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DIVERGENCE OF RIBOSOMAL RNA SEQUENCES
WITHIN ANGIOSPERMAE

by

Michael Harold Vodkin

A Dissertation Submitted to the Faculty of the

COMMITTEE ON GENETICS

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For the Degree of

DOCTOR OF PHILOSOPHY

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THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Michael Harold Vodkin entitled Divergence of Ribosomal RNA Sequences within Angiospermae be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

Frank B. H. Kattermann
Dissertation Director

5 January 1971
Date

After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

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*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.

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SIGNED: Michael Vodkin

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ABSTRACT

Deoxyribonucleic acids (DNA's) have been extracted from nuclei of barley, blue panicum grass, wheat, cotton, and pumpkin. The buoyant densities of these DNA's in cesium chloride are 1.701, 1.702, 1.703, 1.692, and 1.694 gm/cc, respectively. In addition to the main band, pumpkin DNA displays a satellite component with a buoyant density of 1.705 gm/cc. The buoyant densities of the alkaline-denatured DNA's are, respectively, 1.717, 1.719, 1.718, 1.709, and 1.711 gm/cc. The denatured DNA's of barley, wheat, and cotton, when renatured under equivalent conditions of concentration and time, band in cesium chloride at positions closer to their respective native buoyant densities than do those of blue panicum grass and pumpkin, and thus the DNA's of blue panicum grass and pumpkin probably are composed of a larger proportion of unique nucleotide sequences than those of the other DNA's.

Radiolabeled ribosomal ribonucleic acids (rRNA's) have been prepared from the leaves of barley, wheat, cotton, and pumpkin. Polyacrylamide gel electrophoresis was used to resolve the 25 s, 23 s, 18 s, and 16 s rRNA components of barley and to demonstrate that the two cytoplasmic components, 25 s and 18 s, and the two chloroplastic components, 23 s and 16 s, were radiolabeled. Radiolabeled rRNA's from various species were hybridized to barley and cotton DNA's that had been fractionated from a preparative cesium chloride gradient. Hybridization is coincident with the bulk of barley DNA; however, rRNA's

hybridize specifically to those fractions of cotton DNA whose buoyant density and guanine + cytosine (GC) content are greater than those of the bulk of the DNA.

Homologous saturation-hybridization of leaf rRNA's to nuclear DNA's establishes that the combined total of cytoplasmic and chloroplastic ribosomal DNA's (rDNA's) comprises 0.73%, 0.22%, 0.71%, and 1.88%, respectively, of the genomes of barley, wheat, cotton, and pumpkin. Competition experiments with unlabeled root rRNA's establish that about 60% of barley and cotton rDNA's code for cytoplasmic rRNA.

Heterologous saturation-hybridization of leaf rRNA's to nuclear DNA's has been used to estimate the similarity of rRNA nucleotide sequence among monocots, among dicots, and between monocots and dicots. These data can be compared with the DNA homology estimates compiled by other investigators. It is concluded that the nucleotide sequence coding for rRNA is conserved to a greater extent than that of the rest of the DNA.

The thermal stabilities of rRNA-DNA hybrids were measured in terms of Tm, the temperature at which 50% of the rRNA is released from the hybrid. The Tm's of homologous monocot rRNA-monocot DNA hybrids are lower than those of homologous dicot rRNA-dicot DNA hybrids. These data can be interpreted to indicate a heterogeneity of monocot rRNA's and the genes that code for them or a difference in GC content between monocot and dicot rRNA's.

INTRODUCTION

Deoxyribonucleic acid (DNA) comprises the genetic material of all cellular organisms and, as such, it contains information which specifies the primary structure of the many proteins synthesized by cells. The processes by which the nucleotide sequence of DNA specifies the amino acid sequence of a protein involve the transcription of that information into ribonucleic acid (RNA) and the subsequent translation of RNA into polypeptides.

During transcription RNA is synthesized on a DNA template. The specificity of this process depends on the complementary relationship between the deoxyribonucleotide of the template and the corresponding ribonucleotide on the growing RNA chain. Thus, the RNA that has been transcribed is complementary to the segment of DNA from which it was copied. This complementary relationship between RNA and DNA is the underlying principle for *in vitro* hybridization experiments. This technique involves annealing denatured DNA with RNA under appropriate conditions. By monitoring the binding of RNA to DNA, one can measure regions on DNA that are complementary to certain RNA species.

By hybridizing ribosomal RNA (rRNA) to DNA, one can determine the proportion of the genome which is complementary to rRNA. Hybridization experiments can also reveal the similarity among rRNA's in terms of their nucleotide base sequences. Hybridization of the rRNA's of different species to the DNA of a single species provides an experimental approach for comparing the base sequences of those rRNA's. One may conclude that rRNA's which hybridize to the same extent to a DNA are more similar to each other than rRNA's which differ in this regard.

Another method for comparing rRNA sequences from different species involves thermal stability measurements of rRNA-DNA hybrids. After rRNA has hybridized to DNA, the hybrids can be dissociated as a function of temperature (Moore and McCarthy 1967). The temperature at which 50% of the rRNA has dissociated from the hybrid is defined as the Tm. Because rRNA from one species may not be the exact complement to the sequences coding for rRNA (rDNA) of another, some base-pairs in the rRNA-DNA hybrid of these two species will be mismatched. Any mismatching is believed to result in hybrids that have lower thermal stabilities than those obtained with homologous DNA's and RNA's.

Several investigations employing the techniques mentioned above have been performed in order to determine the similarity in base sequence among rRNA's of different species of higher plants (Matsuda and Siegel 1967; Matsuda, Siegel, and Lightfoot 1970; Trewavas and Gibson 1968; and Bendich and McCarthy 1970a). Matsuda and Siegel (1967) and Matsuda et al. (1970) hybridized the rRNA of several Dicotyledonae (dicots) to several dicot DNA's. All dicot rRNA's hybridized to the same extent to a given DNA. Therefore, they concluded that rRNA sequences among dicots have been conserved. Bendich and McCarthy (1970a) hybridized dicot rRNA to DNA's of higher plants and determined the Tm's of the various rRNA-DNA hybrids. They concluded from these studies that the evolution of rRNA of higher plants had been conservative in nature. Trewavas and Gibson (1968), on the other hand, claimed that sequences of rRNA among dicots have diverged to an appreciable extent. They also noted that nucleotide sequences of rRNA between Monocotyledonae (monocots) and dicots have diverged to such an extent that less than 10% of the sequences are common to both monocots and dicots.

In this study, rRNA-DNA hybridizations were performed in order to compare the sequence homology of rRNA's among higher plants. Experiments were performed in which monocot rRNA's were hybridized to monocot and dicot DNA's. The reciprocal experiments in which dicot rRNA's were hybridized to dicot and monocot DNA's were also performed. The amount of rRNA complementary to a given DNA was determined, and the thermal stability of the rRNA-DNA hybrids was measured. Evidence from these experiments shows the following: (1) The nucleotide sequences of rRNA's among dicots have been conserved. (2) The nucleotide sequences of rRNA's among monocots have diverged from each other considerably. (3) There has been considerable divergence of rRNA sequences between monocots and dicots.

LITERATURE REVIEW

Hybridization

Hall and Spiegelman (1961) undertook the first RNA-DNA hybridization studies. A mixture of P^{32} labeled T2 RNA and heat-denatured H^3 labeled T2 DNA was heated and cooled slowly to encourage annealing. After cooling, the solution was centrifuged to equilibrium in cesium chloride (CsCl) in order to resolve RNA from DNA. Fractions were collected and monitored for ultraviolet (U.V.) absorption and radioactivity. The formation of an RNA-DNA hybrid was inferred because some of the P^{32} (RNA) co-banded with H^3 (DNA). No hybrid was detected in a mixture of the nucleic acids which had not been subjected to heating and slow-cooling.

The above experiment suffered from two disadvantages that have since been eliminated. Equilibrium centrifugation is both costly and time-consuming, and DNA-DNA annealing competes with RNA-DNA annealing in liquid-liquid hybridizations. Nygaard and Hall (1964) utilized filtration through nitrocellulose filters to selectively remove RNA-DNA hybrids from free RNA. This technique eliminated the necessity for equilibrium centrifugation. Immobilization of single-stranded DNA on agar (Bolton and McCarthy 1962) or nitrocellulose filters (Gillespie and Spiegelman 1965) was found to prevent the reassociation of single-stranded DNA, thus eliminating competition for DNA in RNA-DNA annealing studies.

The procedure developed for RNA-DNA hybridization by Gillespie and Spiegelman (1965) has since been used by many investigators. It consists of allowing solutions containing denatured DNA to filter through nitrocellulose filters. Under appropriate conditions, nearly all of the DNA binds to the filters. The filters on which the single-stranded DNA has been immobilized are then incubated with labeled RNA. After incubation, the filters are washed and incubated with pancreatic RNAase to remove RNA which has not specifically hybridized to DNA. The filters are then monitored forradioactivity to measure the amount of RNA that has hybridized. In one type of experiment, a fixed amount of DNA is hybridized with increasing concentrations of RNA. When such increases resulted in no further net hybrid formation, the DNA is considered to be saturated with the RNA species in question.

RNA-DNA hybrid formation has been found to be sensitive to, among other variables, ionic strength and temperature (see review by Walker 1969). Church and McCarthy (1968) observed that the RNA-DNA hybrids formed at lower temperatures had a lower thermal stability than those of the hybrids formed at higher annealing temperatures. They concluded that the specificity of hybridization reactions decreases at lower temperatures (nonrestrictive temperatures). Thus, RNA-DNA hybrids formed at lower temperatures contain nucleotide sequences that are only partially homologous to each other. Such partial homologies are expected because Britten and Kohne (1968) have demonstrated that families of similar but not identical sequences of DNA exist in many eukaryotes. Hybridization at a higher temperature (restrictive conditions) apparently discriminates against nucleotide sequences of RNA that are only partially homologous to those of DNA. However, as the degree of homology between RNA and DNA increases, hybridization will occur at restrictive conditions despite a small amount of base-pair mismatching.

Types of rRNA and Their Localization

In addition to the cytoplasmic ribosomes, plants contain chloroplastic ribosomes (Lyttleton 1962). Each type has a characteristic sedimentation coefficient: 80 s for cytoplasmic ribosomes and 70 s for chloroplastic (Boardman, Francki, and Wildman 1966). Loening and Ingle (1967) have separated and characterized the rRNA components of the cytoplasm as 25 s and 18 s and those from the chloroplast as 23 and 16 s rRNA.

The genes coding for rRNA in eukaryotes had been suspected of being localized at the nucleolar-organizer (NO) region (Birnstiel 1967). Ritossa and Spiegelman (1965) proved the validity of this hypothesis for Drosophila melanogaster by hybridizing rRNA to the DNA from populations which contained different numbers of NO regions. They found that the percent of the genome complementary to rRNA was directly proportional to the number of NO regions. Birnstiel et al. (1966) have shown that the ribosomal DNA (rDNA) of Xenopus laevis is also localized at the NO region.

The connection between rDNA and the NO region has not as yet been demonstrated for plants. Chipchase and Birnstiel (1963) isolated nuclei and nucleoli from peas. They hybridized rRNA to the DNA extracted from both organelles. Because the rRNA hybridized equally to both DNA's, they concluded that rDNA was not specifically localized at the NO region. However, the isolation of nucleoli not contaminated by a considerable amount of nuclear debris is a difficult procedure, and it is quite possible that the nucleolar DNA of Chipchase and Birnstiel (1963) was contaminated by significant amounts of nuclear DNA. It would be of interest to repeat the experiment and determine whether nucleolar DNA can be physically distinguished from nuclear DNA.

Attempts have also been made to locate the genes that code for chloroplastic rRNA. Hypothetically, there are three possibilities for the location of the chloroplastic rDNA: exclusively in the nucleus, exclusively in the chloroplast, or distributed in both. Scott and Smillie (1967), Tewari and Wildman (1968), and Jaworski (1969) detected hybridization between chloroplastic rRNA and chloroplastic DNA. Tewari and Wildman (1968) and Matsuda et al. (1970) detected hybridization between chloroplastic rRNA and nuclear DNA. Therefore, chloroplastic rDNA is apparently distributed in both chloroplastic and nuclear DNA. It is also of interest that Jaworski (1969) showed that some cytoplasmic rDNA was found in chloroplastic DNA.

Satellite DNA and Redundancy of rDNA

From a knowledge of the percentage of the genome complementary to rRNA, molecular weight of rRNA, and DNA content per cell, it is possible to compute the number of rRNA genes per cell. In virtually all organisms examined there is, in fact, more than one copy of rDNA per cell. The redundancy of rDNA varies from only a few in bacteria (Kohne

1968) to several thousand in some higher plants (Matsuda and Siegel 1967).

In many organisms the guanine + cytosine (GC) content of rRNA (and thus the strand of rDNA coding for it) differs from that of the bulk DNA. A method capable of distinguishing DNA's on the basis of GC content could theoretically resolve rDNA from bulk DNA. Isopycnic banding of DNA in CsCl operates on the basis of buoyant density (Meselson, Stahl, and Vinograd 1957). The buoyant density, in turn, is dependent on the GC content (Schildkraut, Marmur, and Doty 1962). When nuclear DNA of either oocytes of X. laevis or somatic tissue of Cucurbita pepo is banded in CsCl, a band with a buoyant density greater than that of the rest of the DNA is found (Brown and Dawid 1968, Matsuda and Siegel 1967). It was also demonstrated that the rRNA hybridized exclusively to satellite DNA in both cases. Thus, only the satellite DNA contained rDNA. Even though the rDNA of most organisms cannot be seen as a discrete component in CsCl gradients, rRNA will frequently hybridize to DNA whose buoyant density is usually greater than that of the bulk DNA and whose GC content is just slightly below that of rRNA (Sinclair and Brown 1967).

Evolution of rRNA

The GC content and molecular weight of rRNA from a number of species have been compiled. The GC content of rRNA, as a whole, varies from 39 to 65% in all organisms studied to date (Perry et al. 1970, Attardi and Amaldi 1970). The molecular weight of the larger rRNA subunit varies from 1.1 to 1.7 x 10^6 daltons; the smaller from 0.56 to 0.66 x 10^6 daltons (Ingle 1968, Perry et al. 1970).

In addition to the evolutionary differences in GC content and in molecular weight of rRNA, information concerning differences in its base sequence has also accumulated. The differences in the base sequence of rRNA of a number of species have been compared to differences in the base sequence of the rest of the genome. Species of bacteria within the Bacillus group have been investigated in this regard by Dubnau et al. (1965), Doi and Igarashi (1965), Moore and McCarthy (1967), and Takahashi, Saito, and Ikeda (1967). They hybridized labeled rRNA and either labeled DNA or pulse-labeled RNA (messenger RNA) of one species to DNA of that species (homologous hybridization) as well as to DNA of other species (heterologous hybridizations). They observed that heterologous hybridization values for rRNA, in many cases, were 50 to 80% of the homologous value, whereas similar values for DNA or messenger RNA were frequently less than 10% of the homologous value. They concluded, therefore, that relative to the rest of the genome, rRNA sequences have been conserved. Moore and McCarthy (1967) and Kohne (1968) conducted similar experiments between other genera of bacteria and arrived at the same conclusion. In eukaryotes a similar conclusion was reached for the genera Saccharomyces and Drosophila by Bicknell and Douglas (1970) and Laird and McCarthy (1968), respectively. Thus, the conservation of rRNA relative to the rest of the genome may apply to all cellular organisms.

Several investigators have studied rRNA sequence homology in higher plants. Matsuda and Siegel (1967) hybridized dicot rRNA's to dicot DNA's. The dicot rRNA's hybridized equally to a given DNA and were thus judged to have very similar base sequences. Trewavas and Gibson (1968) hybridized labeled pea (a dicot) rRNA to pea DNA in the presence of excess unlabeled dicot and monocot rRNA's. If sequences in the unlabeled rRNA differ from those in the labeled rRNA, the former should have no effect on the hybridization value (Hall, Nygaard, and Green 1964). When unlabeled dicot rRNA's competed with labeled pea rRNA, the hybridization value was 15 to 75% of the control. When unlabeled monocot rRNA's competed with labeled pea rRNA, the hybridization value was 95% of the control. They concluded that 25 to 85% of the rRNA sequence of pea is conserved among other dicot rRNA's and that less than 5% of the rRNA sequence of pea is conserved among monocot rRNA's. These results were compared with those of Bendich and Bolton (1967), who found a 5 to 55% DNA-DNA homology among dicots and a 0 to 5% DNA-DNA homology between monocots and dicots. Therefore, rRNA sequences among dicots and between monocots and dicots are conserved no more than the DNA sequences which represent the rest of the genome. However, the work of Trewavas and Gibson (1968) has been criticized by two independent groups. Bendich and McCarthy (1970a) suspected that the radiolabeled pea rRNA preparations were dominated by bacterial contamination, and Matsuda et al. (1970) were unable to detect divergence of rRNA sequences among the same dicots tested by Trewavas and Gibson (1968).

Bendich and McCarthy (1970a) attempted to correlate taxonomic relationships to rRNA sequence homology. They annealed labeled pea rRNA to DNA's of plants, animals, and microorganisms and then determined the Tm's of the various rRNA-DNA hybrids. The pea rRNA-pea DNA hybrid displayed the highest Tm, and other hybrids had lower Tm's, depending on the source of rRNA. In general, the more closely related the heterologous source of rRNA was to pea, the higher was the Tm of the hybrid. They concluded that the hierarchy of relatedness of rRNA's among some higher plants paralleled their phylogenetic relatedness.

Although the preponderance of the data favors rRNA conservation relative to the rest of the genome, there is some evidence, although controversial, that such is not the case in the Angiospermae. It is hoped that this study can verify or disprove the controversial data.

MATERIALS AND METHODS

Both seeds and leaves of the following plants were utilized in this study:

Hordeum vulgare L., cv. Betzes (barley)

Panicum antidotale Retz. (blue panicum grass)

Triticum vulgare L., cv. Siete Cerros (wheat)

Gossypium barbadense L., cv. Pima (cotton)

Cucurbita pepo L., cv. Small Sugar (pumpkin).

The first three are monocots; the last two dicots.

Seeds were immersed for 10 minutes in a 1% hypochlorite solution immediately before germination. Monocot seeds were germinated on paper towels at $15^{\circ}C$; dicot seeds in perlite at about $30^{\circ}C$.

DNA Extraction

All procedures were performed at $0\text{-}4^{\circ}C$ unless otherwise noted.

Monocot Seeds

Dry seeds were weighed, ground to a powder in a Waring blender, and mixed with 1-1.5 V/W of 0.4 *M* sucrose-0.01 *M* MgCl-0.05 *M* tris-0.005 *M* mercaptoethanol (pH 8.2) (grinding buffer). The homogenate was filtered through 2 layers of cheesecloth, and the nuclear pellet was collected by centrifugation at 1,000 x *g* for 5 minutes. To solubilize plastid material, the pellet was resuspended several times with 3.5% Triton X-100 (Rohm and Haas) in grinding buffer and collected each time with the same conditions of centrifugation as noted above. The pellet was suspended in a small volume of 1 x SSC (0.15 M NaCl-0.015 M $Na_3C_6H_5O_7 \cdot 2\ H_2O$). Addition of 0.1 volumes of 5% sodium dodecyl sulfate (SDS) in 45% ethanol and the subsequent 10-minute incubation at 60°C lysed the nuclei. The lysate was made to 1.0 M with NaCl and centrifuged for 10 minutes at 12,000 x g. The supernatant was shaken gently for 5 minutes in a separatory funnel with an equal volume of 1% octanol in chloroform. Centrifugation for 3 minutes at 3,000 x g separated the phases. The upper aqueous phase was removed with a wide-mouthed medicine dropper to prevent shearing. The chloroform treatment was repeated as long as denatured protein was visible at the interface. Addition of 2 volumes of ethanol precipitated the DNA, and it was collected by spooling it around a glass rod. The DNA was suspended in 0.1 x SSC, made to 1 x SSC with 10 x SSC, and subjected to further purification described in DNA Purification section.

Dicot Seeds

The above method could not be applied to dicot seeds because of lipid interference. Decorticated dry seeds were ground in a Waring blender, passed through a 60-mesh Tyler screen, and defatted with organic solvents. The DNA was liberated with SDS, as above. The method of defatting and extraction is similar to that described by Ergle and Katterman (1961).

Leaves

Leaves, whether from monocot or dicot, could be treated identically. They were passed through a meat grinder and collected in a chilled mortar. After addition of acid-washed sand, they were further ground with a mortar and pestle in 1 V/W of grinding buffer. The homogenate was then processed exactly as stated for monocot seeds.

Labeling of DNA

All glassware and solutions were autoclaved at 121°C under a pressure of 30 pounds per square inch. Thirty-five grams of decorticated cotton seeds were sterilized as previously described and placed in a Petri dish with 30 ml of Hoagland's nutrient solution and 250 uC of thymidine-H^3-CH_3 (New England Nuclear, sp. act. of 2 C/mM). After 7 hours of occasional stirring at 37°C, the fully imbibed seeds were rinsed with distilled water and placed in 20 ml of Hoagland's solution. They were harvested after an additional 24 hours, at which time at least 95% of the seeds exhibited a hypocotyl of 1 to 3 mm in length. The DNA was extracted according to the methods described in the section Dicot Seeds.

DNA Purification

DNA extracted as described in sections Monocot Seeds, Dicot Seeds, Leaves, and Labeling of DNA was subjected to further purification. The RNA was removed by incubating the DNA solution for 30 minutes at 37°C with pancreatic RNAase (Calbiochem, 5x crystallized) at a final concentration of 50 µg/ml. The stock solution of RNAase was prepared according to Marmur (1961). At the end of the 30-minute period, the RNAase was hydrolyzed by incubation with 100 µg/ml of pronase for 2 hours at 37°C. Previously the pronase had been self-digested for 1 hour at 37°C to remove any contaminating DNAase. The DNA was then subjected to chloroform treatment, precipitated, and resuspended as described in Dicot Seeds section.

Remaining contaminants were separated from DNA by Sepharose 2B (Pharmacia) chromatography. A 2 x 20 cm bed volume was packed in a column and washed with a large volume of degassed 1 x SSC. The DNA, in one ml of 1 x SSC, was applied to the column and eluted with 1 x SSC. Three ml fractions were collected, and absorbance was monitored at 260 nm. The column was equilibrated with 0.02% sodium azide, when not in use.

The DNA fractions were pooled, and an absorption spectrum was read between 220 and 320 nm. The DNA concentration was estimated by assuming that a concentration of 1 mg/ml of pure DNA in a 1-cm path length should read 20 optical density (O.D.) units at 260 nm (Eigner and Doty 1958). [The DNA solutions whose concentrations were also estimated colorimetrically by the diphenylamine method gave the same value.]

Preparative Cesium Chloride Density Gradient Centrifugation

The DNA purified by the methods given in the last section was routinely used for hybridization experiments. In some cases, these DNA's were further purified using preparative CsCl density gradient centrifugation (Flamm, Bond, and Burr 1966). Five hundred ug of DNA in 7.85 ml of 0.05 *M* tris (pH 8.5) was added to 9.8 gm of solid CsCl. The density CsCl was adjusted to 1.700 gm/cc with the aid of a Zeiss refractometer (Ifft, Voet, and Vinograd 1961). The solutions were loaded to the top of 2½" x 5/8" polyallomer tubes. Samples were centrifuged 35,000 rpm for 64 to 68 hours at 20°C, using the Spinco model L-2 ultracentrifuge with the no. 50 angle rotor. The rotor was allowed to stop without braking, and the solutions were pumped through an Isco model UA-2 U.V. analyzer at 2 ml/minute. The sample was continuously monitored at 254 nm. The fraction representing DNA was diluted with 1 x SSC and centrifuged 45,000 rpm in the L-2 ultracentrifuge for 12 hours at 5°C. Any material with a sedimentation coefficient greater than 5 s should have been quantitatively pelleted (Bond et al. 1967). The DNA pellet was resuspended in 1 x SSC.

Denaturation and Renaturation of DNA

The pH of a DNA solution 10-50 µg/ml was raised to 12.0-12.5 by the addition of 0.1 volumes of fresh 1 N NaOH. After 10 minutes the solution was neutralized with 0.15 volumes of 1 M KH_2PO_4 and immediately diluted to approximately 3 ug/ml. To renature, denatured DNA was incubated in 2 x SSC for 2 hours at 68°C.

Analytical Cesium Chloride Density Gradient Centrifugation

Three ug of native, denatured, or renatured plant DNA and 1 ug of marker DNA (*Micrococcus luteus*) in 0.6 ml of 1 x SSC were added to 0.75 gm of solid CsCl, and the density was adjusted to 1.700 gm/cc. The DNA solutions were loaded in a cell containing an Epon centerpiece and centrifuged at 44,000 rpm in the Spinco model E ultracentrifuge equipped with U.V. optics and the four-cell mask and timer accessories.

After 18 to 20 hours, U.V. absorption photographs were taken on Kodak commercial film, and the negatives were traced on a Beckman model RB Analytroldensitometer equipped with a film densitometer accessory. Buoyant densities were calculated by the method of Sueoka (1961).

Labeling of rRNA

Monocot

Seedlings about 3 weeks old were cut off at the base of the shoot. One hundred and twenty leaves (about 10 gm) were placed upright in 20 conical containers (Nielsen and Rohringer 1963). One mC of uridine-5-H^3 (Schwarz Bio Research Inc., sp. act. greater than 20 C/mM) was distributed evenly among the containers. After the leaves had imbibed the labeled base through their shoots, they were kept in a small volume of distilled water for 48 hours. At the end of the period rRNA was extracted.

Dicot

Approximately 10 gm of leaves were excised from 3-week-old plants. Slits 2 mm apart were cut perpendicular to the long axis extending from either side of the midrib to just short of the margin (Zaitlin, Spencer, and Whitfeld 1968). The leaves were placed in a Petri dish 14 cm in diameter and covered with 0.01 $\underline{M}$ KH_2PO_4. One mC of labeled uridine was added and incubation continued for 48 hours.

rRNA Extraction

All glassware was flamed or treated with chromic acid; all solutions were boiled; leaves or roots were rinsed several times with distilled water and blotted dry. They were ground to fine powder with a mortar and pestle at -20°C. Thawing took place in 4 V/W of 0.1% bentonite [prepared by method of Fraenkel-Conrat, Singer, and Tsugita (1961)]-1% sodium pyrophosphate-1% SDS (pH 5.0). Another 4 V/W of phenol, redistilled over zinc and equilibrated with 1 x SSC, was added. The mixture was shaken vigorously in the cold for 15 minutes. Centrifugation for 10 minutes at 5,000 x g separated with the phases. The aqueous phase was submitted to two more phenol treatments.

The RNA was precipitated with 0.1 volumes of 20% potassium acetate (pH 5.0) and 2 volumes of ethanol, and the precipitate was collected by centrifugation at 12,000 x g for 10 minutes. The pellet was suspended in a small volume of 0.5% SDS-0.15 M sodium acetate (pH 8.0) and subjected to phenol treatment until protein was no longer visible at the interface. Phenol was removed from the aqueous layer by shaking three times with 2 volumes of cold diethyl ether. Traces of ether evaporated as nitrogen gently bubbled through the solution. The RNA was precipitated and collected as before.

The RNA was suspended in 1 ml of 0.067 M phosphate buffer (pH 6.8), to which was added 1 ml of 4 M LiCl. After 12 hours rRNA precipitated (Avital and Elson 1969). The precipitate was collected by centrifugation at 12,000 x g for 5 minutes and washed sequentially in 2 M LiCl and 75% ethanol. The RNA was suspended in 1 ml of the phosphate buffer, and an absorption spectrum was determined between 220 and 320 nm. The RNA concentration was estimated by assuming that a concentration of 1 mg/ml of RNA in a 1-cm path length has an O.D. of 25 at 260 nm (Sueoka and Yamane 1962). [The RNA solutions whose concentrations were also estimated colorimetrically by the method of Dische and Schwarz (1937) gave the same values.]

Cotton rRNA, prepared by the above methods, still had a red pigment. It was further purified by the method of Bellamy and Ralph (1968).

Gel Electrophoresis of rRNA

Ribosomal RNA was analyzed electrophoretically according to the method of Bishop, Claybrook, and Spiegelman (1967). The gels were soaked in distilled water for several hours in a Petri dish, transferred to a quartz cuvette (5 mm x 100 mm x 8.5 mm), and scanned at 260 nm on a Gilford Model 240 spectrophotometer equipped with a Model 2410 linear transport device and a Heathkit Servo-Recorder Model EUW-20A.

Gels that were to be monitored for radioactivity were frozen solid, quickly by means of Dry Ice, in a curved spatula and cut into 1.05 mm slices with a bank of evenly spaced stainless steel razor blades. The slices were digested in 0.5 ml of 30% H_2O_2 for 24 hours at 50°C and then counted for radioactivity (Siegel and Huber 1970). Counting was done as noted in section Scintillation Counting.

Annealing of rRNA to DNA

Hybridization was carried out essentially according to Gillespie and Spiegelman (1965). Bac-T-Flex B6 nitrocellulose membranes (Schleicher and Schuell Co.), 47 mm in diameter, were soaked in 2 x SSC and washed under suction with the same buffer. A solution of DNA in 2 x SSC, denatured as described in Denaturation and Renaturation of DNA, was passed through the filters, which were mounted on Millipore sterifil™ aseptic filtration systems. Each membrane was washed with 50 ml of 2 x SSC under gravity. The filters were air-dried for 30 minutes and then in vacuo for 2 hours at 80°C.

Each filter was quartered, inserted into a vial, and incubated for 18 hours at 68°C in 1 ml of 0.1% SDS in 2 x SSC containing appropriate amounts of labeled rRNA. The reaction was stopped by chilling the vials. The filters were washed batchwise in the following solutions for 30 minutes each: 6 x SSC at 50°C, 2 x SSC, 2 x SSC, 20 ug/ml of pancreatic RNAase in 2 x SSC, and 2 x SSC. Filters were oven-dried and counted.

In each experiment a constant amount of DNA was incubated with increasing concentrations of RNA. There was a minimum of six different concentrations, each of which was duplicated. The count from blank filters, without DNA, was subtracted from the others. To calculate the percent hybridization at saturation, the data were transformed and plotted according to Bishop et al. (1969). Different rRNA preparations from the same species, but with similar specific activities, were utilized.

Competition

A series of filters with a constant amount of DNA was incubated with a fixed, saturating concentration of labeled rRNA. Varying concentrations of unlabeled rRNA, up to 15-fold excess, were added to each vial in duplicate. To calculate the percent competition at infinite concentration, the data were transformed and plotted according to Bishop et al. (1969).

Retention

For studies of DNA retention by the filters, known amounts of labeled DNA were mock-hybridized with cold rRNA. For comparison, some filters were counted immediately after embedding and also after hybridization (including washing).

Hybridization of DNA Fractionated on a Preparative Cesium Chloride Density Gradient

DNA (100-200 µg) in 3.25 ml of 0.05 M tris buffer (pH 8.5) was added to 4.25 gm of solid CsCl and the final density adjusted to 1.700 gm/cc. The solution was placed in the polyallomer tube and overlaid to the top with mineral oil. Centrifugation was as described in section Preparative Cesium Chloride Density Gradient Centrifugation.

After centrifugation, 15-drop fractions were collected by piercing the bottom of the tube with a 20-gauge needle. One ml of 1 x SSC was added to each fraction and the O.D. read at 260 nm. Alternate fractions were hybridized with rRNA as described in the previous section, except that filters were incubated in a common beaker with labeled rRNA in 5 ml of 0.1% SDS in 2 x SSC. Some filters were split, and each segment challenged with a different rRNA, as outlined by Brown and Weber (1968).

Thermal Stability of rRNA-DNA Hybrids

Duplicate filters were incubated with a saturating concentration of labeled rRNA and treated as described in Annealing of rRNA to DNA, except that they were not oven-dried or counted. The filters were then incubated at 60°C in separate vials for 5 minutes in 2 ml of 2 x SSC. The filters were transferred to another pair of vials and the incubation was continued at 65°C. The process was repeated in 5°C increments up to 100°C, with 10 minutes allotted for equilibration at each temperature. (Boiling water had a temperature between 97 and 98°C but was plotted as 100°C.) The radioactivity in each pair of vials, representing rRNA melted at each temperature, was counted as described under Scintillation Counting; also the filters were counted after the last incubation to determine if any radioactivity remained on them. The percent of cumulative counts released was plotted against temperature, and the Tm was read directly from the graph.

Scintillation Counting

Samples were counted at optimal gain (65%) and a window setting of 50-1000 on a Packard model 3320 Tri-Carb scintillation spectrometer. Filters were counted in vials containing 10 ml of 0.5% PPO (2,5-diphenyloxazole) in toluene; aqueous solutions were counted in vials with 15 ml of 0.01% POPOP (1,4-bis[2-(5-phenyloxazolyl) benzene])-0.55% PPO in a solution 33% Triton X-100 and 67% toluene. The background was usually 25-30 counts per minute.

RESULTS

Characterization of DNA

The DNA's used in hybridization experiments were characterized by CsCl isopycnic centrifugation in a model E analytical ultracentrifuge in order to determine their buoyant densities and also to detect whether a DNA satellite might be present. Ultraviolet photographs of the DNA's were taken at equilibrium, and densitometer tracings of these photographs were made. The tracings are shown in Figures 1 through 5, and the buoyant densities calculated from these figures are summarized in Table 1. With the exception of pumpkin, no other DNA displays a satellite component. The buoyant densities of the native DNA's from barley, blue panicum grass, wheat, cotton, pumpkin, and pumpkin satellite are 1.701, 1.702, 1.703, 1.692, 1.694, and 1.705 gm/cc, respectively. The buoyant densities for barley, wheat, pumpkin, and pumpkin satellite agree closely with previously reported values (Sinclair and Brown 1967, Matsuda and Siegel 1967). The values for blue panicum grass and cotton DNA's were previously unpublished.

It is of interest to note that the DNA's of the monocot species examined have similar buoyant densities of 1.701 to 1.703 gm/cc and that the DNA's from both dicot species examined are characterized by somewhat lower buoyant densities. Other investigations of monocot and dicot DNA's show a similar relationship of their buoyant densities. Green and Gordon (1967) list the buoyant densities of DNA's from

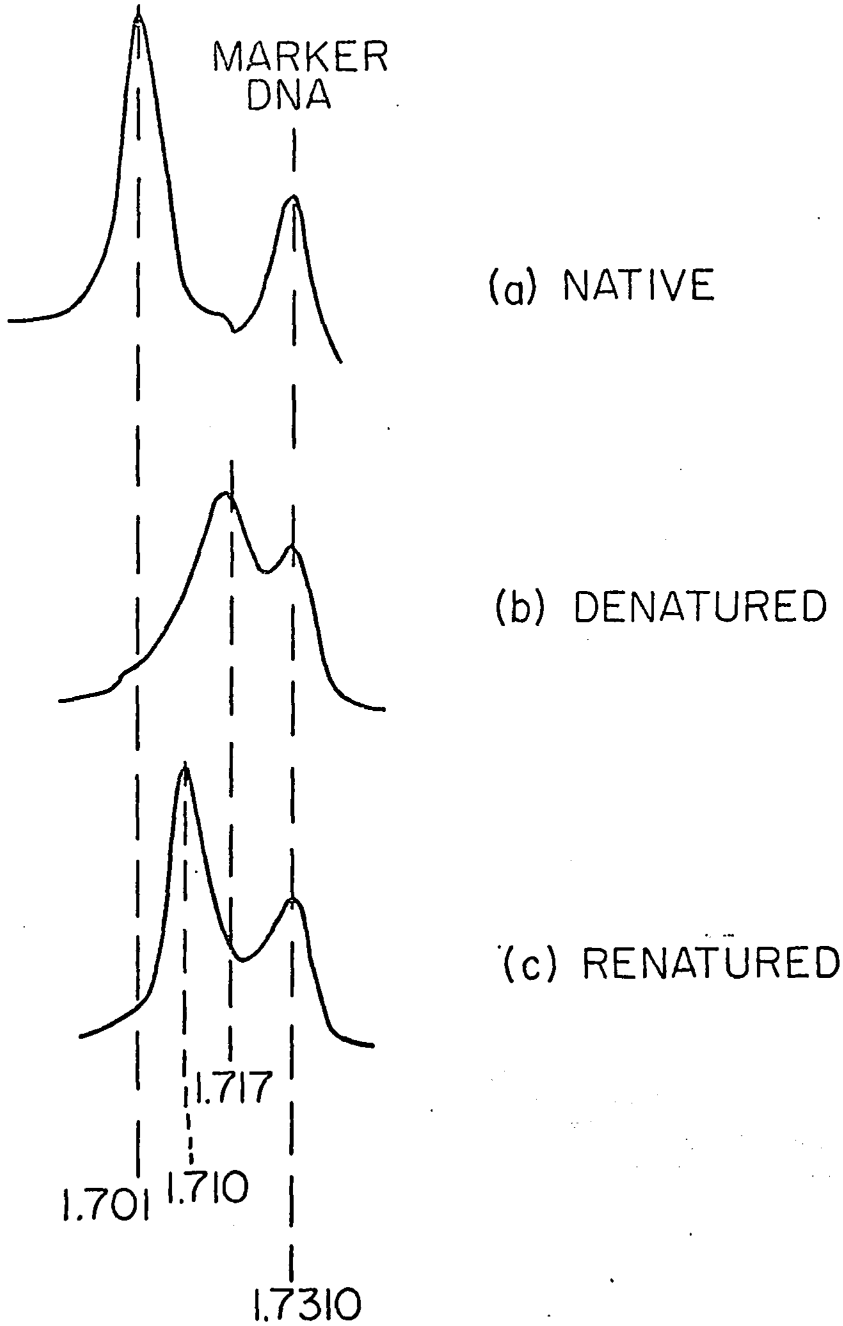

Figure 1. Densitometer tracings of ultraviolet photographs of barley DNA banded by analytical CsCl centrifugation.

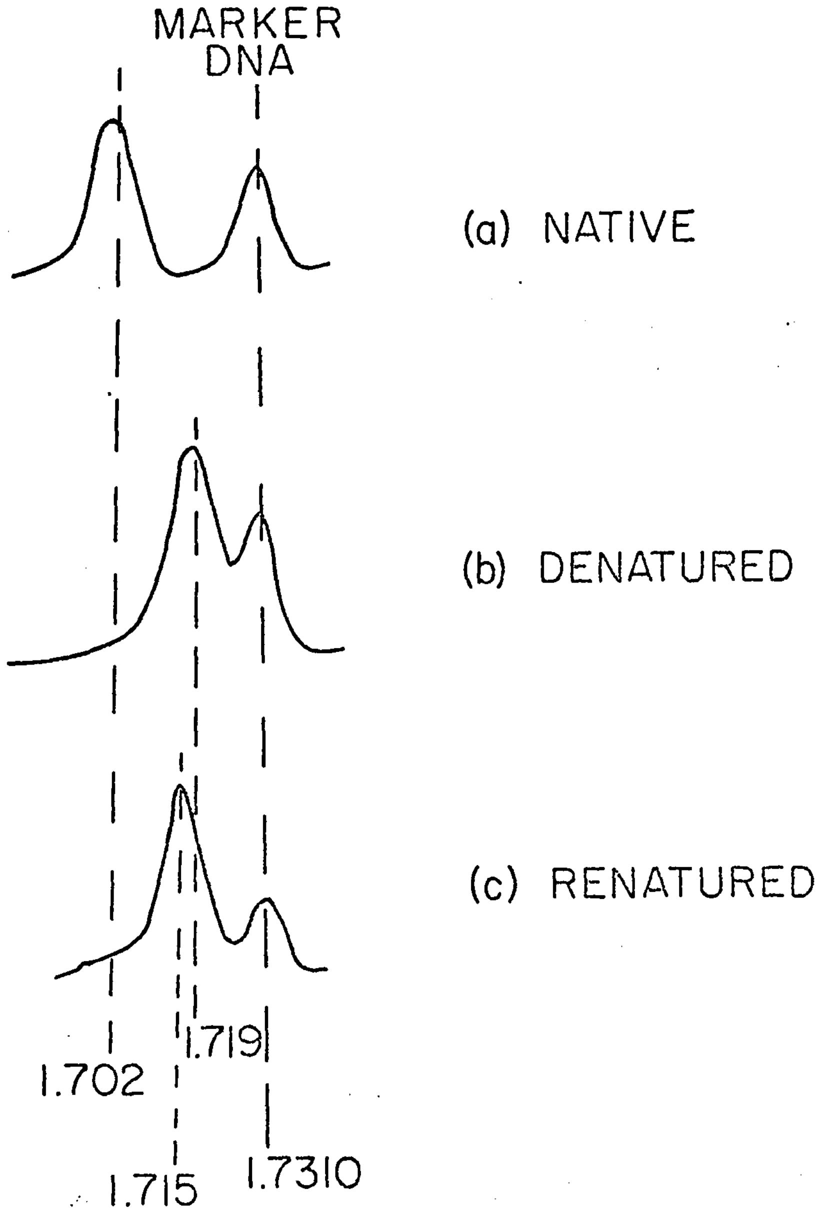

Figure 2. Densitometer tracings of ultraviolet photographs of blue panicum grass DNA banded by analytical CsCl centrifugation.

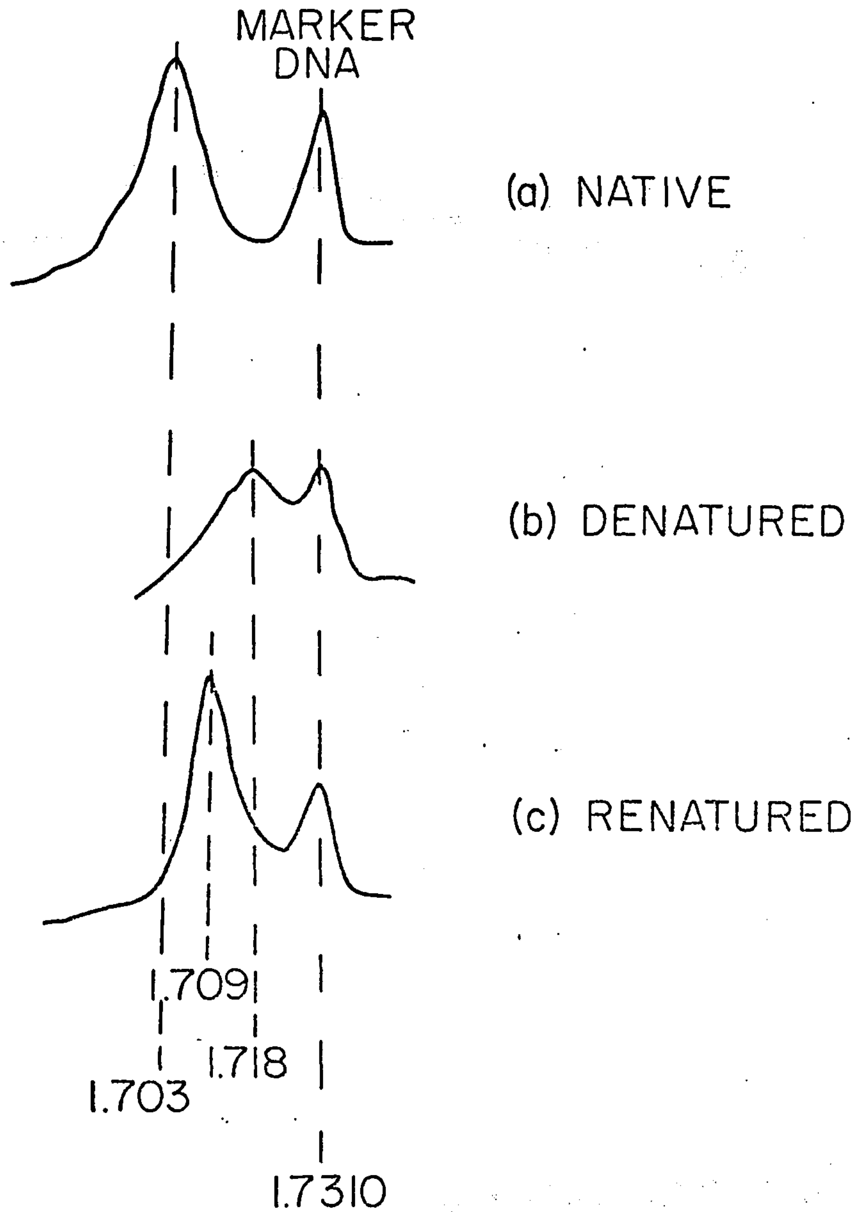

Figure 3. Densitometer tracings of ultraviolet photographs of wheat DNA banded by analytical CsCl centrifugation.

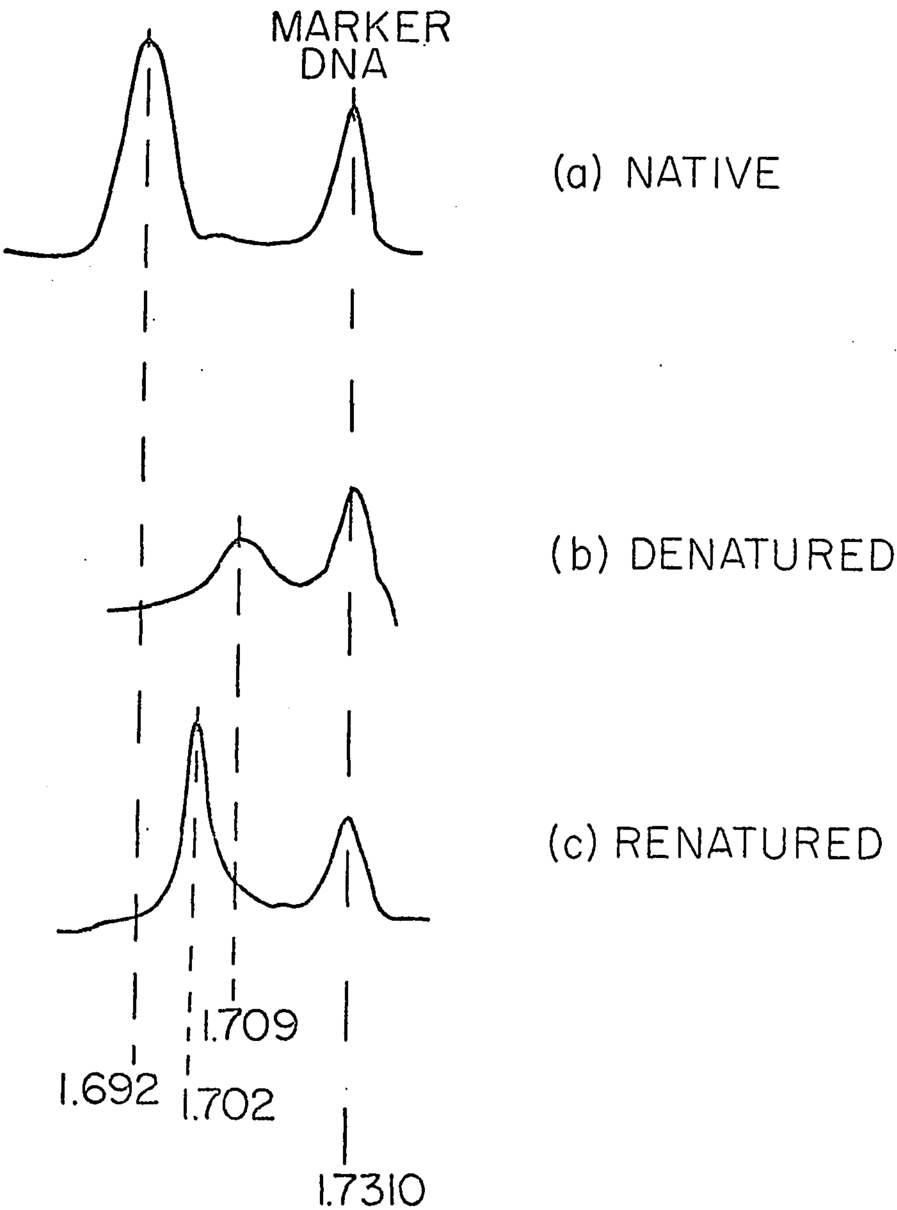

Figure 4. Densitometer tracings of ultraviolet photographs of cotton DNA banded by analytical CsCl centrifugation.

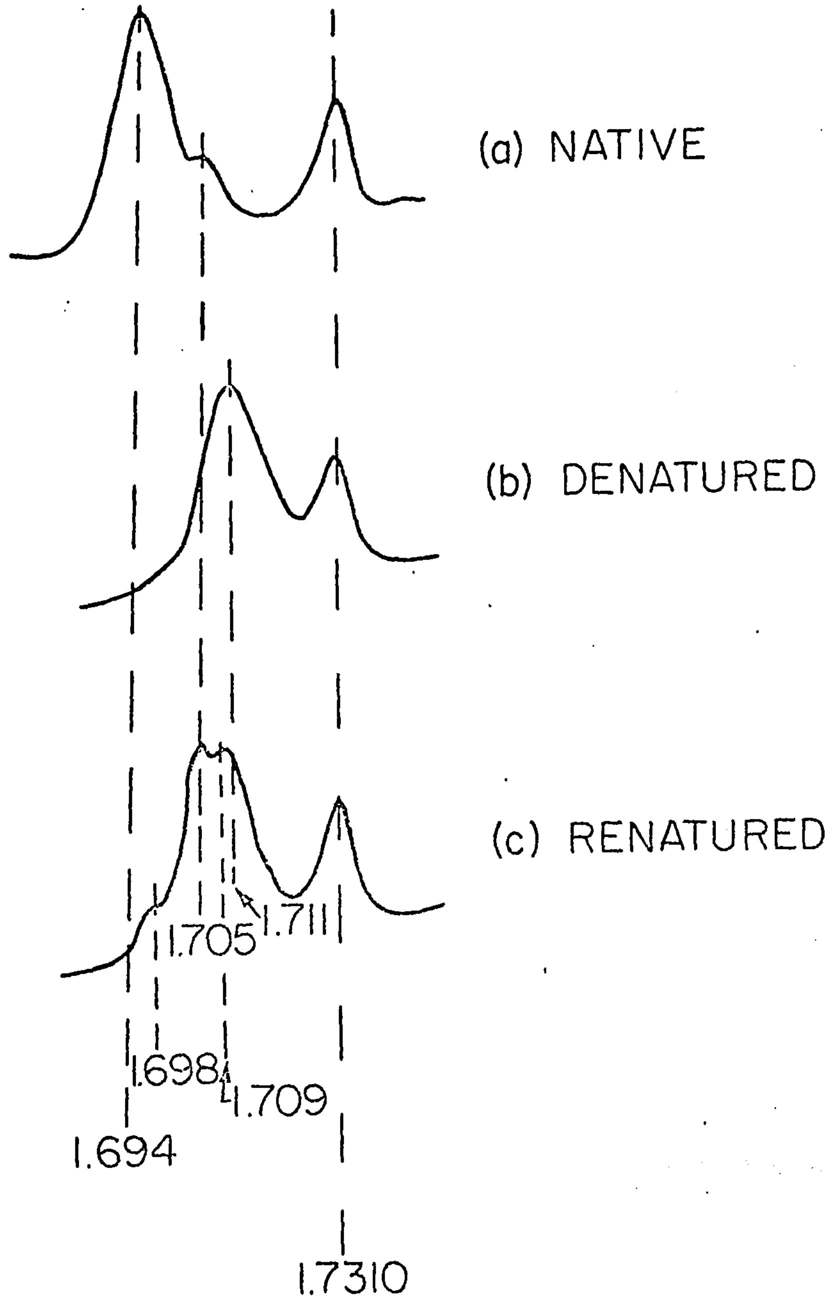

Figure 5. Densitometer tracings of ultraviolet photographs of pumpkin DNA banded by analytical CsCl centrifugation.

Table 1. Buoyant density of plant DNA's.[a]

	Native	Denatured	Renatured
Barley	1.701	1.717	1.710
Blue panicum grass	1.702	1.719	1.715
Wheat	1.703	1.718	1.709
Cotton	1.692	1.709	1.702
Pumpkin	1.694	1.711	1.709, 1.705
Pumpkin satellite	1.705	--	--
B Component of pumpkin	--	--	1.698

a. Plant DNA's (3 μg) and marker DNA (1 μg) were centrifuged to equilibrium in analytical CsCl gradients. U.V. photographs were taken and then traced with a densitometer. Buoyant densities were calculated according to the method of Sueoka (1961) from the densitometer tracings shown in Figures 1 through 5.

several dicots. They vary from 1.690 to 1.697 gm/cc. Bendich and McCarthy (1970b) assign buoyant densities of 1.700 and 1.702 gm/cc respectively to the DNA's of oats and rye (monocots). However, Green and Gordon (1967) and Wells and Ingle (1970) report the buoyant density of onion DNA, a monocot, as 1.688 and 1.691 gm/cc, respectively. Thus, it appears that the difference in buoyant densities between monocot and dicot DNA's may have some taxonomic significance despite the one exception.

The buoyant densities of the DNA's from all five species were examined following alkaline denaturation. The results are shown in Figures 1(b) through 5(b) and are summarized in Table 1. The buoyant densities increased by 0.015 to 0.017 gm/cc over those of native DNA's. Again it is of interest to note that the denatured monocot DNA's have similar buoyant densities (1.717-1.719 gm/cc) and that they are greater than the buoyant densities of dicot DNA's.

The denatured DNA's (3 ug/ml) from all five species were renatured in 2 x SSC for 2 hours at 68°C. The buoyant densities of the renatured DNA's are shown in Figures 1(c) through 5(c) and are summarized in Table 1. Renaturation of DNA involves a transition from the single-stranded state to the double-stranded state. During this transition, the buoyant density of DNA decreases. This decrease is a relative measure of the extent of renaturation. The denatured DNA's of barley, blue panicum grass, cotton, wheat, and pumpkin renatured to different extents. The DNA of wheat renatured to the greatest extent; the DNA's of barley and cotton renatured to a lesser degree than did that of wheat; the DNA's of blue panicum grass and pumpkin renatured less than those of barley, wheat, and cotton. Renatured pumpkin DNA is characterized by a minor component with a buoyant density of 1.698 gm/cc and two components of approximately equal amplitude with buoyant densities of 1.705 and 1.709 gm/cc. The minor component (B component) is a rapidly renaturing fraction currently being investigated by Thornburg (1970). The component at 1.709 gm/cc may represent some DNA which renatured very little under the conditions employed, and the other component may represent DNA that renatured to a greater extent.

Wetmur and Davidson (1968) have noted the effect of various parameters on the renaturation of DNA: DNA concentration, time of reaction, molecular weight of DNA, ionic strength and pH of the solvent, and complexity of the DNA (the number of base-pairs in non-repeating sequences per cell). The DNA's from all species examined were renatured under identical conditions with the exceptions of complexity of DNA and molecular weight of DNA. Because the DNA's of all species were extracted in a similar fashion, it is assumed, but not demonstrated, that the DNA's were of similar molecular weights. Thus, the differences in the renaturation rates of the DNA's examined may reflect differences in the complexity of the DNA's. Because the renaturation rate of DNA is inversely proportional to the complexity (Wetmur and Davidson 1968), the results obtained indicate that either the content of families of repeated nucleotide sequences in barley, wheat, and cotton DNA's may be greater than those in blue panicum grass and pumpkin DNA's or that possibly the genomic content of DNA may be less in the former species than in the latter. Bendich and McCarthy (1970b) have, in fact, noted that a large fraction of barley and wheat DNA's are composed of redundant sequences.

Characterization of rRNA

Ribosomal RNA preparations were routinely subjected to polyacrylamide gel electrophoresis in order to determine the presence and integrity of the individual rRNA components. After electrophoresis, gels were scanned at 260 nm, and the O.D. was recorded. Electrophoretic patterns of rRNA from leaves display 4 O.D. peaks. Figure 6(a) represents a typical profile. The s values have been assigned in accordance with Loening and Ingle (1967). The area under the 25 s peak appears to be at least twice that under the 18 s peak. Since the molecular weight (hence the nucleotide content) of the 25 s RNA is about twice that of the 18 s rRNA, such a relationship would be expected if both components are present in equimolar amounts. However, there is less 23 s than 16 s rRNA, possibly on account of degradation of the former. Figure 6(b) depicts the profile for rRNA from barley roots. Supposedly roots from corn contain no 70 s ribosomes (Hsiao 1964) and therefore should not have 23 s and 16 s rRNA. Shoulders on the light side of the 25 s and 18 s rRNA may be degradation products or bacterial contamination.

In order to compare the O.D. profile with the radioactivity profile, a gel with labeled barley rRNA was fractionated. Labeled rRNA was subjected to electrophoresis as described above. After scanning for optical density, the gel was sliced, and the radioactivity of each

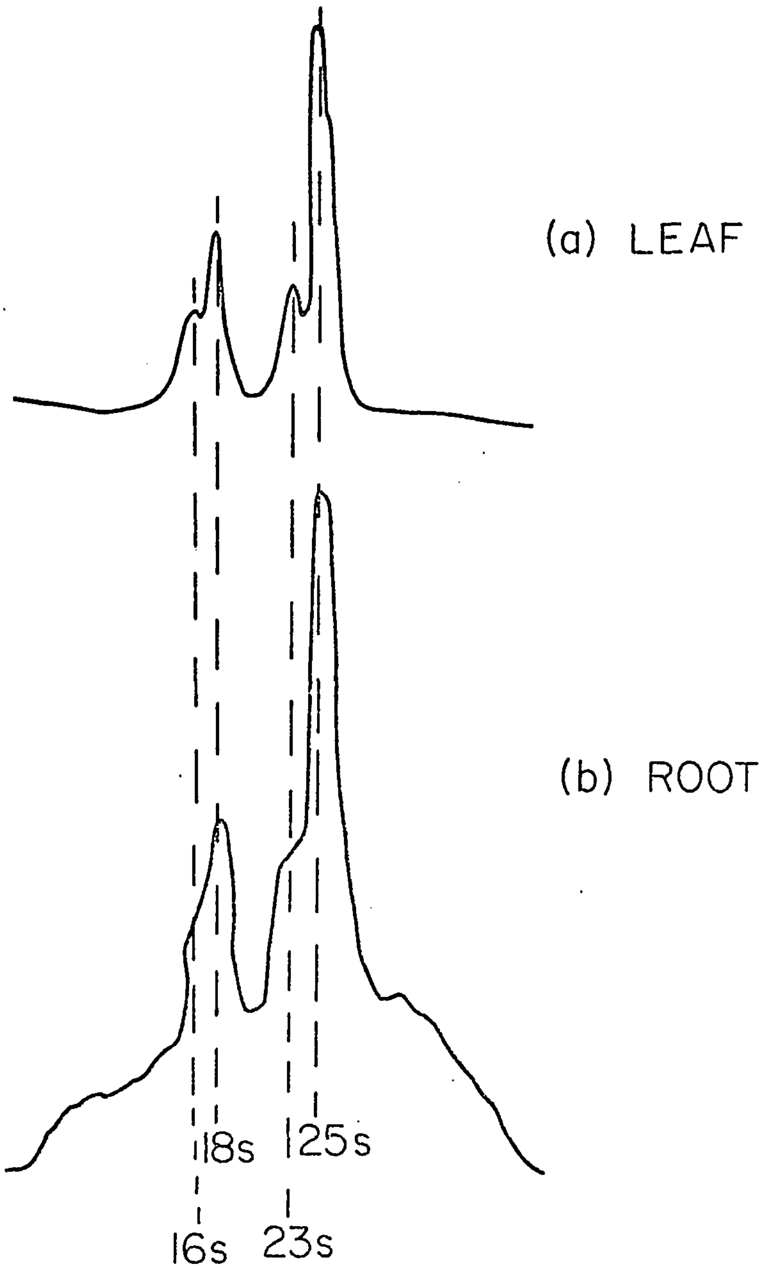

Figure 6. Gel electropherogram of barley leaf and root rRNA's.

fraction was determined. Labeled rRNA from leaf material was expected to have four peaks of radioactivity as well as of optical density. Figure 7 is a comparison of the optical density and radioactivity profiles of barley rRNA components separated by polyacrylamide gel electrophoresis. The four labeled peaks correspond closely to the four optical density peaks. The two additional minor peaks of low specific activity, that are lighter than 16 s, probably represent degradation products. The above data indicate that labeled rRNA is synthesized by plants and not by contaminating bacteria.

Filter Retention of DNA

Gillespie and Spiegelman (1965) monitored the retention of labeled DNA by filters during the course of hybridization experiments. They found that more than 95% of the DNA was still filter-bound after a hybridization experiment. It was decided to perform similar experiments to determine how much DNA was retained by filters under the experimental conditions employed in this study.

Ten or 20 ug of labeled cotton DNA (specific activity of 85 counts/minute/μg of DNA) were embedded in quadruplicate in three separate experiments. Two filters were counted immediately after embedding; the other two were counted after a mock-hybridization with unlabeled rRNA and subsequent washing. Under the experimental conditions employed, 100% of the DNA was embedded on the filters. During the hybridization and subsequent washing, however, 62%, 75%, and 82% of the DNA were retained at the end of the three experiments. Although an average of 27% of the DNA was released from the filters in these experiments, no correction

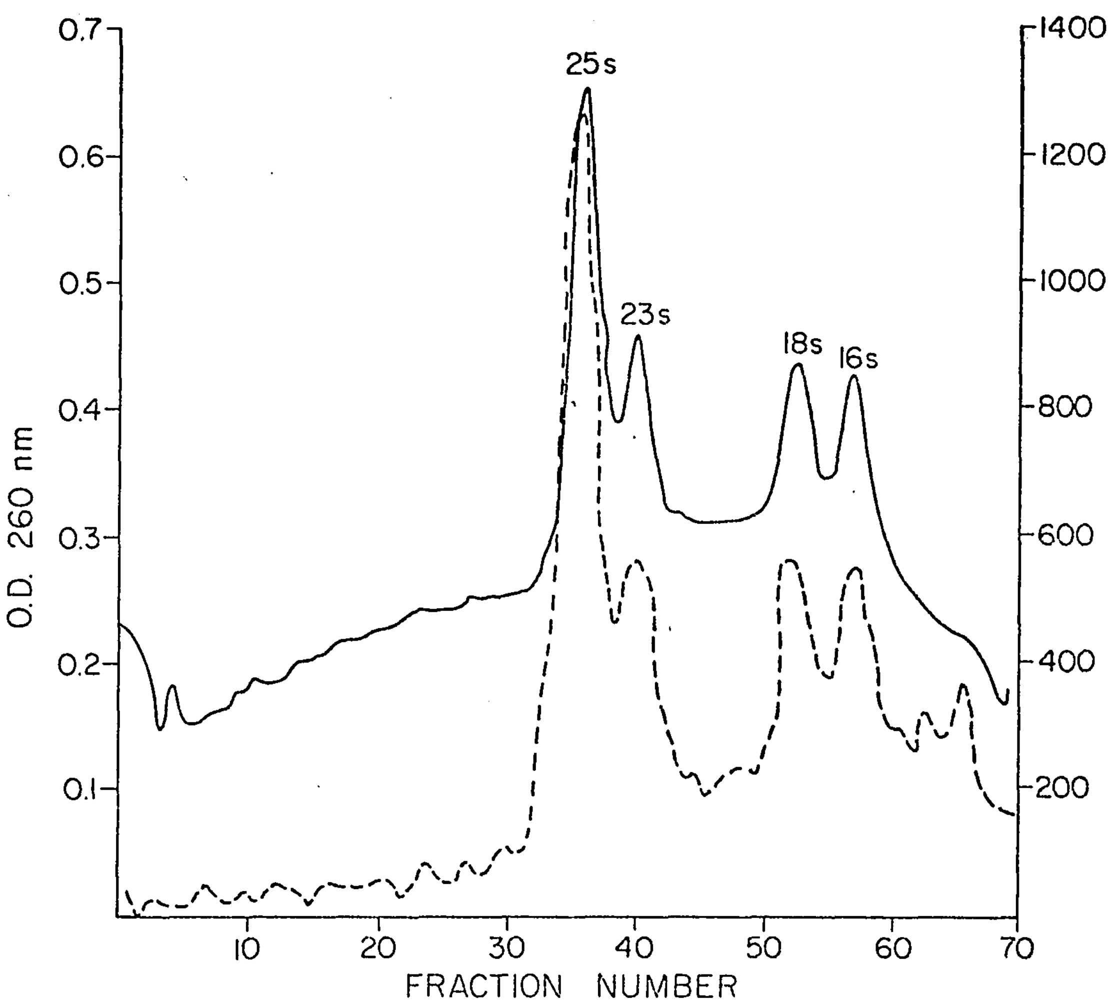

Figure 7. Fractionation of labeled barley leaf rRNA on gel.

for the loss was made in hybridization experiments described in the next section. There is some possibility that rRNA in the incubation mixture might have selectively prevented the release of rDNA by hybridizing to that particular segment of DNA.

Homologous and Heterologous Hybridization Values at Saturation

In order to determine the base sequence homology of rRNA among monocots, among dicots, and between monocots and dicots, monocot and dicot rRNA's were hybridized to monocot and dicot DNA's in saturation-hybridization experiments. Table 2 lists the specific activities of the rRNA's employed for this study, and Figure 8 presents a graph of percent barley DNA hybridized plotted against barley rRNA input. At the higher rRNA inputs, the percent hybridization remains relatively constant. This value is taken as the saturation-hybridization value. The data on Figure 8 are transformed and plotted according to Bishop et al. (1969) on Figure 9. The percent hybridization at saturation can be obtained in Figure 9 by taking the reciprocal of the slope of the linear regression line. The values obtained in Figure 9 are up to 10% higher than those obtained by means of Figure 8.

Table 3 shows the saturation-hybridization values for a number of combinations of rRNA s and DNA's. Each value is the result of one saturation-hybridization experiment, and the data were plotted as in Figure 9. Selected points and, when feasible, entire saturation curves were repeated with both the same and different nucleic acids. A 5 to 12% deviation from the values in Table 3 was observed when five of the

Table 2. Specific activities of leaf rRNA preparations.

Source of rRNA	Counts/minute/ug of rRNA
Barley	1300
Barley	1200
Barley	1470
Wheat	3920
Cotton	7250
Pumpkin	20000
Pumpkin	20550

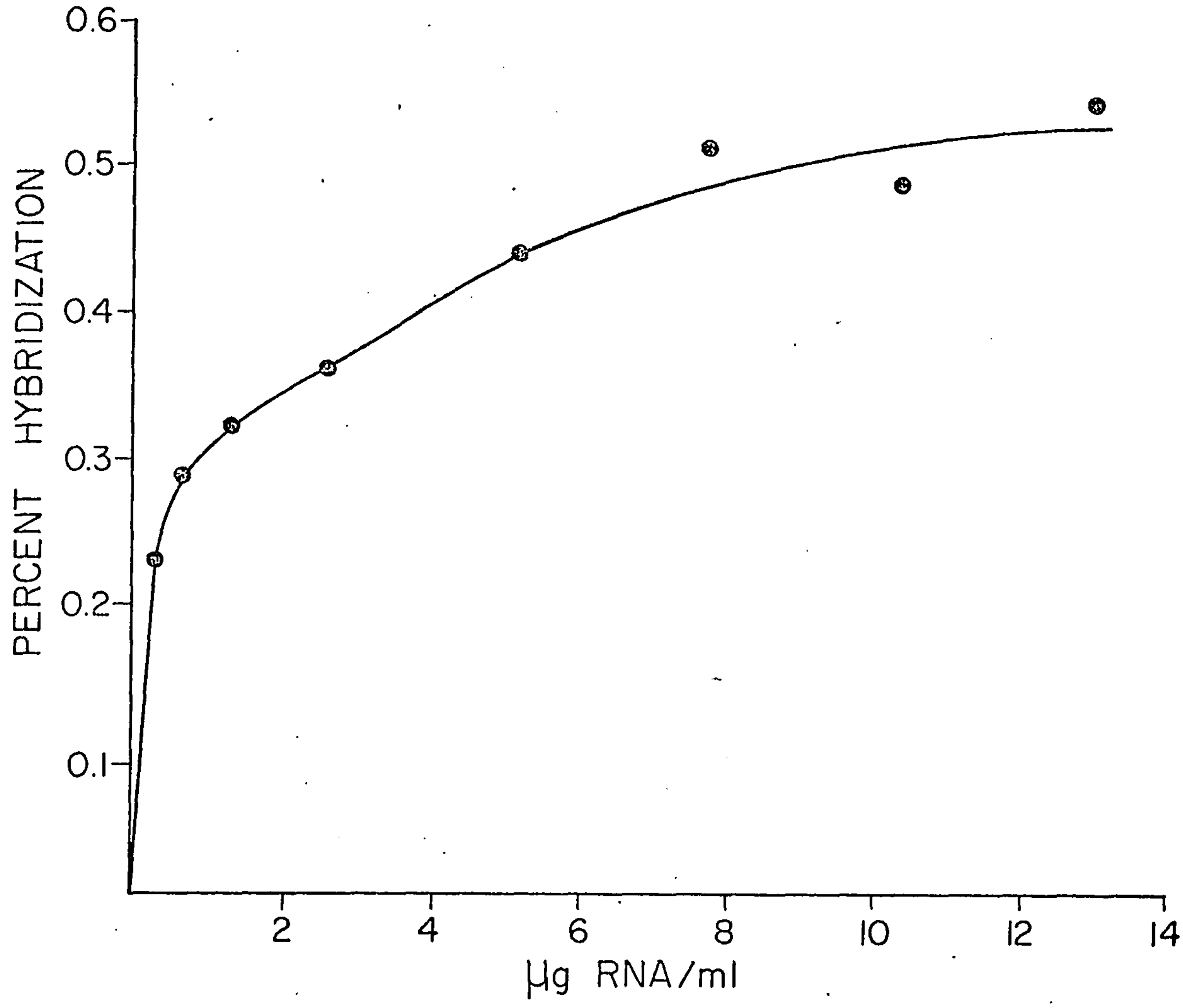

Figure 8. Saturation curve of barley leaf rRNA hybridized to barley seed DNA.

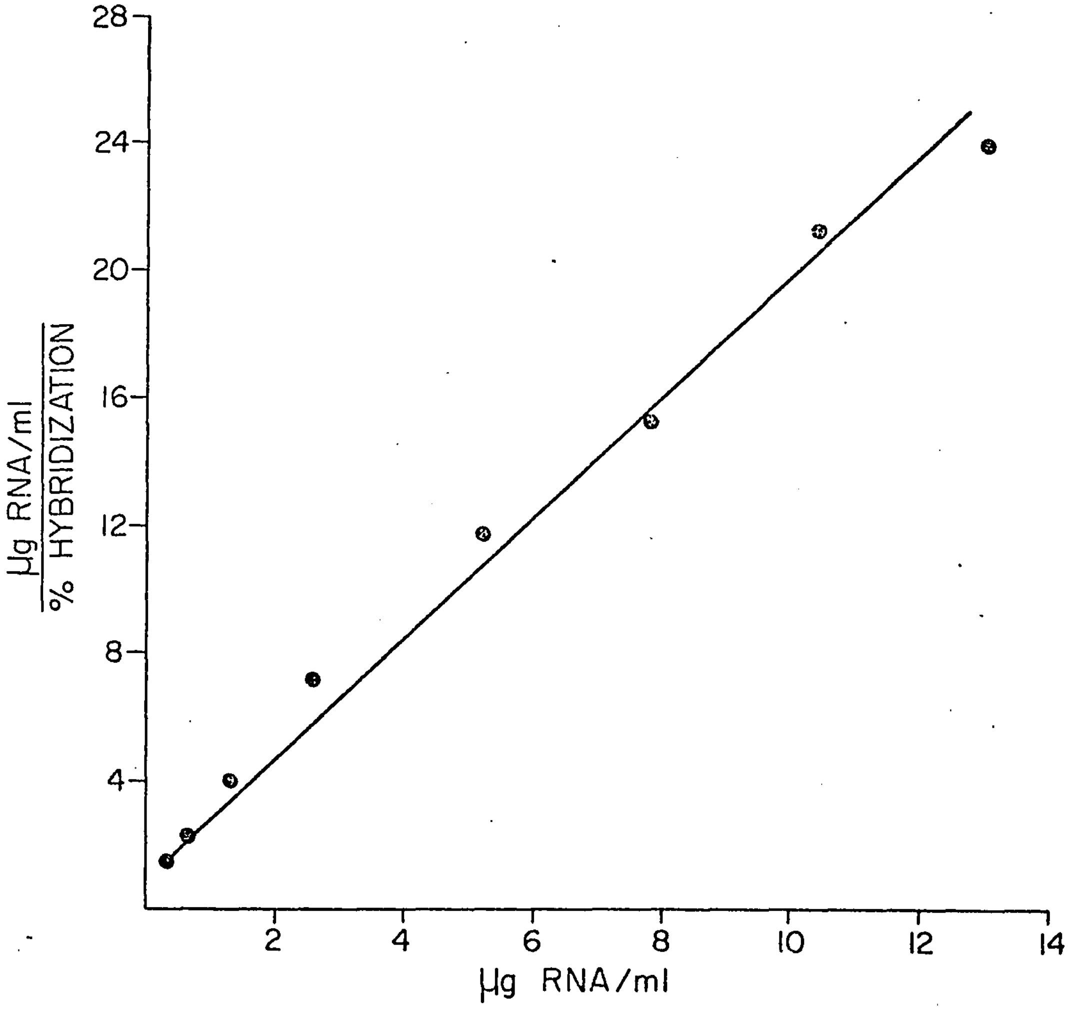

Figure 9. Saturation curve with data of Figure 8 transformed according to Bishop et al. (1969).

Table 3. Percent of DNA hybridized with rRNA at saturation.[a]

Source of rRNA	Source of DNA				
	Monocot			Dicot	
	Barley	Panic Grass	Wheat	Cotton	Pumpkin
Monocot					
Barley	0.73	0.47	0.15	0.55	1.76
Wheat	0.42	0.68	0.22	0.46	1.50
Dicot					
Cotton	0.34	0.23	0.13	0.71	2.07
Pumpkin	0.29	0.26	0.11	0.45	1.88

a. A fixed amount of filter-bound seed DNA was hybridized with increasing concentrations of labeled leaf rRNA in 1 ml of 0.1% SDS in 2 x SSC. After hybridization, filters were washed sequentially with 6 x SSC at 50°C, 2 x SSC, and 20 µg/ml of pancreatic RNAase in 2 x SSC. Filters were oven-dried and monitored for radioactivity; the count from filters without DNA were subtracted from the latter. The saturation value was calculated by the method of Bishop et al. (1969).

saturation-hybridization experiments were repeated with a different rRNA as well as a DNA preparation.

The data in Table 3 reflect several patterns. These patterns can be seen more clearly in Table 4 where the data are presented as the relative amount of binding of DNA to heterologous rRNA's, with the homologous reaction normalized at 100%. With one exception, the two dicot rRNA's hybridize equally to a given monocot or dicot DNA. The exception is that cotton and pumpkin rRNA's hybridize unequally to cotton DNA. Despite this exception a general trend toward the conservation of rRNA sequences between dicots can be noted. This conclusion is in agreement with the findings of Matsuda and Siegel (1967).

The two monocot rRNA's, on the other hand, do not hybridize equally to a given DNA. For instance, the saturation-hybridization value of wheat rRNA hybridized to barley DNA is only 58% of that observed for wheat rRNA hybridized to wheat DNA. Likewise, barley rRNA hybridizes to wheat DNA 68% of the extent that barley rRNA hybridizes to barley DNA. These data suggest that about 60% of the rRNA sequences between the two monocots has been conserved.

When dicot rRNA's hybridize to monocot DNA's, 40-60% of the homologous saturation-hybridization values are observed. In the reciprocal experiments of hybridizing monocot rRNA's to dicot DNA's, 65-95% of the homologous saturation-hybridization values are observed. This disparity between reciprocal hybridizations will be treated in the Discussion chapter.

The hybridization values in Table 3 involve leaf rRNA's, which include the 25 s and 18 s rRNA components of the cytoplasm as well as

Table 4. Relative percent of DNA hybridized with rRNA at saturation.

Source of rRNA	Source of DNA			
	Barley	Wheat	Cotton	Pumpkin
Barley	100	68	77	94
Wheat	58	100	65	80
Cotton	47	59	100	110
Pumpkin	40	50	63	100

the 23 s and 16 s rRNA components of the chloroplast. In order to assess what proportion of rDNA codes for 25 s and 18 s rRNA, competition experiments were performed with unlabeled root rRNA's. In one experiment, a series of filters with barley DNA was hybridized with a fixed, saturating concentration of labeled barley leaf rRNA and increasing concentrations of unlabeled barley root rRNA. The same type of experiment was performed with cotton DNA, labeled cotton leaf rRNA, and unlabeled cotton root rRNA. The data were plotted according to the method of Bishop et al. (1969), in Figures 10 and 11, from which it is concluded that the relative contribution by cytoplasmic rRNA (25 s and 18 s rRNA) to the saturation-hybridization values of barley and cotton are estimated at 58 and 63%, respectively. In control experiments, hybridization of labeled leaf rRNA to DNA was reduced by more than 95% in the presence of excess unlabeled leaf rRNA.

In order to test the purity of DNA, hybridization values were obtained with DNA's that had undergone a purification procedure in addition to those routinely employed. Purified barley and cotton DNA's were submitted to preparative CsCl centrifugation. Those DNA's were then hybridized with a saturating concentration of barley and cotton rRNA's, and the results are presented in Table 5. Because there is relatively little difference between the values in Tables 3 and 5, it was concluded that further purification of DNA did not alter its hybridization properties.

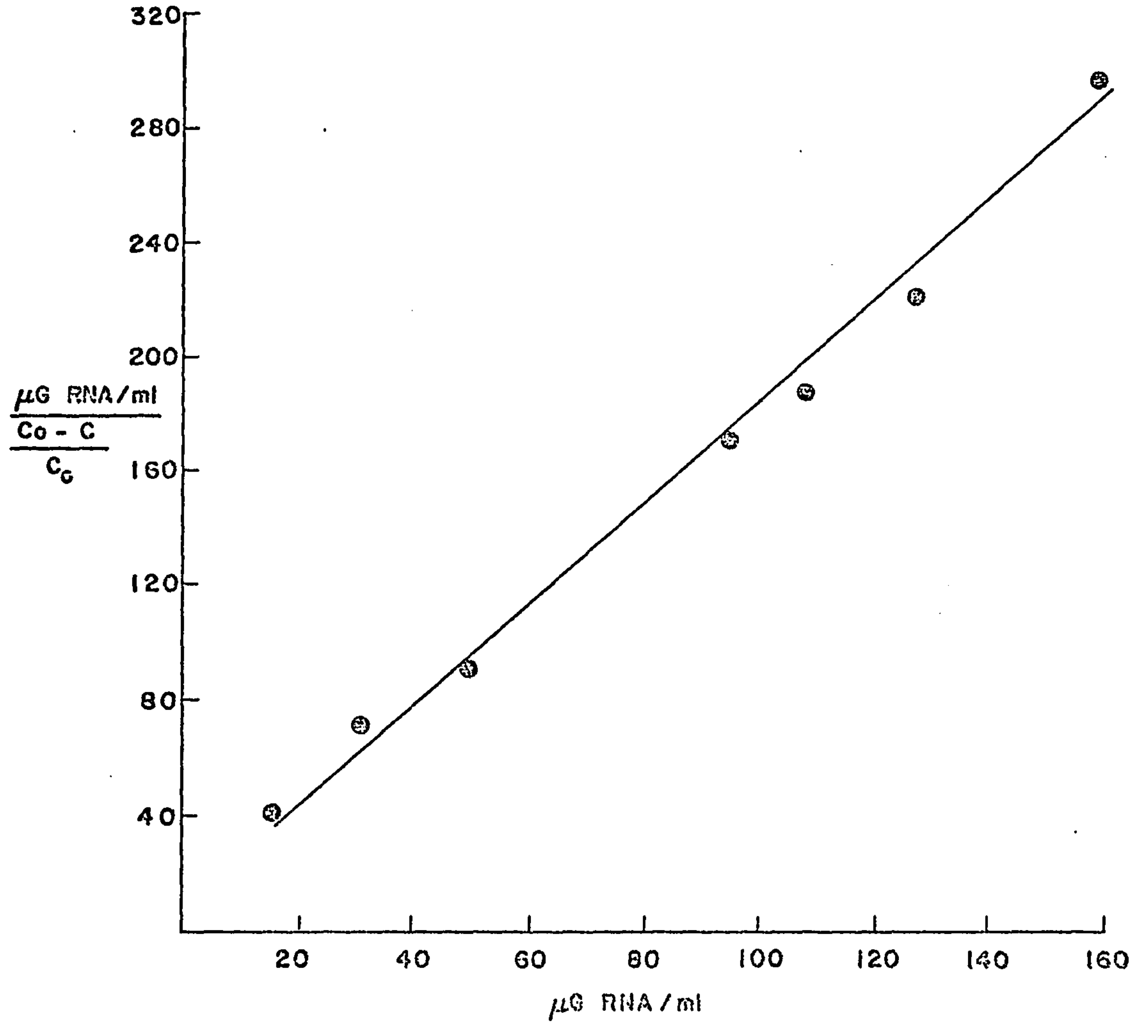

Figure 10. Hybridization of labeled barley leaf rRNA to barley DNA in presence of unlabeled barley root rRNA.

A constant amount of barley DNA was hybridized with a fixed, saturating concentration of labeled barley leaf rRNA in presence of varying, excess concentrations of unlabeled barley root rRNA. The data were plotted according to Bishop et al. (1969).

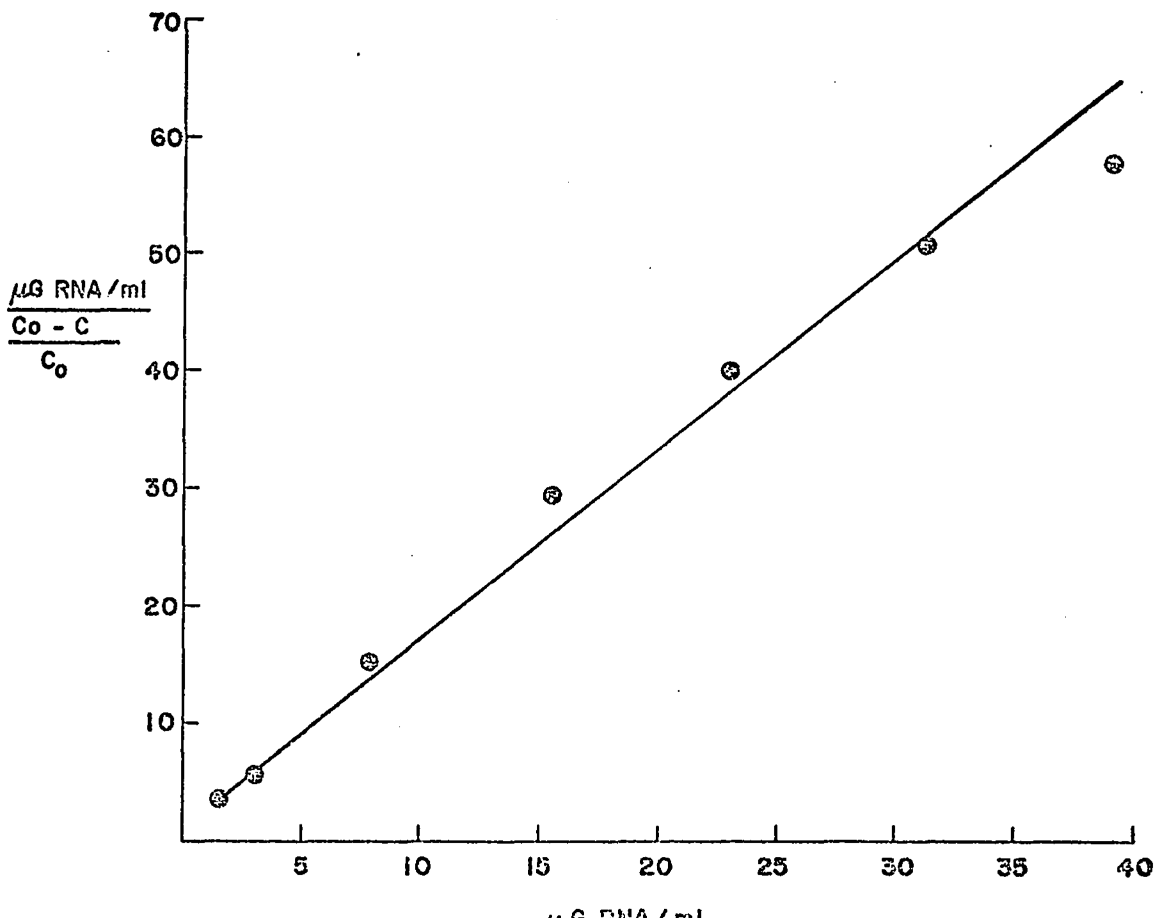

Figure 11. Hybridization of labeled cotton leaf rRNA to cotton DNA in the presence of unlabeled cotton root rRNA.

A constant amount of cotton DNA was hybridized with a fixed, saturating concentration of labeled cotton leaf rRNA in presence of varying, excess concentrations of unlabeled cotton root rRNA. The data were plotted according to Bishop et al. (1969).

Table 5. Percent of DNA hybridized with rRNA after DNA has been purified by preparative CsCl centrifugation.

Source of rRNA	Source of DNA			
	Barley		Cotton	
Barley	(0.73)[a]	0.62	(0.55)	0.54
Cotton	(0.34)	0.42	(0.71)	0.80

a. The values in parentheses were taken from Table 3 for comparison, and those hybridizations were not performed with DNA's that had been subjected to preparative CsCl centrifugation.

Thermal Stability of rRNA-DNA Hybrids

Saturation-hybridization experiments measure the proportion of DNA that can be recognized by the species of RNA under study. Because both homologous and heterologous hybridization experiments have been performed, it is of interest to compare the thermal stabilities of both types of hybrids to determine if there has been a greater extent of base-pair mismatching in the heterologous than in the homologous hybrids. For this purpose, rRNA-DNA hybrids were exposed sequentially to temperatures from 60 to 98°C in 5°C increments. As the temperature increases, rRNA is released from the hybrid and is no longer filter-bound. The cumulative percent of rRNA solubilized was plotted against the temperature. Figures 12 through 15 are selected examples of thermal stability profiles for rRNA-DNA hybrids, and Table 6 lists the Tm for each hybrid. At the end of the experiments, filters retained less than 9% of the rRNA originally bound. Five of the 20 Tm's listed on Table 6 were repeated, and the maximal difference observed between duplicate experiments was 1.5°C.

It can be seen from Table 6 that dicot rRNA-dicot DNA hybrids, on the whole, display the highest Tm's. Within this class of hybrids, the two homologous hybrids have higher Tm's than the two heterologous hybrids (see Tables 6 and 7). The difference in Tm's between the pumpkin rRNA-pumpkin DNA hybrid and the cotton rRNA-pumpkin DNA hybrid is 4.0°C. The difference in Tm's between the cotton rRNA-cotton DNA hybrid and the pumpkin rRNA-cotton DNA hybrid is also 4.0°C. Assuming that 1.5% mismatching lowers the Tm by 1.0°C (Laird, McConaughy, and

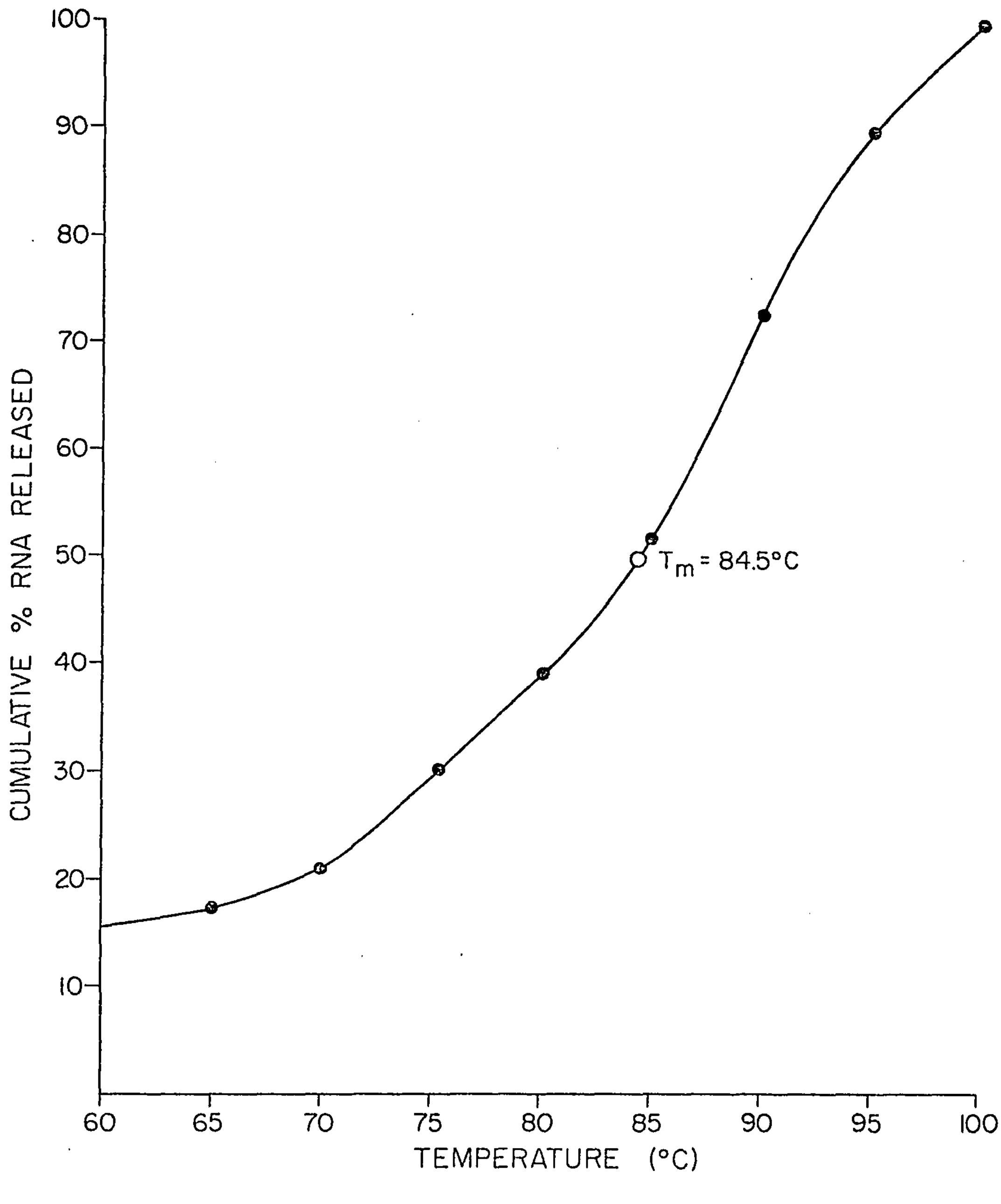

Figure 12. Thermal stability of the wheat rRNA-wheat DNA hybrid.

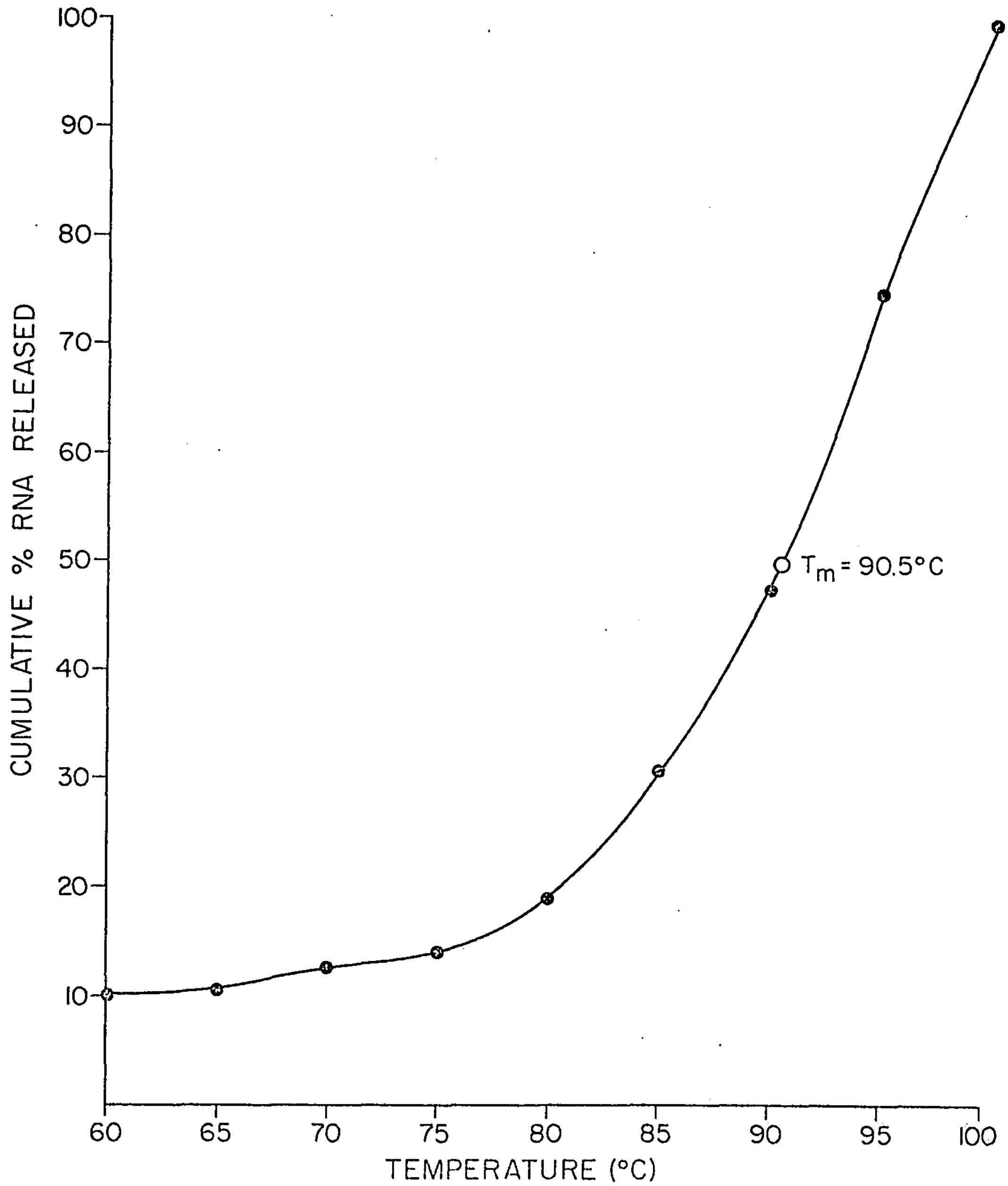

Figure 13. Thermal stability of the cotton rRNA-cotton DNA hybrid.

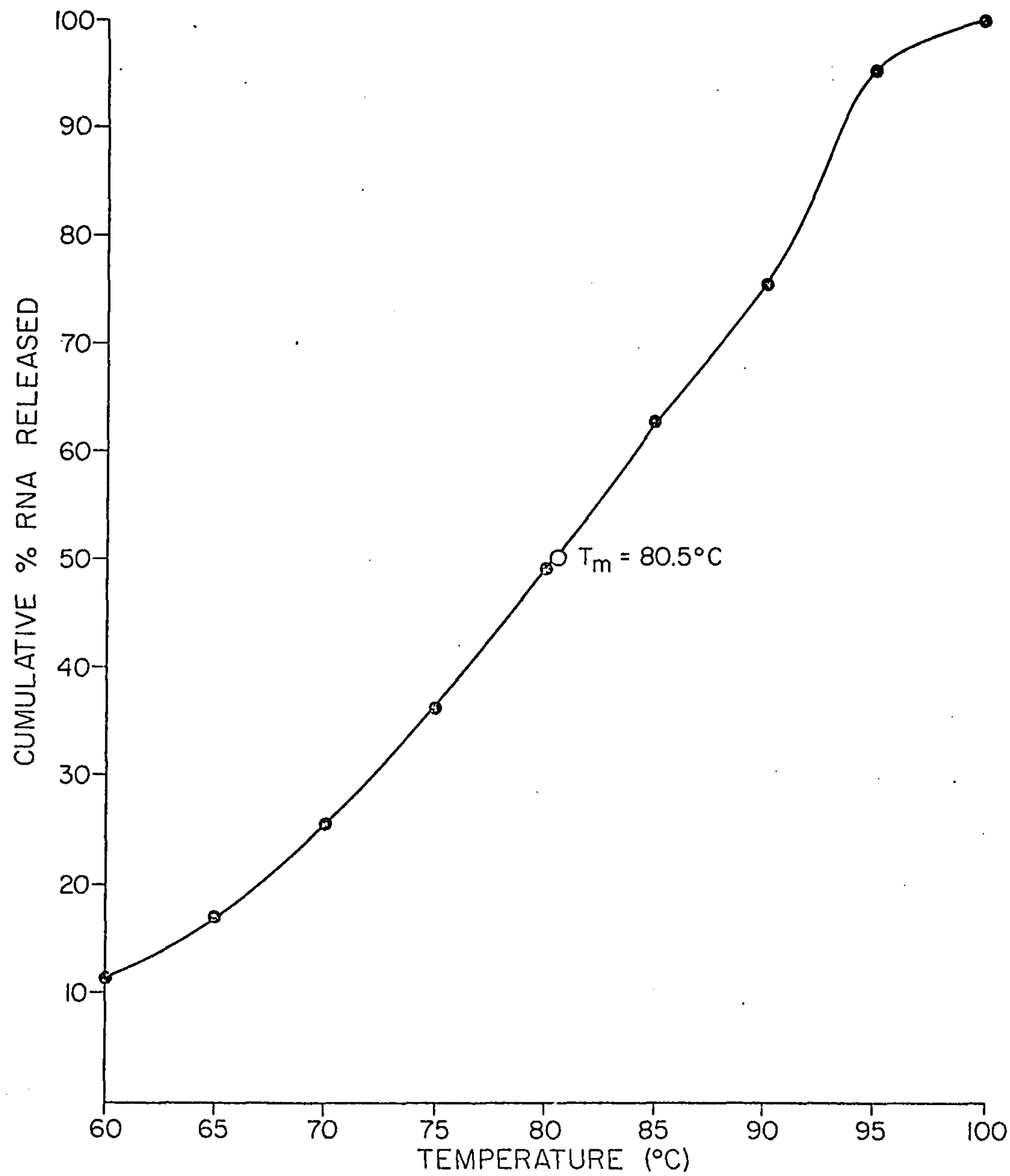

Figure 14. Thermal stability of the barley rRNA-cotton DNA hybrid.

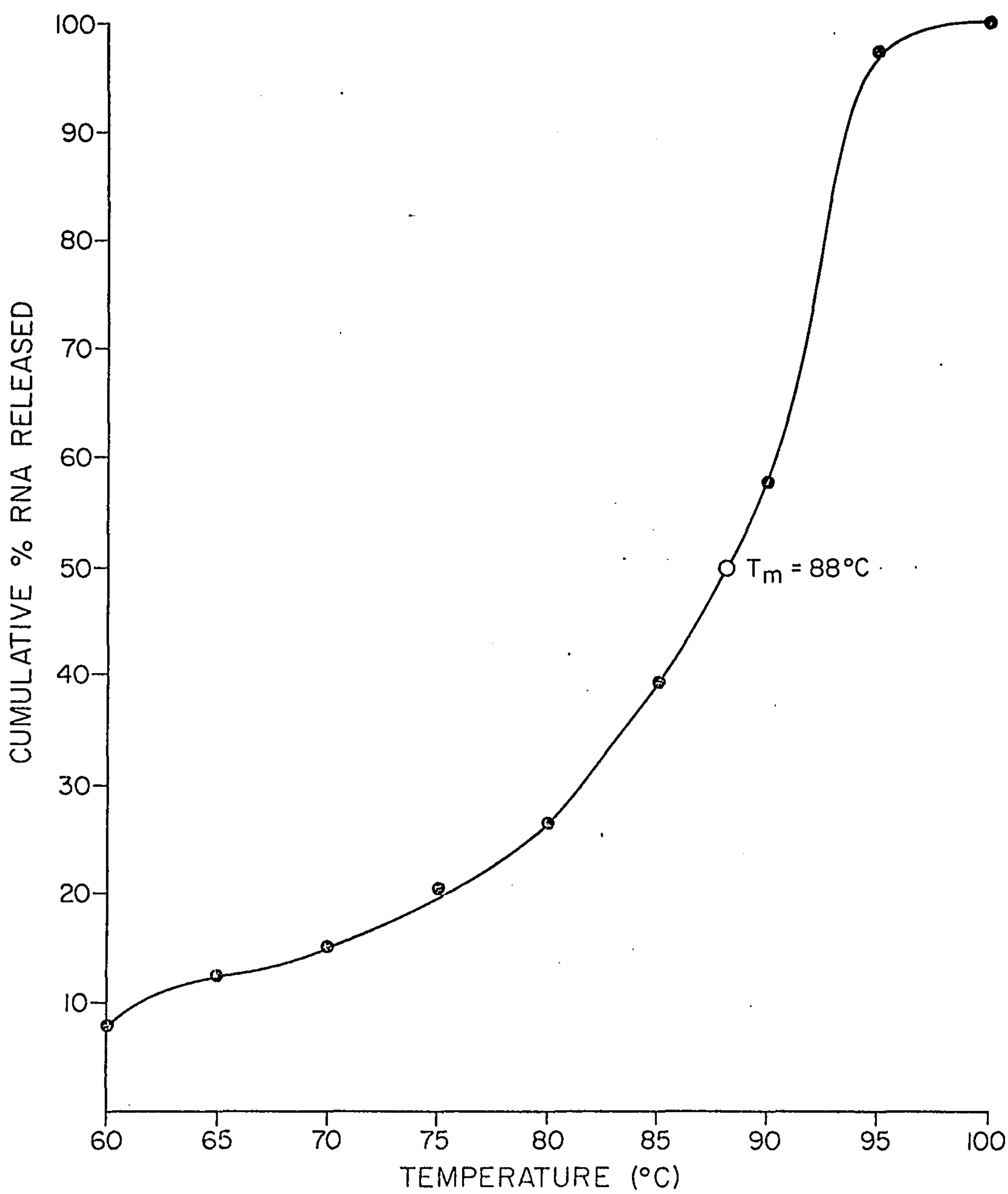

Figure 15. Thermal stability of the cotton rRNA-barley DNA hybrid.

Table 6. Tm of rRNA-DNA hybrids.[a]

Source of rRNA	Source of DNA				
	Monocot			Dicot	
	Barley	Panic Grass	Wheat	Cotton	Pumpkin
Monocot					
Barley	87.0°C	83.5	83.0	80.5°C	83.5
Wheat	87.0	83.0	84.5	82.0	86.0
Dicot					
Cotton	88.0°C	86.5	83.5	90.5°C	88.0
Pumpkin	88.5	85.0	81.0	86.5	92.0

a. Filter-bound seed DNA was hybridized with a saturating concentration of labeled leaf rRNA, as described in Table 3. Duplicate filters of rRNA-DNA hybrids were incubated at 60°C for 5 minutes in vials containing 1 ml of 2 x SSC. The filters were transferred to another pair of vials maintained at 65°C and subsequently to a series of vials ranging in temperatures from 70 to 98°C. The radioactivity released from the filters was monitored, and the cumulative percent rRNA released was plotted against temperature.

Table 7. Difference in Tm's between heterologous and homologous rRNA-DNA hybrids.[a]

Source of rRNA	Source of DNA			
	Barley	Wheat	Cotton	Pumpkin
Barley	--	1.5	10.0	8.5
Wheat	0°C	--	8.5	6.0
Cotton	-1.0	1.0	--	4.0
Pumpkin	-1.5	3.5	4.0	--

a. The Tm of each heterologous rRNA-DNA hybrid listed in Table 6 was subtracted from the corresponding homologous rRNA-DNA hybrid.

McCarthy 1969), which is the best estimate available, these differences translate into a 6% base-pair mismatching in both heterologous dicot rRNA-dicot DNA hybrids. In comparing the Tm's between homologous and heterologous monocot rRNA-monocot DNA hybrids, it can be seen from Tables 6 and 7 that the Tm's show little difference. Thus, there is no evidence of base-pair mismatching in the heterologous monocot rRNA-monocot DNA hybrids, as compared to the homologous hybrids.

The homologous monocot rRNA-monocot DNA hybrids have lower Tm's than the homologous dicot rRNA-dicot DNA hybrids. The thermal stability profiles of the former are broader than those of the latter (compare Figures 12 and 13). Moore and McCarthy (1968) observed a broad thermal stability profile and a relatively low Tm of the rabbit rRNA-DNA hybrid and concluded that the nucleotide sequences coding for rabbit rRNA were heterogeneous. Such a heterogeneity would result in base-pair mismatching in homologous monocot rRNA-monocot DNA hybrids and could explain their relatively low Tm's. Alternatively the GC content of dicot rRNA's could be higher than that of monocot rRNA's. Such a difference in GC content would also cause a decreased Tm for homologous monocot rRNA-monocot DNA hybrids. The effect of GC content on the Tm of DNA-DNA hybrids has, in fact, been quantitated by Marmur and Doty (1959) as a 1°C decrease in Tm per 2.5% decrease in GC content. The maximum decrease in Tm's among homologous monocot rRNA-monocot DNA hybrids and homologous dicot rRNA-dicot DNA hybrids is 9.0°C (see Table 6), which would be equivalent to a 22.5% difference in GC content. However, it is uncertain whether this quantitative analysis is applicable to the

Tm's of rRNA-DNA hybrids. In conclusion, either of the two above hypotheses could explain the lower Tm's of homologous monocot rRNA-monocot DNA hybrids.

The dicot rRNA-monocot DNA hybrids display approximately the same Tm's as those of the corresponding monocot rRNA-monocot DNA hybrids. Therefore, both types of hybrids have the same percentage of base-pair mismatching. By contrast, the monocot rRNA-dicot DNA hybrids have Tm's considerably lower than those of the corresponding dicot rRNA-dicot DNA hybrids. For example, the barley rRNA-cotton DNA hybrid has a Tm 10.0°C lower than that of the cotton rRNA-cotton DNA hybrid. The monocot rRNA-dicot DNA hybrids apparently have 9 to 15% more base-pair mismatching than was observed in dicot rRNA-dicot DNA hybrids.

It should be noted, however, that the Tm analysis refers only to the rRNA and DNA that have hybridized. In the case of homologous hybridizations, all or nearly all of the rDNA sequences have hybridized with rRNA sequences. In the case of heterologous hybridizations, some sequences of rRNA have diverged to such an extent that they no longer anneal to heterologous DNA's. Therefore, the extent of base-pair mismatching in heterologous hybrids as compared to that of homologous hybrids does not take into account widely divergent sequences of rRNA's and can thus over-estimate the proportion of the nucleotide sequence shared by the homologous and heterologous rRNA's.

Hybridization of rRNA to DNA Fractionated From a Preparative CsCl Gradient

As in other organisms investigated, plant rDNA clusters in CsCl gradients with a buoyant density characteristic of the GC content of rRNA (Sinclair and Brown 1967). In order to resolve rDNA from the bulk of DNA, plant DNA's were centrifuged to equilibrium in CsCl, and fractions of the gradient were collected. The optical density at 260 nm of each fraction was determined, and alternate fractions were hybridized with labeled leaf rRNA. By splitting a filter containing the denatured DNA of a single fraction, it was possible to hybridize the same fraction of DNA with various rRNA's. Figure 16 shows that barley rRNA hybridizes to DNA slightly less dense than the bulk of barley DNA, which has a buoyant density of 1.701 gm/cc and a GC content calculated from it as 42 % by the method of Schildkraut et al. (1962). Chen and Osborne (1970) reported a similar observation for wheat rRNA and wheat DNA. In Figure 17, cotton DNA with a buoyant density of 1.692 gm/cc and a calculated GC content of 33% allows for a better resolution of rDNA from the bulk of the DNA. In all cases, homologous and heterologous rRNA's hybridized to DNA of the same buoyant densities.

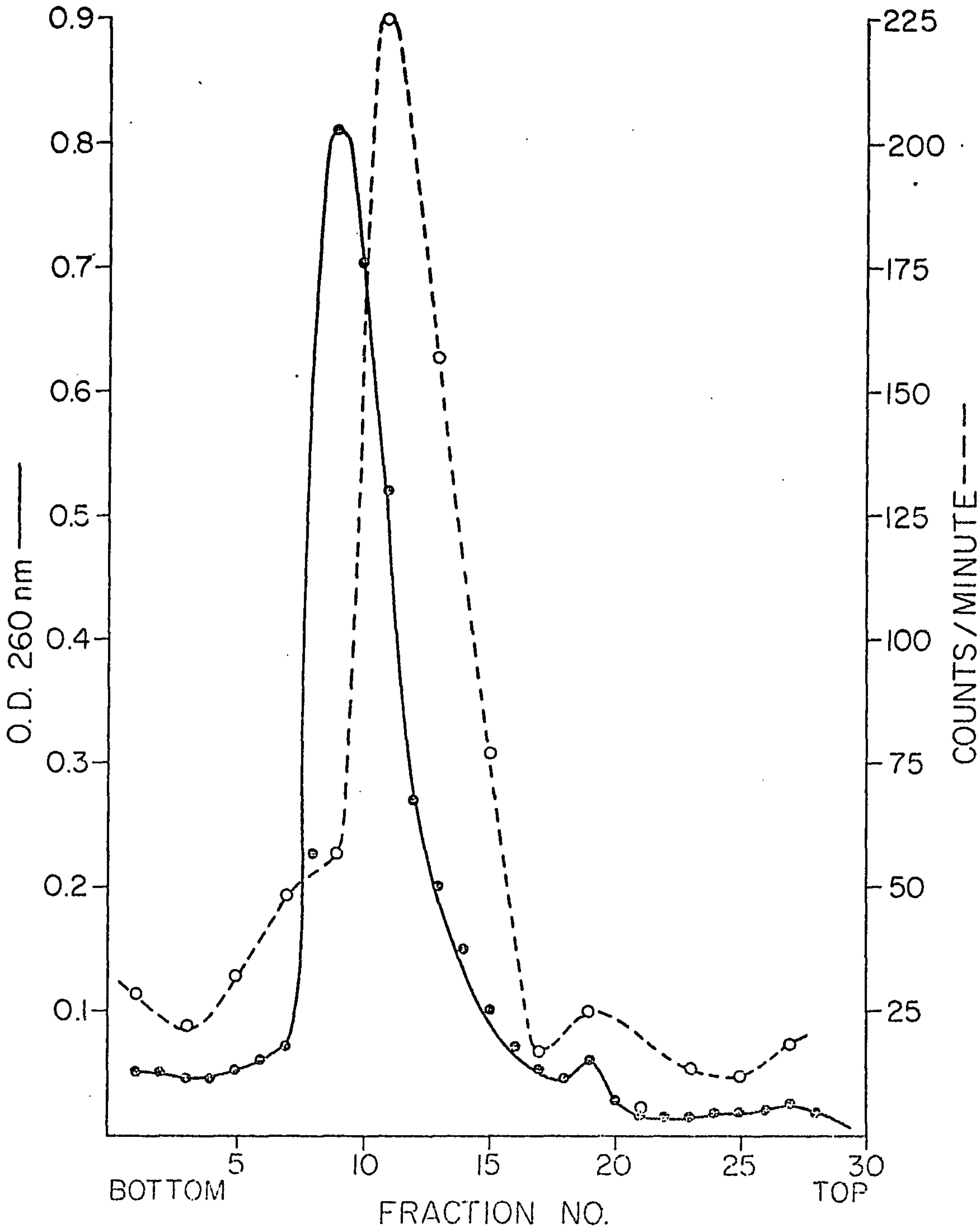

Figure 16. Distribution of rDNA in barley DNA banded on a preparative CsCl gradient.

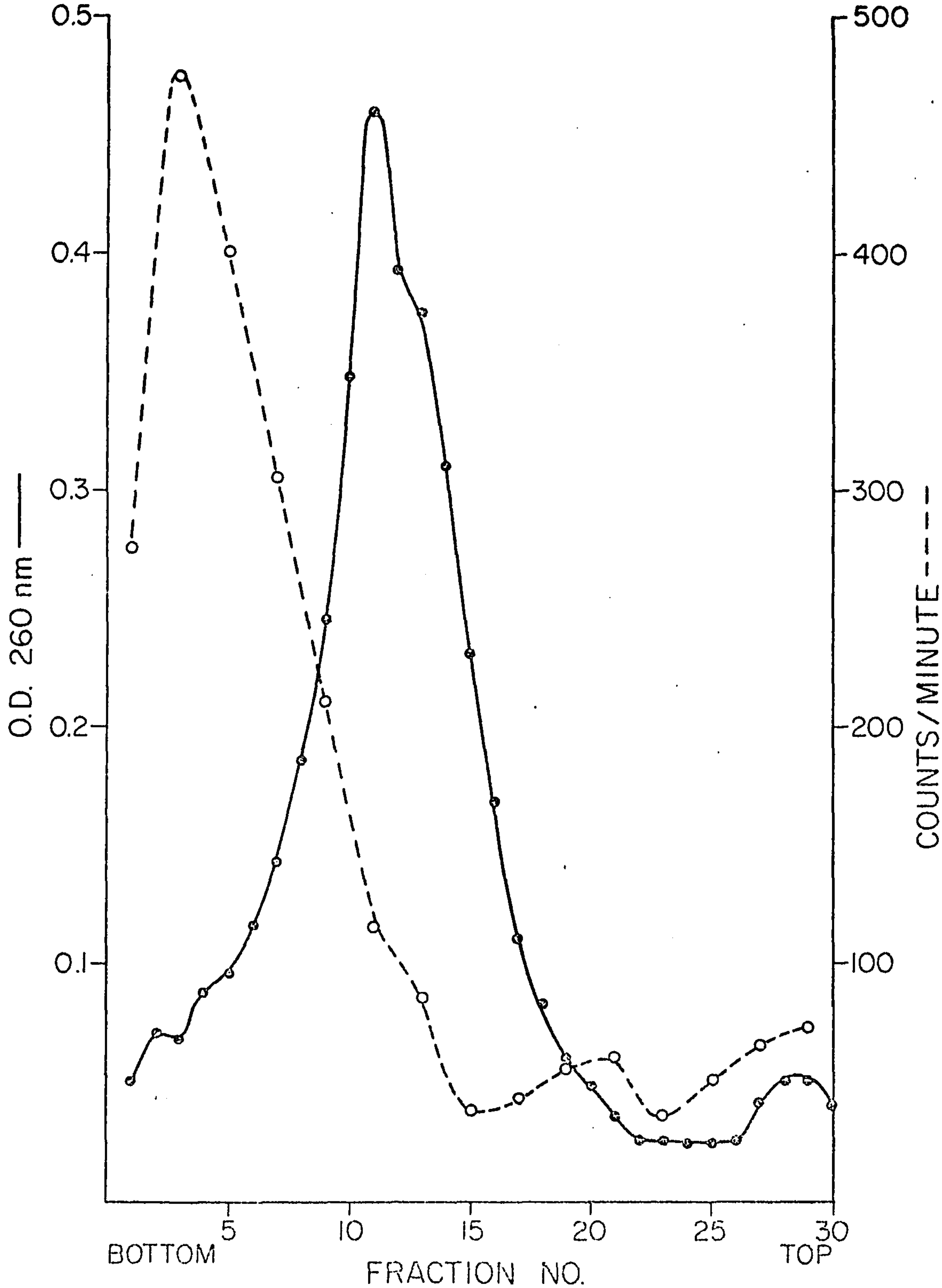

Figure 17. Distribution of rDNA in cotton DNA banded on a preparative CsCl gradient.

DISCUSSION

Accurate values for rRNA-DNA hybridization studies depend heavily on the purity of the nucleic acids. The DNA's used in the present study are judged to be pure on the following basis: a purification procedure, subsequent to those routinely employed, did not alter the hybridization properties of DNA's. Ribosomal RNA's are judged to be pure because they underwent rigorous purification procedures and duplicate preparations from the same species yield essentially the same results.

Labeled leaf rRNA shows no evidence of being synthesized by contaminating bacteria, because peaks of radioactivity corresponded closely to the optical density profile of the four rRNA components separated by polyacrylamide gel electrophoresis. The above criterion for judging freedom from bacterial contamination may be inadequate, because the 23 s and 16 s rRNA components of chloroplasts coincide with the 23 s and 16 s rRNA components of bacteria. However, a fraction of the labeled leaf rRNA behaves as if it were homologous to chloroplastic rDNA in the following experiment: under conditions where excess unlabeled leaf rRNA depresses hybridization of labeled leaf rRNA by more than 95%, excess unlabeled root rRNA depresses hybridization only by 60%. Messenger RNA can also not be detected because representative rRNA preparations hybridized specifically to DNA (rDNA) of a buoyant density greater than the bulk of cotton DNA. Therefore, all four components of leaf rRNA, 25 s, 23 s, 18 s, and 16 s, and only those four components, are judged to be labeled.

The hybridization data reported in this study agree essentially with those from the literature. Chen and Osborne (1970) cite a value of 0.32% for wheat rRNA hybridized to wheat DNA at saturation; the value cited in this study is 0.22%. According to Bendich and McCarthy (1970a), barley contains twice as much rDNA as wheat. Assuming that the true value for the proportion of wheat DNA homologous to wheat rRNA is the average of the value in this study and that of Chen and Osborne (1970), 0.54% of barley DNA should code for barley rRNA. The value cited in this study is 0.73%. Goldberg (1970) assigns for pumpkin DNA a value of 1.95%, which is very close to the value of 1.88% assigned in this study. The fact that various dicot rRNA's generally hybridize equally to a given DNA extended similar observations by Matsuda and Siegel (1967).

The data from Table 3 suggest that there has been some divergence of rRNA base sequences both among monocots and between monocots and dicots. On the basis of reciprocal rRNA-DNA hybridizations, approximately 60% of the nucleotide sequence of barley rDNA is homologous to the nucleotide sequence of wheat rRNA; 60% of the nucleotide sequence of wheat rDNA is homologous to the nucleotide sequence of barley rRNA. From the data of Bendich and McCarthy (1970b), it can be seen that 72% of wheat DNA is homologous to barley DNA and that 48% of barley DNA is homologous to wheat DNA. However, the latter experiments involved the hybridization of redundant sequences at nonrestrictive conditions of hybridization, and the values obtained from them were regarded as overestimates of the true extent of DNA homology. Therefore, the nucleotide sequence of rRNA's of barley and wheat is conserved at least as much and probably more than that of the bulk of the DNA's of barley and wheat.

Because dicot rRNA's hybridize equally to a given DNA, it is concluded that the nucleotide sequence of rRNA's among dicots has been conserved. Bendich and Bolton (1967) found a 5-55% homology in the nucleotide sequence among dicot DNA's. Again these values probably overestimate the true homologies. Therefore, the nucleotide sequence of rRNA's between dicots has been conserved more than that of the bulk of the dicot DNA's. Moreover, the conservation of the rRNA sequence among dicots is greater than that among monocots.

The divergence of the rRNA base sequence between monocots and dicots is difficult to determine. Reciprocal hybridizations between monocots and dicots are unequal. The two estimates of nucleotide sequence conservation between monocot and dicot rRNA's obtained from the reciprocal hybridizations are 40-60% and 65-95%. Bendich and Bolton (1967) found a maximum of 10% nucleotide sequence conservation between monocot and dicot DNA's. Thus, whichever estimate is accepted, the rRNA nucleotide sequences of monocots and dicots have been conserved more than those of the rest of their genomes.

Several models can be invoked to explain the unequal reciprocal hybridizations noted above. One model depends on the heterogeneity of rRNA that may have been observed within monocots. Each monocot may thus have a family of related genes coding for rRNA. Apparently the dicot rRNA hybridizes only to some of these related genes under restrictive conditions. Thus, not all of the monocot rDNA is available to hybridize to dicot rRNA. In the reciprocal experiment involving dicot DNA and monocot rRNA, all of the dicot rDNA is available to hybridize with some of the related monocot rRNA's. Because rRNA is in excess, the saturation-hybridization value can approach 100% of the homologous value. Thus, reciprocal hybridizations, under those circumstances, are expected to yield different estimates of conservation of rRNA sequence.

The above interpretation is subject to qualification, and other explanations are also possible. For instance, unequal specific activities of the four rRNA components can lead to inaccurate estimations of the saturation-hybridization values. Even if the specific activities of the four major rRNA components were equal, unequal labeling of heterogeneous subpopulations of the rRNA components can also cause problems in estimating the saturation-hybridization values. Therefore, all hybridization data should be judged with a certain amount of caution.

SUMMARY

DNA's have been isolated from nuclei of barley, blue panicum grass, wheat, cotton, and pumpkin. Except for the rDNA satellite of pumpkin, all native DNA's show one band in CsCl gradients. Alkaline-denatured DNA's increase in buoyant density by 0.015-0.017 gm/cc. Denatured DNA's, when renatured under identical conditions, decrease in buoyant density by 0.002-0.009 gm/cc. The DNA's of blue panicum grass and pumpkin, when renatured, decrease in buoyant density to a lesser extent than do the other DNA's and are judged to have fewer redundant sequences than the other DNA's.

Labeled rRNA's have been isolated from leaves of barley, wheat, cotton, and pumpkin and are characterized by four optical density peaks with polyacrylamide gel electrophoresis. The four optical density peaks of barley rRNA correspond closely with four radioactivity peaks. Ribosomal RNA's from different species hybridize specifically to those fractions of cotton DNA whose buoyant density and GC content are greater than the bulk of cotton DNA.

The proportion of DNA coding for rRNA in barley, wheat, cotton, and pumpkin amounts to 0.73%, 0.22%, 0.71%, and 1.88% of the respective haploid genomes. Competition experiments with unlabeled root rRNA indicate that about 60% of barley and cotton rDNA's codes for cytoplasmic rRNA; the other 40% codes for chloroplastic rRNA.

On the basis of reciprocal hybridizations and thermal stability profiles, the divergence of rRNA nucleotide sequences has been estimated among monocots, among dicots, and between monocots and dicots. In comparison to DNA homologies determined by Bendich and Bolton (1967) and Bendich and McCarthy (1970b), the rRNA nucleotide sequence among monocots, among dicots, and between monocots and dicots has been conserved relative to that of the rest of the genome.

A comparison of the thermal stability profiles and Tm's between homologous monocot rRNA-monocot DNA hybrids and homologous dicot rRNA-dicot DNA hybrids suggests a possible heterogeneity for monocot rRNA's and the genes that code for them or a difference in GC content between monocot and dicot rRNA's. The heterogeneity noted above might also explain the unequal reciprocal hybridizations between monocot and dicot DNA's and rRNA's.

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