# CHARACTERIZATION OF THE DEOXYRIBONUCLEIC ACID OF VEGETATIVE CELLS OF AZOTOBACTER VINELANDII

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

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1972

This is to certify that the

thesis entitled

CHARACTERIZATION OF THE DEOXYRIBONUCLEIC ACID OF VEGETATIVE CELLS OF *AZOTOBACTER VINELANDII*

presented by

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has been accepted towards fulfillment of the requirements for

Ph. D. degree in Microbiology and Public Health

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Date September 27, 1972

ABSTRACT

CHARACTERIZATION OF THE DEOXYRIBONUCLEIC ACID OF VEGETATIVE CELLS OF *AZOTOBACTER VINELANDII*

By

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A study of the DNA of *Azotobacter vinelandii* was made. Vegetative cells of *A. vinelandii* were found to contain $6.7 \times 10^{-14}$ grams of DNA per cell and two stainable nuclear bodies, while cysts contained one half as much DNA and only one nuclear body. The minimal DNA content per *Azotobacter* cyst is thus an amount eight times that of the *E. coli* genome.

Melting curves of *A. vinelandii* DNA when plotted on normal probability paper show a bimodality, indicating two components with different guanine + cytosine (G+C) contents. Both components are in about equal concentration in the cell, one having 61% G+C and the other 69% G+C.

DNA renaturation experiments on *A. vinelandii* DNA have been shown in this study to deviate from second order kinetics, unlike typical bacteria. Analysis of the data resolved the renaturation curve into the sum of

two second order curves, characterized by $C_o t_{1/2}$ values (the initial concentration of DNA times time at the 50% renaturation point) of 1.3 and 8.9 (corrected for G+C effects). This corresponds to unique nucleotide sequence lengths which are 0.4 and 3.1 times as long as that of the E. coli genome, i.e. $1.2 \times 10^9$ and $7.9 \times 10^9$ daltons, respectively.

Deviation from second order kinetics has also been substantiated by the discrepancy between unique sequence sizes obtained from initial renaturation rates and $C_o t_{1/2}$ values on A. vinelandii DNA. Initial renaturation rates of this DNA have indicated a unique sequence size about half the value of that obtained from the $C_o t_{1/2}$ value of the total Azotobacter renaturation. This rate has been shown to correspond to the expected initial rate when two components of $C_o t_{1/2}$ values of 1.3 and 8.9 are present.

The slow component of the analyzed renaturation curve ($C_o t_{1/2} = 8.9$) was concentrated by allowing the renaturation to proceed to 88% completion and fractionating the DNA on an hydroxylapatite column. The single-stranded DNA, unrenatured at the time of fractionation, consists mainly of the slow component (approximately 85%) since the greater portion of the fast component ($C_o t_{1/2} = 1.3$) has renatured by the time of fractionation.

This concentrated slow component was then allowed to renature to completion. Values for unique sequence size were obtained from this renaturation curve. The curve could also be resolved into two components of $C_o t_{1/2}$ values of 1.3 and 8.9 respectively, as was the case for the total Azotobacter renaturation.

Attempts to separate the two G+C components of A. vinelandii DNA using CsCl density gradients, NaI gradients and both in combination with a relaxation centrifugation technique were only partially successful since the peaks showed considerable overlap. This was attributable to a shearing of the DNA which was inherent in the purification procedures and a model is presented to account for the poor separation.

A number of models are devised to describe the possible organization of the DNA within the cell.

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By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology
and Public Health

1972

Dedicated to my wife, Nancy

ACKNOWLEDGMENTS

I wish to thank Dr. Harold Sadoff for his excellent guidance and for the many stimulating discussions we have had during the course of my graduate work. I am grateful to Dr. Roy J. Britten for the opportunity to visit his laboratory to become acquainted with the techniques of DNA renaturation studies. I also wish to acknowledge the informative discussions I have had with Dr. Donald Brenner concerning this work. Lastly, I wish to thank Brian Berke for his technical assistance in preparation of the figures in this thesis for publication.

Financial assistance from the National Institute of Health and the Department of Microbiology, Michigan State University, is gratefully acknowledged.

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# INTRODUCTION

Azotobacter vinelandii is a nitrogen-fixing soil organism capable of encystment (39, 46, 53). The encystment process can be induced by growth on B-hydroxybutyrate (BHB), butanol and other related compounds (25). Growth of A. vinelandii vegetative cells on BHB results in a final cell division (41), loss of flagella, accumulation of poly-B-hydroxybutyrate and formation of outer cyst layers to form a mature cyst. Cysts are thick-walled, spherical, resting cells which are highly resistant to ultraviolet light, sonication, and dessication (47). Although both cysts and spores are eubacterial resting cells, cysts differ from spores in that they are not heat resistant, contain no dipicolinic acid (46) and loss of calcium has no effect on their viability and dessication resistance.

The suspension of cysts in glucose and nitrogen-free salts solution (Burk's buffer) stimulates germination and outgrowth to form vegetative cells. Such cells are large (3 x 1 μm), rod-shaped, gram negative, highly motile and obligately aerobic.

The life cycle of A. vinelandii has been compared to sporulation in Bacillus species and has been used as

a model system for studying cellular differentiation. DNA levels during encystment have been investigated (41) and the DNA content of the resting Azotobacter cyst has been determined to be $3.4 \times 10^{-14}$ grams while vegetative cells have 2 to 4 times this amount (41).

Similar DNA contents per cell from synchronous cultures of A. vinelandii cells have been reported by Zaitseva (54). Muller and Kern (35) found comparable levels of DNA in radiation resistant mutants of Azotobacter chroococcum.

Presumably the cyst contains the minimal amount of DNA needed for the cell to survive. Nuclear staining (41) has shown the cyst to have one stainable nuclear body, probably corresponding to one genomic unit.

This amount of DNA is about 8 times that of the E. coli genome. It is unlikely that A. vinelandii has a range of biochemical capabilities 8 fold greater than E. coli and this fact, coupled with the high levels of DNA per cell, has led to the present study into the nature of the DNA of A. vinelandii.

A number of other reports have suggested that the DNA of A. vinelandii might differ from other bacteria. Mishra and Wyss (33, 34) have reported difficulty in obtaining A. vinelandii mutants. Gunter and Kohn (19) have shown A. agilis to exhibit multiple-hit survival curves when subjected to x-irradiation, unlike other

bacteria which show single-hit curves. Pochon (39) has described the Azotobacter "nucleus" to be vessicular in nature and similar to the yeast nucleus in appearance.

It was the aim of this research to characterize the DNA of A. vinelandii in terms of: G+C content, unique nucleotide sequence size and renaturation kinetics.

# LITERATURE REVIEW

## The Azotobacteriaceae

Much of the early work on the family Azotobacteriaceae was concerned with the groups' ability to fix nitrogen. Although other organisms such as blue-green algae, purple and green bacteria and some Clostridium and Desulfovibrio species also fix nitrogen, few incorporate as much as the Azotobacter species (21). Six species of Azotobacter have been described in the literature: Azotobacter vinelandii, Azotobacter beijerinkii, Azotobacter chroococcum, Azotobacter insignis, Azotobacter macrocytogenes, and Azotobacter agilis. Although these six species are all classified as Azotobacter, there is considerable evidence to indicate that they should not all be grouped together. DeLey and Park (16) have shown by analysis of DNA base ratios and DNA hybridization among the strains that the Azotobacteriaceae should really be divided into 3 groups. The vinelandii-beijerinkii-chroococcum group consists of organisms having a range of 65.7 $\pm$ 0.8% G+C and hybridizable with Pseudomonas putida DNA to the extent of 40-50%. The insignis-macrocytogenes group has G+C contents ranging from 57-58.6%. These strains are 50-60% hybridizable with Ps. putida DNA. The third group consists of the insignis strain which has 52.9 $\pm$ 0.4% G+C and is

about 30% hybridizable with Ps. putida DNA. DeLey has suggested that only the first group be designated Azotobacter while the other two groups should be termed Azomonas and Azotococcus respectively.

A. vinelandii, the organism used in this study, was first isolated in 1904 by Lipman (26). The organism is a large rod or peanut-shaped cell with an average size of 3 x 1 μm. It is highly motile by peritrichous flagellation (4), produces a soluble pigment which fluoresces apple-green under ultraviolet light (16) and undergoes a life cycle involving a dessication resistant, resting cell known as a cyst.

Relatively little research has been carried out on the DNA of A. vinelandii. Olson and Wyss (37) fractionated the nucleic acids from dormant and germinated cysts of Azotobacter on methylated albumin-kieselguhr (MAK) columns and noted changes in DNA configuration after germination. Pochon (39), in light microscope studies, showed the nuclear body of A. vinelandii to possess a number of chromatinic granules making up a vessicular nucleus. During cell division the nuclear material takes the form of two or four large rods which divide with the cell and then fragment to reform the vessicular nucleus.

DNA--Historical Survey

In the late 1800's, F. Miescher (32), while studying the cell nucleus in animal cells, was the first to isolate and chemically characterize pure DNA from salmon sperm. The purines and pyrimidines comprising DNA were isolated in the late 1800's by Kossel (5) and later the deoxyribonucleotides and deoxyribonucleosides corresponding to the four bases were isolated and characterized. The tetranucleotide model for DNA structure was relatively long-lived. It was suggested in 1909 (20) and disproved in 1948 (21). Briefly this model represented nucleic acids as high molecular weight compounds composed of a large number of tetranucleotides in sequence similar to the amino acids in a protein.

In 1944, Avery, MacLeod and McCarty (3) showed DNA to be the agent responsible for Pneumococcal transformation and in so doing established the chemical nature of genes. Soon thereafter the composition of DNA was further elucidated by the observations of Vischer et al. (48) that adenine and thymine and guanine and cytosine showed equal molar proportions (i.e. adenine and thymine concentrations were always equal as were guanine and cytosine concentrations). The present model for DNA structure, commonly called the Watson-Crick structure (49), can be summarized as follows:

1. The amount of adenine equals the amount of thymine and the amount of guanine equals the amount of cytosine.
2. Adenine pairs only with thymine and guanine pairs only with cytosine.
3. The DNA molecule consists of two right-handed helical polynucleotide chains of opposite polarity (i.e. internucleotide linkage in one strand is 3' to 5' and 5' to 3' in the other).
4. Bases are on the inside of the chain and a purine on one strand always pairs with a pyrimidine on the other.
5. The distance between base pairs is 3.4 Å and there are ten bases per turn of the helix.

## Methods of Studying DNA

Since the Watson-Crick model, many techniques have been developed to study and characterize the DNAs of various organisms. Native DNA molecules undergo a process called denaturation in the presence of a number of agents, viz increased temperature, addition of hydroxyl or hydrogen ions and a large variety of organic reagents. Denaturation involves the transition of native double-stranded DNA to single-stranded DNA through hydrogen bond breakage between purine-pyrimidine pairs. A considerable amount of information about the DNA of an organism can be

obtained from a DNA melting curve (thermal denaturation curve). Typically this technique involves the heating of a solution of DNA (at neutral pH) from room temperature to some higher temperature (usually about 100C) until no further strand separation is observed. Melting curves are carried out in an ultraviolet spectrophotometer where the change in absorbance at 260nm is used to monitor the strand separation. As strand separation proceeds, the absorbance at 260mm increases due to the "unstacking" of the bases in the molecule. Melting curves are sigmoidal in shape and are characterized by the position of their midpoints ($T_m$, the temperature at which half the DNA is denatured) and the steepness of the slope at this point. Changes in ionic strength of the solvent or addition of various denaturing agents will shift the position of the $T_M$ for any DNA. Marmur and Doty (29) have shown that there is a linear relationship between the base composition (G+C%) and $T_M$. Thus the G+C content of a DNA can be determined by obtaining its $T_M$ in a solvent of known ionic strength.

Another parameter obtainable from a DNA melting curve is the degree of hyperchromicity exhibited by the DNA sample. The hyperchromic shift as it is called is represented by the percentage of absorbancy (at 260nm) increase seen following denaturation. This parameter is also dependent on the G+C content of the native DNA,

with DNAs of higher G+C contents having lower hyperchromicities (e.g. a DNA of 63% G+C has a hyperchromicity of 29% while a DNA of 51% G+C has a hyperchromicity of 39%).

DNA melting curves may also be analyzed by the method of Knittel (22). This technique involves plotting thermal melting data on normal probability paper. Gaussian distributions when plotted in this way give a straight line. Melting data yields a straight line and allows for accurate determination of $T_m$ and a rapid determination of the standard deviation of the compositional distribution of the DNA. Such plots may also be used to reveal compositional heterogeneity if a broken line is obtained.

Russel et al. (40) have described another technique which allows for the calculation of the base composition of nucleotide sequences which melt in different portions of the DNA melting curve. Briefly the method consists of obtaining ultraviolet wavelength scans from 245 to 280nm at a number of temperatures along the melting curve. A formula based on the molar extinction coefficients of AT and GC pairs at various wavelengths, is used to determine the base composition of DNA melting during any temperature increment along the melting curve.

Among the most recent techniques employed in DNA research is the study of the kinetics of the reassociation of DNA. This technique was first employed by

Britten (9) and others in the middle 1960's to show the presence of repeated nucleotide sequences in the DNAs of many higher organisms. DNA, when denatured to its single-stranded state and incubated at fairly high salt concentrations, will reassociate (return to its double-stranded form). The process involves the random collision of single-stranded fragments until a complementary sequence of base pairs is encountered and bonding between the base pairs results in double-stranded DNA. DNA renaturation (reassociation) follows second order kinetics (see Appendix B). Marmur et al. (30) originally explored the conditions necessary for efficient reassociation and others (9, 50, 51) have further refined the techniques. The requirements for reassociation are:

1. An adequate concentration of cations (renaturation is blocked below 0.01 M sodium ion),
2. An incubation temperature high enough to weaken intrastrand secondary structure (optimal renaturation temperature is about 25C below the temperature for dissociation of DNA),
3. An incubation time and DNA concentration sufficient to allow an adequate number of collisions to take place,

4. A homogeneous population of DNA fragments about 500 nucleotides in length (the larger the fragment the faster the rate).

A DNA renaturation experiment would thus involve, DNA purification, shearing to small fragment size (about 500 nucleotides), dialysis against a suitable buffer, denaturation of DNA and the monitoring of the reassociation of the strands. The reassociation of DNA can be assayed in a number of ways, each dependent on some detectable difference between double (reassociated) and single (unreassociated) stranded DNA. For example, one of the most commonly used techniques is ultraviolet spectrophotometry (30). This method is based on the fact that dissociated (denatured) DNA absorbs more ultraviolet light than reassociated DNA (at 260nm). Thus renaturation reactions are carried out in water jacketed thermal cuvettes in a spectrophotometer and the absorbance at 260nm is monitored. This technique has the advantage of requiring little DNA (about 80 µg).

Another commonly used technique takes advantage of the fact that single and double stranded DNAs can be separated on an hydroxylapatite (calcium phosphate) column (9). Thus renaturation is followed by removing samples from a DNA solution which is renaturing and fractionating the double and single stranded DNA on hydroxylapatite. This procedure provides an estimate

of how much DNA has renatured. This technique has the advantage that the DNA concentration can be increased greatly over the spectrophotometric method so that slow renaturations can be speeded up. It also allows for fractionation of DNA species of different renaturation rates.

A third technique which may be used is the DNA-agar method (31). Long strands of DNA are physically immobilized on a support substance and radioactivity labeled fragments of single stranded DNA are added. Following various incubation times the unbound labeled fragments are washed away and the bound radioactivity is assayed by standard techniques.

DNA renaturation kinetics can be characterized as follows:

1. The renaturation reaction follows second order kinetics,
2. The maximum rate of renaturation occurs 25C below the dissociation temperature of the DNA,
3. Decreasing the molecular weight of the fragments, decreases the rate of the reaction,
4. Renaturation rate decreases as the genetic complexity of the source increases (i.e. as the unique sequence size increases),

5. The rate is very dependent on ionic strength below 0.4 M sodium ion.

The unique sequence length of the DNA of an organism can be obtained from the results of a renaturation experiment. The % renaturation is plotted on semi-logarithmic paper versus $C_o t$ (the initial DNA concentration in moles of nucleotides/liter times time).* The $C_o t$ value at which 50% reassociation has taken place is termed the $C_o t_{1/2}$ and is characteristic of each organism. This value is indicative of the minimal length of the unique sequence of the organism's DNA or in the case of most bacteria the genome size. E. coli DNA is used as a standard since its genome size has been measured quite accurately by Cairns (13). The shape of a renaturation curve is important in determining whether an organism contains repeated base sequences in its DNA. Non-repeated DNA, characteristic of most procaryotes, gives the typical second order S-shaped plot. Eucaryotic DNAs have been shown to deviate from second order kinetics. This is easily visualized in most eucaryotes where the curve is biphasic. For example, calf DNA shows a biphasic renaturation curve (10). Forty percent of the calf DNA has already reassociated at a $C_o t$ value of 2. Little

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*As a general rule, dissociated DNA of $OD_{260} = 2$ renatured for 1 hour in 1 SSC at the optimal renaturation temperature gives a $C_o t$ of 1.

renaturation takes place between values of 2 and 200. The remaining 60% has a $C_o t_{1/2}$ of about 3000. The rapidly reassociating fraction of calf DNA has a $C_o t_{1/2}$ of 0.03 as compared to 3000 for the slow fraction. Thus the concentration of DNA sequences which reassociate rapidly is 100,000 times the concentration of those which renature slowly. Therefore 40% of the calf genome consists of unique sequences repeated about 100,000 times. To date all procaryotic DNAs studied have shown no redundant DNA, while all eucaryotic DNAs have contained repeated DNA sequences.

The function of repeated sequences in DNA is unknown. A number of possible roles have been suggested. Multiple copies of a gene would allow for higher rates of synthesis. This might be necessary for the synthesis of structural proteins found in large amounts in the cell, or for ribosomal RNA. Multiple similar copies could produce a class of proteins which were similar such as antibody proteins. Britten (8) has also suggested the possibility of highly repetitive DNA sequences having a regulatory role. Finally, Crick (10) has proposed that repeated DNA sequences play a structural role by interacting with the chromosomal proteins in eucaryotes.

Initial rates of a renaturation reaction can be used to obtain unique sequence sizes on non-repeated DNA.

Gillis (18) and Brenner (6) have determined genome sizes for a number of bacteria using this method.

Density gradient centrifugation is of value in studying DNA (42), since the buoyant density of DNA in CsCl is directly proportional to its G+C content. This technique is used to determine the G+C contents of an unknown DNA and to effect the separation of DNAs of varying G+C contents. A modification of the CsCl buoyant density centrifugations is the density gradient relaxation method as described by Anet (1). This technique takes advantage of the facts that the "steepness" of the gradient formed is dependent on the speed of centrifugation and that CsCl diffuses rapidly relative to the diffusion of DNA. Operationally, centrifugation is first performed at a high speed to form the gradient and separate and concentrate the 2 DNA species. The rotational velocity is then brought to the lower value to form the desired density gradient. The final gradient is quickly formed following change over to the slower speed. DNA molecules in such a gradient seek their new densities after relaxation (slow speed) and thus the time required for a density gradient run is shortened. Anet (2) has also described the use of sodium iodide gradients with and without ethidium bromide to separate DNA mixtures. Ethidium bromide intercalates in DNA and

lessens its buoyant density. Anet was thus able to separate more efficiently mixtures of DNAs of different G+C contents, since high G+C DNAs take up less ethidium bromide than lower G+C DNAs.

## EXPERIMENTAL METHODS

### Strains and Cultivation

A. vinelandii (ATCC 12837) was grown from a single isolated cyst from a laboratory stock culture. Cells were grown on Burk's nitrogen-free medium plus 0.5% glucose at 30C on a rotary shaker with 100-200 milliliters of culture medium per 500 milliliter flask. Larger batches were grown in a 3 liter fermenter (New Brunswick Scientific Co., New Brunswick, New Jersey) or in a 100 liter pilot-plant fermenter (Stainless and Steel Products Co., St. Paul, Minn.). Cells were harvested in a continuous-flow centrifuge (The Sharples Corp., Philadelphia, Pa.).

Burk's nitrogen-free medium as described by Wilson and Knight (52) is made up of the following ingredients:

| | |
|---|---|
| $KH_2PO_4$ | 0.2 gm/l |
| $K_2HPO_4$ | 0.8 " |
| $MgSO_4 \cdot 7H_2O$ | 0.2 " |
| $CaSO_4 \cdot 2H_2O$ | 0.1 " |
| $FeSO_4 \cdot 7H_2O$ | 5.0 mg/l |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.25 " |

Glucose was dissolved in distilled water and sterilized by filtration.

E. coli K12 and Pseudonomas aeruginosa were grown on nutrient broth in shake flasks at 30C.

## Determination of DNA Content per Cell

Three methods were employed in determining the DNA content of A. vinelandii cells. These were grown in Burk's medium to late log phase and cell numbers were assessed by direct counts in a Petroff-Hauser counting chamber and by dilution plating. In the first method, the culture was chilled, cells harvested and washed by centrifugation in 0.1M tris- 0.1M NaCl buffer (pH 9) and resuspended in the same buffer. Cold 10% sodium dodecyl sulfate (SDS) was added to give 1% SDS and the suspension was stirred for 20 minutes in an ice bath. An equal volume of cold water-saturated phenol was added and the mixture stirred for another 20 minutes, after which the aqueous and phenol layers were separated by centrifugation. The aqueous layer was precipitated with cold ethanol, the precipitate resuspended in 0.1M NaCl and DNA assayed by the diphenylamine assay of Burton (12).

A second method involved extraction of a known number of cells with 10% trichloroacetic acid (TCA) at 95C for 15 minutes and DNA levels determined by the Burton method.

The third method estimated DNA per cell using a modification of the Schmidt-Thannhauser nucleic acid fractionation (42). Cells were harvested as before and washed twice with Burk's buffer (Burk's medium without

glucose). The cells were resuspended in 7% cold TCA (30 ml) and were stirred for 20 minutes in the cold. The mixture was centrifuged and the pellet reextracted with 30 ml 10% TCA. The pellet obtained from centrifugation was suspended in 5 ml of water and 20 ml of 95% ethanol and centrifuged. One extraction with 25 ml of 100% ethanol, and three extractions with ethanol:ether (3:1) were carried out at room temperature with stirring. The precipitate was then collected by centrifugation and hydrolized for 15 hours at 37C in 1N KOH. Following hydrolysis the solution was neutralized with HCl and the DNA precipitated with 1 volume of 5% TCA and the precipitate washed twice with 5 ml 5% TCA by centrifugation. The final pellet was extracted twice with 5% TCA at 90C for 15 minutes, cooled and centrifuged. Supernatants contained the DNA and were assayed by the diphenylamine method of Burton, an inorganic phosphorus assay and the indole assay.

## Nuclear Staining

Nuclear staining of A. vinelandii cells was performed by a modification of the procedures of Piekarski (38). Cells were air dried on clean slides and fixed in 70% ethanol for 1 hour. Hydrolysis of cellular RNA was then carried out by incubation in 1N HCl for 30 minutes at 60C. After washing with water the cells were

stained with Giemsa stain for 5-10 minutes. Concentrated Giemsa stain is prepared by mixing 0.5 grams Giemsa with 33 ml glycerol and 33 ml methanol and heating for 2 hours at 60C. Diluted Giemsa is used for staining and consists of one drop of concentrated Giemsa stain per 1 ml 0.1 M phosphate buffer pH 6.8. With this technique cell cytoplasm appears sky blue and nuclei dark purple.

## Preparation of DNA

Cells were harvested in late log phase, washed and frozen. A modification of the Marmur technique (28) for DNA purification was used with two additional RNAse incubations (100 µg/ml RNase for 1 hour at 37C) and a pronase treatment using 100 µg/ml of the enzyme. The pronase was self-digested for two hours at 37C to rid it of DNAse prior to treating the DNA overnight at 37C.

DNA purity was monitored by observation of ultraviolet spectra where the 260/280 nm ratio was required to be close to 2. DNA was assayed by a number of methods including Burton's diphenylamine assay (12), Ceriotti's indole assay (14) and a modification of the Schmitt-Thannhauser inorganic phosphorus assay of Schneider (44). RNA was assayed by the orcinol method for pentose (11) and protein was assayed by the method of Lowry (27). DNA was considered pure when RNA and protein levels were less than 1% of the total preparation. DNA preparations were stored at -20C.

## DNA Melting Curves

All DNA melts were carried out in 0.1 x SSC* buffer. DNA solutions with an absorbance of approximately 0.5 (at 260 nm) were placed in jacketed cuvettes (Arthur Thomas Co., Philadelphia, Pa.) of 1 cm light path for temperature control during observation. The cuvettes were placed in an Hitachi DB spectrophotometer and the temperature controlled by an insulated circulating water bath (Sargeant Co., Detroit, Mich.). The temperature of the solution in the cuvette was monitored using a YSI thermister thermometer with the probe cemented into the cuvette plug (Yellow Springs Industries, Yellow Springs, Ohio). The absorbance at 260 nm was read and the temperature raised quickly to about 10C below the onset of the melting region. The temperature was then raised in approximately 1C increments, allowing 10 minutes for equilibration at each temperature. The optical density at 260 nm was read at each temperature and corrected for thermal expansion ($V_t/V_{25C}$). Data were plotted on both rectangular coordinate paper and on normal probability paper by the method of Knittel (22). Scan melts used in the method of Russel (40) were carried out as above and at each temperature values for optical density at 245 to 280 nm in 5 nm increments were obtained.

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*SSC buffer is 0.15M NaCl plus 0.015M sodium citrate pH 7.

## Preparation of DNA for Renaturation

Shearing of DNA was accomplished by two passages through a French pressure cell (American Instrument Co., Silver Springs, Md.) at 15,000 lbs/in$^2$. To minimize DNA denaturation during the shearing process, the DNA was dissolved in 5 x SSC buffer and the pressure cell was cooled in ice before use. Following shearing DNA samples were dialyzed to equilibrium against 0.1 x SSC buffer.

## Renaturation Techniques

The reassociation of DNA was assayed spectrophotometrically. Sheared DNA in 0.1 x SSC buffer was denatured by heating at 100C for 5 minutes. The DNA was immediately diluted with an amount of 10 x SSC buffer which yielded a final concentration of 2 x SSC buffer and an optical density of 1.5 - 2.0 at 260 nm. The DNA was then transferred to a water-jacketed cuvette preheated to the desired renaturation temperature in the spectrophotometer. The total time necessary for mixing and transfer of the DNA to cuvettes was about 2 minutes. Optical densities at 260 nm were read every 5 minutes for the first hour (for initial renaturation rates) and every half-hour thereafter.

## Hydroxylapatite Fractionation of Double and Single Stranded DNA

Sheared DNA in 0.14 M sodium phosphate buffer, pH 7 was renatured in a sealed vial. The sample was placed on a thermal jacketed hydroxylapatite column (Biorad Laboratories, Richomnd, Calif.) maintained at the temperature of renaturation and equilibrated with 0.14 M sodium phosphate buffer, pH 7. The sample was washed through the column with 2 ml portions of the same buffer to stop the renaturation and fractionate double and single stranded DNA. The absorbances of the eluates was monitored at a wavelength of 260 nm. When the absorbance of column eluates had returned to a basal level, the column was washed with 0.4 M sodium phosphate buffer pH 7 in 2 ml aliquots and monitored as above. A fractionation of double (renatured) and single (unrenatured) stranded DNA was obtained by this method. Single stranded DNA was eluted in the 0.14 M phosphate buffer and double stranded DNA was eluted from the column with 0.4 M phosphate buffer.

## Density Gradient Centrifugation

Cesium chloride (CsCl) density gradient centrifugation was carried out by the method of Ganesan and Lederberg (17). Optical grade CsCl was used (Schwartz-Mann Bioresearch, Orangeburg, N.Y.) and the average density in the tubes was 1.72 grams/ml. DNA in SSC buffer was added in amounts from about 75 to 200 µg. A

total of 4.5 ml of CsCl was pipetted into 5 ml capacity nitrocellulose centrifuge tubes and layered with mineral oil. Tubes were spun in an SW39 head in the Beckman Model L refrigerated ultracentrifuge at 30,000 rpm for 80 hours at 15C. Tubes were pierced, drops collected, diluted with distilled water, and the absorbance at 260 nm monitored.

An alternate method involved centrifugation at 41,000 rpm for 24 hours at 15C in the SW 50L head. Fractionation was performed as above.

Both of these methods were tried using the density gradient relaxation method of Anet (1) in an attempt to effect better separation of the DNA components.

Sodium iodide gradients with ethidium bromide (2) were also used in combination with the density gradient relaxation technique (1). In this case DNA samples were spun in a type 50 angle head at 50,000 rpm for 20 hours followed by a centrifugation at 35,000 rpm for 50 hours at 15C. Average density of the gradient was 1.55 grams/ml and a total of 5.6 mls per polyalymer tube was used.

## Analysis of G+C Contents

G+C contents were analyzed spectrophotometrically by the method of Hirshman (20). In this method DNA is heated above the temperature at which it is completely denatured and the absorbance values at 240 to 280 nm in

10 nm increments is determined. From these data one can calculate the G+C content of the denatured DNA.

# RESULTS

## DNA Content per A. vinelandii Cell

DNA content per cell was determined by three methods: a phenol DNA extraction, a hot TCA extraction and a modification of the Schmitt-Thannhauser fractionation with the DNA supernate assayed by three different DNA methods. The phenol extraction gave a value of $6.7 \times 10^{-14}$ grams per cell (corrected for a yield of about 70%). Hot TCA extraction gave $6 \times 10^{-14}$ grams/cell. The third method gave results of $6.6 \times 10^{-14}$ grams (Burton diphenylamine assay), $6.7 \times 10^{-14}$ grams (inorganic phosphate assay) and $6.3 \times 10^{-14}$ grams (indole assay) per cell (Table 1).

## Number of Stainable Nuclear Bodies per Cell

The number of nuclear bodies per cell were determined from the same cultures used for DNA assay by the nuclear staining technique described. Cells in early exponential phase were found to contain as many as four nuclear bodies per cell while late exponential cells had two nuclear bodies. Cysts always contained one nuclear body. The amount of DNA in the cyst is presumably the minimal amount of DNA necessary for maintenance of the cell.

TABLE 1.--DNA content per cell of A. vinelandii.

| DNA Extraction Method | DNA Assay Method | DNA/Cell |
|---|---|---|
| 1. Phenol | Diphenylamine | $6.7 \times 10^{-14}$* |
| 2. Hot TCA | Diphenylamine | $6.0 \times 10^{-14}$ |
| 3. Schmitt-Thannhauser | Diphenylamine | $6.6 \times 10^{-14}$ |
| Schmitt-Thannhauser | Inorganic phosphorus | $6.7 \times 10^{-14}$ |
| Schmitt-Thannhauser | Indole | $6.3 \times 10^{-14}$ |

*Corrected for estimated yield of 70%.

## DNA per Nuclear Body

From the amount of DNA per cell and the number of nuclear bodies per cell, the amount of DNA per nuclear body was calculated to be about $3.3 \times 10^{-14}$ grams. This represents a value about 8 times that of the E. coli genome.

## DNA Melting Curves

DNA melting curves were obtained as described. The results are shown in Figures 1 and 2. All curves appear as typical S-shaped curves when plotted on rectangular coordinates except for the mixed melt of E. coli (G+C = 51%) and Ps. aeruginosa (G+C = 68%) in Figure 1. The hyperchromic shift of A. agilis (Figure 2) is low probably due to low purity.

Figures 3 and 4 are sama data plotted on normal probability paper by the method of Knittel. Figure 3 shows the melting curve of an approximately equal mixture of the DNA of E. coli and Ps. aeruginosa. The normal probability plots in Figures 3 and 4 are straight lines only in the case of E. coli, A. agilis, and Ps. aeruginosa. The mixed melt as expected is biphasic as is the A. vinelandii plot (Figure 4). While the mixed melt has a plateau region where no DNA is melting the A. vinelandii plot shows a break at about 54% of the total absorbance increase.

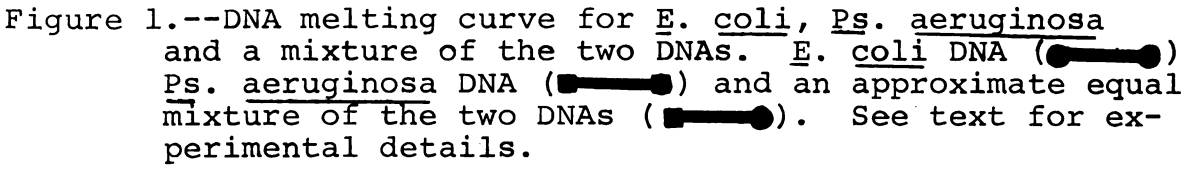


Figure 1.--DNA melting curve for E. coli, Ps. aeruginosa and a mixture of the two DNAs. E. coli DNA (●——●), Ps. aeruginosa DNA (●——●) and an approximate equal mixture of the two DNAs (●——●). See text for experimental details.

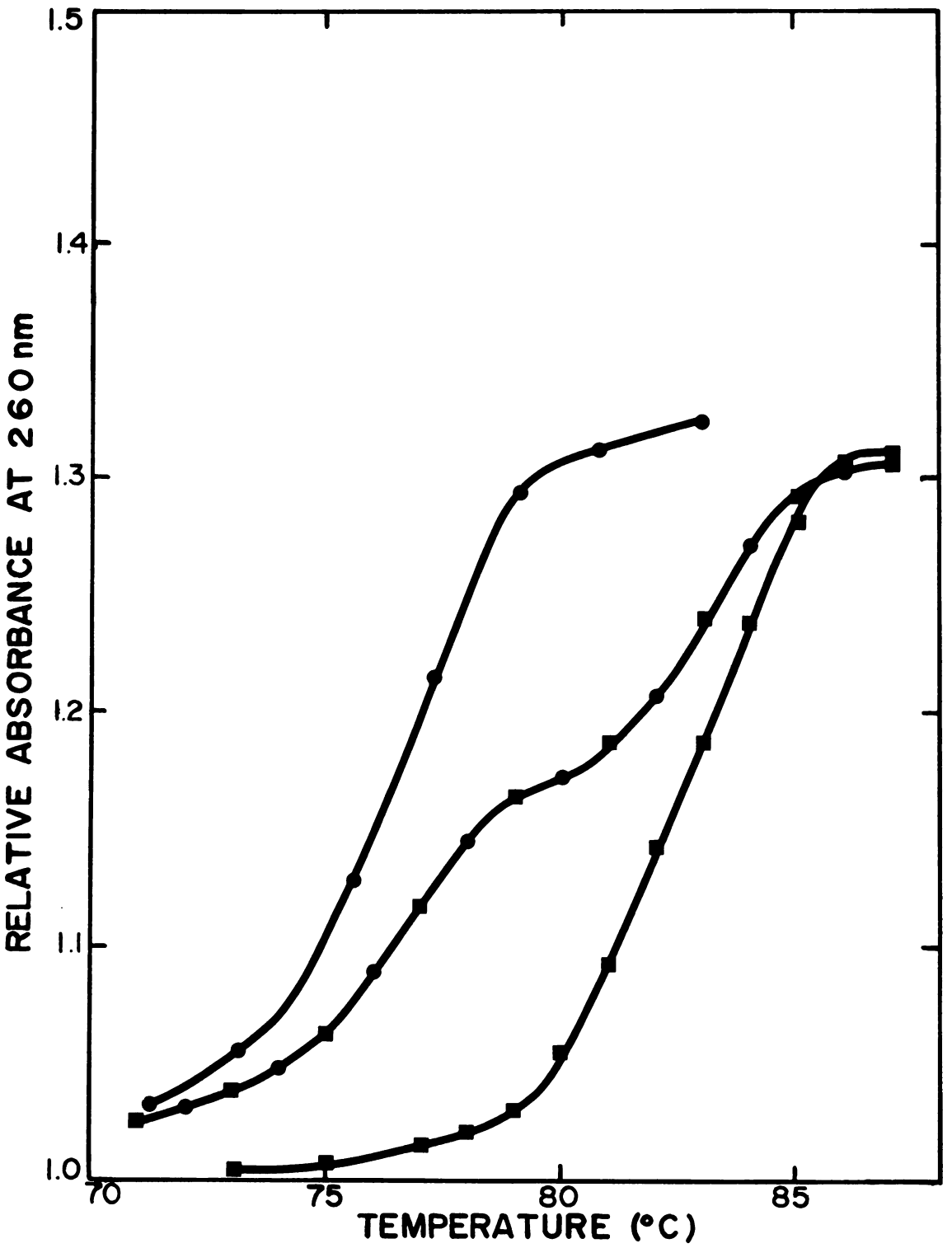
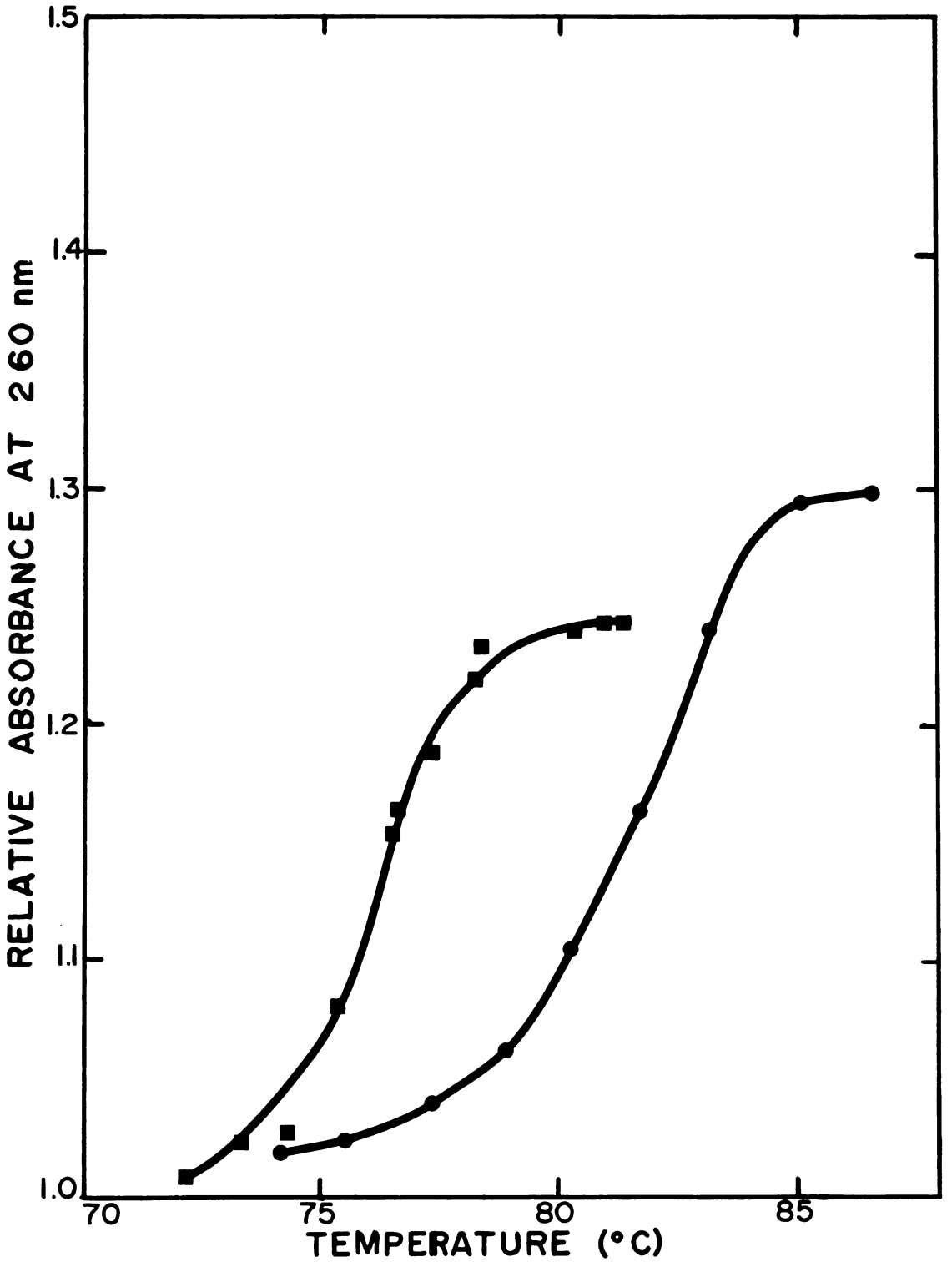

1.5
1.4
1.3
1.2
1.1
1.0
RELATIVE ABSORBANCE AT 260 nm
70
75
80
85
TEMPERATURE (°C)

Figure 2.--DNA melting curve for A. agilis and A. vinelandi DNA A. agilis DNA (▬▬) and A. vinelandii DNA (●▬●). See text for experimental details.


1.5
1.4
1.3
1.2
1.1
1.0
RELATIVE ABSORBANCE AT 260 nm
70
75
80
85
TEMPERATURE (°C)

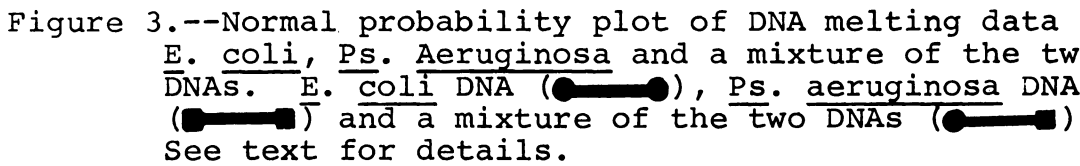


Figure 3.--Normal probability plot of DNA melting data for E. coli, Ps. Aeruginosa and a mixture of the two DNAs. E. coli DNA (●——●), Ps. aeruginosa DNA (■——■) and a mixture of the two DNAs (●——■). See text for details.

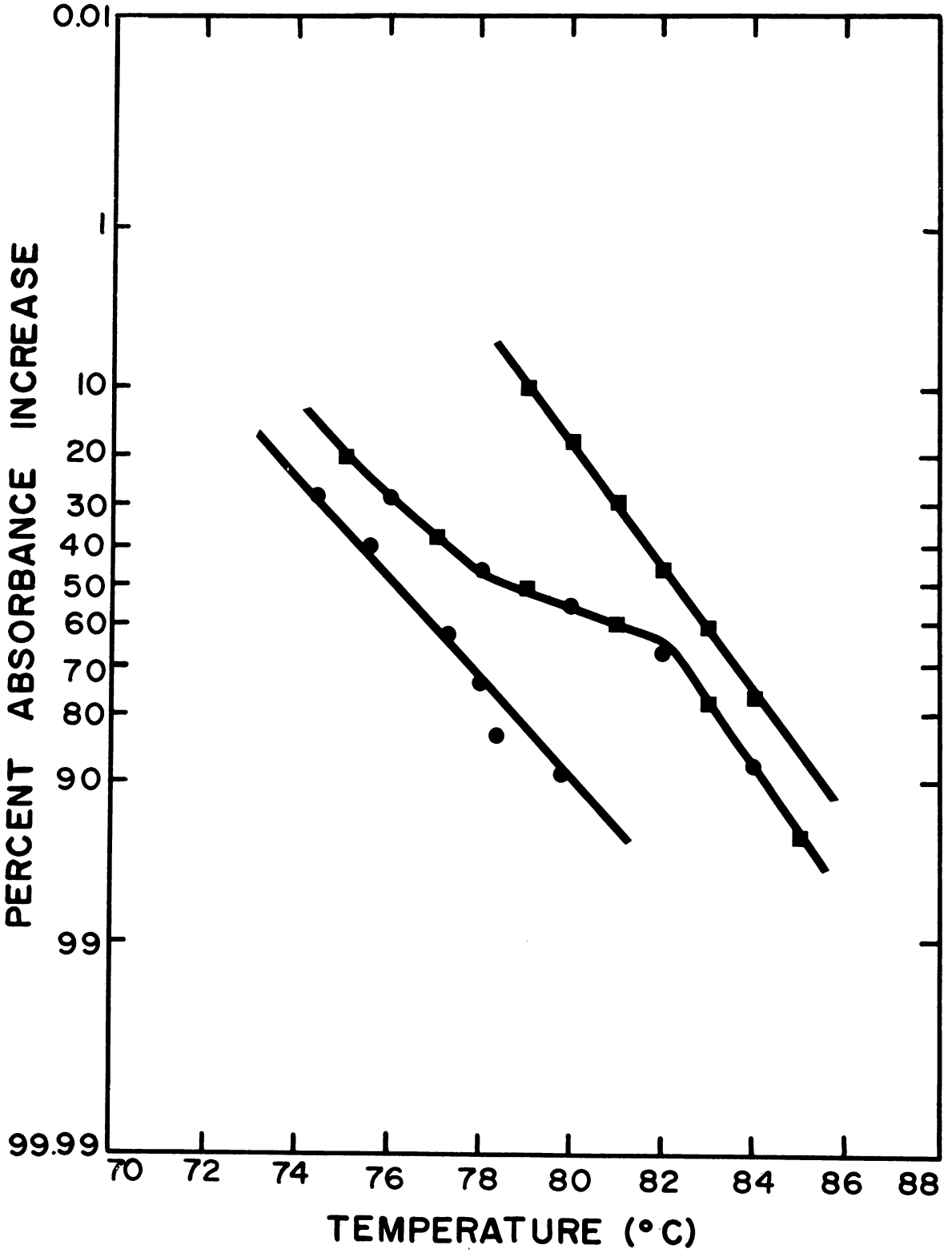
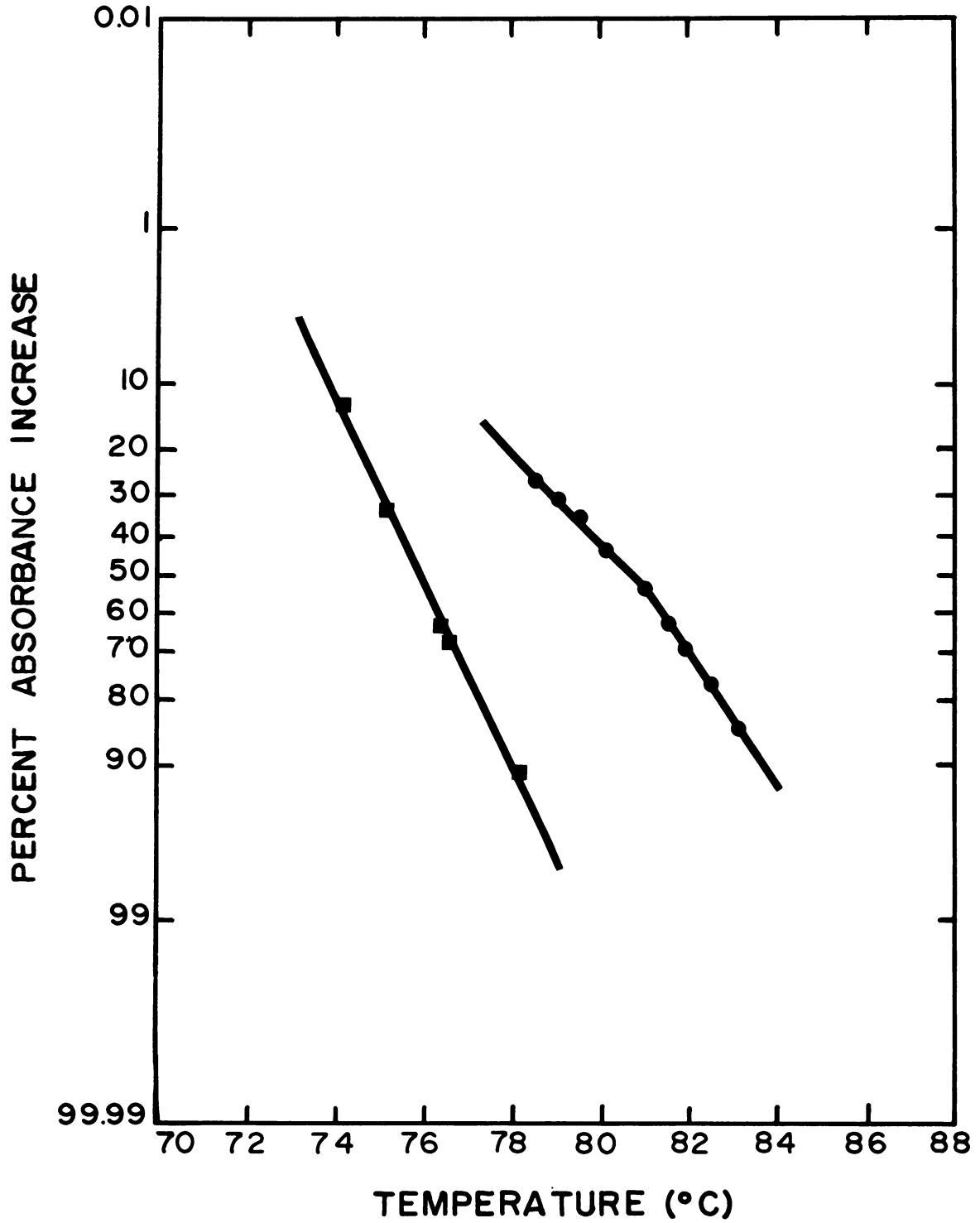

0.01
1
10
20
30
40
50
60
70
80
90
99
99.99
PERCENT ABSORBANCE INCREASE
70
72
74
76
78
80
82
84
86
88
TEMPERATURE (°C)

Figure 4.--Normal probability plot of DNA melting data for A. agilis and A. vinelandii DNA. A. agilis (■) and A. vinelandii (●). See text for experimental details.


0.01
1
10
20
30
40
50
60
70
80
90
99
99.99
PERCENT ABSORBANCE INCREASE
70
72
74
76
78
80
82
84
86
88
TEMPERATURE (°C)

An estimate was made of the approximate difference in the G+C contents of the two DNA components of the A. vinelandii melting curve by setting up a model for a two component mixed DNA melt (see Appendix A). Two straight lines were drawn on probability paper with slopes approximately equal to the slopes of the two components in the A. vinelandii melt. These represented melting curves of homogeneous DNAs with different G+C contents. By varying the distance between the lines (increasing or decreasing G+C differences) and graphically adding them, the following was obtained: with two lines far apart (i.e. large difference in G+C contents) there was obtained a curve like the mixed melt with Ps. aeruginosa and E. coli. As the hypothetical lines were moved closer together the plateau diminished and finally disappeared giving a biphasic curve resembling the A. vinelandii melting curve. Finally by moving the curves even closer the line became indistinguishable from a straight line. By this method of analysis the G+C contents of the two components of the A. vinelandii melting curve appears to differ by 7 to 11% G+C . Since the hyperchromicity of the low G+C component is greater than the high G+C component, the 54-46% split seen on the melting curve is really more closely represented by two components in about equal amounts.

By using scan-melts on the DNA of A. vinelandii and E. coli calculations were possible to determine the

% G+C melting between various temperature increments on the melting curves. Calculations for E. coli extrapolated to a % G+C of about 51%, a value consistent with the true G+C percent of the organism. The Azotobacter calculations however gave anomalous results in that only two temperature increments gave positive values which could be plotted while three increments gave values which were negative. A line drawn through the two positive increment points extrapolated to about 69% G+C, as compared to 65% G+C for A. vinelandii.

## DNA Renaturation Experiments

DNA from E. coli and A. vinelandii was renatured. E. coli was used as a standard since the genome size of the organism is well documented (13) and renaturation kinetic data is also available (10). Figure 5 shows the renaturation values of E. coli superimposed on an ideal second order reaction plot with the same $C_o t_{1/2}$ value. The two curves show good agreement. The $C_o t_{1/2}$ value for E. coli K12 from this curve is about 2.9 in 2 x SSC buffer at the optimal renaturation temperature in this buffer (72C). Using the correction factor for salt concentration given by Britten (7), a value of about 8 was obtained for the $C_o t_{1/2}$ of E. coli in 1 x SSC buffer. This value agrees well with those already reported (10, 50).

Figure 5.--Kinetics of reassociation of E. coli DNA. E. coli data points (◑ ◑ ◑), and ideal second order kinetic curve (▬▬▬). See text for experimental details.

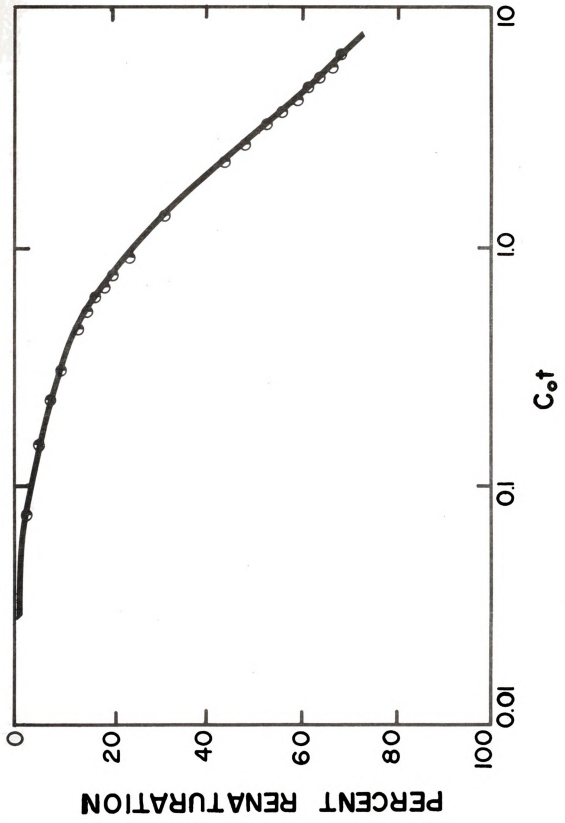

PERCENT RENATURATION
0
20
40
60
80
100
0.01
0.1
1.0
10
$C_o t$

Figure 6 shows the renaturation curve of A. vinelandii compared to an ideal second order reaction with the same $C_o t_{1/2}$ value. These curves do not superimpose well. The A. vinelandii DNA appears to renature faster in the early part of the curve and is well below the ideal curve, while it seems to renature slightly slower than ideal in the last part of the curve. The A. vinelandii renaturation curve was analyzed graphically to obtain the best fit for the experimental curve from the sum of two ideal second order curves. In this analysis it is assumed that the two ideal second order components contributed equally to the reaction. This is substantiated by the melting curve results which showed two components in about equal concentrations. The two best fitting curves thus obtained have $C_o t_{1/2}$ values of 1.1 and 6.8 respectively in 2 x SSC. Figure 7 shows these two curves and their sum compared to the total A. vinelandii renaturation curve. The unique sequence sizes of the two components of the renaturation curve after correction for G+C effects (45), gives values of about $1.2 \times 10^9$ daltons (0.4 times the size of the E. coli genome) and $7.9 \times 10^9$ daltons (3.1 times the size of the E. coli genome).

Initial renaturation rates were calculated for E. coli and A. vinelandii DNA as described by Gillis and DeLey (18). The genome size of A. vinelandii was determined to be $1.5 \times 10^9$ daltons using E. coli as a

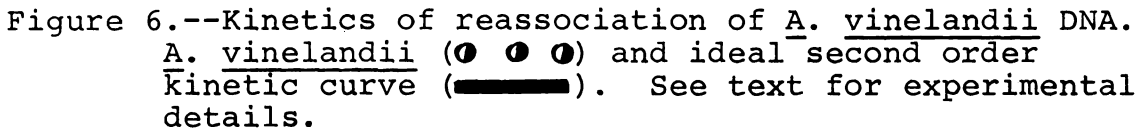


Figure 6.--Kinetics of reassociation of A. vinelandii DNA. A. vinelandii (◐ ◐ ◐) and ideal second order kinetic curve (▬▬▬). See text for experimental details.

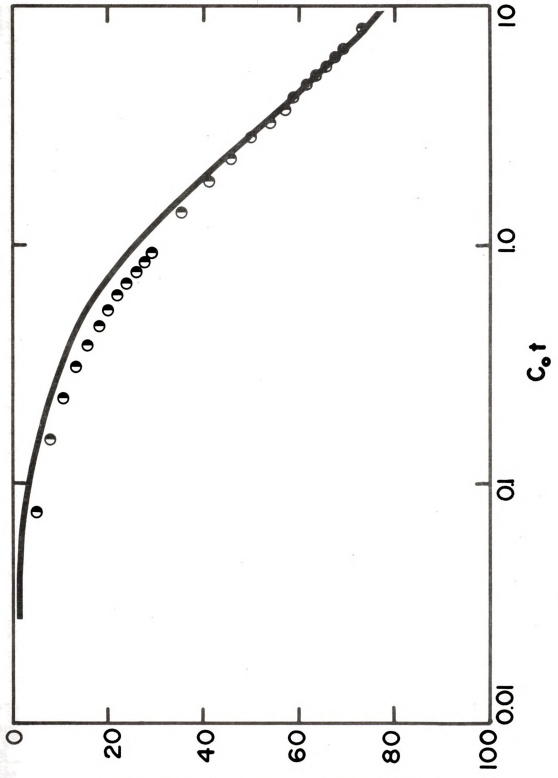
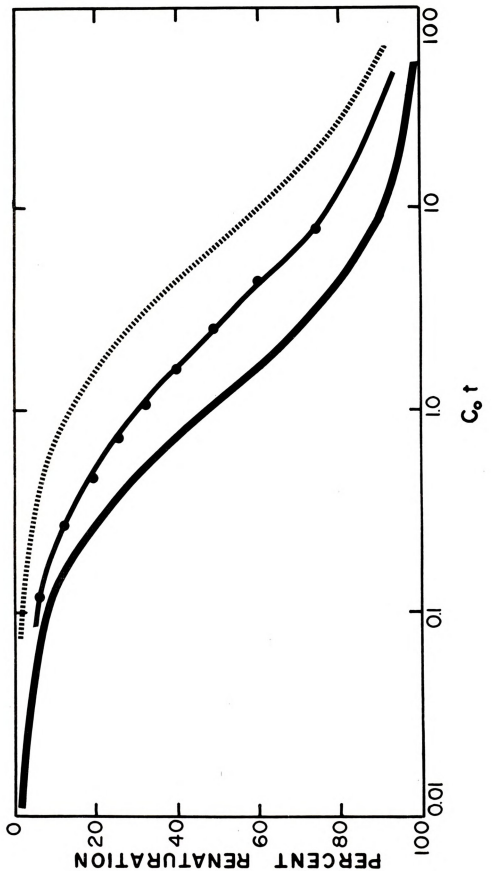

PERCENT RENATURATION
0
20
40
60
80
100
0.01
0.1
1.0
10
$C_o t$

Figure 7.--A comparison of the reassociation kinetics of A. vinelandii with the sum of two ideal second order reaction kinetic curves. Ideal second order kinetics curve of $C_o t_{\frac{1}{2}}$ value = 1.1 (▬▬▬), ideal second order kinetic curve of $C_o t_{\frac{1}{2}}$ value = 6.8 (▬ ▬ ▬), the graphic sum of the two ideal second order curves (▬▬▬) and data points for the renaturation of A. vinelandii (● ● ●). See text for experimental details.


PERCENT RENATURATION
0
20
40
60
80
100
0.01
0.1
1.0
10
100
$C_o t$

standard. The genome size from the $C_o t_{1/2}$ value of the renaturation curve of A. vinelandii (Figure 6) is $2.5 \times 10^9$ daltons. This discrepancy is attributable to the fact that the A. vinelandii renaturation curve is really the sum of two second order curves and thus the initial part of a renaturation would reflect the rapid renaturation of the $C_o t_{1/2} = 1.1$ component. Thus calculations of the unique sequence length based on the value obtained from the initial rate would be low. If a calculation is made of the theoretical genome size for the two component system from the weighted average of the initial renaturation rates of the two components one obtains a value of $1.4 \times 10^9$ daltons, which agrees well with the experimental value.

An additional renaturation experiment for the purpose of determining the respective $C_o t_{1/2}$ values of the two components and their G+C contents was carried out. A. vinelandii DNA in 0.14 M sodium phosphate buffer, pH 7.0 was allowed to renature almost completely at 75C and the double and single stranded DNA was fractionated on hydroxylapatite columns. DNA assays on the two fractions indicated that the DNA had been 88% renatured. From the A. vinelandii renaturation curve and its components (Figure 7) it was then calculated that the single stranded DNA fraction was composed of a fast component ($C_o t_{1/2} = 1.1$) to the extent of 15% of the total and a slow component ($C_o t_{1/2} = 6.8$) of 85%. The single stranded fraction

was then allowed to renature as before in 2 x SSC buffer at 80C. Figure 8 shows this renaturation curve and the theoretical renaturation curve for a 15%:85% mixture of DNAs with $C_o t_{1/2}$ values of 1.1 and 6.8 respectively. The theoretical curve coincides well with the experimental values up to about 20% renaturation when the experimental renaturation begins to level off. Thus the values of 1.1 and 6.8 for the $C_o t_{1/2}$ values of the two component system appear to be consistent with the renaturation of the single stranded DNA fraction.

G+C content analysis was done on the single stranded fractions from the hydroxylapatite separation in order to calculate the G+C contents of the two components of the A. vinelandii renaturation. Fraction 1 (single stranded, non-renatured) was estimated to contain 62% G+C by the method of Hirshman. Solving the simultaneous equations shown below yielded the values of 60.7 and 69.3 for the respective G+C contents of the slow ($C_o t_{1/2}$ = 6.8) and fast component ($C_o t_{1/2}$ = 1.1), a difference of about 8.6% G+C.

Let S = G+C% of the slow component

F = " " " fast "

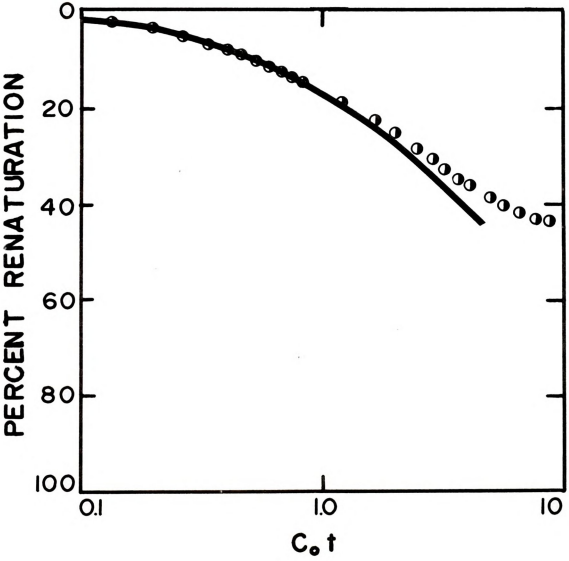
$GC_1$ = " " fraction 1 from hydroxylapatite = 62%

Average G+C content of A. vinelandii = 65%

$GC_1 = 0.15F + 0.85S$

$\frac{S + F}{2} = 65\%$

Figure 8.--Kinetics of reassociation of fractionated A. vinelandii DNA enriched for the slowly renaturing component. Theoretical renaturation curve (——) and renaturation data for enriched DNA (◑ ◑ ◑). See text for experimental details.


PERCENT RENATURATION
0
20
40
60
80
100
0.1
1.0
10
$C_o t$

A previous renaturation of A. vinelandii and E. coli DNA done in Dr. Britten's Laboratory (Carnegie Institute of Washington, D.C.) prior to the experiments just described was analyzed by a computer program at the Carnegie Institute. The renaturation proceeded to about 50% and the computer analyzed curve consisted of two components with $C_o t_{1/2}$ values of 2.6 and 17.3. The conditions under which this experiment was carried out were somewhat different from those conducted at Michigan State University. The DNA was sheared under 50,000 lbs/in$^2$, the solvent used was 0.14 M sodium phosphate buffer + 8 M urea pH 7 (MUP) as opposed to 2 x SSC buffer, and the temperature of incubation was 62C. The computer printout from this experiment indicated two components: the first consisted of 45% of the DNA and had a $C_o t_{1/2}$ of 2.6, while the other component (55%) had a $C_o t_{1/2}$ of 17.3. This analysis coupled with the melting curves described in Figure 4 showing a break in the curve at about 54%, indicated that the higher G+C component is the component of $C_o t_{1/2} = 2.6$. A renaturation done with E. coli DNA indicated a $C_o t_{1/2}$ of about 6. Thus these values are comparable to those determined in 2 x SSC buffer since the 2 components are 0.4 and 2.9 times the size of the E. coli genome.

Density Gradient Centrifugation

The physical separation of the two components of A. vinelandii DNA was attempted using CsCl density gradient centrifugation. It was anticipated that these two components could be reasonably purified to allow renaturations to be done separately on each component. Figure 9 shows the results of this trial. Highly sheared A. vinelandii gives one peak while "unsheared" DNA is just barely resolvable into two peaks (these peaks are probably real since they have been observed in repeating the experiment). The difference in density between the two peaks is 0.0084 grams/$cm^3$ which corresponds to an 8.4% G+C content difference.

The two components of A. vinelandii could not be separated more efficiently in other attempts and this is believed attributable to the shearing implicit in purification of DNA. Shearing has the effect of increasing the overlap of the two DNA peaks due to increased diffusion rates of sheared fragments in the gradient and the increased compositional heterogeneity of the fragments. The final result is one intermediate peak. The areas under the two initial peaks remains the same but the width of the peaks increases with shearing. The validity of such a model was tested by attempting to separate mixtures of sheared and unsheared E. coli (51% G+C) and Ps. aeruginosa (68% G+C) DNA on CsCl gradients. The

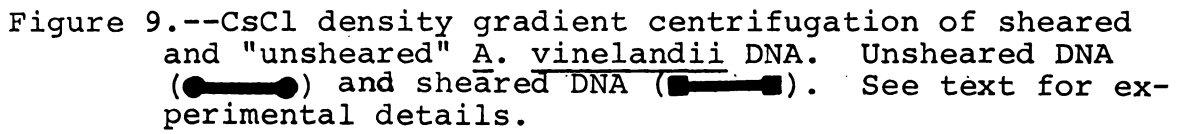
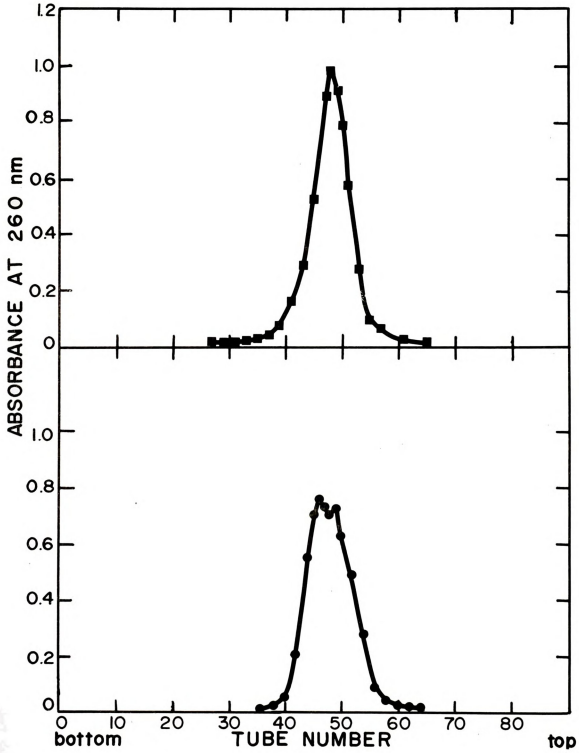
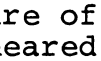
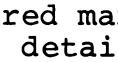


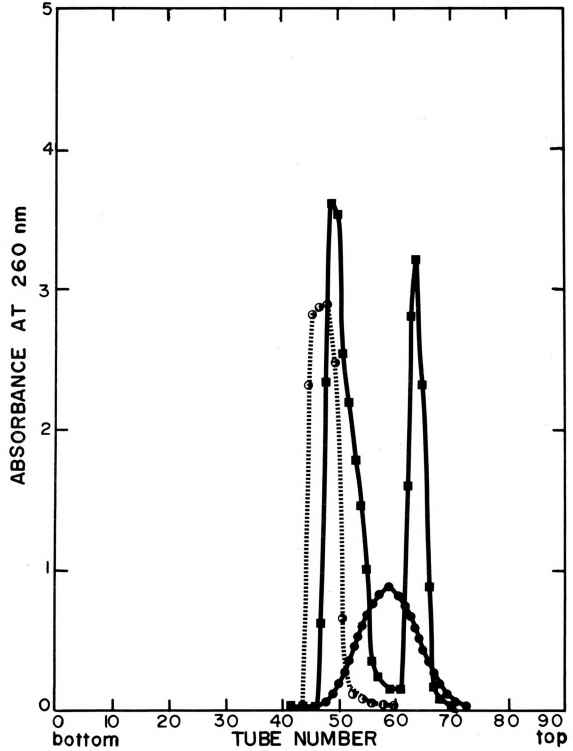
Figure 9.--CsCl density gradient centrifugation of sheared and "unsheared" A. vinelandii DNA. Unsheared DNA (●——●) and sheared DNA (■——■). See text for experimental details.


ABSORBANCE AT 260 nm
1.2
1.0
0.8
0.6
0.4
0.2
0
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
bottom
TUBE NUMBER
top

results are shown in Figure 10 and the results appear to be in agreement with the theoretical considerations.

Other methods, including relaxation centrifugation, sodium iodide gradients and a combination of these two methods did not result in any better separation of the two DNAs.

Figure 10.--CsCl density gradient centrifugation of a mixture of E. coli and Ps. aeruginosa DNA sheared and "unsheared". Unsheared mixture of the two DNAs (  ), sheared mixture of the two DNAs (  ) and Ps. aeruginosa unsheared marker DNA (  ). See text for experimental details.


ABSORBANCE AT 260 nm
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
90
bottom
TUBE NUMBER
top

## DISCUSSION

The purpose of the research described in this thesis was to characterize the DNA of A. vinelandii. A. vinelandii contains a large amount of DNA per cell (about $6.6 \times 10^{-14}$ grams). Nuclear staining has indicated two nuclear bodies per cell in late log phase and thus each nuclear body contains about $3.3 \times 10^{-14}$ grams of DNA. Stained cells were observed in the light microscope and thus the resolution was limited. The nuclear bodies of A. vinelandii appear vessicular when stained but the resolution is not sufficient to distinguish the number of vessicles present per nucleoid. Pochon (39) first described this vessicular nucleoid and suggested that it consisted of four vessicles. The unit of DNA comprising the genome of A. vinelandii is thus uncertain. However one would expect the cyst to contain one genomic unit, which consits of one nuclear body.

The significance of such a large amount of DNA per nuclear body in A. vinelandii is unknown. It is unlikely that the organism has seven times the range of biochemical capability of E. coli. The DNA/protein ratios in both bacteria are comparable. These facts suggested

the possibility of repeated sequences in DNA or the presence of multiple chromosomes.

## Melting Curves

The DNA melting curves of A. vinelandii are atypical of bacteria in that a linear relationship between temperature and the % absorbance increase, is not obtained, when the data are plotted on probability paper. Melting curves of A. vinelandii DNA are bimodal, indicating two populations with differing G+C contents. The difference in G+C content between the two populations is estimated at about 8% by analysis of melting curves, CsCl density gradient centrifugation and spectrophotometric analysis of a mixture of the two components. E. coli K12, Ps. aeruginosa, and A. agilis were used as control DNAs and all were found to give a straight line plot on normal probability paper. Mixed melting curves of E. coli and Ps. aeruginosa DNA show a bimodality in probability plot melting curves. The plateau region is due to the large difference in G+C content between the two DNAs (about 18% G+C difference). The E. coli DNA melts almost completely before the Pseudomonas DNA starts to melt. The plateau region thus represents the temperature span between the end of the E. coli melt and the beginning of the Ps. aeruginosa melt. DNAs with more nearly equal G+C contents show no plateau, but only a break in the

curve as seen in the A. vinelandii melting curve. The break in the A. vinelandii curve comes at about 54% completion of DNA denaturation. However, after correcting for the differential hyperchromicities of the two G+C components, about equal amounts of the two components were found to be present.

The results of the scanning melts on A. vinelandii DNA were not very informative. They served only to indicate that A. vinelandii DNA was atypical in its denaturation kinetics.

## DNA Renaturations

DNA renaturation experiments on A. vinelandii DNA showed a deviation from the ideal second order kinetics normally observed for E. coli and all other procaryotes thus far studied. By taking advantage of the fact that ideal second order $C_o t$ curves have a slope of 100 and they are fully defined by their $C_o t_{1/2}$ values, it was possible to construct, by trial and error, the sum of two second order curves to obtain the best fit to the experimental curve. The two curves obtained in this manner had $C_o t_{1/2}$ values of 1.1 and 6.8 respectively. These values were corrected for G+C effects by the method of Seidler (44) in which each % G+C above 51% increases the $C_o t_{1/2}$ value by 0.018%. The resulting $C_o t_{1/2}$ values were 1.3 and 8.9. Thus it would appear that A. vinelandii has

equal amounts of DNA of two unique sequences which are 0.4 and 3.1 times the size of the E. coli genome. Initial rates for the renaturation of A. vinelandii DNA confirmed these unique sequence sizes as did renaturation of DNA which had been fractionated on hydroxylapatite columns.

When the separation of the two DNA components on CsCl density gradients failed, the DNA fractionation experiment was devised. This experiment took advantage of the differential rates of renaturation of the two DNA components. Thus at the time when the renaturing A. vinelandii DNA was fractionated on hydroxylapatite, 88% of the total DNA had reassociated. However this 88% of the total was comprised of 96% of the fast component ($C_o t_{1/2}$ value = 1.1) and 81% of the slow component ($C_o t_{1/2}$ value = 6.8). The unrenatured DNA therefore consisted of 15% fast renaturing component and 85% slow renaturing component. This procedure, in effect, yielded a purification of the slow renaturing component from a 50-50 mixture to an 85-15 mixture. A renaturation of this concentrated slow component was again resolvable into the weighted average of two second order curves of $C_o t_{1/2}$ value = 1.1 and 6.8. The deviation from the hypothetical sum after about 15% renaturation (Figure 8) was due to the nature of the renaturation experiment and hydroxylapatite fractionation. Bacterial DNA

renaturations generally proceed to 90-95% reassociation. However, the reaction had already proceeded to 88% renaturation by the time the fractionation of renatured and denatured DNA was performed. The additional 42% renaturation (of the 12% left to renature) brought the total to 93% of the DNA renatured.

Another important factor which must be considered in assessing the extent of the renaturation reaction is the distinction between renaturation reactions monitored by hydroxylapatite fractionation and those observed by spectrophotometric means. The hydroxylapatite method measures the fraction of DNA pieces that have reassociated regions and thus bind to hydroxylapatite. The optical method however measures the fraction of the DNA actually base paired. Thus it is possible that in the fractionation experiment lengths of single stranded DNA which eluted from hydroxylapatite at low ionic strength had their complements in that DNA which bound to the column.

## Density Gradient Centrifugations

Density gradient centrifugations gave mostly qualitative information. Two approximately equal peaks were observed varying in density which corresponded to 8.5% G+C in A. vinelandii. The model of two DNA peaks becoming one when sheared was substantiated by the E. coli - Ps. aeruginosa density gradient experiment.

## Models for Cellular Organization of the DNA

Three models (Figure 11) may be constructed to describe the cellular organization of the DNA in A. vinelandii. They are the following: the multichromosomal--redundancy model, the monochromosomal--redundancy model and the multichromosomal--non-redundancy model.

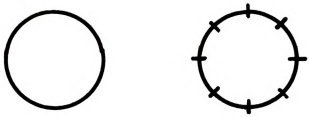
In the multichromosomal--redundancy model each nuclear body has a DNA content of about $3.1 \times 10^7$ nucleotide pairs. Unique sequence sizes of $2 \times 10^6$ nucleotide pairs and $1.4 \times 10^7$ nucleotide pairs correspond to the $C_o t_{1/2}$ values of the two components. Thus if we consider each nuclear body as two separate chromosomes we would have two equal size chromosomes with eight fold redundancy in the small sequence chromosome and no redundancy in the large sequence chromosome.

The monochromosomal--redundancy model would consist of one large chromosome (the nuclear body) having eight copies of the small unique sequence and one copy of the large.

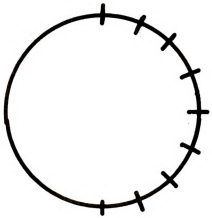
The final model, multichromosomal--non-redundancy, would involve one large chromosome of the higher unique sequence size and eight small chromosomes.

Which, if any, of these models represents the nuclear array in A. vinelandii is not now known. Nuclear staining would tend to exclude the final model as no small staining bodies were seen. However, if an artifact

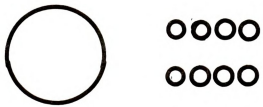
Figure 11.--Models for the possible organization of DNA in A. vinelandii. Drawn approximately to scale. See text for experimental details.



MULTICHROMOSOMAL-REDUNDANCY



MONOCHROMOSOMAL-REDUNDANCY




MULTICHROMOSOMAL-NON-REDUNDANCY

of the staining procedure caused clumping of chromosomes, they would be difficult to resolve and they might even appear as large vessicular structures.

The shearing of DNA in purification produces fragments of about $5 \times 10^4$ nucleotide pairs in length and mechanical shearing produces pieces of about $5 \times 10^2$ nucleotide pairs. Since both of these nucleotide lengths are well below either unique sequence size, little information would be obtained on the nuclear array of A. vinelandii by sedimentation studies.

## Approaches to Resolving the Validity of the Final Model

The establishment of the validity of the final model could be approached by autoradiography as employed by Cairns to visualize the E. coli chromosome (13). This method would necessitate labeling of Azotobacter vinelandii DNA with tritiated thymidine to obtain high specific activity DNA, followed by gentle lysis and radioautography. Unfortunately this method has many difficulties. A. vinelandii do not take up tritiated thymidine well and high specific activities may not be obtainable. In addition, chromosome lengths in the models are greater than E. coli and thus the probability of shearing is greatly increased.

Another method sometimes used to visualize DNA with the electron microscope is the Kleinschmidt

Method (23). Here DNA is allowed to absorb to a basic protein film, such as cytochrome c. The complex is then transferred to electron microscope specimen grids which are shadowed with platinum. Obtaining molecular sizes of DNA using this method is suitable for only rather small DNAs such as viral DNAs. Since the A. vinelandii chromosomes are so much larger than viral and even bacterial DNAs, it is unlikely that this method could be applied successfully here. Even if the DNA could be prepared without shearing its large length would be difficult to contain in the confines of one hole in a specimen grid.

## SUMMARY

The DNA content per cell of A. vinelandii was determined to be $6.7 \times 10^{-14}$ grams. Cells have two stainable nuclear bodies and thus $3.3 \times 10^{-14}$ grams per nuclear body. DNA melting curves of A. vinelandii when plotted on normal probability paper were biphasic indicating two DNAs of different G+C contents. The two DNA components have been shown to be about 61% and 69% G+C and are in about equal amounts in the cell. DNA renaturation curves for A. vinelandii deviate from second order kinetics and were analyzed to be the sum of two ideal second order reaction curves characterized by $C_o t_{1/2}$ values of 1.1 and 6.8. These values correspond to unique sequence sizes of $2.0 \times 10^6$ and $1.4 \times 10^7$ nucleotide pairs respectively.

Attempts to separate the two components by CsCl density gradient centrifugation were not completely successful and a model is provided to explain this result. Three models are presented for the possible organization of DNA within the cell.

# LIST OF REFERENCES

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# APPENDICES

# APPENDIX A

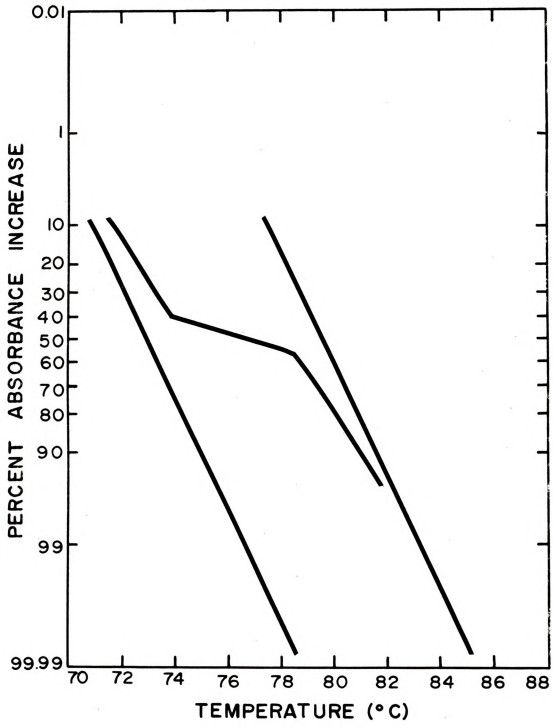
## A MODEL FOR THE ESTIMATION OF G+C CONTENT DIFFERENCES OF THE TWO COMPONENTS OF *A. VINELANDII* DNA

## A MODEL FOR THE ESTIMATION OF G+C CONTENTS DIFFERENCES OF THE TWO COEFFICIENTS OF A. VINELANDII DNA

The following discussion is aimed at clarifying the general method used to assign a range of values to the G+C content differences between the two components of the DNA of A. vinelandii. This method is based on a model for a two component mixed DNA melting curve. The simplifying assumption is made that the compositional distribution of nucleotide pairs is the same in both components and thus when normal probability plots are made the slopes of the lines representing the two components are the same. By varying the distance between the lines (i.e. the difference in G+C content) and graphically adding them, three types of summation curves are obtained. Figure 12 shows the sum of two components with large G+C content differences. Figures 13 and 14 are sums of curves with decreasing differences in G+C content, respectively. Figure 13 is similar to the experimental curve of A. vinelandii. Thus by changing the difference in G+C content between the two curves, the range of G+C content differences which gives a biphasic curve as opposed to a linear or plateaued curve. In this way a range of G+C differences

can be attributed to the components of the experimental curve.

Figure 12.--The theoretical DNA denaturation curve for a mixture of two DNAs with a large difference in G+C content.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PERCENT ABSORBANCE INCREASE
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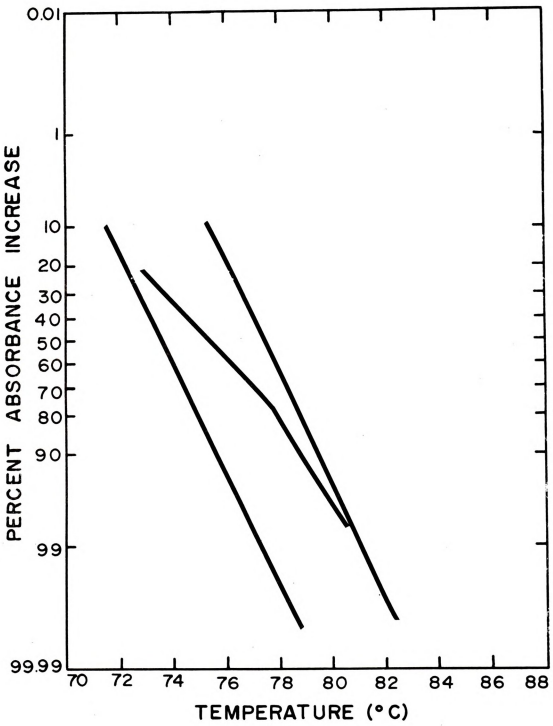
TEMPERATURE (°C)

Figure 13.--The theoretical DNA denaturation curve for a mixture of two DNAs with an intermediate difference in G+C content.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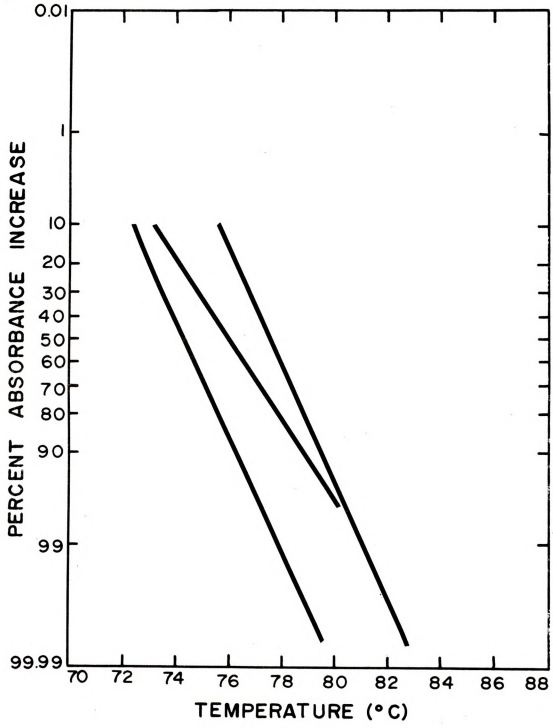
TEMPERATURE (°C)

Figure 14.--The theoretical DNA denaturation curve for a mixture of two DNAs with a small difference in G+C content.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# APPENDIX B

## SOME ASPECTS OF THE THEORY OF RENATURATION OF DNA

SOME ASPECTS OF THE THEORY OF

RENATURATION OF DNA

Plotting DNA Reassociation
Data--the $C_o t$ curve:

DNA renaturation has been observed to be a second order phenomena. The reassociation of a pair of complimentary sequences is concentration dependent relying on the diffusion of DNA fragments and the subsequent collision and pairing of the fragments.

The general equation for a second order reaction is as follows:

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

- $c$ = the concentration of DNA in unpaired strands
- $t$ = time in seconds after initiation of the reaction
- $K$ = the reaction rate constant for any particular circumstances
- $C_o$ = the total concentration of DNA in all single stranded regions

Rearranging we have:

$$\frac{dc}{c^2} = -Kdt$$

Integrating this equation from t = 0 to any future time, t, and from c, the concentration of unpaired strands

to $C_o$, the total concentration of DNA, we then have:

$$\int_{c}^{C_o} \frac{dc}{c^2} = \int_{0}^{t} -Kdt$$

Following integration and evaluation between these limits we have:

$$\frac{c}{C_o} = \frac{1}{1 + KC_o t}$$

This is an ideal second order reaction equation and when plotted gives the typical S-shaped renaturation curve (the "$C_o t$" curve). Such a plot has the advantage of allowing the comparison of reactions carried out at different concentrations and by plotting $C_o t$ on a logarithmic scale measurements over a long time period may be presented. The central 2/3 of an ideal second order reaction plotted in this manner approximates a straight line with a slope of 100.

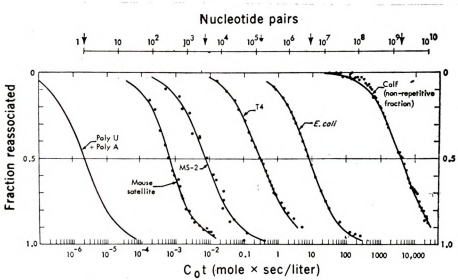
Dependence of Renaturation Rate on Complexity (unique sequence size):

In DNAs without repeated sequences the complexity unique sequence size and genome size are all equal. The rate of renaturation of DNAs sheared to the same piece size and renatured under comparable conditions is

inversely proportional to the unique sequence size. This may be illustrated by the following example:

$DNA_1$ has a unique sequence size 1/2 that of $DNA_2$. Both DNAs are sheared to the same size fragments. DNAs are allowed to renature under the same conditions.

Since renaturation depends on the random collision of two complimentary fragments, $DNA_1$ would renature faster since the mate to any given fragment is diluted less by non-mates than in the case of $DNA_2$. This relationship has been shown experimentally by Britten (10) and the data are presented below:




Reassociation of double-stranded nucleic acids from various sources. The genome size (*25*) is indicated by the arrows near the upper nomographic scale. Over a factor of $10^6$, this value is proportional to the $C_0t$ required for half reaction. The DNA was sheared (*18*) and the other nucleic acids are reported to have approximately the same fragment size (about 400 nucleotides, single-stranded). Correction has been made (*19*) to give the rate that would be observed at 0.18*M* sodium-ion concentration. No correction for temperature has been applied as it was approximately optimum in all cases. Optical rotation was the measure of the reassociation of the calf thymus nonrepeated fraction (far right). The MS-2 RNA points were calculated from a series of measurements (*28*) of the increase in ribonuclease resistance. The curve (far left) for polyuridylic acid + polyadenylic acid was estimated from the data of Ross and Sturtevant (*29*). The remainder of the curves were measured by hypochromicity at 260 nm; a Zeiss spectrophotometer with a continuous recording attachment was used.

APPENDIX C

*PHYSIOLOGICAL STUDIES OF ENCYSTMENT*

*IN AZOTOBACTER VINELANDII*

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Reprinted from Journal of Bacteriology, 1971, 105:185-189.

JOURNAL OF BACTERIOLOGY, Jan. 1971, p. 185-189
Vol. 105, No. 1


# Physiological Studies of Encystment in *Azotobacter vinelandii*[1]

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Received for publication 3 August 1970

*Azotobacter vinelandii*, in late exponential growth phase, encysts when the glucose in the medium is replaced with β-hydroxybutyrate. A final cell division then occurs without apparent deoxyribonucleic acid (DNA) synthesis, resulting in a reduction from two to one nucleoids per cell and a final DNA content of $3.2 \times 10^{-14}$ g per cell. This is also the DNA content per cyst. A β-hydroxybutyrate dehydrogenase is derepressed by the addition of the inducer and is identical to the enzyme in acetate-grown cells in its *p*H optimum, Michaelis constant for substrate, temperature-activity response, and mobility during electrophoresis in acrylamide gel.

Cysts of *Azotobacter vinelandii* are resting cells which are analogous to endospores of bacilli in that they are considerably more resistant than vegetative cells to deleterious physical and chemical agents (17). Cultures of this organism may be induced to encystment by growth on agar plates of Burk's nitrogen-free medium supplemented with 0.2% *n*-butyl alcohol as the carbon source (18, 23). Alternatively, cultures may be grown to late exponential phase in liquid medium with glucose as the carbon source; the cells are then induced to encystment by the replacement of the glucose with either β-hydroxybutyrate (BHB) or crotonate (9). This two-step process can be carried out in relatively large culture volumes and has been applicable to the study of the time course of this unique cellular differentiation process at the physiological (9) or fine structural level (6). We have been concerned with the biochemical events which occur upon the induction of encystment and have examined the immediate effects of the addition of BHB on cell growth, cell division, and enzyme induction.

## MATERIALS AND METHODS

**Strain and cultivation.** *A. vinelandii* ATCC 12837, which was used throughout these experiments, was cultivated in Burk's nitrogen-free medium (22) at 30 C. In the two-step method for cyst production (9), cultures were grown to late exponential phase ($2 \times 10^8$ cells/ml) with 1% glucose as the carbon source. The cells were centrifuged from the medium aseptically, suspended in sterile Burk's medium with 0.2% BHB as the carbon source, and incubated with aeration at 30 C. Aqueous solutions of both glucose and BHB (sodium salt) were sterilized separately by autoclaving.

**Preparation of cell extracts.** Vegetative and encysting cells of *A. vinelandii* were suspended in 0.05 M tris (hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.5) containing $10^{-3}$ M ethylenediaminetetraacetic acid (EDTA), and were then disrupted in a sonic oscillator (Measuring & Scientific Equipment, Ltd.). The chelating agent (EDTA) was necessary to achieve rapid and reproducible cell rupture.

**Analytical procedures.** The nitrogen content of vegetative and encysting cells was determined by the micro-Kjeldahl analysis (19). Deoxyribonucleic acid (DNA) was determined by the method of Burton (2). Cells were extracted with 10% trichloroacetic acid at 95 C for 15 min, and the deoxynucleotides were assayed by using highly polymerized calf thymus DNA as a standard. Proteins were estimated spectrophotometrically (20). Compounds containing amino sugars were assayed by the Rondle and Morgan (15) procedure with glucosamine as the standard. The D-β-hydroxybutyric acid dehydrogenase of encysting cells was assayed by the procedure of Jurtshuk et al. (7). The rate of oxidation of BHB was equal to the rate of reduction of nicotinamide adenine dinucleotide (NAD), measured at 340 nm. One unit of enzyme oxidized 1 μmole of substrate per min at 37 C, and specific activity was expressed in units of protein per mg.

**Photomicroscopy.** Time-lapse phase-contrast photomicroscopy of encysting *A. vinelandii* was carried out with a Zeiss Photomicroscope by using slide cultures of the organism on Burk's nitrogen-free agar medium containing 0.2% BHB. Nuclear staining of *A. vinelandii* at various stages in its life cycle was performed by the procedures of Piekarski (14). Direct cell counts, which were made with a Petroff-Hauser counting chamber, corresponded well with viable counts obtained by standard plating techniques.

**Gel electrophoresis.** The relative mobilities of BHB

[1] Journal article 5177 from the Agricultural Experiment Station, Michigan State University.

[2] Public Health Service predoctoral trainee, grant GM-01911.

dehydrogenases in acrylamide gel electrophoresis at *p*H 8.3 and 4 C were tested by the method of Davis (4). Vertical gels of 5% Cyanogum 41 (10 by 20 by 0.3 cm) were prepared in a vertical gel electrophoresis cell (E-C Apparatus Corp., Philadelphia, Pa.). Tris-glycine buffer (*p*H 8.3) was employed, and a constant current of 6 ma was maintained. Bromophenol blue, the anionic marker, migrated to within 1 cm of the anionic end of the gel in 1.75 hr. The BHB dehydrogenases were stained specifically by coupling nitroblue tetrazolium reduction to the oxidation of the substrate (16).

## RESULTS

**Growth and encystment.** The two-step encystment procedure (9) was scaled-up to a 3-liter volume in the following manner. Cultures of *A. vinelandii* were grown in 100-ml volumes of Burk's medium (1% glucose) per 500-ml Erlenmeyer flask. When a cell concentration of $2 \times 10^8$ per ml was achieved (approximately 18 hr at 30 C with shaking), 200 ml of culture was introduced into 2,800 ml of the same medium at 30 C in a 3-liter New Brunswick fermentor. The culture was stirred at 400 rev/min and aerated at 3 liters/min. Samples of this culture were taken at intervals for analyses of turbidity, cell count, total nitrogen, DNA, and BHB dehydrogenase. When the cell concentration reached $2 \times 10^8$ per ml, the cells were harvested by centrifugation and suspended in fresh Burk's nitrogen-free medium containing 0.2% BHB. Stirring, aeration, and sampling were continued through the initial stages of encystment.

Figure 1 presents growth data for *A. vinelandii* under the conditions described. After the induction of encystment by BHB, the culture appeared to undergo one cell doubling. This was apparent from both the turbidity and total nitrogen data and was substantiated by time-lapse phase-contrast micrography (Fig. 2). A cluster of six vegetative cells on a slide culture was observed over a period of 8 hr. These cells had been grown in liquid Burk's nitrogen-free medium containing glucose. They were washed once in buffer and spread over the BHB-containing medium. The initiation of cell division was readily apparent at 2.5 and 3.5 hr. At 8 hr, five of the cells had divided, and their progeny appeared to be in the early stages of encystment. At that time, the sixth cell was in its final division.

Concomitant with the last cell division, an arrest of nitrogen fixation occurred. The total nitrogen of the culture remained constant with approximately 95% in the cellular material. DNA synthesis stopped at the time of the last cell division. The DNA content per cell dropped from $15 \times 10^{-14}$ g per cell in exponentially growing cultures to $3.4 \times 10^{-14}$ g per encysting cell (Fig. 3). The latter value is precisely the DNA content per cyst of *A. vinelandii*. Nuclear staining showed that cells in early exponential growth had as many as four nucleoids, whereas late exponential-phase cells had two. Cysts contained only one nucleoid.

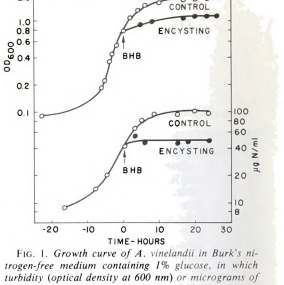


FIG. 1. *Growth curve of A. vinelandii in Burk's nitrogen-free medium containing 1% glucose, in which turbidity (optical density at 600 nm) or micrograms of nitrogen per milliliter are plotted as functions of time. Cells in late exponential growth were induced to encystment with 0.2% BHB with subsequent inhibition of nitrogen fixation and cell growth.*

An alternative procedure for initial extraction (3) and then DNA analysis was performed to validate the observations of DNA content per cell. A 100-ml culture of *A. vinelandii* was grown in Burk's medium (1% glucose) to a concentration of $1.8 \times 10^8$ cells/ml. The culture was chilled to 4 C; the cells were harvested, washed in 0.1 M Tris-0.1 M NaCl buffer (*p*H 9.0), and resuspended in 18 ml of the same buffer. Two milliliters of cold 10% sodium dodecyl sulfate was added, and the suspension was stirred for 20 min in an ice bath. An equal volume (20 ml) of water-saturated phenol was added, and the mixture was slowly stirred for an additional 20 min. The aqueous and phenol layers were separated by centrifugation, and the DNA in the aqueous layer was precipitated by the addition of three volumes of ethanol. The DNA precipitates were collected and suspended in 0.1 M NaCl. The total DNA recovered from $1.8 \times 10^{10}$ cells was 840 μg, amounting to $4.7 \times 10^{-14}$ g/cell. The amino sugar content of the DNA preparation was used as a measure of its contamination with cell envelope constituents (21). The DNA preparation contained an equivalent of 25 μg of glu-

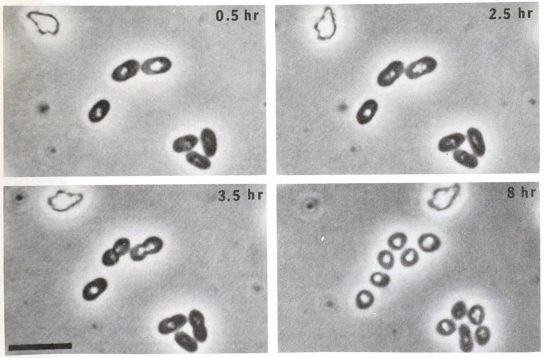


FIG. 2. *Time-lapse, bright phase-contrast photomicrographs of encysting A. vinelandii. Cells were grown to late exponential growth, and slide cultures were prepared on Burk's agar containing 0.2% BHB. The marker indicates 5 nm.*

cosamine whereas the supernatant solution contained 1860 $\mu$g of glucosamine.

The addition of BHB to late exponentially growing cultures of *A. vinelandii* resulted in the induction of a soluble $\beta$-hydroxybutyric acid dehydrogenase which was NAD dependent. This enzyme was not found in young cells; growing on glucose however, within 1 hr of the addition of BHB, a specific activity of 0.03 units/mg of protein was noted in cell extracts (Table 1). Because of the relationship of BHB metabolism to encystment, we considered the possibility that the dehydrogenase was a unique "encystment enzyme" analogous to several which occur in cells with the onset of sporulation in *Bacillus* species (1, 5). A soluble BHB dehydrogenase has been studied in cells of *A. vinelandii* grown on acetate as the sole source of carbon and energy (7). We grew 3-liter cultures of the organism in Burk's nitrogen-free medium containing 1% sodium acetate, harvested the cells, and prepared extracts. The specific activity of the BHB dehydrogenase was 0.025 units/mg. This enzyme was compared to that occurring during encystment by observing enzyme activities over the temperature range 26 to 48 C, pH responses, Michaelis constants for substrate, and relative electrophoretic mobilities in acrylamide gels. The results of the

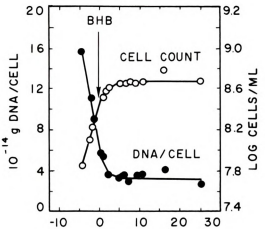


FIG. 3. *Cell concentration and DNA content per cell of encysting A. vinelandii as a function of time.*

comparisons are presented in Fig. 4 and Table 2 and indicate that the two enzymes are very similar.

## DISCUSSION

The replacement of glucose with BHB in the two-step induction of encystment is similar in

TABLE 1. *Specific activity of β-hydroxybutyrate dehydrogenase upon induction of encystment of Azotobacter vinelandii*

| Time after addition of BHB (hr) | Specific activity (units/mg) |
|---|---|
| 0 | 0 |
| 1 | 0.030 |
| 1.5 | 0.027 |
| 2 | 0.023 |
| 3 | 0.023 |
| 4 | 0.021 |

many respects to a metabolic shift-down (8). At the time of its replacement by BHB, about 50% of the original glucose still remained in the medium (9). These cells must have shifted from carbohydrate metabolism to lipid metabolism with the attendant need for gluconeogenesis. Marcromolecular synthesis and cell division which was in progress at the time of the induction of encystment appeared to be completed without the initiation of new rounds. Control cultures which were maintained in the glucose-containing medium continued to divide and achieved final population levels of $1.1 \pm 10^9$ cells/ml. Approximately 1% of these cells formed cysts.

The total nitrogen in the culture increased 1.25-fold after induction of encystment, and the total cell count increased 1.4-fold, a value consistent with the 1.8-fold increase in optical density. A reduction in the DNA content per cell was achieved in this process, the final level equaling the DNA content of mature cysts. A derepression of BHB dehydrogenase synthesis occurred, and relatively high levels of the enzyme were present in cells within 1 hr of induction of encystment. The specific activity of this enzyme during encystment was equal to that in cells grown on acetate. A complete cessation of nitrogen fixation took place approximately 3 hr after the addition of BHB.

The shift-down per se did not induce encystment. This metabolic state occurs after glucose exhaustion from the medium; however, under these conditions, relatively few cysts are produced (9). The specificity of the inducers of encystment, (*n*-butanol, crotonate, or BHB) suggest that unique metabolic sequences may be involved in cyst formation. In some unknown manner, the metabolites of *n*-butanol, which stop normal growth, control the formation of cyst components. Even though key intermediates may exist, they must be transitory because ultimately 90% of all exogenously added BHB is oxidized to $CO_2$ (9).

The BHB dehydrogenase which was elaborated during encystment was identical to the enzyme which occurred in *A. vinlandii* during vegetative growth on acetate on the basis of the following parameters. The two soluble dehydrogenases were indistinguishable in their *p*H optima, Michaelis constants, electrophoretic mobilities in acrylamide gels, and in their activation energies. We conclude that BHB dehydrogenase is not an encystment-specific enzyme in the sense that protease (1) or glucose dehydrogenase (5) is related to the sporulation of *Bacillus cereus*. Rather, the enzyme is induced when the cells shift to the metabolism of lipid materials.

The DNA content per cell of *A. vinelandii* varied with the stages of its life cycle. Cells in mid-exponential growth contained $15 \times 10^{-14}$ g of DNA/cell and were multinucleate. During encystment a minimal value of $3.4 \times 10^{-14}$ g of DNA/cell was observed for mononucleate cells. This latter value amounts to about 2.5% of the dry weight of the encysting cells, assuming that they contain 8% nitrogen. The reduction in nucleoids per cell after exponential growth has been observed in other bacteria and in metabolic shift-down experiments in which cell growth rate is altered (8, 10). Cysts contain approximately 10 times

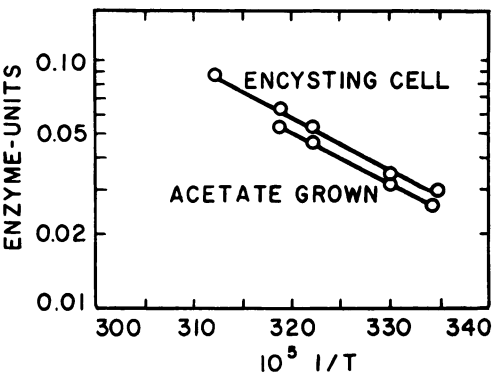


FIG. 4. *Arrhenius plot of the effect of temperature over the range 25 to 48 C on the activities of 0.045 units of BHB dehydrogenase from acetate grown cells and 0.055 units from encysting A. vinelandii. The activities are expressed as equivalent units (37 C).*

TABLE 2. *Properties of β-hydroxybutyrate (BHB) dehydrogenase of Azotobacter vinelandii*

| Source | *p*H optimum[a] | $K_m$ (mM)[b] | $R_m$[c] |
|---|---|---|---|
| Encysting cells . . . . . | 9.0 | 4.0 | 0.38 |
| Acetate-grown cells . . | 9.0 | 5.5 | 0.38 |

[a] The enzyme (0.05 units) was assayed over a *p*H range of 7.5 to 11.0 in 0.05 M Tris-glycine buffer.

[b] Michaelis constants for either enzyme were determined with 0.02 to 0.05 units of enzyme over a BHB dehydrogenase concentration range of 0.001 to 0.03 M.

[c] Mobility relative to bromophenol blue in electrophoresis on 5% acrylamide gel at *p*H 8.3.

as much DNA per nucleoid as does *Escherichia coli* (10). Similar DNA contents for synchronous cultures of *A. vinelandii* have been reported by Zaitseva et al. (24). Müller and Kern (13) observed that the DNA contents of various radiation resistant mutants of *A. chroococcum* ranged from $10^{-14}$ to $19 \times 10^{-14}$ g/cell. Despite these corroborative findings, we considered the possibility that the high values of DNA content per cell were artifacts of the assay procedure. If the hot trichloroacetic acid used to hydrolyze the cell DNA also hydrolyzed a part of the cell envelope, the values for the DNA assay might have been inordinately high. Therefore, we extracted DNA from 100 ml of a culture in late exponential growth phase and, after a standard DNA assay, calculated the DNA per cell. To detect contamination of the DNA preparation by the cells' lipopolysaccharide, we monitored hexosamine content of our precipitated DNA and the aqueous phase from which it was precipitated. Only 1.3% of the hexosamine-containing material originally present in the aqueous phase was precipitated with the DNA. Assuming a 70% yield of DNA on extraction, we calculated that late exponential cells of *A. vinelandii* contain $6.7 \times 10^{-14}$ g of DNA/cell. Since these cells contained an average of two nucleoids per cell, the individual nucleoids must have contained $3.4 \times 10^{-14}$ g of DNA. This value is identical to that which we have obtained for encysting cells and cysts of *A. vinelandii* by our DNA assay. The relatively large mass of the *A. vinelandii* nucleoid suggests that there may be considerable redundancy in its genome and may account for the difficulty in obtaining mutants of this organism (11, 12).


## ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grant AI-01863 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. E. B. was a predoctoral trainee, supported by Public Health Service grant GM-01911 from the National Institute of General Medical Sciences.


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