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Characterization of Deoxyribonucleic Acid from Actinomycetes

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CHARACTERIZATION OF DEOXYRIBONUCLEIC ACID FROM ACTINOMYCETES

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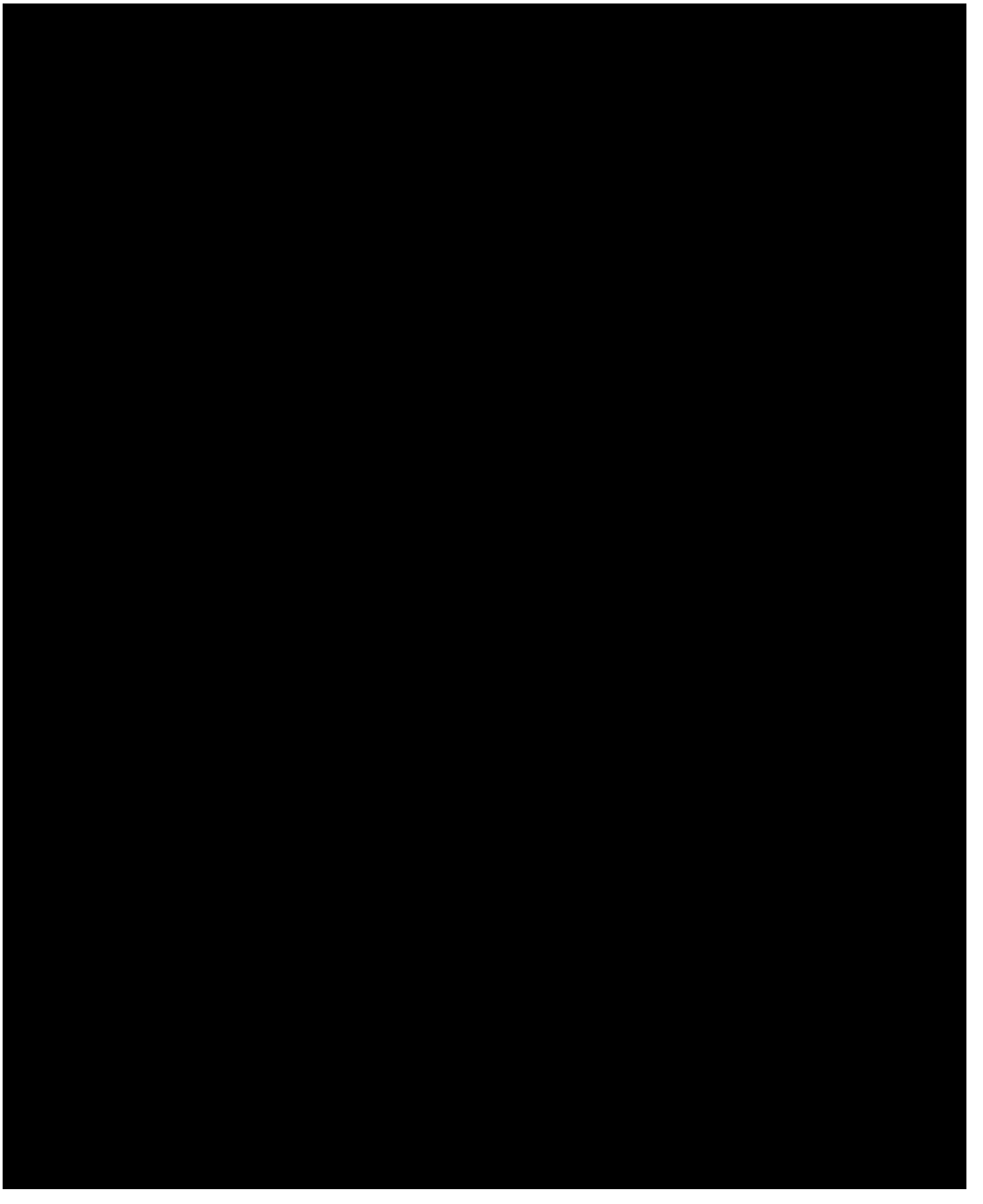

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Introduction

The genetic potential (genotype) of an organism is encoded in the linear order of the four nucleotide bases in its deoxyribonucleic acid (DNA). These sequences are translated into co-linear sequences of amino acids in structural or enzymic proteins, which directly, or indirectly, constitute the phenotype of the cell. Accordingly, evolutionary divergence from a common ancestor proceeds as the progeny accumulate base substitutions in their DNA. Recent evidence strongly indicates that remnants of an organism's evolutionary history are retained, inscribed in the genetic determinants themselves. Because of our increased understanding of the molecular architecture of DNA, approaches to microbial classification other than classical determinative systematics can be developed. In fact, the evolutionary approach to bacterial classification, long hindered by the lack of a recognized fossil record, now seems feasible at several molecular levels. The documentation of this "fossil record" inscribed in the molecules of cells was reviewed by Mandel (1969). An approach to an evolutionary classification could therefore be formulated for a group of microorganisms by analyzing their DNA.

The relationships among representatives of the bacterial genera Streptomyces and Nocardia are of particular interest because of their special relevance for industry, medicine

and agriculture; moreover, their taxonomic status remains a subject for active study and debate. Accordingly the first portion of this dissertation describes the isolation of DNA and the determination of the base composition of DNA from selected actinomycetes. In addition, I have modified the method of Warnaar and Cohen (1966) for quantitative assay of reassociation between denatured DNA fixed to nitrocellulose membrane filters and free, labeled, denatured DNA. This approach has been used to assess, on a molecular level, the relationships among these organisms.

Review of the Literature

Our present concepts about the structure of the DNA molecule are based largely on the model suggested by Watson and Crick. The physical and chemical nature of DNA has been reviewed extensively by Felsenfeld and Miles (1967), Edwards and Shooter (1966), Josse and Eigner (1966) and Kit (1963). What has emerged most clearly is the enduring validity of the Watson and Crick model.

Renaturation of DNA. Since the discovery that two strands of DNA can be physically separated and specifically reassociated (Marmur and Lane, 1960; Doty et al., 1960), it has been established that this reaction can be extremely valuable for comparing related nucleic acids as well as for analyzing ribonucleic acid (RNA) transcribed from DNA. There are two general methods with which to study the reassociation of DNA: reassociation carried out with denatured DNA from one source immobilized in an agar matrix or on a membrane filter surface, and reassociation performed with both test nucleic acids free in solution. Brenner (1970) pointed out that both methods could yield specific and reproducible data when properly applied.

Free Solution Reassociation. This system initially posed two major difficulties: (1) each species of nucleic acid present was free to react with itself, and (2) the product of

the heterologous reaction was difficult to quantitate and almost impossible to isolate (Gillespie and Spiegelman, 1965; Marmur et al., 1963). However, recent progress in understanding the kinetics of free solution reassociation has resulted in a revival of interest in the system. It is now apparent that specific duplex formation is a function of the initial concentration of each species and the time of incubation (Britten and Kohne, 1968; 1967). Furthermore, the use of hydroxyapatite to fractionate single-stranded and double-stranded DNA has probably been a major factor in the re-emergence of free solution systems. Duplex nucleic acid molecules are bound by hydroxyapatite in 0.12 M sodium phosphate buffer (pH 6.8) while single-stranded nucleic acids are not (Bernardi, 1965; Miyazawa and Thomas, 1965). In 0.4 M sodium phosphate buffer (pH 6.8), the double-stranded molecules are eluted. Thermal stability profiles of reassociated DNA duplexes are generated by washing the hydroxyapatite with an elution series of increasing temperatures. The advantages of hydroxyapatite have been summarized by Brenner et al. (1969b): (1) It was not necessary to immobilize the unlabeled DNA, and reassociated (hybrid) DNA did not leach out of the agar or from the filter in thermal elution studies. (2) The binding of labeled bacterial DNA fragments to unlabeled DNA from the same source was routinely 20% to 40% in agar, 10% to 70% on filters and from 75% to 95% in free solution. (3) Unlabeled DNA was not immobilized, thus its absorbancy could be assayed providing a valuable internal control. (4) Kinetics in free solution were typically

uncomplicated second-order, whereas kinetics in agar and filters were more complex. Brenner et al. (1969b) did point out one disadvantage; competition experiments could not be done with hydroxyapatite. Moreover, citrate and potassium ions greatly diminished the ability of hydroxyapatite to bind DNA (Brenner, personal communication). Formamide and dimethyl sulfoxide could not be used with hydroxyapatite unless their concentration was less than 1% because they apparently destroyed the cross-linking of the hydroxyapatite (McConaughy et al., 1969).

Immobilized DNA. Systems which allowed single-stranded DNA to be immobilized and yet remain available for binding complementary polynucleotides overcame many of the disadvantages of the early free solution systems. Possibly the most obvious advantage was that single strands of DNA in or on an insoluble matrix could not self-reassociate to form duplexed regions (Bolton and McCarthy, 1962).

Denhardt (1966) modified the technique of Gillespie and Spiegelman (1965) whereby single-stranded, high molecular weight unlabeled DNA was nearly irreversibly bound to a nitrocellulose membrane filter. This was accomplished by slowly filtering dilute, denatured DNA solutions dissolved in a salt solution composed of 0.9 M NaCl and 0.09 M sodium citrate through the filters. Thorough drying fixed the DNA to the filters. Interestingly, the exact reason why denatured DNA but not double-stranded DNA or any kind of RNA was bound to nitrocellulose filters is not known (Wohlhieter et al., 1966; Fishman and Schiff, 1968). By incubating the DNA-filters in

a solution containing free denatured, sheared, labeled DNA, Denhardt was able to detect DNA by DNA interactions. Denhardt's method relied on a pre-incubation of DNA-filters in a solution containing 0.02% each of ficoll, polyvinylpyrrolidone and bovine serum albumin to prevent non-specific binding of the denatured labeled DNA. Warnaar and Cohen (1966) simultaneously described a similar method which required no albumin pre-incubation. Warnaar and Cohen's procedure made use of the fact that single-stranded DNA was eluted from nitrocellulose with buffers of low ionic strength and high pH (10^{-3} M tris hydroxymethylaminomethane, pH 9.4) whereas the hybridized DNA was not. Moreover, the background levels of non-specifically bound DNA were about one order of magnitude lower than the method of Denhardt (Warnaar and Cohen, 1966).

Both Denhardt (1966) and Warnaar and Cohen (1966) showed that with increased volume and increased temperature, the extent of renaturation of homologous DNA dropped substantially. No explanation was offered and the assumption apparently was that any duplexes formed were of exact fit. This error went unnoticed for about two years with the subsequent publication of data that overestimated DNA sequence homology due to non-specific conditions (Mandel, 1969; Johnson and Ordal, 1968). This occurred even though Martin and Hoyer (1966) had established that with decreasing incubation temperature, the degree of duplex formation did increase, but with a decrease in thermal stability indicating non-specific duplex formation. In fact, previous agar-gel work (reviewed by

Hoyer and Roberts, 1967) showed that the formation of specific duplexes depended on the same parameters as the free solution reactions. Data from thermal elution or thermal chromatography, for example, provided a measure of the stability of the DNA duplexes. Well matched complexes should display thermal elution profiles coincident with those of native molecules of similar sizes (Mandel, 1969). The thermal stability of a reassociated duplex is characterized by its Tm,e (elution temperature at which 50% of the DNA duplexes has been dissociated). The differences between the Tm,e value of an interspecific duplex and that of the homologous reference reaction has been designated the ΔTm,e value. There appears to be a direct correlation between ΔTm,e and the percent of unpaired bases in an interspecific duplex. Laird et al. (1969) reviewed studies with artificial polymers and presented experimental data based upon natural DNA polymers which suggested that a 1.5 C decrease in thermal stability resulted from 1% unpaired bases within a DNA duplex. It must be noted that the melting temperature (Tm,e) obtained by the release of DNA fragments due to complete strand separation is not theoretically nor experimentally equivalent to the Tm measured optically (Brenner et al., 1969a). Practically, the Tm,e and optical Tm agree rather closely (Brenner et al., 1969a; Kingsbury et al., 1969 and Johnson and Ordal, 1968).

Scientists using agar-gel pioneered reassociation at two or three incubation temperatures followed by thermal elution (Hoyer and Roberts, 1967). This technique was aptly applied

to filters by Martin and Hoyer (1966). In addition, Martin and Hoyer (1966) showed that the ratio of binding at 40 C to that obtained at 60 C could discern remote relationships not detected by direct hybridization or thermal elution. The full impact of their work was not appreciated until it was substantiated by Johnson and Ordal (1968) using filters and by Brenner and Cowie (1968) using hydroxyapatite and agar-gel. As inferred by Martin and Hoyer (1966) and postulated by McCarthy (1967), the studies of Johnson and Ordal (1968) and those of Brenner and Cowie (1968) confirmed the existence of a class of nucleotide sequences which could reassociate at more non-exacting incubation temperatures but could not reassociate at more exacting incubation temperatures. McCarthy (1967) suggested that the duplexes formed at non-specific incubation temperatures were remotely related and could therefore be a measure of evolutionary divergence. This suggestion with its subsequent confirmation raised the status of DNA reassociation from a laboratory curiosity to a powerful tool for discerning molecular relationships.

The methods using filter-immobilized DNA have a number of inherent disadvantages which limit their application. Because the total reassociation of free DNA with DNA fixed to filters rarely exceeds 50%, Brenner et al. (1969b) inferred that the observed binding may not be representative of the entire genome. Leaching of reassociated DNA from filters can also occur. This apparently was not a problem in the original work of Gillespie and Spiegelman (1965), Denhardt (1966) and Warnaar and Cohen (1966). However, in some

instances, leaching from the DNA-filters seriously limited the assays (Okanishi and Gregory, 1970).

To overcome this undesirable elution of fixed DNA at high temperatures, McConaughy et al. (1969), using a method developed by Bonner et al. (1967), added formamide to the incubation mixture. They found that 1% formamide reduced the optical Tm of Bacillus subtilis DNA by 0.72 C. By this method, high specificity and rates of reaction were achieved utilizing incubation temperatures of 37 C or less. The thermal elutions were complete at 40 to 50 C with no loss of fixed DNA. McConaughy and co-workers compared the rates of reaction in free solution with the reaction rates on filters both with and without formamide and concluded that the information obtained with both systems was identical. They also showed that this system could be adapted for use with hydroxyapatite provided the reassociating solution containing formamide was diluted with 0.12 M sodium phosphate buffer (pH 6.8) so that the formamide concentration was below 1% before application to the hydroxyapatite. Legault-De'mare et al. (1967) showed that the temperature of renaturation of DNA on membrane filters could be lowered if 30% (v/v) dimethyl sulfoxide (DMSO) was incorporated into the incubation solution. Rogul et al. (1970) used DMSO as a solvent for the Denhardt method. The high background obtained with the original Denhardt procedure was considerably reduced and specific binding could be obtained at lower incubation temperatures. The modified system employing DMSO, however, gave an unacceptably large experimental error.

Specificity of DNA Reassociation. The parameters affecting DNA reassociation have been adequately summarized by McCarthy and Church (1970), Brenner (1970), Mandel (1969) and Brenner et al. (1969a). Their importance cannot be overemphasized, and they are therefore repeated here. (1) GC pairs exhibit greater thermal stability than AT base pairs; thus, if a given DNA duplex contains more GC pairs, its thermal stability will be higher. Moreover, the sites for initiation of reassociation appear to involve sequences rich in G and C. McCarthy and Church (1970) pointed out that initial reaction products were rich in GC pairs. (2) The size of the DNA fragments affects DNA reassociation in free solution, larger fragments reassociating faster than the smaller ones (Britten and Kohne, 1966). Moreover, below a chain length of about 15 nucleotides (in bacteria) there was no specific duplex formation (McConaughy and McCarthy, 1967). Ideally, we want to compare specific DNA sequences at the exclusion of all others; however, most current methods for producing DNA sequences generate a random population of fragments of different size. In fact, Brenner et al. (1970) noted that the DNA fragments used in their experiments sedimented as a broad band in Cs_2SO_4 and alkaline sucrose density gradients. Production of fragments is usually accomplished by mechanical shearing. The most common method involves passing DNA through a needle valve. The size of the fragments produced is governed by the pressure drop produced. Brenner et al. (1970) reported production of fragments with the average molecular weight of 1.25×10^5 daltons by the use of a

50,000 lb. inch^{-2} pressure drop. Unless modified, the ordinary French press will withstand up to about 20,000 lb. inch^{-2} and will generate fragments in the range of 3 to 5 x 10^5 daltons. Because the rate of reassociation is inversely proportional to the viscosity of the solution (Wetmur and Davidson, 1968), the reaction can be affected by chain length. This effect can be controlled by using uniformly sheared DNA fragments. Large fragments may produce other undesirable effects. If a particular fragment contained an internal sequence capable of forming a duplex with the other DNA species under the conditions employed, this reaction and other reactions may be influenced by the effect of free terminal stretches of single-stranded DNA (Walker, 1969). (3) The most common procedure for producing a single-stranded DNA is heating aqueous solutions to 4 C to 5 C above the Tm followed by quick-cooling and increasing the salt concentration. For organisms of GC content less than 50%, this method is probably acceptable. However, for DNA samples of high GC content, high molecular weight and high concentration, complete strand separation may not occur at 100 C even in dilute buffers. Mandel (1969) stated that if separation was not complete and cross-links occurred, intrastrand and interstrand reassociations would decrease the number of available sites for interspecific duplex formation. Brenner et al. (1969a) described a simple method for removing cross-linked and partially reassociated DNA from the fragment preparation. A hydroxyapaptite column was equilibrated at a suitable temperature (about 30 C below the Tm) with 0.14 M

sodium phosphate buffer (pH 6.8). DNA fragments dissolved in this buffer were passed through the column. Single-stranded DNA passed through the column while cross-linked DNA was bound. Heating DNA during denaturation and during reassociation may also produce undesirable effects. Greer and Zamenhof (1965) showed that DNA can be depurinated and ultimately degraded by heating at high temperatures in dilute buffers. Shapiro and Klein (1966) observed that cytidine and cytosine are deaminated at 95 C in a variety of aqueous buffers. Because most DNA studies are done in a saline-citrate buffer, Shapiro and Klein's observation that the rate of deamination increased with increasing molarity of citric acid-citrate buffers is disturbing. A logical alternative to heat denaturation is denaturation by NaOH. Another approach could be the use of denaturants such as formamide and dimethyl sulfoxide. In any event, the choice of methods for producing and fragmenting single-stranded DNA can drastically influence DNA reassociation. (4) The rate of reassociation is highly dependent on salt concentration; moreover, the thermal stability of reassociated DNA increases as the ionic strength increases. Brenner (1970) pointed out that one can easily shift the mid-point temperature of strand separation by 20 C or more by changing the salt concentration. (5) The optimal temperature for reassociation is about 30 C below the Tm of a given DNA (Marmur et al., 1963). (6) To obtain meaningful reassociation data, the concentration of labeled and unlabeled DNA must be carefully chosen. For studies in which one DNA species was immobilized

in agar or on a filter, a 50:1 ratio of unlabeled to labeled DNA was sufficient to provide an excess of available sites for the labeled DNA to reassociate. Most reported experiments used incubation times varying between 15 to 20 hr. In free solution reactions, the DNA concentrations and incubation times were especially critical. Because duplexes composed of two labeled DNA strands cannot be distinguished from duplexes composed of a labeled and an unlabeled DNA strand, a large excess of unlabeled DNA was used (usually from 4,000 to 8,000 fold excess). This reduces the extent of reassociation between two labeled DNA strands.

The Cot concept. Britten and Kohne (1967, 1968) showed that specific hybrid formation was a function of the initial concentration of each DNA species and the time of incubation. They introduced the acronym Cot which was an abbreviation for the product of initial concentration (c_o) and time (t). The units were given in moles of nucleotides per liter times seconds. Cot controled reassociation of DNA when the temperature, salt concentration and fragment size were defined. If we assume that 1 μg of DNA has an absorbance at 260 nm of 0.024, the Cot units are readily calculated, using the initial A_{260} and incubation time (Britten and Kohne, 1966):

$$\text{Cot} = 1/2(A_{260})\ (\text{incubation time in hr}) =$$
$$(\text{moles of nucleotide})\ (\text{seconds})\ (\text{liter})^{-1}$$

It is generally assumed that renaturation of DNA follows second order reaction kinetics because the process involves the collision of two complementary strands. A graphic

representation of the relationships between the extent of reassociation and Cot allows an investigator to decide whether the reaction rates deviate significantly from second order kinetics. For this purpose, the percent reassociation is shown on the ordinate in arithmetic units and the Cot on the abscissa in logarithmic units. The curve generated by an uncomplicated second-order reaction is reasonably symmetrical, sigmoid-shaped and makes a relatively straight transition from the completely denatured state to the completely reassociated state over a 100-fold range in Cot values (Britten and Kohne, 1968).

Additional information about genome structure can be inferred from the time course of DNA reassociation. A useful point is the Cot value at which half of the initially denatured DNA has reassociated. This point has been designated by $Cot/\frac{1}{2}$ or $\frac{Cot}{2}$ (Britten and Kohne, 1968). Some of the implications of Cot/2 can best be symbolized mathematically:

The rate of disappearance of denatured DNA should be:

$$-\frac{dc}{dt} = kc^2$$

where c = concentration of denatured DNA
t = time of renaturation

By integrating and evaluating over $t=0$ ($c=c_o$), we obtain

$$\frac{c}{c_o} = \frac{1}{1 + k(c_o t)} \quad .$$

When the DNA is half-renatured

$$\frac{c}{c_o} = \frac{1}{2} = \frac{1}{1 + k(c_o t)} \quad .$$

By solving for (c_0t) at half-renaturation, we obtain $c_0t = 1/k$ or, $Cot/2 = 1/k$

DNA can be characterized by the value of Cot/2. Because k has been found to be inversely proportional to the complexity of the DNA, Cot/2 is directly proportional to the genome size. Cairn's measurement of the size of the Escherichia coli genome (4.5×10^6 nucleotide pairs) has been used frequently as a reference value. Thus if an organism's DNA has a Cot/2 twice that of E. coli DNA, the organism has a genome size of 9×10^6 nucleotide pairs. Britten and Kohne (1968) pointed out that the linear relationship between the Cot/2 and genome size was true only in the absence of repeated sequences.

Mandel (1969) suggested that closely related organisms should contain the same amount of genetic informational capacity. The estimation of genome size by renaturation kinetics has the advantage of measuring only the informational length of a particular DNA species, not its physical length (Falkow et al., 1969). Kingsbury (1969) studied some selected bacterial DNA samples by optical reassociation kinetics in 0.15 M NaCl - 0.015 M sodium citrate (SSC) or in 0.12 M sodium phosphate buffer. By this method, Chlamydia trachomatis had a genome size of 6×10^5 nucleotide pairs based on the size of the E. coli genome (4.5×10^6 nucleotide pairs). No correction was made for differences in GC content. Moore and McCarthy (1969) showed that the genome size of extreme halophiles was similar to that of E. coli and contained no rapidly renaturing fraction. The genome size of

B. subtilis was found to be close to that of E. coli (McConaughy et al., 1969). Renaturation kinetics in SSC at 60 C gave a genome size of 9.2×10^9 daltons for Saccharomyces cerevisiae (Bicknell and Douglass, 1970). The authors found few, if any, repeated sequences. The Cot/2 for E. coli was found to be 4.86 compared to 16.0 for S. cerevisiae.

Falkow et al. (1969) discussed how pure R-factor DNA could be recovered using reassociation kinetics on hydroxyapatite. The value of hydroxyapatite was the fact that DNA sequences could be fractionated on a preparative scale. Using this method, Kohne (1968) isolated ribosomal RNA (rRNA) cistrons from E. coli and Proteus mirabilis. Likewise, Brenner et al. (1970) isolated and characterized transfer RNA (tRNA) cistrons from E. coli.

Britten and Kohne (1968) emphasized that the Cot/2 measured by optical methods is different from the Cot/2 measured in hydroxyapatite. In fact, the latter method gave a Cot/2 of about 50% of the Cot/2 determined optically. This was to be expected because the fraction of fragments reassociated is measured by hydroxyapatite while the fraction of total strand length reassociated is determined optically. Nevertheless, by using standards of known genome size to calibrate each system, excellent agreement between the results obtained by the two methods was obtained.

Applications of DNA Reassociation to Actinomycete Taxonomy. The DNA from 30 actinomycetes was tested in the Bolton-McCarthy agar-gel system to determine the degree with

which they could bind ^{32}P labeled Streptomyces griseus DNA (Yamaguchi, 1967). A relatively high concentration (200 µg/ml) of DNA was used during the 5 min heating in 0,1 x SSC to denature the DNA. The salt concentration during incubation was 0.4 M Na^{+1} and the temperature was 65 C. For these high GC organisms, the conditions were quite non-exacting. Moreover, there was a conspicuous absence of any high GC, non-homologous DNA controls. Not unexpected, therefore, was the author's conclusion that the actinomycetes were genetically a homogeneous group and that DNA homology studies were not particularly useful in taxonomic studies.

Tewfik and Bradley (1967) tested DNA samples from 12 streptomycetes to determine the extent of their reassociation with labeled DNA from S. venezuelae and S. rimosus. The agar-gel system was used. Although the stated incubation temperature was 60 C, the low binding to appropriate controls suggested that, in fact, a more stringent temperature was used. With S. venezuelae as the reference (40% absolute binding), the remaining streptomycetes showed 37 to 88% relative binding. In a separate test, three nocardiae showed from 24 to 44% homology with S. venezuelae. These comparisons were generally corroborated by the studies of Enquist and Bradley (1968) using the membrane filter technique of Warnaar and Cohen (1966). A range of 39 to 77% homology was obtained by Tewfik and Bradley (1967) with these 12 strains when S. rimosus DNA (34% absolute binding) was used as the reference. In both instances, reciprocity was exhibited and trends evident in one system were evident in the other.

Monson et al. (1969) used a modification of the filter method of Warnaar and Cohen (1966) to solve a frustrating taxonomic issue. Most of the genetic studies on streptomycetes have been done with cultures erroneously designated as S. coelicolor. To determine whether these cultures were genetically homogeneous with the S. violaceoruber nominifer, DNA hybridization was done and selected pairs of mutants were crossed. The reference DNA preparations were from type cultures of S. coelicolor Muller and S. violaceoruber 14980. An exacting incubation temperature of 75 C was used accompanied by adequate controls. The results definitively established that S. coelicolor and S. violaceoruber were different; moreover, the cultures used by Bradley, Hopwood and Sermonti as well as Actinopycnidium caeruleum were closely related to the S. violaceoruber 14980 type culture and were distinct from the type cultures for S. coelicolor and S. violaceus.

Farina and Bradley (1970) used similar methods in combination with thermal elution to analyze DNA from a group of actinomycetes which form sporangia. Their results separated this group into two clusters: the first contained Actinoplanes, Dactylosporangium, and Ampullariella; the second group contained Planomonospora, Spirillospora and Streptosporangium. Again type cultures were used for the study. Using S. venezuelae as the reference, all of the genera examined in the families Actinoplanaceae and Streptosporangiaceae showed little homology with this reference (10 to 20% relative binding). Only DNA from S. albus (type culture for the genus Streptomyces), Streptoverticillium

baldaccii and Microellobosporia flavea appreciably bound the S. venezuelae reference. Of interest was the significant binding of DNA samples from different families to the reference. The Tm,e of these intra-family duplexes was about 5 to 6 C lower than the homologous duplexes. The fact that the taxonomy of the sporangia-forming actinomycetes established by cell-wall analysis was corroborated by Farina and Bradley's (1970) analysis confirmed the usefulness of both methods for actinomycete systematics.

Evolution and Nucleic Acids. During their divergence from a common ancestor, two organisms each accumulate base substitutions in their DNA. These base substitutions are reflected in experimental nucleic acid reassociation studies by base mismatching. The relationship between decrease in thermal stability and the fraction of mismatched bases has emerged from several studies (Laird et al., 1969). Although DNA base composition is useful as a first approximation of relatedness (DeLey, 1969), it cannot be used as a quantitative measure of divergence. However, nucleic acid homology measurements, because they reflect the number of nucleotide changes that have occurred since the species diverged, do provide a quantitative measure of evolutionary divergence (Laird et al., 1969). The measurement of DNA species divergence can be complicated by the existence of repetitive DNA sequences (Britten and Kohne, 1968).

Conserved rRNA Loci. The evolution of base sequences takes place at different rates at different sites in the

genome (McCarthy, 1967). Conserved genes could have great significance as to the possible common ancestry of highly divergent organisms or groups. For example, it was found that in the genus Bacillus, whose various members differ in GC by as much as 20%, there was little overall genetic homology between any individual species, but that rRNA, tRNA and antibiotic resistance loci were highly conserved in all species tested (Doi and Igarashi, 1966; Dubnau et al., 1965). It was of considerable interest that DNA base sequences of rRNA and tRNA were shown to be conserved relative to the total DNA in enterobacteria, myxobacteria and yeast (Schweizer et al., 1969; Midgely, 1968 and Moore and McCarthy, 1967). Moreover, there is evidence that multiple sites on the genome exist for both 16 and 23S rRNA synthesis and that these multiple sites are contiguous in bacteria (Cutler and Evans, 1967). Midgely (1968) estimated that there were 45 cistrons responsible for rRNA synthesis in E. coli. Interestingly, the author found an equal transcription rate for all the cistrons in unit time. Avery and Midgley (1968) found that the 16S and 23S rRNA mutually and completely competed for their respective sites of hybridization. In Saccharomyces cerevisiae, Schweizer et al. (1969) estimated that there were 140 cistrons for rRNA and 320 to 400 cistrons for tRNA. Their results suggested that there were separate cistrons for all three classes of rRNA and tRNA. Ritossa and Spiegelman (1965) found evidence for several hundred rRNA cistrons in Drosophila melanogaster. Wood and Luck (1969) concluded that the 25S and 19S rRNA genes in mitochondrial

DNA of Neurospora crassa were repeated at least four times. Kohne (1968) isolated and characterized E. coli rRNA cistrons by taking advantage of their higher relative concentration and hence, more rapid renaturation rate.

Although ribosomal cistrons appear to be relatively resistent to evolutionary change compared to other cistrons, such changes are by no means precluded (Moore and McCarthy, 1967). Aronson and Holowczyk (1965) concluded that the ribosomal RNA fraction of Pseudomonas aeruginosa and E. coli were heterogeneous based on an analysis of pancreatic ribonuclease digests. The authors suggested that in each organism there were several rRNA cistrons differing slightly in base sequence. A comparative study was made of the arrangement of base sequences in the rRNA cistrons of rabbit DNA (Moore and McCarthy, 1968). It was concluded that the cluster of rRNA cistrons in a mammalian DNA, which represented an evolutionary or an historical series of tandem duplications, exhibited intercistronic base sequence divergence. By enzymic digestion and subsequent gel electrophoresis of rRNA from representative bacteria and mammals, Pinder et al. (1969) found that the structure of rRNA had differentiated appreciably in the course of evolution. Significantly, the authors showed that the overall structure as opposed to the nucleotide sequence tended to be conserved during evolution. In contrast to previous results, these scientists concluded that no evidence existed for heterogeneity in an rRNA population from a given species. However, recent work by Muto (1970) suggested that 16S rRNA from E. coli showed hetero-

geneity. If heterogeneity indeed exists, then a number of interesting questions are posed: does diversity in rRNA imply diversity in ribosome populations? In higher organisms, is a change in the population of rRNA involved in a regulatory process during differentiation?

An interesting analysis of apparent marker evolution rate versus genetic map position in B. subtilis was accomplished by Childon and McCarthy (1969). Because the genome of B. subtilis has been shown to be replicated in sequential order from one end to the other, Childon and McCarthy (1969) tested the hypothesis that the resulting gene-dosage effect might influence the rate of evolution of genes near the origin and terminus. By the use of two assays, that is, the relative efficiency of heterologous transformation and the decrease in the thermal stability of heteroduplex DNA formed by two strands originating from different species, the authors concluded that the rate of marker evolution was directly influenced by its map position. Significantly, it also appeared that gradients of conservation of base sequence occurred on both sides of the loci for ribosomal RNA.

Neutral Mutations. DNA reassociation studies reviewed by King and Jukes (1969) suggested that there was considerable latitude at the molecular level for random genetic changes that have no effect upon the fitness of the organism. It has been proposed that most changes in amino acid sequence undergone by several proteins during evolution have been the result of a non-Darwinian process: the random fixation of neutral or near neutral amino acid substitutions (King and Jukes,

1969; Kimura, 1969; Kimura and Ohta, 1969; Kimura, 1968; Wright, 1966 and Freese, 1962). The following lines of evidence have been cited by King and Jukes (1969) to support this conclusion: (1) data on structure of cytochrome c, insulin, alpha and beta chains of hemoglobin and serum albumin from a wide variety of species indicated that these proteins had undergone amino acid substitutions at a constant rate during evolution. This was not expected by a mechanism involving the selection of advantageous mutations; (2) neutral amino acid substitutions (in function) have been found in several proteins; (3) the neutral allele-random fixation model suggested by Kimura (1969) was consistent with rates of amino acid substitution in several proteins; (4) the Treffers mutator gene produced a trend toward DNA of GC content higher than the original parent and (5) a significant correlation existed between the number of synonymous codons for each amino acid and its respective occurrence frequency in a large number of proteins. This would suggest that the structure of the genetic code itself may exert an important influence on the evolution of these proteins. Arnheim and Taylor (1969) tested the hypothesis that there was a relationship between the rate of evolutionary change and the degree of neutral allelic variation in populations by using data on hemoglobin variants of man. Their conclusion was that such a relationship existed; however, they stressed the need for more data before a strong conclusion could be reached.

An interesting case for neutral or near-neutral mutations having altered electrophoretic mobility but not catalytic

activity was presented by Shaw (1965). Shaw calculated that 75% of all possible single base mutations would not be detected by a change in electrophoretic mobility. From her compiliation of data, it appeared that enzymes differed widely in the molecular alterations which they could tolerate.

Laird et al. (1969) presented a thorough discussion of DNA reassociation data and the rate of fixation of nucleotide substitutions in evolution. Their conclusions supported the ideas of Walker (1969) in that the rate of evolution of DNA from a number of higher organisms was 2 to 5 times greater than inferred from comparative amino acid sequences of selected proteins. This conclusion seems compatible only with the neutral mutation-random fixation hypothesis.

Clarke (1970) took issue with the neutral mutation hypothesis reviewed by King and Jukes (1969). He pointed out several weaknesses in their arguments which, if true, may cast doubt upon the validity of the hypothesis. Unfortunately, Clarke did not discuss the significant discrepancy of DNA sequence divergence as compared to protein sequence divergence. Apparently Clarke did accept King and Jukes (1969) idea that natural selection was the editor and not the composer of the genetic message.

Materials and Methods

Stock Cultures. The organisms used in this study were primarily members of the genera Streptomyces and Nocardia (Table 1). The stock streptomycete cultures were propagated on tomato paste-oatmeal agar medium (TPO) of the following composition: 20 g Contadina tomato paste; 20 g Heinz baby oatmeal; 15 g Difco agar and 1 liter deionized water. The pH of the medium was adjusted to 6.8 with 1 N NaOH. Members of the genus Nocardia were propagated on peptone-yeast extract agar medium (PY) of the following composition: 5 g Difco peptone; 3 g Difco yeast extract; 15 g Difco agar and 1 liter deionized water. Both media were autoclaved at 121 C for 15 min. The cooled, molten media were dispensed into petri dishes. All the streptomycete stock cultures were incubated at 30 C for 7 to 14 days, and the nocardial stock cultures were incubated at 30 C for 3 to 7 days.

Mass Culture of Actinomycetes for Isolation of DNA. For streptomycetes, spores were scraped from a 7 to 14 day old culture grown on TPO agar plates and were suspended in 25 ml of PY broth. For nocardial cultures, growth from a 3 to 7 day old culture on PY agar plates was suspended in 25 ml of PY broth. The inoculum was homogenized with a Potter-Elvehjem tissue grinder. About 10 ml of this suspension was added to 1 liter of PY broth. The seeded medium was incubated on a rotory shaker at 27 to 30 C for 15 to 24 hr. The resulting

Table 1

Principal Cultures

Culture Designation	Source
Actinopycnidium caeruleum	H. Lechevalier, Rutgers University
Escherichia coli B	Univ. Minnesota collection
Mycobacterium rhodochrous M370	R. Gordon, Rutgers University
M. smegmatis VAC 433	N.M. McClung, University of South Florida
Mycobacterium sp. $17C_2$ III swine	R. Manion, Minneapolis Veterans Hospital
Myocobacterium sp. scotochromogenic	R. Manion, Minneapolis Veterans Hospital
Mycobacterium sp. CDC avian	R. Manion, Minneapolis Veterans Hospital
M. stercoides A406	E. Mankiewicz
M. tuberculosis $H37R_v$	R. Manion, Minneapolis Veterans Hospital
Myxococcus xanthus FB	M. Dworkin, University of Minnesota
Nocardia canicruria N1574	ATCC 11048
N. corallina N78	ATCC 4273 as *Mycobacterium rhodochrous*
N. corallina NS5	J.B. Clark, University of Oklahoma
N. corallina N76	ATCC 4276 as *M. rhodochrous*
N. erythropolis N2	J.N. Adams, University of South Dakota
Streptomyces aureofaciens S10762	ATCC 10762
S. cinnamomeus S1285	ATCC 11874

S. coelicolor Muller S352	S.A. Waksman, Rutgers University
S. coelicolor S2419	NRRL - B2419 as S. canescus
S. erythreus S233	Univ. Minnesota collection
S. fradiae S347	Univ. Minnesota collection
S. griseus S104	E. McCoy, University of Wisconsin
S. griseus S1945	E. McCoy, University of Wisconsin
S. rimosus S10970	ATCC 10970
S. venezuelae S13	Univ. Minnesota collection
S. venezuelae S86	Univ. Minnesota collection
S. violaceoruber S1	G. Sermonti
S. violaceoruber S16	NRRL-B-1257
S. violaceoruber S199	Univ. Minnesota collection
S. violaceoruber S307	Univ. Minnesota collection
S. violaceoruber S3443	R. Gordon, Rutgers University
S. violaceoruber S3740	R. Gordon, Rutgers University
S. violaceoruber S14980	ATCC-14980

mycelial growth was harvested by centrifugation (2000 x g for 15 min) and was subsequently washed three times with saline-EDTA (0.15 M NaCl and 0.1 M sodium ethylenediamine tetraacetate, pH 8.0). The EDTA and high pH retarded deoxyribonuclease activity. The washed mycelial growth was frozen at -20 C in plastic bags and stored until needed.

Lysis of Actinomycetes for DNA Isolation. For streptomycetes, 2 to 3 g of washed, wet packed mycelia were suspended in 25 ml saline-EDTA solution in a 500 ml glass-stoppered flask. One ml of the enzyme lysozyme (Calbiochem, 200 mg/ml) was added; the flask was shaken at 42 C for one hr. Next 2 ml of the enzyme pronase (Calbiochem, 10 mg/ml) was added and the flask shaken at 42 C until lysis began as indicated by an increase in viscosity accompanied by a marked decrease in turbidity. To bring lysis to completion, 2 ml of 25% (w/v) sodium dodecyl sulfate (SDS) was added and the flask was gently shaken by hand at ca. 25 C for 1 or 2 min. Next, the flask was heated in a 60 C water bath for 10 min with occasional shaking followed by slow cooling to ca. 25 C. Most of the nocardia and several streptomycetes were insensitive to lysozyme; however a pretreatment of the washed mycelia with acetone and diethyl ether rendered most of these organisms susceptible to the enzyme. About 2 to 3 g of washed, wet-packed mycelia were shaken with 30 ml acetone on a wrist action shaker for 30 min at ca. 25 C. The mycelia were then collected by centrifugation at 2000 x g for 10 min and the supernatant fluid was discarded. The pellet was suspended in 30 ml diethyl ether, shaken on a wrist-action shaker for 30

min at ca. 25 C, and centrifuged at 2000 x g for 10 min. The supernatant fraction was poured off and the mycelia were suspended in 25 ml saline-EDTA and treated as previously described for the streptomycetes. After lysis, the Marmur (1961) procedure for the isolation of DNA from microorganisms was followed.

Extraction of DNA from Actinomycetes. All volumes indicated here are based upon 2 to 3 g of cells in 25 ml saline-EDTA; therefore, the values were adjusted accordingly when more or less material was used. Lysed cells were mixed with 7 ml of 5 M sodium perchlorate solution to give a final concentration of 1 M sodium perchlorate. The high salt concentration served to dissociate proteins from nucleic acids. An equal volume of Sevag's mixture (chloroform-isoamyl alcohol; 24:1 v/v) was added and the mixture shaken for 30 min at ca. 25 C on a wrist action shaker. Centrifugation at 8000 x g for 10 min separated the emulsion into 3 layers; chloroform at the bottom, denatured protein in the middle and a top aqueous layer containing the nucleic acids. The chloroform denatured proteins while the isoamyl alcohol reduced foaming and aided in separation and maintenance of the layers in the centrifuged deproteinized solution. The aqueous layer was carefully collected and about 2 volumes of 95% ethanol layered over it. The precipitated nucleic acids which collected at the interface of the two layers were spooled onto a glass rod. After the precipitate had dissolved (about 15 min), 0.5 ml 10 x SSC (1 x SSC buffer contained 0.15 M NaCl and 0.015 M sodium

citrate; 10 x SSC contained 10 times this concentration; 0.1 x SSC contained 1/10 this concentration; see Gillespie and Spiegelman, 1965) was then added to bring the salt concentration to 1 x SSC. Nucleic acids readily dissolve in solutions of low ionic strength; however, the high salt concentration was necessary to stabilize the DNA and to decrease the shearing effects of thermal agitation. The citrate chelated divalent cations. To digest ribonucleic acid (RNA), ribonuclease (Calbiochem, bovine pancreas) was added to a final concentration of 50 μg/ml and the mixture incubated at 37 C for 30 min. The Sevag's deproteinization step was then repeated with 15 ml of Sevag's mixture until little or no protein collected at the interface. The DNA was again precipitated with 2 volumes of 95% ethanol and dissolved in 4.5 ml 0.1 x SSC. One ml of 3 M acetate solution was added to give a final concentration of 0.3 M acetate. Next, 6.0 ml isopropanol was added slowly with continuous shaking; isopropanol selectively precipitated the DNA while RNA fragments remained in solution. The DNA was collected on a glass rod and dissolved in 4.5 ml distilled water. Next 0.5 ml 10 x SSC was added to give a solution of 1 x SSC. The final DNA solution was stored over a few drops of chloroform at 5 C.

Determination of Buoyant Density of DNA. A concentrated stock cesium chloride (CsCl) solution was prepared each week by dissolving 15 g CsCl (Matheson Coleman and Bell, 99% purity) in 7 ml 0.02 M THAM buffer (tris-hydroxymethylaminomethane) pH 8.5. The final solution was passed through a membrane filter (0.45 μm) to remove any insoluble material.

The absorbance at 260 nm of the filtered CsCl solution was measured; if the absorbance exceeded 0.05, the CsCl was repurified by filtration or recrystalization from hot ethanol. The technique of Meselson et al. (1957) was followed. The density of the CsCl solution was brought to about 1.718 g cm^{-3} by mixing 0.84 ml of the concentrated CsCl stock solution with 0.23 ml of a 0.1 x SSC solution containing 0.5 to 2.0 µg of the DNA to be examined plus about 0.5 µg of the standard reference DNA (E. coli DNA or S. coelicolor DNA in 0.1 x SSC). It has been determined by Schildkraut et al.(1961), that the refractive index of the CsCl-DNA solution is linearly related to the density as follows:

$$\rho_{25\ C} = 10.8601(n_D 25\ C) - 13.4974$$

where

$\rho_{25\ C}$ = density at 25 C

$n_D 25\ C$ = refractive index at 25 C

The refractive index was measured with a Bausch and Lomb, Abbe-3L refractometer. The refractive index was adjusted to 1.401 which corresponded to an average density of 1.718 g cm^{-3}. When a final adjustment of density was required, it was accomplished by adding either solid CsCl or 0.1 x SSC. Next 0.75 ml of the DNA-CsCl solution was carefully injected into a Spinco 12 mm analytical centrifuge cell equipped with a 1^{o} negative wedge, quartz, upper window and a plane quartz lower window. The centerpiece was routinely the Kel-F, cesium resistant type. The loaded cell was placed in an An-D two place rotor with a 7.0 g counterbalance. Centrifugation was done in a Spinco Model E analytical ultracentrifuge at 44,770

rev min^{-1} at 25 C. The CsCl gradient was formed after ca. 10 hr running time. By 18 hr, equilibrium was very nearly achieved. Using quartz optics and an ultraviolet (UV) light source, UV absorption photographs were taken on Kodak commercial film. The resulting ultraviolet absorption films were traced with either the Beckman model RB Analytrol film densitometer or with the Gilford Model 2410 Linear Transport scanning attachment and the Gilford Model 2400 spectrophotometer (Gilford Instruments; Oberlin, Ohio). The width of the scanning beam was maintained at 100 μm. The buoyant density of each unknown DNA was then calculated with respect to the position of an internal standard of either E. coli B DNA (1.7100 g cm^{-3} or S. coelicolor Muller DNA (1.7313 g cm^{-3}). The calculations were performed using the equation of Schildkraut et al. (1961):

$\rho_x = 0.0092(r^2 - r_o^2) + \rho_{std}$

ρ_{std} = density of standard reference DNA. This was taken to be 1.7100 g cm^{-3} for E. coli DNA and 1.7313 g cm^{-3} for S. coelicolor Muller DNA

ρ_x = density of sample DNA in g cm^{-3}

r_o = distance of the standard DNA band from the central axis of rotation.

r = distance of the sample DNA band from the central axis of rotation.

Because the value of ρ_{std}, r_o and r are known, the value of ρ_x can be calculated. When indicated, DNA was denatured by diluting the stock DNA preparation to 0.1 x SSC and boiling for 5 min followed by quick cooling in an ice bath.

Measurement of Thermal Denaturation of DNA. The mid-point of the hyperchromic shift at 260 nm (Tm) of DNA solutions heated in 0.1 x SSC was determined using the Gilford Model 2000 or Model 2400 Multiple Sample Absorbance Recorder equipped with a Beckman monochrometer, a linear temperature programming unit, a linear thermosensor for measuring the temperature of the samples, an automatic cuvette positioner and an Haake thermoregulated circulator. Three DNA samples could be run at one time enabling one to use internal standards. All samples were prepared in 0.1 x SSC with 0.1 x SSC as the blank unless otherwise noted. The A260 of each sample was in the range of 0.3 to 0.4 which corresponded to approximately 12.5 to 16.5 μg DNA/ml respectively (assuming 1 μg of DNA has an A260 = 0.024). After the recorder was adjusted to proper zero and 100% and the heating system calibrated according to the Gilford operation manual, the samples were placed in the cuvette heating block. An auxilliary offset control was used to space the recorded absorbance plots of the three samples. The temperature of the cuvette holder was automatically recorded for each reading. The heating rate was linear from 50 C to 90 C and was complete in about 60 min.

Alternatively, a Zeiss PMQII spectrophotometer equipped with a flow-through cuvette holder attached to a thermoregulated circulator was used. A260 readings were taken manually at 1 to 5 min intervals and the temperature at each reading was read from a thermometer in the circulator. The heating rate was controlled by a Neslab temperature programmer and was routinely set to give a 1 degree rise in 5 min to allow

equilibration of the samples and the circulator.

Preparation of ^{14}C-labeled DNA from Streptomycetes. An inoculum of 7 to 14 day old spores was prepared in 50 ml of sterile 0.15 M NaCl. After the inoculum was homogenized, 10 to 20 ml were added to 1 liter of sterile, cooled medium of the following composition: (solution A) 20.0 g glucose; 0.5 g $MgSO_4 \cdot 7\ H_2O$; 2.0 g NH_4NO_3; 1.0 g asparagine; 5.0 g peptone and 800 ml deionized water in a 1 liter flask; (solution B) 5.0 g K_2HPO_4 and 200 ml deionized water in a 500 ml flask. Solutions A and B were autoclaved separately and subsequently mixed aseptically. The inoculated medium was shaken at 30 C until the A260 reached 0.3 to 0.5 (usually 12 to 24 hr). At this time rapid growth of the organism was in progress. Next, 500 μC of 2-^{14}C-labeled uracil (New England Nuclear) in 5 ml sterile deionized water was added and the culture again shaken at 30 C. Samples were taken immediately after label addition and at 1 hr intervals afterwards to follow the incorporation of label into the mycelia. When the uptake of the label began to plateau, the mycelia were harvested by centrifugation, washed three times with saline-EDTA and the DNA isolated as described previously.

Shearing and Denaturation of ^{14}C-labeled DNA. The concentration of a ^{14}C-labeled DNA was adjusted to 0.5 to 1 mg DNA/ml in 2 x SSC. The DNA was then sheared by twice passing the solution through a French pressure cell (American Instruments Co. Inc., AMINCO, Silver Springs, Md.) at 10,000

lb in^{-2}. This produced fragments of DNA having molecular weights of approximately 5 x 10^5 daltons or less (McCarthy and Bolton, 1963). The sheared DNA was dialysed overnight against 0.1 x SSC and was then denatured by heating in boiling water for 5 min followed by quick chilling in an ice-water bath. The salt concentration was brought to 2 x SSC by adding the appropriate amount of 10 x SSC. The concentration of single-stranded, ^{14}C-labeled DNA fragments was determined by comparing the count min^{-1} of the fragments to the specific activity of the native labeled DNA preparation.

The Membrane Filter Technique for Assay of DNA Reassociation. The procedure for DNA-DNA reassociation on membrane filters was derived from that of Warnaar and Cohen (1966), in that buffers of low ionic strength and high pH were used to elute the single-stranded, unhybridized DNA from the membrane filter. After investigating a number of variables, the following protocol was developed. Dilutions from the stock solutions of DNA were made to give 20 µg DNA/ml in 0.1 x SSC. Next, 2.5 ml of this sample was pipetted into a small screwcap tube, heated in boiling water for 5 min and was then quickly cooled in ice-water. This process denatured the DNA. Denaturation was generally monitored by observing the change in A260. When the 2.5 ml samples had cooled, 2.5 ml of 12 x SSC was added to give 5 ml of approximately 6 x SSC solution containing 10 µg DNA/ml.

Schleicher and Schuell (Keene, N.H.) type B-6, size

25 mm, pure nitrocellulose membranes and Millipore HAWP size 25 mm membranes were used. The filters were soaked for 1 to 2 min in 6 x SSC and washed by suction with 10 ml of 6 x SSC. The previously prepared denatured DNA solution (5 ml of the 6 x SSC solution containing 50 μg DNA total) was passed slowly through the filter using standard Millipore apparatus. Maximum retention of the input DNA occurred when gravity filtration was used. The efficiency of binding of the denatured DNA to the membranes was monitored by comparing the A260 of the filtrate to the A260 of the solution prior to filtration. The filters were placed in scintillation vials without caps and were air dried for at least 4 hr followed by 2 hr in a vacuum oven at 80 C. By tightly capping these vials containing the dried filters and storing them in a dry place, the filters could be kept for extended periods before carrying out the subsequent reassociation process.

The dried filters were placed in clean scintillation vials and 1.50 ml of 1.25 x SSC buffered with 0.02 M THAM-HCl was added. The pH of this solution was 8.0. Next, 0.05 ml of a dilution of sheared, labeled, denatured DNA to give 1 μg was carefully pipetted in each vial, mixed by gentle rotation, and each vial was tightly capped. Depending on the experiment, the mixture was incubated at 60, 70 or 75 C for 15 hr to 20 hr. The filters were then carefully removed and rinsed briefly with 0.003 M THAM-HCl, pH 9.4 (Warnaar and Cohen, 1966). After this rinse, both sides of the filter were washed by suction with 40 ml of the same buffer. The filters were washed at a rate not exceeding 3 ml/min. The washed filters were removed

and dried by air or with an infra-red lamp and then counted in a Packard Tri-Carb Scintillation counter. Binding was expressed as count $min^{-1}\mu g^{-1}$ DNA on the filter. Filters which had no DNA bound to them, yet had undergone the entire procedure served as controls for non-specific binding of the labeled DNA. E. coli DNA served as one nonhomologous control while Myxococcus xanthus DNA served as a high GC yet nonhomologous control. Salmon sperm DNA (Calbiochem, grade A) was often included as a nonhomologous control.

The final washing of the reannealed DNA on the filter was a critical step. The standard wash method previously described was used for most of the subsequent experiments; however, the method was tedious and limited the number of assays which could be run. Therefore, for some of the later experiments, an alternative wash procedure was devised. After incubation, the filters were removed from the reaction mixture, rinsed briefly with 0.003 M THAM-HCl, pH 9.4 and then immersed for 10 min in a vial containing 10 ml 1 x SSC at the incubation temperature. After air drying, the filters were assayed as described previously.

The thermal stability of the reassociated DNA duplexes formed was also determined. Filters were prepared, loaded with DNA and incubated at various temperatures as before. After reassociation was complete, the filters were removed, rinsed briefly with 0.003 M THAM-HCl, pH 9.4 and immersed in 10 ml 1 x SSC at the incubation temperature for 10 min. The labeled DNA was then eluted by incubating the filters for 15 min in 2.5 ml 1 x SSC in scintillation vials at temperature

increments of 5 C. Next 15 ml of Triton X-100, toluene scintillation fluid [666 ml toluene, 333 ml Triton X-100 5.5 g 1,4-bis-2-(5-phenyloxazolyl) benzene and 125 mg 2,4-diphenyloxazole] was added to the vials and the radioactivity determined. Up to 30% aqueous salt solution can be counted in this fluid with only a small loss in efficiency due to quenching. The results were expressed as the relative amount of ^{14}C-DNA released, that is, as the ratio:

$$\frac{\text{count min}^{-1} \text{ released at a given temperature}}{\text{total homologous count min}^{-1}} \times 100\%$$

Results

CsCl Density Gradient Analysis. Purified DNA from selected actinomycetes, most of which had A260/A280 ratios in the range of 1.8 to 2.0, were individually mixed with a reference E. coli DNA and centrifuged in CsCl as described previously (p 30 of this thesis). Because a linear relationship exists between the buoyant density of DNA and its base composition (Schildkraut et al., 1962), the following relationship was used to calculate the mole fraction of guanine plus cytosine of the various DNA samples:

$$\rho = 1.660 + 0.098\ (GC)$$

where

ρ = buoyant density in g cm^{-3}

GC = mole fraction guanine plus cytosine

At least three determinations were made with most streptomycete DNA samples while two determinations were generally made with other samples of actinomycete DNA. The buoyant densities of the actinomycete DNA samples studied ranged from 1.7214 to 1.7312 g cm^{-3} (Table 2). All DNA preparations showed only a single, symmetrical band in the CsCl gradient. The calculated %GC of the streptomycete DNA preparations ranged between 70.1 and 72.6, those of nocardiae were between 62.6 and 70.7, and those of the mycobacteria were between 64.3 and 70.3. Myxococcus xanthus FB was determined to have a GC content of 68.5%.

Table 2

Buoyant Density and Base Composition of Selected Actinomycete DNA Preparations

Source of DNA	Buoyant Density ($g\ cm^{-3}$)	GC (%)
S. coelicolor Muller S353	1.7312 ± 0.0003	72.6
S. griseus S1945	1.7312 ± 0.0005	72.6
S. aureofaciens S10762	1.7311	72.6
S. coelicolor S2419	1.7309 ± 0.0007	72.3
S. cinnamomeus S1285	1.7307	72.1
S. fradiae	1.7304	71.8
S. violaceoruber S1	1.7304 ± 0.0004	71.8
S. violaceoruber S307	1.7303 ± 0.0007	71.7
S. violaceoruber S3740	1.7303 ± 0.0003	71.7
S. violaceoruber S199	1.7302 ± 0.0003	71.6
Actinopycnidium caeruleum VAC 342	1.7300	71.4
S. griseus S104	1.7300	71.4
S. violaceoruber S14980	1.7299 ± 0.0006	71.3
S. violaceoruber S3443	1.7299 ± 0.0002	71.3
S. violaceoruber S16	1.7298 ± 0.0006	71.2
N. coralline NS5	1.7293	70.7
S. venezuelae S13	1.7287 ± 0.0002	70.1
Mycobacterium sp. $17C_2$ III swine	1.7289	70.3
Mycobacterium sp. scotochromogenic	1.7278	69.2
N. opaca N76	1.7267	68.1
Mycobacterium sp. CDC avian (A)	1.7266	67.9

M. smegmatis VAC433	1.7264	67.7
M. fortuitum	1.7255	66.8
Nocardia opaca N109	1.7252	66.5
Mycobacterium stercoides A406	1.7242	65.5
M. rhodochrous 155C	1.7231	64.4
M. tuberculosis $H37R_a$	1.7230	64.3
N. corallina N305	1.7228	64.2
N. erythropolis N2	1.7214	62.6
Myxococcus xanthus FB	1.7271	68.5

[a] Standard deviations were calculated only on trials of 3 determinations or more.

DNA Reassociation: Immobilization of Denatured DNA on Membrane Filters. The amount of input unlabeled DNA retained by both Millipore and Schleicher and Schuell (SS) membrane filters was dependent on the ionic strength of the solvent. Solvent concentrations of 2x, 4x, 6x, 8x and 10x SSC were tested and 6x SSC proved the best for DNA retention. The amount of input DNA retained by the filter varied inversely with the speed of filtration. Gravity filtration gave optimum retention of input DNA. Millipore filters retained only 50% of the input DNA, while SS filters retained almost 100% of the input DNA at optimum conditions. Millipore membranes gave virtually the same results in DNA reassociation experiments as did the SS filters, but for ease of quantitation the majority of the assays were conducted with SS membrane filters.

Quantitative Nucleic Acid Relationships. ^{14}C-labeled, sheared, denatured S. venezuelae S13 DNA (specific activity between 3000 to 7000 count min^{-1} μg^{-1} depending on the preparation) was reassociated with a number of unlabeled, denatured actinomycete DNA samples bound to nitrocellulose membrane filters. The fraction of the labeled S. venezuelae S13 DNA bound to each membrane filter was calculated and expressed as a per cent of the fraction bound to the filter with unlabeled S. venezuelae S13 DNA attached to it (the homologous reference system). This fraction is presented as the relative per cent bound DNA. The following three controls were included: (1) Myxococcus xanthus FB DNA as an unrelated DNA with a high (68%) GC content, (2) Escherichia coli B DNA

as an unrelated DNA with a moderate (50%) GC content and (3) a membrane filter with no DNA attached, to measure non-specific binding of the ^{14}C-labeled DNA to the membranes.

Many investigators have stressed the importance of the temperature of incubation on DNA reassociation (McCarthy and Church, 1970). McCarthy (1967) has pointed out that temperatures significantly lower than the optimum permit distantly related nucleotide sequences to reassociate. To examine the effect of incubation temperature on the relative amounts of hybrid duplexes formed, a series of experiments was done at 60 C and 70 C (Table 3). When the temperature of incubation was raised from 60 C to 70 C, the reassociation of homologous S. venezuelae S13 DNA dropped about 5%. Furthermore, these data emphasize the importance of the incubation temperature on the extent of duplex formation. Examination of the control filters suggested that the 70 C incubation was high enough to exclude any non-related sequence reassociation, i.e., duplex formation between all but well matched sequences was precluded. The extent of reactions at 60 C was, in general, higher than the corresponding 70 C reactions, but the difference seemed variable from reaction to reaction. It was assumed that these differences were due to the reaction of distantly related sequences.

In the 70 C reaction, the biologically recombinable pair S. violaceoruber S199 and S16, while having identical GC composition, were seen to be quite different by S. venezuelae S13. Also, DNA from S. venezuelae S86 and from S. griseus S104, two organisms which are morphologically similar, reacted

Table 3

DNA Reassociation with Selected Actinomycete DNA Preparations

Unlabeled DNA Source	% Relative Binding at an Incubation Temperature of		Divergence Index (DI)
	60 C	70 C	
S. venezuelae S13	100(1168)[b]	100(920)[b]	1.00
S. aureofaciens S10762	95[c]	32[c]	0.34
S. violaceoruber S199	70	52	0.74
S. venezuelae S86	98	88	0.90
S. rimosus S10970	91	77	0.85
S. griseus S104	99	88	0.89
S. cinnamomeus S1285	87	66	0.76
S. erythreus S233	80	54	0.68
S. fradiae S347	70	26	0.37
S. griseus S1945	72	38	0.53
S. violaceoruber S16	75	37	0.49
N. opaca N76	50	37	0.74
N. corallina NS5	37	36	0.97
N. corallina N78	41	40	1.00
N. erythropolis N2	25	23	0.92
Mycobacterium rhodochrous M370	--[d]	4	----
M. tuberculosis $H37R_a$	--	5	----
Myxococcus xanthus FB	10	5	0.50
Escherichia coli B	5	1	0.20
Salmon sperm	2	2	1.00

Blank filter	2	1	----

---

[a] Streptomyces venezuelae S13 is the homologous reference system.

[b] The actual binding in count min^{-1}

[c] The average of 4 to 6 trials. Standard deviation was less than 5%

[d] Not done

the same by S. venezuelae S13 reference DNA. DNA from Nocardia, a different genus in another actinomycete family, showed between 20 to 40% reaction with the reference DNA indicating a significant degree of relatedness; however, mycobacterial DNA (organisms in the same family as Nocardia) showed no reaction with S. venezuelae S13 DNA. Finally, some of the streptomycete DNA samples bound less reference DNA than did DNA from nocardiae.

By comparing the reactions at 60 C and 70 C, another important parameter was provided; that is, an estimate of base sequence similarity to or divergence from the homologous reference DNA. I have chosen to call incubation temperatures that allowed distantly related sequences to react non-exacting. This is to contrast exacting incubation conditions that allowed only closely related sequences to react. By dividing the amount of relative binding at exacting conditions by that at non-exacting conditions, a useful ratio was obtained. This ratio has been called the Divergence Index (DI). This ratio has been called the thermal binding index (TBI) by Brenner et al. (1969a). DI values are useful in gauging the presence or absence of closely related genetic material. A value close to 1.00 indicates that all of the sequences that bind the reference DNA are virtually identical to the reference, whereas a value approaching 0.00 indicates the test DNA shares almost no regions of similarity with the reference DNA.

The streptomycetes used in this study seemed to show a large spectrum of relatedness to the reference DNA with DI values ranging from 0.33 to 1.00. Interestingly, although

the nocardial DNA preparations bound less than 40% of the streptomycete reference DNA, the DI values suggested the existence of small but significant amounts of conserved nucleotide sequences with respect to the S. venezuelae S13 reference DNA. These may be ancestral sequences indicative of common origin or they may represent evidence for relatively recent gene transfer.

DI values were also determined for a number of closely related streptomycetes (as determined by classical determinative methods) using S. violaceoruber 14980 ^{14}C-labeled DNA (specific activity 4000 count min^{-1} μg^{-1}). The exacting incubation condition was 75 C to preclude all but closely related sequences; the non-exacting condition was kept at 60 C. Within this group, the DI values ranged from 0.66 to 1.00 (Table 4). This suggested a significant amount of sequence conservation among the ten organisms examined. S. coelicolor Muller and S. violaceoruber 14980 were, however, distinctly different by this method. Interestingly, the organisms fell in two groups, those clustering around the reference DNA and type culture for the specific epithet S. violaceoruber and those clustering around S. coelicolor Muller, the type culture for the specific epithet S. coelicolor.

Thermal Stability of Intra- and Interspecific DNA Duplexes. The thermal stability of reassociated DNA fragments formed at various incubation temperatures may be used as an index of the extent and specificity of sequence pairing. Previously reassociated DNA fragments were dissociated and eluted

Table 4

DNA Reassociation Among Streptomycetes[a]

Unlabeled DNA Source	% Relative Binding at an Incubation Temperature of		Divergence Index (DI)
	60 C	75 C	
S. violaceoruber S14980	100(660)[b]	100(520)[b]	1.00
S. violaceoruber S3443	100	96	0.96
S. violaceoruber S1	100	85	0.85
S. violaceoruber S3740	100	97	0.97
S. violaceoruber S199	100	95	0.95
S. violaceoruber S307	89	83	0.93
S. violaceoruber S16	100	88	0.88
S. griseus 1945	65	43	0.66
S. coelicolor Muller S352	57	38	0.67
S. coelicolor S2419	48	37	0.77
Myxococcus xanthus FB	18	10	0.55
Escherichia coli B	16	9	0.56
Blank filter	5	4	----[d]

[a] Streptomyces violaceoruber S14980 is the homologous reference system

[b] The actual binding in count min^{-1}

[c] The average of 4 trials. Standard deviation was less than 5%

[d] Not done

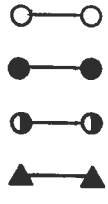
from the filters by increasing temperature stepwise, and assaying the eluted fractions by measurement of radioactivity. The thermal midpoint of elution (Tm,e) is defined as that temperature at which 50% of the reassociated fragments have become dissociated by strand separation and eluted.

Thermal elution studies were done to determine the degree of incomplete matching in the duplexes formed with S. venezuelae S13 reference DNA under exacting and non-exacting conditions. N. opaca N76 and S. violaceoruber S16 were chosen because both had a similar extent of reassociation of 70 C, yet were members of different taxonomic families. Moreover, samples of DNA from these two organisms have different GC contents. Myxococcus xanthus FB DNA was included as a non-related, high GC control.

At the very non-exacting incubation temperature of 50 C (Fig. 1), the majority of the reassociated duplexes was either non-specific or very loosely matched. The homologous reassociation showed a small degree of specific duplex formation, however, most homologous duplexes were of very low stability. The Tm,e for the homologous reaction was 57 C, for S. violaceoruber S16 the Tm,e was 54 C and for N. opaca N76 the Tm,e was also 54 C. Very little discrimination occurred at this temperature as demonstrated by the Tm,e of 54 C for the non-related DNA of Myxococcus xanthus FB.

At the incubation temperature of 60 C (Fig. 2), the reactions became more specific. The Tm,e for the homologous reaction was 85 C, for S. violaceoruber S16 the Tm,e was 77 C and for N. opaca N76 the Tm,e was 73 C. The conditions were

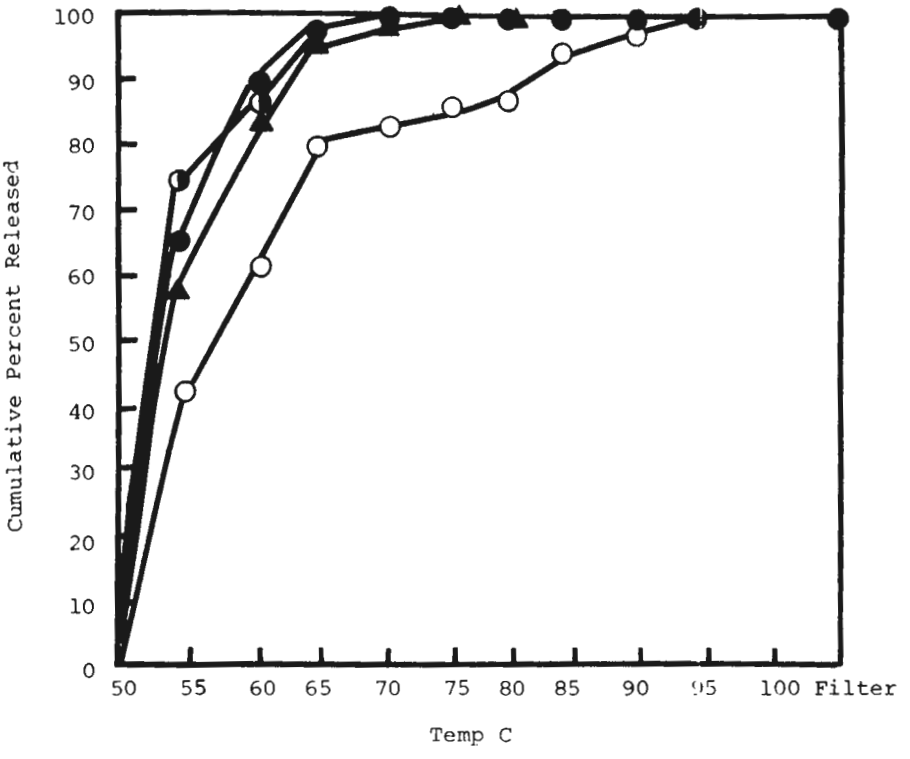
Fig. 1 Thermal stability of DNA duplexes formed between S. venezuelae S13 DNA and DNA of selected actinomycetes. The top graph is the integral form of elution or cumulative percent released versus the temperature of elution. The Tm,e is read directly from this plot by determining the temperature at which 50% of the DNA duplexes are dissociated. The incubation temperature was 50 C. The specific activity of the input labeled DNA was 7000 count $min^{-1} \mu g^{-1}$. The absolute homologous binding was 10.9%.



: S. venezuelae S13 (labeled)
: S. violaceoruber S16
: N. opaca N76
: Myxococcus xanthus FB

The bottom graph is the differential form of elution or relative percent released versus temperature of elution. The conditions are the same as stated above.

: S. venezuelae S13 (labeled)
: S. violaceoruber S16
: N. opaca N76
: Myxococcus xanthus FB


Cumulative Percent Released
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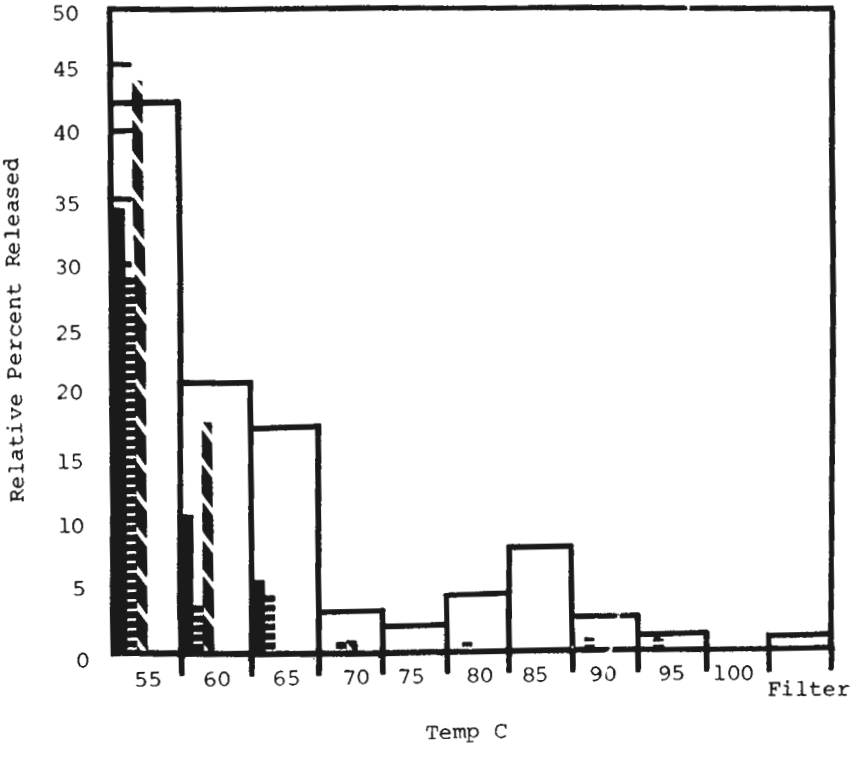
50 55 60 65 70 75 80 85 90 95 100 Filter
Temp C


Relative Percent Released
50
45
40
35
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20
15
10
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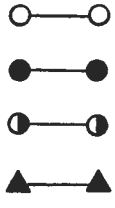
55 60 65 70 75 80 85 90 95 100
Filter
Temp C

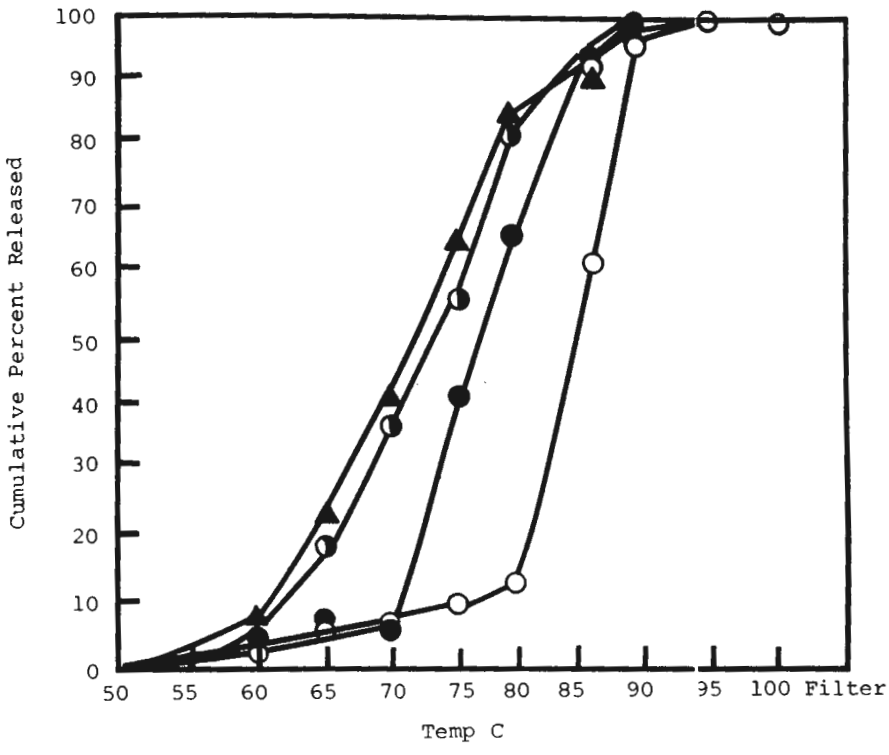
Fig. 2 Thermal stability of DNA duplexes formed between S. venezuelae S13 DNA and DNA of selected actinomycetes. The top graph is the integral form of elution or cumulative percent released versus the temperature of elution. The Tm,e is read directly from this plot by determining the temperature at which 50% of the DNA duplexes are dissociated. The incubation temperature was 60 C. The specific activity of the input labeled DNA was 7000 count $min^{-1}\mu g^{-1}$. The absolute homologous binding was 36%.



: S. venezuelae S13 (labeled)
: S. violaceoruber S16
: N. opaca N76
: Myxococcus xanthus FB

The bottom graph is the differential form of elution or the relative percent released versus temperature of elution. The conditions are the same as stated above.

: S. venezuelae S13 (labeled)
: S. violaceoruber S16
: N. opaca N76
: Myxococcus xanthus FB


Cumulative Percent Released
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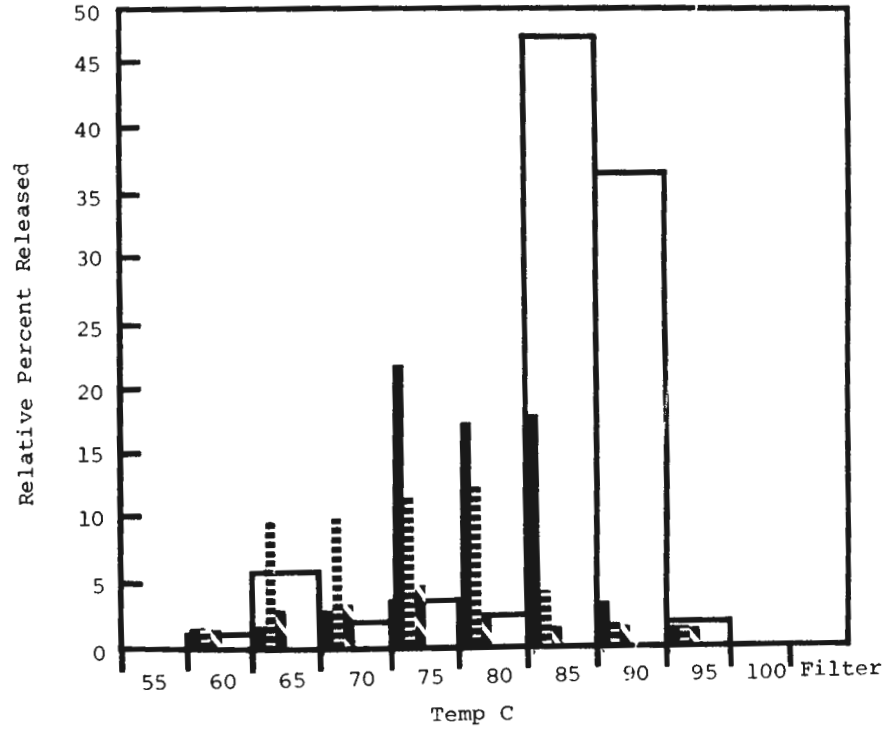
90
80
70
60
50
40
30
20
10
0
50 55 60 65 70 75 80 85 90 95 100 Filter
Temp C

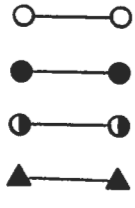

Relative Percent Released
50
45
40
35
30
25
20
15
10
5
0
55 60 65 70 75 80 85 90 95 100 Filter
Temp C

such that the extremely low stability duplexes formed during the 50 C incubation were lost. The extent of binding of Myxococcus xanthus (15%) still suggested that the 60 C incubation was non-exacting.

At an incubation temperature of 70 C (Fig. 3), most of the low stability duplexes formed at 60 C were lost. The Tm,e of the homologous reference DNA remained 85 C. The majority of the reference DNA bound by S. violaceoruber S16 formed duplexes having a Tm,e about 7 C lower than the homogous DNA. Interestingly, N. opaca N76 formed duplexes with S. venezuelae S13 DNA that were 5 C lower in thermal stability. The extent of reaction of N. opaca N76 and S. violaceoruber S16 at this exacting incubation temperature was virtually the same, yet the thermal stability of the duplexes were distinctly different. Furthermore, as can be seen by the elution profile, N. opaca N76 formed a significant number of duplexes with stabilities approaching those of S. venezuelae S13 duplexes. These data correlate well with the predictions obtained from DI values.

The thermal stability profiles of DNA duplexes formed between S. venezuelae S13 reference DNA and representatives of two important streptomycete species are presented in Fig. 4. The difference between S. coelicolor S352 and S. violaceoruber S199 and S14980 was quite striking. The obvious biphasic elution profile for S352 is in contrast to the integral "Gaussian-like" dissociation curves for the S. violaceoruber strains. The DI for S. violaceoruber S199 (0.74) suggested the existence of closely related sequences compared to S.

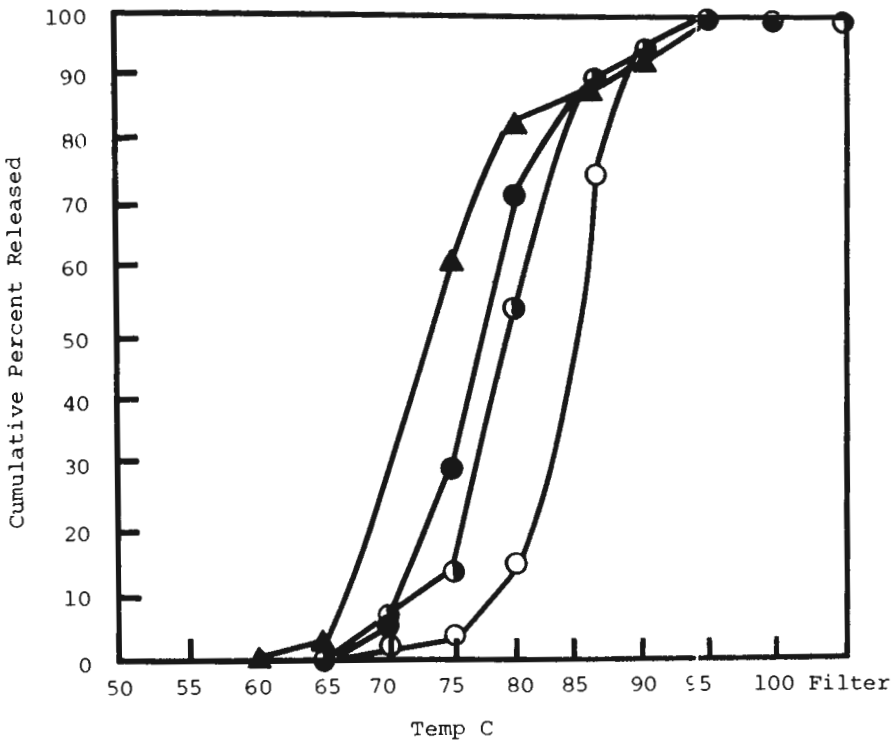
Fig. 3 Thermal stability of DNA duplexes formed between S. venezuelae S13 DNA and DNA of selected actinomycetes. The top graph is the integral form of elution of cumulative percent released versus the temperature of elution. The Tm,e is read directly from this plot by determining the temperature at which 50% of the DNA duplexes are dissociated. The incubation temperature was 70 C. The specific activity of the input labeled DNA was 7000 count $min^{-1}\mu g^{-1}$. The absolute homologous binding was 25%.



: S. venezuelae S13 (labeled)
: S. violaceoruber S16
: N. opaca N76
: Myxococcus xanthus FB

The bottom graph is the differential form of elution or the relative percent released versus temperature of elution. The conditions are the same as stated above.

: S. venezuelae S13 (labeled)
: S. violaceoruber S16
: N. opaca N76
: Myxococcus xanthus FB


Cumulative Percent Released
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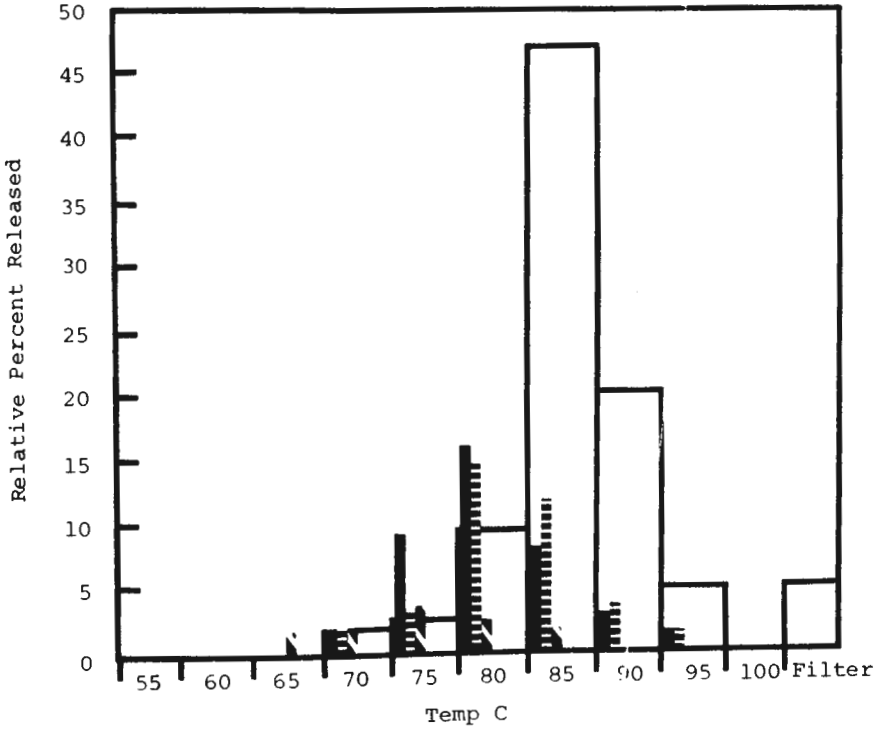
50 55 60 65 70 75 80 85 90 95 100 Filter
Temp C


Relative Percent Released
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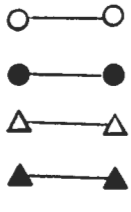
55 60 65 70 75 80 85 90 95 100 Filter
Temp C

venezuelae S13. This prediction again was borne out by the thermal stability profiles. The Tm,e of S199 was only 1 C lower than the homologous Tm,e. On the other hand, S. violaceoruber S14980 had a Tm,e of 79 C, about 6 C lower than the Tm,e of S. venezuelae S13. There seemed to be little doubt that S. coelicolor S352 was distinct from S. violaceoruber S199 and S14980.

Yamaguchi (1965) concluded from cell wall analyses that the genus Actinopycnidium was not separable from the genus Streptomyces. Monson et al. (1969) confirmed this conclusion using direct DNA reassociation. It was of interest to determine the stability of the duplexes formed between the DNA of S. violaceoruber S14980 and Actinopycnidium DNA. It is interesting that at 60 C (Fig. 5) extensive low stability duplexes were formed with the reference DNA S. violaceoruber S14980. The results are strikingly similar to the 50 C incubation with S. venequelae S13 DNA as the reference. A significant number of high stability complexes were formed by the DNA from the closely related S. violaceoruber S199 and DNA from Actinopycnidium strains.

At 70 C (Fig. 6), almost all the low stability duplexes were removed and, as demonstrated by the very tenacious binding, only high stability complexes remained. The Tm,e of all DNA samples was almost identical at 96 C. The sequences of Actinopycnidium DNA which reacted with the reference S. violaceoruber S14980 DNA were closely related as seen by duplex stability and by position of elution. This substantiates

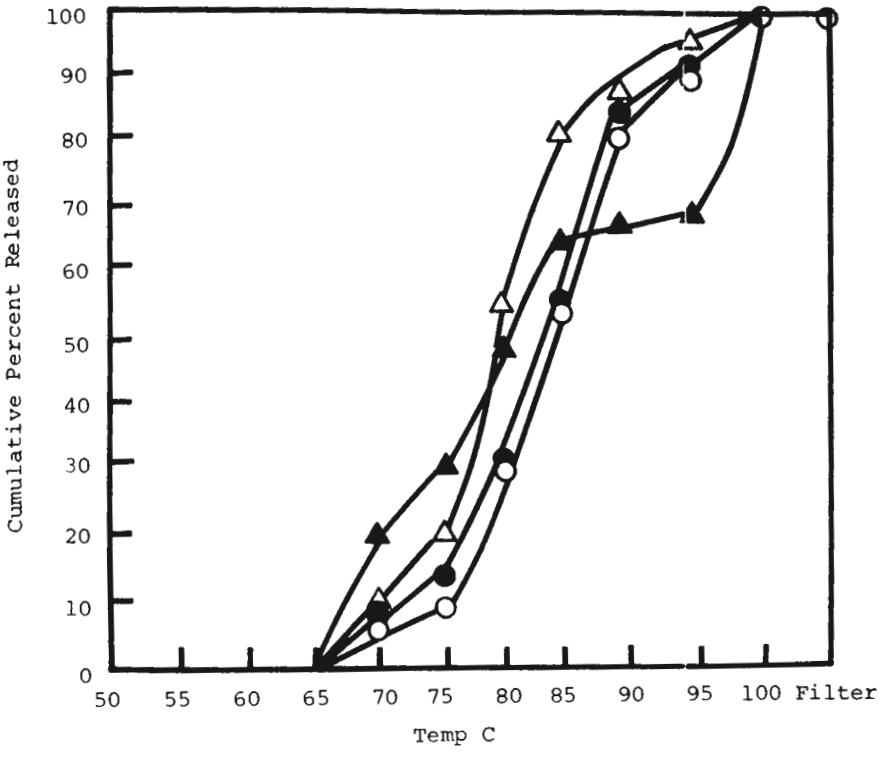
Fig. 4 Thermal stability of DNA duplexes formed between S. venezuelae S13 DNA and DNA of selected actinomycetes. The top graph is the integral form of elution or cumulative percent released versus the temperature of elution. The Tm,e is read directly from this plot by determining the temperature at which 50% of the DNA duplexes are dissociated. The incubation temperature was 70 C. The specific activity of the input labeled DNA was 3000 count $min^{-1}\mu g^{-1}$. The absolute homologous binding was 12.5%.



: S. venezuelae S13 (labeled)
: S. violaceoruber S199
: S. violaceoruber S14980
: S. coelicolor Muller S352

The bottom graph is the differential form of elution or the relative percent released versus temperature of elution. The conditions are the same as stated above.

: S. venezuelae S13 (labeled)
: S. violaceoruber S199
: S. violaceoruber S14980
: S. coelicolor Muller S352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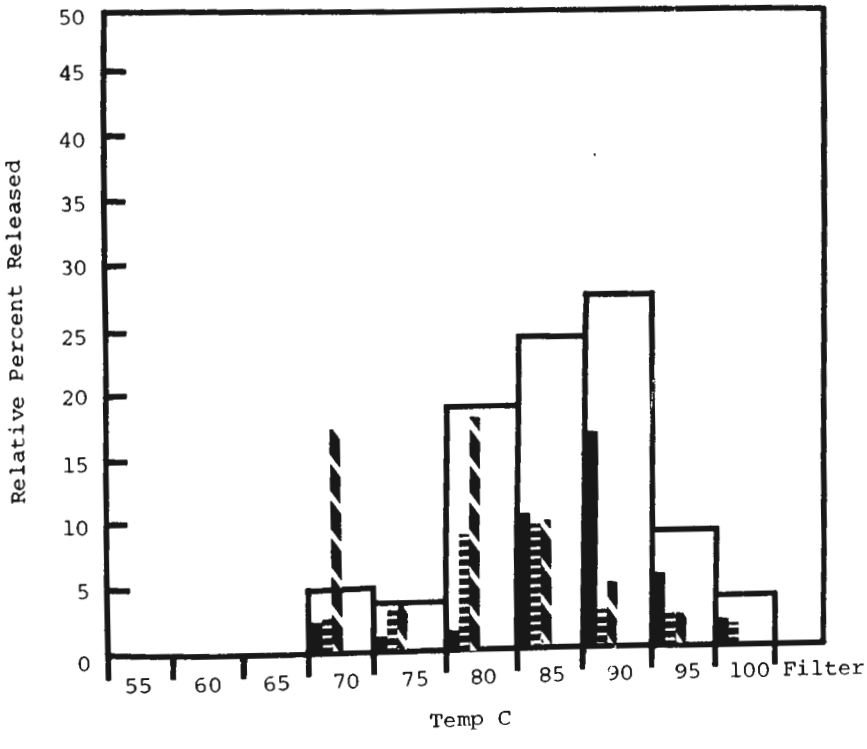
Temp C


Relative Percent Released
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55 60 65 70 75 80 85 90 95 100 Filter
Temp C

Fig. 5 Thermal stability of DNA duplexes formed between S. violaceoruber S14980 DNA and DNA of selected streptomycetes. The top graph is the integral form of elution or cumulative percent released versus the temperature of elution. The Tm,e is read directly from this plot by determining the temperature at which 50% of the DNA duplexes are dissociated. The incubation temperature was 60 C. The specific activity of the input labeled DNA was 4000 count $min^{-1}\mu g^{-1}$. The absolute homologous binding was 18%.


: S. violaceoruber S14980 (labeled)


: S. violaceoruber S199


: Actinopycnicium caeruleum VAC 342


: A. caeruleum VAC 449

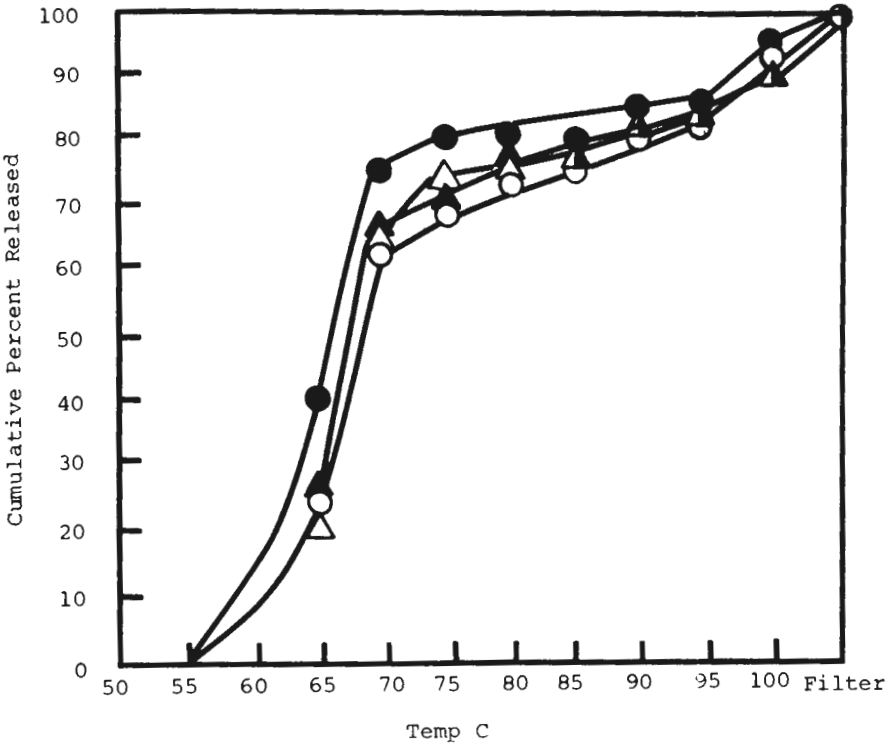
The bottom graph is the differential form of elution or the relative percent released versus temperature of elution. The conditions are the same as stated above.


: S. violaceoruber S14980


: S. violaceoruber S199


: A. vaeruleum VAC 342


: A. caeruleum VAC 449


Cumulative Percent Released
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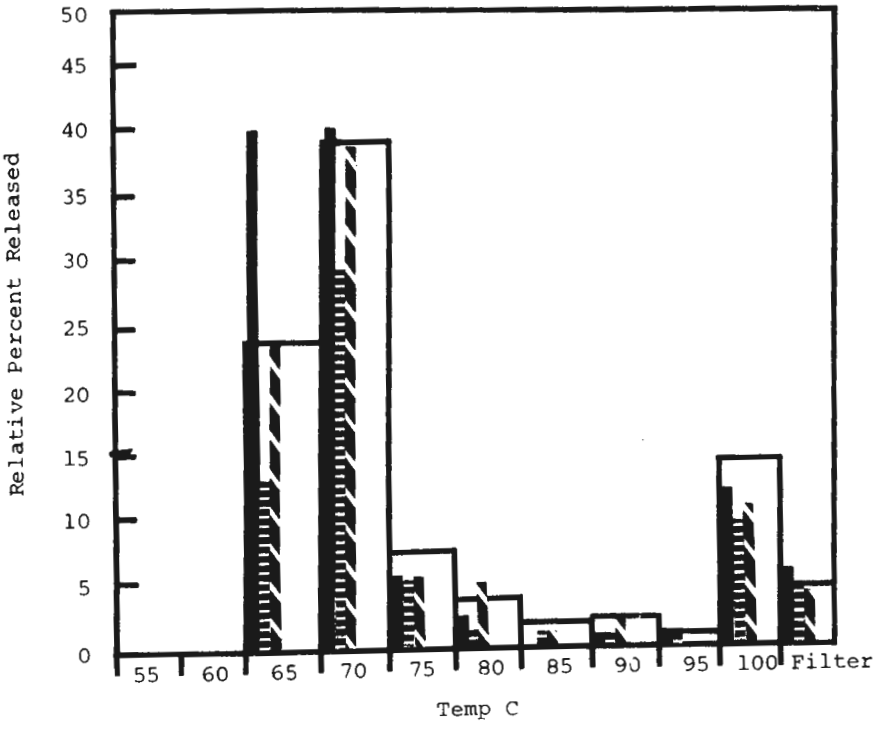
50 55 60 65 70 75 80 85 90 95 100 Filter
Temp C


Relative Percent Released
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40
35
30
25
20
15
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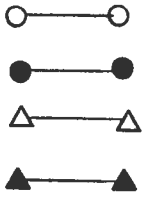
5
0
55 60 65 70 75 80 85 90 95 100 Filter
Temp C

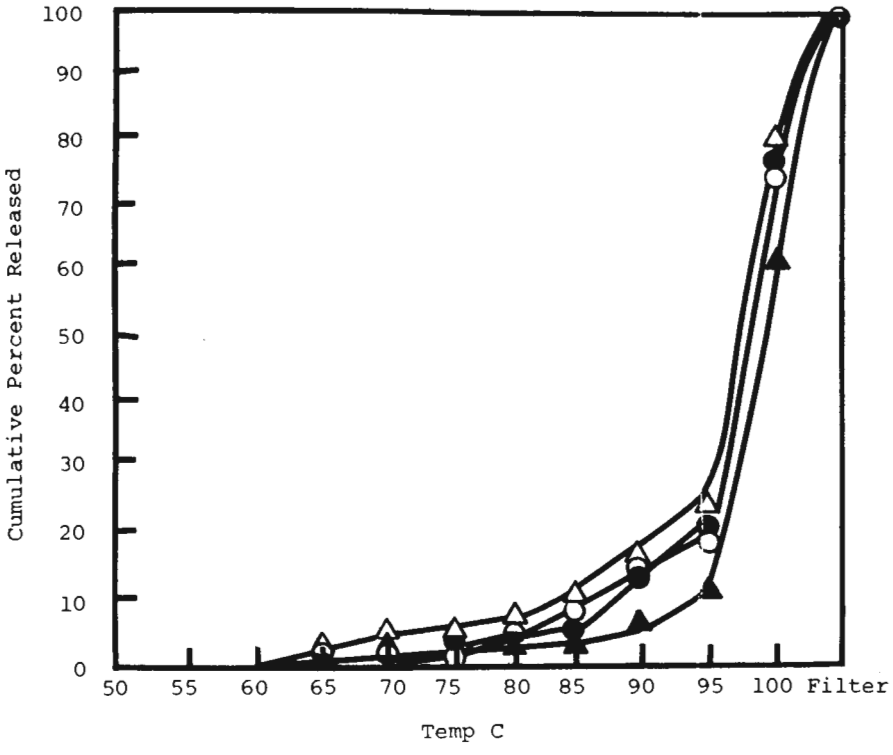
Fig. 6 Thermal stability of DNA duplexes formed between S. violaceoruber S14980 DNA and DNA of selected streptomycetes. The top graph is the integral form of elution or cumulative percent released versus the temperature of elution. The Tm,e is read directly from this plot by determining the temperature at which 50% of the DNA duplexes are dissociated. The incubation temperature was 70 C. The specific activity of the input labeled DNA was 4000 count $min^{-1}\mu g^{-1}$. The absolute homologous binding was 9.1%.



: S. violaceoruber S14980 (labeled)
: S. violaceoruber S199
: Actinopycnidium caeruleum VAC 342
: A. caeruleum VAC 449

The bottom graph is the differential form of elution or the relative percent released versus temperature of elution. The conditions are the same as stated above.

: S. violaceoruber S14980 (labeled)
: S. violaceoruber S199
: A. caeruleum VAC 342
: A. caeruleum VAC 449


Cumulative Percent Released
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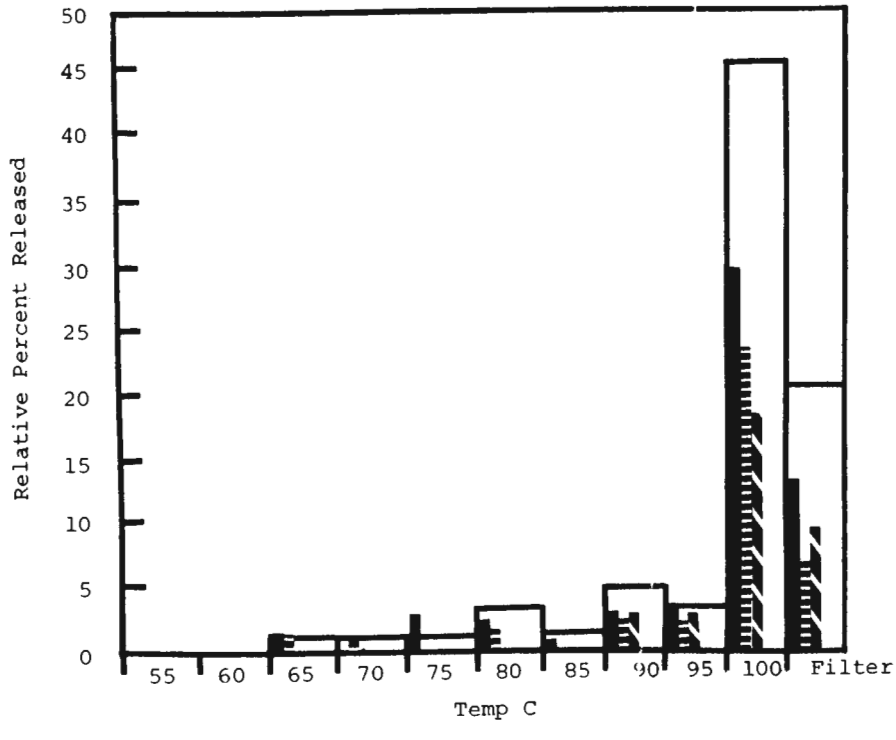
50 55 60 65 70 75 80 85 90 95 100 Filter
Temp C


Relative Percent Released
50
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20
15
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55 60 65 70 75 80 85 90 95 100 Filter
Temp C

the observations of Monson et al (1969).

A Graphical Approach to Nucleotide Divergence in Actinomycetes. If one assumes that the reduced thermal stability of the "distantly related" sequences reflects the proportion of unpaired bases within the interspecific duplex, then it is possible to assess the relative degree of base sequence divergence within a group of organisms.

When surveying the DNA from a large group of micro-organisms, it was time consuming to determine the thermal stability of each reaction at a number of incubation temperatures. A graphical approach was derived which indicated relationships between test organisms and provided information regarding the degree of base sequence divergence without resorting to time consuming thermal elution experiments. The data were obtained using two incubation temperatures, exacting and non-exacting. DI values were calculated as described earlier (Table 3 and 4). Previous thermal elution experiments suggested that there was a direct correlation between ΔTm,e (the difference between Tm,e values of an interspecific duplex and that of the homologous reference reaction) and the DI; the lower the DI, the greater the ΔTm,e.

DI values can be interpreted at the molecular level in terms of the distribution of nucleotide divergence (Fig. 7). Nucleotide divergence occurring more or less randomly throughout the genome was designated dispersed divergence. This was in contrast to localized divergence or localized conservation where changes occur in specific regions only.

Fig. 7 Distribution of mutations and the effects of different patterns of nucleotide divergence on reassociation assays.

DISTRIBUTION OF MUTATIONS	EFFECT ON REASSOCIATION	
	DIVERGENCE INDEX	EXACT BINDING
DISPERSED 	CA. 0	APPROACHES 0
LOCALIZED 	CA. 1	APPROACHES 100
CONSERVED 	CA. 1	APPROACHES 0

The duplexes formed during non-exacting conditions were most easily thought of as being composed of both incompletely matched and closely matched sequences. Under the more exacting incubation conditions, the duplexes formed were those of closely matched sequences.

Symbolically, let:

a = percent of incompletely matched duplexes

b = percent of closely matched duplexes

c = percent of unreacting DNA sequences

Then the non-exacting incubation gives (a + b) while the exacting incubation gives (b).

$$\text{The Divergence Index (DI)} = \frac{b}{a+b}$$

The values of (a+b) and (b) are subject to the following limitations:

$$100\% \geq (a+b) \geq 0$$

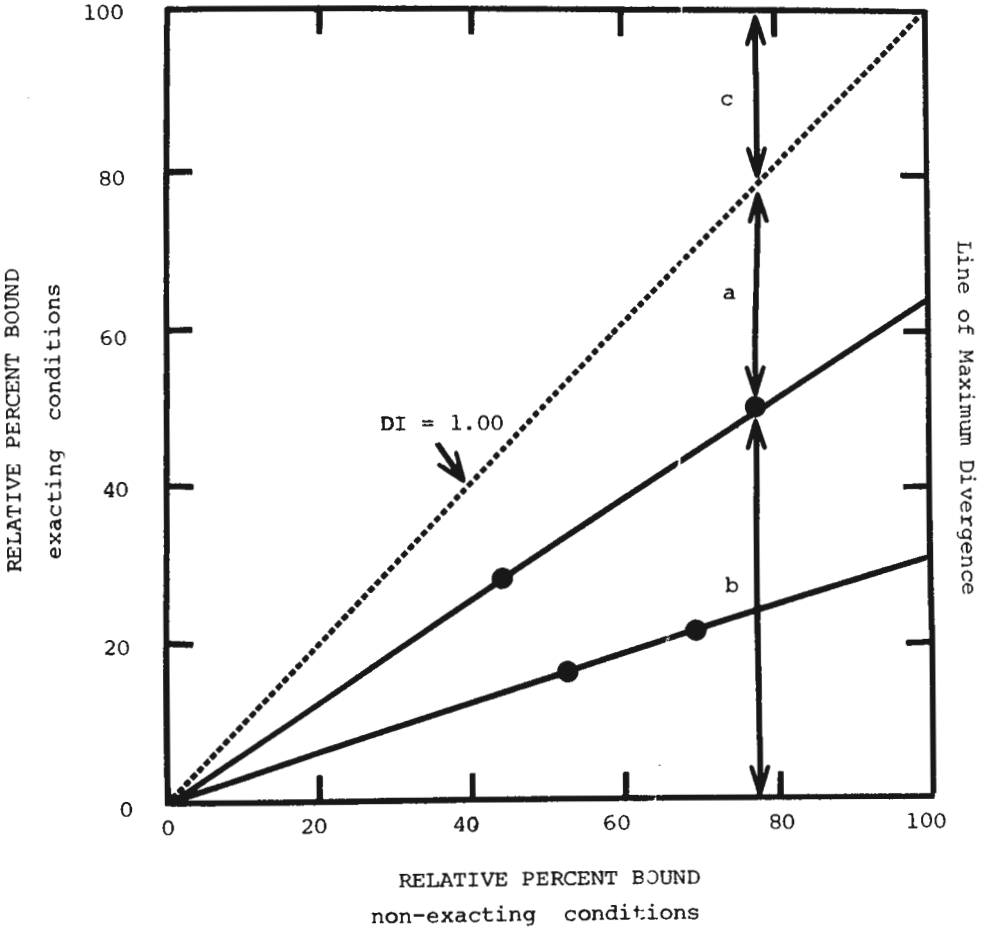
$$b \leq (a+b)$$

The total number of sequences available to react is defined by:

$$a + b + c = 100\%$$

When binding at exacting conditions (b) was plotted against binding at non-exacting conditions (a+b), a graphical presentation of the data relative to the reference DNA was obtained (Fig. 8). The diagonal (45^{o}) separates the graph in two parts, the upper portion contained values of c (non-related DNA). These sequences have diverged to the point that even at non-exacting conditions they did not react. The DI for a given test DNA with respect to the reference DNA was

Fig. 8 Determination of divergence patterns. a = percent of incompletely matched duplexes; b = percent of well matched duplexes; c = percent of unreacting DNA sequences. The Divergence Index (DI) is defined as b/(a+b). All points on a line drawn through the origin have the same DI. The line for DI = 1.00 divides the graph into an upper half containing values of "c" and a lower half containing values of "a" and "b". Points near the line of maximum divergence indicate that the respective DNA preparations have undergone extensive dispersed divergence.


100
80
60
40
20
0
0
20
40
60
80
100
RELATIVE PERCENT BOUND
exacting conditions
RELATIVE PERCENT BOUND
non-exacting conditions
DI = 1.00
a
b
c
Line of Maximum Divergence

given by the slope of the line drawn from its characteristic point through the origin. Any point on a given line had the same DI. The vertical distance from a point to the 45° diagonal gave the percent of distantly related sequences while the vertical distance from the diagonal to 100% above the point gave the percent of unrelated sequences. Insight into the mechanism of evolutionary divergence can be gained by considering the nature of nucleotide reassociation between DNA preparations containing different degrees of partially matched pairing but essentially no totally unmatched sequences, i.e., as $c \rightarrow o$, $(a+b) \rightarrow 100\%$. Graphically maximum divergence was indicated by the vertical line from the 100% point on the abscissa, that is, the upward projection from the point of 100% binding under non-exacting conditions. Any point on or near this line of maximum divergence indicated that sequences in the test DNA had diverged from the reference DNA to the point that any more changes in these nucleotide sequences would result in loss of their ability to reassociate to a detectable extent. Thus, organisms whose DNA had undergone essentially all dispersed divergence with respect to the reference DNA fell on or near the line of maximum divergence. DNA samples exhibiting localized divergence or sequence conservation were found near the 45° line.

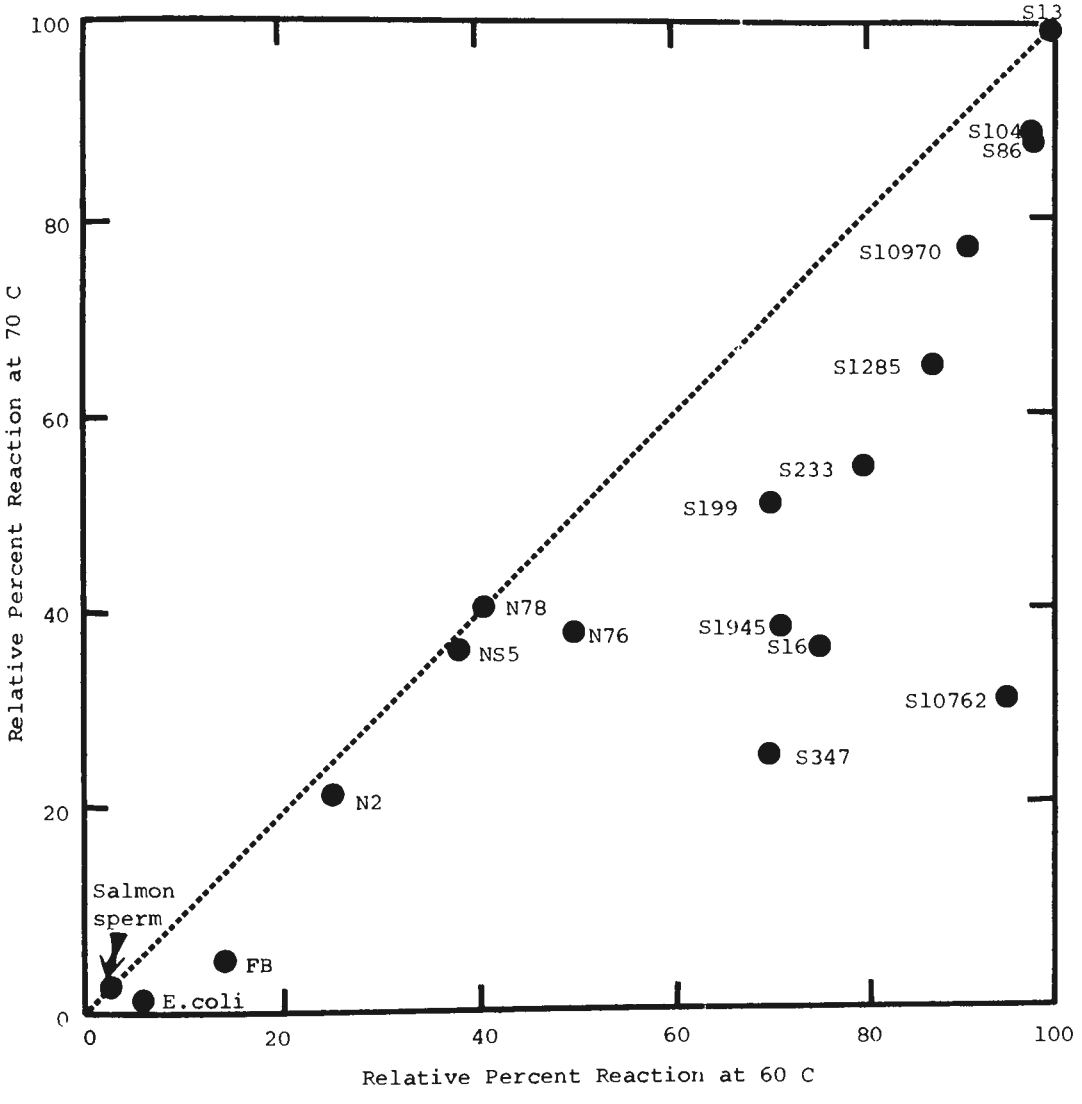
It is imperative that the limitations placed upon (a+b) and (b) be observed. Misleading data and erroneous interpretations arise when inappropriate "exacting" and "non-exacting" conditions are selected. In this study, the choice of conditions was appropriate as indicated by thermal elution

studies (Fig. 1 - 6).

Base Sequence Divergence in Actinomycete DNA. The degree of divergence from the reference S. venezuelae S13 DNA was quite varied (Fig. 9). Organisms like S. griseus S104 and S. venezuelae S86 exhibited only a small number of unreacting sequences, while the nocardial DNA samples showed between 50 to 80% totally unrelated sequences. It was of interest that in the Nocardia those sequences that did react were closely related to the reference as indicated by their high DI value and position on the graph. S. aureofaciens S10762 appeared to have undergone almost all dispersed divergence with respect to the reference DNA; furthermore it showed virtually no totally unrelated DNA. Interestingly, it exhibited only 30% closely related sequences. The remaining streptomycete DNA samples showed a marked diversity with respect to the reference, but all showed a significant amount of relatedness. Of the sequences that reacted, the nocardiae N76, N_2, NS5, and N78 along with streptomycetes S104 and S86 showed less than 10% distantly related sequences.

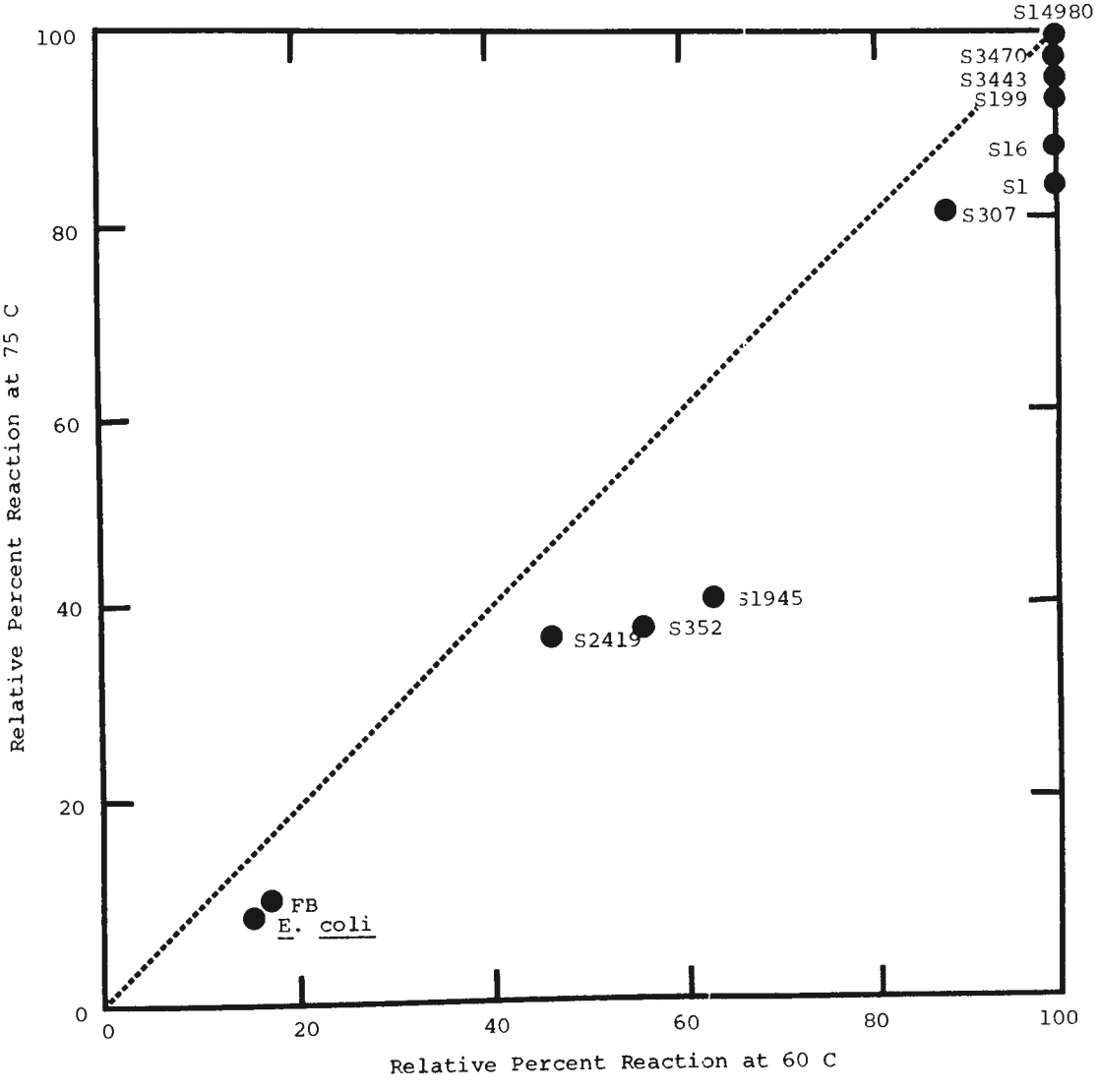
Using more exacting conditions (75 C) and S. violaceoruber S14980 as the reference, it was possible to show this marked divergence between S. coelicolor cultures and S. violaceoruber cultures (Fig. 10). Of the S. violaceoruber DNA samples studied, only S307 appeared to have diverged to any extent; it exhibited less than 10% distantly related sequences and about 10% totally unreactive sequences. The S. coelicolor DNA samples were found to have about 40% closely related sequences and almost 50% totally unreacting sequences.

Fig. 9. Determination of divergence patterns with respect to S. venezuelae S13. The DNA reassociation data was obtained from Table 3. The diagonal line (Divergence Index = 1.00) divides the graph in two parts, the upper half representing unreacting DNA sequences and the lower half representing reacting DNA sequences. All points on a line drawn through the origin have the same Divergence Index (D_I). Points near the diagonal (D_I = 1.00) indicate that the respective DNA preparations exhibit sequence conservation with respect to S. venezuelae S13. Points near the line of maximum divergence (see previous figure) indicate that the respective DNA preparations have undergone extensive dispersed divergence. The organism designations are given in Table 1.


S13
S104
S86
S10970
S1285
S233
S199
N78
N76
S1945
S16
NS5
S10762
S347
N2
Salmon
sperm
FB
E.coli
100
80
60
40
20
0
0
20
40
60
80
100
Relative Percent Reaction at 70 C
Relative Percent Reaction at 60 C

Interestingly S1945 showed about the same divergence from S. violaceoruber 14980 as from S. venezuelae S13.

Fig. 10 Determination of divergence patterns with respect to S. violaceoruber S14980. The DNA reassociation data was obtained from Table 4. The diagonal line (Divergence Index = 1.00) divides the graph in two parts, the upper half representing unreacting DNA sequences and the lower half representing reacting DNA sequences. All points on a line drawn through the origin have the same Divergence Index (DI). Points near the diagonal (DI = 1.00) indicate that the respective DNA preparations exhibit sequence conservation with respect to S. violaceoruber S14980. Points near the line of maximum divergence indicate that the respective DNA preparations have undergone extensive dispersed divergence. The organism designations are given in Table 1.


S14980
S3470
S3443
S199
S16
S1
S307
S1945
S352
S2419
FB
E. coli
100
80
60
40
20
0
0
20
40
60
80
100
Relative Percent Reaction at 75 C
Relative Percent Reaction at 60 C

Discussion

The purpose of this study was to examine the relationships of selected actinomycetes on a molecular level. Rather than deal with phenetic relationships which are based on the overt characteristics of organisms as they are presently observed, I chose to examine phylogenetic or evolutionary relationships. The ultimate goal was to probe the evolutionary pathways that gave rise to the actinomycetes as we observe them today. The major premise underlying this work was that the evolutionary history of an organism is retained, inscribed in the sequence of nucleotide bases in DNA. It seemed logical to begin the analysis by comparing total, gross nucleotide composition of the organisms.

CsCl Density Gradient Analysis of Actinomycete DNA. Marmur et al. (1963) concluded that a necessary, but not sufficient condition for substantial genetic relatedness between pairs of organisms was overall similarity in the DNA base composition. In practice, however, the principal value of the %GC content is its use as an exclusionary determinant in the formulation of taxonomic groups. Because similiarity in %GC does not necessarily indicate genomic similarity, only differences in %GC are meaningful. For example, the GC ratios of DNA from Saccharomyces cerevisiae, Bacillus subtilis and Homo sapiens are all about the same. In the final analysis, DNA base composition is useful as a first approximation of

relatedness, but it cannot be used as a quantitative measure of evolutionary divergence.

The calculated mole percent guanine plus cytosine (%GC) of the streptomycete DNA samples used in this study fell between 70.1 and 72.6. This is in relatively good agreement with previous determinations based on thermal denaturation and buoyant density values (Monson et al., 1969; Tewfik and Bradley, 1967 and Bradley, 1966).

The values for nocardial and mycobacterial DNA preparations ranged between 62.6 and 70.7 %GC. These values correlate well with the studies of Wayne and Gross (1968), Tewfik and Bradley (1967) and Jones and Bradley (1964). Whereas the streptomycete DNA preparations were rather homogeneous in %GC, significant diversity was found in both nocardial and mycobacterial DNA samples. A bimodal clustering effect was observed for values of %GC in these DNA preparations: the nocardia gave two distinct clusters, the 62 to 64 %GC group and the 68 to 70 %GC group. N. opaca N109 fell directly between these clusters. In agreement with Wayne and Gross (1968), the mycobacterial DNA preparations exhibited a low %GC group in the 64 to 66 %GC range and a high %GC group with values in the 67 to 70 %GC range. Accordingly, my limited study corroborates the observations of Jones and Sneath (1970) who suggested that the genus Streptomyces was a rather homogeneous group, while the genera Nocardia and Mycobacterium were quite heterogeneous.

It should be realized that of the routine methods used, only hydrolysis and chromatography and possibly the depurina-

tion method give direct estimates of base composition. The calculation of nucleotide composition from measurements of buoyant density in CsCl or Tm is predicated on an empirically established relationship with chemical data. The correspondence of values derived by the different techniques may differ because of the uncertainty of the formulae used in the interconversion of thermal transition and buoyant density data to chemical composition (Gasser and Mandel, 1968). The GC content of DNA can be useful for diagnostic and taxonomic purposes only when the determinations are truly comparative. It must be emphasized that the techniques, chemical supplies and equipment used in different laboratories and even in a given laboratory can differ enough to produce minor discrepancies in observed %GC results. Investigators must refrain from placing undue weight on differences in GC content determined in various laboratories by diverse methods (Mandel et al., 1970). Nevertheless, Jones and Sneath (1970) suggested that a difference in %GC of 5% implies a species difference, whereas a difference of less than 2% is not of taxonomic significance.

DNA Reassociation in Actinomycetes. If we assume that the evolutionary development of an organism is reflected in the sequence of bases in its DNA, then comparison of DNA sequences of organisms should give a complete phyletic evaluation of their present relatedness. Although this proposal is extremely provocative, the complete, direct sequence analysis of a DNA molecule is not currently possible. Fortunately, the complementary nature of the DNA double helix itself

can be used to circumvent these technical difficulties. By determining the extent of inter-specific DNA reassociation, the relative number of sequences held in common can be calculated. By measuring the thermal stability of these inter-specific DNA duplexes, the exactness of fit can be estimated. Using these two parameters, it is possible to assess quantitative relatedness (extent of reaction), and also to estimate the degree of evolutionary divergence (sequence mismatching).

At first, it seemed that "one number" would define the absolute relatedness of one organism to another. However, since the recognition that DNA samples from related organisms often contain identical sequences, a spectrum of partially matched sequences, and totally dissimilar sequences, the inadequacy of the "one number" concept became obvious (Brenner, 1970). Although somewhat of a dilemma at first, the analysis of this spectrum of sequence matching provided a basis for deducing relationships among actinomycetes.

The streptomycetes, a homogeneous group by total base composition, showed diversity in DNA reassociation studies. Significantly, all streptomycete DNA examined reassociated measurably with S. venezuelae S13 DNA. This underscores the total evolutionary relatedness of the streptomycetes. S. griseus S104 and S. venezuelae S86 have diverged only slightly compared to the reference S. venezuelae S13. S. fradiae S347, on the other hand, has diverged to the point where over 30% of its DNA does not react with the reference DNA.

Most of the genetic studies on streptomycetes have been done with cultures designated as S. coelicolor. The results

in this study confirm the observations of Monson et al. (1969) in that the cultures historically called S. coelicolor are virtually identical with the S. violaceoruber nominifer and are clearly distinct from the S. coelicolor nominifer. These studies also indicate that considerable divergence can occur within groups of closely related organisms.

One of the most significant facts to emerge from this study was that nocardial DNA exhibited significant reassociation with S. venezuelae S13 DNA; furthermore, thermal stability experiments suggested these inter-familial DNA duplexes were closely matched. It is tempting to suggest that these sequences represent ancestral remnants unchanged through the evolution from a common ancestor. An equally intriguing interpretation is that these sequences have been recently transferred in some type of genetic exchange. In any event, the nocardia examined in this study now contain DNA sequences very similar to sequences found in S. venezuelae S13. In contrast, the mycobacterial DNA samples studied showed no reaction with S. venezuelae S13. The conclusion reached was that mycobacteria have diverged to such an extent that they are not presently related to S. venezuelae S13.

The graphical determination of divergence patterns was extremely useful. It not only visualized the relationships of the test DNA samples to the reference DNA, but it also provided this data without resorting to time consuming thermal elution experiments. It is imperative, however, that the limitations placed upon (a+b) and (b) be observed in using this graphical approach. Misleading data and erroneous

interpretations arise when inappropriate "exacting" and "non-exacting" conditions are selected. Moreover, it must be emphasized that the "evolutionary divergence" measured is divergence from the reference DNA. For a survey to have evolutionary significance, many reference cultures must be studied including the type cultures of the species and genera involved.

Although it is experimentally possible to estimate the degree of divergence of DNA sequences with respect to a given reference DNA, two major factors are missing from the analysis. First, the genome size of organisms in question should be known for truly comparable studies. Second, a fixed reference point in evolutionary time for the divergence of a particular organism should be established. It is now possible to estimate genome size by renaturation kinetics. However, for the organisms used in this study no such data are available, although preliminary experiments with S. venezuelae S13 DNA suggest that the DNA contains no rapidly reassociating portions and follows second-order reassociation kinetics (p 113 of this thesis). At present, no reference point in evolutionary time for microbial divergence is available. In fact, it is not readily possible to distinguish recent gene transfer from conserved ancestral sequences. Jones and Sneath (1970) suggested that bacterial evolution could follow reticulate modes of change with numerous partial fusions of phyletic lines. This concept presents severe conceptual and practical problems for analysis of DNA reassociation data. On the other hand, Mandel (1969) suggested that

such reticulation of diverging lines was a minor problem due to the relative rarity of genetic interaction in nature. This issue still remains to be resolved. The influence or importance of the so-called neutral mutation theory of evolution on DNA reassociation data has not been thoroughly examined. If this concept is valid, then it is conceivable that phenetically similar organisms might possess substantial amounts of genomic diversity. In such instances, there would be a significant degree of base sequence mispairing during DNA reassociation. In practice, there is little specific reassociation of DNA between organisms of phenetic similarities below 50%. This could very well indicate that the neutral mutation concept is valid.

From a deterministic point of view, no formal guidelines exist with which to correlate reassociation data to taxonomic groupings. It does seem clear that when DNA from two organisms cannot reassociate, these organisms are not presently related. Brenner (1970) suggests that when the extent of interspecific DNA reassociation is virtually 100% and the thermal stability of the duplexes is identical to that of the reference reaction, the organisms in question are in the same species. In this regard, a unit of classification applicable to DNA reassociation data is the "genospecies" (Ravin, 1963). This concept involves a group of strains potentially able to contribute to or share in a common gene pool. Phenotypically, this results in a cluster of satellite strains around a central core. Probably even more useful in the present framework of taxonomy would be the recognition of the type culture concept in the

arbitrary definition of species. If this were done, then reassociation data using the type cultures as reference DNA preparations would establish meaningful and comparable relationships (Monson et al., 1969).

Ideally, microbial taxonomy should be based on phylogenetic relationships (Brenner et al., 1969a). Very little is presently known of the evolutionary relations among bacteria. However, the technical tools developed and presented in this thesis may make the approach to a natural classification feasible. Mandel (1969) predicted that the next few years will see the emergence of a new specialty, "Quantitative Systematics". Hopefully, the time is fast approaching when the relationships of microorganisms will be integrated with the main lines of organic evolution.

II. Characterization of DNA from Spores of *Streptomyces venezuelae* S13

Introduction

Searches for industrially useful fermentation products synthesized by streptomycetes and developmental studies on these fermentation processes have generally been focused on the vegatative stage of growth. Consequently, little is known about potentially useful substances made by, or present in, streptomycete spores. Because sporulation can often be correlated with the gain or loss of ability to make novel substances (Schaeffer, 1969), the search for useful fermentation products should be extended to include streptomycete spores. Furthermore, an analysis of secondary metabolism and sporulation may provide insight into the biochemical mechanisms regulating this seemingly simple differentiation process (Kornberg et al., 1968).

I based my search for novel spore products upon the observations of Tewfik and Bradley (1967) who noted that the DNA from Streptomyces venezuelae S13 spores possessed unique properties. Somewhat similar discrepancies between DNA preparations from spores and vegatative cells have been described for Bacillus subtilis (Halvorson et al., 1967).

Evidence is presented in this section that a substance unique to spores of S. venezuelae is strongly complexed with the spore DNA. A partial purification and characterization of the suspected substance is also described.

Review of the Literature

Endospore formation in eubacteria, conidiation in actinomycetes and lower fungi and ascosporogenesis in yeast are commonly designated sporulation. In spite of the conspicuous differences existing among them, they are all intracellular differentiation processes subdividing the cell by new membrane formation and they remain unexpressed as long as rapid growth is possible (Schaeffer, 1969).

This intracellular differentiation can often be correlated with gain or loss of the ability to produce novel substances such as antibiotics, exoenzymes, toxins and low molecular weight compounds. In 1945, Schatz and Waksman discovered that spontaneously arising asporogenous variants of S. griseus also lost the ability to produce streptomycin. This same phenomena was also found in S. lavendulae when streptothrycin production was studied (Waksman and Schatz, 1945). Schaeffer (1969) pointed out that these observations may not be due to the ability of the strains to conidiate, but may be due to a change in the branching habits of the growing mycelium.

Other metabolic changes often coincident with the onset of sporulation include changes in respiratory rate, appearance of tyrosinase and formation of carotenoids (Cochrane, 1963). Cochrane (1963) suggested that in Aspergillus flavus, the appearance of conidia was coincident with a marked fall in mycelial

nitrogen; moreover, during spore formation, mycelial phosphorus declined as much as 80%. Cantino and Horenstein (1955) reported that during morphogenesis in Blastocladiella emersonii, the formation of the resistant sporangium was accompanied by synthesis of chitin, melanin and β-carotene.

Melanins - or a class of insoluble black pigments - are usually assumed to be products of tyrosinase action (Cochrane, (1963). The pigment of S. scabies is probably of this class, although the correlation of pigment production with sporulation was tenuous (Cochrane, 1963). A similar melanin-like pigment was described by Mencher and Heim (1962) in S. lavendulae. In this regard, it is interesting that Gregory and Huang (1964) postulated that the gene for tyrosinase production in S. scabies was borne by a small plasmid.

The peptolides, sporidesmin and sporidesmolides, though not endowed with antibiotic activity, are extremely cytotoxic in very low concentration (Wright, 1968). These mycotoxins are produced by isolates of Pithomyces chartarum (Done et al., 1961). Sporidesmolides were not isolated from cultures which did not sporulate (Dingley et al., 1961); furthermore, these peptolides were located at the surface layers of the fungal spores (Bertaud et al., 1963). Lower fungi produce a number of toxins whose possible relation to sporulation has not been examined to date (Wright, 1968).

Bradley and Ritzi (1968) using electron microscopy, showed that the wall of spores from S. venezuelae was inlaid with many tiny rods extractable with xylene and alcohol. The rods were not present in vegetative mycelial walls.

Protease and nuclease production in actinomycetes was discussed by Cochrane (1961) and Waksman (1959) but no insight was given whether sporulation was involved or not. The extracellular wall-lytic enzymes (L-alanine amidases) of S. albus seem to be produced exclusively by the aerial mycelium when sporulation is initiated (Ghuysen et al., 1962).

Novel cytological and biochemical features of sporulation of bacilli and clostridia have been reviewed by Schaeffer (1969). In Bacillus species besides the well known dipicolinic acid and N-succinyl glutamic acid, other new low molecular weight compounds found only in sporulating cells have been described (Srinivasan, 1965).

Bonsen et al. (1969) found 3-L sulfolactic acid to be a major constituent of spores of B. subtilis. The compound was completely absent from vegetative cells during growth, but large amounts accumulated just before the development of refractile spores. The 3-L sulfolactic acid accounted for 5% of the dry spore weight. The compound was completely and rapidly released into the medium upon germination. It was not found in B. megaterium, B. cereus or B. thuringiensis.

Teichoic acids were found to be absent from the spores of B. licheniformis and B. subtilis (Chin et al., 1968). Apparently the spores of B. licheniformis lacked the enzymes responsible for teichoic acid synthesis. However, in B. subtilis, portions of the teichoic acid synthesizing machinery were present, yet in the spore no product was made.

Warth and Strominger (1969) working with the peptidoglycan of vegetative cells and spores of B. subtilis, showed

that the lactam of muramic acid, a sugar not previously found in nature, was a unique spore constituent. Moreover, the cross-linking was found to be 19% in spore peptidoglycan compared to 41% in vegetative peptidoglycan; D-alanine was the carboxy terminus of peptide substituents in spores while meso-diaminopimelic acid residues were found in vegetative cells; the meso-diaminopimelic acid residues in the vegetative cells were amidated but were not amidated in the spore. The authors proposed that the synthesis of the spore peptidoglycan is carried out by an entirely different set of enzymes than those used for synthesis of the peptidoglycan in vegetative cell walls.

Dipicolinic acid has not been found to date in any actinomycete spore with the exception of the unusual and atypical heat resistant "endospores" of two thermophilic actinomycetes described by Cross et al. (1968). These spores contained 3.6% dipicolinic acid on a percent dry weight basis.

DNA having a buoyant density in CsCl heavier than that from vegetative cells has been isolated from spores of B. cereus (Douthit and Halvorson, 1966) and B. subtilis (Halvorson et al., 1967). The heavy DNA also exhibited a higher melting point. The heavy DNA was double-stranded, contained no abnormal sugars or bases and had the same base composition as the vegetative DNA. It was unable to bring about genetic transformation and did not compete with vegetative DNA in genetic transformation. The heavy DNA formed during sporulation and disappeared upon germination

(Halvorson et al., 1967).

In an effort to explain this phenomenon, Szulmajster et al., (1969) studied the B. subtilis culture used by Halvorson. The authors found that a mutant (Clb-1) occurred spontaneously during the normal growth of the wild type culture. The mutant apparently excreted a substance which killed the wild type cells and allowed the slower growing Clb-1 mutant cells to overtake the culture. The DNA of Clb-1 had a buoyant density in CsCl of 1.714 g cm^{-3} compared to normal B. subtilis DNA of 1.703 g cm^{-3}. Both DNA preparations had an identical base composition of 42% by chemical analysis. To date, no explanation of the increased buoyant density of the mutant has been proposed, although W. Steinberg (personal communication) suggested that teichoic acids may be bound to the DNA.

Evans and Spizizen (1970) partially characterized spore DNA from B. subtilis 168, PR97 and BR43. They discovered two density classes of spores, one with an average density of 1.340 g cm^{-3} and one having a density of 1.280 g cm^{-3}. The spore fractions were physiologically distinct populations that differed from each other with respect to heat resistance and rate of germination. Interestingly, DNA from both spore populations had novel properties that readily distinguished them from vegetative cell DNA. The spore DNA samples from both spore populations had low affinities for methylated albumin kieselguhr (MAK) columns and exhibited an unusually high hyperchromicity upon denaturation. More important, however, the spore DNA samples had certain other characteristics

which set them apart from each other. DNA from heavy spores was active in transformation, had the same buoyant density in CsCl and had the same Tm as vegetative DNA. DNA from light spores was biologically inactive and had an increased buoyant density in CsCl and an increased Tm compared to vegetative DNA. No explanation was given for the aberrant DNA properties.

Preliminary investigations by Tewfik and Bradley (1967) have established that S. venezuelae S13 spore DNA displayed a buoyant density appreciably less than that of the mycelial DNA. Bednar and Frea (1967) sought, but did not find, any differences in the buoyant density of DNA from spores and vegetative mycelia of S. fradiae.

Interaction of Substances with DNA. A substance may have many modes of interaction with DNA involving a number of bonds. The reason for these varied interactions is the complex nature of solvated DNA. DNA in solution can manifest a) polyanionic character; b) hydrogen bonding capacities; c) hydrophobic bond capabilities; d) resonance and pi-interactions and e) dipole interactions (Felsenfeld and Miles, 1967; Ts'o, 1969). Moreover, DNA in solution can exist in many conformations. Thus in analyzing interaction of substances with DNA, the multi-faceted nature of DNA in solution must be remembered.

At physiological pH, DNA carries a high density of negative charge. For this reason DNA can associate with various species of cations, especially the alkaline earth

cations such as Mg^{+2} (Fishman et al., 1967). The most rigorous analysis of cation binding to DNA has been done by Shapiro et al. (1969). These authors used a novel method of competitive equilibrium dialysis to quantitate the binding. They concluded that Ca^{+2}, spermine $^{+4}$, basic amino acids and their derivatives, Na^{+1}, Li^{+1}, Cs^{+1} and K^{+1} were bound with equal affinity to DNA preparations of all base compositions. Polylysine, however, reacted preferentially with DNA richer in adenine and thymine (AT) pairs. This reaction was reversible and cooperative with one lysine binding per nucleotide. In 2 M tetramethyl ammonium chloride, this effect was reversed so that polylysine reacted selectively with DNA rich in guanine and cytosine (GC) pairs. Tetramethyl and tetraethylammonium ions were bound more tightly to AT rich DNA than to GC rich DNA. When the binding of the series mono-lysine to tetra-lysine was examined, all were bound equally tightly to all DNA samples regardless of base composition.

Fishman et al. (1967), Eichhorn et al. (1966) and Kit (1963) showed that the divalent cations Mg^{+2}, Ba^{+2}, Mn^{+2}, Co^{+2}, Ni^{+2} and Zn^{+2} increased the Tm of DNA whereas Cu^{+2}, Cd^{+2} and Pb^{+2} decreased the Tm. All of these cations affected the buoyant density of DNA in CsCl but usually were displaced by Cs^{+1}. The cations Hg^{+1} and Ag^{+1} didn't affect Tm but did increase the buoyant density in CsCl.

The concept of intercalation was introduced by Lerman (1961) to explain certain observations on the interaction of acridine dyes with DNA. He suggested that planar molecules

possessing fused ring systems might insert (intercalate) between adjacent base-pairs of the DNA double helix. As a result, the base-pairs above and below the molecule become separated by over twice their normal distance; an event that requires partial uncoiling of the helix. Lerman (1961) proposed that the energy for this process came from charge interactions. Ts'o (1969) suggested from entropic considerations, that hydrophobic bonding may play a role in the intercalation process.

Waring (1966) has reviewed the characteristic lines of evidence which indicate the intercalation phenomenon: a) viscosity showed a dramatic increase (3 to 4 times greater than normal DNA) due to physical lengthening and stiffening of the molecule; b) sedimentation coefficient (S) was lowered compared to free DNA due to a reduction in the mass per unit length of the DNA molecule; c) x-ray diffraction patterns showed a complete disappearance of the layer-line array indicative of double-helical DNA with retention only of the meridional spot corresponding to the 3.4 Å separation between base pairs; d) small angle x-ray scattering showed a diminished mass per unit length; f) fluorescence depolarization and flow dichroism studies showed that the DNA base-pairs relative to the helical axis remained unaffected by the presence of the intercalated molecules and that these molecules were in a plane perpendicular to the helical axis; g) the amino groups of the intercalated molecules were shielded from electrophilic attack by nitrous acid and h) the Tm showed an increase indicative of helix stabilization.

The original intercalation model proposed by Lerman (1961) suggested overlap of the acridine dye with both members of a base pair with the subsequent prediction that denatured DNA will bind little if any acridine. However, Blake and Peacocke (1968) have shown that there were as many binding sites for acridine in denatured DNA as for native DNA and that the binding constants were similar to those for the native duplex.

The compounds for which an intercalation mechanism has been proposed are many (Waring, 1968). Usually, the mechanism is chosen on the basis of one or, at most, a few of the major characteristics of intercalation. The following list is not inclusive, but it serves to show the variety of agents implicated in intercalation. Wagner (1969) suggested from circular dichroism data that lysergic acid diethylamide intercalated with calf thymus DNA. Wright (1968) reviewed the literature on mycotoxins and concluded that aflatoxins bind to DNA. Moreover, there appeared to be a differential affinity for DNA with the four classes of aflatoxin. Complex formation was observed between the rosaniline dye, crystal violet, and DNA or RNA purified from Escherichia coli (Adams, 1968). The evidence was based upon spectral shifts of the dye when DNA was added. The 5-methyl phenazinium cation was found to have two types of interaction with DNA (Ishizu et al., 1969). The interaction involving a small number of ligands with strong binding was characterized as intercalation, while the other interaction involving many ligands with weak binding was characterized as charge complexing.

Miracil D (Burroughs-Wellcome) and a series of its derivatives were tested to determine the nature of the complex formed with DNA (Hirschberg et al., 1968). The authors concluded that the proximal nitrogen on the Miracil D side chain interacts with a phosphate residue on the DNA backbone and the heterocyclic ring system intercalates with base residues in the DNA. Kersten and Kersten (1969) reviewed the inhibitors acting on DNA synthesis and reported that the following classes of antibiotics bound to DNA: acridines, anthracyclines, quinacrines, ethidium bromide, actinomycins, chromomycin, mithramycin and luteoskyrin. Stewart (1968) examined in detail the effect of intercalated acridine orange on the Tm of DNA. He noted that acridine orange broadened the thermal transition of DNA. The conclusion was that the dye dissociates from DNA when the DNA denatures; the broadening was the result of the transfer of acridine orange molecules from denatured to native DNA. The nogalamycin family of antibiotics was studied by Reusser and Bhuyan (1967), Bhuyan (1967) and Bhuyan and Smith (1965). The results were that nogalamycin bound to A, T or both in DNA; however, the intercalation mechanism was not ruled out. Kersten et al. (1966) studied the properties of complexes between DNA and antibiotics which affect RNA synthesis. They concluded that anthracyclines like cinerubin, daunomycin and nogalamycin behave like acridine dyes giving the typical intercalation symptoms. However, unlike acridines they were persistently bound at high ionic strength. The authors observed binding to DNA but without such intercalation

characteristics for actinomycins C and D and for the chromomycin-like antibiotics (chromomycin A_3, olivomycin and mithramycin). These compounds all caused a decrease in buoyant density (CsCl) but, with the exception of the actinomycins, had no effect on the Tm. Muller and Crothers (1968) provided extensive data showing that the actinomycin chromophore was intercalated between the base pairs in the DNA complex. Moreover, binding was shown to occur adjacent to any GC pair. However, binding at a given site produced a distortion of the helix that greatly disfavored binding of another actinomycin closer than six pairs away. The authors pointed out that several forms of the complex existed at equilibrium due to conformational changes within the cyclic peptide rings of actinomycin. It was suggested that each peptide ring interacted with one strand of the double helix. Wells (1969) concluded that the presence of guanine moieties in a DNA was not, in itself, a sufficient requisite to cause binding of actinomycin D. He suggested that the DNA structural considerations may be of paramount importance. The presence of guanine may induce a suitable configuration to permit the binding of actinomycin, hence explaining the observed dependence of actinomycin binding on guanine.

Yielding (1967), O'Brien et al. (1966) and Kurnick and Radcliffe (1962) examined the reaction between DNA and the antimalarial drugs quinacrine (Atabrine), chloroquine (Aralen), quinine and 4-aminoquinoline. The conclusions reached were that these compounds all have properties suggesting intercalation with DNA. Charge-complexing was also

strongly implicated as an equally important parameter in the ability of this group of drugs to bind to DNA. Interestingly, of the antimalarials tested, the presence of the diamino-butane side chain greatly increased the tenacity of binding.

Steroidal diamines (primarily of the irehdiamine A group) were also suspected of intercalating with DNA (Mahler et al., 1966). The authors suggested that a phosphate bridging, charge-neutralizing interaction was the primary event, but that intercalation could be a secondary process. Diquaternary amines were most effective as stabilizing agents while mono-amines were ineffective.

Interestingly, neutral hydrocarbons including pyrene, 3,4-benzpyrene, dibenzanthracene, coronene, tetracene, pentacene and 20-methylcholanthrene have been shown to bind to DNA (Isenberg and Baird, 1969; Lesko et al., 1968). Pyrene, 3,4 benzpyrene and dibenzathracene bound to DNA by an intercalation mechanism whereas the others bound by an unknown process. Ts'o (1969) pointed out that in view of the hydrophobic stacking properties of bases in DNA, it is not surprising that many hydrophobic compounds of great biological importance, such as steroids and the polycyclic carcinogens, interact strongly with nucleic acids.

Antibiotics which bind covalently to DNA constitute an interesting class of compounds. Most of these substances have been classified as alkylating agents. Mitomycin is generally taken as the representative and most studied example (Kersten and Kersten, 1969). Contrary to the review of Waring (1966), Kersten and Kersten, (1969) presented evidence

that the inhibitory effect on DNA synthesis by mitomycin was not caused by cross-linking of the DNA. They suggested that the quinone ring of mitomycin plays an important role in the inhibitory effect on DNA synthesis. These authors did not discard, however, the evidence that mitomycin does produce cross-links. Their point was that derivatives of mitomycin, lacking the aziridine (the alkylating portion) ring, were still active inhibitors of DNA synthesis. Other antibiotics implicated as alkylating agents include phleomycin, carcinophylin, streptonigrin, hydroxyurea and nalidixic acid (Kersten and Kersten, 1969).

It is fair to say that DNA has not yet been isolated entirely free of amino acids despite rigorous efforts at their removal (Bendich and Rozenkranz, 1963). To date, it is not clear whether these amino acid residues are present as such in covalent linkage or as covalently attached peptides or small proteins. Bendich and Rosenkranz (1963) reported that some eleven amino acids were obtained upon hot 6N HCl hydrolysis of bull sperm DNA. These amounted to about 0.1% of the mass of DNA with one-third of the amino acids being serine and threonine. It was interesting to note that these authors found that serine could be obtained as O-phosphoserine. In this regard, Massie and Zimm (1965) suggested that the genophore of B. subtilis may consist of subunits having a molecular weight of 250 x 10^6 held together by serine, threonine or small peptide linkers. Salser and Balis (1969) found that amino acids were extremely tightly bound to E. coli B and K-12 DNA. Moreover, the amount was a function

of growth conditions; it varied with changes in the composition of the media and with phases of the growth cycle. Interestingly, under conditionsof protein labeling and inhibition of DNA synthesis, the cell protein was highly labeled but the DNA associated amino acids were not. Attempts to remove the amino acids with CsCl banding, gel filtration in 5 M urea, 2 N HCl or 0.5 N $HClO_4$ were not successful. Significantly, serine was obtained as O-phosphoserine. The authors pointed out that no abnormal amounts of basic amino acids were found.

As reviewed by Yarus (1969) a surprising number of proteins could bind specifically to DNA thus apparently recognizing nucleotide sequences. Among the most studied proteins were: DNA polymerase, restriction and modification enzymes, repair enzymes, ligases, repressors, RNA polymerase, initiators, membrane attachment sites, and antibodies. Bhagavan and Atchley (1965) isolated a DNA-protein complex from B. subtilis by precipitation with 0.01 M $MgCl_2$. The complex was more soluble at higher and lower $MgCl_2$ concentrations. Butler and Godson (1963) working with B. megaterium also used a medium containing 0.01 M $MgCl_2$ in preparing the DNA-protein complex. Alberts et al. (1968) isolated a number of DNA-binding proteins from E. coli. Unexpectedly, the vast majority of these proteins were negatively charged indicating a binding mechanism more complex than charge interaction.

Jacob, Brenner and Cuzin (1953) proposed that the contact of DNA with the cell membrane was important in the replication of bacterial DNA. Goldstein and Brown (1961)

found evidence for the preferential location of replicating DNA in contact with large aggregates of material in lysed cells of E. coli. Ganesan and Lederberg (1965) later showed that a large amount of the replicating DNA of B. subtilis was linked to cell membranes, an association which could be reduced by pronase treatment. Davern (1966) has shown that pronase released DNA from lighter fractions during CsCl density gradient sedimentation of E. coli spheroplasts lysed by detergent. By the action of sonication, pronase and ribonuclease treatment of detergent lysed cells, Porter and Fraser (1968) concluded that protein was active in the binding of membrane components to DNA. Tremblay et al. (1969) have devised a simple method for isolation of a cell membrane-DNA complex from bacteria. The method exploited the ability of portions of the cell membrane to which DNA was attached to adhere to the hydrophobic surfaces of crystals. The crystals were formed using Mg^{+2} and the detergent sarkosyl. Separation was accomplished by slow speed sucrose gradient centrifugation.

Young and Jackson (1966) examined the extent and significance of contamination of DNA by teichoic acid in B. subtilis. The authors discovered that teichoic acids were solubilized by lysozyme. The data suggested that teichoic acids were not completely removed from DNA by precipitation with ethanol, phenol extraction or by density gradient centrifugation. Significantly, teichoic acids did not affect buoyant density of the DNA. The teichoic acids could be separated from DNA by 4% agarose or methylated

albumin kieselguhr columns. Moreover, unlike DNA, teichoic acids were soluble in and removed by cold perchloric acid.

Materials and Methods

Isolation of Streptomycete Spores. Streptomycete cultures were propagated on tomato paste oatmeal agar. The cultures were incubated at 30 C for 4 to 12 days. The aerial mycelia were scraped from well sporulating growth, suspended in distilled water and immediately lyophilized. Lyophilized spores were stored in sterile plastic bags at -20 C. Some spore preparations were not lyophilized but were stored at 4 C in 95% ethanol for varying periods of time (overnight to 4 weeks). The usual procedure was to harvest these alcohol soaked spores by filtration or centrifugation and to wash them in 4 volumes of anhydrous acetone. The acetone was removed by air drying.

Rupture of Streptomycete Spores. Streptomycete spores were quite resistant to lysis by lysozyme, pronase and sodium dodecyl sulfate. Consequently, an alternative form of rupture was developed. Equal amounts of lyophilized spores and acid-washed 20 μm glass beads (3 M Co., Minneapolis, Minn.) were mixed in a pre-cooled mortar and ground manually (generally from 5 to 10 min). Pulverized dry ice was added during grinding to keep the mixture dry and cold. The grinding process was quite critical to the extraction of DNA; too much grinding sheared the DNA while not enough grinding did not rupture the spores. The ground spores were suspended in

10 ml saline-EDTA and the suspension was treated with 2 mg pronase followed by incubation at 42 C for 1 to 2 hr. The spore suspension was then transferred to a ground-glass stoppered flask and 4 ml 25% SDS was added followed by incubation at 60 C for 15 min with occasional shaking. After the incubation period, the flask was cooled slowly to ca. 25 C and DNA was isolated as described earlier.

Broth cultures of the streptomycetes were also grown and the mycelia harvested and washed as previously described for mass culture of streptomycetes. This material was lyophilized and lysed in the same manner as the spores; DNA was then isolated as described earlier. This DNA was used as a control when analyzing the corresponding spore DNA.

Buoyant Density Determinations. The procedure for determination of buoyant density in CsCl has been described earlier (p 30 of this thesis). For some experiments spore DNA was denatured in 0.1 x SSC by boiling for 5 min followed by quick-cooling in an ice-water bath. The sample was then immediately prepared for centrifugation.

Preparative Density Gradient Centrifugation of DNA in Cesium Chloride. The procedure was carried out in the SW50L rotor of the Spinco L2-65B preparative ultracentrifuge. A 4 ml amount of CsCl stock solution of density 1.799 g cm^{-3} (pH 8.5) was added to cellulose nitrate tubes containing 0.2 ml of the test DNA solution. Included with the test DNA solution was 1 µg of ^{14}C-labeled S. coelicolor Muller DNA for a reference. The CsCl solution was then overlaid with

mineral oil to within 1/8 inch of the top of the 5 ml tube. The samples were centrifuged for 40 hr at 40,000 rev min^{-1} at 25 C. The rotor was brought to an unbraked stop and the tubes carefully removed. The bottom of the tubes was punctured and 10 drop fractions were collected in 1.0 ml water. Each fraction was assayed for radioactivity and for absorbance at 260 nm.

Thermal Denaturation of DNA. This procedure has been described previously. (p 33 of this thesis).

Determination of DNA Base Composition by Depurination. The nucleotide composition of selected DNA samples was determined by spectrophotometrically assaying for free purines released by acid-induced depurination according to the method of Huang and Rosenberg (1966). DNA solutions were precipitated with 95% ethanol and collected by spooling on a glass rod. Enough precipitated DNA was dissolved in 2 ml of 1 x SSC to give a concentration of 200 to 300 µg DNA/ml. Visking dialysis tubing (¼ in. by 10 in.) was boiled twice for 10 min in saline-EDTA, rinsed and boiled a third time for 10 min in deionized water and soaked in 1 x SSC overnight to remove any ultraviolet absorbing substances. One-half ml of the DNA dissolved in 1 x SSC was carefully placed in the pre-washed dialysis tubing. Special care was taken not to allow any DNA to contaminate the outside of the tubing. The tubings were then looped into individual acid-washed test tubes (13 x 100 mm) containing 5.0 ml 1 x SSC with the pH adjusted to 1.58 with HCl. The ends of the tubing were secured with a rubber

band and the test tubes were covered with aluminum caps. After gentle rolling action for 24 hr at 37 C, the absorbance of the dialysates was determined at 265 nm and 280 nm in a Zeiss PMQII spectrophotometer. A sample containing 1 x SSC but no DNA at pH 1.58 was used as the blank.

The mole fraction of G+C can be expressed as a function of the absorbance ratio at two given wavelengths. Huang and Rosenberg (1966) provided the following formula:

(1) $$R = \frac{(1-X_G)a + X_G c}{(1-X_G)b + X_G d}$$

where $R = A265/A280$

X_G = mole fraction guanine

a = molar absorptivity of adenine at 265 nm

b = molar absorptivity of adenine at 280 nm

c = molar absorptivity of guanine at 265 nm

d = molar absorptivity of guanine at 280 nm

Solving for X_G from equation (1)

(2) $$X_G = \frac{a - (bR)}{(a-c) + (d-b)R}$$

The values assigned to a,b,c and d at a pH of 1.58 were determined by Huang and Rosenberg (1966). Equation (2) then becomes:

$$X_G = \frac{13.1 - (5.0R)}{5.8 + 1.9R}$$

It is a simple matter then to solve for the mole fraction guanine, which is taken to be equal to the mole fraction cytosine.

Reassociation Between Spore and Mycelial DNA Preparations. Reassociation was assessed by measuring the extent of *in vitro* duplex formation. *S. venezuelae* S13 mycelial DNA was labeled with uracil-2-^{14}C as described previously on p 34 of this thesis. The specific activity was between 3000 count $min^{-1}\mu g^{-1}$ and 7000 count $min^{-1}\mu g^{-1}$ depending on the preparation. The membrane filter technique of Warnaar and Cohen (1966) as modified by Monson et al. (1969) was used. Thermal elution of spore-mycelial DNA duplexes was done as described earlier on p 37 of this thesis.

Kinetics of Reassociation of Spore and Mycelial DNA Duplexes in Free Solution. DNA samples at a concentration of 40 to 70 μg/ml were denatured in 5 M $NaClO_4$ in quartz cuvettes placed in a Beckman DU spectrophotometer equipped with a Gilford multisample absorbance recorder, linear thermosensor and a Haake circulating water bath. Denaturation was accomplished by increasing the temperature of the cuvette chamber until no change in A260 was observed. After denaturation was judged to be complete, the temperature was rapidly lowered to 52 C (25 C lower than the Tm in 5 M $NaClO_4$) by circulating -5 C ethylene glycol through the cuvette chamber. The temperature dropped to 60 C within 5 min. Ethylene glycol-water (70:30) at 52 C was added to the circulator and the temperature quickly stabilized at 52 C by 10 min after denaturation. During the cooling procedure and for the next hour, absorbance readings at 260 nm were automatically taken at 2 min intervals. Reassociation was subsequently monitored by A260 readings every 40 min. The pro-

portion of the DNA which had renatured at any given Cot (concentration of nucleotides in moles per liter, times duration of incubation time in seconds) value was determined by relating the decrease in absorbance at 260 nm to the maximal decrease possible (Wetmur and Davidson, 1968):

$$\% \text{ Reassociation} = \frac{A_f - A}{A_f - A_i} \times 100\%$$

where A_i = A260 of native DNA at 25 C

A_f = A260 of denatured DNA

A = A260 of reassociating DNA at a given time

Cot values were determined by the following formula:

Cot = A260/2 x time of incubation in hr

The value of 0.024 for the A260 of 1 μg of DNA was used to calculate relative concentrations (Brenner, 1970)

Spectral Analysis of DNA. The spectra of certain unusual DNA samples were analyzed in the ultraviolet (UV) and visible region using a Beckman DK-2A ratio recording spectrophotometer or a Beckman DB spectrophotometer equipped with a Sargent recorder. For analysis in the UV region, the DNA samples were diluted to 20 μg/ml, whereas in the visible region DNA samples were not diluted (500 μg DNA/ml or greater). Matched quartz cuvettes were used for all measurements. The blank consisted of an SSC solution of the same concentration as the DNA samples. If the samples to be analyzed were in SSC of a pH different from the blank, then the blank was adjusted to that pH by adding an equal amount of acid or base (1 N HCl or 1 N NaOH). David Peterson (Dept. Microbiol., Univ. Minnesota, Minneapolis) assisted in calibrating the

DK-2A and in interpretation of the spectra.

Sephadex Column Chromatography. Certain DNA samples were analyzed by Sephadex G-100 chromatography. G-100 Sephadex (Pharmacia, Piscataway, N.J.) was hydrated in 1 x SSC, packed in a 1.5 x 30 cm column that was then equilibrated for 30 hr with 5 x SSC. DNA in 1 x SSC was heated in a boiling water bath for 10 min and 0.5 ml was applied to the column. The column was washed with 5 x SSC at room temperature (ca. 25 C). Fractions of 1.0 ml were collected at a flow rate of 10 ml/hr. The absorbance at 260 nm was monitored using an ISCO flow cell and U2-A recorder (Instrumentation Specialties Co., Lincoln, Nebr.). The void volume of the column was determined for 0.5 ml of 0.2% Blue Dextran (Pharmacia, Piscataway, N.J.) which was used as an excluded reference.

For analysis of certain streptomycete spore products, Sephadex G-75 and G-25 were each hydrated in distilled water and packed in 1.5 x 30 cm columns. The void volume was determined using 0.2% Blue Dextran as an excluded reference. Distilled water was routinely used as the elutant. Columns were monitored using the ISCO flow cell and U2-A recorder as previously described. The flow rate for G-75 colums was maintained at 25 to 30 ml/hr by a 20 cm column of water; for G-25 columns the flow rate was 50 ml/hr maintained by a 20 cm column of water.

Column Adsorption Chromatography. Column adsorption chromatography was used to isolate pigmented products from

streptomycete spores. The adsorbent used was silicic acid (SiliCar, 100-200 mesh; Mallinckrodt Chemical Works, St. Louis, Mo.). A 2 x 40 cm glass column with a fritted glass disc to hold the adsorbent was used. The column was wet-packed using a slurry of hexane and the adsorbent. The column height was generally 10 to 15 cm. The top of the column was covered with a 1 cm layer of 20 μm glass beads to prevent disruption of the adsorbent surface. The spore pigments in 10 ml butanol were layered on the column. When the sample entered the column bed, 25 ml hexane was added. Elution with hexane continued until the hexane eluate was clear and dried leaving no residue. Next, the column was eluted with butanol until the eluate was clear, Finally, the majority of pigment was recovered when the column was eluted with a 0.1% solution of HCl in methanol (acidified methanol).

Paper and Instant Thin Layer Chromatography. One dimensional (descending) paper chromatography was done on Whatman No. 1 paper. The solvent system used was n-butanol, acetic acid, water (4:3:1). Samples were dried onto the paper under a stream of warm air from a hair dryer. The paper was placed in a chamber equilibrated with the proper solvent system. Most runs were complete by 10 hr with a solvent advance of about 30 cm. Completed chromatograms were placed in a fume hood to dry. When dry, the chromatogram was first examined under long wave UV (320 nm) and short wave UV (253 nm) using respectively the Blacklight and Mineralight (Ultraviolet Products, Inc., San Gabriel, Calif.). The

following reagents were subsequently used to locate and characterize the substances separated: ninhydrin spray (Gelman Products, Ann Arbor, Mich.), Gelman aniline phthalate spray, and an alkaline silver nitrate dip.

Instant Thin Layer Chromatography (ITLC) was done using a Gelman ITLC chamber and Gelman ITLC paper (either silica gel SG or silicic acid SA). Solvent systems employed were chloroform, methanol, 58% ammonia (2:2:1); n-butanol, acetic acid, water (4:3:1); hexane, methanol (1:1); ethyl acetate, 0.1% HCl in methanol (1:1). Samples were applied with a 10 μliter capillary pipette and dried under a stream of warm air. The chamber was prepared and equilibrated as described in the Gelman ITLC manual. Runs were usually complete within 90 min with a solvent advance of 15 to 20 cm. Completed chromatograms were removed and dried in a fume hood. The chromatograms were analyzed in the same manner as the paper chromatograms with the exception that no alkaline silver nitrate dip was used in ITLC experiments.

Standard solutions of authentic amino acids were prepared by dissolving 2 mg of each of the 21 common amino acids in 2 ml of 50% isopropanol pH 2.5 with HCl. The purified amino acids were purchased as a complete kit from Calbiochem. Ethanolic solutions (1 mg/ml) of glucose, glycerol, arabinose, D-galactose, D-mannose, N-acetyl-D-galactosamine, D-galactosamine, D-glucosamine, ribose, ribitol, DL -α,ε-diaminopimelic acid and ethidium bromide were also prepared. Solutions of 5-methyl cytosine, uracil, thymine, adenine, adenosine, cytosine, guanine and thymidine were prepared by mixing 2 mg

of the desired compound in 2 ml 10% isopropanol containing 1% concentrated HCl. The polyamines spermine, spermidine and putrescine were prepared as 1 mg/ml solutions in 50% ethanol containing 1% concentrated HCl.

Mr. Miles Sharpley (Dept. Biology, Virginia Commonwealth Univ., Richmond) provided a mixture of the four mycotoxins produced by Aspergillus flavus grown on autoclaved rice grains. The mycotoxins were dissolved in chloroform and stored at 4 C.

Paper Electrophoresis. Six strips of Whatman 3 MM, 2 cm x 20 cm filter paper was secured to the rack of an electrophoretic cell (Buchler Instruments) and wetted with about 4 ml of barbital-sodium barbital buffer, pH 8.6 (ionic strength = 0.075; commercial buffer, Buchler Instruments). Next 1 liter of the buffer was added to fill each well to the proper level. After equilibrating for 2 hr with the current on and cover closed, the strips were spotted with 10 µliter samples. A current of 3 ma cm^{-2} at a potential of 100 volts was applied for 3 hr. The strips were air dried and examined under short and long wave UV light as described for paper chromatography.

Chemical Assays. Colorimetric determinations of protein content were performed with the Folin phenol reagent as described by Lowry et al. (1951) with bovine serum albumin as the standard. DNA was assayed colorimetrically with the diphenylamine reagent according to the method of Burton (1956). Total phosphate was determined by the method of Bartlett (1959) using KH_2PO_4 as the standard.

Enzyme Assays. Sensitivity to deoxyribonuclease I (DNase I; Calbiochem) of selected DNA preparations was monitored with a Zeiss PMQII spectrophotometer. DNA was dissolved in 0.5 M sodium acetate, 0.01 M $MgSO_4$ buffer (acetate-Mg buffer) pH 6.0. DNase I (bovine pancrease) was made up to 1 mg/ml in acetate-Mg buffer. Finally, 100 µg of enzyme was added to 2.0 ml DNA solution and the increase in A260 with time was measured. The temperature was maintained at 30 C.

Crude Pigment Isolation From Streptomycete Spores. Pigmented products were isolated from whole cell preparations of spores or mycelia by extracting them first with 100 ml of 95% ethanol and then with 100 ml acetone. The solvents were collected by filtration and subsequently evaporated to dryness. Alternatively, the yellow chloroform layer from the Marmur DNA extraction procedure was used as a source of crude pigments. A third isolation method which proved most successful involved washing the whole spores with 0.1 N KOH or NH_4OH with subsequent extraction of the KOH wash with n-butanol. The butanol layer contained the pigmented products.

Results

Aberrant Spore DNA and Sporogenesis. Tewfik and Bradley (1967) reported that DNA extracted from the spores of S. venezuelae S13 exhibited a significantly lighter buoyant density in CsCl than the DNA extracted from the mycelia. In early attempts to verify this observation, DNA was isolated from a number of spore preparations. The yields of DNA for these early studies were quite low (often less than 200 μg DNA/ml) because not enough spores were available for efficient extraction. Several pre-treatments of spores including lyophilization, washing with ethanol or washing with ethanol and acetone were used in an attempt to increase the efficiency of spore rupture. Significantly, depending on the pre-treatment of the spores, buoyant density values were obtained ranging between 1.7209 g cm^{-3} to 1.7300 g cm^{-3}. Moreover, there seemed to be a decrease in buoyant density with the age of the spore. To test this hypothesis, DNA isolated from spores of varying chronological age was compared to DNA obtained from mycelia using the following parameters: buoyant density in CsCl, direct reassociation of spore DNA with mycelial DNA and thermal denaturation in 0.1 x SSC and 5 M $NaClO_4$. Interestingly, the DNA of the 6, 8 and 10 day old spore samples was distinctly yellow in color. The DNA from 3 day old spores and from 10 day solvent extracted spores was colorless as was mycelial DNA. Microscopic examination of spore samples stained by crystal violet before DNA extraction

Table 5

Effect of Spore Age on the Physical Properties of Isolated DNA

Sample	CsCl ($g\ cm^{-3}$)	Tm		Binding to Mycelial DNA
		0.1 x SSC	5 M $NaClO_4$	
Mycelial DNA	1.7275	85 C	89 C	100%
Spore DNA				
3 days	1.7275	-[a]	-	100%
6 days	1.7215	87 C	89 C	72%
8 days	1.7072	88 C	89 C	30%
10 days	1.7082	88 C	89 C	60%
10 days[b] (solvent)	1.7272	85 C	89 C	100%
8 days[c] (denatured)	1.7222	-	-	-

[a] -: not done

[b] 10 day old spores were washed with ethanol and acetone prior to DNA extraction

[c] The 8 day old spore DNA preparation was denatured in 0.1 X SSC

extraction showed that the 3 day old aerial mycelium consisted mainly of aerial hyphae with no visible spores. However, at 6, 8 and 10 days few, if any, aerial hyphae were seen; the preparation was almost entirely free spores. As the spores aged, their DNA became progressively less dense (Table 5). Significantly, DNA from spores washed with ethanol and acetone prior to DNA extraction behaved like mycelial DNA. When the 8-day spore DNA was heat denatured, it formed a symmetrical band in the CsCl gradient and gave a 15 mg cm^{-3} increase in buoyant density indicating that the DNA was double-stranded. In 0.1 x SSC, the Tm of spore DNA increased as the spores aged. It should be noted that for the 8 and 10 day old spore sample, the Tm of the DNA was probably higher than 88 C because the spore DNA had not completely denatured at 100 C. The Tm of DNA from 10-day old spores that had been washed in ethanol and acetone prior to DNA extraction was virtually identical to the Tm of mycelial DNA. When the Tm determinations were done in 5 M sodium perchlorate, a different result was obtained. Both spore and mycelial DNA samples gave the same Tm values. This was a possible indication that something was removed or altered by the sodium perchlorate and heat.

The capacity of mycelial DNA to reassociate with mycelial or spore DNA on membrane filters was measured by direct reassociation assays. These results established that the mycelial reference DNA bound less effectively to spore DNA than to the homologous mycelial DNA. Moreover, this reduced binding capacity continued to decline with age of the spore.

It should be noted that the 10-day old spore sample washed with ethanol and acetone prior to DNA extraction showed 100% binding to mycelial DNA.

Chemical Determination of Base Composition of Spore DNA. To examine the possibility that the overall nucleotide composition of the spore DNA had changed, the base composition of spore DNA was determined by acid-induced depurination (Table 6). The values for E. coli and B. cereus DNA were consistent with literature values. The most obvious result was that there was no discernible difference between mycelial DNA and the aberrant spore DNA samples. The aberrant nature of spore DNA was apparently not due to a change in its nucleotide composition. Moreover, no novel material was detected in the dialysate during the prolonged dialysis of the spore DNA against 1 x SSC (pH 1.58).

Thermal Stability of Reassociated Spore-Mycelial DNA Duplexes. Previous experiments suggested that labeled mycelial DNA reassociated less effectively with spore DNA than with the homologous mycelial DNA. The thermal stability of the duplexes formed between spore and mycelial DNA was also examined. Spores grown for 13 days (240 plates) were harvested and lyophilized as described previously (p 92 of this thesis). The spores were divided in two portions and DNA was extracted from each portion. The spore DNA so obtained was labeled 13 day #1 and 13 day #2. Mycelial DNA was obtained in a similar manner. The chemical and physical characterization of these DNA samples will be described in the

Table 6

Chemical Analysis of the Nucleotide Composition of *S. venezuelae* S13 Spore DNA

DNA Sample	$\frac{A265^a}{A280}$	Mole Fraction Guanine Plus Cytosine
Escherichia coli	1.707	0.51
	1.691	0.51
Bacillus cereus	1.908	0.38
	1.931	0.36
Mycelial DNA	1.411	0.71
	1.401	0.72
	1.415	0.71
	1.426	0.70
8-day spore DNA	1.409	0.71
	1.411	0.71
	1.421	0.705
	1.409	0.71
10-day spore DNA	1.410	0.71
	1.425	0.70

[a] Absorbance of dialysate at 265 nm divided by dialysate absorbance at 280 nm

following sections. Suffice it to say that both spore DNA preparations were yellow in color and clearly aberrant. The results shown in Fig. 11 indicated that of the duplexes formed between both spore DNA samples and mycelial DNA (about 65% relative reassociation for both) the majority were of lower stability. In fact, few high stability complexes were formed. The results shown are the average of two trials. Although the general trend of duplex stability was the same, the thermal stability seemed to vary unpredictably with duplicate samples of spore DNA. The standard error for spore DNA samples was 8% while for mycelial DNA samples, the standard error was 3%.

Renaturation Kinetics of Spore and Mycelial DNA. The renaturation of denatured DNA can be considered to be a second-order reaction with a maximum rate at a temperature ca. 25 C below the Tm of the DNA. Because spore DNA gave not only low total reassociation with mycelial DNA but also formed duplexes of low stability, it was of interest to examine the kinetics of reassociation. Experiments of this sort reported in the literature are usually done in a solvent of 1 x SSC or 0.12 M sodium phosphate buffer. Unfortunately, the high Tm of mycelial DNA and even higher Tm of spore DNA in these buffers presented a serious problem. Even if total strand separation could have been effected, the incubation temperature would have had to be between 75 C and 85 C to reach the optimum rate of renaturation. Initial experiments were attempted in 1 x SSC and 0.1 x SSC; however, these experiments were not successful. Evaporation, degradation

Fig. 11 Thermal stability of DNA duplexes formed between S. venezuelae S13 mycelial DNA and spore DNA. The DNA duplexes were formed at 70 C. The specific activity of the input labeled DNA was 3000 count $min^{-1}\mu g^{-1}$. The absolute homologous binding was 12%. The top graph is the integral form of elution or cumulative percent released versus the temperature of elution. The Tm,e is read directly from this plot by determining the temperature at which 50% of the DNA duplexes are dissociated.

○—○ : S. venezuelae S13 mycelial DNA (labeled)

●—● : S. venezuelae S13 spore 13 day #1

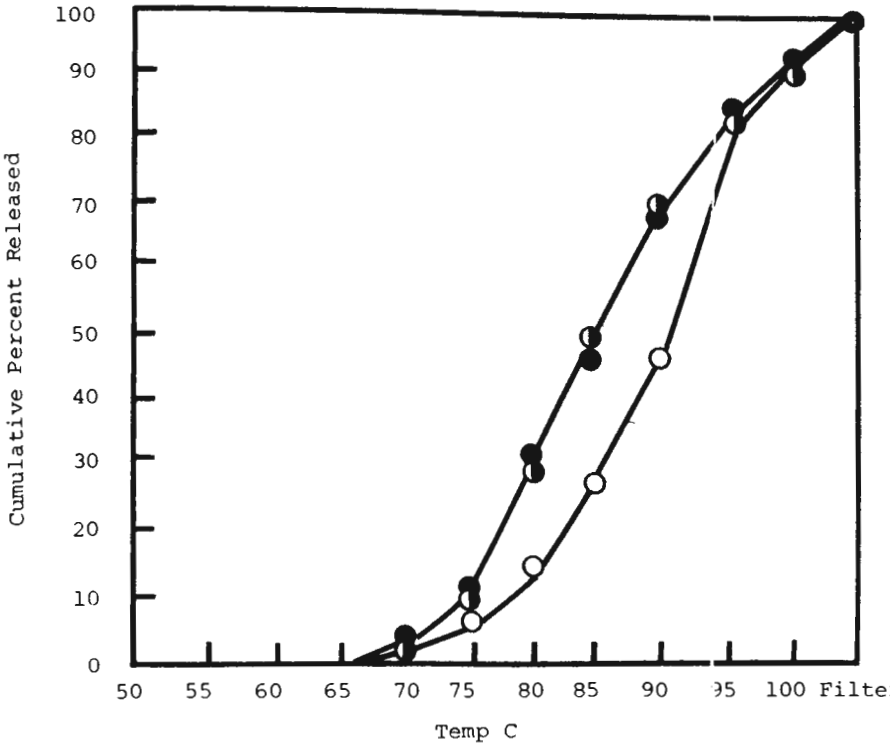
◑—◑ : S. venezuelae S13 spore 13 day #2

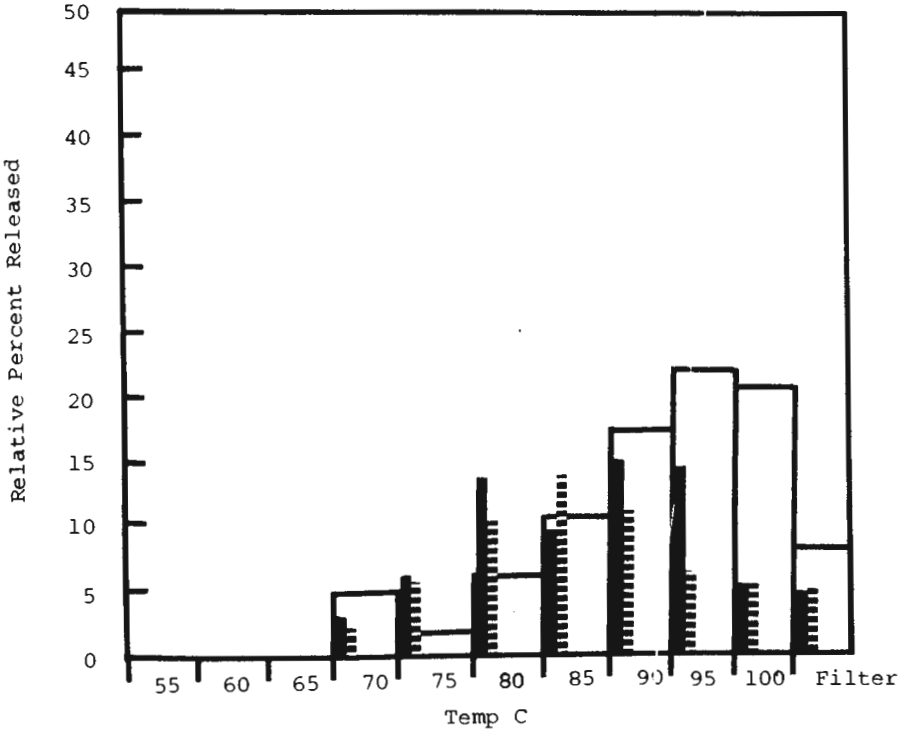
The bottom graph is the differential form of elution or the relative percent released versus temperature of elution. The conditions are the same as stated above.

▭ : S. venezuelae S13 mycelial DNA (labeled)

◧ : S. venezuelae S13 spore 13 day #1

▯ : S. venezuelae S13 spore 13 day #2


Cumulative Percent Released
100
90
80
70
60
50
40
30
20
10
0
50 55 60 65 70 75 80 85 90 95 100 Filter
Temp C


Relative Percent Released
50
45
40
35
30
25
20
15
10
5
0
55 60 65 70 75 80 85 90 95 100 Filter
Temp C

of DNA and spectrophotometer sensitivity to high temperatures were some of the problems encountered. Another solvent system (5 M $NaClO_4$) proved useful. The perchlorate anion was known to be a denaturant of DNA (Kit, 1963); moreover the rate of DNA reassociation was practically independent of salt concentration over 1 M in Na^{+1} (Wetmur and Davidson, 1968). Furthermore, both spore and mycelial DNA melted at the same temperature in 5 M sodium perchlorate. By lowering the concentration of SSC to 0.05 x SSC in 5 M $NaClO_4$, a Tm of about 77 C was reached for both spore and mycelial DNA. This meant that complete strand separation of both DNA preparations was possible and more important, renaturation could be carried out at the relatively low temperature of 52 C (Tm - 25 C). With this system it was possible to examine the rate of renaturation of spore and mycelial DNA.

The raw denaturation and reassociation data for spore 13 day #1 and mycelial DNA are shown in Fig. 12. The data for spore 13 day #2 and mycelial DNA are shown in Fig. 13 The average of both reassociation experiments is plotted semilogarithmically (% reassociation versus log Cot) in Fig. 14 The Tm of spore 13 day #1 and spore 13 day #2 was 77 C; the same Tm was observed for mycelial DNA. Unlike denaturation in 0.1 x SSC, spore DNA and mycelial DNA melted sharply with the same width of transition in 5 M $NaClO_4$.

The sigmoid shape of the Cot plot (Fig. 14) and the slope of the linear portion suggested that the rate of renaturation of mycelial DNA followed second-order kinetics. On the other hand, the Cot plot established that spore DNA

Fig. 12 Denaturation and renaturation of S. venezuelae S13 mycelial and spore DNA in 5 M $NaClO_4$ at an incubation temperature of 52 C. Note change of scale from temperature to time and back to temperature on abscissa. All measurements taken at a wavelength of 260 nm.

●——● : S. venezuelae S13 mycelial DNA

●·····● : S. venezuelae S13 spore 13 day #2 DNA

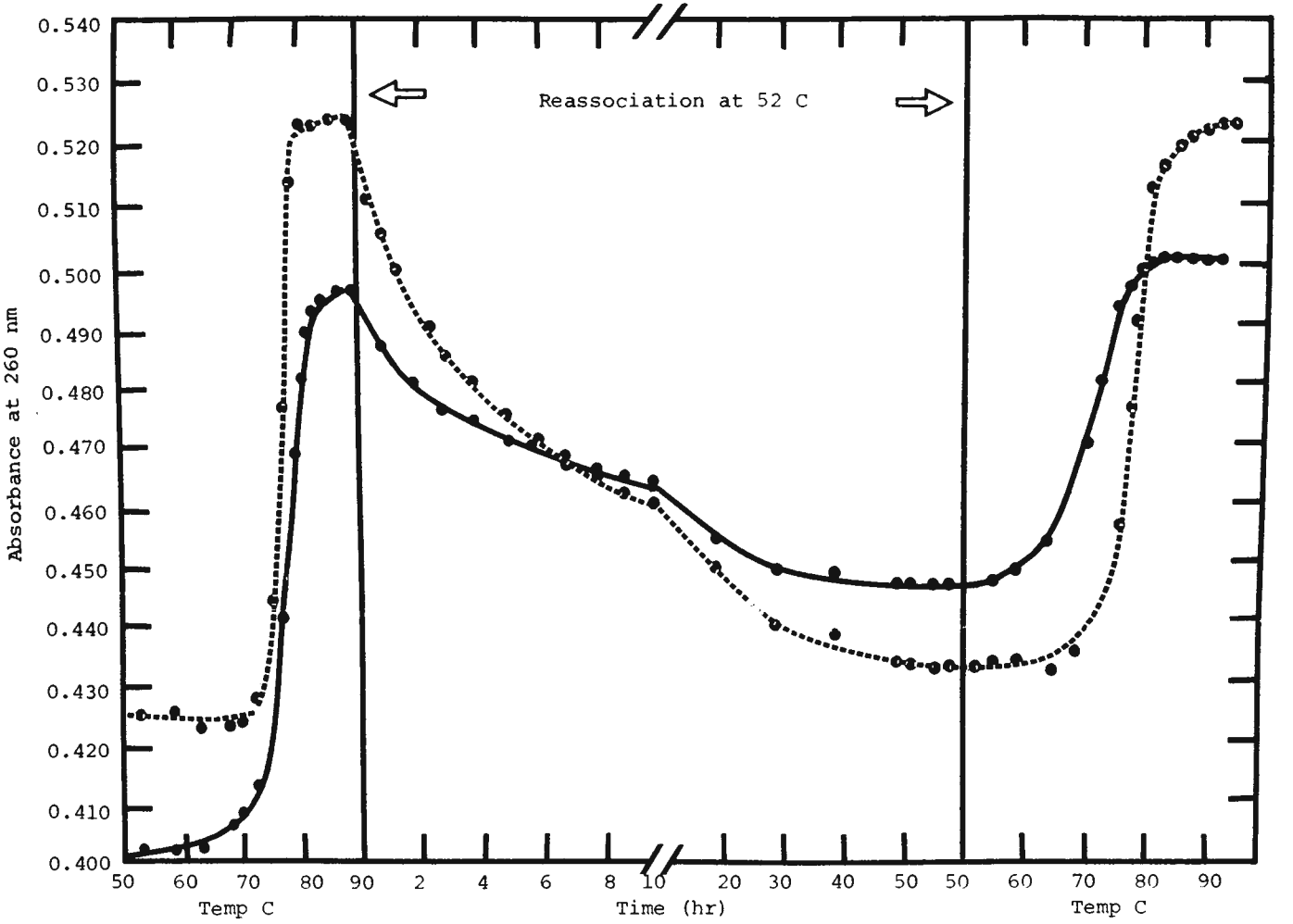

Reassociation at 52 C
Absorbance at 260 nm
0.540
0.530
0.520
0.510
0.500
0.490
0.480
0.470
0.460
0.450
0.440
0.430
0.420
0.410
0.400
50 60 70 80 90
Temp C
2 4 6 8 10 20 30 40 50
Time (hr)
50 60 70 80 90
Temp C

Fig.13 Denaturation and renaturation of S. venezuelae S13 mycelial and spore DNA in 5 M $NaClO_4$ at an incubation temperature of 52 C. Note change of scale from temperature to time and back to temperature on abscissa. All measurements taken at a wavelength of 260 nm.

●——● : S. venezuelae S13 mycelial DNA

●·····● : S. venezuelae S13 spore 13 day #1 DNA

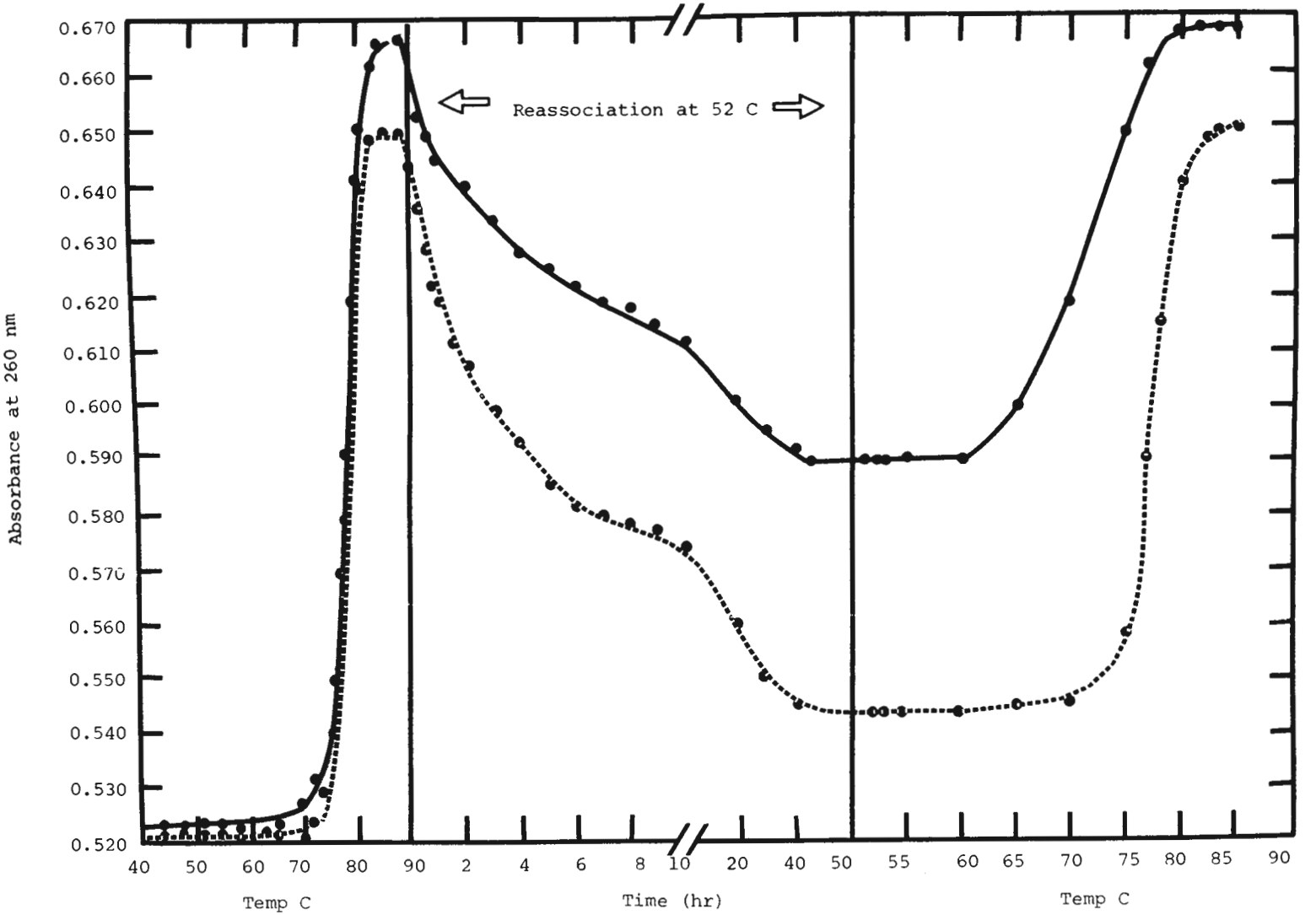

Absorbance at 260 nm
0.670
0.660
0.650
0.640
0.630
0.620
0.610
0.600
0.590
0.580
0.570
0.560
0.550
0.540
0.530
0.520
Reassociation at 52 C
40 50 60 70 80 90 2 4 6 8 10 20 40 50 55 60 65 70 75 80 85 90
Temp C
Time (hr)
Temp C

Fig. 14 Renaturation Kinetics of denatured DNA from S. venezuelae S13 spore and mycelial DNA. Denatured DNA from both sources was allowed to renature at 52 C in 5 M $NaClO_4$. These data are averaged from two trials (see Fig. 12 and Fig. 13)

●—● : S. venezuelae S13 mycelial DNA

○—○ : S. venezuelae S13 spore DNA

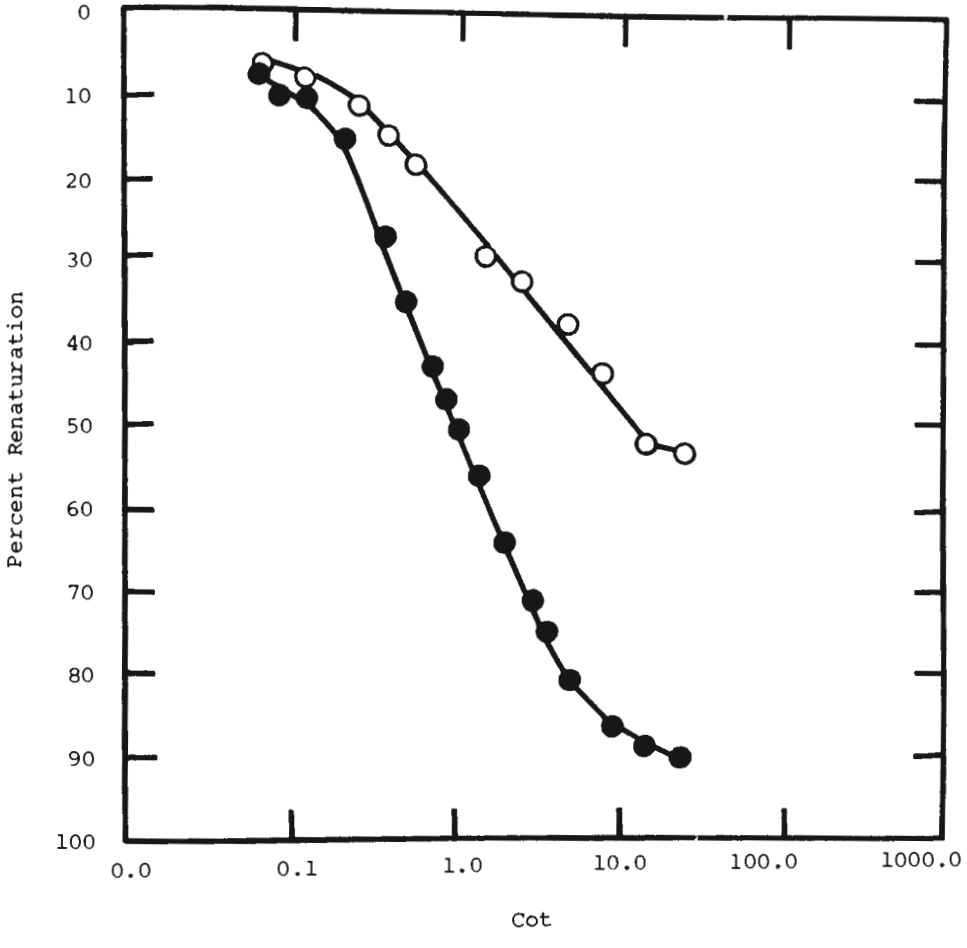
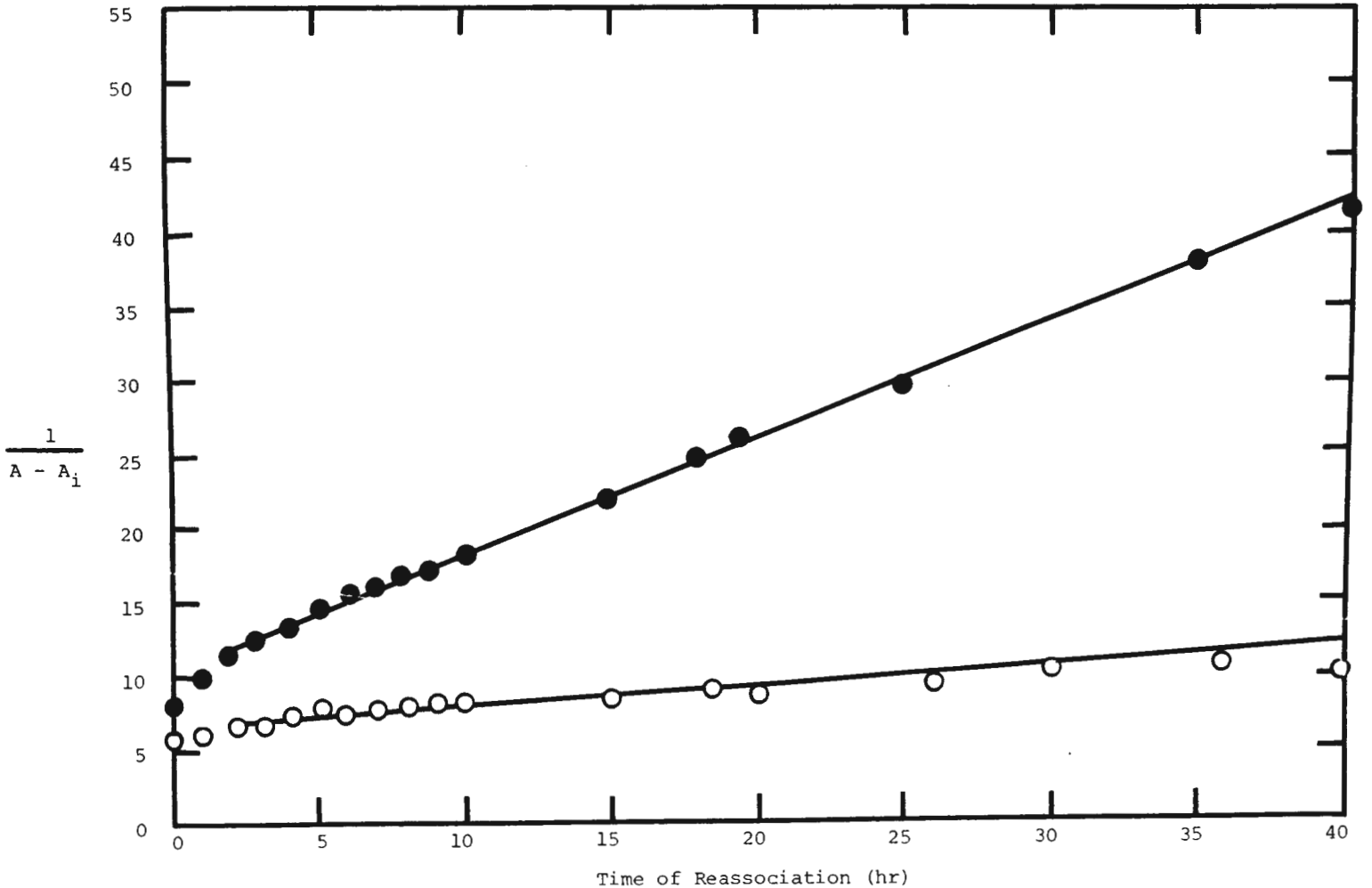

Percent Renaturation
0
10
20
30
40
50
60
70
80
90
100
0.0
0.1
1.0
10.0
100.0
1000.0
Cot

Fig. 15 Renaturation of S. venezuelae S13 mycelial DNA and spore DNA in 5 M $NaClO_4$. $1/A - A_i$ = reciprocal of change in absorbance. Data is obtained from Fig. 13.

●——● : S. venezuelae S13 mycelial DNA

○——○ : S. venezuelae S13 spore DNA


55
50
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
1
A - A_i
Time of Reassociation (hr)

renatured slower and to a lesser extent than did mycelial DNA. To establish more rigorously the kinetic order of reassociation, the data were plotted in such a way as to yield a linear relationship between the concentration of denatured DNA and the time of reassociation. Such a relationship was found when the reciprocal of change in absorbance at 260 nm was plotted against time of incubation in hours (Fig.15). Because the change in absorbance is directly proportional to the concentration of denatured DNA (Wetmur and Davidson, 1968). the linear relationship found strongly suggests that the reassociation of mycelial DNA was indeed a bimolecular reaction, Spore DNA definitely renatured more slowly than mycelial DNA; however, the data suggest that the spore DNA reaction was also second-order.

Even after 40 hr incubation, the spore DNA did not exceed 55% reassociation compared to the 92% reassociation observed for mycelial DNA. Significantly, the Tm of the renatured spore DNA was 5 to 7 C lower than the native spore DNA preparation. Renatured mycelial DNA gave essentially the same Tm as native mycelial DNA.

Spectral Analysis of Spore and Mycelial DNA. The spectral analysis of spore and mycelial DNA in the ultraviolet (UV) region (220 nm to 320 nm) showed few, if any significant differences (Fig.16). The spore DNA used in this study was the 8-day spore DNA characterized in the experiment on the effect of spore age and DNA (Table 5). The DNA was definitely yellow in color and clearly aberrant compared to mycelial DNA.

Fig.16 Ultraviolet spectra of spore and mycelial DNA from *S. venezuelae* S13. The solvent was 1 x SSC, pH 7.0

———— : *S. venezuelae* S13 mycelial DNA

····· : *S. venezuelae* S13 8-day spore DNA

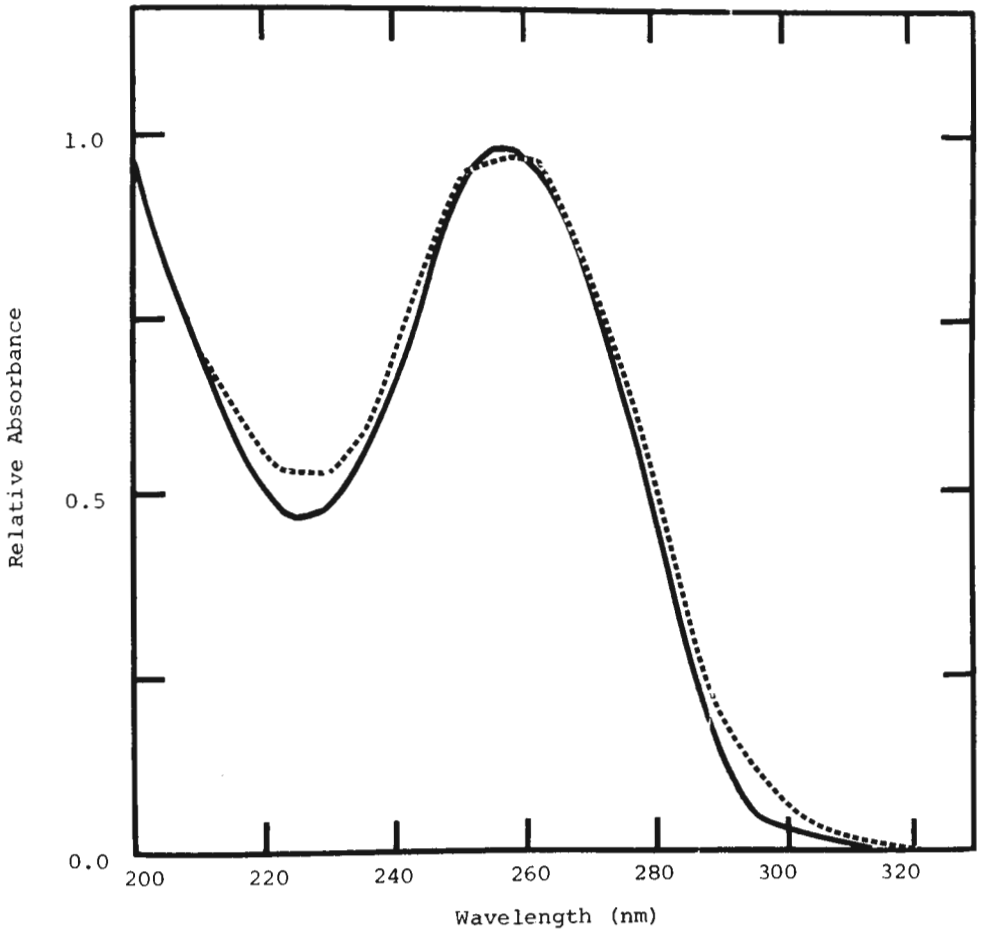
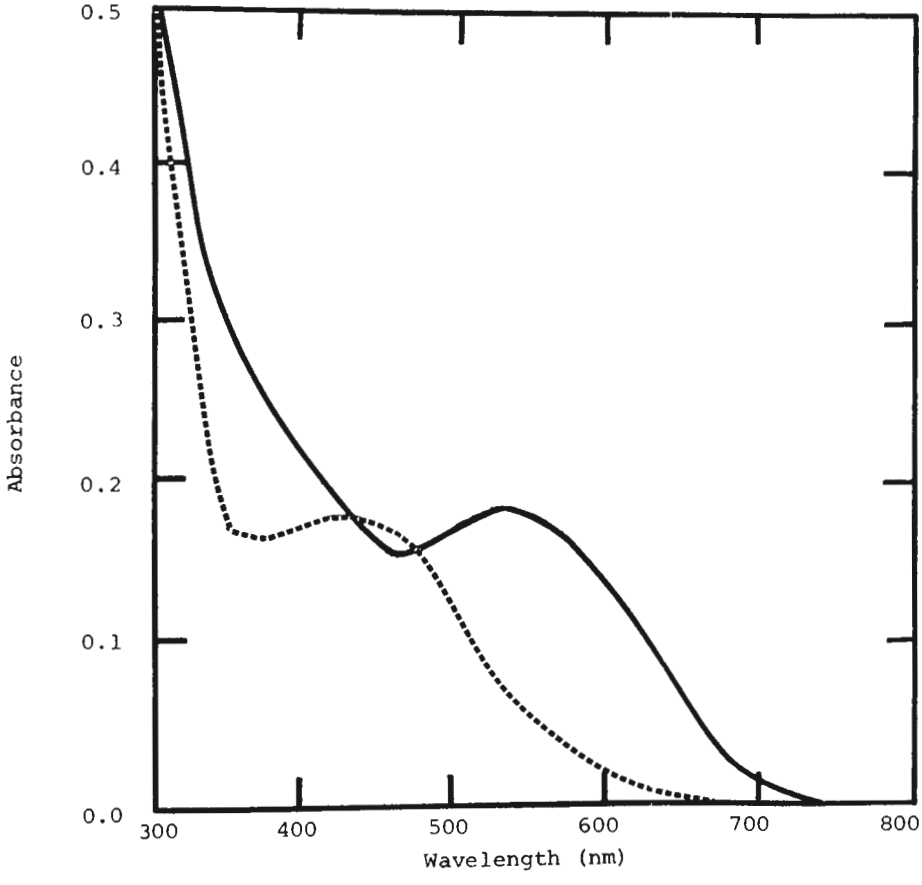

Relative Absorbance
1.0
0.5
0.0
200
220
240
260
280
300
320
Wavelength (nm)

Fig.17 Visible absorption spectrum of S. venezuelae S13 spore DNA in 1 x SSC at pH 3 (·····) or pH 13 (———).


0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
300
400
500
600
700
800
Wavelength (nm)

In the UV region, there seemed to be a minor shift of the major peak from around 257 nm for mycelial DNA to 260 nm for 8-day spore DNA. The A260/A280 ratio for spore DNA was 1.81 for mycelial DNA, the ratio was 1.83. The major peak of both samples advanced to 264 nm at a pH of 14 with the A260/A280 for both becoming 1.71. At an acid pH of 2.0, depurination occurred with the major peak for spore DNA shifting to 277 nm and the corresponding mycelial DNA peak shifting to 278 nm. Spore DNA routinely gave A260/A280 ratios between 1.8 and 1.9.

The visible spectrum of concentrated spore DNA was noteworthy (Fig. 17). At a pH near neutrality and below, the spore DNA was distinctly yellow and transparent. The spectrum showed a weak, broad absorbance in the 400 to 450 nm range. In basic solutions (pH 12.5), the DNA changed to a light pink color with a weak absorbance near 540 nm. Significantly, the color change was reversible, and remained reversible even after heating the DNA in 1 N KOH at 100 C for 30 min. At DNA concentrations less than 100 µg/ml, no color could be detected even with the most aberrant DNA. Mycelial DNA gave no absorbance in the visible region at any pH.

Sensitivity of Spore DNA to Ribonuclease, Pronase and Deoxyribonuclease. The evidence thus far indicated that something may be bound to or associated with the spore DNA. It was suggested that DNA could be associated with the spore DNA; therefore, an experiment was devised in which the spore DNA was treated with pancreatic ribonuclease and T1 ribo-

nuclease. S. venezuelae S13 mycelial DNA, yeast RNA (grade A, Calbiochem) and 13 day #2 spore DNA were used. The RNA and DNA samples in 1 x SSC were denatured by boiling at 105 C in a glycerol-water bath for 7 min followed by quick cooling. Next, 0.1 ml of a 1 mg/ml solution of pancreatic RNase (Calbiochem) was added to each sample in a screw cap tube and the contents were quickly and thoroughly mixed. Then 1 ml from each tube was placed in a separate dialysis bag prepared exactly as in the depurination assay (p 94 of this thesis). The tubing was looped into a 13 by 80 mm test tube with 2 ml 1 x SSC. Incubation was carried out for 2 hr at 37 C. The A260 and A280 of the dialysate was then read (Table 7). A similar experiment was conducted with T1 ribonuclease. The results with ribonuclease show that no UV absorbing material was detectable in dialysates from either spore or mycelial DNA during the dialysis with pancreatic or T1 ribonuclease (Table 7). The dialysis assay was sensitive enough to detect the hydrolysis of yeast RNA by both enzymes.

The 13 day #1 spore DNA was digested with pronase (Calbiochem) at 37 C for 1 hr. The buoyant density of 13 day #1 spore DNA before pronase digestion was 1.6997 g cm^{-3}; after pronase digestion, the buoyant density was calculated to be 1.7002 g cm^{-3}. The difference of 0.0005 g cm^{-3} was hardly significant because experimental error for previous buoyant density experiments ranged between $\pm$0.0002 and $\pm$0.0007 g cm^{-3}. Moreover, pronase is used during the isolation of DNA. This proteolytic enzyme did not seem to affect the aberrant buoyant density of spore DNA.

Table 7

Sensitivity of Spore DNA to Ribonuclease

Nucleic Acid Sample	Pancreatic RNase Dialysate Absorbance at A260	Pancreatic RNase Dialysate Absorbance at A280	T_1 RNase Dialysate Absorbance at A260	T_1 RNase Dialysate Absorbance at A280
S13 mycelial DNA (initial A260=0.84)	0.068[a]	0.069	0.068	0.065
13 day #2 spore DNA (initial A260=0.97)	0.039	0.040	0.037	0.037
Yeast RNA (initial A260=1.2)	0.372	0.292	0.564	0.320
1 x SSC blank[b]	0.026	0.030	0.028	0.030

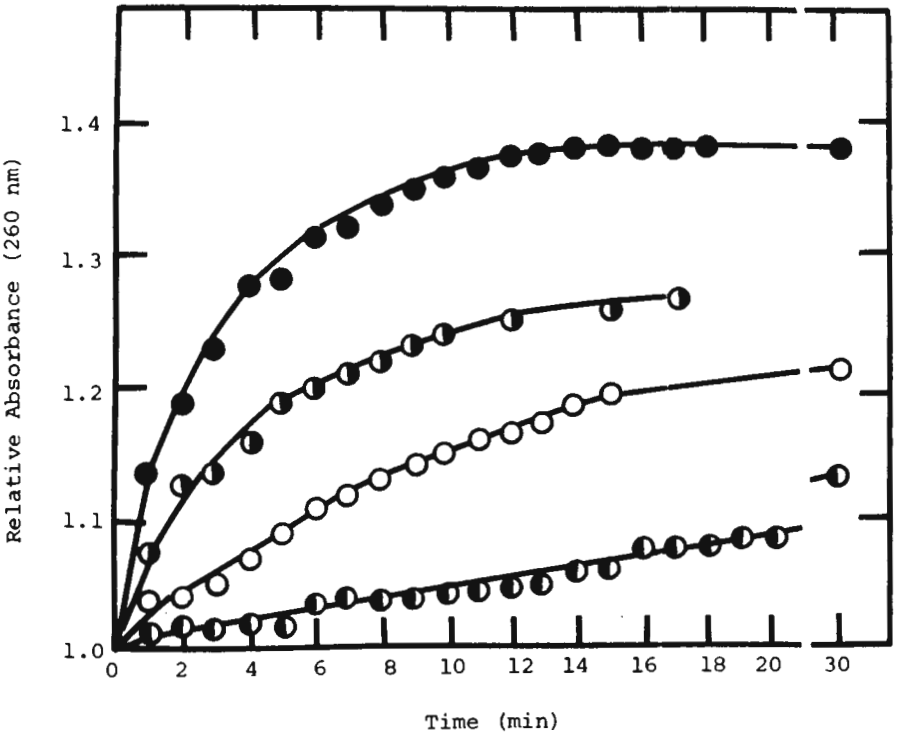
[a] Average of two determinations

[b] Dialysis tubing with 100 µg of each respective RNase in 1 x SSC

Mycelial DNA and spore DNA responded differently to pancreatic DNase 1 (Calbiochem) in acetate-Mg buffer, pH 6. The rate and extent of hydrolysis of spore DNA depended on the time that the DNA was in the acetate-Mg buffer prior to the addition of 0.1 ml (100 μg) of DNase to a 2.0 ml solution of DNA. Even after 14 hr in acetate-Mg buffer prior to addition of DNase, the rate of hydrolysis of spore DNA was clearly different from that for mycelial DNA. Mycelial DNA was consistently sensitive to DNase regardless of prior exposure to the buffer.

Paper Electrophoresis of Spore and Mycelial DNA. The 8-day spore DNA (buoyant density 1.7197 g cm^{-3}) and mycelial DNA were spotted on strips of Whatman 3 MM paper and subjected to 3 ma cm^{-2} at 100 volts for 3 hr. The buffer was barbital-sodium barbital, pH 8.6. Several trial runs were made with a sample of spore DNA shaken overnight with 2 M LiCl. The untreated 8-day spore DNA remained at the origin; moreover, no compounds were removed from the DNA that were detectable with UV light or ninhydrin. Significantly, the spore DNA appeared red or maroon under long wave UV light. Mycelial DNA, like spore DNA remained at the origin: likewise, no compounds were detected with UV or ninhydrin. Mycelial DNA appeared dark purple under long wave UV light. Interestingly, the 8-day spore DNA treated with LiCl showed a yellow band migrating toward the cathode with short wave UV light. The dark red absorbance characteristic of spore DNA remained at the origin. To determine the nature of the cathode-migrating yellow band, the strips were sprayed with 2% H_2SO_4. The

Fig. 18 Susceptibility of S. venezuelae S13 mycelial DNA (●——●) and spore DNA to hydrolysis by deoxyribonucelase I. Spore DNA was incubated in acetate-Mg buffer for 0.5 hr (◐——◐), 1.5 hr (○——○) or 14 hr (◑——◑) prior to the addition of 100 µg (0.1 ml) of enzyme to 2.0 ml DNA solution. The concentration of DNA used was 20 µg/ml. The temperature was maintained at 30 C.


Relative Absorbance (260 nm)
1.4
1.3
1.2
1.1
1.0
0
2
4
6
8
10
12
14
16
18
20
30
Time (min)

yellow band did not char, indicating that it was not organic. Experiments with 2 M LiCl alone produced the same yellow band. Apparently the band was the Li^+ ion front or a complex produced by LiCl interaction with the barbital buffer. It is also possible that the LiCl contained this material as a contaminant.

Spore DNA routinely gave no color with ninhydrin even after heating at 105 C for 10 min. The red absorbance observed with long wave UV light was quite striking when compared to the dark purple absorbance of mycelial DNA. Significantly, when the spore DNA reacted with ninhydrin at room temperature (ca.25 C), the red absorbance under longwave UV was lost.

Chemical Analyses of Spore DNA. Colorimetric determinations of protein content were performed with the Folin phenol reagent as described by Lowry et al. (1951) using bovine serum albumin as a standard. DNA was assayed colorimetrically with the diphenylamine reagent according to the method of Burton (1956) using salmon sperm DNA (Calbiochem, grade A) as a standard. Total phosphate was determined by the method of Bartlett (1959). KH_2PO_4 was used as a standard for the phosphate assay.

When the concentration of DNA was calculated on the basis of the extinction coefficient at 260 nm and on the basis of the diphenylamine test, both spore and mycelial DNA samples gave essentially the same values (Table 8). Apparently, whatever was bound to or associated with the spore DNA did not interfere with the diphenylamine test nor with the absorbance at 260 nm. The amount of Folin positive material

Table 8

Chemical Analyses of Spore DNA Samples

DNA Sample	DNA Concentration By: A260[a] (µg/ml)	Diphenylamine (µg/ml)	% Folin Reacting Material	%P By[b] Chemical Assay
13 day #1 spore DNA	380	365	12.0	14.5
13 day #2 spore DNA	370	370	11.0	14.0
8-day spore DNA	180	175	12.5	15.0
Mycelial DNA	567	570	1.3	10.5
Salmon sperm DNA	720	720	1.1	10.0

[a] A260/0.024 = concentration of DNA in µg/ml

[b] $\%P = \frac{\mu g\ P}{\mu g\ DNA} \times 100\%$; where P in µg/ml = $\frac{\mu moles\ PO_4}{ml} \times \frac{95\ \mu g\ PO_4}{1\ \mu mole} \times \frac{0.33 \mu g\ P}{1\ \mu g\ PO_4}$

was distinctly different for spore and mycelial DNA. Spore DNA samples showed about 12% more Folin reacting material than did mycelial DNA. Furthermore, total phosphorus analysis based on Bartlett's method for total phosphate indicated that spore DNA contained about 4% more phosphorus (in absolute terms) or about 40 to 50% more phosphorus in relative terms than did mycelial DNA.

Acid Hydrolysis and Chromatography of an Aberrant Spore DNA. DNA extracted from 7-day old spore was used in the following experiments. This DNA gave a buoyant density of 1.7074 g cm^{-3}, a Tm 3 C higher than mycelial DNA, 2% more phosphorus (absolute value) than mycelial DNA and about 11% more Folin positive material. Spore DNA and mycelial DNA were diluted to give an A260 of 15 which corresponded to 625 µg DNA/ml in 1 x SSC. Next, 1 ml of each DNA solution was placed in a 13 x 100 mm soft glass test tube and 1 ml of concentrated HCl was added to give ca. 6 N HCl. Immediately after the addition of acid, the tubes were sealed and placed in an oven at 105 C. After 12 hr the acid was removed by flash evaporation. Then 0.5 ml distilled water was added and the hydrolyzed samples were frozen (-20 C) until needed. Descending chromatography was done using butanol, acetic acid, water (4:3:1) as previously described (p 99 of this thesis).

All the ninhydrin positive spots for both spore and mycelial DNA were visualized only after heating at 100 C. The one exception to this was a faint blue-brown spot at Rf = 0.23 which appeared at room temperature (Fig. 19). This was probably glycine as the standard glycine solution reacted

Fig. 19 Descending paper chromatography of DNA acid hydrolysates. The solvent system used was butanol, acetic acid, water (4:3:1).

● : Spot not held in common

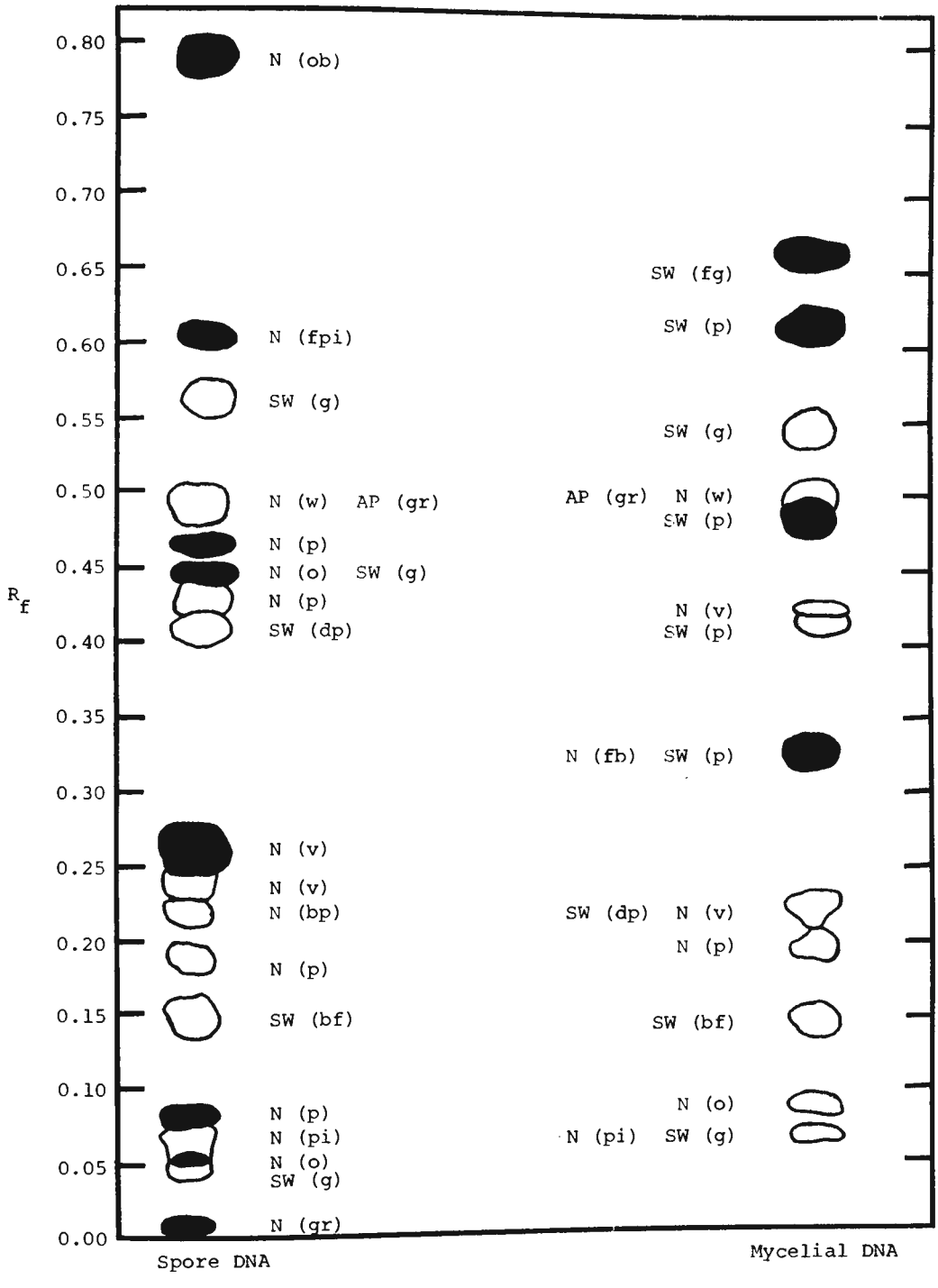
N : Ninhydrin positive

AP : Aniline phthalate positive

SW : Short wave ultraviolet light

Colors: (in parentheses)

pi - pink
g - green
w - white
gr - grey
p - purple
o - orange
dp - dark purple
v - violet
bp - brownish purple
bfl - blue fluorescence
fb - faint blue
fg - faint green
fpi - faint pink
ob - orange brown


0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
R_f
N (ob)
N (fpi)
SW (g)
N (w) AP (gr)
N (p)
N (o) SW (g)
N (p)
SW (dp)
N (v)
N (v)
N (bp)
N (p)
SW (bf)
N (p)
N (pi)
N (o)
SW (g)
N (gr)
Spore DNA
SW (fg)
SW (p)
SW (g)
AP (gr) N (w)
SW (p)
N (v)
SW (p)
N (fb) SW (p)
SW (dp) N (v)
N (p)
SW (bf)
N (o)
N (pi) SW (g)
Mycelial DNA

in a similar manner. With the exception of methionine, all the standard amino acids developed color at about 25 C with ninhydrin. Both ribose and deoxyribose migrated to R_f = 0.48 and showed a white spot with ninhydrin and a dark grey spot with aniline phthalate. Thus, the aniline phthalate positive spot for both DNA samples was most likely deoxyribose or ribose. Spots absorbing shortwave UV light corresponding to standard solutions of adenine (0.61), guanine (0.22) were seen in mycelial DNA but not in spore DNA. Spots corresponding to uracil (0.46) were seen in both DNA preparation (cytosine is rapidly converted to uracil in strong mineral acids). Thymine gave a blue absorbance with shortwave UV light and had an R_f value of 0.55. Both DNA preparations showed a light green spot with short wave UV at this R_f. Spore DNA showed at least five distinctly different spots from those obtained with mycelial DNA. On the other hand, mycelial DNA showed four spots not seen with spore DNA. Of the standard solutions tested, the following were not detected in the DNA samples: glucose, glycerol, arabinose, D-galactose, D-mannose, D-galactosamine, D-glucosamine, ethidium bromide, 5-methyl cytosine, cytosine and none of the standard amino acids except glycine. No reducing sugars (with the exception of the spot at R_f = 0.48) were seen in either DNA sample with the aniline phthalate spray. Alkaline silver nitrate treatment showed one spot with spore and mycelial DNA which corresponded to ribose and deoxyribose.

When native spore and mycelial DNA were run with this solvent, nothing was seen with ninhydrin, aniline phthalate,

silver nitrate or UV. When observed with longwave UV light, the spore DNA appeared a deep maroon or dark red at the origin while mycelial DNA appeared a dark purple. Under short wave UV, both showed a dark purple absorbance. Chromatography was done using Instant Thin Layer Chromatography paper and equipment manufactured by Gelman Products (Ann Arbor, Mich.). The results with this system were not very reproducible, but as in paper chromatography, both spore DNA and mycelial DNA exhibited a variety of spots. Interestingly, with silicic acid support and a solvent composed of $CHCl_3$, CH_3OH, 58% NH_4OH (2:2:1), very few ninhydrin positive spots were seen. Hydrolyzed spore DNA gave two UV absorbing spots which were quite distinct and different from the hydrolyzed mycelial DNA. A spot with $R_f = 0.86$ which appeared red under longwave UV was very obvious while a faint blue spot ($R_f = 0.18$) appeared with ninhydrin. No ninhydrin reacting material was visible until the chromatogram was heated at 100 C. No aniline phthalate reacting material was seen.

When the solvent system was butanol, acetic acid, water (3:1:1), two spots unique to spore DNA appeared: with ninhydrin, an orange spot ($R_f = 0.40$) and with longwave UV, a red spot ($R_f = 0.44$). Again, no aniline phthalate reacting material was seen.

The native spore and mycelial DNA showed no movement with both solvents. Significantly, after reaction with ninhydrin at room temperature, the red absorbance of spore DNA under longwave UV disappeared. The red spots under

longwave UV found in spore hydrolysates were also lost when sprayed with ninhydrin.

Solvent Extraction of Spore and Mycelial DNA. No chromatographically identifiable compounds nor characteristic color were extracted from spore DNA by a number of solvents and conditions including acetone, ethanol, ethanol-ether (1:1), ethyl acetate, butanol, 8 M urea, 5%, cold trichloroacetic acid or 1 N KOH at 100 C for 30 min. Treatment of spore DNA overnight with 0.5 M HCl at ca. 25 C followed by descending paper chromatography with butanol, acetic acid, water (4:3:1) as the solvent produced some interesting results. Both spore and mycelial DNA showed characteristic spots on shortwave UV corresponding to adenine and guanine. Mycelial DNA showed no spots upon ninhydrin or silver nitrate treatment. Spore DNA, on the other hand, showed two definite silver nitrate positive bands (R_f = 0.25, 0.34) and one faint silver nitrate reacting spot at R_f = 0.48. A faint, pink ninhydrin spot appeared after 10 min at 100 C at R_f = 0.20. No reducing sugars were detectable using aniline phthalate spray.

Binding of Spore Products to Added DNA. Experiments were carried out to determine whether the aberrant properties of spore DNA were due to a spore product which could be non-specifically bound to any DNA or whether it was specific for spore DNA. In these studies, 12 day old spores were lyophilized, the dry spores and frozen mycelia were mixed, ground manually together and the DNA extracted (Table 9). The

Table 9

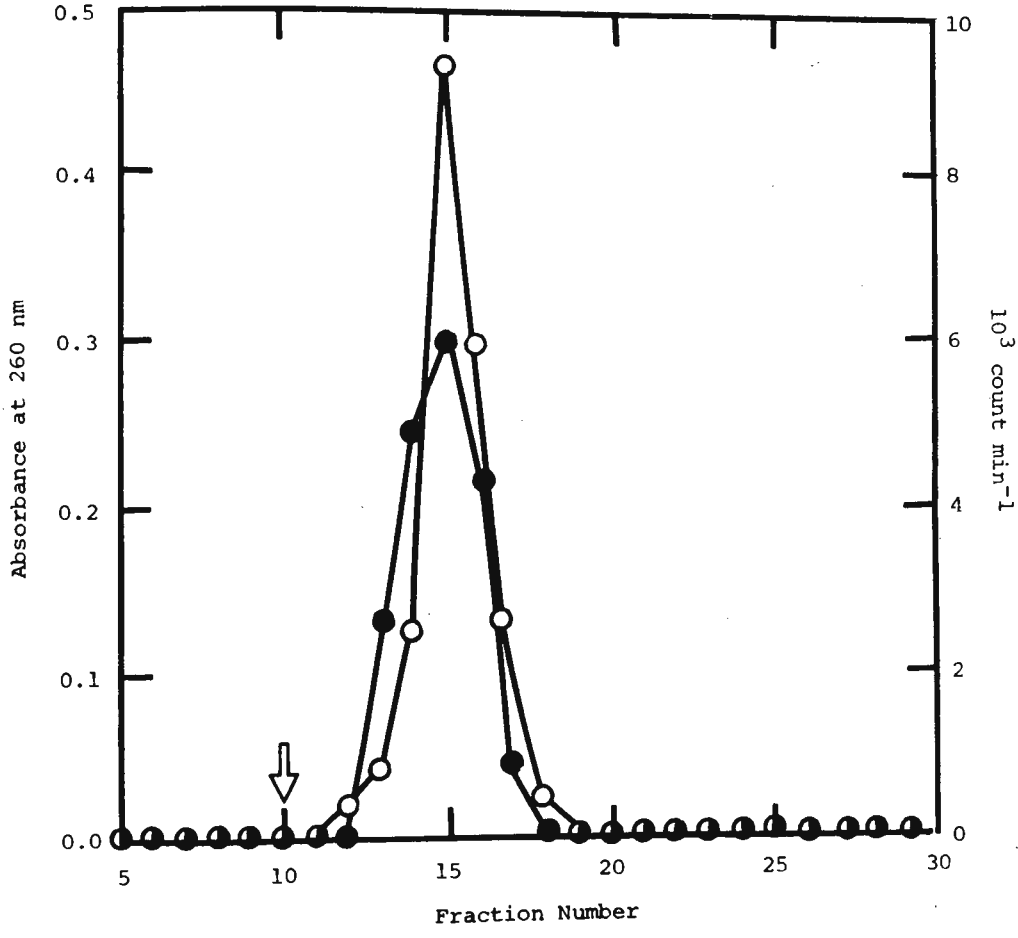
Alteration of Normal Mycelial DNA by Disrupted Spores

Analysis	Mycelial DNA	DNA Mixture	Spore DNA
A260/A280	1.83	1.84	1.85
Buoyant Density in CsCl ($g\ cm^{-3}$)	1.7287	1.7143	1.6997
Tm (0.1 x SSC)	86.0 C	87.0 C	89.5 C
% Folin Positive Material	1.3	9.2	12.0
DNA Concentration By: Diphenylamine	990 µg/ml	580 µg/ml	365 µg/ml
A260	1000 µg/ml	600 µg/ml	385 µg/ml
% Phosphorus	10.3	12.3	14.1

resulting DNA gave only one symmetrical band in the CsCl gradient. Moreover, the buoyant density of this mixture was intermediate between that of the unmixed 13 day spore DNA and that of the unmixed mycelial DNA. The Tm of the DNA from the mixture in 0.1 x SSC was intermediate between the Tm values of each DNA alone. The amount of Folin positive material and the total phosphorus concentration was intermediate between the values for pure spore DNA and pure mycelial DNA. DNA reassociation between the mixture and mycelial DNA followed by thermal elution revealed a bimodal elution profile; one portion having a Tm,e corresponding to mycelial DNA and the other with a Tm,e 8 C lower than mycelial DNA.

When the labeled, purified DNA from another streptomycete (S. coelicolor Muller) was added to lyophilized, manually disrupted spores and the extracted DNA fractionated on a CsCl gradient, similar results were obtained (Fig. 20). The added labeled DNA had the same buoyant density as the spore DNA (the buoyant density of S. coelicolor DNA is normally 1.7313 g cm^{-3}; the buoyant density of 13 day #1 spore DNA was 1.6997 g cm^{-3}). The UV absorbance of the labeled DNA was less than 0.05 so the spore DNA alone contributed to the A260 peak. It appeared that whatever was bound to the spore DNA could be partitioned to added DNA. Significantly, added DNA was altered only if it was mixed with freshly disrupted dehydrated spores, but was not altered if it was mixed with manually disrupted spores that had been stored for 12 hr at 4 C after grinding.

Fig. 20 Alteration of the buoyant density of ^{14}C-labeled DNA from S. coelicolor Muller by ruptured S. venezuelae S13 spores. The position of the untreated ^{14}C-labeled S. coelicolor Muller DNA is marked by an arrow. The position of the treated labeled DNA is marked by closed circles. The absorbance at 260 nm of the spore DNA is denoted by open circles. The A260 of the input labeled DNA was less than 0.05 so that the spore DNA alone accounts for the absorbance at 260 nm.

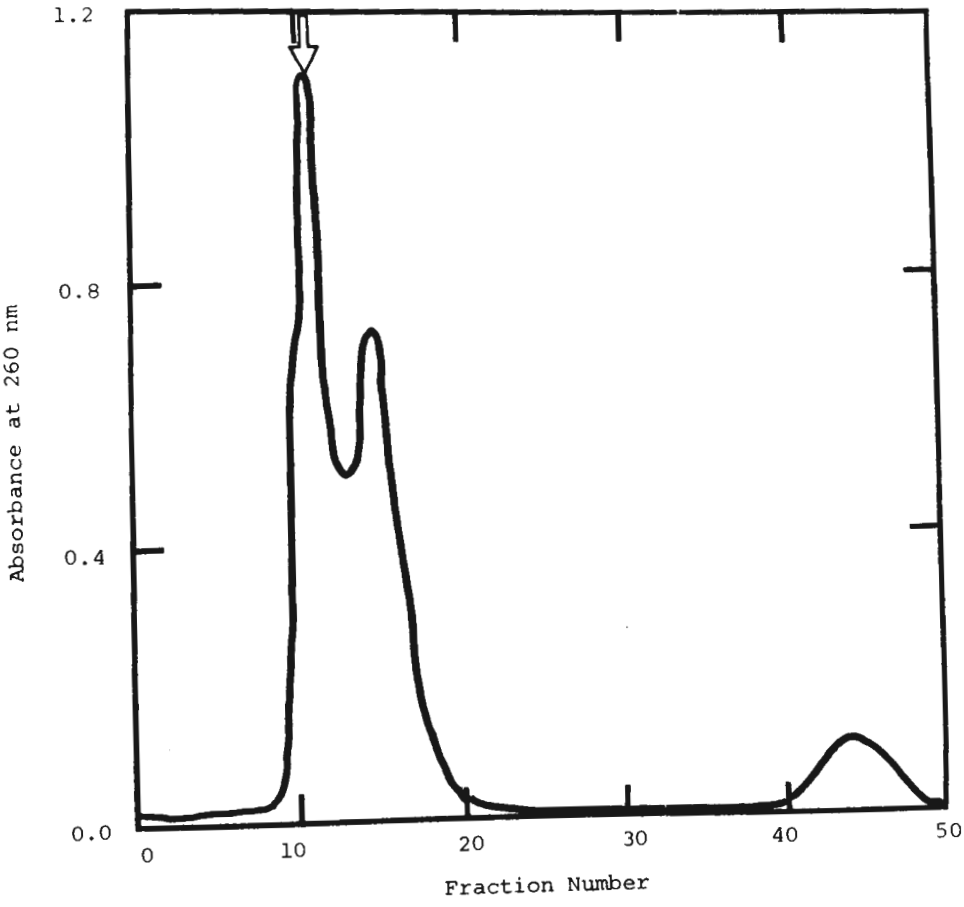

0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 260 nm
10
8
6
4
2
0
10^3 count min^{-1}
5
10
15
20
25
30
Fraction Number

Yeast RNA (Calbiochem, grade A) and salmon sperm DNA (Calbiochem, grade A) were added to ground 13 day old spores. The DNA was extracted in the usual manner. Compared to the yields of DNA from spores alone, the yields of DNA obtained from spores were excellent when additional nucleic acid was provided; moreover, the isolated DNA was quite yellow. Preparative CsCl centrifugation of the spore DNA, RNA, spore DNA plus RNA, spore DNA plus RNA subsequently treated with pancreatic RNase and spore DNA plus salmon sperm DNA provided the following information. Yeast RNA alone was quite heterogeneous and gave a number of peaks near the bottom of the centrifuge tube. No change was seen in the general appearance of the gradient profile when spore DNA plus RNA wasrun i.e., the spore DNA did not change its position in the gradient when the RNA was added. Ribonuclease digestion of spore DNA plus RNA yielded a gradient profile indistinguishable from that of spore DNA alone, again indicating that adding RNA to ground spores did not alter the aberrant spore DNA. When salmon sperm DNA was mixed with spores, the resulting DNA was very yellow and a yield of over 2 mg/ml was obtained. This suggests that adding carrier DNA to spores could be a good method for isolation of the binding material. The buoyant density profile in CsCl revealed a broad band from the top to the bottom of the tube. Tm analysis in 0.1 x SSC showed that although the native salmon sperm DNA and the spore plus salmon sperm DNA mixture gave the same Tm, the spore plus salmon sperm DNA mixture began melting earlier and gave a broad melting transition. A very slight transition

was seen in the area where spore DNA would be expected to melt.

Sephadex G-100 Column Chromatography of Spore DNA. Previous results indicated that heat and increased salt concentration might remove whatever was bound to the DNA, therefore an attempt was made to isolate this material by Sephadex G-100 column chromatography. Spore DNA in 1 x SSC was heated in a boiling water bath for 10 min and then passed through a Sephadex G-100 column equilibrated with 5 x SSC. Two major peaks of UV-absorbing material passed quickly through the column and a minor peak was retarded by the G-100 Sephadex (Fig. 21). When mycelial DNA was similarly treated, only one peak emerged and this coincided with the first peak (the void volume). Chemical analyses showed that the first two peaks were predominantly DNA but that there was some Folin positive material in the second peak. The small third peak gave a negative diphenylamine test and contained most of the input Folin positive material. UV analysis of this peak gave a nucleotide-like spectrum with a broad maximum at 250-255 nm. The first two peaks could be eliminated by treatment with DNase but the third peak was not affected. Pronase did not alter the behavior of the DNA samples during G-100 Sephadex column chromatography. Native spore and mycelial DNA passed through the column as a single peak approximately coincident with the second peak shown in Fig. 21 . This indicated that the first peak was probably denatured DNA whereas the second peak was partially denatured material. This

Fig. 21 Separation of components from denatured spore DNA by Sephadex G-100 gel chromatography. The elutant was 5 x SSC. The arrow indicates the void volume.


1.2
0.8
0.4
0.0
Absorbance at 260 nm
0
10
20
30
40
50
Fraction Number

impression was strengthened by slow-cooling of the denatured DNA prior to applying to the column. The same general profile was generated but the first peak was considerably reduced in size while the second peak was correspondingly larger. Characterization of the small third peak has been unsuccessful to date. Initial attempts to alter mycelial DNA by adding fractions of the third peak to it have been unsuccessful.

Anomalous Spore DNA Preparations. An interesting anomaly was discovered when DNA was extracted from 7-day old spores that had been lyophilized and stored at -20 C for 6 months (Table 10). Initial isolation of DNA from the freshly lyophilized 7-day spores yielded the expected aberrant DNA; however, the DNA obtained from stored spores was not yellow and did not exhibit an aberrant Tm or buoyant density. Unexpectedly, the DNA from the stored spores had more Folin positive material than did the original aberrant DNA. The thermal denaturation profile, although giving the same Tm as mycelial DNA, had a slightly broader transition width with a shallower slope. This was not unlike the Tm of salmon sperm DNA added to spores. These same alterations were observed with a substantial number of spore samples. Moreover, almost 25% of the spore samples that were stored in the cold failed to yield high molecular weight DNA upon extraction.

By storing the lyophilized spores over a desiccant in a sealed container at -20 C, the loss of the aberrant character was delayed. In this regard, it was found that the DNA

Table 10

Anomolous Spore DNA Preparations

Spore Sample[a]	Buoyant Density ($g\ cm^{-3}$)	Tm in 0.1 x SSC (C)	% Folin
Fresh 7-day spores	1.7094	88.5	12.1
7-day spores stored 6 months	1.7291	87	17
7-day spores partially hydrated	1.7297	87	1.8
8-day spores	1.7291	86.5	20
12-day spores	1.7285	87	23
8-day spores	1.7282	87	--[b]
9-day spores	1.7285	87	--
10-day spores	---	87	--
11-day spores	---	87	--

[a] Each entry represents a different spore preparation with the exception of the first three 7-day old spores which are representative of three functions of the same initial population of spores. The age of the spore given is the time from inoculation to time of harvesting. With the exception of the first three entries, all spore samples were stored for 6 to 12 months at -20 C prior to DNA isolation.

[b] not done

extracted from freshly lyophilized spores that had been partially rehydrated, was not aberrant with respect to buoyant density, Tm, chemical analysis and DNA reassociation. It is probable that the storage effect was due, in part, to rehydration. This effect may have significance in germination of the spores.

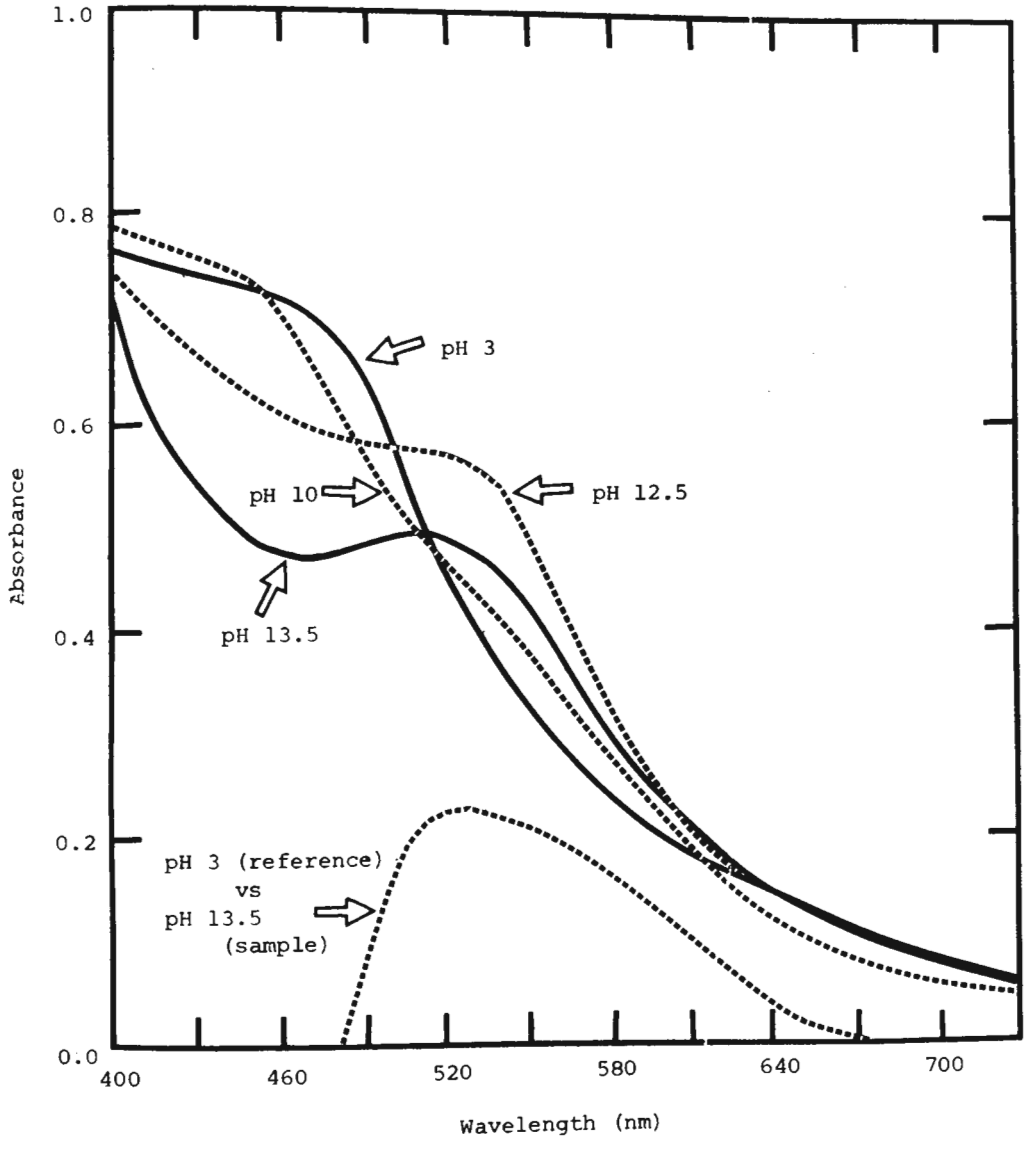
Analysis of Selected Streptomycete Spore DNA Samples. DNA was extracted from lyophilized spores of selected streptomycetes in a preliminary search for other novel spore DNA specimens. Interestingly, the DNA from 13 day old spores of S. violaceoruber 14980, 199 and S16 was distinctly blue in color, while the spore DNA of S. violaceoruber S307 was a red-violet color. The Tm in 0.1 x SSC for all the mycelial DNA preparations was 85 to 87 C while the Tm's for spore DNA samples were 5 to 10 C lower that the corresponding mycelial DNA preparations. Buoyant density determinations with S199 spore DNA showed a 2 mg cm^{-3} increase in buoyant density over mycelial DNA; moreover, when S199 spores were washed in ethanol and acetone, the buoyant density remained 2 mg cm^{-3} heavier than the S199 mycelial DNA. These results indicate that spore DNA in several S. violaceoruber strains had properties directly opposite to aberrant spore DNA in S. venezuelae S13. Most significant, however, is the fact that the spore DNA was aberrant.

Characterization of a Possible DNA-binding Pigment from S. venezuelae S13 Spores. Until it was discovered that spore DNA had distinct pH indicator activity, little progress was

made in isolating any suspected DNA-binding pigments from spores. Addition of acetone or chloroform extracts of whole spores to mycelial DNA had failed to alter the DNA by the criteria of buoyant density and Tm. However, extracts from the chloroform layer during DNA isolation were bright yellow in color and thus were first examined for the yellow-pink pH indicator pigment. Upon adding chloroform to the air dried Sevag's extract followed by 0.1 N KOH, two layers formed; the top aqueous layer was pink, the bottom chloroform layer was yellow-brown and opaque. Addition of 1 N HCl caused the pink layer to become yellow. The yellow-brown chloroform layer became yellowish and transparent. A number of solvents were tested for ability to extract the suspected DNA-binding pigment from the aqueous layer. Butanol was found to be efficient in extraction, volatile enough for ease in solvent removal and only slightly soluble in water.

The crude pigment extracted with butanol was soluble in chloroform, ethyl acetate, distilled water, water made pH 3 with 1 N HCl, water made pH 10, 12 and 13 with 1 N KOH and 1% sodium carbonate. When butanol was added to the aqueous or chloroform solutions, the pigment was extracted into the butanol layer. The visible spectra for the crude butanol pigment dissolved in water at various pH's are shown in Fig. 22 . The correspondence of these spectral peaks to those of spore DNA is striking (see Fig. 17 of this thesis, p117). In dilute solutions no marked UV absorption peaks were seen. A slight shoulder was seen in the rapidly rising absorbance profile at the 260 nm to 280 nm range. After

Fig. 22 Absorption spectrum of a crude butanol extract from S. venezuelae S13 spores. The extract was air-dried and dissolved in distilled water at the indicated pH.


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
400
460
520
580
640
700
Wavelength (nm)
pH 3
pH 10
pH 12.5
pH 13.5
pH 3 (reference)
vs
pH 13.5
(sample)

250 nm the absorbance rose rapidly off scale.

A pigment with the same spectral characteristics could be isolated from whole spores by homogenizing the spores in 0.1 N KOH followed by extraction of the pigment with butanol. Examination of this crude butanol extract using instant Thin Layer Chromatography (Gellman Products; Ann Arbor, Mich.) with a chloroform, methanol (100:3) solvent revealed an extraordinary number of compounds in this extract. Many exhibited bright yellow, blue or orange fluorescence under UV, while others gave no UV absorbances or fluorescence but had strong ninhydrin reactions. No reducing sugars were detected using aniline phthalate spray. A pigment having yellow-pink color transition was found at the origin and one with orange-red transition was found at $R_f = 0.35$.

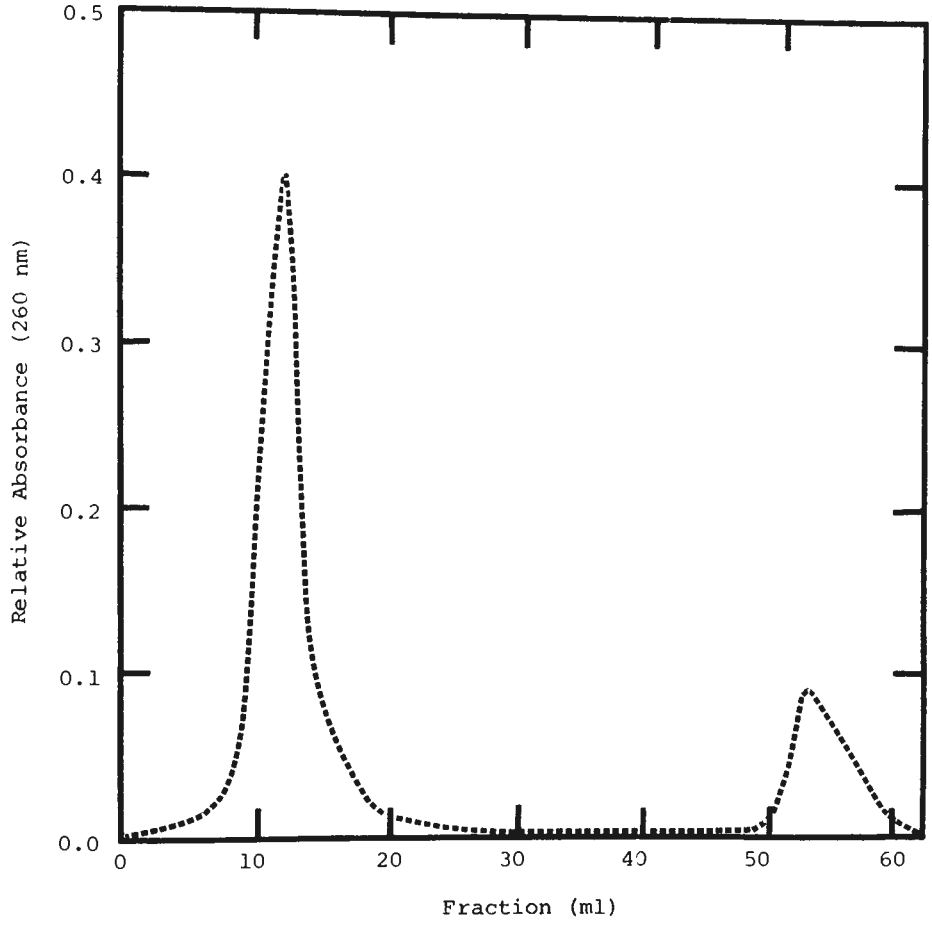
Mr. Miles Sharpley (Biology Dept., Virginia Commonwealth University, Academic Division, Richmond) provided a mixture of Aspergillus flavus toxins which was run in the same system. Four distinct spots were seen using longwave UV light: a blue fluorescing spot at $R_f = 0.40$, a blue-green spot $R_f = 0.43$, a green spot at $R_f = 0.51$ and a similar colored spot at $R_f = 0.60$. No compounds giving these appearance under UV light nor having these R_f values were found in the butanol extract of spores. When the standard amino acids were run in a solvent composed of butanol, acetic acid, water (3:1:1) two distinct clusters of ninhydrin reacting spots were found in the regions $R_f = 0.22$ to 0.40 and $R_f = 0.50$ to 0.80. The butanol extract gave strong ninhydrin reactions in both these regions suggesting presence of most amino acids. Spots

having the appearance under shortwave UV light and with R_f values corresponding to adenine and guanine were quite prominent. With this solvent the suspected pH indicator pigment remained at the origin.

An attempt was made to purify the crude butanol extract using Sephadex G-75 column chromatography. A 1.5 by 50 cm column was used and distilled water, pH 5.5 was used as the eluant. The butanol extract was air dried and dissolved in water, pH 5.5. A solution containing 0.2% blue dextran plus 0.002 M cytosine was used to calibrate the void and bed volumes of the column. Blue dextran emerged in about 12 ml while cytosine emerged in about 50 ml. When 2 ml of the aqueous pigment solution was chromatographed, two peaks were obtained (Fig. 23). The first peak containing very strong pH indicator activity emerged in the void volume while a smaller colorless peak with no detectable pH indicator activity emerged in the bed volume. When the void volume pigment was concentrated by flash evaporation and run again, the same two peaks were observed. The small bed volume peak increased in size when the crude pigment was adjusted to pH 3 with 1 N HCl or the pH 13 with KOH. When the smaller bed volume peaks were pooled and concentrated by flash evaporation, pH indicator activity with the same spectral characteristics as the crude extract was found. Passage of this material through the G-75 column gave one peak coincident with the bed volume, i.e., no high molecular weight material was found.

When air dried, concentrated crude butanol extract was made to pH 13 with 1 N KOH a flocculent precipitate formed.

Fig. 23 Elution profile of crude butanol extract of S. venezuelae S13 spores. The butanol extract was air-dried and dissolved in water, pH 5.5. Sephadex G-75 was used; the eluant was distilled water, pH 5.5. The excluded reference, 2% blue dextran, emerged in 12 ml while cytosine emerged in 50 ml.


0.5
0.4
0.3
0.2
0.1
0.0
Relative Absorbance (260 nm)
0
10
20
30
40
50
60
Fraction (ml)

Removal of this precipitate by Millipore filtration and subsequent passage of the pH indicator filtrate through a G-75 column revealed only the low molecular weight component. The precipitate was not soluble in water or ethyl acetate but was readily soluble in butanol, benzene and hexane. The precipitate did not have significant pH indicator activity. The low molecular weight pigment did not pass through dialysis tubing during overnight dialysis against distilled water at either 4 C or 25 C. The low molecular weight pigment was concentrated by flash evaporation and its visible and UV spectra were determined (Figs. 24 and 25). A sample of this pigment was dissolved in water pH 5.5 and divided in three portions. One portion was made to pH 3 with HCl, one was made to pH 13 by addition of KOH and the remaining portion was kept at pH 5.5. Passage of these samples through Sephadex G-75 showed a single sharp peak at the bed volume (50 ml) for the pH 5.5 sample, a broad peak beginning at 40 ml for the pH 3 tube and two peaks for the pH 13 portion - one sharp, large peak at 50 ml and a small peak excluded in the void volume. These results are suggestive of an aggregation phenomenon at extremes of pH.

An initial attempt was made to determine the molecular weight of the G-75 bed volume pigment using Sephadex G-25. A single peak emerged after the void volume and before the cytosine reference peak. Initial extimates place the molecular weight in the 1,000 to 5,000 range.

When TPO medium was scraped free of spores and the used medium extracted with butanol, a pH indicator pigment similar

Fig. 24 Ultraviolet absorption spectrum of partially purified, low molecular weight pigment from S. venezuelae S13 spores. This is 1/10 dilution of the sample shown in the previous Figure. (Fig. 23). The pH was adjusted to pH 3.0 (O—O) with HCl and to pH 13 (●—●) with KOH.

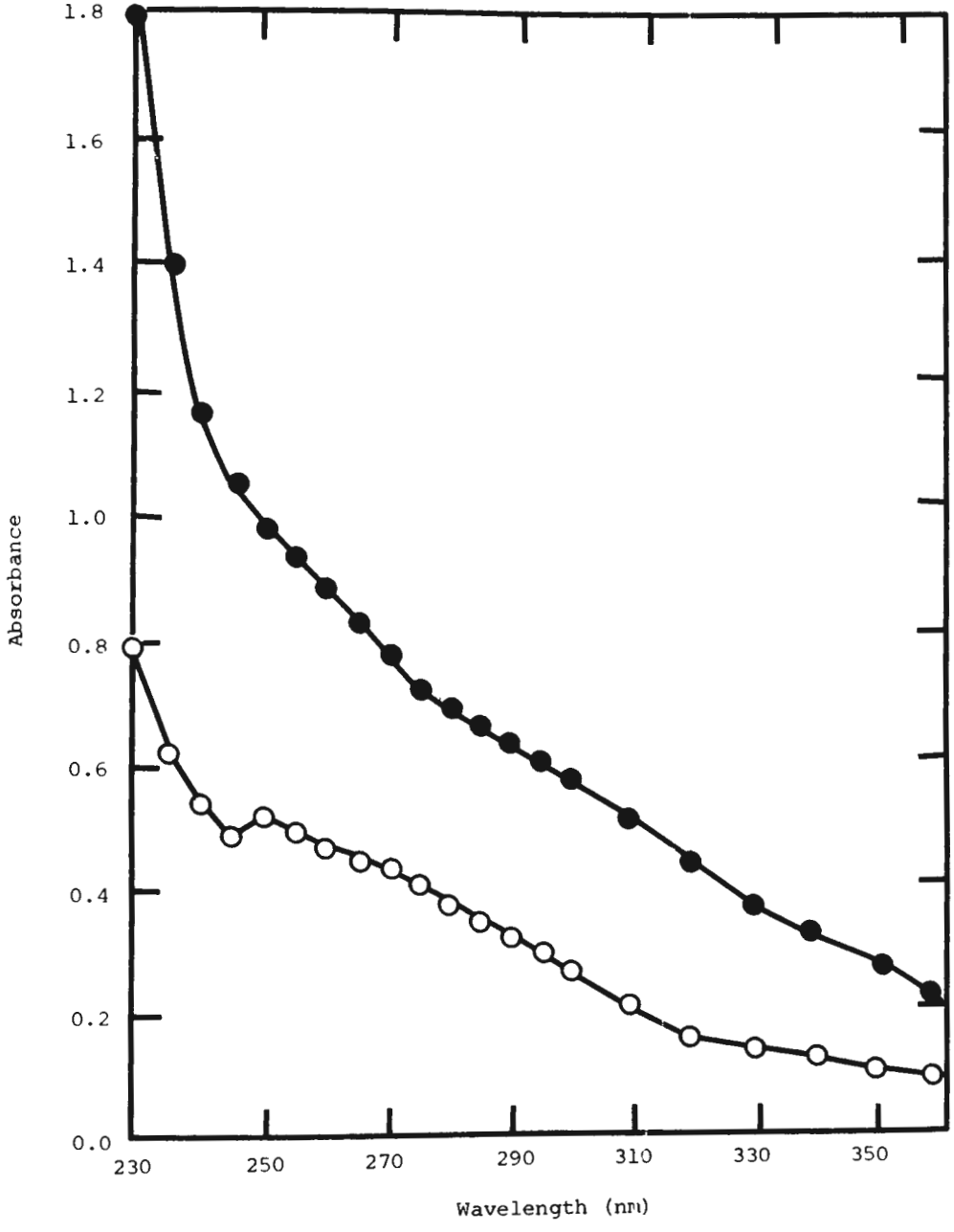
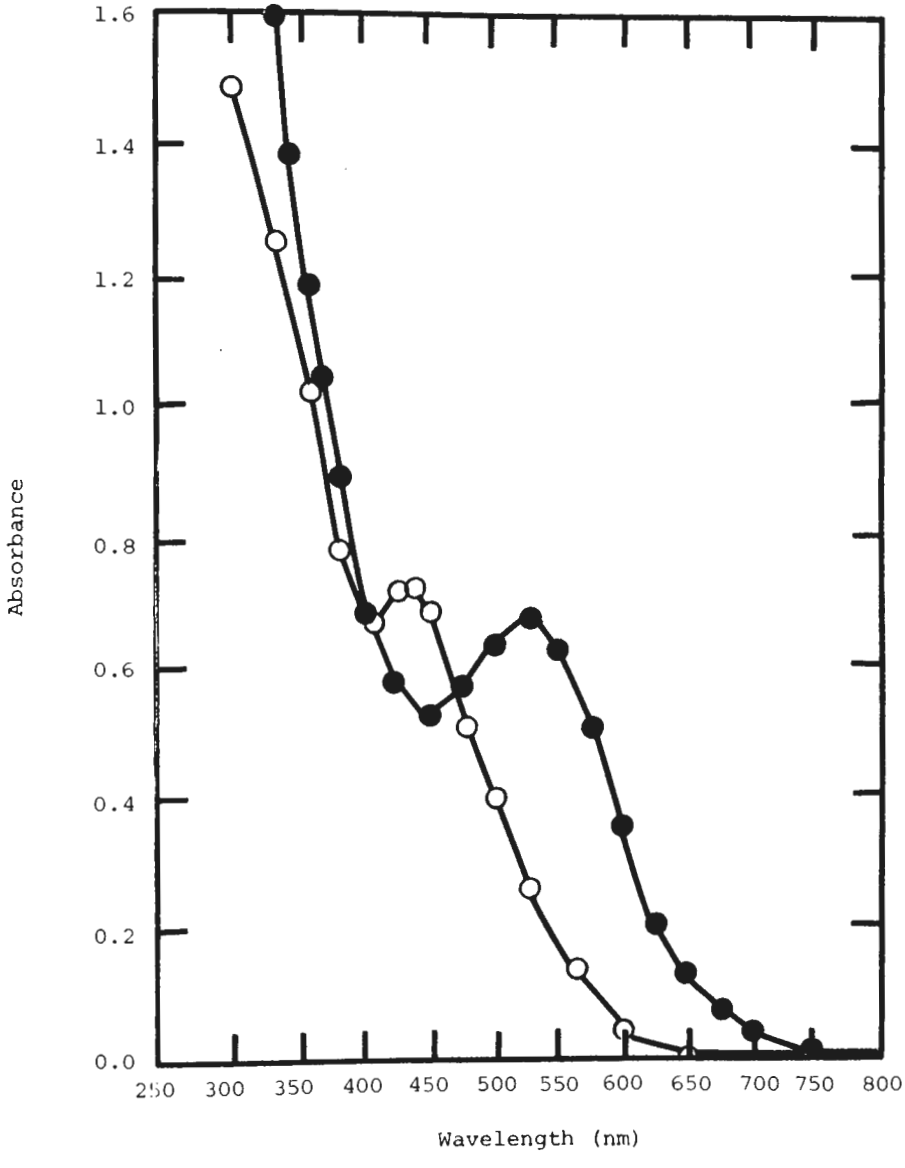

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
230
250
270
290
310
330
350
Wavelength (nm)

Fig. 25 Visible absorption spectrum of partially purified, low molecular weight pigment from S. venezuelae S13 spores. The pH was adjusted with HCl to pH 3.0 (O——O) or with KOH to pH 13 (●——●).

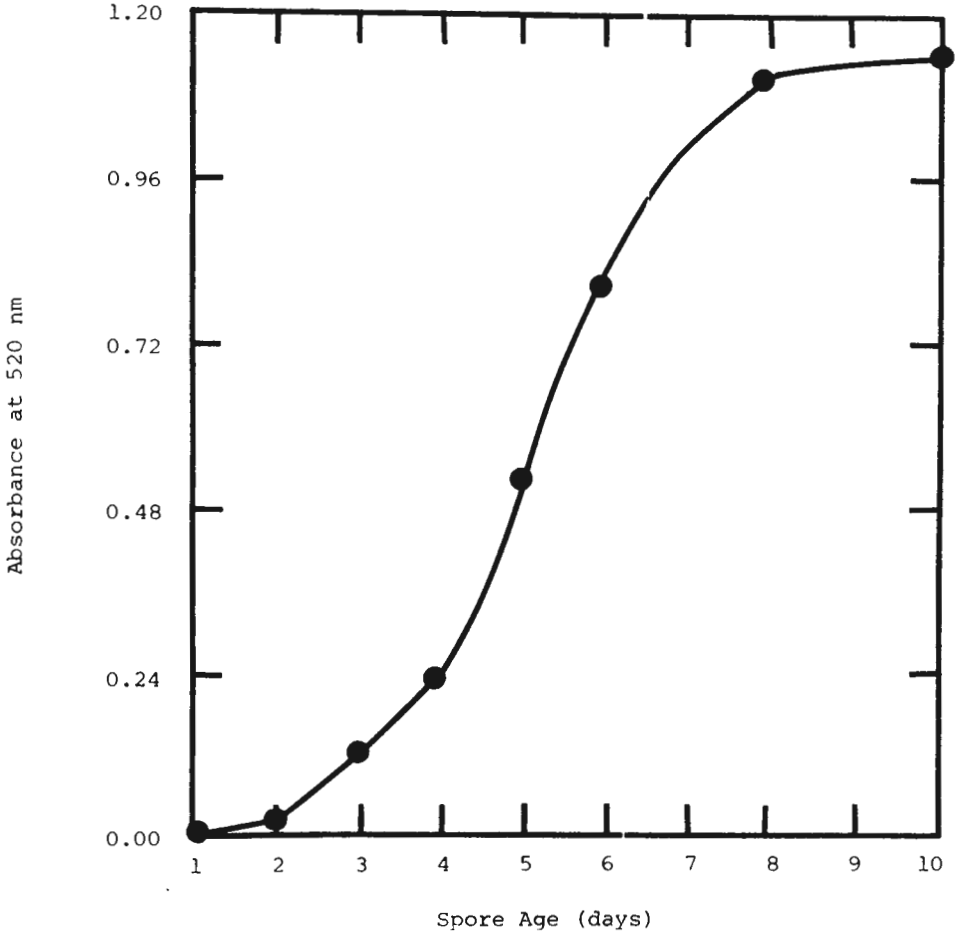

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
250
300
350
400
450
500
550
600
650
700
750
800
Wavelength (nm)

to that found in the spores was found. The major difference was that the pigment found in the medium had no high molecular weight component in G-75 chromatography. No pH indicator pigment could be isolated from fresh TPU medium. Work with the medium pigment was complicated by an orange, low molecular weight compound apparently derived from the tomato paste used in the growth medium. Attempts to remove the contaminating pigment by dialysis against water were partially successful because the pH indicator pigment apparently adhered or bound to the dialysis membrane while the orange pigment passed through.

The pH indicator pigment was found to be associated only with spores and not with mycelia. When 15 g of broth grown mycelia were extracted with KOH and butanol, no pigment was detected in the butanol layer. On the other hand, pigment production in spores was a function of spore age (Fig. 26). The aberrant nature of spore DNA also followed a similar time course.

To obtain the pigment in larger quantities, a bulk purification scheme using silicic acid columns was devised. Previous chromatography experiments showed that when paper, silica gel, silicic acid and alumina were used as supporting material, the pigment remained at the origin regardless of solvent system used. Only when 0.1 N HCl was added to the solvent, did any migration take place. This suggested that the pigment was binding to the support material. Accordingly the pigment in crude butanol extracts was bound onto a silicic acid column. Hexane removed a yellow non-polar pigment which

Fig. 26 Time course of pigment production in S. venezuelae S13 spores. Forty plates of TPO medium were inoculated with S. venezuelae S13. On the day indicated, 4 plates were removed and the surface growth was gently removed by scraping. The aerial mycelial so obtained were extracted with 2 ml 1 N KOH in a tissue homogenizer. Next, 4 ml butanol were added to this mixture and then shaken for 5 min at 25 C. The butanol layer was decanted and filtered through Whatman #4 filter paper. The A520 of this fluid was read using KOH saturated butanol as the blank.


1.20
0.96
0.72
0.48
0.24
0.00
Absorbance at 520 nm
1
2
3
4
5
6
7
8
9
10
Spore Age (days)

when concentrated by flash evaporation, displayed very slight pH indicator activity. No other colored products were eluted with benzene, chloroform or butanol, but when acidified methanol was added, a yellow pigment quickly eluted. This pigment had very strong yellow-purple pH indicator activity. Using silica gel as a supporting medium, thin layer chromatography of the pigment with hexane, acidified methanol (1:1) showed a sharp spot at $R_f = 0.45$ with a small spot at $R_f = 0.5$. Both spots showed pH indicator activity when exposed to NH_4OH fumes. When this pigment was passed through a Sephadex G-75 column, only a low molecular weight form emerged.

Because very little pigment was available, only one attempt thus far has been made to bind the pigment to DNA. Salmon sperm DNA (Calbiochem, lyophilized, grade A) was mixed with a glass rod in a tube containing dried crude, butanol extract. No water was used at this step because previous results with whole spores suggested that water inhibited binding of the spore material to DNA. The dry DNA was mixed until it appeared a mottled brown color. Next, 5.0 ml 1 x SSC was added and the DNA was shaken at ca. 25 C overnight. The following day the dissolved DNA was reprecipitated with ethanol. The DNA was distinctly yellow at this stage. The DNA was dissolved in 1 x SSC and dialyzed against 1 x SSC overnight at 4 C. The DNA was yellow and turned a light pink when KOH was added. No further characterization of this DNA has been done to date.

Antibiotic Sensitivity Experiments Using Crude Butanol Extracts from Spores. B. subtilis B-1, Staphylococcus aureus S-1 and E. coli CSH-2 grown in Penassay broth (Difco) were obtained from Dr. J.D. Punch. A 0.1 ml sample of an 18 hr broth culture was mixed aseptically with 10 ml melted, sterile Penassay agar and poured into plastic petri dishes. Next, 0.1 ml crude butanol extract was applied to sterile Schleicher and Schuell (Keene, N.H.) paper antibiotic-testing discs and allowed to air dry. The discs were placed on the seeded agar surface and the plates were incubated at 37 C for 18 hr. Three pH values for the extract were used: pH 3, pH 7 and pH 8. At pH 3 a 3 mm zone of inhibition was seen around each disc; at pH 7 and 8 no zones of inhibition were seen. When sterile water, pH 3 was added to discs, a similar sized zone was seen suggesting that the observed inhibition was due only to H^+ ions and not to the extract. The plates were observed at 24 and 48 hr with no observable change in results.

When low molecular weight pigment from TPO medium was used in the same assay, no inhibition of the test organisms was observed. Only one pH (7.5) was used for this experiment.

Discussion

Aberrant DNA from bacterial spores has been reported in the genus Bacillus; however, the properties of spore DNA from S. venezuelae S13 are distinctly different from those of Bacillus endospore DNA. Briefly, the DNA from Bacillus spores is more dense than vegetative DNA whereas S. venezuelae spore DNA is definitely lighter than the vegetative DNA. S. venezuelae spore DNA is totally aberrant, becoming more so with spore age, while Bacillus spore DNA shows two density classes of DNA which vary with spore age. The abnormal hypochromicity seen for Bacillus spore DNA is not apparent in DNA from streptomycete spores. S. venezuelae spore DNA is distinctly yellow and changes to pink at alkaline pH. Bacillus spore DNA, on the other hand, shows no such visible color. S13 spore DNA is relatively insensitive to DNase while Bacillus spore DNA is quite sensitive. Finally, solvent treatment of streptomycete spores restores the DNA to normal properties while solvent treated Bacillus spores remain aberrant.

There are some overt similarities, however. The Tm of both spore DNA preparations is increased over vegetative DNA. The base compositions of the spore DNA samples are identical to the corresponding vegetative DNA compositions. Pancreatic and T1 RNase have no effect on the aberrant DNA properties; the non-specific protease, pronase, is also ineffective. The ultraviolet spectrum of both spore DNA samples compared to

vegetative DNA is unchanged. Finally, both spore preparations are lyophilized prior to DNA extraction.

The studies with S. venezuelae S13 spore DNA indicate that its aberrant properties are due to a spore-produced substance bound tenaciously to the DNA. This substance is a Folin-positive, phosphate containing compound which has few, if any common amino acids or reducing sugars in its structure. It most likely contains a conjugated system of double-bonds asscciated with a moiety responsive to pH, the pK of which is near 12. Moreover, this conjugated system involves an amino-group or similar ninhydrin reacting molecule. The substance has secondary or tertiary amine groups in its structure. The material is bound uniformly over each DNA molecule, i.e., it is not bound at a single site on the genome. It is bound to single-strands of DNA, that is, the binding is not solely dependent upon the duplex structure of DNA. The DNA-binding substance is probably associated with the DNA phosphate backbone in such a way to stabilize the DNA helix. This association is stable in CsCl and in 0.1 x SSC at high temperature. A solution of 5 M $NaClO_4$ displaces the stabilizing effect of the complex, but probably does not remove the binding material; moreover, in this solvent, the binding material markedly interferes with the hydrogen bonding ability of the nucleotide bases. The stubstance can bind to DNA other than S. venezuelae spore DNA under suitable conditions. Finally, as the spores mature, more of this material is bound to the DNA.

The lack of sensitivity to both pancreatic and T1 RNase

in addition to the light buoyant density of the spore DNA effectively rules out the presence of a DNA-RNA hybrid. In fact, the light buoyant density alone excludes many of the possible DNA-binding materials including most cations, short chain polyamines, teichoic acids and most proteins. Significantly, the streptomycetes themselves produce many DNA-binding compounds including a variety of antibiotics affecting buoyant density and Tm (Kersten et al., 1966). A possible model system applicable to streptomycete spore DNA may be a class of streptomycete-produced antibiotics called anthracyclines. These antibiotics are virtually the only class of compounds which when bound to DNA, have properties similar to the S. venezuelae spore DNA complex. These properties include: a progressive decrease in DNA buoyant density and corresponding increase in Tm with increasing antibiotic concentrations; a persistant binding in CsCl and in 0.1 x SSC at high temperatures; a more effective binding to DNA of high %GC than to DNA of low %GC and finally, once bound these compounds are extremely difficult to remove. Their mode of binding has not been definitely characterized although most investigators agree that intercalation is involved. These antibiotics do not, however, have the spectral characteristics of streptomycete spore DNA nor do they contain phosphate. Unlike the substance from S. venezuelae spores, anthracyclines bind to DNA in aqueous solutions. These facts limit the usefulness of these compounds as a model system for studying the streptomycete spore DNA complex.

There are a number of reasons to believe that the pH

indicator pigment isolated from whole S. venezuelae spores is the substance bound to the spore DNA. First, the spectral characteristics of the pigment in acidic and basic solutions corresponds very closely to similarly treated spore DNA. Second, the pigment is produced only by spores and not by mycelia. Third, the time course of pigment production in spores parallels the production of aberrant DNA. Fourth, the chromogenic ability of the pigment, like S. venezuelae spore DNA, is destroyed by ninhydrin. Finally, the pigment can be bound to DNA. Although no structural characterization of the pigment is available, some of its physical properties are useful in deducing the probable nature and significance of the compound. S. venezuelae S13 often produces a brown soluble pigment. The brown-black pigment is sometimes referred to as "melanin" or "melanin-like". The pigment isolated from S. venezuelae spores is not of this class because unlike melanin, the spore pigment is soluble in water, yellow in color at pH's up to 12 where it changes to a pink color and finally is not precipitated from alkaline solutions by HCl.

One of the most obvious properties of the isolated pigment is its existence in two molecular weight forms. The high molecular weight pigment has both polar and non-polar characteristics being soluble in water, butanol and benzene. The low molecular weight pigment is soluble in water and butanol, but not in benzene. The high molecular weight pigment can be most easily thought of as being composed of a complex of a non-polar material (membrane fragment?) with

the polar pigment. The nature of the binding between the polar and non-polar components is such that it can be easily disrupted by base and the activity of silicic acid. The spores themselves excrete the low molecular weight pigment into the media. It appears that most of the pigment isolated from the spore itself is complexed with the non-polar fragment.

The apparent association of the suspected DNA-binding pigment with a membrane-like fragment is significant. Electron micrographs of S. venezuelae S13 spores show a membrane associated with the genome (Bradley and Ritzi, 1968). The pigment could link the DNA to the membrane in some manner. This association could be a mechanism for protection of the spore DNA from dessication. In this capacity, the pigment may attach to the DNA when water is absent and be removed when water is present. This same mechanism could also be invoked to explain the phenomenon of germination. During the quiescent stage of the spore's life cycle, the pigment may be bound to the DNA, repressing most functions. When conditions are suitable for germination (when water is present), the pigment is released, de-repressing the system, and outgrowth occurs.

It is also conceivable that there could be no in vivo function of the pigment-DNA complex. The complex may be formed only as an artifact of the DNA extraction procedure. In any event, however, a compound which binds to DNA in the manner observed with S. venezuelae spore DNA will have experimental utility. If specific base sequences or DNA conforma-

tion are required for effective binding of this compound, it could prove useful in probing the structure and function of nucleic acids.

If further work proves that the DNA binding function of the pigment is indeed an artifact of the extraction procedure, the role of pigment in sporulation and germination must not be overlooked, if for no other reason than the pigment is produced only by spores. If the pigment has a function in sporulation, it should be possible to find similar pigments in related streptomycetes. Although pigmented DNA has been isolated from spores of selected streptomycetes, no work has been done on characterization of these spore pigments with respect to the S. venezuelae S13 pigment.

It is conceivable that the pigment may function in the extensive membrane rearrangement that occurs during sporulation or in germination. Moreover, the lipophilic nature of the pigment could suggest a transport function. For example, molecules that cannot pass through the membrane could be transported out of the thick walled, hydrophobic spore by attachment to the pigment. The pigment may also be involved somehow in the commitment of the cell to sporulate. If the pigment proves not to have a direct functional role in sporulation, its biosynthesis may nonetheless be necessary. For example, its synthesis could result in reduced internal pools of certain compounds thereby de-repressing or repressing certain enzymes. The pigment could function as a biosynthetic scavenger, eliminating toxic products by incorporating them in its structure. The compound's pigmented properties

could also suggest a function in shielding the spore from certain harmful wavelengths of light.

Summary

Deoxyribonucleic acid (DNA) analyses were used to assess on a molecular level, the relationships among representatives of the genera Streptomyces, Nocardia and Mycobacterium. The methods developed in this study have been used for routine analysis of DNA from a large number of actinomycetes and have given reliable and reproducible data.

DNA isolated from various actinomycetes was characterized by buoyant density determinations in CsCl from which the mole fraction guanine plus cytosine (GC) content was calculated. All the streptomycete DNA preparations studied had buoyant densities in the range of 1.7287 to 1.7312 g cm^{-3} which corresponded to GC compositions of 70% to 73% GC respectively. The nocardial DNA preparations tested fell in two groups, one with a GC content in the range of 62 to 64% GC and another in a 68 to 70% GC group. The mycobacterial DNA tested had GC values overlapping those of the nocardial DNA specimens; moreover, mycobacterial DNA exhibited a bimodal clustering of GC values, 64 to 65% GC and 67 to 70% GC. All DNA preparations examined by equilibrium buoyant density centrifugation in CsCl contained a single component with no satellite bands.

The method of Warnaar and Cohen for assay of DNA/DNA reassociation on membrane filters was modified for studying

reassociation of DNA in high GC organisms. DNA isolated from selected actinomycetes was tested for homology with Streptomyces venezuelae S13 mycelial DNA by direct reassociation experiments. Unlabeled DNA from the various actinomycetes was immobilized on Schleicher and Scheull nitrocellulose B-6 membrane filters and then incubated for 15 to 20 hr at 70 C with ^{14}C-labeled DNA. The measure of relatedness was the relative percentage of renaturation of a denatured test DNA with labeled, denatured homologous DNA. Unrelated DNA having GC contents of 50 and 70% were included as controls. The streptomycetes studied were relatively homogeneous in that measureable interspecific duplexes were formed between the reference DNA and all streptomycete DNA examined. Significantly, the results also suggested that S. venezuelae S13 was related to the nocardial specimens examined but was not related to the mycobacterial cultures studied. The results agreed generally with prior agar-gel studies on DNA reassociation and with previous classifications.

Nucleotide sequence divergence in DNA extracted from streptomycetes and nocardiae was determined by measuring the extent of renaturation at 60 C and 70 C. The use of thermal elution of labeled, renatured duplexes from filters substantiated the existence of a class of nucleotide sequences which can reassociate at 60 C but cannot reassociate at the more exacting 70 C incubation temperature. The use of exacting incubation conditions (70 C) permitted the formation only of those DNA duplexes that exhibited a high

degree of thermal stability and hence, closely related to the reference DNA. The non-exacting 60 C incubation allowed those sequences to associate which were distantly related. The ratio of binding at 70 C to the binding at 60 C was designated the Divergence Index (DI). The DI was useful for gauging the presence or absence of closely related genetic material and for determining divergence patterns. The conclusions obtained from this method were corroborated by the much more time consuming thermal elution method. The divergence studies suggested that the streptomycetes contain a wide spectrum of related sequences compared to the reference DNA. Interestingly, the nocardiae examined seemed to have a small but significant amount of conserved nucleotide sequence compared to the S. venezuelae S13 reference.

During these studies on actinomycete DNA it was realized that DNA from S. venezuelae S13 spores had novel properties. As spores aged the buoyant density in CsCl decreased from 1.727 to 1.707 g cm^{-3}, the midpoint of thermal denaturation (Tm) in 0.1 x SSC increased from 85 to 88.5 C, and the apparent reassociation with mycelial DNA decreased from 100 to 30%. Spore DNA in 5 M $NaClO_4$ had the same Tm as mycelial DNA. Spore DNA (1.707 g cm^{-3}) after heat denaturation showed a single band in CsCl (1.722 g cm^{-3}). Spore DNA was resistant to pancreatic deoxyribonuclease I, but became progressively sensitive after treatment with 0.5 M sodium acetate. Chemical nucleotide analysis of spore and mycelial DNA showed no detectable difference in

GC content. The aberrant nature of spore DNA was not affected by pronase or ribonuclease. Washing the spores with ethanol and acetone prior to DNA extraction restored the isolated DNA to normal buoyant density and Tm values. When alcohol and chloroform extracts of spores were dried and mixed with authentic DNA preparations, no change in Tm or buoyant density was found. Spore DNA was yellow in color at pH values below 12 and pink in color at values above 12. Spore DNA heated in high salt showed three characteristic peaks in Sephadex G-100 column chromatography, but only one peak was found with comparably treated mycelial DNA. Attempts to characterize these peaks have been inconclusive to date. Chemical analyses of spore DNA showed 15 to 20% Folin positive material, 40 to 50% more phosphorus than mycelial DNA and no detectable sugars. It appeared that whatever was bound to the spore DNA could be partitioned to added DNA. Experiments of this type were successful only if the test DNA was added to freshly disrupted, dehydrated spores with crushed dry ice. Rehydration of spores showed loss of this binding activity. No chromatographically identifiable compounds or characteristic color were extracted from spore DNA by a number of solvents and conditions including: acetone, ethanol, ethanol-ether, ethyl acetate, butanol, 8 M urea, 5% cold trichloroacetic acid and 1 N KOH at 100 C for 30 min.

A crude pigmented fraction was isolated from spores which had similar chemical characteristics as aberrant spore DNA. This pigment could not be demonstrated in mycelia;

moreover, pigment production seemed to be directly correlated with the age of the spores. The data suggested that this pigment is probably bound to spore DNA and is responsible for the aberrant characteristics of S. venezuelae S13 spore DNA.

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