

RIBOSOMAL CISTRON VARIABILITY IN COTYLEDONS
OF GERMINATING PUMPKIN (CUCURBITA PEPO L.)

by

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ABSTRACT

Between the second and fourth day of germination the DNA and RNA content of developing cotyledons of pumpkin (Curcubita pepo L.) approximately doubled. The DNAs of cotyledons between the second and sixth day of germination were examined for their proportion of coding for ribosomal RNAs (rRNA) of chloroplastic and cytoplasmic ribosomes (CHL-rDNA and CYT-rDNA, respectively). Hybridization experiments were conducted which showed that root rRNAs could be used to determine CYT-rDNA and that CHL-rDNA could be calculated as the difference in the hybridization values obtained using cotyledonary and root rRNAs.

DNAs extracted from whole cotyledons or isolated nuclei from light-grown pumpkins yielded similar results. Amplification of rDNA like that reported in Xenopus oocytes was not found. Instead, fluctuations in coding for rRNAs were related to nuclear DNA replication. Similar experiments using DNAs extracted from etiolated pumpkins indicated that a high proportion of CHL-rDNA was present in the DNA extracted from isolated nuclei. DNA extracted from whole cotyledons, however, showed very low proportions of CHL-rDNA. The reasons for this difference were discussed.

INTRODUCTION

Cells of most organisms contain more than one copy of the genes which code for ribosomal ribonucleic acids (rRNA). This redundancy varies from a few copies in the procaryote genome to thousands of cistrons per cell in the more complex deoxyribonucleic acid (DNA) of eucaryotes. Compilation of data (5) from studies involving a wide phylogenetic range of species indicates that somatic tissues of higher animals contain a few hundred rDNA (the DNA which codes for rRNA) copies per cell while higher plants attain the greatest proportion of rDNA cistrons reaching as high as 27,500 copies per genome in Chinese cabbage.

Selective replication of ribosomal cistrons in amphibian oocytes during oogenesis (8) suggested that the proportion of rDNA in other organisms may vary during a growth cycle. In this respect, fluctuations in rDNA content have been reported to occur in the vegetative cell cycle of Chlamydomonas reinhardi (28) and in the germinating wheat embryo (15). Ingle and Sinclair (30), however, could find no difference in the percent of hybridization during germination of either the wheat or maize embryo.

Massive synthesis of rRNA in Xenopus oocytes later in oogenesis following selective rDNA synthesis (8) was suggestive of a relationship between the replication of extra rDNA copies and a requirement for RNA synthesis. A rapid and selective synthesis of rDNA upon transfer of Tetrahymena pyriformis (22) to enriched media indicated that such a relationship was possible, but conclusive evidence is not available. In higher plants, the only available evidence suggests that the proportion

of rDNA remains constant under conditions when the metabolic rates vary considerably (30).

Since little is known about the relationships of rDNA coding to rRNA production, the study reported herein was undertaken to further this understanding. The developing cotyledon of pumpkin (Cucurbita pepo L.) was selected because the cotyledons emerge in a near-synchronous fashion on the third day of germination and begin greening immediately upon exposure to light. Furthermore, the nucleic acid content is known to undergo dramatic changes during germination. The DNA content usually doubles between the second and third day, without a proportionate increase in cell number, and remains constant through the seventh day. In addition, total RNA is known to increase 5 to 7 fold between the second and fourth day (43).

Studies were also made to determine the effect of light and dark growth conditions on the proportion of DNA coding for both chloroplastic and cytoplasmic ribosomal RNAs (CHL-rRNA and CYT-rRNA, respectively). Tewari and Wildman (52) and Matsuda, Siegel, and Lightfoot (39) found that the genes coding for chloroplastic ribosomal RNAs (CHL-rDNA) are distinct from those coding for cytoplasmic ribosomal RNAs (CYT-rDNA) and that both groups of genes are found as multiple copies in nuclear DNA. Only CHL-rRNA coding has been found in chloroplast DNA (51), but the number of cistrons is quite small (5).

LITERATURE REVIEW

Satellite DNA and Redundant rDNA

The isolation of rDNA cistrons has been achieved in some organisms utilizing isopycnic centrifugation in CsCl. In plants rDNA has a higher molar guanine and cytosine content (G + C about 50%) than that of bulk DNA (G + C about 35% to 40%) and will band at a higher density (38). Ribosomal cistrons which will not form a satellite in CsCl can be shown to lie on the heavy side of the main band DNA by collecting fractions from the gradient and hybridizing these with rRNA (41). Satellite DNA has been found in yeast (46), Xenopus (5), Chlamydomonas (49), and in higher plants, such as pumpkin, Chinese cabbage, and pinto bean (38).

The rDNA of Xenopus laevis was shown to be associated with the nucleolus (13) by the discovery that the amount of satellite rDNA was proportionate to the number of nucleoli. Matsuda and Siegel (38) found satellite DNA only in plants which contained a high hybridization value for rRNA. The pumpkin satellite, which banded at 1.707 g cm^{-3} , was over a hundred times richer in rDNA than the bulk DNA. In addition, they found that rRNAs from tobacco, Chinese cabbage, and pinto beans hybridized equally well to a given plant DNA, but DNAs differed in the amount of rRNA binding. From these results they concluded that the base sequences of rRNAs of widely divergent species were similar, but plant DNAs could vary widely in their proportion of rDNA. In a later study (39), which supported their previous findings, it was also shown that the base

sequences coding for CHL-rRNAs were distinct from those coding for CYT-rRNAs. The rDNAs for each type of RNA appeared to be evolutionarily conserved, and the buoyant densities of both were identical to that of the pumpkin satellite DNA.

Although results from the hybridization of heterologous and homologous rRNAs to rDNAs suggest a conservation of rRNA cistrons among eucaryotes, there are limits to homogeneity (5). Base on buoyant density and molecular weight data (14), it appears that the smaller cytoplasmic ribosomal subunit is more similar among species as diverse as tobacco, sea urchin, chicken, and mouse than the larger subunit. The fact that the molecular weight of the rRNA precursor in mammals is twice that in plants taken together with the interspecific hybridization data implies that the ribosomal cistron itself may be quite similar but that the spacer DNA (DNA segments interspersed between rRNA cistrons) has been considerably altered.

In support of this, Brown, Wensink, and Jordan (11) found a similar buoyant density, base composition, and nucleotide sequence as judged by reciprocal hybridization of isolated rRNA from Xenopus laevis and X. mulleri. However, utilizing the 40S precursor RNA which contained spacer sequences as well as rRNA, they found that hybridization was best using an homologous system. Similarly, Grierson and Loening (27) indicated a possible diversity of rDNA in tissues of the same plant. Using gel electrophoresis, they found that the 80S (cytoplasmic ribosome) precursor of the root had a molecular weight of 2.7×10^6 daltons whereas that of the leaf was 2.9×10^6 daltons. It is possible, though, that an undetected cleavage of spacer DNA occurred in the root.

Location of rDNA

A great deal of autoradiographic work has been done to determine the location of rDNA genes. From such studies, the rDNA appears to be located within the nucleolus and on specific chromosomes at regions usually associated with nucleolar formation, termed nucleolar organizer regions or NOR (13, 41, 48). Cytological work with Phaseolus coccineus and P. vulgaris (1, 6) has shown regions of rDNA at the NOR which consists of a heterochromatic arm connected to satellite DNA with diffuse strands of chromatin. A nucleolus was associated with some of the chromosomes at the NOR site.

Biochemical evidence for locating rDNA at the NOR rests on work done with mutants whose genomes vary in NOR complement. Wallace and Birnstiel (55) used hybridization saturation curves to show a proportionate increase in rDNA content with increasing numbers of NOR. The anucleolate mutant exhibited no satellite DNA and no rRNA hybridization. Previous experiments utilizing Drosophila mutants with one to four NOR on the X chromosome produced similar results (47). Yeast, which does not lend itself to the cytologic study required to establish the presence of NOR, has been shown to have 70% of the rDNA complement on chromosome I by separating whole chromosomes of wild type and aneuploid mutants on sucrose gradients (23) and hybridizing the chromosomes with rRNA. Phillips, Kleese, and Wang (44) used two wild types and one mutant strain of corn containing an extra piece of heterochromatin in the NOR to locate rDNA. Upon crossing these three species they found that the NOR was inherited with the expected Mendelian

frequency. The nucleolus of the mutant was 64% larger and contained twice the rDNA of the single heterochromatic NOR.

Replication and Stability of Redundant rDNA

Replication of rDNA during the cell cycle varies with the organism studied. For example, the slime mold, Physarum (58), synthesizes rDNA after bulk DNA synthesis has begun, but in Chlamydomonas reinhardi (28) the rDNA complement builds up before nuclear DNA synthesis. Several models for rDNA replication, particularly concerning Xenopus (7, 12, 56), have been presented, but the mechanism of rDNA replication and its control are not well understood.

A great deal of evidence (10, 18, 35) has been presented for the Xenopus oocyte which suggests that the chromosomal rDNA first synthesizes rRNA and that this rRNA transcribes extrachromosomal rDNA using an RNA-dependent DNA polymerase. Briefly, the above workers have shown that the banding in CsCl of satellite rDNA being formed during amplification is sensitive to RNase. Upon isolation of the RNAs present in this satellite band, they recovered a piece of RNA which was not complementary to rRNA; in fact, it was not even complementary to the H strand (the DNA strand which transcribes the rRNA precursor molecule). This piece of RNA was associated with the L strand (DNA complementary to the H strand) and would bind only to RNA made with E. coli polymerase which transcribes both strands of DNA including spacer regions. These results led them to state that the L strand as well as the H strand is transcribed into an RNA whose only function would be as a template for DNA synthesis.

Other studies for selective rDNA synthesis suggest a more complex process is involved in the amplification process than was first suspected. Using two species of Xenopus and the progeny of their reciprocal crosses, Brown and Blackler (7) found that the rRNA of either homozygous parent was equally complementary to the progeny irrespective of the maternal species. But upon hybridization with rRNA transcribed by E. coli polymerase, the spacer RNA of only one species, X. laevis, hybridized best to the DNA from all progeny regardless of its sex in the cross. These results indicate a dominance of the X. laevis rDNA to that of X. mulleri.

The bobbed mutant of Drosophila (50) may be another case of rDNA amplification. In this mutant a portion of the rDNA genome is lost thus impairing rRNA synthesis. Reversion to the wild type complement of rDNA occurs spontaneously after several generations. The imbalance is thought to be corrected through synthesis and incorporation into the genome of extra rDNA.

An analogous situation may exist in hyacinth. Timmis, Sinclair, and Ingle (54) utilized several euploids and aneuploids of Hyacinthus orientalis which had different NOR complements in hybridization experiments with rRNA. They found no proportionate relationship in the rDNA number with the number of NOR present. However, comparing the average rDNA number of all the euploids with that of all the aneuploids, they showed a definite decrease in rDNA number in the aneuploid genome. It is possible that rectification of the rDNA complement of the lost chromosomes accounts in part for these results.

Amplification of rDNA in Xenopus laevis, occurring at pachytene in the oocyte (4), reaches a maximum of 2×10^6 cistrons per cell. These nonchromosomal genes persist throughout oogenesis but are dispersed during gastrulation with the resultant somatic tissue containing 1 to 2.5×10^3 cistrons per cell (5). This phenomenon focused attention on the stability of redundancy in plant tissues. Howell (28) established that a periodic fluctuation in rDNA content occurs during the vegetative cell cycle of Chlamydomonas reinhardi. In higher plants Chen and Osborne (15) reported that the embryo of ungerminated wheat contains 30% more rDNA than a wheat embryo after 48 hours of germination, presumably due to gene deletion. However, they also show a concurrent synthesis of DNA. If there is a synchronous replication of DNA during this time period, a dilution of the rDNA cistrons by replicating DNA could account for their results. Ingle and Sinclair (30), in comparing three-day-old wheat or corn embryos to the ungerminated seed, detected no difference in the percent of rRNA hybridization.

Redundant rDNA and the Synthesis of rRNA

If an organism is transcribing rRNA at maximum capacity, any demand for additional massive amounts of rRNA would be limited by the number of rDNA templates available. With this in mind, studies have been done which try to relate rDNA content to rRNA synthesis. No direct relationship of gene dosage to rRNA synthesis was derived from studies with Xenopus (32). In crosses of the wild type (2 NOR) with anucleolate (no NOR) or partial nucleolate (1/2 NOR) mutants the amounts of rRNA synthesized were equivalent to the homozygous wild type. But in hybrids

of the two mutants a heterozygous cross in which only half of the rRNA cistrons of one genome and none of the other were present resulted in a reduction of rRNA synthesis by approximately half, while the homozygous anucleolate hybrid had no substantial rRNA synthesis. Apparently a full haploid complement of genes is required for rRNA synthesis to remain at the level of the wild type, and the amount of rDNA in excess of this haploid complement does not effect an increased amount of rRNA synthesis.

Work with a protozoan, Tetrahymena pyriformis, may suggest that rDNA synthesis can respond to metabolic requirements. Upon transfer from an inorganic to an organic medium, the protozoan exhibits an increased growth rate. Engberg, Mowat, and Pearlman (22) indicated that replication of rDNA began within 90 minutes after transfer to the enriched medium and before bulk DNA synthesis commenced. This was accompanied by morphological changes within the nucleolus. Although rDNA was expected to replicate after bulk DNA, the transfer may have induced asynchrony resulting in the higher values for redundant genes (28).

Studies by Ingle and Sinclair (30) point out that artichoke tissues, such as leaf or tuber tissue, which vary in metabolic rates and should have varied requirements for rRNA did not have detectable differences in rDNA content. More recently, Miksche and Hotta (40) demonstrated that DNA from the root tips of conifer seedlings reannealed at a slightly slower rate than the DNA from whole seedlings. This indicated that the root tip may have fewer redundant genes than the whole seedling and suggested that slight differences in DNA content may exist in different tissues.

Chloroplast DNA and the Effect of Light
on rRNA Synthesis

Chiang and Sueoka (17) used differential ^{14}N - ^{15}N labelling and the resulting density shift to demonstrate the semi-conservative replication of chloroplast DNA (CHL-DNA) before nuclear DNA (N-DNA) synthesis in Chlamydomonas. Studies with tobacco (52) and Euglena (51) chloroplasts indicate that the chloroplast DNA contains coding for only the 70S ribosome (chloroplastic rRNA) and that the cistrons are not highly redundant. However, Bartels and Hyde (2) using wheat and Wells and Sager (57) using Chlamydomonas demonstrate that CHL-DNA exhibits a faster renaturation rate than nuclear DNA which is indicative of redundancy. This repetitious CHL-DNA has not been shown to be rDNA, but such a possibility would be attractive in view of studies on light-induced RNA synthesis.

Hybridization of RNA extracted from pea stems grown in the dark and in the light to pea DNA indicated the presence of a new species of RNA in the light-grown stem, but this species was not identified (53). Ingle (29) concluded, using gel electrophoresis profiles of rRNA from developing radish cotyledons, that CHL-rRNA accumulates in the cotyledon after the CYT-rRNA with maximum incorporation of labelled uridine into CHL-rRNA occurring after emergence of the cotyledon. He did not, however, relate this synthesis to a light-induced phenomenon. Brown and Haselkorn (9), using Euglena, detected an undefined increase in the mRNA population of light-grown cells, and autoradiograms indicated that label was going into the chloroplast. But they could not show an increase in CHL-rRNA in the light-grown Euglena. After noting a consistently

MATERIALS AND METHODS

DNA was obtained from cotyledons of seeds and seedlings of pumpkin, Cucurbita pepo L. var. Small Sugar (Dessert Seed Co., El Centro, California). Pumpkin cotyledons and roots, and leaves and roots of tobacco, Nicotiana tabacum L. var. tabacum, were utilized as sources of labelled and unlabelled RNAs.

Approximately 75 pumpkin seeds were placed lengthwise in 5 rows over 2 liters of vermiculite in an 8" x 12" x 2 3/4" plant tray (Carolina) and were overlaid with another liter of vermiculite. This planting procedure was followed, since Pegelow (43) found earlier that random planting led to inconsistent germination. Initial and subsequent daily watering of plants was generally made between 9:30 and 11:00 a.m. in growth chambers maintained at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with a photoperiod (fluorescent and incandescent lights) from 8:00 a.m. to 8:00 p.m. of about 800 ft-c in intensity at plant height. Under these conditions, seedling emergence occurred by the morning of the third day. Once emergence occurred, the plants were watered with a modified Hoagland's nutrient solution consisting of 1 ml 1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 ml 1 M KNO_3 , 1 ml 1.5 M $\text{Ca}(\text{NO}_3)_2$, 1 ml 1 M KH_2PO_4 :1 M K_2HPO_4 (1:1), and 1 ml of micromix (3.75 g H_3PO_3 , 2.25 g $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 0.075 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.075 g MnO_3 , 0.33 g ZnSO_4 , made to a volume of 3 liters) for each liter of nutrient solution.

Tobacco plants, 6 to 8 weeks old, were obtained from Jeffery Hubert, Department of Agricultural Biochemistry, The University of

Arizona, and transferred to modified Hoagland's nutrient solution. They were grown hydroponically at room temperature ($23^{\circ}\text{C} \pm 3^{\circ}\text{C}$) for about three weeks until vigorous growth began. The plants were then labelled with $\text{H}_3^{32}\text{PO}_4$ and RNA was extracted.

DNA Extraction, Purification, and Storage

Whole cotyledons as well as purified nuclei were used as sources of DNA in the hybridization experiments. In order to prepare whole cotyledonary or total DNA, the cotyledons were first ground and converted to a pigment-free acetone powder prior to DNA extraction using a modification of a procedure suggested by Katterman (Dr. Frank Katterman, Department of Agronomy and Plant Genetics, The University of Arizona, personal communication). In contrast, nuclear DNA was obtained directly from washed nuclei using a slight modification of the procedure outlined by Matsuda *et al.* (39). Unless otherwise indicated, all operations were at 0°C to 4°C , and centrifugations were for 5 minutes using a Sorvall SS-34 rotor at 7,000 rpm in a Sorvall RC-2 B centrifuge.

Total DNA

To prepare acetone powders for total DNA extraction, cotyledons from three trays (20 to 40 g fresh weight) were harvested, weighed, and stored at -60°C prior to grinding. Eight representative cotyledons were also weighed and stored for subsequent DNA and RNA extraction.

After grinding the frozen cotyledons to a fine powder in a mortar chilled to -20°C , 80 ml of ice-cold ethanol:ether 2:1 (v/v) were added and the mixture was ground for an additional 5 minutes followed by filtration through a large Buchner funnel. The residue was then washed

with further aliquots of ethanol:ether (600 ml total) followed by 300 ml acetone. The final residue was then vacuum and air dried overnight followed by storage of the dried powder at -20°C .

DNA's were obtained from the acetone powders by first grinding the powders with sand in an ice-cold mortar using 5 ml of grinding medium (0.14 M NaCl, 0.1 M disodium ethylenedinitrilotetra-acetate, 2% (w/v) sodium dodecyl sulfate, pH 8.2) per gram of powder (usually 3 to 6 g) for 5 minutes until a thick paste resulted. The paste was transferred to a large beaker and incubated at 60°C for 1 hour with occasional stirring. After cooling to room temperature, the mixture was centrifuged and the supernatant fraction collected. The pellet was reextracted with about 5 ml chloroform:octanol (1%, v/v, octanol in chloroform), centrifuged, and the upper aqueous phase was collected and combined with the first. Freshly prepared pronase at a concentration of 5 mg/ml in 1 x SSC (0.15 M NaCl, 0.01 M trisodium citrate, pH 7.2) was predigested at 37°C for 30 minutes and added to the supernate to a final concentration of 1 mg/ml. Following further incubation at 37°C for 30 minutes, the solution was washed repeatedly with an equal volume of chloroform:octanol and centrifuged until no white precipitate was found at the interface. Subsequently, the DNA was precipitated with 95% ethanol, pelleted, and redissolved in 0.1 x SSC. Volumes were kept minimal (5 to 15 ml) for ease of handling and to retain the spooling quality of the DNA in 95% ethanol.

Further DNA preparation was done according to Marmur (37), except that ribonuclease digestion was followed by pronase, as described above, before washing with chloroform:octanol. In addition,

isopropyl precipitation steps were not included, but the DNA was purified further by gel exclusion chromatography using Sepharose 4B columns (Pharmacia Fine Chemicals Inc.). In this final purification step, DNAs ranging in concentration from 0.16 mg/ml to 1.49 mg/ml in 1 x SSC were applied to 12 x 1.5 cm columns of Sepharose 4B in volumes ranging from 1.5 ml to 0.3 ml, respectively, taking care not to overload the column by applying more than 10 A_{260} units. The DNAs were eluted with 1 x SSC and collected as 1-ml fractions. The A_{260} profile of the eluate was determined, and DNA fractions were pooled.

The concentrations of DNA were estimated by assuming that a solution of 1 mg DNA/ml has an $A_{260} = 20$ for a 10 mm light path (20, 36). DNA samples were also assayed with the diphenylamine reaction as outlined by Giles and Meyers (24). Deoxyadenosine was used as a standard assuming that 0.411 μ g deoxyadenosine is equivalent to 1 μ g DNA. All hybridization experiments utilized the A_{260} estimated values for DNA concentration. Estimates of DNA concentration by the diphenylamine test and A_{260} were similar, indicating that the DNA samples were free of major RNA contamination.

Nuclear DNA

Nuclei from freshly harvested cotyledons were isolated and washed according to the procedure of Matsuda *et al.* (39). The resultant pellets of isolated nuclei were stored at -60°C for no more than two weeks prior to DNA extraction. To extract DNAs, the nuclei pellets were first resuspended in saline EDTA (0.15 M NaCl, 0.1 M ethylenediamine-tetra-acetate, pH 8.0) and incubated at 60°C for 20 minutes with

occasional shaking. The solution was then brought to 1 molar with 5 M HClO_4 and shaken gently at room temperature for 30 minutes, as outlined by Marmur (37). Subsequent washing was as described for total DNA preparation.

Storage of DNA

All samples of DNA were stored in screw cap vials over ice at 0°C to 4°C , and experiments were conducted as soon as possible after purification. After no more than two weeks of such storage, a few drops of chloroform:octanol were added to each vial to retard bacterial growth. Any subsequent hybridization experiments with DNA stored over chloroform required reprecipitation with 95% ethanol redissolving in $0.1 \times \text{SSC}$.

Because this laborious storage method was not satisfactory for maintenance of DNA integrity for longer than a month, a second method of storage was attempted. A known aliquot of DNA was lyophilized to dryness and stored in a screw cap vial at -20°C for two months. The DNA was redissolved in $0.1 \times \text{SSC}$, and hybridization values were compared to those obtained before lyophilization.

Procedure for Labelling Plants with RNA Precursors

Labelled pumpkin root and cotyledonary RNAs were prepared using tritiated uridine (20 to 40 Ci/m mole) obtained from International Chemical and Nuclear Corporation (ICN) or New England Nuclear Corporation (NEN). Carrier-free $\text{H}_3^{32}\text{PO}_4$ (ICN) was used to prepare labelled tobacco RNAs.

Prior to labelling, roots of 3-day-old pumpkin seedlings were washed and inserted through 3/4-inch holes that were drilled in stiff,

opaque or foil-covered plastic sheets. The seedlings were supported on the plastic platform by wrapping the hypocotyl with flexible strips of foam plastic, and the roots were quickly placed in nutrient solutions to avoid drying. Normally, about 16 plants were used to prepare labelled root or cotyledonary RNAs.

Labelled pumpkin root RNAs were prepared by quickly blotting adhering nutrient solution from roots and then placing them in a shallow parafilm dish that contained 0.5 mCi ^3H -uridine in about 1 ml solution. The roots were allowed to absorb the labelled solution for 2 minutes, then 10 ml of nutrient solution was added, and the seedlings were then shaken gently for 30 hours at 25°C under fluorescent lighting of about 400 ft-c. Further additions of nutrient solution were made as needed to prevent drying of the roots. Following the 30-hour incubation, the labelled solution was further diluted, and the plants were cultured with root aeration for an additional 20 hours in the growth chamber.

For preparation of labelled cotyledonary RNAs, plants, grown hydroponically in 2 liters of solution, were labelled from early on the third morning until early in the morning of the fourth day. One cotyledon per plant was marked with a pinprick and 5 or 10 μl of a 0.2 mCi ^3H -uridine solution (containing 0.1%, v/v, Triton-x-100) were applied to the abaxial surface at nearly hourly intervals throughout the third day, and the plants were harvested early on the fourth morning.

In order to prepare labelled tobacco RNAs, vigorously growing plants were transferred to -P nutrient solutions and 2 mCi $\text{H}_3^{32}\text{PO}_4$ were added directly to each solution in the glazed clay pots. Roots and young

leaves were harvested three days later and extracted for RNAs. RNAs were also obtained from similarly treated but otherwise unlabelled plants.

Extraction and Purification of RNA

RNAs were obtained both by direct extraction of tissue and from isolated ribosomes. In either method, adhering, unincorporated labelled uridine was first removed by vigorous washing. Roots were washed by shaking first in 400 ml 0.05% sodium hypochloride and later in 600 ml 1% (w/v) sodium dodecyl sulfate (SDS). They were then blotted and cooled in an ice-cold mortar. Cotyledons were washed in 75 ml 1% (w/v) SDS, blotted, and cooled in a mortar. All later procedures were at 0°C to 4°C and centrifugations were at 7,000 rpm in a Sorvall SS-34 rotor for 5 minutes, unless stated otherwise.

Extraction from Tissue

For direct RNA extractions, plant tissues (5 to 10 g fresh weight) were ground in sand with 10 ml buffer (1%, w/v, SDS, 1%, w/v, sodium pyrophosphate, pH 8.0) to a paste and rinsed into a centrifuge tube with 5 ml buffer. An equal volume of water-saturated, redistilled phenol was quickly added, and the mixture was vigorously shaken for at least 5 minutes and then centrifuged. After extracting with phenol three times, the upper aqueous phase containing RNA was washed three times with about 3 volumes ether from a freshly opened can to remove residual phenol. The dissolved ether was then removed by evacuation and the solution centrifuged to remove insoluble materials. The dissolved RNA was precipitated with 95% ethanol at -20°C overnight. The resultant pellet following centrifugation was redissolved in 1 to 2 ml 0.015 M

Sorenson's phosphate buffer, pH 7.0, and reprecipitated in 1.5 M NaCl. The pellet, which contains ribosomal RNAs and other high molecular weight RNAs was redissolved and stored at -20°C .

Concentrations of RNAs were determined by assuming that 1 mg RNA/ml has an A_{260} of 25 in a 10 mm light path.

Extraction from Isolated Ribosomes

Ribosomal RNAs were prepared from isolated ribosomes according to the method of Matsuda et al. (39).

RNA Characterization

Resolution of the various RNA species was made using the agarose-polyacrylamide gel electrophoresis method of Peacock and Dingman (42). Agarose gels were made by heating 2.45 ml H_2O , 4 ml acrylamide solution (15%, w/v, acrylamide; 0.75%, w/v, bisacrylamide) and 8.33 ml 3 x E buffer (0.12 M trizma base, 0.06 M sodium acetate, 0.003 M disodium EDTA, pH 7.2 with glacial acetic acid) to 37°C . To this was added 10 ml of 1.25% (w/v) agarose (BioRad Laboratories), 20 μl tetramethylethylenediamine, and 0.2 ml 10% (w/v) ammonium perchlorate. The agarose had been previously treated with activated charcoal and filtered to remove impurities. To effect resolution, approximately 15 μg RNA were applied to each gel and electrophoresis with a Heathkit IP-17 power supply was performed at 5 ma per gel for 1.25 hours in 1 x E buffer with 0.2% (w/v) SDS. The gels were scanned at 260 nm on a Gilford 240 spectrophotometer, using the Gilford #2410 linear transport and a Brown-Honeywell recorder. The gels were then frozen and sliced into 1.25-mm sections and transferred to scintillation vials. One-half ml of 30%

H₂O₂ was then added, and the gel slices were incubated at 45°C overnight before addition of 10 ml Triton-toluene fluor consisting of 16.5 g PPO (2,5-diphenyloxazole), 0.3 g POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene), 2 liters toluene, and 1 liter Triton-x-100. Counting was done in a Packard 3320 Tri-carb scintillation counter. Radioactivity was plotted on the optical density scans, areas under the peaks were cut out and weighed, and specific activity of each RNA species was determined.

DNA-RNA Hybridization

Hybridizations were conducted using a modification of the method of Gillespie and Spiegelman (25). Nitrocellulose membranes (Schleicher and Schuell, B-6, 25 mm), numbered with a soft lead pencil, were soaked for a few seconds in 2 x SSC and were placed numbered side down in a Millipore microanalysis filter holder. Five ml of 2 x SSC were added to each membrane and suction was applied to maintain a 1 ml/min flow rate. Membranes were then turned numbered side up and again washed with 5 to 10 ml 2 x SSC. At this time DNA was alkali-denatured by bringing 4 ml of a 1 x SSC solution of DNA to pH 12.6 with 1 M NaOH. After being held at that pH for 10 minutes, the solution was brought to pH 6.5 with 1 M KH₂PO₄ and was applied immediately to the nitrocellulose membranes, using 5 µg DNA per membrane. The DNA solution was allowed to drip through the membrane without suction to allow for maximum binding of DNA to the membrane. Two ml of 2 x SSC were then added and a very mild suction was applied. DNA-embedded membranes were air dried for 1 hour and were placed in a vacuum oven at 80°C for 4 hours. Membranes were then removed from the oven, cooled to room temperature

for 5 to 10 minutes, and carefully placed in snap-cap glass vials 1 inch in diameter (one membrane per vial) containing 2 ml of RNA solution. The RNA solutions, usually prepared 2 to 3 hours in advance, were made with 0.1% (w/v) SDS in 2 x SSC and were placed in the glass vials (2 ml/vial) and stored at 0°C to 4°C until used. The vials were then covered with cellophane, capped, and placed in a 65°C to 67°C water bath for 14 hours to allow for maximum annealing of RNA to DNA. The vials were then removed from the water bath and quickly cooled to room temperature. Membranes were washed on each side in the filter holder with 5 to 10 ml 2 x SSC to remove unbound RNA, and then each membrane was incubated at 37°C in 5 ml ribonuclease (predigested at 10 mg/ml 2 x SSC at 80°C for 10 minutes) at 20 µg/ml 2 x SSC to digest nonspecifically bound RNA. The membranes were then washed on each side with 10 ml 2 x SSC, air dried for 15 to 30 minutes, and dried at 80°C for 4 hours in a vacuum oven. The membranes were transferred to scintillation vials, 10 ml of toluene fluor (10 g PPO, 0.5 g POPOP, 2 liters toluene) were added, and counting was done in a Packard 3320 Tri-carb scintillation counter.

Determination of Total Nucleic Acids

Total nucleic acids from eight cotyledons, extracted by the method of Cherry (16), were estimated (in mg/ml) by multiplying the absorbance difference at 260 nm and 290 nm by 0.057. DNA concentration was determined by the diphenylamine method as modified by Kupila, Bryan, and Stern (33), and RNA content was determined by subtraction of the DNA from the total nucleic acid content.

RESULTS AND DISCUSSION

As presented by Pegelow (43), dramatic changes in nucleic acid metabolism occur during the early development of the pumpkin cotyledon which suggest that there may be certain periods when the demand for rRNA is high. As an indication of the metabolic events occurring within the time period for the experiments studied here, four of the eight representative cotyledons selected from the samples utilized for DNA extractions were analyzed for nucleic acid content. These results are shown in Figure 1.

The RNA content was found to more than double between the second and fourth days and thereafter to decline in cotyledons exposed to light, in good agreement with Pegelow. Dark-grown cotyledons did not attain this peak period of RNA content; instead the RNA almost doubled and remained at a constant level through the sixth day. While Pegelow noted a tripling in DNA content between the second and third days, the light-grown cotyledons examined here showed a doubling in DNA content by the fourth day. Although not doubling, the DNA content of dark-grown cotyledons is not very different from the DNA content of light-grown cotyledons. The fluctuations observed on day 3 may be due to the use of only two replicate samples; other points are means of 4 to 6 replications.

Because the nucleic acid metabolism seems to be most active between days 2 and 4, this period was chosen for a closer examination of the rDNA content. Extractions of DNA from isolated nuclei, termed

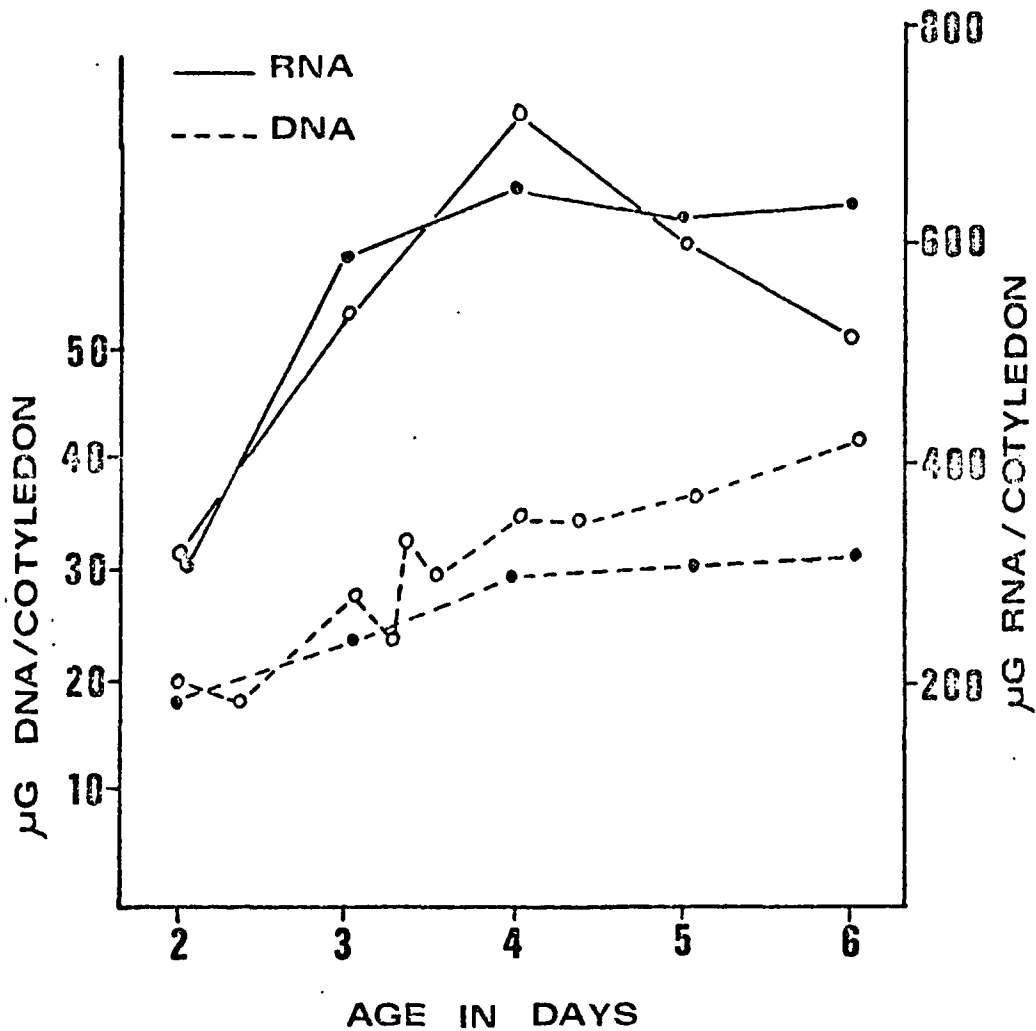


Figure 1. RNA and DNA Content of Etiolated and Light-grown Pumpkin Cotyledons during Development

Extraction and quantitation of total nucleic acid and determination of DNA content by the diphenylamine method were as described in Materials and Methods. Total RNA was determined by subtracting DNA from the total nucleic acid. The points represent the mean of at least two replications. Symbols: open circles: light-grown; closed circles: etiolated.

nuclear DNA (N-DNA), and extractions of DNA from whole cotyledons, termed total DNA (T-DNA), were designed to separate nuclear events from those in the cytoplasm. The total DNA was expected to contain N-DNA and DNAs present outside the nucleus. The age of the cotyledon was measured in days from the initial watering period early in the morning on day 1. Each day began at 8:00 a.m., designated as zero hour, when the lights came on, and all hourly divisions of a day were measured from this time.

Characterization of RNAs

In early studies, RNAs were extracted directly from plant tissues, but later experiments utilized RNAs extracted from isolated ribosomes. The integrity and purity of each RNA preparation was determined with agarose-polyacrylamide gel electrophoresis, and S values were assigned according to Loening and Ingle (34). The radioactivity tracings and UV absorption profiles of these RNAs are found in Figures 2, 3, and 4. Figures 2 and 3 represent the cotyledonary RNAs obtained by direct extraction from cotyledons and from isolated ribosomes, respectively, while Figure 4 profiles an RNA extracted directly from tobacco roots. The slight amount of label in the DNA and in the 4S regions of Figures 2 and 4 were removed by salt precipitation before use in hybridization.

Certain characteristic features of these RNA samples should be noted. The cotyledonary RNAs (cotyl-RNA) contain subunits for both 70S and 80S ribosomes; the large and small RNA molecules of each are indicated on the abscissa as 25S and 18S (for the 80S or cytoplasmic ribosome) and as 23S and 16S (for the 70S or chloroplastic ribosome). In addition, the 70S ribosome is known to yield a degradation product of

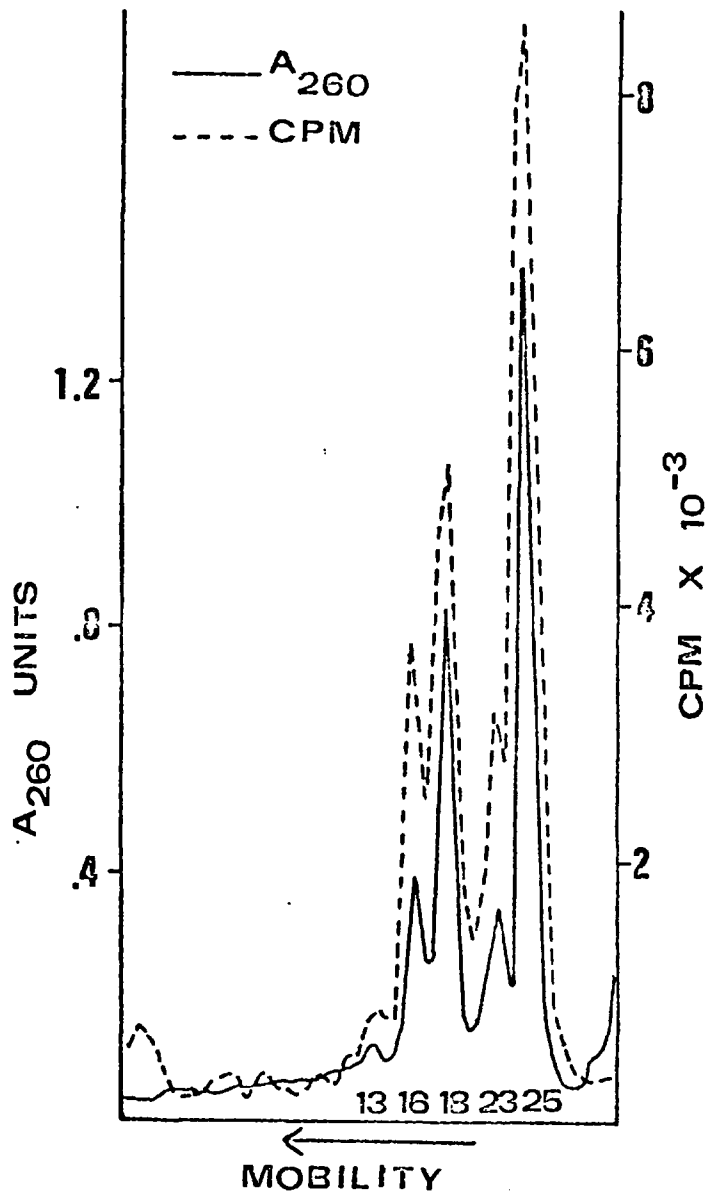


Figure 2. Fractionation of Labeled Pumpkin Cotyledonary RNA Extracted from Whole Cotyledons

Fractionation was done on agarose-polyacrylamide gels. RNAs were labelled with ³H-uridine, extracted directly from pumpkin cotyledons, and purified as outlined in Materials and Methods. Approximately 15 to 20 μ g RNA were run at 5 ma/gel for 75 minutes. The calculated specific radioactivities of RNAs of 70S ribosomes (23S, 16S, 13S) and 80S ribosomes (25S, 18S) were 4200 cpm/ μ g and 4000 cpm/ μ g, respectively.

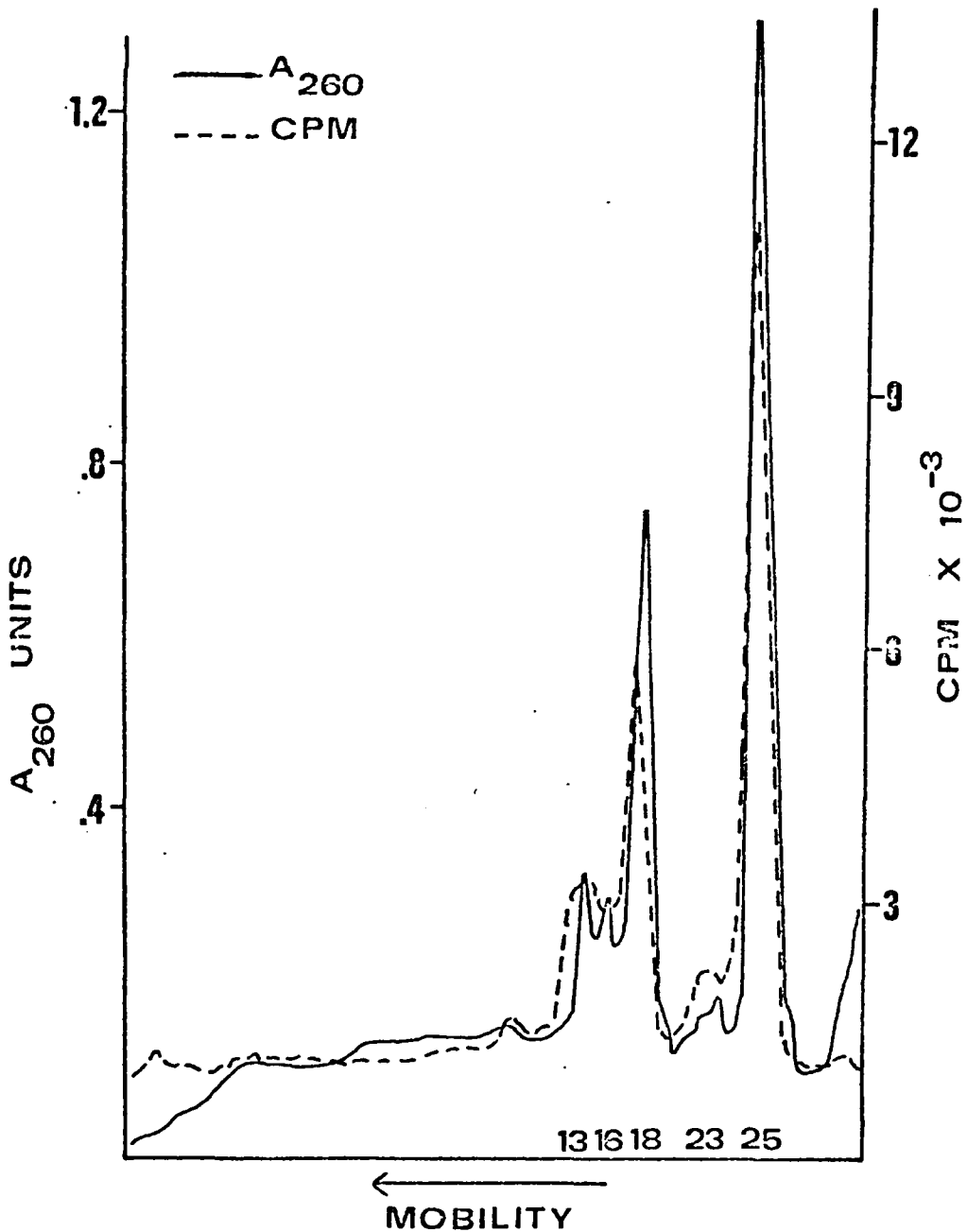


Figure 3. Fractionation of Labeled Pumpkin Cotyledonary RNA Extracted from Isolated Ribosomes

Fractionation was done on agarose-polyacrylamide gels. Extraction from isolated ribosomes labelled with ³H-uridine, purification, and fractionation were as outlined in Methods and Materials and in Figure 2. The calculated specific radioactivities are 7800 cpm/ μ g for RNAs of 80S ribosomes and 6300 cpm/ μ g for RNAs of 70S ribosomes.

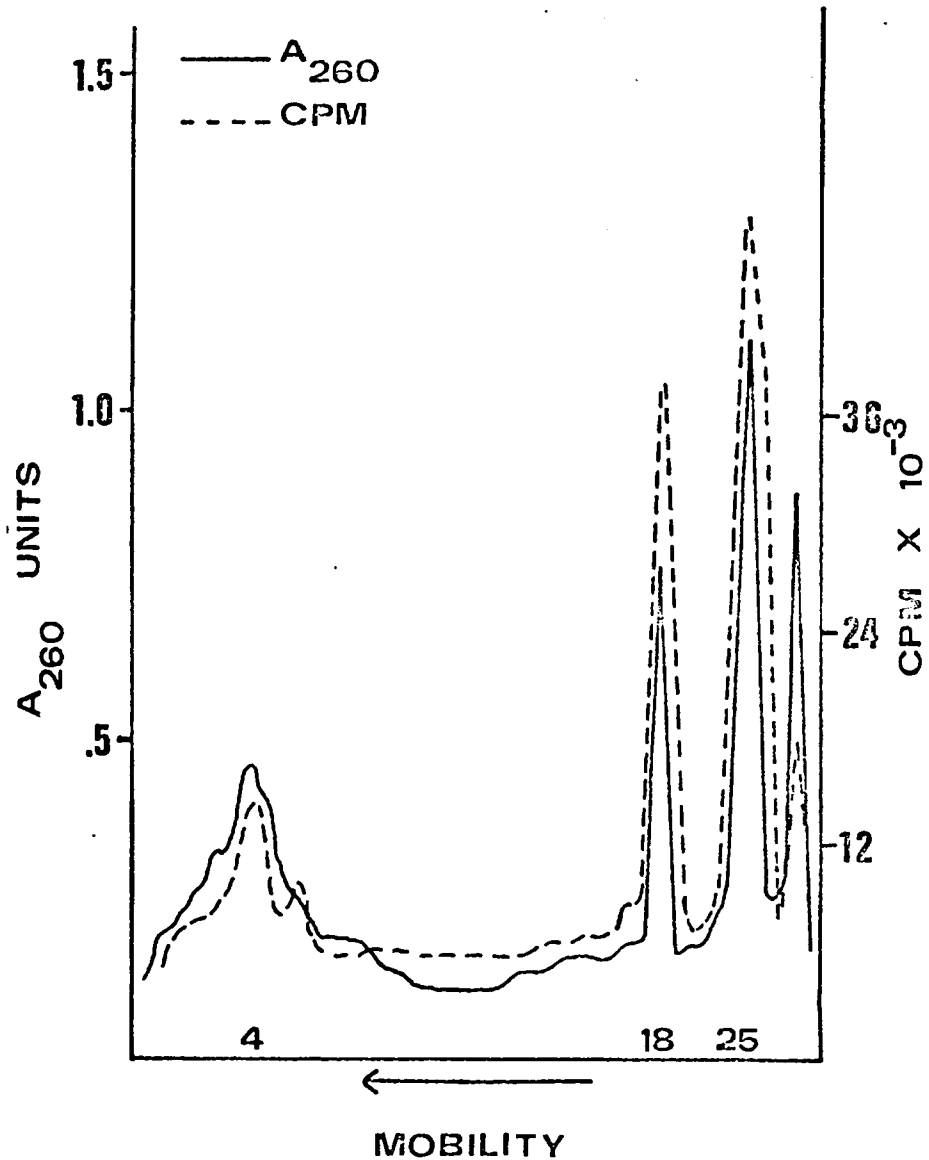


Figure 4. Fractionation of Labelled Tobacco Root RNAs

Fractionation was done on agarose-polyacrylamide gels. RNAs were labelled with $H_3^{32}PO_4$, extracted directly from tobacco roots, purified, and fractionated as outlined in Materials and Methods and in Figure 2. The A_{260} ratio of 25S:18S was 1.86:1; and the calculated specific radioactivities were 3600 cpm/ μ g and 4100 cpm/ μ g for the 25S and 18S RNAs, respectively.

13S (34). The more dispersed RNA components of 70S ribosomes in Figure 3 were taken to mean that some degradation occurred during the pelleting of ribosomes due to RNase attack (34). Roots have RNAs from only the 80S ribosome in agreement with Matsuda et al. (39). If peaks in the 23S and 16S region were found or if the OD peaks did not coincide with the radioactivity peaks, the root RNA was considered contaminated with bacterial ribosomes and was discarded. In all these figures, radioactivity and OD peaks were coincident.

The specific radioactivities of the RNAs of green tissues were determined by pooling the areas of 25S and 18S RNAs for CYT-rRNA and those of 23S, 16S, and 13S for CHL-rRNA. It should be noted that the specific activities indicated in these figures, although similar, are not equal. This may be due to the technique of calculating specific activities, particularly when the high background produced in agarose gels made the placement of a base line arbitrary. As an example, when specific activities were calculated in Figure 3, using a blank gel as the base line, values of 7800 cpm/ μ g for RNAs of 80S ribosomes and 6300 cpm/ μ g for RNAs of 70S ribosomes were obtained. If the base line was shifted to the lowest point on that particular gel, the specific activities were calculated to be 7600 and 8800 cpm/ μ g, respectively.

Determination of Saturating Amounts of RNA

The amount of RNA required to saturate a given DNA sample can be determined by reacting the DNA with increasing amounts of RNA until all sites on the DNA are full and the percent of DNA hybridized does not increase. Ideally, saturation curves should be run for all DNA samples,

but due to the large number of samples such saturation curves were not made. Instead, a sample from 4-day-old cotyledons, expected to have the greatest amount of rDNA coding from preliminary experiments, was utilized in all saturation hybridization experiments.

The curves in Figure 5 show clearly that cotyl-RNAs hybridize to a greater extent to DNA from 4-day-old cotyledons than to DNA from 2-day-old cotyledons, and in both cases the cotyl-RNA has a higher hybridization value than root RNAs. In addition, it can be seen that RNAs extracted directly from whole tissues do not approach saturation even after the addition of 24 μ g of RNA. Considering the labelling profile of the RNAs used (Figure 2, the pumpkin root is not shown), the above results suggest that RNAs extracted by this method are not pure rRNAs. One or more labelled RNAs are present in amounts undetectable by UV absorption which continually add to the hybridization value upon the addition of increasing amounts of RNA. This other RNA, of unknown specific activity, may be a single RNA species or a population of RNAs of which several possibilities exist. A population of messenger RNAs may be responsible for this continual rise in hybridization. Alternatively, a population of poly U sequences (since labelling was done with ^3H -uridine), analogous to the poly A sequences found in animal systems (19), may be present in pumpkin cotyledons which would also produce these results.

Bendich and Bolton (3) and Goldberg, Bemis, and Siegel (26) showed that DNAs of widely divergent plants, as well as plants within the genus Cucurbita, were different, suggesting that messenger RNAs were also different. But Matsuda et al. (39) showed that rRNAs were

Figure 5. Hybridization of Pumpkin Total DNA with Labelled RNAs Extracted Directly from Pumpkin Cotyledons and Roots

Five μg total DNA (extracted directly from cotyledons) of 2-day-old (open circles) or 4-day-old (closed circles) pumpkin seedlings were reacted with the indicated amounts of ^3H -uridine-labelled RNAs for 14 hours at 66°C as outlined in Materials and Methods. The indicated points are the means of three or four replications. The specific radioactivities were 4000 cpm/ μg and 3400 cpm/ μg for cotyledonary and root RNAs, respectively; and the net cpm bound were 600 and 430 cpm for 2-day and 4-day total DNAs reacted with 24 μg cotyledonary RNA, and 250 cpm for 4-day total DNA reacted with 12 μg root RNA.

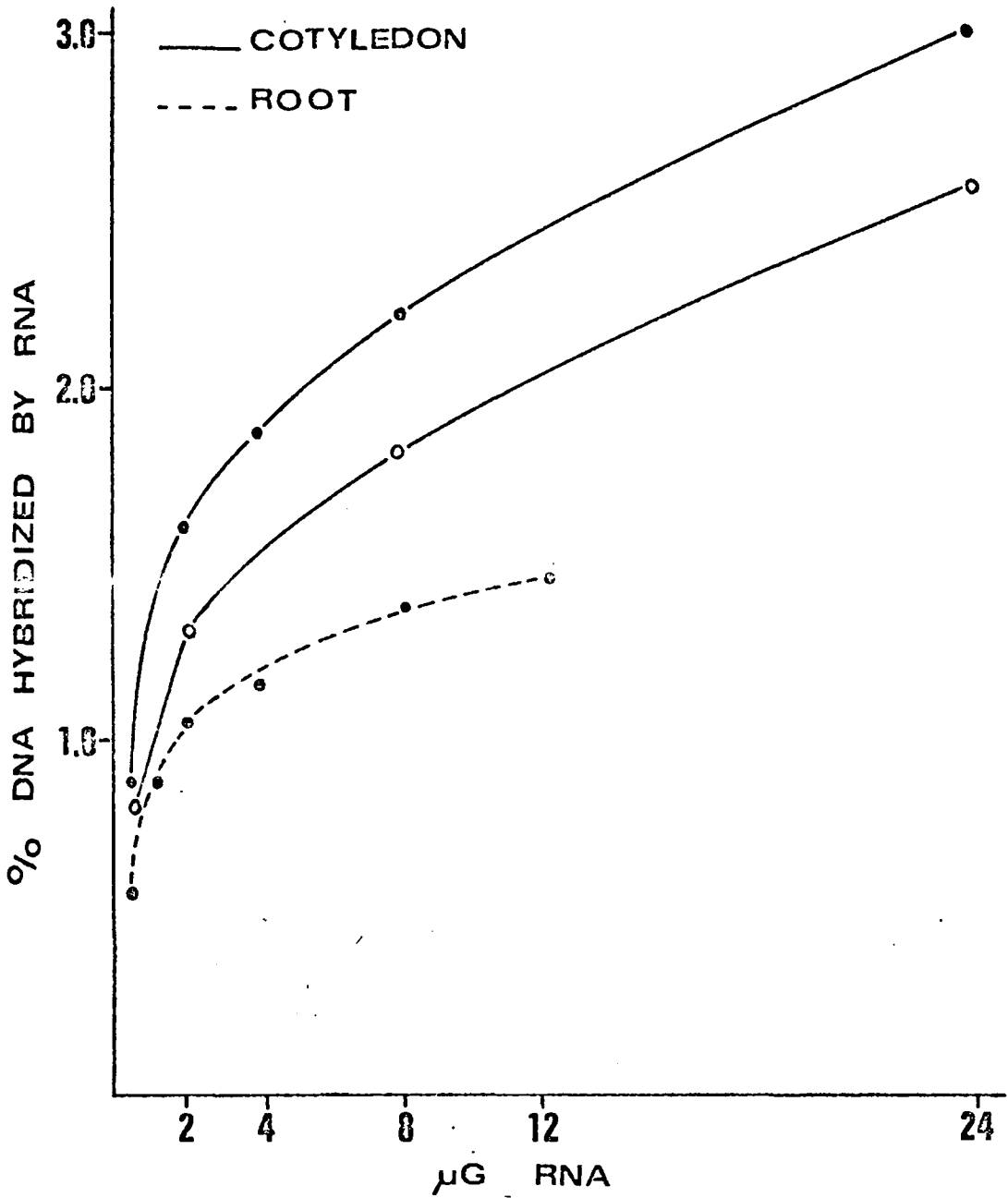


Figure 5. Hybridization of Pumpkin Total DNA with Labelled RNAs Extracted Directly from Pumpkin Cotyledons and Roots

similar in widely divergent plants; therefore, to reduce the effect of these non-ribosomal sequences or RNAs, attempts were made to attain saturation with tobacco RNAs rather than pumpkin RNAs. As shown in Figure 6, this strategem did not have the expected effect. The tobacco leaf RNA did not approach saturation and, in fact, had an almost identical curve to that obtained with homologous RNA-DNA hybridization. The root RNA (Figure 4), however, did approach saturation. This not only suggests that some RNA sequences are conserved in diverse plants in addition to rRNA sequences but that these conserved sequences may be associated only with greening tissue.

In earlier work, Matsuda et al. (39) showed that saturation could be achieved with RNAs extracted from isolated ribosomes. This method was used to rid the RNA of these contaminating sequences, and saturation was achieved (Figure 7). Root RNAs extracted by this method were too badly degraded to characterize on agarose gels, so the tobacco root rRNAs of Figures 4 and 6 were judged to be pure enough for use in hybridization. Because the saturation curve is asymptotic, a judgment of the saturating amount of RNA was made. In all experiments designed to measure the percent of rDNA in the genome, then, 5 μ g DNA were hybridized with 4 μ g tobacco root RNA or 8 μ g pumpkin cotyl-rRNA and the same RNA samples of Figures 3 and 4 were used.

Determination of the Percent DNA Coding for RNAs of 70S and 80S Ribosomes

The percent of DNA coding for CYT-rRNA can be determined in a straightforward manner using roots as a source of CYT-rRNA. But since the isolation of chloroplastic ribosomes is difficult, some method other

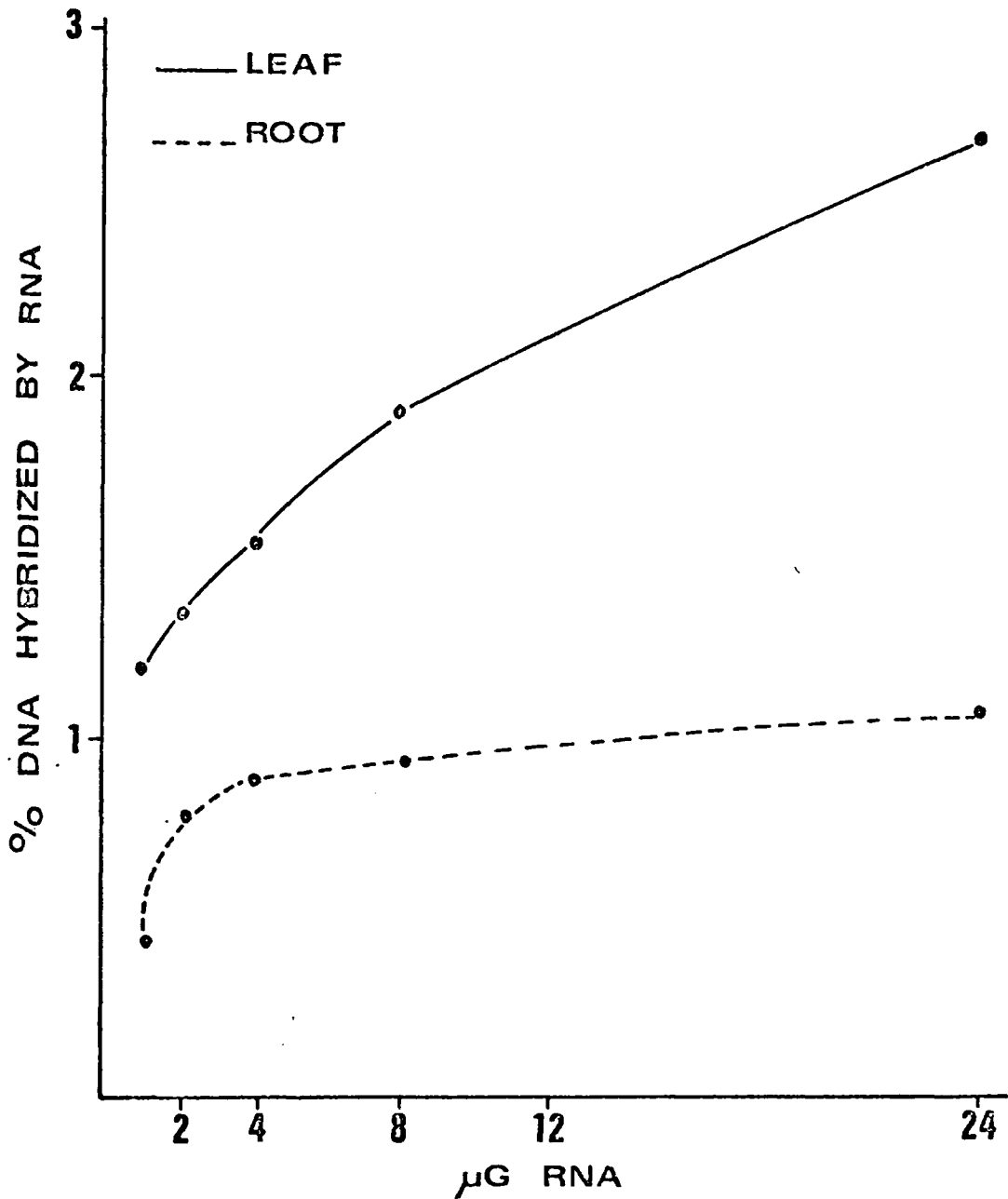


Figure 6. Hybridization of Pumpkin Total DNA with Labelled RNAs Extracted Directly from Tobacco Leaves and Roots

Five μg total DNA were reacted with the indicated amounts of RNAs labelled with $\text{H}_3^{32}\text{PO}_4$ as outlined in Materials and Methods and in Figure 5. Each point is the average of two replications. Specific radioactivities of 7900 and 16,000 cpm/ μg yielded 1100 and 960 cpm bound at 24 μg RNA input for leaf and root RNAs, respectively.

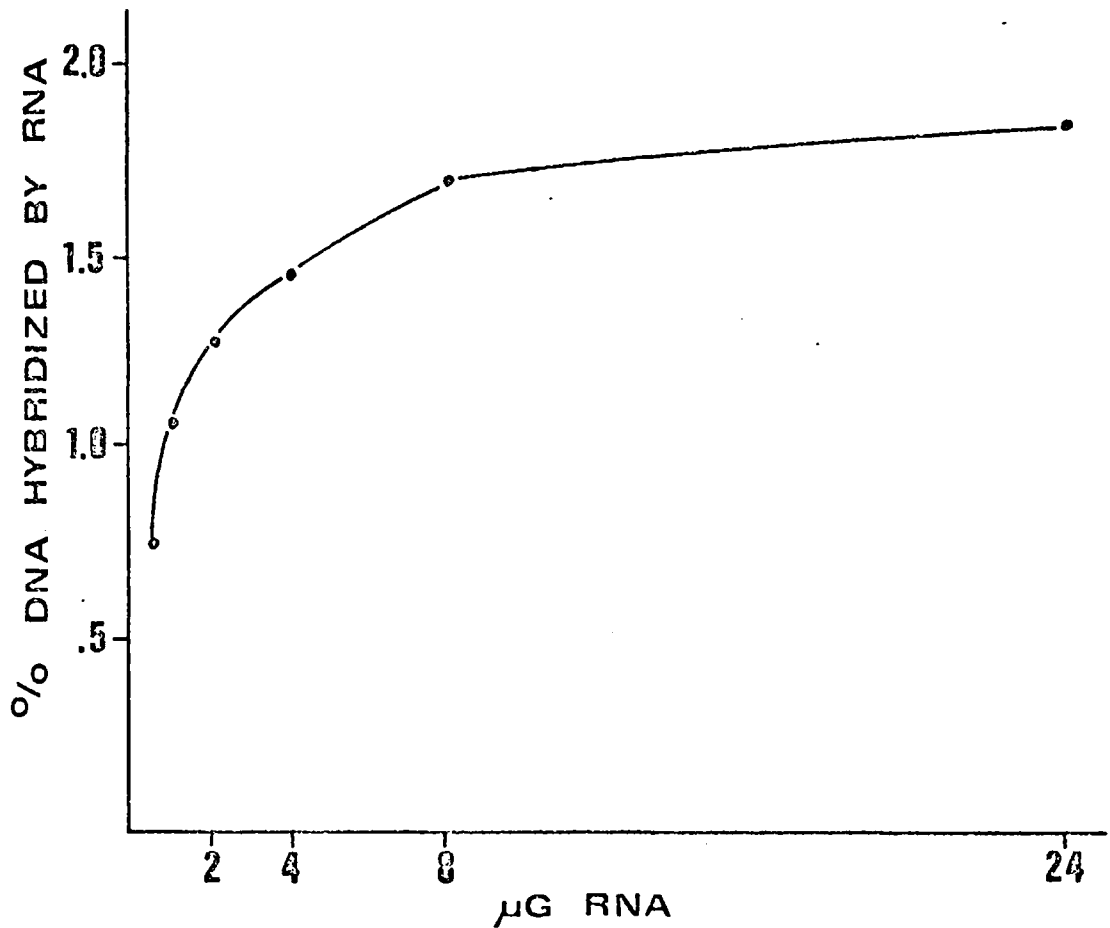


Figure 7. Hybridization of Pumpkin Total DNA with Labelled RNAs Extracted from Isolated Ribosomes

Five μg DNA were reacted with the indicated amounts of ^3H -uridine-labelled rRNA as outlined in Materials and Methods and in Figure 5. Each point represents the average of three replications. The specific radioactivity of 6300 cpm/ μg yielded 580 cpm bound with the addition of 24 μg RNA.

than hybridization to purified CHL-rRNA must be found. Matsuda *et al.* (39) demonstrated that the RNAs of 70S and 80S ribosomes of leaves, which hybridized to a greater extent than the RNAs of the roots, could be used to determine CHL-rDNA content. If the RNAs of 70S and 80S ribosomes occupy separate sites on the DNA and if they are of equal specific activity, the difference between the hybridization values obtained with leaf or root RNAs would represent the contribution of the CHL-rRNA.

The data in Figure 8 suggest that the RNAs of 70S and 80S ribosomes occupy different sites on the DNA and that the subtraction method of estimating CHL-rDNA content is a valid one. In Figure 8, 5 μ g of DNA were reacted with 4 μ g of cotyl-rRNA (Figure 3) and additional indicated amounts of unlabelled tobacco root RNA. In the absence of competing RNA, a value of 1.5% of the DNA complementary to rRNA (CHL-rRNA and CYT-rRNA) was obtained. But upon the addition of competing unlabelled root RNAs (CYT-rRNA), this value decreased and approached 0.6% in a manner not unlike that shown in Figure 7. This occurred because the unlabelled CYT-rRNA could compete with only some of the cotyledonary RNAs for the DNA sites, implying that the CYT-rRNAs and CHL-rRNAs occupy separate sites on the DNA.

If the specific activities of the CHL-rRNA and CYT-rRNA of the cotyledon are assumed to be identical, a direct subtraction of 1.5% - 0.6% = 0.9% should mean that 0.6% of the DNA codes for CHL-rRNA and 0.9% of the DNA codes for CYT-rRNA. It can be seen that a subtraction of Figure 6 from Figure 7 (using the same DNA and RNA samples) yields (1.7% - 0.9% = 0.8%) 0.8% coding for CHL-rRNA and 0.9%

Figure 8. Hybridization of Pumpkin DNA with Labelled Cotyledonary RNA and Competing Amounts of Unlabelled Tobacco Root RNA

The effect of unlabelled tobacco root RNA on the reactivity of total DNA from 4-day-old pumpkin cotyledons with labelled rRNA from isolated ribosomes of pumpkin cotyledons is shown (see Figure 3). The tobacco root RNA was extracted directly from tobacco roots, purified, and fractionated as outlined in Materials and Methods. Five μg DNA and 4 μg ^3H -rRNA from pumpkin cotyledons were reacted with the indicated amounts of unlabelled tobacco root RNA as outlined in Materials and Methods and Figure 5. Each point represents the average of two replications; 490 cpm were bound with no unlabeled RNA added, while 190 cpm were bound upon the addition of 24 μg of unlabelled root RNA.

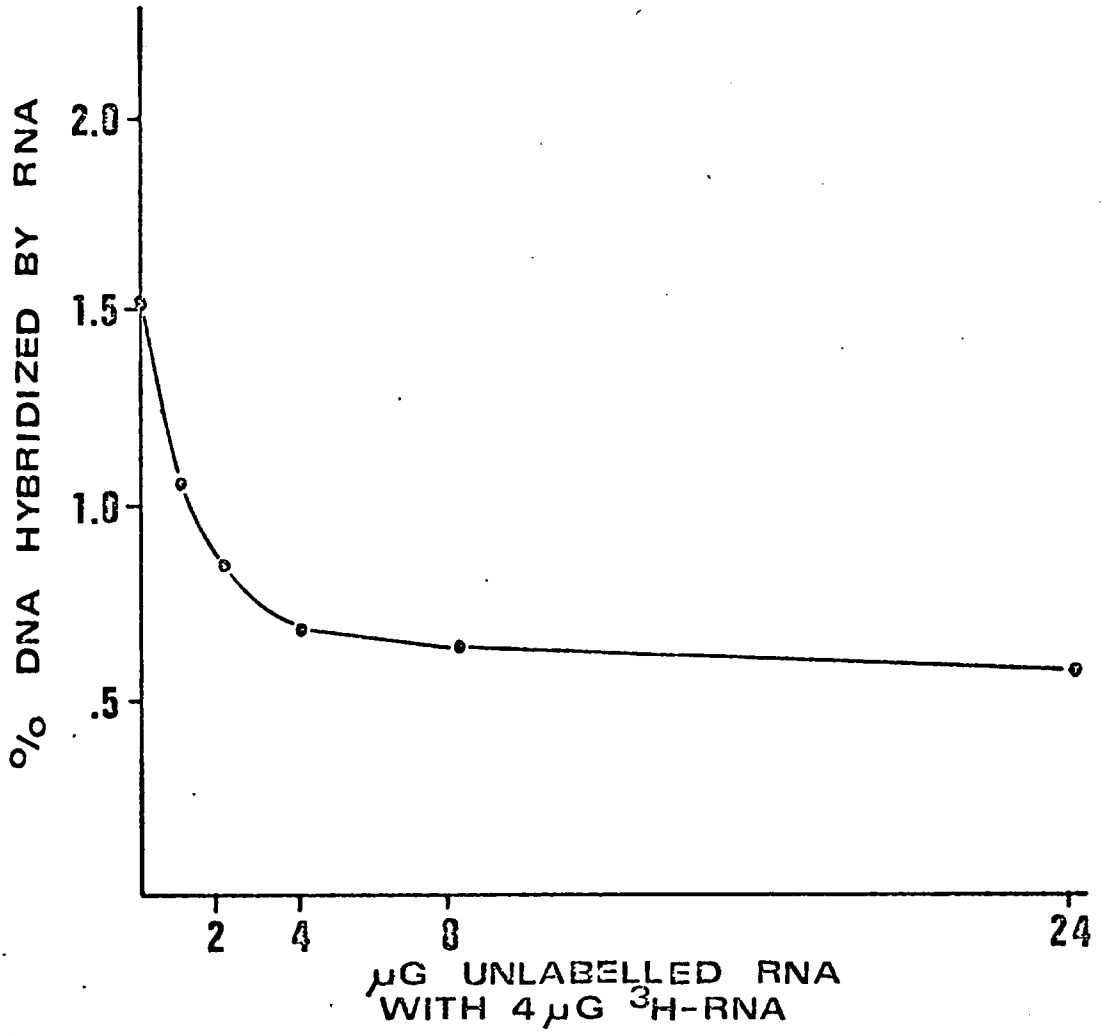


Figure 8. Hybridization of Pumpkin DNA with Labelled Cotyledonary RNA and Competing Amounts of Unlabelled Tobacco Root RNA

coding for CYT-rRNA. In both cases, the same value for CYT-rRNA was obtained, and the difference of 0.2% for CHL-rRNA is believed to be due to the CHL-rRNA not being at saturating levels. (See Figure 7 and compare hybridization values, using 4 and 8 μ g RNA.)

It was recognized early in this study that there are many variables in the hybridization technique which are not understood, and several of these inherent errors are discussed at length in a review by Kennell (31). To reduce some of these errors, care was taken to treat all samples alike throughout extraction and hybridization. It should be noted that filters were air dried for 1 hour and then dried in a vacuum oven for 4 more hours in contradiction to the directions of Gillespie and Spiegelman (25), but this procedure was continued in order to keep all hybridization conditions identical. All filters were well dried before being placed in the oven, and every replicate hybridization yielded similar cpm bound. In addition to an identical hybridization technique, the same RNA samples (Figures 3 and 4) were used to demonstrate more effectively relative changes in rDNA content.

The purity of the DNA was also of concern; therefore, each DNA sample was passed through a Sepharose 4B column. DNA fractions which elute first in the gel exclusion chromatography technique were pooled, and the resultant DNA concentrations were determined with A_{260} and diphenylamine reagent methods. These two methods yielded very similar results which indicated that major amounts of RNA were no longer present in any DNA sample. Hybridizations were carried out within two weeks of the last chloroform:octanol treatment to assure the integrity of the DNA. Pegelow (43) found that as DNA was stored, its hybridization value

decreases over a period of 1 to 3 months. A second method of storage, lyophilization, was attempted on one sample of DNA. Before lyophilization, the DNA sample had a value of 1.98% coding for the cotyledonary RNA of Figure 2. Two months later, after lyophilization and storage at -20°C , 1.93% of the DNA coded for this same sample of cotyl-RNA. This strongly suggests that lyophilization may be a reliable method of storing DNAs for long periods of time.

Changes in rDNA Content during Cotyledon Development in the Light

As shown in Figure 1, a gradual doubling of the DNA content occurred between the second and fourth days. In addition, fluctuations in the DNA content were noted in samples harvested early on the third day. The data suggested that the developing cotyledon was not as synchronous a system as was indicated by Pegelow (43). The results presented in Figure 9, however, were reassuring. These data were collected from separate experiments involving two groups of plants grown at separate times. Harvests were made on the third day at 0, 3, and 9 hours and at 0, 3, and 6 hours after the lights came on. These data (Figure 9) show clearly that, although the two groups contained different proportionate amounts of CHL-rDNA and CYT-rDNA, the relative changes in their content followed a similar pattern. The CYT-rDNA first increased by about 0.2% (percent of the DNA coding for rRNA) and then decreased concomitantly with an accumulation of CHL-rDNA. This was taken to mean that the system was synchronous and that an average of the data collected from plants grown at separate times was indicative of general trends in the rDNA content of the population. The data presented in

Figure 9. Hybridization of DNAs from Two Groups of Cotyledons Grown at Separate Times in the Light with Labelled RNAs

Total DNAs from developing pumpkin cotyledons were reacted with saturating amounts of ^3H -uridine-labelled RNAs. Groups A and B were planted at separate times; Group A was harvested at 0, 3, and 9 hours and Group B was harvested at 0, 3, and 6 hours after the lights came on on the third day. Five μg DNA were reacted with 4 μg tobacco root RNA (Figures 4 and 6) or 8 μg pumpkin cotyledonary rRNA (Figures 3 and 7) as outlined in Materials and Methods and in Figure 5. The determination of the % DNA hybridized by RNA of 70S ribosomes was as described in Table 1.

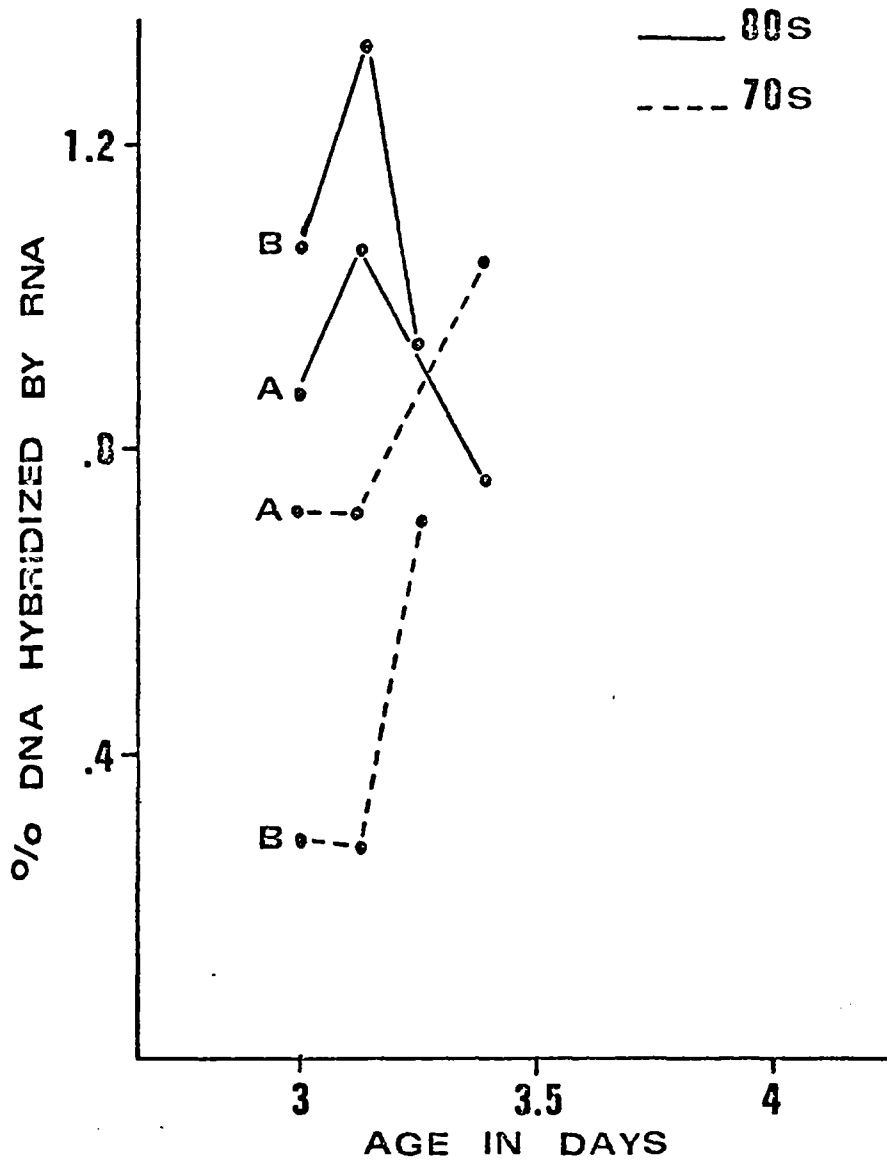


Figure 9. Hybridization of DNAs from Two Groups of Cotyledons Grown at Separate Times in the Light with Labelled RNAs

Table 1 and in Figures 10 and 11 were gathered from various groups of plants which were not grown simultaneously.

Since studies have reported conflicting results concerning fluctuations in rDNA content during plant development, the principal goal of this study was to determine whether or not the rDNA content is stable during the development of pumpkin cotyledons. It was concluded from Figures 10 and 11 that changes do occur. Early on the third morning, immediately after the lights came on, the CYT-rDNA content increased from about 1% to 1.2% and then dropped to 0.8%. This was accompanied by a doubling (0.5% to 1%) of the CHL-rDNA content followed by a return to its original value. Furthermore, this occurrence was found in both the N-DNA and the T-DNA.

If portions of the genome replicate early in S (before bulk DNA synthesis), an increase in the cistron content and a return to its original level would be expected. On the other hand, a drop in the DNA content followed by a return to the original level would indicate that other parts of the nucleus replicated prior to its replication. Reasoning that a synchronous population of cells in the cotyledon could relate a selective accumulation or loss of rDNA to events in the nucleus, several inferences were drawn from these data.

In Figures 9, 10, and 11, a portion of the CYT-rDNA replicated prior to the CHL-rDNA. The CYT-rDNA then experienced a sudden decrease in proportion to the rest of the genome as the CHL-rDNA content doubled. Both types of rDNA returned to their original levels as a second group of CYT-rDNA cistrons replicated.

Table 1. Hybridization of Total and Nuclear DNAs from Light-grown Pumpkin Cotyledons with Labelled RNAs

The hybridization reactivity of total and nuclear DNAs from pumpkin cotyledons grown in the light with the RNAs are described in Figures 3 and 7 and in Figures 4 and 6. The conditions of hybridization were outlined in Materials and Methods and in Figure 9. The percent hybridization obtained with tobacco roots (80S) were subtracted from those obtained with the cotyledonary rRNA for an estimate of the CHL-rDNA indicated here as 70S.

Age of Cotyledon		% DNA Hybridized by RNA					
		Total DNA			Nuclear DNA		
Days	Hours	Number of Samples	80S	70S	Number of Samples	80S	70S
2	48	2	0.95	0.03	2	1.06	0.39
2.33	56	-	--	--	1	1.12	0.44
3	72	2	0.97	0.50	1	1.07	0.65
3.13	75	2	1.20	0.50	1	1.16	0.63
3.25	78	1	0.76	1.03	-	--	--
3.33	80	1	0.93	0.71	1	1.20	0.88
3.5	84	-	--	--	1	0.88	1.11
4	96	2	1.03	0.52	2	1.07	0.54
4.33	104	1	0.75	0.83	-	--	--
5	120	2	1.00	0.48	1	0.95	0.66
6	144	2	1.15	0.82	1	0.86	0.68

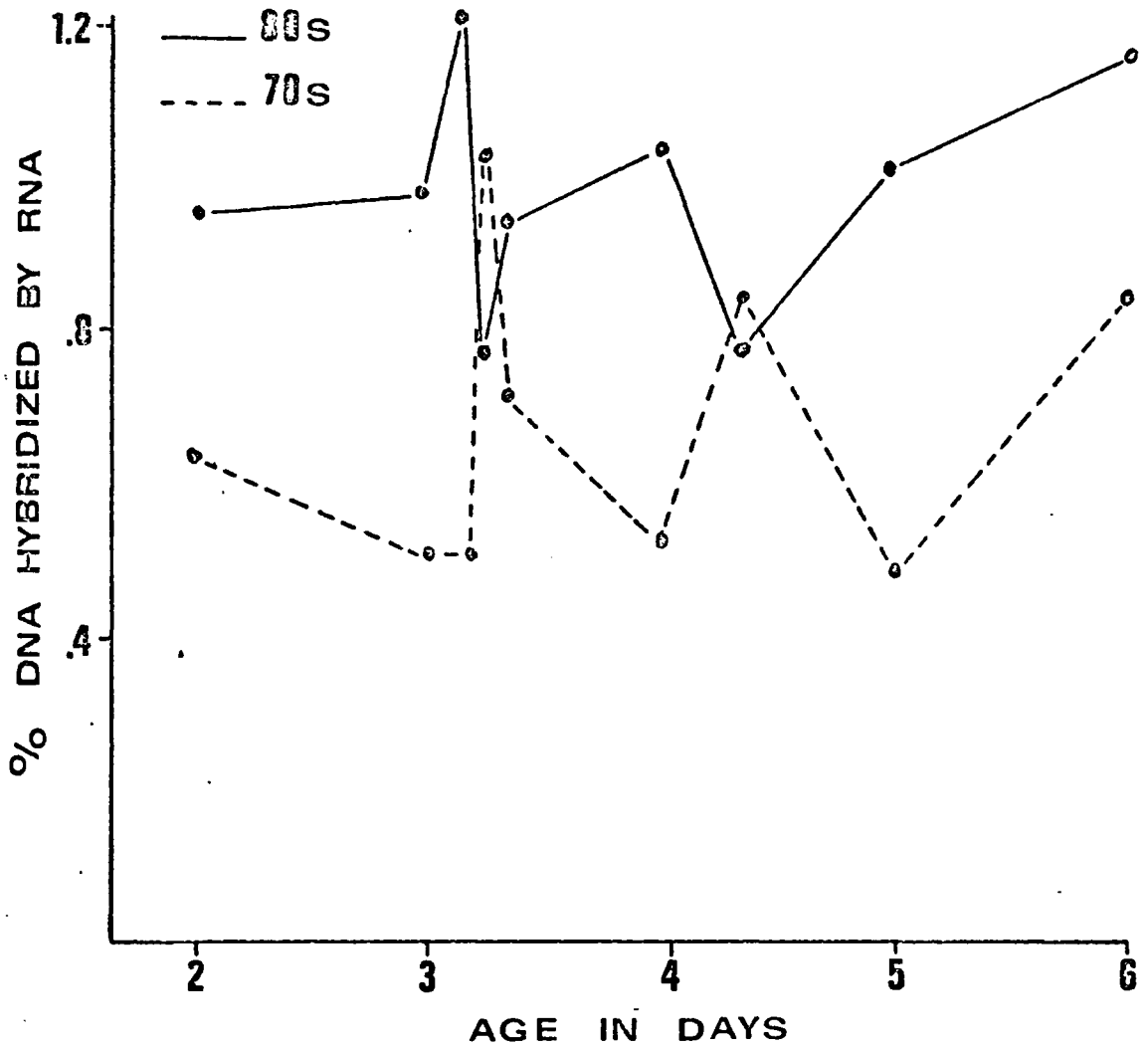


Figure 10. The rDNA Content of Total DNAs Extracted from Developing Light-grown Pumpkin Cotyledons

Hybridization of total DNAs extracted from light-grown pumpkin cotyledons was done as described in Figure 9. Method of calculation of 70S values is given in Table 1; data are from Table 1.

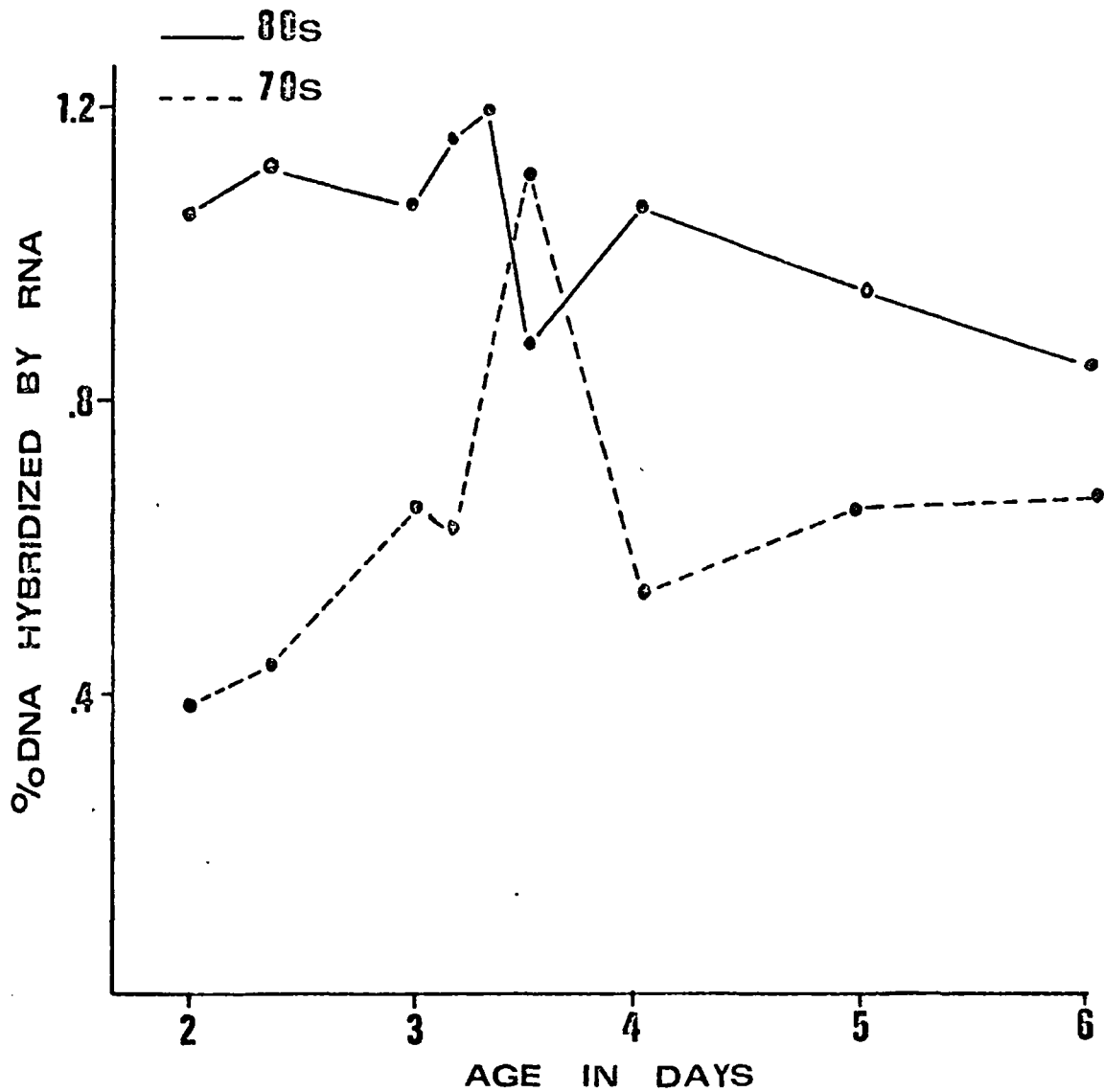


Figure 11. The rDNA Content of Nuclear DNAs Extracted from Developing Light-grown Pumpkin Cotyledons

Hybridization of nuclear DNAs extracted from isolated nuclei of light-grown developing cotyledons was done as described in Figure 9. Method of calculation of 70S values is given in Table 1; data are from Table 1.

The changes occurring only in the total DNA (Figure 10) during the fourth day are represented by only one sample, and speculations that this represents another series of replications are difficult to justify. Two replicates of the values at day 6 (Figure 10), however, indicate a rather significant increase (0.2%) in CHL-rDNA content that was not observed in the N-DNA (Figure 11). Although suggestive of a contribution by chloroplast DNA to the rDNA content, this does not seem likely. The above-noted increase would involve a few thousand cistrons, yet only 1 or 2 CHL-rDNA cistrons have been reported for chloroplast DNA (5).

Except for the sharp fluctuations discussed above, the CYT-rDNA and CHL-rDNA seem to represent relatively constant proportions of the genome, about 1% and 0.6%, respectively. Massive amplification of rDNA, as found in Xenopus, was not found during the development of pumpkin cotyledons; but changes in rDNA content of short duration, probably due to synchronous DNA replication, did occur. In concurrence with Ingle and Sinclair (30), it was concluded that the rDNA content is stable during pumpkin cotyledon development. It is possible that the 30% decrease in rDNA reported by Chen and Osborne (15) was a case of synchronous cell division (as seen in Figures 10 and 11) and not a selective degradation as was proposed.

Changes in rDNA Content in Developing Cotyledons Grown in the Dark.

Several workers (9, 21, 53) have pointed to an apparently light-induced phenomenon of increased RNA synthesis but have not yet defined any specific RNA species that is affected. Poulson and Beevers (45) have also noted a higher polysome content in light-grown tissues which

is indicative of protein synthesis. The above work suggested that tissues exposed to light may, then, have a greater need for ribosomes and, consequently, rRNA. A comparison of pumpkin cotyledons grown in the dark and in the light was made to see if the rDNA content was stable.

As an indication of the rDNA content, the percent of the DNA coding for CYT-rRNA (Figures 10, 11, 12, and 13) on days 2, 3, 4, 5, and 6 were averaged together, and the resultant means (one for each figure) were used to calculate the number of CYT-rDNA cistrons. The DNA content was taken to be 4.7×10^{-12} g/cell in a 3-day-old pumpkin cotyledon (Sherry Keener, Department of Microbiology, The University of Arizona, personal communication). The average percent of DNA coding for CYT-rRNA in Figure 10, for example, is 1.02%. Assuming that the DNA is one molecule, this means that the molecular weight of the CYT-rDNA is 2.88×10^{10} ($4.7 \times 10^{-12} \times 6 \times 10^{23} \times 0.0102 = 2.88 \times 10^{10}$). If the molecular weight of ribosomal RNA is taken to be 2×10^6 , the number of CYT-rRNA cistrons is calculated to be 14,400 per cell ($2.88 \times 10^{10} / 2 \times 10^6 = 1.4 \times 10^4$). Similar calculations using averages from the other figures yielded values ranging from 14,400 to 15,600 cistrons per cell, indicating that the CYT-rDNA content is stable during the development of the pumpkin cotyledon under either light or dark growth conditions.

It can be seen in Figure 12 that the CHL-rDNA content of etiolated cotyledons was similar to the values reported for light-grown cotyledons up to and including the third day. The calculated cistron content for Figures 10, 11, and 12 ranged from 8,300 to 9,110 cistrons per cell.

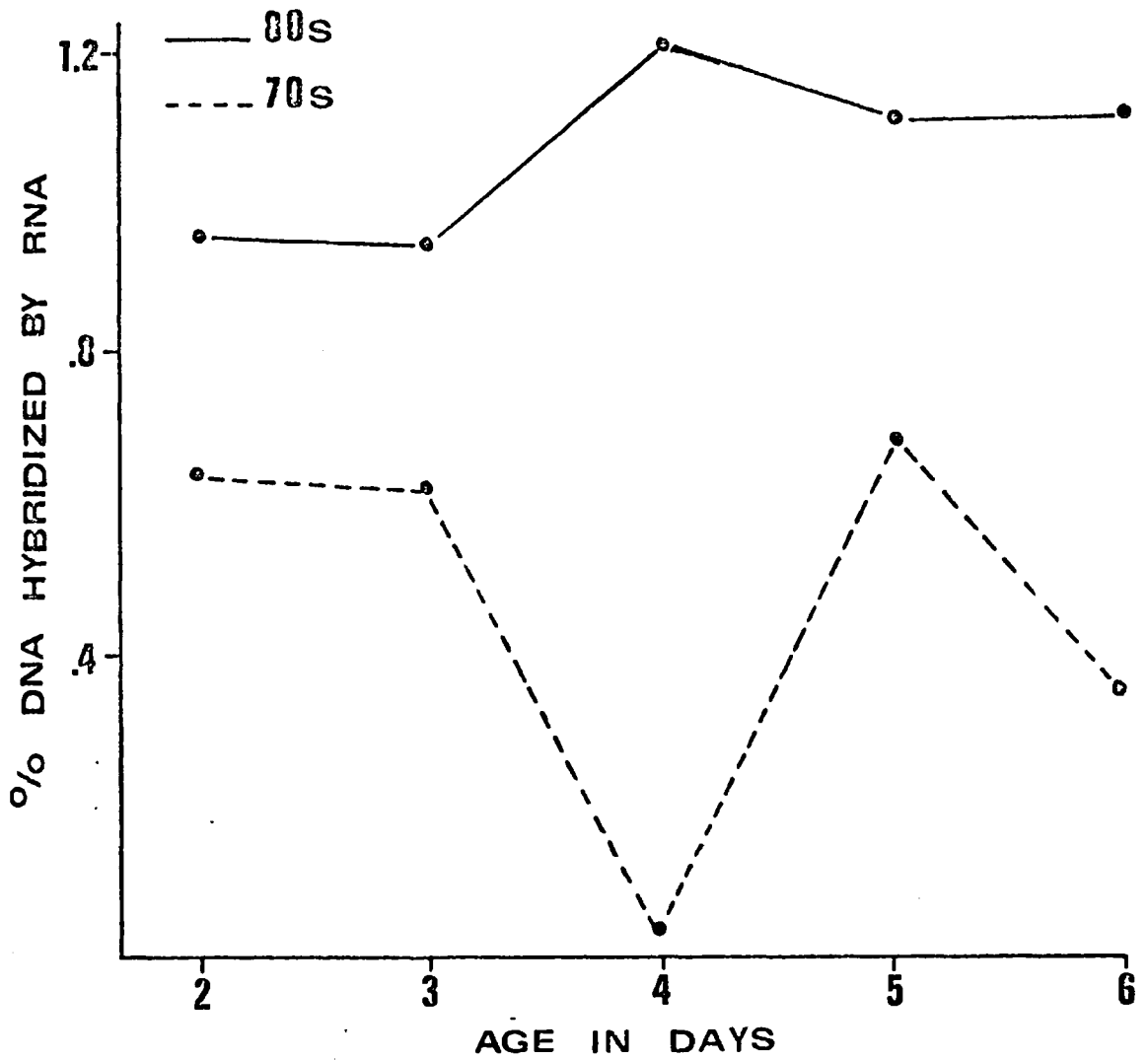


Figure 12. The rDNA Content of Total DNAs Extracted from Developing Etiolated Pumpkin Cotyledons

Hybridization conditions are outlined in Figure 9. Method of calculation of 70S values is given in Table 1; data are from Table 2.

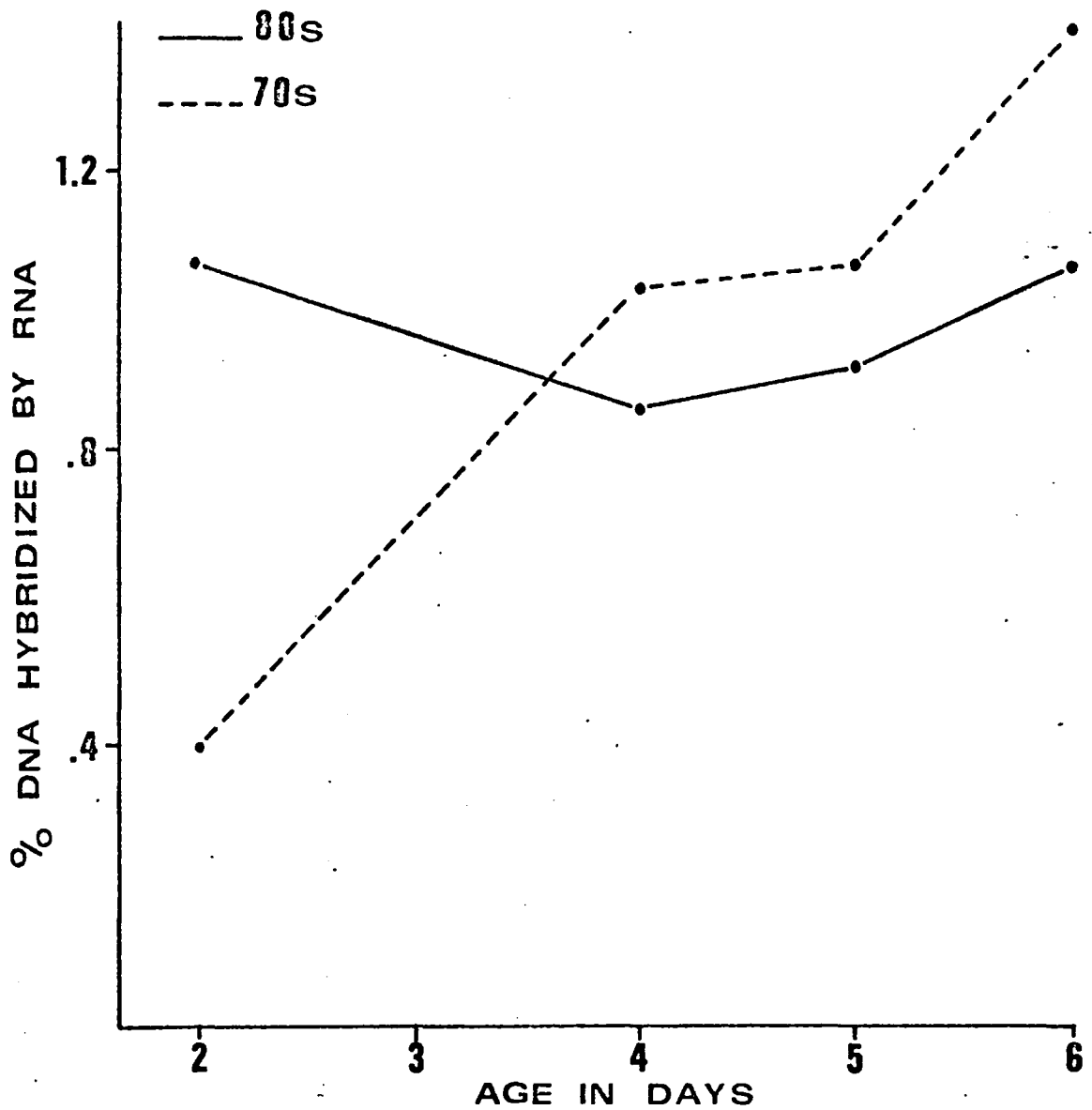


Figure 13. The rDNA Content of Nuclear DNAs Extracted from Developing Etiolated Pumpkin Cotyledons

Hybridization conditions are outlined in Figure 9. Method of calculation of 70S values is given in Table 1; data are from Table 2.

After the third day, however, vast differences were seen in CHL-rDNA content.

The results in Figure 13 show that the CHL-rDNA content in the nuclear DNA extracts nearly doubled (from 0.6% to 1%) by the fourth day. This high proportion of CHL-rDNA persisted through day 5 and increased to 1.35% of the DNA by the sixth day. The CHL-rDNA cistron number, then, more than doubled in contrast to the light-grown tissues (Figures 10 and 11). The total DNA extraction procedure (Figure 12, Table 2), however, indicates a very low proportion of CHL-rDNA during this time period. Since the CYT-rDNA content was constant in both Figures 12 and 13, a selective loss of CHL-rDNA seems to have occurred during the total DNA extraction procedure.

Several possible reasons for a low CHL-rDNA yield were considered. Since this phenomenon occurred only in the etiolated cotyledonary DNA, the lack of exposure to light may have had an effect on the processing or properties of CHL-rDNA such that it was washed out along with the pigments while preparing the acetone powders or that it was now more susceptible to DNase attack. It is also possible that CHL-rDNA is replicated in small units of 1 or 2 cistrons in length and that such short pieces within the nucleus may be more easily lost unless the nuclei are first isolated. If the CHL-rDNA sedimentation properties had changed, then altered recovery efficiencies in the total or the nuclear preparations could explain these results. The reason for the discrepancy between total and nuclear DNA extraction results has not been resolved.

It does seem clear, from Figure 13, that the CHL-rDNA content is twice the value found in light-grown cotyledons. This apparent

Table 2. Hybridization of Total and Nuclear DNAs from Dark-grown Pumpkin Cotyledons with Labelled RNAs

The hybridization reactivity of total and nuclear DNAs from pumpkin cotyledons grown in the dark with the RNAs are described in Figures 3 and 7 and in Figures 4 and 6. Hybridization conditions were as outlined in Materials and Methods and in Figure 9. Determination of 70S (CHL-rDNA) is as described in Table 1

Age of Cotyledon		% DNA Hybridized by RNA					
		Total DNA			Nuclear DNA		
Days	Hours	Number of Samples	80S	70S	Number of Samples	80S	70S
2	48	2	0.95	0.63	2	1.06	0.39
3	72	2	0.94	0.62	-	--	--
4	96	2	1.20	0.04	1	0.87	1.01
5	120	1	1.11	0.67	1	0.91	1.05
6	144	2	1.12	0.36	1	0.79	1.37

increase could be the result of asynchronous CHL-rDNA replication or an altered ability to recover CHL-rDNA. If the increase is an actual one, then these data suggest the possibility of a response in the CHL-rDNA content to the dark growing conditions.

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