISCUSSION

The purpose of the studies reported here is to characterize the intrastrand self-annealing material in MAK fractionated strands of Bacillus subtilis DNA. These studies show that both the L and H strand fragments contain molecules which are capable of self-annealing and that the H molecules from the trailing end of the MAK profile are biologically active. The self-annealing material is able to be fractionated from the rest of the DNA by hydroxyapatite chromatography. The molecules fractionated in this manner contain both base paired regions and single stranded regions.

The total amount of self-complementary sequences in the strands represents 5-10% of the DNA and is not restricted to any particular area of the chromosome. These molecules contain som structural gene sequences as evidence by their transforming activity and hybridizability to pulse labeled log-phase RNA. In addition cistrons for stable RNA species are also present in this fraction as shown by their hybridizability to labeled ribosomal RNA. However, the self-annealing material is also rich in non-transcribed and non-transforming DNA, suggesting the presence of regulatory sequences in the population.

The separation of alkali denatured B. subtilis DNA into two fractions can be accomplished by methylated albumin kieselguhr chromatography using an intermettent salt gradient. The complementarity of these fractions has been well substantiated (Rudner et al, 1968; Karkas et al, 1968; Rudner et

al 1968a; Rudner et al, 1969). The residual transforming activity of denatured DNA from B. subtilis and other transformable bacteria has been investigated in several laboratories and has been shown to be associated with "native-like" molecules containing covalent crosslinks which resist denaturation (Albert, 1968, Albert and Doty, 1968; Chevallier and Bernardi, 1968; Mulder and Doty, 1968; Rownd et al, 1968). MAK chromatography has been shown to resolve most of these molecules in a minor fraction which elutes ahead of the L fraction (Rudner and Remeza, 1973). These molecules renature spontaneously and show no increase in transforming activity following thermal renaturation. No experiments have ruled out the possibility that this fraction also contains molecules with intrastrand crosslinks which are capable of very rapid renaturation.

Another population of molecules with distinctly different reassociation kinetics has also been described (Rudner and Remeza, 1973). These molecules, from isolated strand fragments, are capable of aelf-annealing following thermal renaturation at 68^{o}. The reassociation is shown to occur rapidly and be independent of the DNA concentration, typical of a unimolecular reaction. These results show that the self-complementary sequences could occur on the same molecule, therefore, they are intrastrand associations. This ruled out the possibility that the self-annealing material from the isolated strands is due to cross contamination of one strand with the other (Rudner and Remeza, 1973).

The kinetic data also makes some predictions about the closeness of the self complementary sequences. It suggests that there are no intrastrand crosslinks involved which allow resistance to denaturation. It also suggests that the self complementary sequences are close enough together so that it is statistically much more likely that they will end up on the same molecule than on two separate molecules from the same strand. If

this were not the case, the experiments showing the restoration of transforming activity as a function of DNA concentration would show both kinds of kinetics; uni and bimolecular reactions.

The presence of self annealing material was assayed in four ways: 1) elution on hydroxyapatite columns, 2) transforming activity, 3) thermal denaturation profile and, 4) resistance to S_1 nuclease.

Bernardi (1969) has shown that hydroxyapatite is able to discriminate between nucleic acids having a rigid secondary structure and those having a random disordered structure. When either of the isolated strands (L or H) are self-annealed and applied to an HA column, one main fraction, designated I, elutes at 0.15 - 0.18 M Na PO_4. The first peak is single stranded as shown by its thermal denaturation profile, buoyant density, and its electron microscopic appearance. The recovery of material in this fraction is often incomplete. A second smaller fraction, designated II, elutes at 0.2 - 0.22 M N a P. This fraction of DNA has been designated as a native-like fraction since its biological properties are similar to native DNA and its chromatographic behavior is identical. The second peak contains the DNA which is capable of self-annealing. Both the work reported here and earlier studies (Rudner and Remeza, 1973; Galloway and Rudner, 1974) prove that fraction II has secondary structure as shown by its hyperchromic rise in the range of 80^{o} - 95^{o}, buoyant density, transforming activity, and resistance to S_1 nuclease.

Molecules which elute in the second peak are imperfect, containing both single stranded and double stranded regions, as evidenced by their thermal denaturation profiles. Exact determinations on the number of nucleotides which must be base paired for a fragment of DNA to elute in the native-like fraction have not been made. It has been shown that a fragment with 45 base pairs will elute from HA as native DNA, and that

the size of the single stranded tail (about 1000 nucleotides) did not affect the molarity at which the fragment eluted (Wilson and Thomas, 1973).

From the melting profiles of the self annealed material it is clear that the % helicity in these fractions is more substantial. Estimations on the percent of helicity present, show ca. 20%. Given a single stranded molecular weight of 2.9×10^6, the amount of DNA involved in helical structures is 5.8×10^5, which represents around 1.0×10^3 bases or 1000 base pairs/molecule. Temperature absorbance measurements of self-annealed MAK strands prior to fractionation on HA show a hyperchromic rise which is consistent with the amount obtained in HA fractionated material. There is also a finite amount of DNA which is able to form secondary structure as shown by the experiments of Rudner and Remeza (1973), in which the single stranded peak of HA eluted DNA is subjected to a second period of thermal renaturation and rechromatographed on HA. Only one peak of single stranded material is eluted. This suggests that the formation of secondary structure is due to the specific pairing of self complementary sequences in the same molecule rather than a fortuitous reaction of which all single stranded DNA is capable.

Hydrosyapatite chromatography is most useful as a means of isolating the self-annealed material. It cannot be used solely as an assay for this material since it is not specific for intrastrand base pairing. As mentioned previously the entire molecule which contains helical regions will elute in this fraction, leading to a large overestimation in the amount of self-annealing material. If cross contamination between the L and H strands has occured a biomolecular duplex will form upon renaturation which will also elute in this fraction. Care has been taken to use only the early eluting L strand and the late eluting H strand of the original MAK fractions to avoid this problem.

Both the L and H strands show the same ability to self anneal as shown by hydroxyapatite chromatography, with fraction II representing 20 - 35% of the input DNA. There was no observable difference in either the relative proportions of I and II or their elution molarities for the L and H strand DNA (see Figures 4 and 5). It is reasonable to assume that both strands would have the same amount of self-annealing material. If one strand contains self complementary sequences the other strand must also contain them, for example:

L - C A T T G A C T G G T C A A T
H G T A A C T G A C C A G T T A

It is hard to directly measure the complementarity of L II to H II since this depends on its ability to form a duplex molecule, however, under renaturation conditions the self-annealed structures are formed preferentially. It seemed reasonable that if the self-annealed DNA of the L and H strand was complementary, then the material which was not capable of forming secondary structure, fraction I would also be complementary. The formation of duplexes between L I and H I could be assayed by temperature absorbance measurements or by transforming activity. Both of these assays showed that the HA fractionated (L I + H I) formed duplexes less efficiently that the MAK fractionated (L + H). The hyperchromic rise of melted HA LI + HI duplex is 44% that of the MAK L + H duplex and the transforming activity of the H A LI + HI duplex is 58% that of the MAK L + H duplex. Several possibilities which may account for the differences in duplex formation were investigated e.g. 1) hydroxyapatite chromatography is responsible, 2) the size of the HA fractionated molecules is different from the MAK fractionated molecules and, 3) the HA fractions are less complementary than the MAK fractions.

If hydroxyapatite either inactivated or inhibited the DNA from forming

duplexes in some inknown manner the fractionated DNA would be irreversibly incapable of forming duplexes. Also the passage of pre-formed duplex through a hydroxyapatite column should also inactivate the DNA. The latter type of experiment, forming and L + H duplex from the MAK fractionated strands, and chromatographing this on hydroxyapatite, gave two peaks; the first, a minor peak of single stranded DNA which did not reassociate and a second major peak, containing the duplex DNA. The transforming activity of the MAK L + H duplex was equal to that of the HA eluted MAK duplex in peak II.

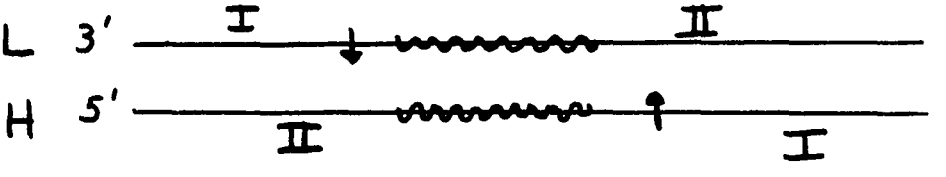
It was also thought possible that a preexisting duplex would be protected from inactivation by HA but that the self-annealed strands would be more susceptible. The reconstitution experiments show that this was not the case. Both the L and the H strands were self annealed individuallyand fractionated into their component parts. All of the fractions were then added back in the proportions at which fractions I and II eluted using an equal amount of total L and total H. The duplex formation was assayed both by temperature absorbance measurements and by transforming activity. The melting profile given in Figure 11 shows that the reconstituted duplex had the same hyperchromic rise as the MAK L + H duplex. This criteria shows that the HA chromatography is not responsible for the decreased ability to form duplexes.

The second possibility was that the HA strands were smaller than the MAK strands. The work of Martinson and Wagenar (1974) has shown that single stranded RNA can be degraded by hydroxyapatite. Although there has been no evidence reported that this occurs with single stranded DNA, the possibility was investigated by looking at the size of the fractionated strands by sedimentation velocity experiments. The fractionated strands were sedimented in alkaline conditions to minimize the effects of

conformational differences. The data in Table 3 shows that the S values for the MAK H, HI and HII fractions were very close being 21.4 S, 20.1 S, and 20.2 S respectively. This gives a molecular weight difference of 10% between the HA and the MAK fractionated DNA, showing that there is a small amount of shear which may be attributed either to the hydroxyapatite or the additional physical manupulations. The differences in the molecular size are consistent with the decreased levels of transforming activity but are insufficient to explain the poor efficiency of the HA LI + HI duplexes.

All of these results lead to the conclusion that hydroxyapatite fractionation in no way leads to the decreased ability to form duplexes seen in the HA fractionated strands. There is no thermodynamic explanation to account for single stranded DNA without self-complementary sequences being less efficient at finding the complementary sequences than those molecules which do contain self-complementary sequences. The only possible explanation, then, is that the single stranded fractions of L and H are less complementary when the self annealing DNA has been removed than when this material is present, either in the MAK strands or the reconstituted mixtures. This then says that the self annealed fraction is not entirely complementary. Since the sequences which are self-complementary must have a self-complementary component on the other strand the non-complementarity must be due to the single strand ends of the molecules.

The following model could explain the decreased complementarity which is observed in the HA fractionated L + H duplexes:



The arrows represent the position of the breaks in the molecule. The molecules marked II will elute in the second fraction of HA eluted DNA because of the presence of self-complementary sequences designated by  . The molecules marked I will elute in the single stranded fraction. The complementary sequences can in this way end up in either HA fraction. This model makes two predictions about the breaks that give rise to these molecules. One, a cleavage across both strands in the same place is not very common, and secondly, that there may be a polarity in the breakage points that leads to a non-random assortment of molecules. If the breakage points were entirely random, cleavage at the same points on the opposite strands should occur as frequently as those shown, giving a population of complementary molecules in the HA fraction I.

Chromosomal breakage in unfractionated and MAK fractionated pneumococcus DNA has been investigated (Roger, 1968; Roger et al, 1966; Roger et al, 1966a). From boundary sedimentation studies they concluded that chromosomal breakage occurs during extraction of the DNA at predetermined regularly spaced intervals. Gabor and Hotchkiss (1966, 1969) also concluded that pneumococcal DNA preparations are very homogenous based on studies of the rate of phenotypic expression of different markers when different heteroduplexes are used. These studies support a model of non-random breakage points. No mechanism to explain such polarity has yet been proposed but the data is consistent with the model. This model satisfactorily explains the cecreased ability of the HA fractionated strands to form duplexes as judged by thermal denaturation profiles and transforming activity. The work of Stewart (1969) on B. subtilis DNA does not support a model of unique break points. He finds that the DNA which is capable of transforming for a given marker is heterogeneous with respect to its molecular weight, buoyant density, and thermal stability. His data, however, does

not rule out the possibility that there are also preferred sits. The markers assayed, as shown in Table 2, are widely scattered throughout the B. subtilis chromosome with pur A16, leu 8, met 5 being located at the beginning, middle and end of the chromosome respectively (Oishi et al 1964). The self annealing material is capable of transforming all of these markers. All other markers that were assayed, thr 5, his ^{B}2-try 2 linkage, showed the same ability to be transformed by the self annealed fraction (Rudner and Remeza, 1973). This indicated that the self complementary sequences are not restircted to any one locus but are present throughout the chromosome.

The transforming activity of both the MAK H strand and HA HII is higher for the pur A marker than for any other locus. This marker has been reported to be relatively resistant to denaturation (Rudner et al, 1967). The pur A16 marker has also been reported to renature more rapidly and to a greater extent than other markers in purified phage PBSH DNA (Haas and Yoshikawa, 1969). As this marker is situated at the origin of the chromosome it may contain either an inter or intra strand crosslink which is required for replication (Doty et al, 1959). Its resistance to denaturation suggests that this DNA is distinct from the self annealing material. This DNA however must be somewhat different from the crosslinked DNA which elutes in the minor peak ahead of the L strand.

In many of the markers which were assayed, the mutations are known to occur in the structural genes. Transformation to prototrophy then, must involve integration of a piece of DNA containing the structural gene. Since the self annealed material is capable of transforming these markers, structural genes must be present in the population. The possibility that the structural genes are single stranded regions contiguous with regulatory regions of self complementary DNA was considered.

The assay of transforming activity along the buoyant density profiles shown in Figure 19 indicates that the transforming activity actually is associated with the DNA which bands at a native-like density rather than the shoulder DNA which bands with a higher buoyant density. This was found to be true of all markers assayed. Although this correlation mainly reflects the requirement for a double stranded structure for transformation it also suggests that structural gene material is present in the self-complementary sequences.

The levels of transforming activity for the H II fraction are lower than those for an L+H duplex, either from MAK or HA fractionated strands. An average transforming activity compared to the initial transforming activity for these fractions is, 10% for H II, 28% for HA LI + H I, and 45% for MAK L + H. This in part reflects the different efficiencies of forming helical structures but also may reflect the extent to which structural gene material is present in these fractions. The % helical structure for these fractions as measured by the hyperchromic increment from 80-95^{o}C in a melting profile is 23% for H II, 37% for HA L I + H I and 76% for MAK L+H.

The ratio between the transforming activity and the % helicity for these fractions is not constant, giving .52 for H II, .76 for HA L+H, and .60 for MAK L+H. This suggests that factors other than the degree of helicity are important, namely the degree to which structural gene material is present in the fraction. The data suggests that the hydroxyapatite purified LI and HI strands contain the most structural gene material. The fact that it never reaches the MAK duplex level even at high DNA concentrations shows some sequences are missing from the population. The H II fraction has the lowest ratio indicating that this fraction is rich in non-transforming DNA such as regulatory sequences. The MAK fractionated DNA which still contains the self annealing activity shows a lower ratio of

transforming activity/% helicity than the purified HA duplex.

The lower transforming activity of the H II fraction could be explained by an alternate hypothesis. The type of secondary structure present in this fraction may not be entirely satisfactory to the exo and endonucleases which may have a regulatory role in allowing DNA to enter a competent cell. Hence, the self-annealing molecules may have regions of helicity interspersed with single strand regions. This could lead either to degradation of the molecule or a reduced ability to enter the cell. The difference in the ratio of transforming activity/% helicity between the MAK and HA duplexes cannot rule out this idea.

The studies on the effect of S_1 nuclease on the transforming activity also support the hypothesis that the NDA which is involved in the self-annealing, although it primarily contains non-transforming DNA, does to some extent have structural gene sequences. If the structural genes were only in the single strand regions, treatment with S_1 nuclease would abolish the transforming activity. The data in Table 6 shows that this is not entirely the case. Although S_1 nuclease reduces the transforming activity of the H II fraction two to three fold, it does not destroy it entirely. Therefore the S_1 nuclease resistant core must contain some structural genes.

Under the conditions of the assay it is not possible to entirely rule out the presence of single stranded regions. High salt (0.15M) was used to reduce the activity of the enzyme on native DNA templates. The salt acts both by stabilizing the DNA structure to prevent fraying of the ends which can serve as a substrate, and by reducing the endonucleolytic activity. These conditions were chosen to preserve the integrity of the loop-like structures. It is possible that a loop followed by a single stranded stretch and another loop would remain undegraded. Electron microscopic studies would be useful in determining the nature of the S_1 resistant cores.

The literature presented in the introduction on the probable existence of secondary structure in intragenic cistrons is consistent with the above mentioned data which concludes that the self-complementary sequences exist within both what is presumably regulatory sequences, and parts of the neighboring structural genes. The single strand tails which are contiguous with these sequences and elute in the second fraction of HA eluted DNA contain the bulk of the structural genes.

The temperature absorbance measurements shown in Figure 12 are useful in determining the extent of helical formation in the self annealing fraction which elutes in the second peak of HA fractionated DNA. Both HA chromatography and the transformation assay indicate the presence of secondary structure but give no information as to what percent of the molecule is involved in base pairing.

Double stranded DNA in aqueous solution is stabilized by interactions between complementary bases and interactions between neighboring bases (stacking). Be denaturing DNA these forces are disrupted and this is reflected by changes in viscosity, optical rotation, buoyant density, light scattering, and extinction coefficient. An easily observable parameter is the hyperchromicity at the absorption maximum of 260 nm which accompanies thermal denaturation of DNA. This change in absorbance takes place in the range of 80-95^{o}C and involves an increase in absorbance of about 40% for native DNA. All other fractions can be compared to the native to estimate the % helicity.

The data show that the HA fractionated self-annealing material from the L and H strand show an equal amount of helical structure and show a hyperchromic rise that is 22-25% that of native DNA. This is consistent with the hyperchromic rise of 5-10% shown for the self annealed MAK L or H strand, since 25-40% of the DNA elutes in peak II following self anneal-

ing. This leads to the estimate that 5-10% of the genome contains self-complementary sequences.

The midpoint of the transition in absorbance is called the melting temperature (Tm), and is characteristic of a given DNA. The melting temperature is dependent on several factors: the base composition of the DNA, the compositional distribution, the presence of unusual bases, the ionic strength of the solvent, the presence of metal ions, and the sticking of DNA to ligands or proteins. Marmur and Doty (1959) established a linear relationship between the Tm and the GC content of DNA. The relationship found is GC = (Tm-69.3) 2.44 for DNA; with a GC content between 30 and 70%.

If the nucleotide composition is roughly homogeneously distributed throughout the chromosome, the melting temperature will reflect the average composition. Doty et al (1960) investigated the compositional heterogeneity by looking at the change in temperature (AT) between 17 and 83% of the absorbance rise and correcting for a two thirds transition width of 3 found for poly d (AT). The DNA of bacteria and higher organisms though, shows a wider melting width than the 3 predicted by synthetic polynucleotides (AT for E. coli DNA is 4-5% and the AT for calf thymus DNA is 9-10) (Doty et al, 1959). DNA's with a wider melting range than these observed values reflect heterogenity of base composition distribution.

The DNA of the self-annealing fractions showed no significant difference in Tm from that of native B. subtilis DNA. This shows that the overall base composition of the regions involved in base pairing is the same as the total DNA. This argues against any hypothesis that the self-annealing DNA may be located in either high AT or GC rich regions. There was no observably difference in the melting width of the transition, so no compositional heterogeneity could be detected either.

Rudner and Remeza (1973) looked at the thermal stability of the self

annealed H fraction, an L+H hybrid and native DNA by heating at the appropriate temperature for 15 minutes, quickly cooling and assaying for transforming activity. The melting temperature of all three samples was found to be the same. This is in agreement with the conclusion that the self annealing material does not have any unusual base composition. A direct analysis of the base composition of the DNA fractions further supports the fact that there is no particular AT or GC richness in the self annealing material.

S_1 nuclease, an enzyme isolated from Aspergellus oryzae by Ando (1966) can be used to detect the presence, and to some extent, measure the amount of secondary structure in DNA. It specifically splits the phosphodiester bonds of single stranded DNA giving 5' deoxynucleotides. Sutton (1971) has shown that this enzyme is capable of making endonucleolytic cleavages but has a predominately exonucleolytic mechanism. The nuclease degrades in both the 5'→3' and the 3'→5' direction. It can degrade tails of a duplex as well as unpaired loop regions. Beard et al (1973) have shown that S_1 is capable of converting circular superhelical SV40 DNA to a linear duplex. The first site of cleavage occurs at a region that is probably unpaired due to the constraints of a superhelical structure. The second cleavage occurs opposite the nick. Weigand et al (1974) constructed a heteroduplex λ DNA which contained a deletion loop. The S_1 nuclease was able to degrade the unpaired loop and leave the rest of the duplex intact. S_1 nuclease is also useful in determining the extent of helix formation in reassociation experiments. Caution must be taken in the use of this enzyme with duplexes which may not be perfectly helical and certainly in investigations on the nature of intrastrand secondary structure.

The ionic strength of the reaction mixture is very important in controlling the specificity of this enzyme. In salt concentrations of

0.01 to 0.2 M, hydrolysis of single stranded DNA occurs to 95% completion. Vogt (1973) looked at the ability of S_1 to introduce nicks into native DNA. He found that in 0.3 M NaCl only one nick per ten molecules was introduced, whereas in 0.05 M salt, one nick per two strands was found. A sufficiently high salt concentration must be used to prevent nicking of native DNA. Similarly, the endonucleolytic activity of S_1 on single strand DNA is greater in low salt. It has also been shown that lowering the enzyme concentration lessens its endonucleolytic activity on duplex and single strand DNA (Crosa et al, 1973).

The data in Figure 20 shows that 56% of the HII fraction remains resistant to S_1 nuclease after 60 minutes incubation. This value is higher than the 24% helicity estimated from the thermal denaturation profiles. This suggests that under the assay conditions used, the endonucleolytic activity was greatly reduced so that unpaired loop regions and single strand sequences between the areas of secondary structure are not degraded. In retrospect, it appears that S_1 nuclease is not suitable for studying intrastrand secondary structure. In minimizing the endonucleolytic activity of this enzyme the remaining structure contains extensive single strand regions thereby obscurring the paired regions. This suggests however, that the secondary structures may more closely resemble the type of structures found in plasmid DNA by Sharp et al (1973) than the hairpin structures with very small turnaround regions found in eukaryotic DNA by Wilson and Thomas (1974).

The biological nature of the self-annealed material was investigated with regard to its hybridizability to RNA. Margulies et al (1970) and Oishi (1969) have shown that the 16S, 23S, and 5S ribosomal RNA hybridizes exclusively with the H fraction of MAK fractionated B. subtilis DNA, specifically in the late eluting region of the absorbance profile of the

MAK H strand. In a later study by Margulies et al (1970a), they showed that total messenger RNA synthesized after a nutritional shift down is preferentially transcribed from one strand of DNA. In B. subtilis 90% hybridizes to the MAK fractionated H strand and 10% to the L strand. When RNA was hybridized to fractions along the MAK elution profile, the greatest hybridizability was to the late eluting MAK H strand. Despite the diversity in the mRNA species, it appears these molecules are transcribed from the DNA molecules which are the latest eluting. It is significant that the biologically active self-annealing material also elutes in this region.

The data in Table 8 shows the characteristic asymmetry ratio of the hybridizability of pulse labeled log phase RNA to the L and H strands and confirms that the late eluting H strand DNA hybridizes preferentially. Under conditions of limiting DNA used here there is a great difference between the hybridizability of the MAK H strand and the HA HI strand. This would suggest that there are transcribed sequences present in the MAK DNA which are eliminated by removal of the self-annealing material. The experiments (Table 9 and Figure 21) using high amounts of DNA show less of a difference between the hybridizability of the two DNAs, nevertheless, a difference between the two populations does exist even at saturating levels of DNA.

This data may be interpreted to mean that the population of DNA sequences which fractionate with the self-annealing material rather than the single strand material is not entirely distinct. There is some randomness involved which will allow a break to occur which causes the DNA to fractionate with HI. As the DNA concentration is increased the statistical probability of including these sequences which are capable of hybridizing increases. If the process were entirely random the levels of hybridization of the HI fraction would eventually reach those of the MAK

fraction at a high enough DNA concentration. This, in fact, does not occur, indicating that a certain population of transcribed molecules always elutes with the self-annealing fraction.

The self-annealing material retains a certain amount of hybridizability even after some of the single stranded regions have been removed by treatment with S_1 nuclease. The low levels of hybridizability of the HII fraction suggest that this fraction contains non-transcribed sequences also. HI hybridized to pulse labeled RNA more than twice as efficiently as HII indicating that the majority of structural gene material fractionates with the DNA which is not capable of self-annealing.

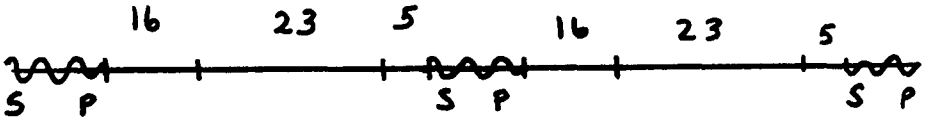
The isolated components of stable RNA do not hybridize uniformly to the chromatographic fractions of the DNA. Each of the RNA cistrons has a different probability of fractionating with the self-annealing material. Since all of the RNA species are able to elute with HI, the secondary structure which causes the DNA to elute with HII must either be outside the cistron or only in a fraction of the cistron so that it has single stranded pieces capable of eluting with HI.

Colli and Oishi (1969) have shown that the genes for 16S and 23S RNA in B. subtilis are physically linked. Both in E. coli and in B. subtilis studies using rifampicin to inhibit the initiation but not the elongation of RNA synthesis have shown that the rRNA molecules are derived from a transcriptional unit consisting of 16S, 23S, and 5S rRNA genes in that order (Doolittle and Pace, 1970; Doolittle and Pace, 1971; Pato and Von Meyenburg, 1970). Oishi and Sueoka (1965) and Dubnau et al (1965) have used density labeling of synchronously replicating B. subtilis to map the RNA genes. They found two widely scattered regions of the chromosome containing the rRNA clusters.

Zimmerman and Leventhal (1967) have determined a chain elongation

rate of 75 to 85 nucleotides/second for RNA polymerase synthesizing rRNA molecules. This rate is twice that of the rate of synthesis of most genes. One mechanism to explain the efficient synthesis of rRNA would suggest the existence of a very efficient promotor or series of promoters for the rRNA transcriptional unit. It is possible that these promoters are capable of forming the type of secondary structure studied here. Secondary structure maps of ribosomal RNA and DNA have been well characterized in eukaryotes (Wellauer and Dawid, 1974; Wellauer et al, 1974). In Xenopus laevis the 40S transcriptional unit codes for a 28S gene, transcribed spacer, and an 18S gene interspersed with non-transcribed spacer. The 28S and spacer regions show extensive secondary structure whereas the 18S gene is entirely devoid of secondary structure. The situation is not as well characterized in prokaryotes. There is evidence that 60 to 70% of the bases in rRNA molecules are base paired, with the 16S having a complex series of hairpin loops (Pace, 1973), but there is very little information as to the structure of the DNA coding for the rRNA.

Based on the differential hybridizability of the RNA components to the self-annealing DNA (see Table 11) and consistent with the literature on the transcription unit, a model is proposed to explain the data:



Since all of the genes are capable of fractionating with hybroxyapatite peak I, there must be little if any secondary structure within the cistrons. The spacer (S) and possible promoter (P) regions outside the genes must contain secondary structure. The 16S and 5S species are much more likely to end up on a molecule containing secondary structure than is the gene

coding for the 23S species. This model makes no predictions about the necessity for predetermined breaks.

The work described herein has described a first step in the isolation of regulatory sequences from a prokaryotic DNA. Clearly this work could be extended to further elucidate the structure of the self annealing molecules, and to obtain a more homogeneous population.

One likely avenue of investigation could be the electron microscope appearance of the self annealed structures. Although a relative newcomer to the field of electron microscopy, nucleic acids can now be visualized with ease. Both single and double stranded nucleic acids can be mounted on EM grids either by the basic film technique of Kleinschmidt (1968) or without a basic protein film (Koller et al, 1969). Secondary structures of various RNAs have been determined, e.g. mouse L cell rRNA (Wellauer et al, 1974) xenopus laevis rRNA (Wellauer and David, 1974) tRNA genes (Wu and Davidson, 1973) etc.

The secondary structures of DNA have been less well characterized other than for various viral DNAs (Lang et al, 1967). The technology is available to study the structure of self annealing molecules of B. subtilis DNA. These studies could provide useful information as to the type of structures formed, e.g. hairpin loops, cloverleaves, etc.; the homogeneity of structures, the reproducibility, the amount of bases in the loop, and so on.

Various methods could be used to attempt to purify the self annealing molecules. One possibility relies on the recognition of self-complementary sequences by regulatory proteins. A specific protein could be bound to the DNA and then the DNA be mildly digested with DNase to leave a fragment of DNA which is longer than the piece "just protected" by the protein. This fragment could then be dissociated from the protein, strand separated

(perhaps by gel electrophoresis) and each strand self annealed. If self annealed molecules can be obtained in this way, a further step towards isolating a homogenous regulatory element will have been achieved.

The use of restriction endonucleases has proved an invaluable tool in the isolation of defined segments of DNA. It is conceivable that they could be used in the isolation of self annealed structures. By using a combination of enzymes, it might be possible to isolate a class of self annealing molecules in which the self complementary sequence which allowed the structure to reassociate was one which could be cleaved by a certain enzyme.

The overall experimental design would be to cleave total B. subtilis DNA with an enzyme and separate the fragments in agarose gels. The DNA could be eluted from various cuts of the gel and denatured. This could be diluted enough so that when renatured, only single strand associations could occur. This structure could then be separated by hydroxyapatite. Those regions of the gel which yielded self annealing molecules could be redigested with another enzyme and treated as above. If the ability to self anneal was completely destroyed it is good evidence that the self complementary sequence was recognized by the enzyme. With over 50 available restriction enzymes (R. Roberts, personal communication, July 1975) the variations on this type of experiment are limited only by the investigators ability to extract or beg for enzymes.

The conundrum of intrastrand secondary structure has not been answered fully by the work presented here or by the experiments proposed. This work has presented a method towards the isolation of regulatory elements and has characterized the self-annealing structures found in B. subtilis DNA. It is not clear that there will be one answer as to the structure and function of intrastrand secondary structure, as simplicity seems to be

a property of biologists rather than of biological organisms.

SUMMARY

The studies presented here show that B. subtilis DNA contains a population of molecules which are capable of self annealing to form intrastrand secondary structures. MAK chromatography was used to separate the DNA strands, designated as L and H. After self annealing at 68°, the molecules containing secondary structure could be isolated from the rest of the DNA by HA chromatography. The L and H strands contained the same amount of secondary structure as judged by HA elution profiles.

Thermal renaturation profiles indicated that this material contained both helical and single strand regions and that the helical structures represented 5-10% of the genome. The thermal stability of the self annealed material indicated that its base composition was not very different from that of native B. subtilis DNA. Direct conformation of this was obtained by base composition analysis. The buoyant density of the self annealed DNA from the H strand was 1.710 g/cm^3, similar to renatured DNA, with a shoulder from 1.716 g/cm^3 to 1.722 g/cm^3 like that of denatured DNA. The molecular weight of the fragments capable of forming secondary structure is the same as the rest of the H strand fragments.

The transforming activity of the chromatographically fractionated strands was used as an assay of these secondary structures as well as an indication of which genes were present in a fraction. Only the self annealed H DNA showed an increased transforming activity (6-10% of native) above the MAK value, and this was not restricted to any one marker indicating the presence of self annealing sequences throughout the chromosome.

The low levels of transforming activity associated with this DNA and with duplexes from HA purified L and H strands led to an investigation

of the possible inactivation of DNA by hydroxyapatite. HA was not found to be responsible for the low level of transforming activity or duplex formation and it was concluded that the self annealing material contains many non-transforming sequences and that the HA L+H duplex is less complementary than the MAK L+H duplex.

The hybridizability of self annealed H strand to pulse labeled and stable RNA was investigated to determine the type of sequence present in the population. It was found that the self annealing H DNA hybridized only 20-30% as well as the total MAK strand to a pulse labeled RNA indicating the presence of many non-transcribed sequences. Hybridization to ribosomal RNA indicated that these sequences were present in the self annealed DNA but the different genes were there in different frequencies, reflecting either their own secondary structure or closeness to a promoter or spacer with secondary structure.

Appendix A Growth Media

1 x Spizizen

0.044 M KH_2PO_4

0.080 M K_2HPO_4

0.0034 M Na Citrate

0.001 M $MgSO_4$

Add water to 1 liter

Minimal media for plates

200 ml 10 x Spizizen

20 g glutamic acid

800 ml water

40 g Bactoagar

10 g dextrose

1 liter water

Mix the two liters after autoclaving

TBAB plates

33 g Tryptose Blood Agar Base (Difco)

5 g Bactoagar

1 liter water

1 x SSC

0.15 M NaCl

0.015 M Na citrate

Sterlini and Mandelstam medium

Basal salts

1×10^{-4} M KH_2PO_4

1×10^{-6} M $FeCl_3$

1×10^{-4} M $MnSO_4$

4×10^{-4} M $MgSO_4$

1×10^{-2} M NH_4Cl

7.5×10^{-4} M Na_2SO_4

1.2×10^{-3} M NH_4NO_3

1×10^{-4} $CaCl_2$-$6H_2O$

Additions

0.86% vitamin free casamino acids

0.024 M D,L alanine

0.019 M L-glutamic acid

0.008 M L-asparginine

0.0001 M L- tryptophan

0.0001 M $CaCl_2$- 6 H_2O

0.2% glucose

100 ug/ml uridine

Adjust to pH 7.1 with 5N KOH

Add water to 1 liter

Resuspension medium

1×10^{-4} M KH_2PO_4

1×10^{-2} M NH_4Cl

7.5×10^{-4} M Na_2SO_4

1.2×10^{-3} M NH_4NO_3

4×10^{-7} M $FeCl_3$

$1\ 10^{-4}$ M Mn Cl_2

1×10^{-4} M tryptophan

1.2×10^{-2} M L-glutamic acid

4×10^{-2} M $MgSO_4$

2×10^{-4} M $CaCl_2$

Add H_2O to 1 liter

Adjust to pH 7.1 with 5N KOH

Adjustment medium

1 vol. 2 x Sterlini and Mandelstam medium

1 vol. Resuspension medium

Appendix B Calculation of the % helicity from a thermal denatuation profile

1. Measure the initial absorbance at 25^0.
2. Determine the change in absorbance (Δ) by measuring the distance from the baseline to the absorbance at 80^0, using the calibrations of the plot.
3. Add the Δ to the initial value to get A^{80}.
4. Follow steps 2 and 3 to determine A^{95}.
5. Use the following formula to determine the % helicity:

$$\frac{100\ (\ A^{95} - A^{80}\ )}{A^{95} - (\ A^{95}/\ 1.38\ )}$$

Appendix C Calculation of the buoyant density from a CsCl density gradient centrifugation

1. The radius from the center of rotation to the end of the tube is 8.1 cm. Since the angle is 26^0, the hypotenuse of the triangle is 8.1 cm/ sin 26 = 18.48 cm.
2. Since the entire sample is 3 cm, one can divide by the number of fractions and determine the distance of the DNA band from the bottom of the tube.
3. When subtracted from the hypotenuse, a new radius from the center of rotation to the DNA band can be determined from the relation: radius = hypotenuse x sin 26^0.

4. The radius from the center of rotation to the DNA marker is determined in the same way.
5. When the buoyant density of the marker is known, and w^2 is known, the buoyant density of the other DNA can be determined using the formula:

$$p = p_o + 4.2\ w^2\ (\ r^2 - r_o^{\ 2}\ )\ x\ 10^{-10}\ g/cm^3$$

Appendix D Calculation of the molecular weight from sedimentation velocity centrifugation.

1. Using the tracing of the photograph obtained from the analytrol, measure the distance from the meniscus to the plateau and divide by two to get the midpoint.
2. Multiply by the magnification factor (0.114) which can be calculated from the known geometry of the cell and the size of the tracing.
3. Subtract this value from 7.3 cm, the distance from the center of rotation to the outer reference hole, to get x, the distance of the macromolecule from the center of rotation.
4. Plot the natural log of x against time and determine the slope, d lnx/ d t.
5. Determine S from the relation:

$$S = \frac{d\ \ln x}{d\ t}\ x\ \frac{1}{w^2}$$

6. Determine the molecular weight of the DNA from the S value using the following relations:

native DNA $S = 0.0882\ M^{0.346}$

alkaline DNA $S = 0.0528\ M^{0.400}$

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Table 1

The specific activities of radioactively-labeled nucleic acids

Nucleic acid	Radioactive Label	Spec. act. (CPM/ug)
DNA	^{32}P (KH_2PO_4)	* 4.1×10^5
DNA	^{32}P (KH_2PO_4)	* 3.8×10^5
DNA	^{3}H thymidine	5.1×10^4
DNA	^{3}H adenine	2.4×10^4
RNA (pulse labeled log)	^{3}H uridine	5.5×10^4
RNA (pulse labeled log)	^{3}H uridine	2.6×10^4
RNA (act. D chased)	^{3}H uridine	2.0×10^4
RNA (16S + 23S)	^{3}H uridine	4.2×10^4

* The specific activity of the ^{32}P labeled DNA given here was determined two days after extraction following purification by MAK chromatography. The specific activity of the DNA at the time of a given experiment is given in the legend.

Table 2

Transforming activity of L + H duplexes from MAK, HA, and reconstituted HA fractionated strands

	% of initial transforming activity			
DNA duplex	purA16	leu-8	metB5	thr-5
	(2.3×10^5)	(8.3×10^4)	(1.7×10^5)	(3.2×10^5)
Native	100	100	100	100
MAK L+H	44.2	37.1	39.8	46.0
HA LI+HI	37.6	31.1	23.9	31.0
HA (LI+LII)+(HI+HII)	45.5	33.0	35.3	44.0
MAK L+H, after HA	41.0	32.6	34.8	43.1

The duplexes were prepared by annealing an equal amount of L and H strand fractions at 20 ug/ml at 68^0 for 2 hours in 2 x SSC. The samples were assayed at 0.5 ug DNA/ml in 1 x SSC.

Table 3

Sedimentation values and molecular weight assignments of native and fractionated strands of B. subtilis DNA

DNA fraction	S x 10^{-13}	MW*
Native	25.0	12.0 x 10^6
Denatured	21.9	3.5 x 10^6
MAK L	18.5	2.3 x 10^6
MAK H	21.4	3.3 x 10^6
HA HI	20.1	2.8 x 10^6
HA HII	20.2	2.9 x 10^6

All of the samples were run at 24,000 RPM in 1 x SSC. The denatured samples were run at pH 12.7 and the native sample at pH 7.0.

* The molecular weight assignments for these values were determined based on the equations given by Studier (1965): native DNA, $S = 0.882\ M^{0.346}$; alkaline DNA, $S = 0.0528\ M^{0.400}$.

Table 4

The composition of native and fractionated strands of B. subtilis DNA

	Composition (moles %)				Molar ratio	
Fraction	A	G	C	T	Pu/Py	A+T/G+C
Native	28.9	21.0	21.6	28.7	0.99	1.35
MAK L	30.8	21.5	22.4	25.8	1.08	1.28
MAK H	28.3	19.6	22.9	28.9	0.92	1.35
HA HI	24.9	22.1	24.3	28.7	0.89	1.15
HA HII	29.6	22.5	20.3	28.0	1.07	1.34
HA HII-S_1*	31.3	23.0	20.0	25.7	1.18	1.32

One ug of ^{32}P labeled DNA (spec. act., 1 x 10^5 CPM/ug) and 500 ug of calf thymus DNA were digested to mononucleotides. A 50 ul aliquot containing 0.1 ug ^{32}P-DNA and 50 ug of calf thymus DNA were spotted onto the chromatograph.

* Before digestion to 5' mononucleotides the sample was digested with S_1 nuclease for 1 hour. The aqueous layer was removed and dialysed against 0.02 M Tris-HCl, 0.05 M $MgCl_2$, pH 7.2.

Table 5

The buoyant densities of native and fractionated strands of *B. subtilis* DNA

DNA fraction	Buoyant density (g/cm^3)
Native	1.703
Denatured	1.720
Renatured	1.707
MAK H	1.720
MAK L	1.718
HA HI	1.723
HA HII	1.710, 1.716-1.722
HA LI	1.719
HA LII	1.709, 1.715-1.722

The buoyant densities of the fractionated DNA samples were made in comparison with a ^3H native *B. subtilis* DNA. The samples were spun in a 50 Ti rotor at 39,000 RPM at 19^0 for 65 hours.

Table 6

The effect of ionic strength and temperature on the digestion of DNA by S_1 nuclease

		% TCA ppt. in 60 min.	
NaCl molarity	Temp.	Native	Denatured
0.05	50	76.0	4.3
0.05	37	79.1	6.8
0.10	50	80.3	15.5
0.10	37	84.7	14.4
0.15	50	82.6	17.1
0.15	37	84.9	19.6
0.20	50	92.1	24.1
0.20	37	90.4	19.2

The enzyme assay shown here was with the enzyme obtained from Miles Laboratories. The reaction mixture contained 0.5 ug ^3H DNA, 10 ug sheared denatured calf thymus DNA, 0.3 mM Na acetate, pH 4.5, and 10^{-6} M ZnCl. The ionic strength and temperature was as indicated. After 60 minutes the samples were divided into two and TCA precipitated. Before the addition of enzyme, 0.1 ml was removed for an initial value.

Table 7

The effect of S_1 nuclease digestion on the transforming activity of native and HA HII DNA

	% of initial transforming activity			
DNA	purA16	leu-8	metB5	thr-5
	($1.2x10^5$)	($6.3x10^4$)	($6.9x10^5$)	($2.6x10^5$)
Native	100	100	100	100
Native-S_1	93.6	82.6	88.3	84.1
HA H II	14.2	6.8	8.2	9.7
HA H II-S_1	7.8	0.7	3.6	4.7

Unlabeled DNA (10 ug/ml) was incubated for 60 minutes in the S_1 assay mixture, but only the samples denoted S_1 contained the enzyme. The DNAs were all assayed at 0.5 ug/ml.

Table 8

The differential decrease in hybridizability of HA purified L and H strands of B. subtilis DNA to ^{3}H- pulse labeled log phase RNA

Chromatographic fraction	Input ratio RNA:DNA	CPM complexed after RNase	^{3}H-RNA hybridized %	Asymmetry ratio H/L	% of original MAK fraction
MAK					
L	100:1	4,992	0.58		
H	100:1	17,763	2.10	3.62	100
total L	50:1	665	0.50		
total H	50:1	2,295	1.45	2.90	100
early L	50:1	561	0.42		
late L	50:1	865	0.63		
early H	50:1	1,167	0.92		
late H	50:1	3,096	2.44	3.20	100
HA					
total LI	50:1	448	0.34		68
total HI	50:1	686	0.49	1.44	30
early LI	50:1	453	0.34		81
late LI	50:1	548	0.40		63
early HI	50:1	331	0.26		28
late HI	50:1	1.156	0.91	1.59	37

The reaction mixture (0.10 ml) for the 50:1 ratio contained 0.1 ug of DNA and 5.35 ug of ^{3}H-RNA (specific activity, 25,709 CPM/ug). The reaction mixture (0.10 ml) for the 100:1 ratio contained 0.1 ug of DNA and 10.7 ug of the same ^{3}H-RNA. The reactions were incubated in 6 x SSC at 68^{0} for 18 hours.

Table 9

The differential hybridizability of MAK and HA fractionated H strands of B. subtilis DNA to pulse-labeled ^{3}H-RNA

DNA fraction	ug DNA(a) immobilized	CPM complexed (b) after RNase	%^{3}H-RNA hybridized	% of original MAK fraction
MAK H	6.7	16,288	41.35	100
HA HI	9.0	9,238	23.45	56.7
HA HII	0.8	184	0.47	1.1
HA HII_D*	8.6	2,890	7.33	17.7
HA $(I+II)_D$*	8.0	12,627	32.05	77.5
HA HII - S_1 D	6.5	2,101	5.35	12.9

* Thermal denaturation (100^0, 10 min.) prior to immobilization on nitrocellulose membranes.

(a) Actual amount of ^{32}P-DNA (ug) immobilized from an input of 10 ug of DNA/membrane. Spec. act. of the DNA: MAK H, 18,931; HI, 14,351; and HII, 16,559 (CPM/ug).

(b) Each annealing mixture contained0.71 ug of pulse-labeled ^{3}H-RNA in 2 x SSC incubated at 66^0 for 16 hours (spec. act. of the RNA, 55,500 CPM/ug).

Table 10

Hybridizability of MAK and HA purified H strands of B. subtilis DNA to ribosomal RNA components

RNA Component	Chromatographic fraction	Input ratio DNA:RNA	CPM complexed after RNase	% DNA hybridized	% of original MAK fraction
16S + 23S	MAK H	10:1	6,255	0.75	100
16S + 23S	HA HI	10:1	4,444	0.53	71

The reaction mixtures (4.0 ml) contained 20 ug of DNA and 2 ug of ^{3}H RNA (16S + 23S). The specific activity of the RNA was 41,649 CPM/ug).

Table 11

The hybridizability of MAK and HA fractionated H strands of B. subtilis DNA to the isolated components of stable ^{3}H-RNA

DNA fraction	RNA fraction	ug DNA immobilized	CPM complexed after RNase	% ^{3}H RNA Hybr. per 10ug DNA	% of original MAK fraction
MAK H	23 S	9.1	3249	28.2	100.0
HA HI	"	8.7	1598	14.5	48.7
HA HII *	"	9.4	1134	9.5	31.9
MAK H	16 S	9.1	1605	15.4	100.0
HA HI	"	8.9	1570	15.3	99.4
HA HII *	"	9.3	1622	15.7	101.9
MAK H	5 S	8.7	987	7.3	100.0
HA HI	"	8.1	521	6.2	84.9
HA HII *	"	8.8	593	6.5	89.0
MAK H	4 S	8.6	944	9.5	100.0
HA HI	"	9.2	917	8.9	93.7
HA HII *	"	8.9	529	4.3	45.3

* Thermal denaturation (100°, 10 minutes) prior to immobilization on nitocellulose filters.

Each annealing mixture (total vol. 1ml) contained 10 ug of ^{32}P labeled DNA applied to a nitrocellulose filter and 0.5 ug ^{3}H RNA in 2 x SSC annealed at 66° for 16 hours. The specific activity of the RNA was: 23S, 25,328; 16S, 19510; 5S, 20,112; and 4S, 18184.

Figure 1. The Sobell model for recognition of DNA sequences. Taken from Sobell, H.M. 1972. Proc. Nat. Acad. Sci. 69: 2483. This model showed the recognition of self-complementary sequences by dimeric proteins. If a duplication of the sequence occured tandem to the sequence, a rearrangement of the hydrogen bonds could form a cloverleaf for recognition by tetrameric proteins.

Figure 1

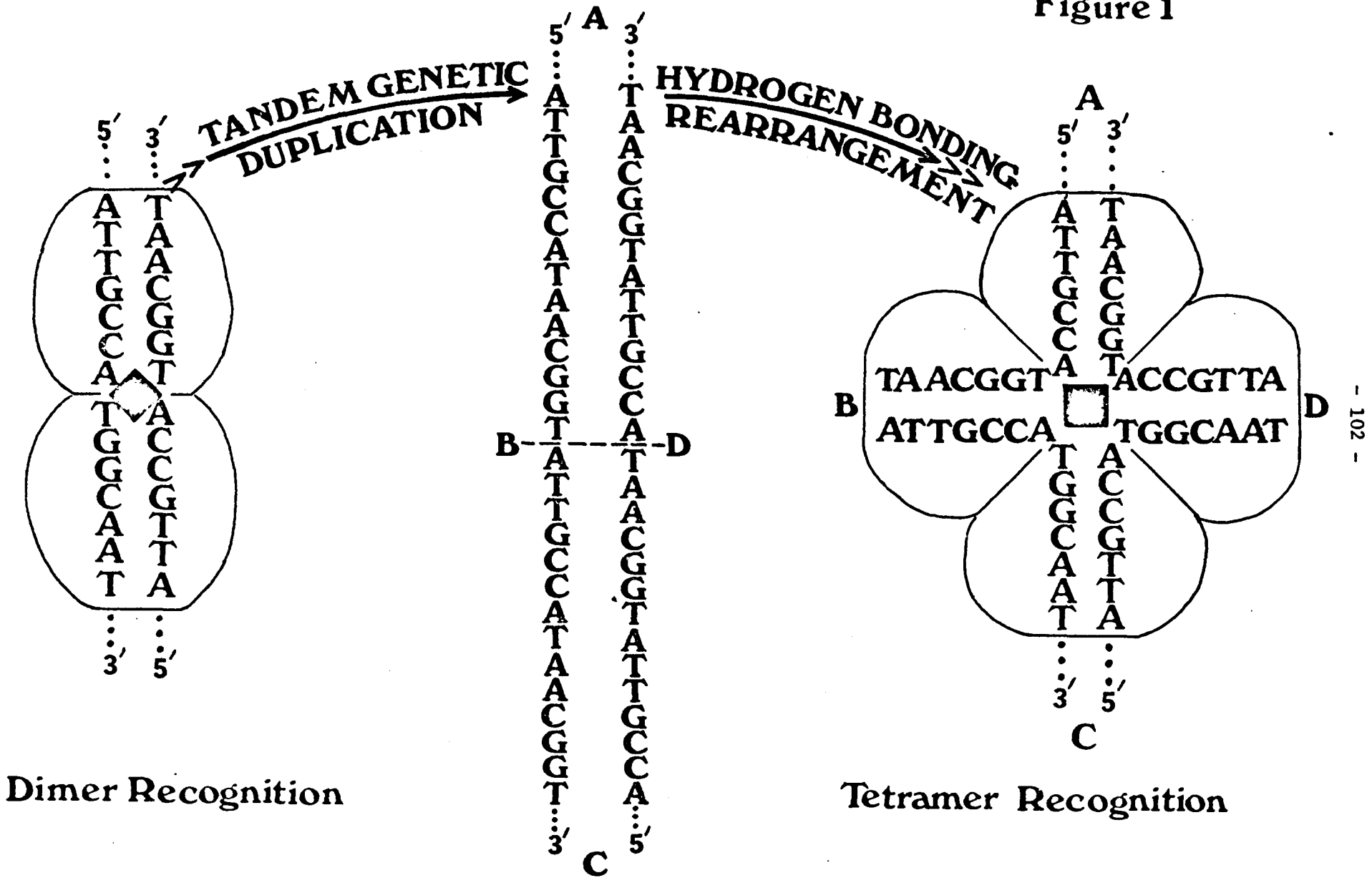

TANDEM GENETIC DUPLICATION
HYDROGEN BONDING REARRANGEMENT
A
B
C
D
5′
3′
ATTGCCATAACGGGTTAACCGGTTA
TAACGGTATTGCCATTGGCAATAACCGTTA
TAACGGTACCGTTA
ATTGCCATGGCAAT
Dimer Recognition
Tetramer Recognition

Figure 2. Fractionation of nucleic acids by Poly-L- lysine kieselguhr (PLK) chromatography. A 50 ml lysate of ^{3}H-adenine labeled B. subtilis, strain Mu8u5ul6, which had been incubated with lysozyme (2 ug/ml, 1 hr., 37^{0}), SLS (2%, 10 min., 60^{0}), and RNase A (50 ug/ml, 30 min., 37^{0}) was mixed with 10 g kieselguhr in 0.4 M NaCl in 0.02 M KH_2PO_4 and 20 mg polylysine. The mixture was added to a column and washed with starting buffer until no UV absorbing material eluted. A linear gradient, 0.4 to 4.0 M NaCl in 0.02 M KH_2PO_4 was started at tube 24, indicated by the arrow. Fractions 42 to 53 were pooled and used as a labeled native DNA marker.

Figure 2

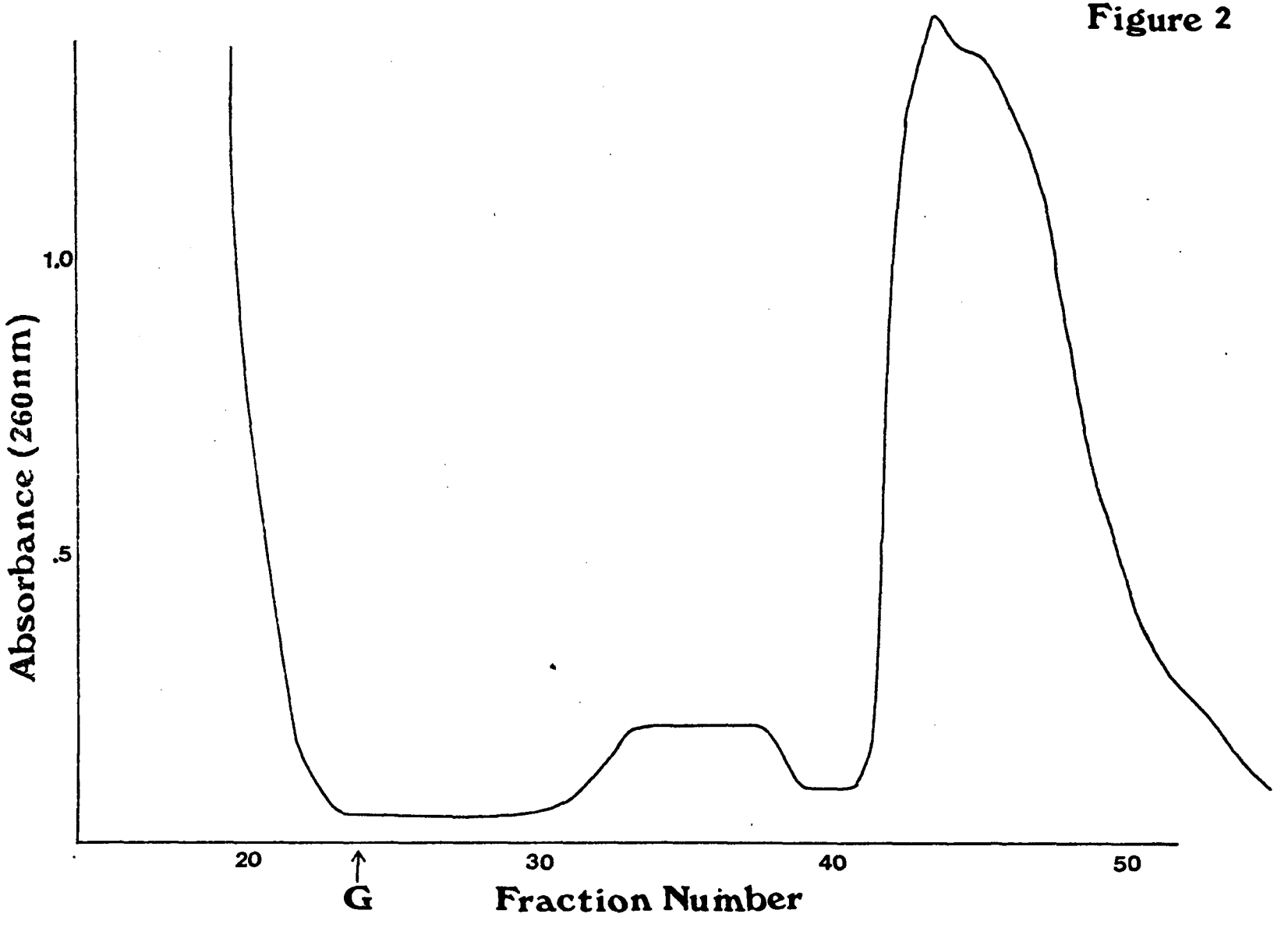


Figure 3. Fractionation of alkali denatured DNA by methylated albumin kieselguhr (MAK) chromotography. Alkali denatured DNA (2.75 mg) from B. subtilis, strain A26u$^-$, was applied in 0.7 M NaCl in 0.05 M $NaPO_4$ buffer, pH 6.8, to a column with 1/2 the standard recipe. The DNA was eluted with 500 ml of gradient 0.7 - 1.5 M NaCl. The gradient was interrupted at tube 39 and reestablished at tube 46. Fractions of 5.6 ml were collected. The total recovery was 71.5% and the ratio of L/H was 1.03.

Figure 3

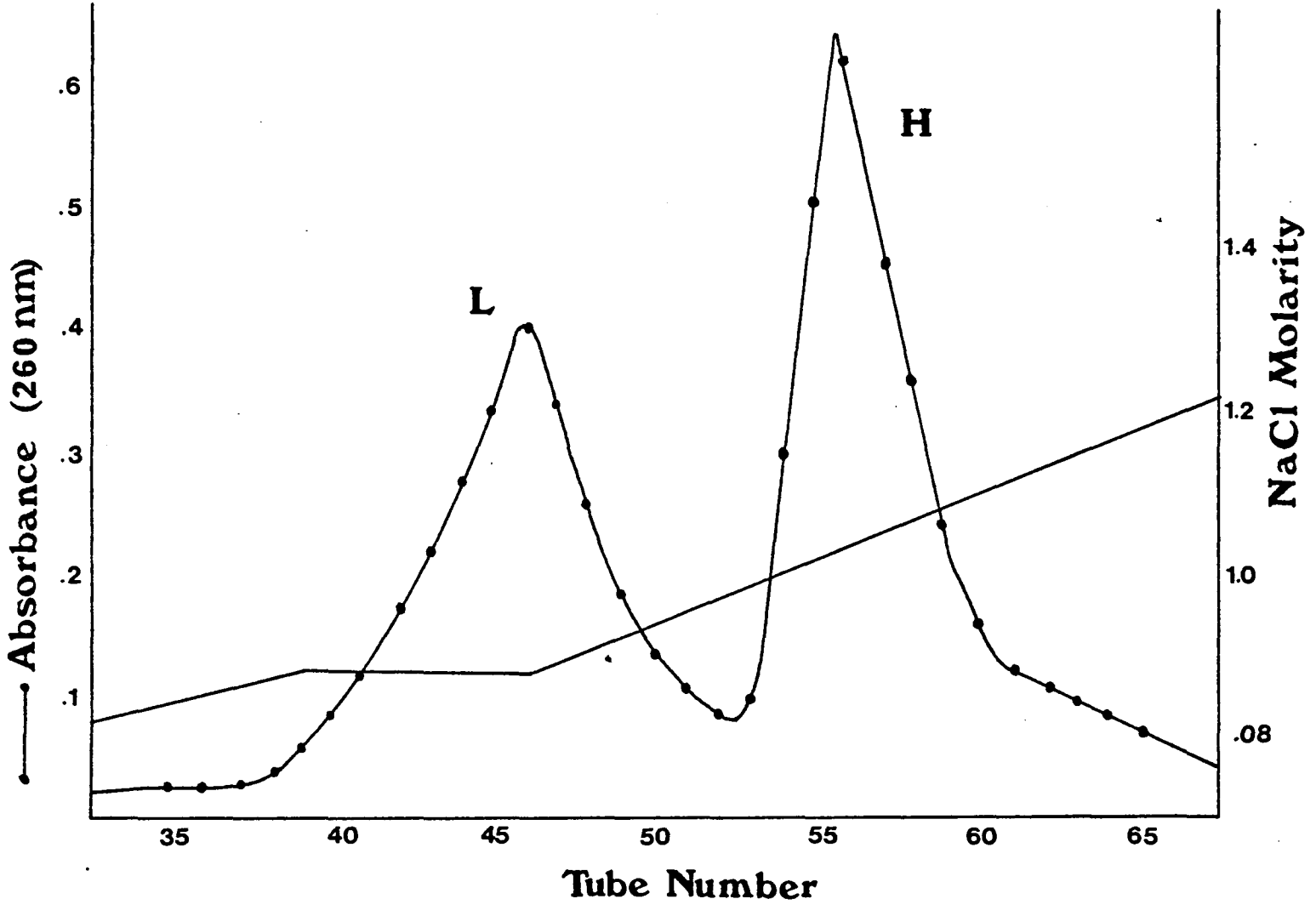

Absorbance (260 nm)
.6
.5
.4
.3
.2
.1
L
H
1.4
1.2
1.0
.08
NaCl Molarity
35
40
45
50
55
60
65
Tube Number

Figure 4. Fractionation of the self-annealed MAK H strand by hydroxyapatite (HA) chromatography. Self-annealed H strand DNA (0.254 mg) was applied in 0.05 M $NaPO_4$ buffer to a column 1 cm in diameter, packed to a height of 5 cm. The DNA was eluted with 300 ml of a linear gradient, 0.05 - 0.5 M $NaPO_4$. The recovery as a % of input was: total, 58.0; HI, 41.1; and HII, 13.6.

Figure 4

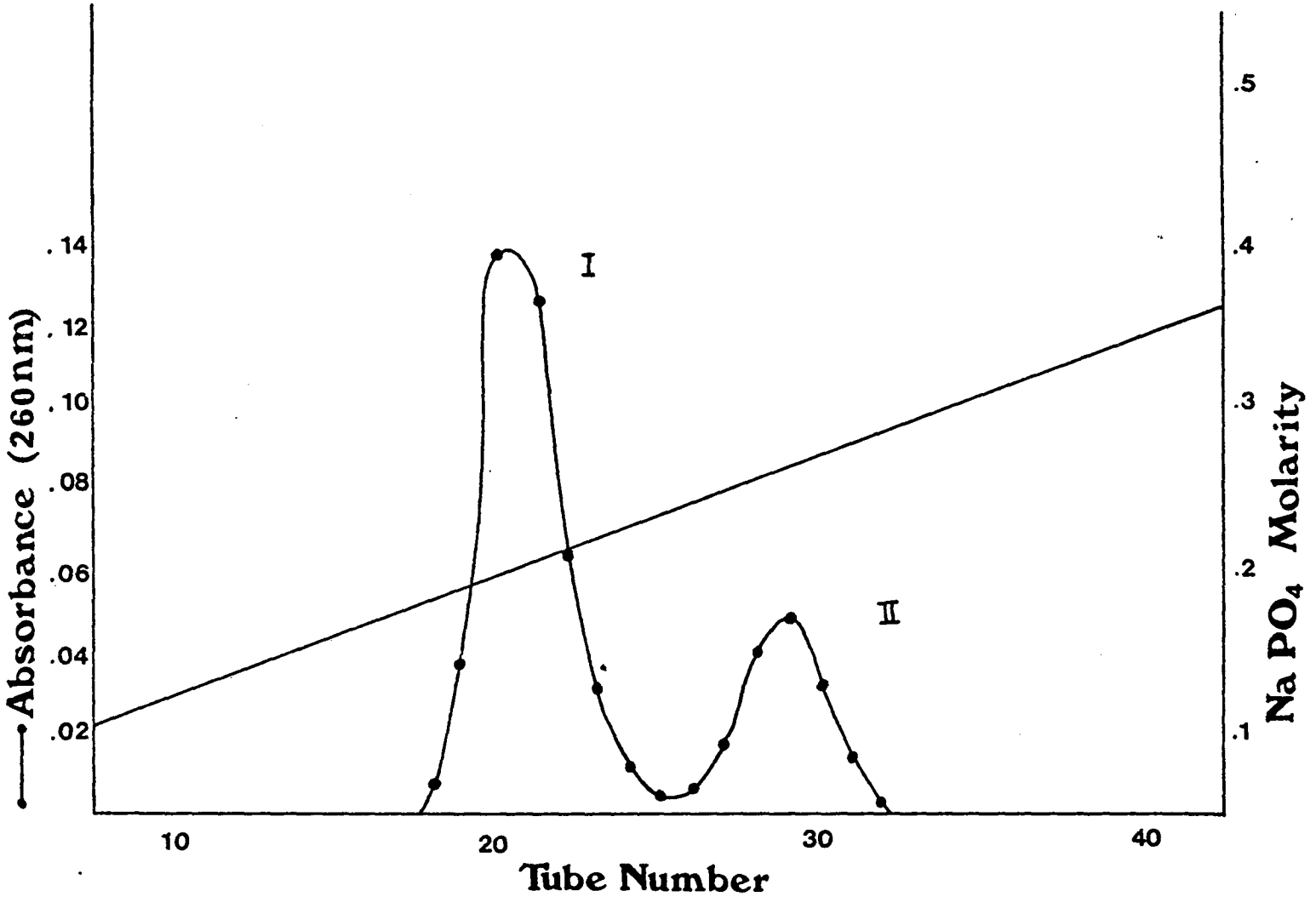

Absorbance (260nm)
.14
.12
.10
.08
.06
.04
.02
I
II
.5
.4
.3
.2
.1
Na PO4 Molarity
10
20
30
40
Tube Number

Figure 5. Fractionation of the self-annealed MAK L strand by hydroxyapatite (HA) chromatography. Self-annealed L strand DNA (0.22 mg) was applied in 0.05 M $NaPO_4$ buffer to a column 1 cm in diameter, packed to a height of 4 cm. The DNA was eluted with 250 ml of a linear gradient, 0.05 - 0.5 M $NaPO_4$. The recovery as a % of input was: total, 65.5; HI, 45.0; and HII, 20.3.

Figure 5

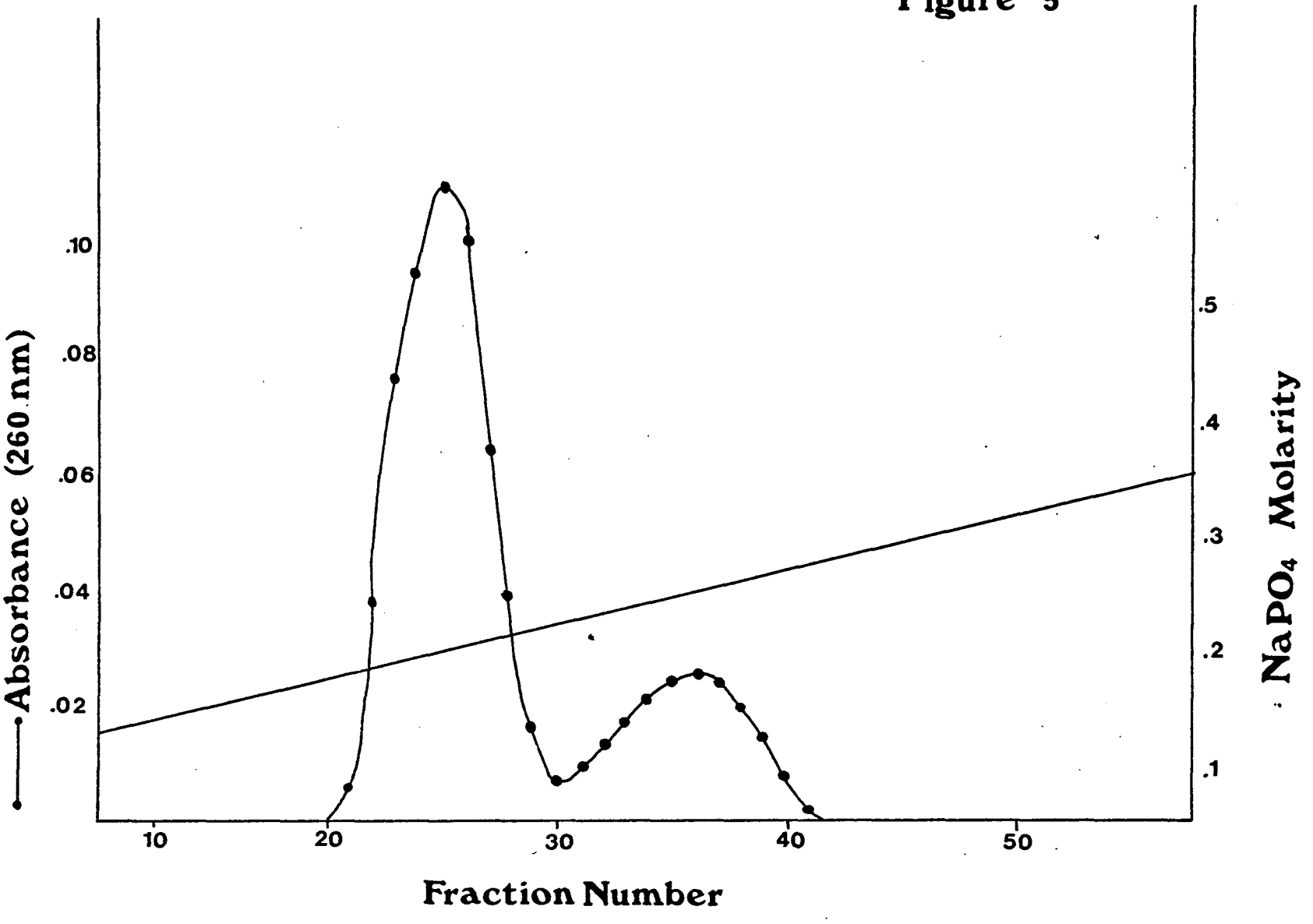

Absorbance (260 nm)
.10
.08
.06
.04
.02
10
20
30
40
50
Fraction Number
.5
.4
.3
.2
.1
NaPO4 Molarity

Figure 6. Fractionation of RNA by methylated albumin kieselguhr (MAK) chromatography. Actinomycin D treated RNA (1.44 mg)in 0.3 M NaCl - 0.05 M $NaPO_4$ was added to a column containing 1/2 the standard MAK recipe. The RNA was eluted with 600 ml of a linear gradient, 0.3 to 1.4 M NaCl in 0.05 M $NaPO_4$ buffer. The gradient was interrupted at tube 69 and reestablished at tube 76. Fractions of 4.0 ml were collected. The total recovery was 80.7%.

Figure 6

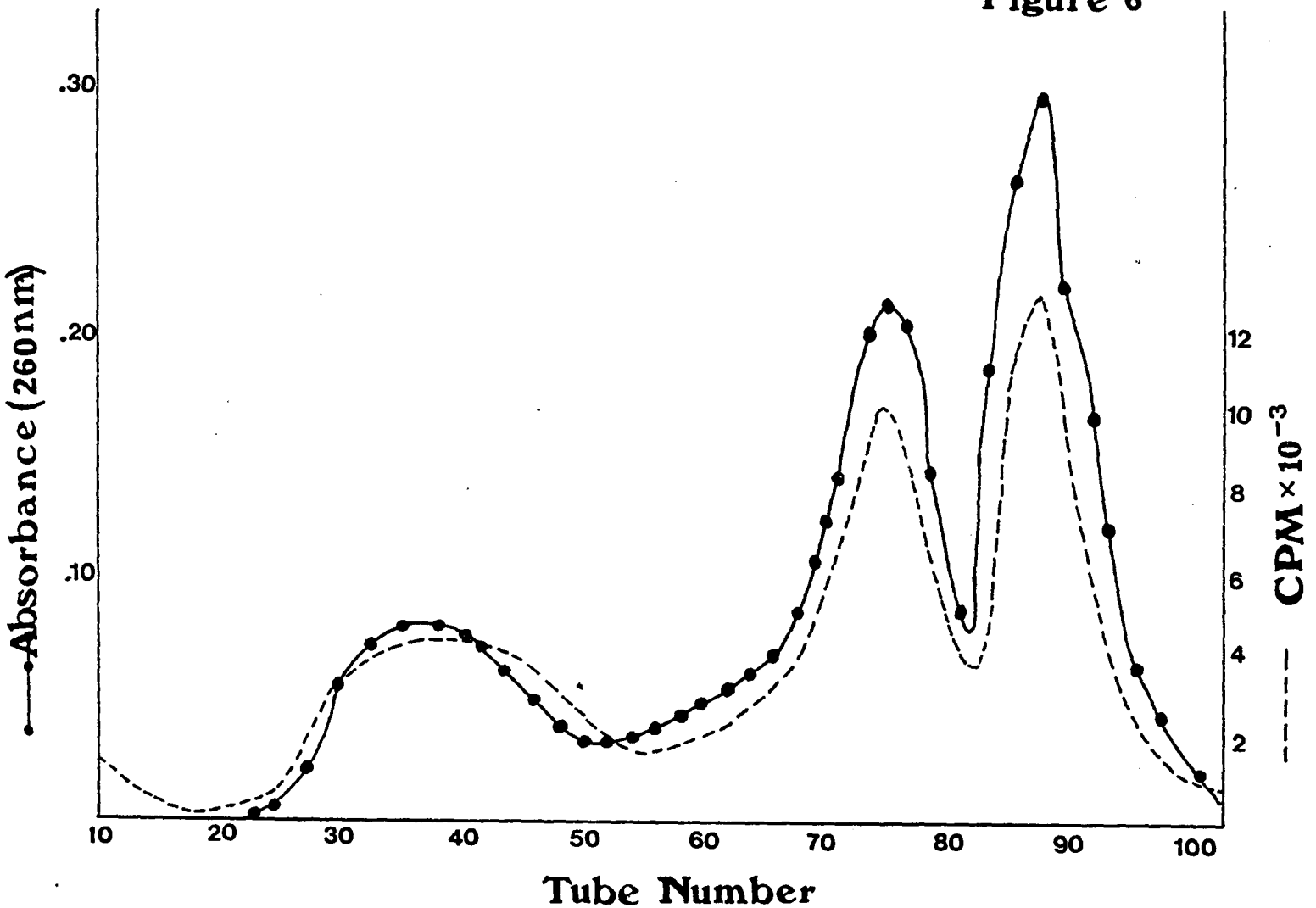

Absorbance (260nm)
.30
.20
.10
10
20
30
40
50
60
70
80
90
100
Tube Number
CPM ×10⁻³
12
10
8
6
4
2

Figure 7. Transforming activity along the MAK elution profile of denatured B. subtilis DNA. Alkali denatured DNA (3.7 mg) was fractionated as in figure 3. The gradient was interrupted at tube 21 and reestablished at tube 24. The recovery was 76.3% with an L/H ratio of 1.15. Transformation was done with 0.2 ug DNA/ ml, assaying for the threonine - 5 marker using frozen BD-170 recipient cells. The transforming activity of native DNA at 0.2 ug/ml was 1.8×10^5 transformants/ ml of recipient cells.

Figure 7

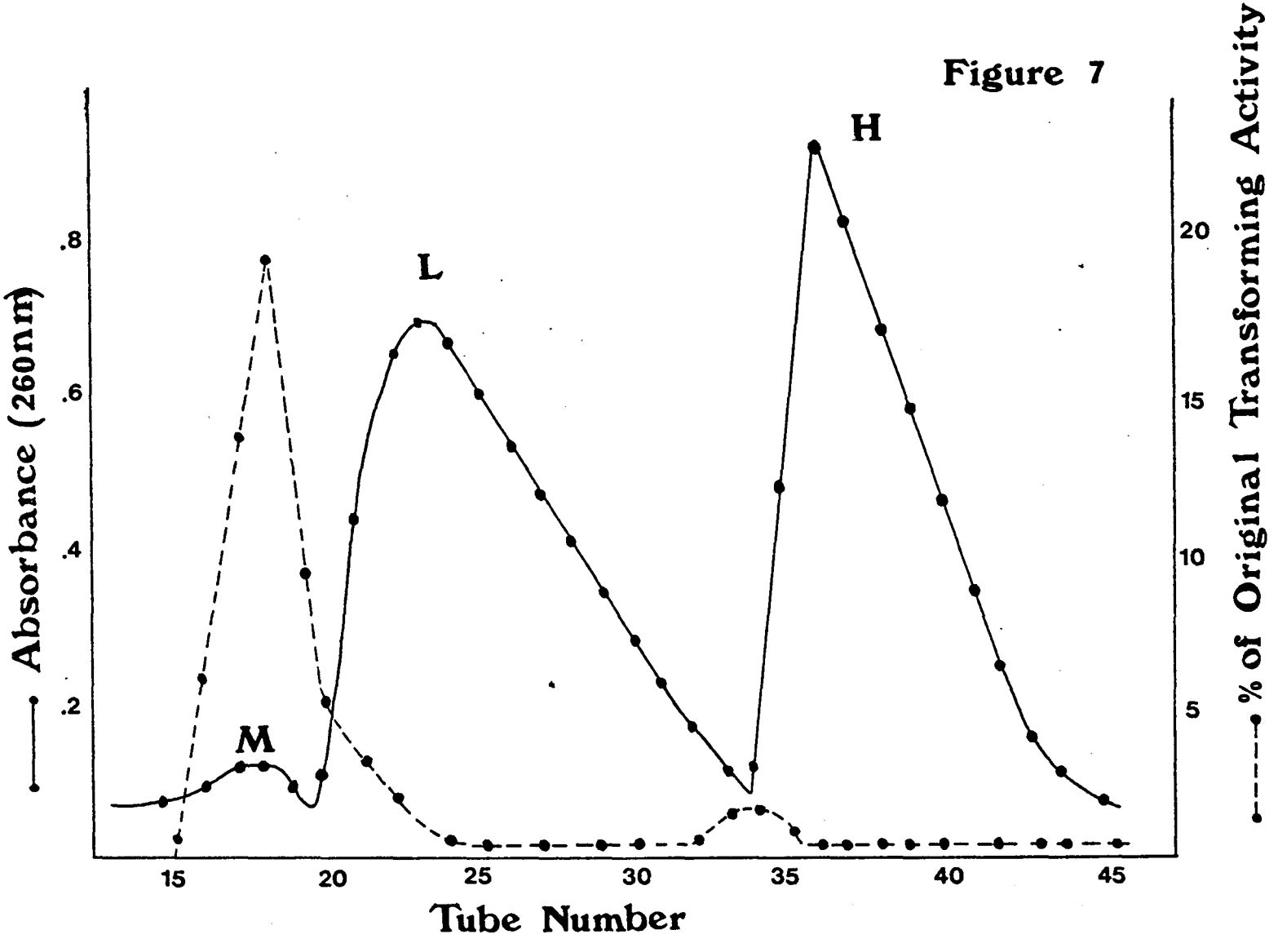

H
L
M
Absorbance (260nm)
% of Original Transforming Activity
.8
.6
.4
.2
20
15
10
5
15
20
25
30
35
40
45
Tube Number

Figure 8. Transforming activity after self-annealing individual fractions along the MAK elution profile. The alkali denatured DNA was the same as that described in figure 7. The fractions were heated at 68^0 for 2 hours and adjusted to 5 ug/ml. Transformation was assayed as in figure 7. The transforming activity of native DNA at 0.5 ug/ml was 2.3×10^5 transformants/ ml of recipient cells.

Figure 8

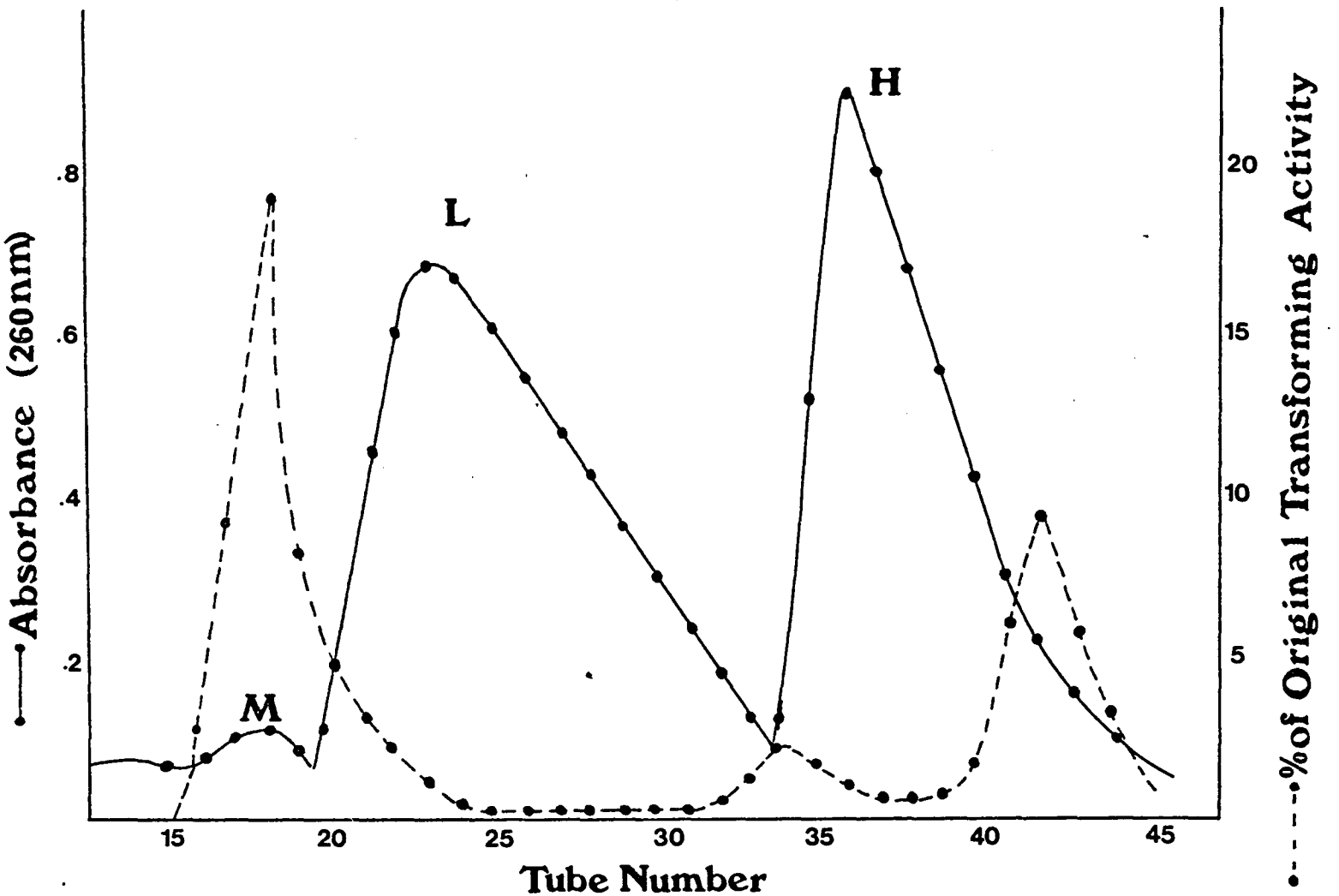

Absorbance (260nm)
.8
.6
.4
.2
M
L
H
15
20
25
30
35
40
45
Tube Number
20
15
10
5
% of Original Transforming Activity

Figure 9. Transforming activity along the HA elution profile of self-annealed MAK H strand DNA. Self-annealed MAK H strand DNA (0.32 mg) was applied to a column 1 cm in diameter packed to a height of 6 cm. The DNA was eluted with 250 ml of a linear gradient 0.05 - 0.5 M $NaPO_4$ buffer. The recovery as a % of input was: total, 72.3; HI, 47.0; and HII, 23.0. The column was assayed for transformation directly using 0.1 ml of DNA with 0.9 ml of BD-170 recipient cells to assay for the threonine marker. The transforming activity of native DNA at a concentration of 0.5 ug DNA/ml was 2.8×10^5 transformants/ml of recipient cells.

Figure 9

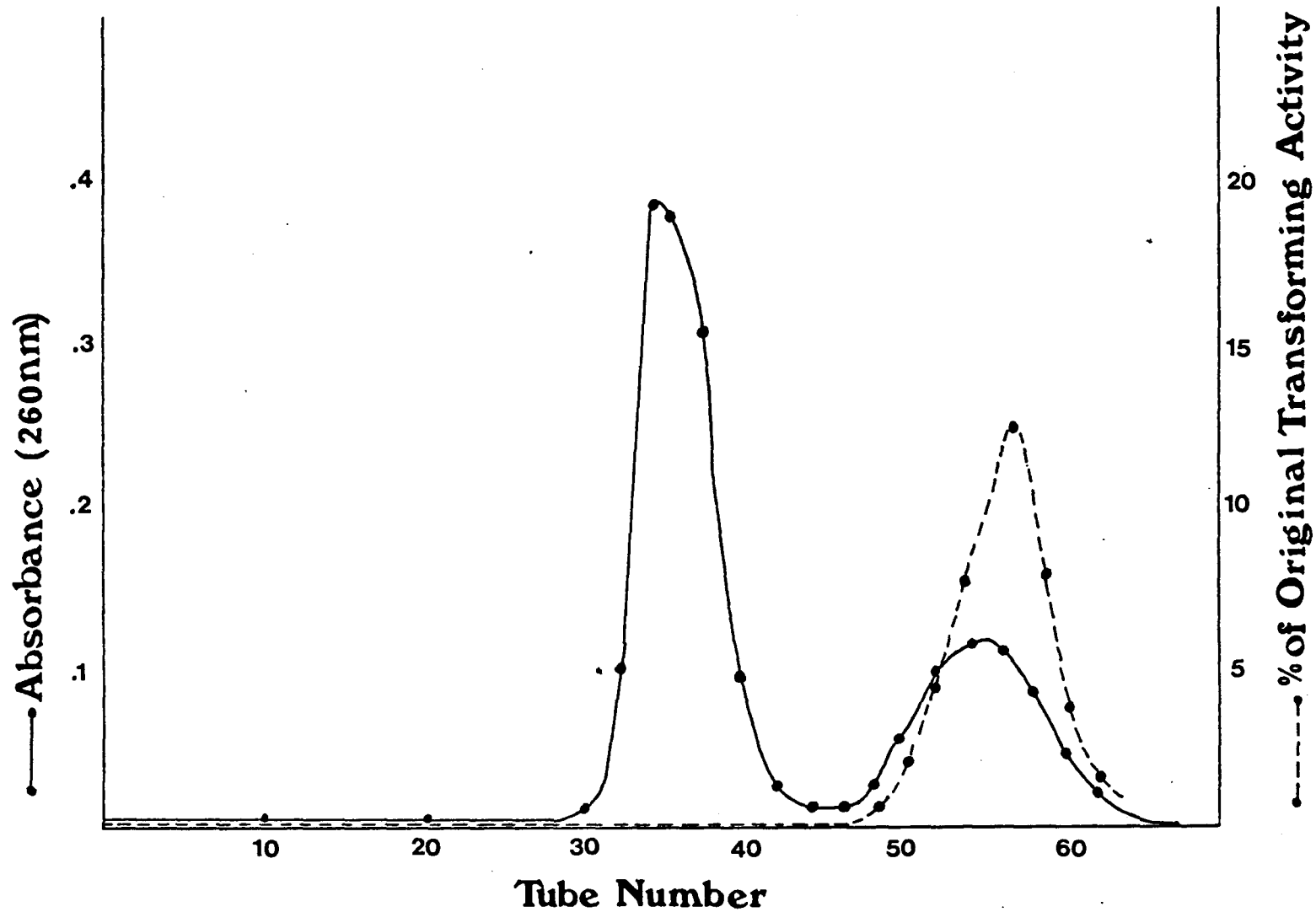

Absorbance (260nm)
.4
.3
.2
.1
10
20
30
40
50
60
Tube Number
% of Original Transforming Activity
20
15
10
5

Figure 10. Melting profiles of MAK fractionated DNA after annealing. Both of the MAK strands were annealed individually as well as together at a concentration of 20 ug/ml in 2 x SSC for 2 hours at 68^0. The samples were then diluted to 1 x SSC and melted out as described in Materials and Methods. The % hyperchromicity of the fractions was: H, 6.2; L, 5.6; and H+L, 76.0.

Figure 10

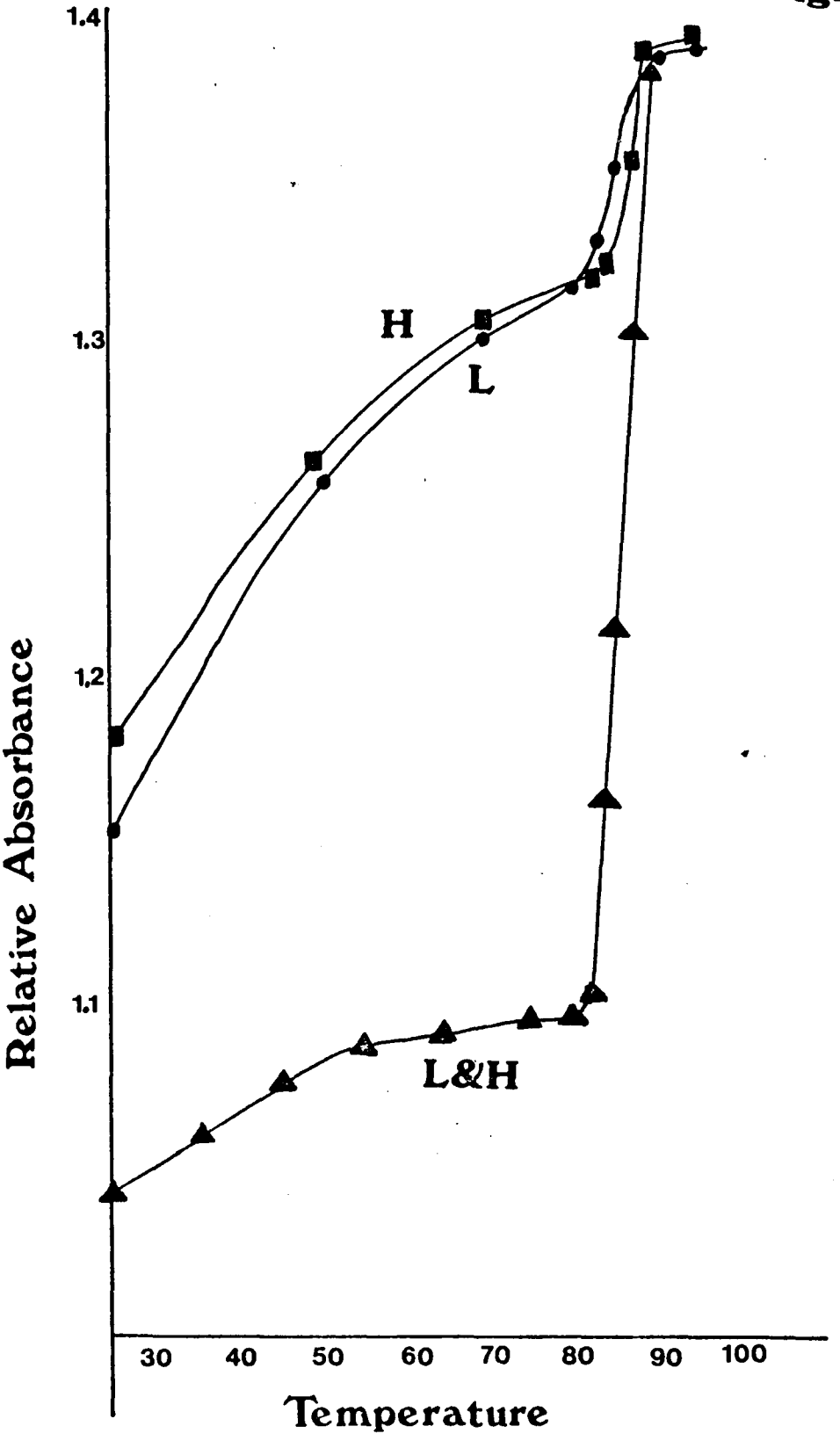

1.4
1.3
1.2
1.1
Relative Absorbance
H
L
L&H
30
40
50
60
70
80
90
100
Temperature

Figure 11. Melting profiles of peak I of HA fractionated self-annealed DNA. Fractions LI and HI were melted out individually and after they had been annealed together. The duplex was annealed at a concentration of 20 ug/ml in 2 x SSC for 2 hours at 68^0 and then diluted to 1 x SSC before melting out. The % hyperchromicity of the fractions was: 0 for HI and for LI; and 46.0 for HI + LI.

Figure 11

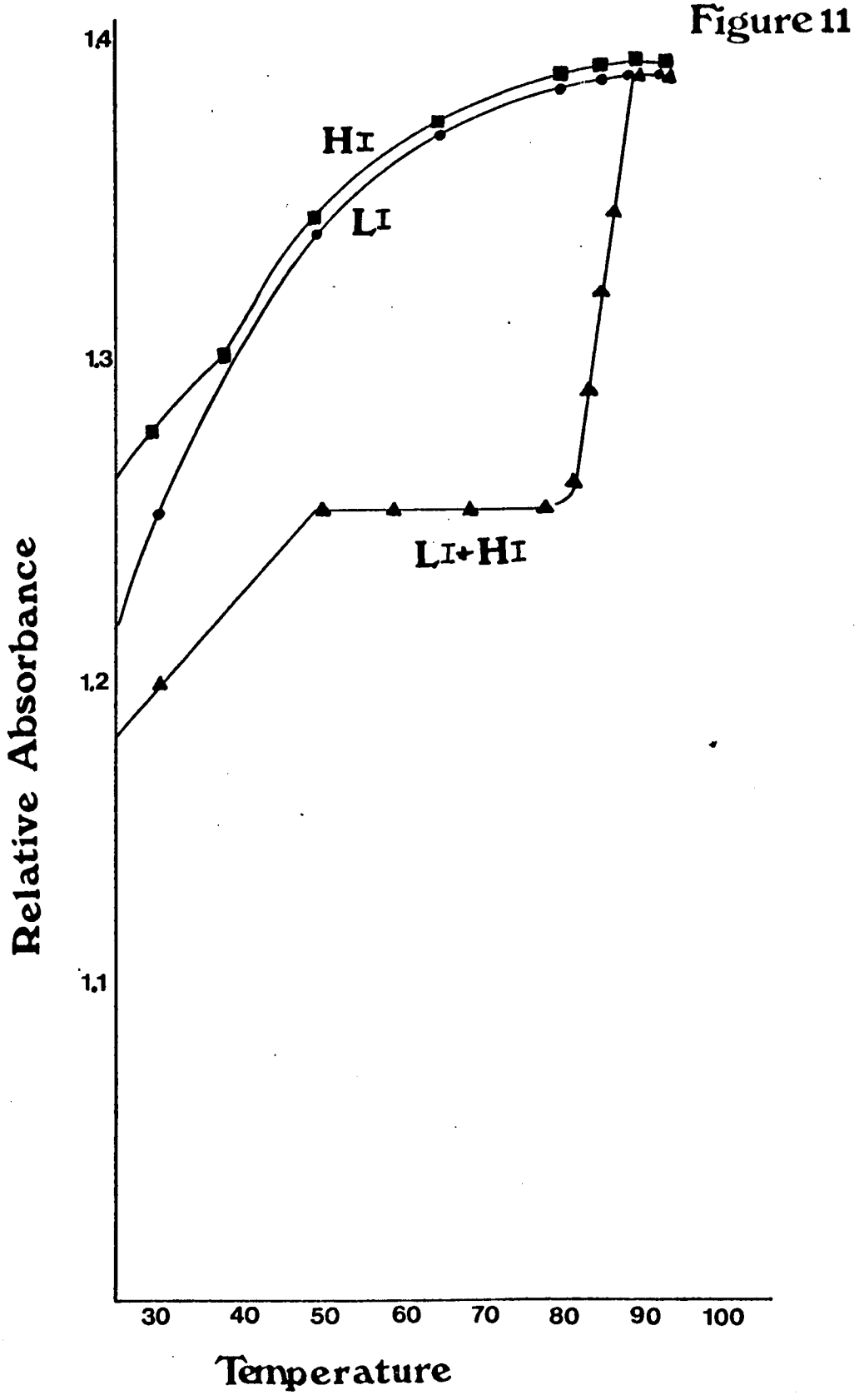

1.4
1.3
1.2
1.1
HI
LI
LI+HI
Relative Absorbance
30
40
50
60
70
80
90
100
Temperature

Figure 12. Melting profiles of peak II of HA fractionated self-annealed DNA. Fractions LII and HII were adjusted to 1 x SSC and melted out individually. The % hyperchromicity of the fractions was: HII, 26.3; and LII, 24.2.

Figure 12

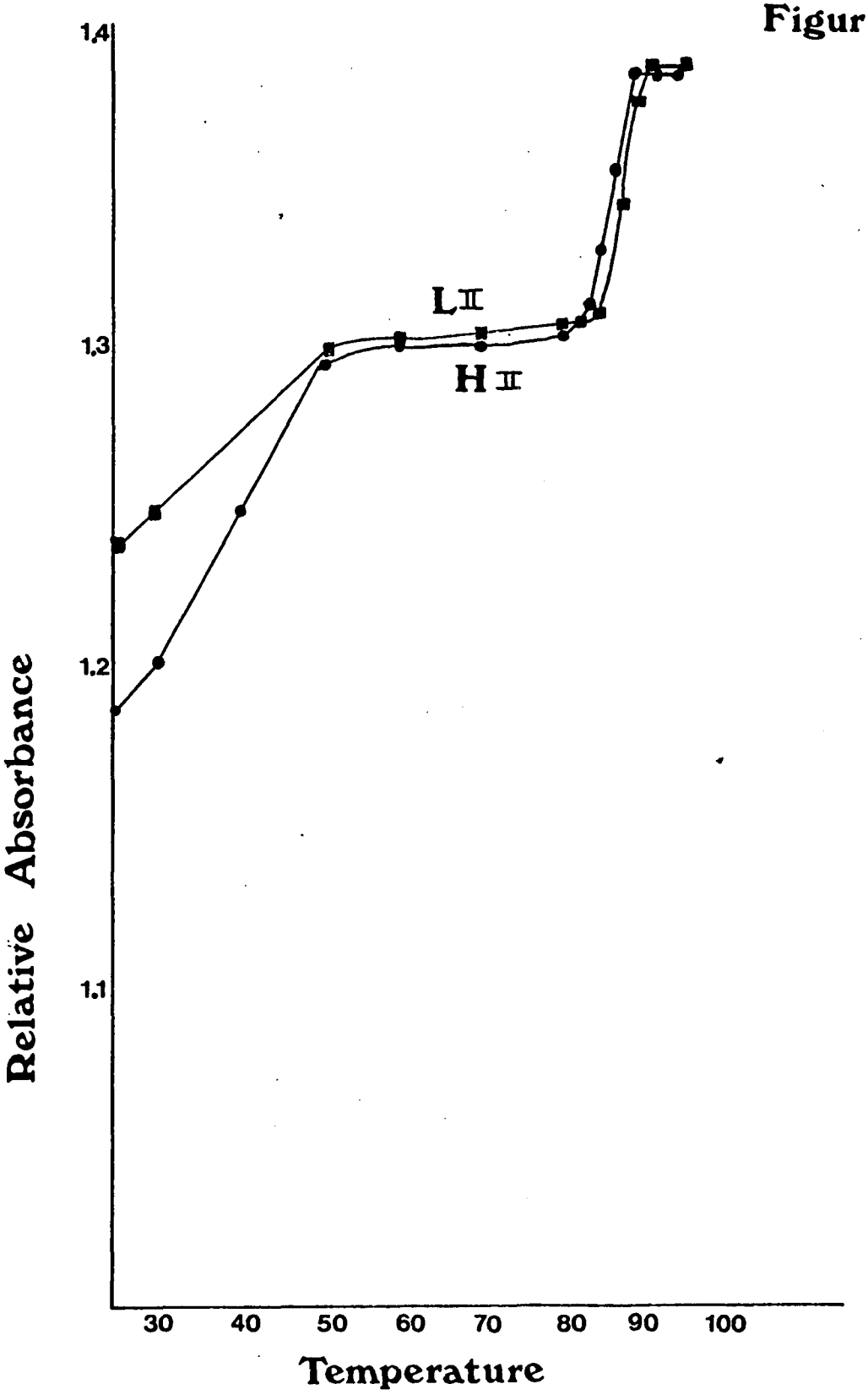

1,4
1,3
1,2
1,1
L II
H II
Relative Absorbance
30
40
50
60
70
80
90
100
Temperature

Figure 13. Melting profiles of MAK, HA, and reconstituted HA L + H duplexes. Equal amounts of the MAK L and H strands and of the HA LI and HI strands were annealed together in 2 x SSC for 2 hours at a concentration of 20 ug/ml. The reconstituted DNA duplex contained LI and LII as well as HI and HII in the ratio at which they eluted from the column, with an equal amount of total L and total H. The samples were adjusted to 1 x SSC and melted out. The % hyperchromicity of the duplexes was: MAK L + H, 44.7; HA HI + LI, 22.4; and HA LI + LII + HI + HII, 40.8.

Figure 13

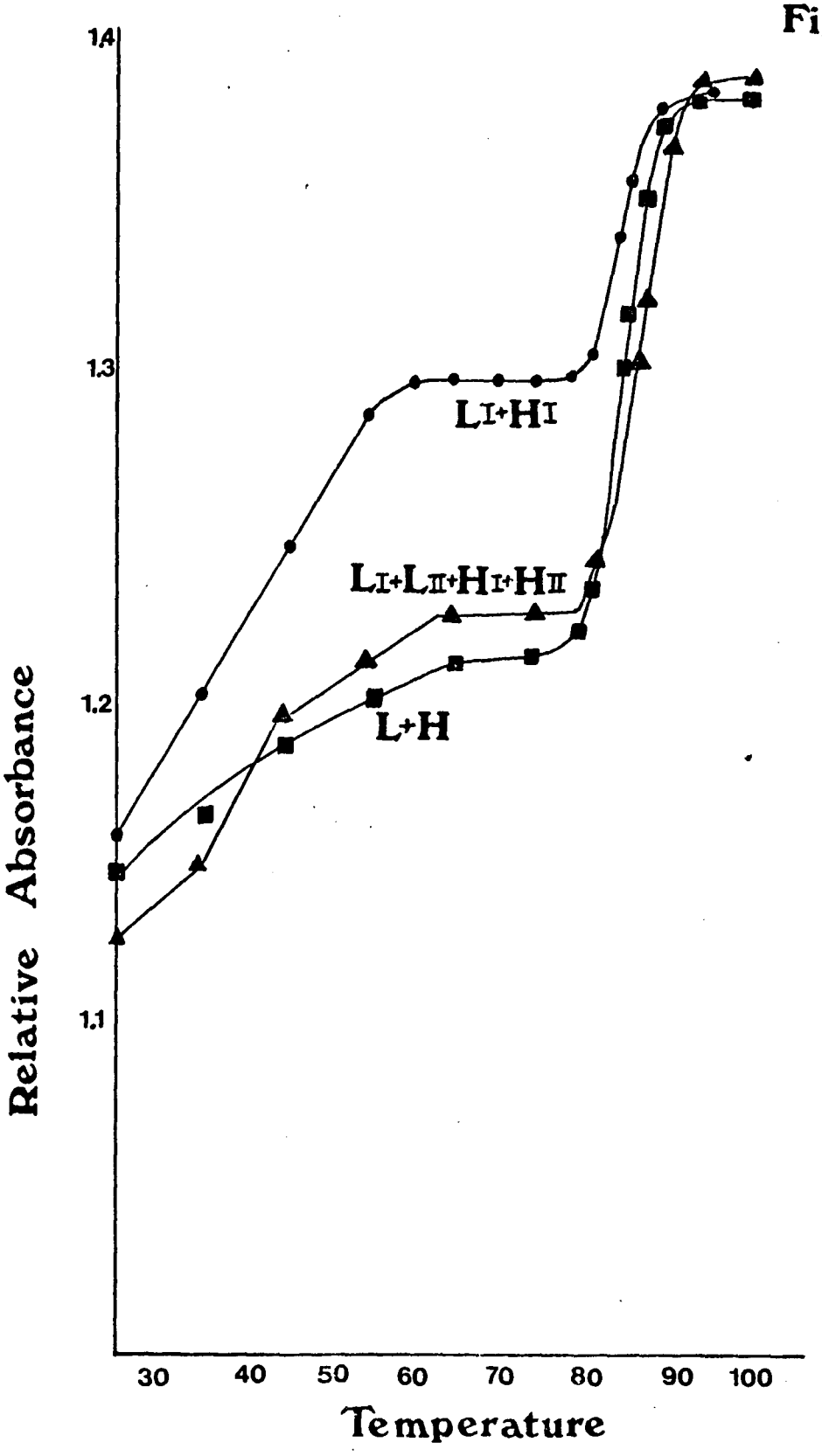

1.4
1.3
1.2
1.1
Relative Absorbance
LI+HI
LI+LII+HI+HII
L+H
30
40
50
60
70
80
90
100
Temperature

Figure 14. Sedimentation coefficients along the HA elution profile of self-annealed MAK H strand DNA. Self-annealed MAK H strand DNA (0,655 mg) was applied to a column 1 cm in diameter packed to a height of 12 cm. The DNA was eluted with a linear gradient, 0.05 - 0.5 M $NaPO_4$ buffer. The recovery as % of input was: total, 59.1; HI, 31.3; and H II, 27.2. Each of the fractions were dialysed against 1 x SSC, 0.2 N NaOH. The fraction was sedimented at its original concentration in a Beckman Model E analytical ultracentrifuge at 24,000 RPM. The sedimentation coefficient is indicated by the bars.

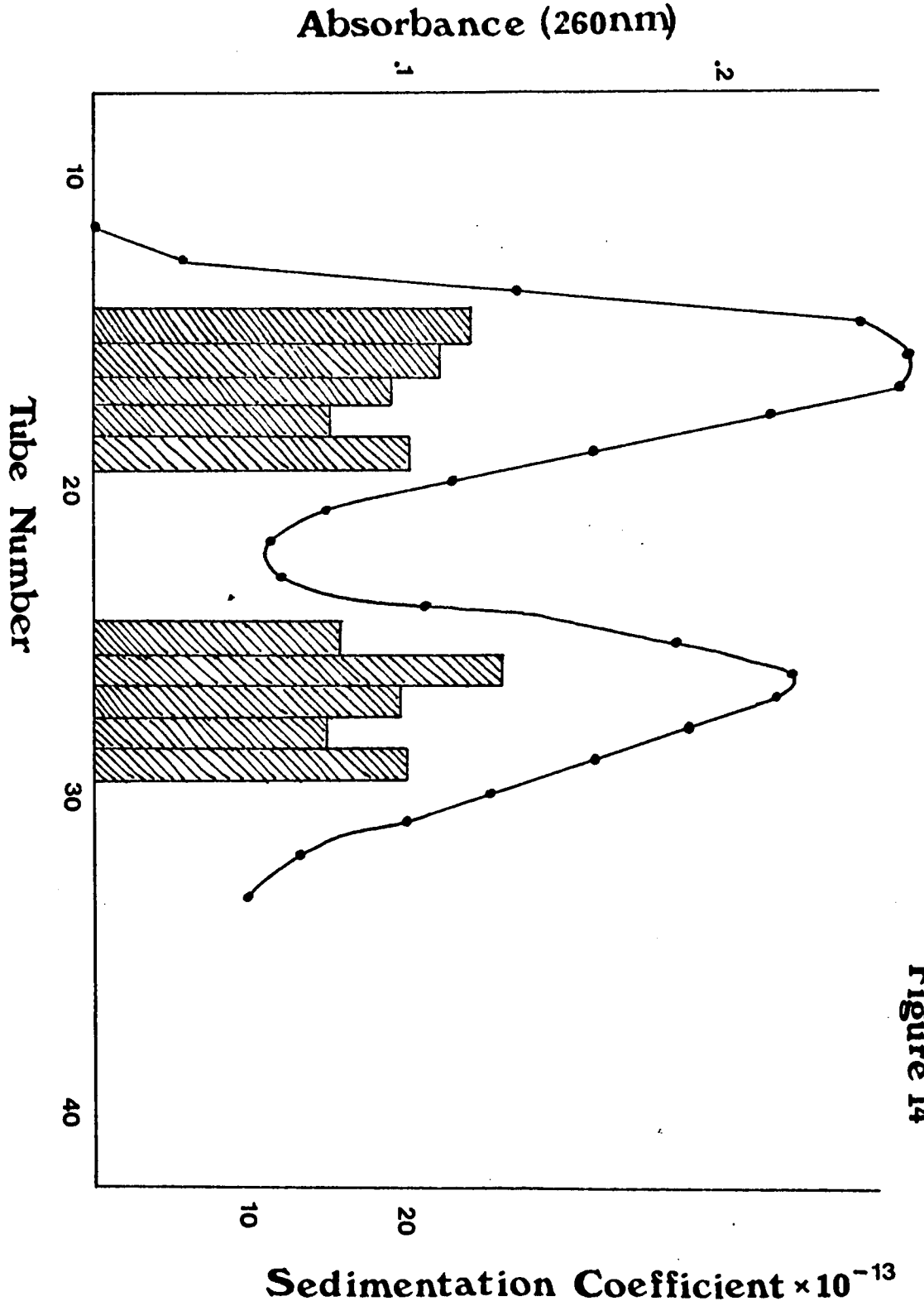

Absorbance (260nm)
.1
.2
10
20
30
40
Tube Number
10
20
Sedimentation Coefficient × 10⁻¹³

Figure 14

Figure 15. CsCl density gradient centrifugation of MAK H strand DNA. MAK H strand DNA (60 ug) and 50,000 CPM of a ^{3}H-adenine labeled native B. subtilis DNA were mixed with solid CsCl and adjusted with 1 x SSC to give an initial density of 1.705 g/cm^{3}. The sample was then spun in a 50 Ti rotor in a Beckman Model L65B centrifuge for 65 hours at 19^{0}. The tube was pierced with a syringe and 20 drop fractions were collected and analysed for radioactivity and for absorbancy at 260 nm.

Figure 15

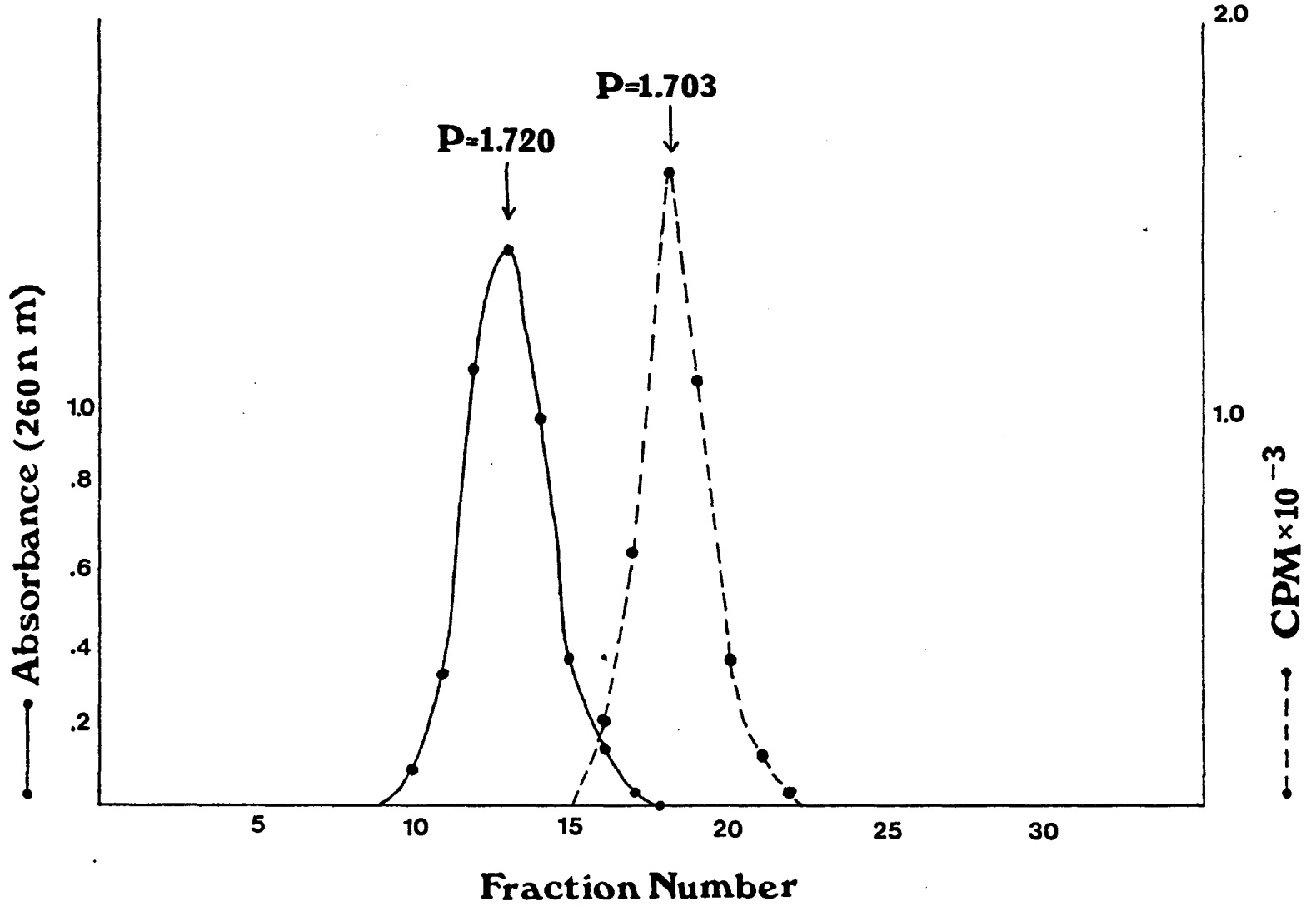

P=1.720
P=1.703
Absorbance (260 n m)
CPM×10⁻³
1.0
.8
.6
.4
.2
2.0
1.0
5
10
15
20
25
30
Fraction Number

Figure 16. CsCl density gradient centrifugation of the self-annealed MAK H strand DNA. Self-annealed MAK H strand DNA (42 ug) was centrifuged and analysed as described in figure 15.

Figure 16

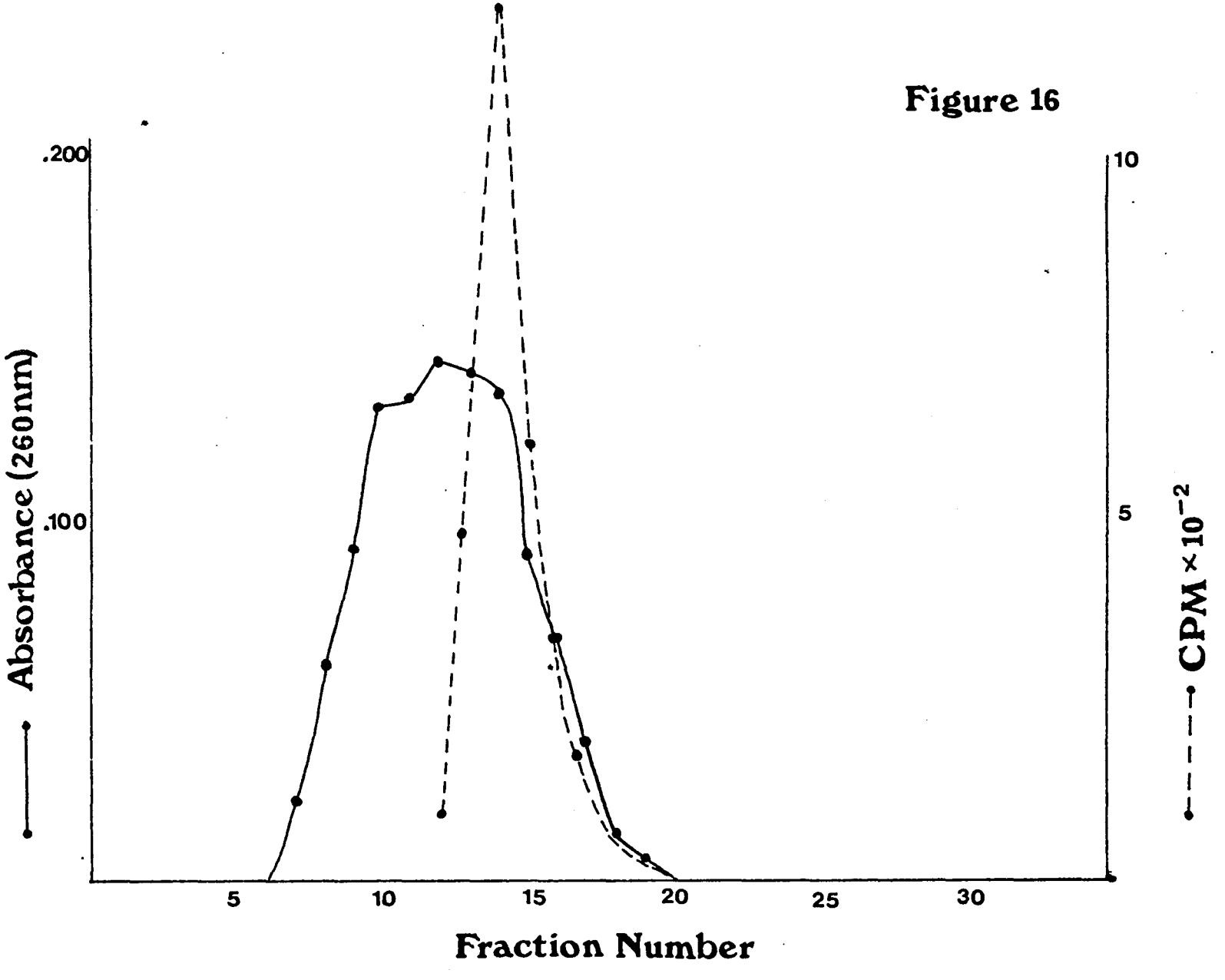

.200
.100
Absorbance (260nm)
10
5
CPM ×10⁻²
5
10
15
20
25
30
Fraction Number

Figure 17. CsCl density gradient centrifugation of HA fractionated HI DNA. HA HI DNA (38 ug) was centrifuged and analysed as described in figure 15.

Figure 17

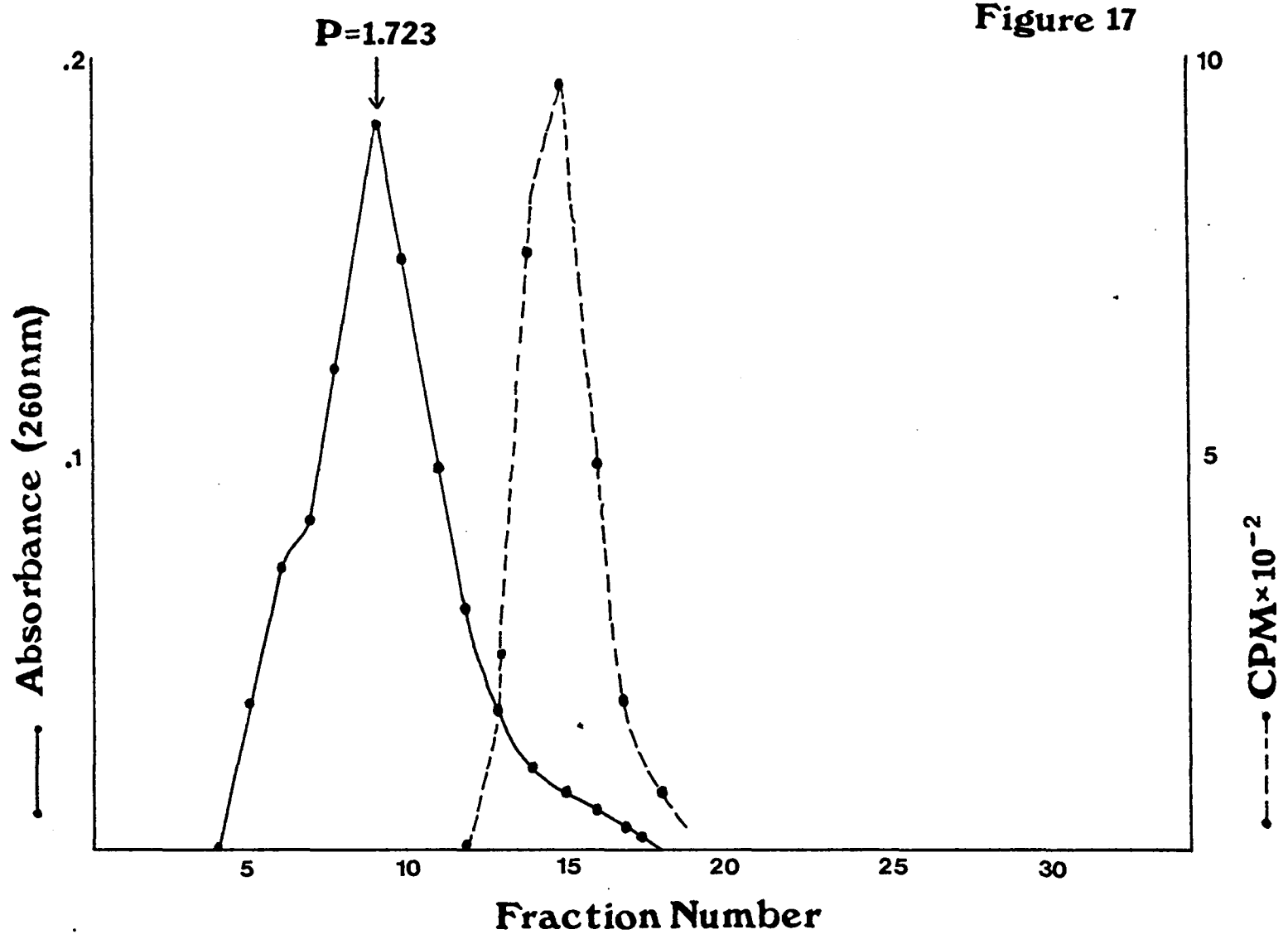

P=1.723
.2
.1
Absorbance (260nm)
10
5
CPM×10⁻²
5
10
15
20
25
30
Fraction Number

Figure 18. CsCl density gradient centrifugation of HA fractionated HII DNA. HA HII DNA (32 ug) was centrifuged and analysed as described in figure 15.

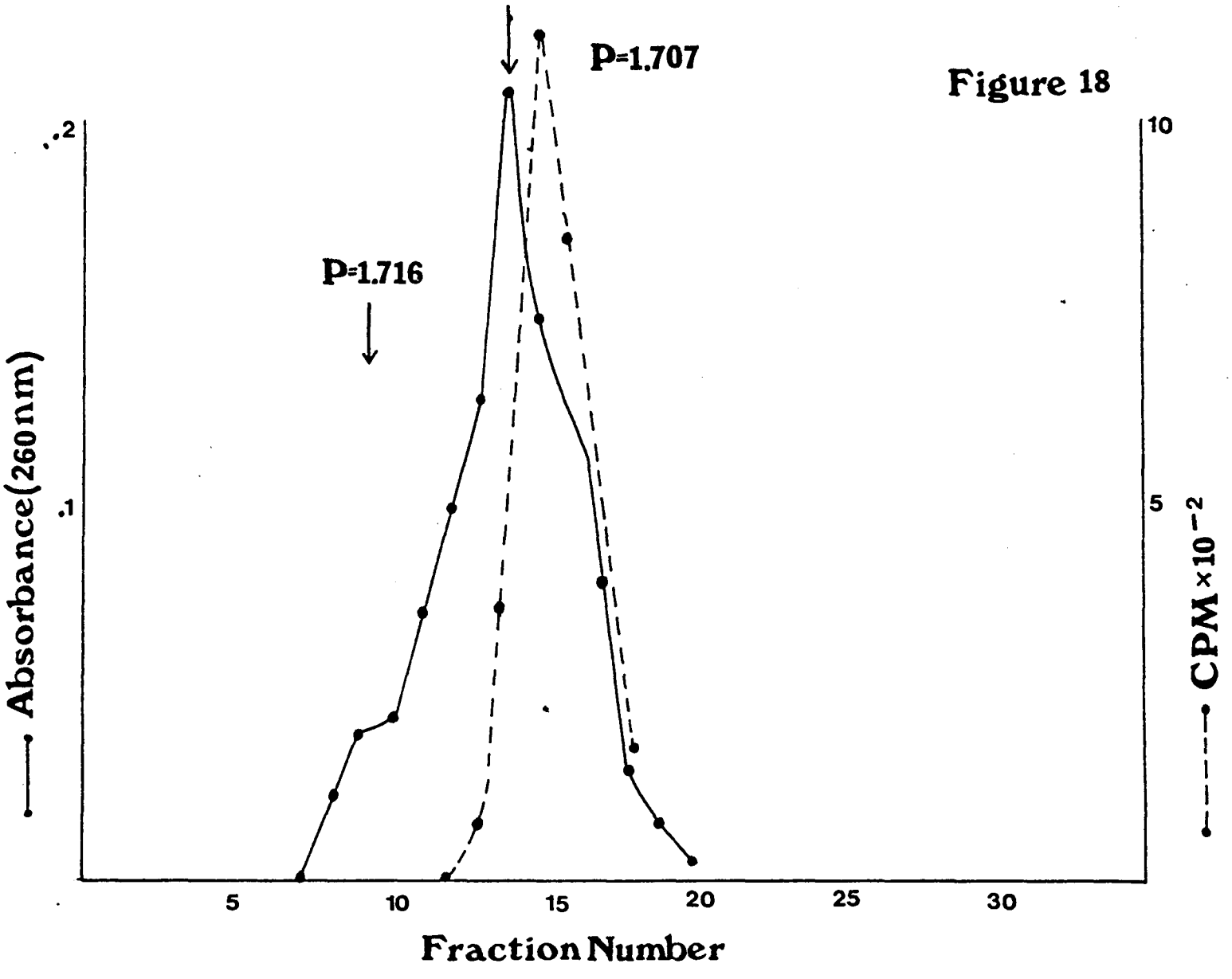

P=1.707
P=1.716
.2
.1
10
5
Absorbance(260nm)
CPM ×10⁻²
5
10
15
20
25
30
Fraction Number

Figure 18

Figure 19. Transforming activity along the CsCl density gradient of HA HII DNA. HA HII DNA (27 ug) was centrifuged and analysed in figure 15 except that the native marker was omitted and the remainder of each of the gradient fractions were diluted with 0.1 x SSC. An aliquot of 0.1 ml was removed and added to 0.9 ml of frozen competent Mu8u5u16 recipient cells. All three markers were assayed and the one shown here is leucine.

Figure 19

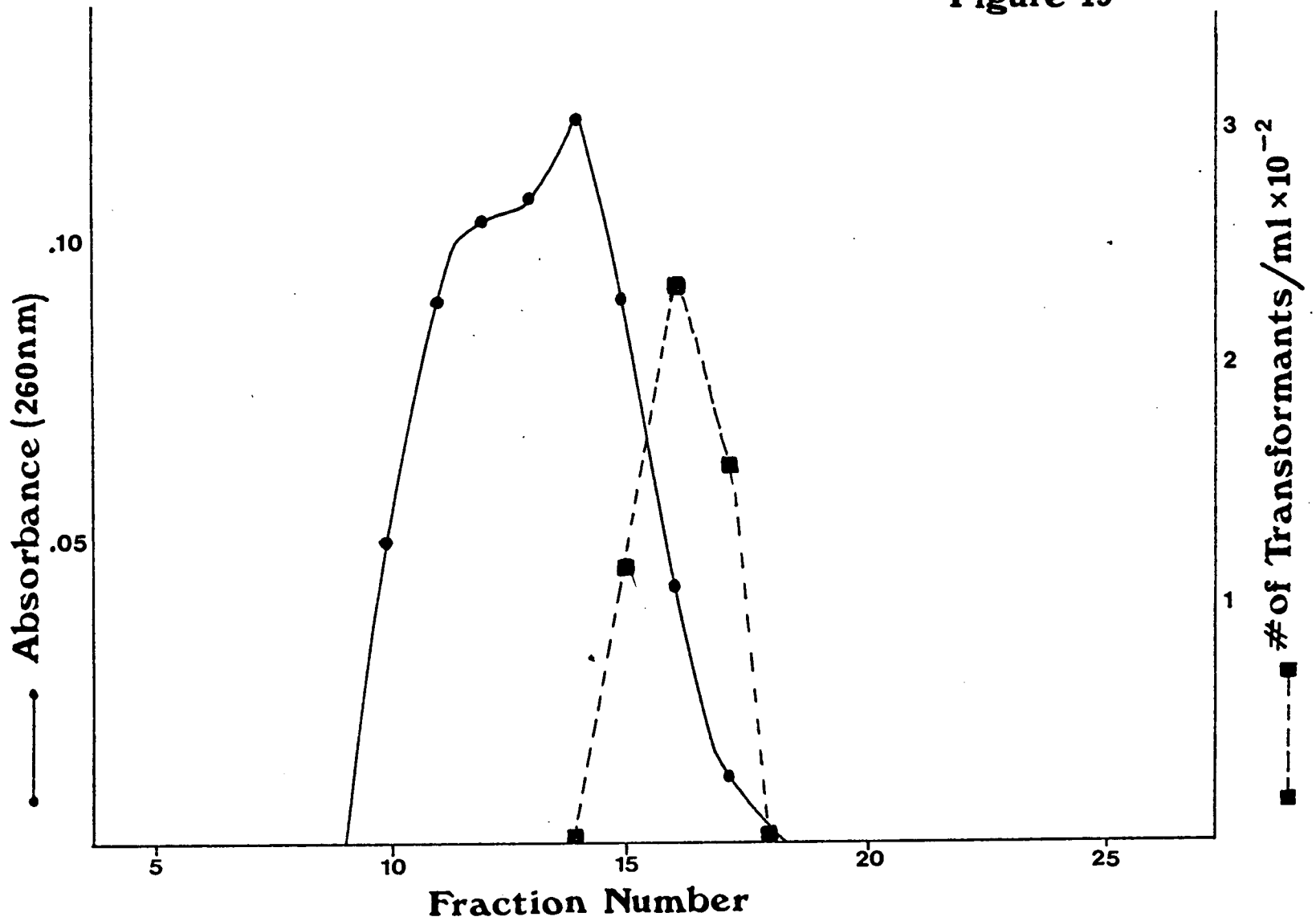

Absorbance (260nm)
.10
.05
5
10
15
20
25
Fraction Number
# of Transformants/ml ×10⁻²
3
2
1

Figure 20. Kinetics of degradation of MAK and HA fractionated DNA by S_1 nuclease. These assays were done using the commercial enzyme obtained from Miles Laboratories. Each reaction mixture (2.5 ml) contained 0.3 mM Na acetate, pH 4.5, 1×10^{-6} M ZnCl, 0.1 M NaCl, 50 ug sheared denatured calf thymus DNA, 1 ug ^{3}H-thymidine labeled DNA (spec. act. 51,196 CPM/ug), and 500 U (as determined by Miles Labs.) of enzyme. An aliquot of 0.1 ml was removed for the initial time point. The reaction mixtures were incubated at 37^{0}. Aliquots of 0.2 ml were removed in triplicate and precipitated with an equal volume of 10% TCA.

Figure 20

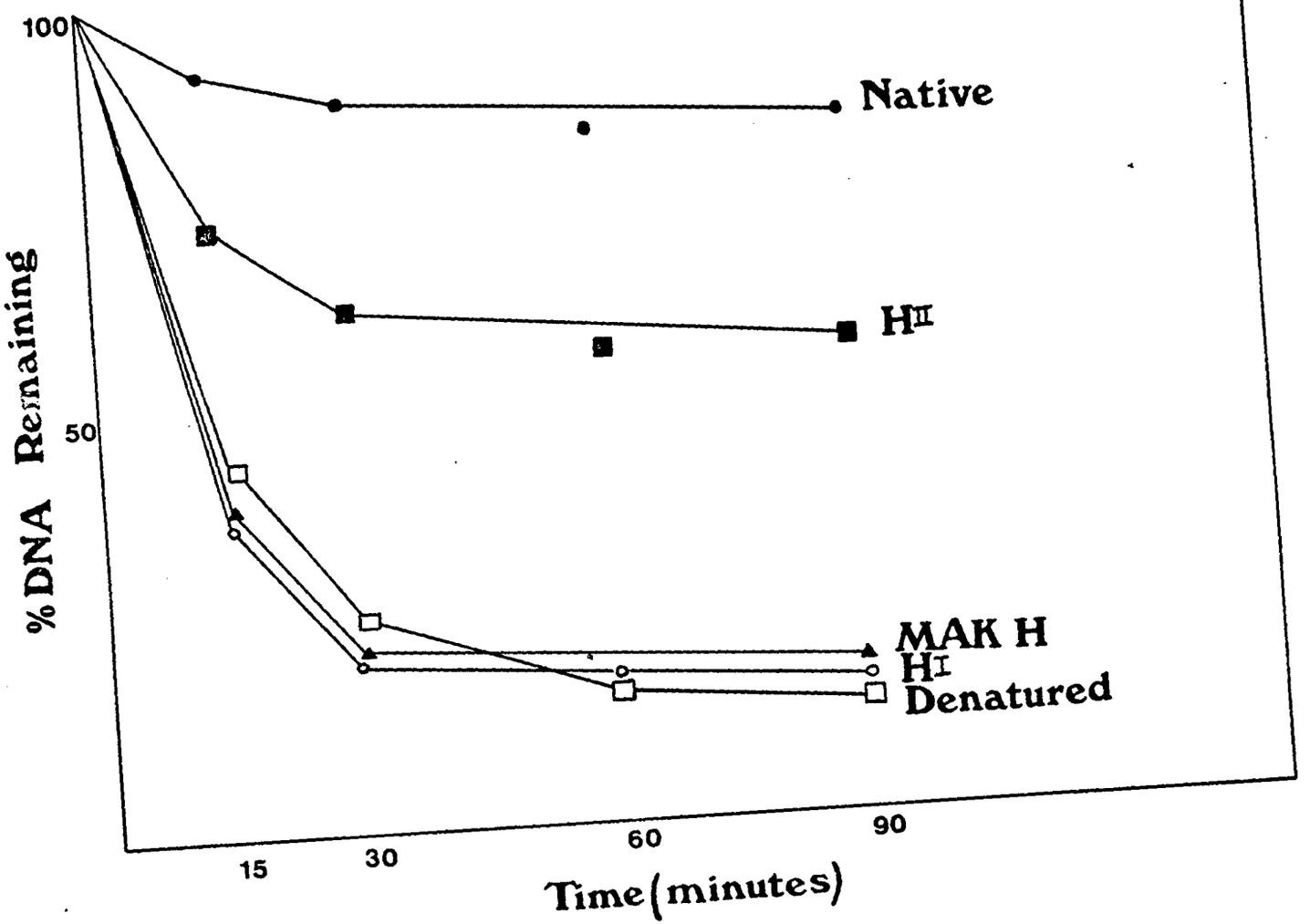
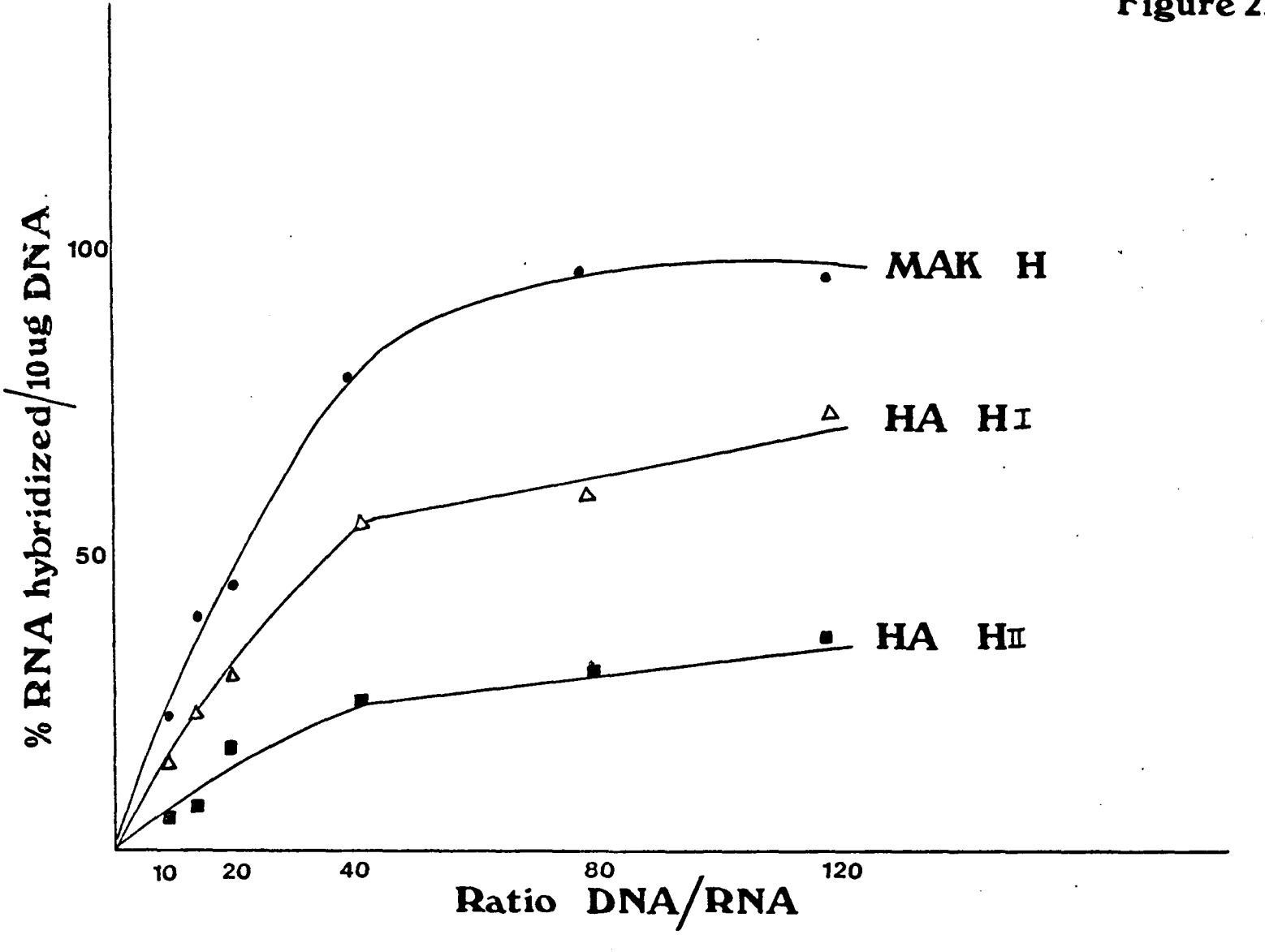

100
50
% DNA Remaining
Native
HII
MAK H
HI
Denatured
15
30
60
90
Time (minutes)

Figure 21. Hybridization of membrane immobilized DNA fractions to pulse labeled log phase RNA as a function of DNA concentration. ^{32}P-labeled DNAs, MAK H (spec act.,18,931 CPM/ug), HA HI (spec. act, 14,351 CPM/ug), and HA HII (spec. act., 16,559 CPM/ug) were bound (10 ug) to a nitrocellulose filter and hybridized to a ^{3}H- uridine labeled pulsed log phase RNA (spec. act., 55,500 CPM/ug). The reaction mixtures for the ratios lo, 14, 20, and 40: 1 all contained 10 ug of DNA and 1.0, 0.7, 0.5, and 0.25 ug of RNA, respectively. The reaction mixtures for the ratios 80 and 120:1 both contained 0.25 ug of RNA and 20 and 30 ug of DNA, respectively.

Figure 21


% RNA hybridized/10ug DNA
100
50
MAK H
HA HI
HA HII
10
20
40
80
120
Ratio DNA/RNA