THE SYNTHESIS OF RIBOSOMAL RIBONUCLEIC ACID IN
DEVELOPING PRIMARY LEAVES OF PHASEOLUS AUREUS

by

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APPENDIX 1: The synthesis of ribosomal RNA in different organisms: structure and evolution of the rRNA precursor.


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SUMMARY

In the Introduction the reasons for studying ribosomal RNA synthesis are outlined and the literature concerning ribosomal RNA metabolism is reviewed.

The results show that the major part of the RNA from young etiolated leaves is cytoplasmic ribosomal RNA. Several unidentified minor RNA components are also present. Chloroplast ribosomal RNA which constitutes about 30 per cent of the total ribosomal RNA in mature leaves is not synthesised until about two days after germination.

Light stimulates cell division and the accumulation of chloroplast and cytoplasmic ribosomal RNA but both types of RNA also accumulate in the dark in the absence of cell division. The first detectable effect of light on RNA synthesis is an increased rate of processing of macromolecular precursors to cytoplasmic ribosomal RNA. This response occurs less than two hours after the onset of light treatment.

The 1.1 x 10^6 molecular weight chloroplast ribosomal RNA is unstable. Old as opposed to newly synthesised molecules are hydrolysed \textit{in vivo} at two specific sites. The resulting fragments dissociate and can be detected separately in polyacrylamide gels. The fragments can be held together by magnesium ions which presumably stabilise base paired regions between the RNA fragments. The 1.3 and 0.7 x 10^6 molecular weight cytoplasmic ribosomal RNAs and the 0.56 x 10^6 molecular weight chloroplast ribosomal RNA are all stable during electrophoresis. Hidden breaks can be revealed in the polynucleotide chains of the cytoplasmic ribosomal RNAs by heating the molecules in solution prior to electrophoresis.
Specific molecular aggregates of ribosomal RNA can be formed by heating RNA solutions and under certain conditions of RNA extraction and fractionation.

Rapidly labelled RNA heterogeneous in size has a DNA-like base composition. Six distinct RNA components with molecular weights of 2.9, 2.7, 2.5, 1.45, 1.0 and 0.45 x 10^6 are super-imposed on the background of polydisperse RNA separated on acrylamide gels. They are metabolically unstable and the four largest molecules resemble ribosomal RNA in base composition. Labelling kinetics suggest that they are macromolecular precursors to ribosomal RNA. All these molecules hybridise preferentially to DNA fractions containing the genes for cytoplasmic and chloroplast ribosomal RNA which are denser than the bulk of the DNA when banded in caesium chloride.

Young leaves and roots synthesise precursors with molecular weights of 2.7 and 2.5 x 10^6. Competitive hybridisation studies suggest that these RNAs contain sequences of both the 1.3 and 0.7 x 10^6 molecular weight cytoplasmic ribosomal RNAs plus different amounts of non ribosomal RNA which are removed during processing of the precursors to form mature cytoplasmic ribosomal RNAs. RNAs of molecular weight 1.45 and 1.0 x 10^6 may be processing intermediates and the possible stages in precursor processing are discussed.

An additional precursor with a molecular weight of 2.9 x 10^6 is synthesised in leaves grown in the light. It is not clear whether this molecule is a precursor to chloroplast or cytoplasmic ribosomal RNA. It is suggested that the 0.45 x 10^6 molecular weight RNA may be cleaved from one end of this precursor during processing.
INTRODUCTION
The aim of this work was to learn more about the synthesis and function of ribosomal RNA (rRNA) and the factors regulating rRNA synthesis during plant development. The importance of RNA metabolism is outlined below, together with the reasons for selecting the synthesis of rRNA as a model system.

Previous work has shown that phytochrome, activated by red light, regulates photomorphogenesis in leaves. This process includes cell division and expansion (Dale and Murray, 1968, 1969) and the development of chloroplasts and photosynthetic enzymes (Graham, Grieve and Smillie, 1968). It was thought that increased rRNA synthesis would accompany these developmental responses and it was of interest to investigate the possible regulatory role of light in RNA synthesis.

Chloroplasts contain a protein synthesising apparatus which is different from that of the cytoplasm (Boulter, Ellis and Yarwood, 1971). The two types of rRNA can be distinguished by their size (Loening and Ingle, 1967). There is evidence that chloroplast rRNA is synthesised within the chloroplast using organelle DNA as template (Scott and Smillie, 1967) and recent studies by Ingle (1968b) suggest that chloroplast and cytoplasmic rRNA synthesis are controlled separately.

Many experiments on leaf photomorphogenesis have been carried out with the genus Phaseolus. After preliminary investigations, P. aureus was selected for this work; it has a number of advantages over other members of the genus. Because of their small size the seedlings can be easily manipulated in the dark and they can be grown conveniently under sterile conditions. Rapid germination and primary leaf development mean that the time between experiments is relatively short.
The role of RNA in growth and differentiation

In 1902 Haberlandt (see Steward, 1970) proposed that plant cells were totipotent. Nearly 60 years later Steward and his colleagues (Steward, Mapes and Mears, 1958) demonstrated that a normal plant would develop, under the appropriate conditions, from a single differentiated cell. During development changes in enzyme complement confer new properties on a cell and differentiating cells differ from each other in structure, size, chemical composition and metabolic activity. All these properties are ultimately determined by the genetic information of the cell. Steward's experiments show that all the genetic information is present within one cell and yet it is clear that in an intact plant such a cell does not express all of the genetic information it contains. The processes of development and differentiation must therefore involve the selective control of gene expression.

Genetic information, determining the production of enzymes (Beadle and Tatum, 1941) is embodied in deoxyribonucleic acid (DNA) (Avery, MacLeod and McCarty, 1944). The linear sequence of nucleotide triplets in the DNA (Crick, 1963; Crick et al., 1961) is directly related to the linear sequence of amino acids in a protein (Yanofsky, Carlton, Guest, Helinski and Henning, 1964). The majority of genes in a eucaryotic cell are situated within the nucleus, separated from the sites of synthesis of proteins, the ribosomes (Zamecnik and Keller, 1954), which are present in the cytoplasm.

The physical separation of genes from the site of enzyme synthesis makes it necessary to postulate the presence in the cytoplasm of a transcript of the genetic message. When analysis of ribosomes showed that they consisted of ribonucleic acid (RNA)
and protein it was suggested that the genetic message was in the form of RNA. The existence of large numbers of ribosomes suggested the possibility that the RNA component of these particles specified the synthesis of many different proteins. With the demonstration of the uniformity of size and base composition of ribosomal RNA (rRNA) it became clear that these particular molecules did not contain sufficient information to account for the synthesis of more than a few proteins. Nevertheless the idea that genes were transcribed into a messenger RNA (mRNA) continued to receive support. Genetical and biochemical studies with bacteria lead to suggestions as to the properties of mRNA (Jacob and Monod, 1961). It was further suggested, on theoretical grounds, that other RNA molecules (transfer RNAs) might act as adaptors during the process of translating the nucleic acid sequences into a linear assembly of amino acids (Crick, 1958). Upon this framework an overwhelming amount of evidence was accumulated, much of it from in vitro studies, which clarified the role of rRNA, tRNA and mRNA in protein synthesis. Many important experiments, such as those leading to the cracking of the genetic code (Nirenberg and Matthaei, 1961) were performed with synthetic polyribonucleotides because natural mRNAs were not available. Most of the evidence supports the view that the genetic message is in the form of RNA (Watson, 1963). The reasons for the lack of detailed information about the properties of mRNA are discussed below. There is no good evidence to support the view (Pelc, 1958) that DNA acts as an intermediate in gene expression.

The regulation of gene expression

In the majority of cases the flow of genetic information
can be represented in the following way (Crick, 1970)

\[
\text{DNA} \quad \xrightarrow{(i)} \quad \text{RNA} \quad \xrightarrow{(ii)} \quad \text{PROTEIN}
\]

In principle this process can be controlled at the transcription stage (i) or the translation stage (ii). There is evidence that both types of control operate. In the case of the lac genes of \textit{Escherichia coli} the genetic operator model of negative transcriptional control by means of a reversible association of a repressor protein with a site near the start of the structural gene has recently been shown to be substantially correct by \textit{in vitro} studies (de Crombrugghe \textit{et al.}, 1971). Positive transcriptional control elements operating in association with RNA polymerase have also been described (Burgess \textit{et al.}, 1969). Genetic control was postulated to account for the rapid response of procaryotes to changes in the environment. A large number of experiments with plants and animals (Harris, 1968; Marcus, 1971) indicate that in some circumstances control of protein synthesis occurs at the cytoplasmic level rather than the genetic level and depends on translational control of stable mRNA. It is clear however that some mechanism must exist for the control of transcription in eucaryotes for some DNA, e.g. mouse satellite DNA, is never transcribed (Flamm, Walker and McCullum, 1969).

A further possible mechanism for the regulation of protein synthesis has emerged from the study of RNA synthesis in mammals. The observation that large RNA molecules are continually being synthesised in the nucleus but fail to enter the cytoplasm led Scherrer (Scherrer, Latham and Darnell, 1963) to propose a theory of post-transcriptional template selection by analogy with the processing of the precursor to rRNA (pre rRNA) described overleaf.
Recently studies of the transcription of the genes of SV40 virus integrated into host cell DNA have suggested that giant nuclear RNA may be a precursor to smaller RNA molecules that are found in the cytoplasm (Wall and Darnell, 1971).

**Characterisation of messenger RNA**

Detailed characterisation of a mRNA is a pre-requisite for studying the regulation of its synthesis, processing, transport and translation. In the original formulation of the genetic operator model (Jacob and Monod, 1961) it was suggested that mRNA would be rapidly labelled, have a DNA-like base composition, be found in association with ribosomes, be heterogeneous in size and metabolically unstable. These are general properties expected of a population of different mRNAs. They are not distinct criteria sufficient to characterise a mRNA and other kinds of RNA may share many of these properties. For example, rapidly labelled RNA would be expected to contain large numbers of growing chains of rRNA and tRNA and after short incubation times these molecules would be heterogeneous in size. Furthermore, certain individual mRNAs would be expected to have a base composition unlike that of the bulk of the DNA, as has been found for the presumptive mRNA for silk (Suzuki and Brown, 1972) and predicted for the mRNA for histones (Kedes and Birnstiel, 1971). Individual mRNAs would be expected to be homogeneous in size because of the colinearity of gene and gene product, and this has been shown to be the case with the silk and globin mRNAs, although globin mRNA contains two chains of slightly different length coding for the α and β chains of the protein.

If stable mRNAs are functioning in the cytoplasm then they would be expected to be associated with ribosomes or polysomes.
and this criterion will aid in their identification as messengers. However it is necessary to show that such RNAs are not fragments derived from rRNA. Cytoplasmic mRNAs may be stored in an inactive form and in this case there is no reason to expect that they are bound to ribosomes. Proof of the identity and function of a mRNA could come either from a knowledge of its base sequence and comparison with the amino acid sequence of the protein it codes for or by the demonstration that the RNA directs the synthesis of a specific identified protein.

The characterisation of mRNA is simplified by studying cells in which a large proportion of the total synthetic activity is devoted to the production of a single protein. There are perhaps a dozen cases where this strategy is likely to lead to the identification of mRNAs such as those for silk, lens protein and plant storage proteins. There is now good evidence that the mRNAs for both the α and β chains of globin can be isolated in a chemically pure form (Pemberton et al., 1972; Lingrel, 1972; Lockard and Lingrel, 1969).

Even in such special cases the positive identification of a mRNA represents a formidable task. In less specialised cells mRNA may be expected to be heterogeneous in size, base composition and stability. It is also present in very small amounts and meaningful studies on the regulation of its metabolism and function are therefore extremely difficult.

The case for studying ribosomal RNA metabolism

There are certain genes which function in all cells that have particular advantages for the study of transcriptional and post-transcriptional control. The products of these genes, rRNA, 5sRNA and tRNA, are involved in protein synthesis itself
and nearly all cells require the RNA products of these genes in large amounts. It has been calculated (Loening, 1970) that in order to manufacture ribosomes at the rate required for cell division, the cell must have multiple sites of synthesis of rRNA. This is achieved partly by having multiple gene copies in the nucleolus and partly by transcribing each gene at several different points simultaneously. The presence of multiple gene copies and their different base composition compared to the bulk of the DNA makes it possible to isolate the ribosomal genes in pure form. The homogeneity and abundance of the RNA products makes isolation and characterisation of these molecules comparatively easy. Furthermore, nucleoli contain a RNA polymerase distinct from other nuclear RNA polymerases (Roeder and Rutter, 1969) suggesting some specificity of the nucleolar enzyme which may have regulatory implications.

In addition to these operational advantages ribosome synthesis is of general interest because a study of ribosome assembly may further understanding of the structure and function of the ribosome. Also, the apparent requirement for new ribosomes during processes such as gamete formation (Siersma and Chiang, 1971) and responses to growth substances (Trewavas, 1968) suggests that the act of ribosome synthesis may be involved in cell regulatory processes.

**Ribosomal RNA structure and function**

Ribosomes are composed of two subunits of unequal size. Each subunit contains a backbone of RNA with a large number of ribosomal proteins associated with it. The rRNA certainly plays a structural role in the organisation of the ribosome. If it has other functions these remain to be discovered. The results
from early studies of rRNA suggested it consisted of a large number of short chains. With improved techniques for studying RNA, notably the inhibition of endogenous ribonucleases during RNA isolation, it became clear that most of the rRNA in the ribosome is present as two molecules, one for each ribosomal subunit. A low molecular weight RNA component, the 5s RNA from the larger subunit was discovered by Monier (Rosset, Monier and Julien, 1964). The physical properties of rRNA suggest that each molecule is a single polynucleotide chain with a high degree of internal base pairing (Cox, 1970).

Prokaryotic ribosomes from bacteria and blue-green algae sediment at about 70s. The ribosomes of chloroplasts are similar in this respect. The finding that the rRNAs from bacteria, blue-green algae and chloroplasts all have very similar molecular weights of 1.1 and $0.56 \times 10^6$ lends support to the view that chloroplasts may have evolved from captured blue-green algae (Loening and Ingle, 1967). The rRNAs from higher plant mitochondria have molecular weights of 1.2-1.3 and $0.7 \times 10^6$ and are present in an 80s ribosome (Harmey and Leaver, 1972). The ribosomes from the cytoplasm also sediment at about 80s. The rRNA from the larger subunit ranges in molecular weight from about $1.7 \times 10^6$ (28s) in mammals to $1.3 \times 10^6$ (26s) in plants. It has been suggested that the tendency for this molecule to be larger in the more recently evolved animals may be related to the development of more complex regulatory mechanisms during the evolution of the higher animals (Loening, 1968). In contrast to this it was also shown that the size of the smaller rRNA appears to have been conserved with a molecular weight of $0.7 \times 10^6$ (18s) in most eucaryotes.
There are many reports of the detection of other RNA molecules in ribosomal preparations. These are often derived from intact chains of rRNA in vivo or in vitro. Their physiological significance is not clear. The chloroplast 1.1 x 10^6 molecular weight rRNA has been shown to be unstable and breaks into a small number of discrete fragments (Ingle, 1968). Another example is the "28s-associated" or "7s RNA". This molecule is separated from the larger rRNA of animals (Pene, Knight and Darnell, 1968) and plants (Payne and Dyer, 1972) by treatments known to disrupt hydrogen bonding. It appears to be synthesised as an integral part of the pre rRNA and the rRNA of the larger subunit of the ribosome. The molecular integrity of the larger rRNA is lost during or soon after ribosome maturation although the 7s RNA remains hydrogen bonded to the larger fragment (Weinberg and Penman, 1970).

The organelle ribosomes are functionally different from those of the cell cytoplasm (Boulter, Ellis and Yarwood, 1971) and this is reflected in the differences in size of the rRNAs. Within one class of ribosomes however there is no evidence for variation in structure or sequence of the RNA from one organism which could have regulatory significance. The molecules of rRNA of one species are remarkably homogeneous in size and base composition. There is evidence from the examination of the products of nuclease digestion and from end group analysis that they may not all be identical. The significance of this evidence is difficult to evaluate in relation to the loss of integrity of rRNA after synthesis.

The genes for ribosomal RNA

The ribosomal genes can be titrated by DNA-RNA hybridisation
techniques, (Yankofsky and Spiegelman, 1963; Gillespie and Spiegelman, 1965). Genes for the larger and smaller rRNAs are present in equal amounts in the genome. Bacteria have about five or six genes for each rRNA (Birnstiel, Chipchase and Speirs, 1970). Animal mitochondria may have only one gene for each rRNA (Dawid, 1972; Aloni and Attardi, 1972). In bacteria the genes for the 1.1 and 0.56 x 10^6 molecular weight rRNAs are clustered together on the chromosome (Birnstiel, Chipchase and Speirs, 1970). All eucaryotes contain multiple gene copies. The estimates range from a few hundred to several thousand per genome. The higher plants in particular seem to have very large numbers of these genes. For example, chinese cabbage may have 3 x 10^4 (Birnstiel, Chipchase and Speirs, 1970). The wide variation in number between species probably reflects true biological variation.

A number of experiments suggest that in eucaryotes the genes are clustered in the nucleolus. Ritossa and Speigelman (1965) showed by DNA-RNA hybridisation that in mutants of Drosophila the amount of ribosomal DNA per cell was proportional to the number of nucleolar organisers. Ritossa also demonstrated (Ritossa et al., 1966) that "bobbed" mutants of Drosophila which mapped at the nucleolar organiser region had partial deletions of the ribosomal genes. Studies with a deletion mutant of Xenopus laevis showed a correlation between absence of the nucleolus and a failure to synthesise rRNA (Brown and Gurdon, 1964). This work was extended by Birnstiel (Birnstiel et al., 1966) who isolated pure ribosomal genes from normal Xenopus and showed them to be absent from anucleolate mutants. Isopycnic caesium chloride centrifugation of DNA from X. laevis oocytes demonstrated
that a single satellite DNA containing gene clusters homologous to both the 0.7 and 1.5 x 10^6 molecular weight rRNAs banded at a density of 1.724 g.cm^-3. Hybridisation of the rRNAs separately to the ribosomal DNA sheared to different sizes showed that the genes for the two rRNAs alternate on the nucleolar DNA (Birnstiel et al., 1968). The repeat unit of the DNA has a molecular weight of about 10^7. Approximately one half of the ribosomal DNA contains sequences not complementary to rRNA. Part of this "spacer" DNA is transcribed during the synthesis of the pre RNA which has a molecular weight of 2.6 x 10^6 (Loening, Jones and Birnstiel, 1969) but certain regions of the DNA appear never to be transcribed (Birnstiel et al., 1968). The non-transcribed regions vary in sequence quite dramatically between closely related species although the ribosomal cistrons themselves are very similar (Brown, Wensink and Jordan, 1972).

Miller and Beatty (1969) have isolated from amphibian nucleoli ribosomal genes in the act of being transcribed. Electron micrographs appear to confirm the presence of multiple genes and the simultaneous transcription of each repeating unit at a number of different points. The silent (non-transcribed) spacer DNA appears to be of variable length.

**Synthesis of ribosomal RNA in the Hela cell**

During a study of the properties of rapidly labelled RNA in Hela cells a distinct RNA component was detected above the background of polydisperse RNA sedimenting between 20s and 100s (Scherrrer and Darnell, 1962). This peak sedimented at 45s and could be detected in RNA extracts after five minutes of labelling cells with radioactive precursors of RNA. After 15 minutes the 45s RNA was more prominent and after 30 minutes a second component
of 32s was detected at about the same time as radioactive 18s RNA appeared. On the basis of experiments with actinomycin D Perry (1964, 1966) suggested that the 45s RNA was converted to the 32s RNA and that these two molecules were macromolecular precursors to rRNA. Perry also concluded that the site of synthesis of the pre rRNA was the nucleolus. This was confirmed by Penman and his colleagues (Penman, Smith and Holtzman, 1966; Holtzman, Smith and Penman, 1966) who showed that the 45s and 32s pre rRNAs were found in the nucleolus. Initially there was controversy concerning the true size of the 45s pre rRNA (see Loening, 1970, for a discussion of this point). Most workers now agree that it has a molecular weight between 4.1 and 4.5 x 10^6. The combined molecular weights of the mature rRNAs is 2.45 x 10^6. This discrepancy implies non-conservative processing of the pre rRNA.

In 1968 Willems et al. deduced from the 32P base composition analysis of the 45s pre rRNA that it contained non-rRNA sequences rich in guanine and cytosine. Analysis of the 45s and 32s pre rRNAs by Amaldi and Attardi (1969) showed that between 30 and 50 per cent of the 45s RNA was not rRNA and 30 per cent of the 32s RNA was unlike rRNA. Competitive hybridisation experiments showed that the 45s pre rRNA contained sequences for both the 18s and 28s rRNAs while the 32s pre rRNA contained only the 28s rRNA sequence (Jeanteur and Attardi, 1969). An alternative approach was used by Wagner, Penman and Ingram (1967) who carried out quantitative analysis of the alkali-stable 2-O-methyl dinucleotides of the pre rRNAs and compared their frequency with those from the mature rRNAs. The results support the conclusion that the 45s pre rRNA contains both rRNA sequences.
Figure 1.

The steps of processing of the rRNA precursor in the HeLa cell.

The rRNA regions are indicated in black and the excess RNA in white. "7"s RNA, which is associated with "28"s and with "32"s RNA has been omitted from the diagram. (Adapted from Weinberg and Penman 1970)
The 45s pre rRNA is methylated shortly after synthesis. About 1.5 per cent of the molecule is methylated and about 80 per cent of the methyl groups are present on the ribose (Vaughan, Soeiro, Warner and Darnell, 1967; Lane and Tamaoki, 1969). The excess RNA is unmethylated and is discarded during processing of the pre rRNA (Weinberg and Penman, 1970); an additional methyl group is added to 18s rRNA during processing (Zimmerman, 1968). The role of the methyl groups in ribosome structure and function is not understood.

Recent studies employing polyacrylamide gel electrophoresis have revealed other nucleolar RNAs in addition to the 45s and 32s molecules. These include a 41s and 20s RNA. The scheme shown in Figure 1 for the biogenesis of rRNA was proposed by Weinberg and Penman (1970) and relates specifically to the situation in the HeLa cell. The results of a comparison of the fingerprints of methyl-labelled nucleolar RNAs with those from rRNA confirms the role of the intermediates in the processing scheme (Maden, Salim and Summers, 1972).

Processing of the pre rRNA to smaller molecules occurs in the nucleolus and appears to involve both conservative and non-conservative steps. At the same time the RNA molecules become associated with proteins (Warner and Soeiro, 1967); some of these are ribosomal proteins but others are unique to precursor ribonucleoprotein particles. During ribosome maturation the 28s rRNA remains in the nucleolus for much longer than the 18s rRNA which is rapidly transported to the cytoplasm. The non-conserved RNA of the precursor has never been detected separately. There is little evidence to suggest a role for this RNA. It may serve to determine the secondary and tertiary structure of the rRNA during the addition of proteins and the
assembly of the ribosomal subunits. The amount of excess RNA in pre rRNAs from different organisms is too variable for it to be mRNA for ribosomal proteins (Loening, 1970). The fact that this RNA is not detected separately also argues against this hypothesis. It is difficult to exclude the possibility that part of the excess RNA serves as mRNA for a small number of proteins. The 5s RNA is not transcribed as part of the pre rRNA (Brown, 1968; Perry and Kelley, 1970).

Polycistronic transcription of the genes for rRNA has regulatory advantages, ensuring as it does that the 28s and 18s rRNAs are synthesised in equimolar amounts. The synthesis of rRNA in procaryotes is achieved with a variation of this mechanism. The feature in common between rRNA synthesis in procaryotes and eucaryotes is that the initial gene product is larger than that finally incorporated into ribosomes.

Ribosomal RNA synthesis in procaryotes

In bacteria a polycistronic precursor to rRNA is not produced. The initial transcription products of the rRNA genes have molecular weights of about 1.15 and 0.7 x 10^6 (Hecht and Woese, 1968). After a few minutes these are converted to the rRNAs of molecular weight 1.1 and 0.56 x 10^6. The pre rRNAs are undermethylated with respect to the mature rRNAs (Lowry and Dahlberg, 1971) where about 1.5 per cent of the molecule is methylated. In contrast to the situation in eucaryotes, about 80 per cent of the methyl groups in bacteria are present on the bases. Equimolar synthesis of the two rRNAs is achieved by virtue of the fact that the two types of cistrons are arranged alternately. After a single initiation event an RNA polymerase molecule transcribes a gene for both the 1.1 and 0.56 x 10^6
molecular weight rRNAs (Bremer and Berry, 1971; Kossman, Stamato and Pettijohn, 1971). Either cleavage of the transcription product occurs during synthesis or alternatively the inter-cistronic divide is not transcribed.

Recent studies with blue-green algae (Grierson and Smith, 1972) have shown that no polycistronic precursor is synthesised. Pulse-chase experiments suggest that a \( 1.2 \times 10^6 \) molecular weight RNA is rapidly converted to the \( 1.1 \times 10^6 \) molecular weight rRNA. RNAs with molecular weights of \( 0.8 \) and \( 0.7 \times 10^6 \) exist separately for a long time. The processing sequence appears to be \( 0.8 \ 0.7 \ 0.56 \).

**Ribosomal RNA synthesis in eucaryotes**

In every eucaryotic organism so far examined a high molecular weight pre rRNA of sufficient size to contain the sequences of both rRNAs had been detected (Perry et al., 1970; Grierson et al., 1970). In most cases there is no direct evidence that the pre rRNAs are polycistronic. It is very difficult to formally distinguish between synthesis of one polycistronic molecule and two separate pre rRNAs with similar molecular weights (see Tiollais, Galibert and Boiron, 1971). Even if it is assumed that all eucaryotic pre rRNAs are polycistronic it is quite clear that there are considerable differences in rRNA biosynthesis between species. The most recently evolved organisms have pre rRNAs with the largest molecular weights and it has been suggested that the pre rRNA has increased in length during the course of evolution (Perry et al., 1970; Grierson et al., 1970). There are exceptions however for closely related plant species may have pre rRNAs of different sizes. Where detailed evidence is available this shows
that the structures of the pre rRNAs and processing intermediates are different in man and rat (compare Weinberg and Penman, 1970 and Egawa, Choi and Busch, 1971).

In plants pre rRNAs have recently been identified on the basis of pulse-chase experiments and $^{32}$P base compositions (Leaver and Key, 1970; Rogers, Loening and Fraser, 1970; Grierson et al., 1970). There is no detailed information concerning the properties of these pre rRNAs or the processing stages. In situations where two precursors can be detected in one organism it is possible that these are synthesised separately (Leaver and Key, 1970).

Regulation of ribosomal RNA synthesis

The synthesis of rRNA could be controlled by regulating (i) the number of transcription sites available, (ii) the rate of chain initiation by RNA polymerase molecules, (iii) the rate of chain growth of the pre rRNA, (iv) the rate of processing of the pre rRNA or (v) the rate of transport of rRNA to the cytoplasm.

The number of DNA sites available for transcription is regulated in two ways in frogs. Amphibian oocytes preferentially synthesise large numbers of ribosomes. The normal complement of rRNA genes in a cell is insufficient to sustain this massive synthesis and the ribosomal genes are amplified about 100 times to provide sufficient sites for rRNA synthesis (Brown, 1966). Mature amphibian eggs which have accumulated a store of ribosomes do not synthesise further rRNA until after gastrulation. Crippa (1970) has isolated from such cells what appears to be a repressor protein. This molecule binds specifically to ribosomal DNA and when injected into eggs preferentially inhibits rRNA synthesis compared to soluble RNA synthesis. Studies with plants (Ingle and Sinclair, 1972)
suggest that gene amplification does not feature in the regulation of rRNA synthesis. There is no evidence concerning the operation of repressor proteins in the nucleolus of any eucaryote other than *Xenopus*.

Nucleoli do contain a RNA polymerase which differs in several respects from that of the nuclear sap (Roeder and Rutter, 1969). It seems likely that this enzyme is under separate physiological control and may serve as a regulatory site for the control of rRNA synthesis. At the present time however there is not much known about the molecular details of the initiation of rRNA synthesis or the factors affecting the rate of chain growth in nucleoli.

There is evidence that post-transcriptional events could regulate rRNA production. For example, methionine starvation leads to the formation of undermethylated pre rRNAs which are not processed to rRNA but accumulate (Vaughan *et al.*, 1967). Similar effects are produced by puromycin although in this case accumulation of the pre rRNA is not due to formation of a defective RNA (Soeiro *et al.*, 1968). Presumably protein molecules, perhaps ribosomal proteins, become limiting. As one might expect cycloheximide has a similar effect. Synthesis and processing of the pre rRNA is decreased and in particular the 45s to 32s transformation is affected before synthesis is reduced (Willems, Penman and Penman, 1969). In systems which are not rapidly synthesising rRNA natural "wastage" of newly labelled pre rRNA may occur so that not all the rRNA sequences reach the cytoplasm (Cooper, 1969; Papaconstantinou and Julka, 1968; Fantoni and Bordin, 1971).
MATERIALS AND METHODS
Growth of plants

Seeds of *Phaseolus aureus* were bought from Valvona and Crolla, Leith Walk, Edinburgh and stored at 0°C until required. Unless otherwise stated seedlings were germinated and grown under sterile conditions. Seeds were rinsed for five to ten minutes in methylated spirits or 95 per cent ethanol, followed by five to ten minutes in a solution of sodium hypochlorite made by diluting a stock solution of 43 per cent sodium hypochlorite 1:10 with distilled water. Seeds were finally rinsed three times with sterile distilled water and planted in growth tubes inside a room sterilised by ultra-violet light, or under a "Pathfinder" hood.

The growth tubes were glass "pyrex" tubes 20 cm long and 3.5 cm internal diameter. These were filled to a height of about 5 cm with moist sand (three parts sand to one part distilled water by volume) and plugged with cotton wool. Sterilisation of the tubes was by autoclaving for 15 minutes at a pressure of 15 p.s.i. Germination of seeds was found to be more rapid and more uniform if there were air spaces in the sand. This was achieved by causing the water in the sand to boil after autoclaving by rapidly releasing the steam from the autoclave.

Between four and ten plants were grown in each tube, depending on the intended length of the growth period. During growth for up to ten days plants required no additional water. In some experiments growth tubes with side arms plugged with "suba-seal" rubber caps were used. This allowed the injection of solutions of radiochemicals through the rubber caps directly into the sand without opening the tubes.
Figure 2.

Absence of procaryotic rRNA from leaves of plants grown with aseptic techniques.

Plants were grown in darkness and labelled from 28-60 hours after germination with $^{32}$P orthophosphate. RNA was extracted and fractionated on 2% gels. Electrophoresis was for 3 hours.

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$^{32}$P

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Absorbance 265 nm.
Figure 3.

Emission spectrum of "warm white" fluorescent tubes used in growth rooms, measured at the plant level.
Sterility

The need to ensure tissue sterility in studies of nucleic acid metabolism using radioisotopes has recently been emphasised (Lonberg-Holm, 1967). Bacterial rRNAs can readily be distinguished from plant cytoplasmic rRNAs by their different mobilities on polyacrylamide gels (Loening and Ingle, 1967). Radioactive nucleic acid preparations from *P. aureus* grown under the conditions described here contained no detectable rRNA of bacterial size either in short term or in long term labelling experiments (Figure 2). An exception was found only in leaves known to be accumulating chloroplast rRNA which is similar in size to that of bacteria (Loening and Ingle, 1967). The peculiar properties of this chloroplast rRNA (described later) readily distinguish it from that of bacteria.

Light sources

Dale (1968) has shown that the ratio of light/dark period has little effect on the rate of RNA synthesis, protein synthesis, cell division and growth in primary leaves of *P. vulgaris*. In these experiments plants were routinely grown in cycles of 12 hours dark, 12 hours light, or in complete darkness at 22°-23°C. In the experiments where the initial effect of light on RNA metabolism was studied, the precise details of the time of transfer from dark to light and the length of the period of illumination are stated where the experiments are described. The light source was 100 watt "warm white" fluorescent tubes. The emission spectrum measured with a spectroradiometer (Agricultural Specialities Corporation, Beltsville, Maryland, U.S.A.) is shown in Figure 3. The incident energy at the plant level was 400 foot candles approximately (measured with a
Figure 4.

Transmission spectrum of green safelight.
photometer: Evans Electroselenium Ltd., Halstead, Essex). When radiochemicals were applied to dark grown plants manipulations were performed under a green safelight. This was a Kodak "beehive" safelamp with a 25 watt tungsten bulb fitted with an Ilford gelatin "Spectrum Green" (604) filter held between two glass plates. The transmission spectrum of the safelight (shown in Figure 4) is within the range of photomorphogenically inactive light (Withrow and Price, 1957). Exposure of plants of P. vulgaris to 15 minutes illumination with a similar safelight has no detectable photomorphogenic effect (Dale, pers. comm.).

Radiochemicals

All radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks. $^{32}$P orthophosphate, specific activity 20-40 Ci per mg P, was dissolved in dilute hydrochloric acid. $^3$H (methyl) labelled methionine, specific activity 5.4 Ci per mM, was dissolved in water. $^3$H uridine, uniformly labelled and 5'-labelled, specific activity 25 Ci per mM, was dissolved in water. All isotopes were stored at 0-4°C. Aliquots were removed with a sterile syringe as required. Hydrochloric acid was removed from $^{32}$P orthophosphate by routinely evaporating the solution to dryness before use and dissolving the orthophosphate in sterile distilled water. This was done only with other isotopes if they were required at higher concentrations than supplied.

Labelling of plants

Unless otherwise stated all labelling experiments were performed with intact plants. Leaves were normally labelled by direct application of radiochemicals, dissolved in a small volume of distilled water, to the leaf epidermis, using a micro-pipette.
Legend for Figure 5.

Leaves of plants grown for four days in the light were labelled by application of isotope directly to the leaves (a, b, d, e) or to the roots (c).

There were twelve leaves per treatment.

a) 80μCi ³²P dissolved in 25μl water applied to each leaf.

b) 80μCi ³²P dissolved in 25 μl 1% glycerol, 1·5% dimethyl sulphoxide, 0·5mM citrate pH4 applied to each leaf.

c) 2mCi ³²P was injected into the sand surrounding the roots.

d) 100μCi ³H uridine dissolved in 25μl water applied to each leaf.

e) 80μCi ³²P dissolved in 25μl 1% glycerol, 1·5% dimethyl sulphoxide, 0·5% tween, 0·5mM citrate pH4 applied to each leaf.

Labelling was for three (a, b, d, e) or four (c) hours. Electrophoresis was for 3 hours in 2·2% gels.

Radioactivity

Absorbance 265 nm.
Figure 5. Comparison of different methods of labelling leaf nucleic acids. (Legend opposite)
Tween and glycerol were used to encourage the solution to spread more evenly over the leaf surface and low pH and dimethyl sulphoxide to increase uptake of the isotope by the tissues. These agents were found to be unnecessary (Figure 5). With care and practice radiochemical solutions could be applied evenly to the leaf epidermis to give quantitatively reproducible patterns of incorporation. Injection of $^{32}$P orthophosphate into the sand also labelled leaves efficiently but it was more difficult to reproduce comparable pulse times between experiments and this procedure was not normally used.

Detached leaves floated on solutions of radioisotopes did continue to synthesise RNA judged by the incorporation of $^{32}$P orthophosphate and $^3$H uridine. However it was noticeable that synthesis and processing of the precursor to ribosomal RNA (pre rRNA) was slowed down. Moreover, the extent of this inhibition varied with the age and physiological state of the leaves.

Roots and hypocotyls were labelled either by direct injection of radioisotope into the sand or by removing seedlings from the sand and incubating intact seedlings in radioactive solutions. In a few experiments excised roots, 1.0-1.5 cm long, and hypocotyls, 2.5 cm long, were used.

**Extraction of nucleic acids**

A modification of the methods described by Kirby (1965) was used for nucleic acid extraction. Leaves and roots were ground with an ice chilled mortar and pestle or with a teflon in glass hand-driven homogeniser in 5-10 volumes of ice cold reagent: 1 per cent triisopropynaphthalene sulphonate, 6 per cent 4-aminosalicylate, 1 per cent sodium chloride plus 6 ml phenol mixture. An equal volume of phenol mixture (500 g phenol, 70 ml m-cresol,
10 g 8-hydroxyquinoline, saturated with water, kept at room temperature) was added and the phases emulsified by shaking and/or stirring for a short time. The mixture was centrifuged at about 3,000 x g for ten minutes at 4°C and the phenol layer was then removed with a Pasteur pipette. The aqueous phase and interfacial precipitate were then further deproteinised by a second phenol treatment after the addition of 0.15 volumes of 3M sodium chloride to drive protein into the phenol layer. After shaking and centrifuging the mixture as before, nucleic acids were precipitated from the aqueous phase at 0-4°C overnight by the addition of 2-2.5 volumes of ethanol. Occasionally shorter precipitation times were used. However this can result in selective loss of soluble RNA. In one experiment, for example, more than 80 per cent of the soluble RNA was left in solution after one hour, although more than 95 per cent of the rRNA was precipitated.

The resulting precipitate was collected by centrifugation and further purified by dissolving in 0.15M sodium acetate containing 0.5 per cent sodium dodecyl sulphate and adjusted to pH 6 with acetic acid, at a concentration of less than 500 μg per ml and reprecipitating with 2 volumes of ethanol at -20°C for at least two hours. This removes detergents and phenol. Selective loss of soluble RNA was not observed presumably due to the combination of higher RNA concentration with lower pH and lower temperature.

In some experiments where quantitative recoveries of nucleic acids from homogenised tissues was required the interfacial precipitate left after the second phenol deproteinisation was re-extracted at 4°C by shaking with 2 ml homogenisation medium
Legend for Figure 6.

Nucleic acids were extracted from leaves of 44 hour old plants in the cold. The phenol residue and interfacial precipitate were then re-extracted with 1 ml of medium at 55°C for two minutes. After precipitation and purification aliquots of control RNA (a) and hot phenol extracted RNA (b) were subjected to electrophoresis for 3½ hours in 2·2% gels.
Figure 6.

Formation of aggregates of RNA during hot phenol extractions. (Legend opposite)
plus 0.3 ml 3M sodium chloride. The mixture was centrifuged as before and the supernatant added to the first and nucleic acids precipitated as described.

The efficiency of the extraction of RNA and DNA by this method was investigated by re-extracting the interfacial precipitate with homogenising medium plus sodium chloride at 60°C. Less than five per cent of the total nucleic acid was extracted by this method. There was no significant enrichment of radioactive pre rRNA over cytoplasmic rRNA, although the hot phenol extracted RNA did contain significantly more radioactive polydisperse RNA. The ratio of DNA to cytoplasmic rRNA was increased also but this was noticeable only with young leaves up to two days old. Nucleic acid extraction at elevated temperatures did cause aggregation of rRNA however (Figure 6). For this reason both DNA and RNA were normally prepared by the cold extraction procedure. When RNA was extracted in the presence of magnesium 5 mM magnesium acetate was added to all reagents except the phenol mixture.

Deoxyribonuclease treatment

RNA molecules are fractionated on polyacrylamide gels by molecular weight. DNA is not fractionated in the same way and runs as a homogeneous component which on 2.4 per cent and 2.6 per cent gels has a mobility similar to that of the pre rRNA. In more dilute gels the pre rRNA runs ahead of the DNA in the normal E buffer but in low salt and magnesium buffer the DNA obscures the pre rRNA. It is necessary in many cases therefore to remove DNA from nucleic acid extracts prior to gel fractionation in order to analyse the pre rRNA region. The most effective method of quantitatively removing all the DNA is by treatment with
Deoxyribonuclease.

Stock solutions of the enzyme (Sigma electrophoretically purified) were stored at -20°C at a concentration of 250 or 500 μg per ml dissolved in 0.05M 2-(Nmorpholino)-ethane-sulphonic acid adjusted to pH 7 containing 0.002M magnesium acetate and 10 per cent dimethylsulphoxide.

Total nucleic acid preparations were collected by centrifugation, resuspended in 75 per cent ethanol containing 0.1M sodium chloride to remove sodium dodecyl sulphate and recentrifuged at 0°C. Alcohol was removed by briefly draining the pellet and the nucleic acid was dissolved in sterile buffer, taking care to leave no undissolved nucleic acid on the side of the tube, at a concentration less than 500 μg per ml.

Deoxyribonuclease from the stock solution was then added to a final concentration of 10-20 μg per ml. Digestion was at 0°C for about 10 minutes. At the end of this time an equal volume of sodium acetate/sodium dodecyl sulphate solution was added and the RNA precipitated with 2 volumes of ethanol at -20°C for at least 1 ½ hours.

This treatment completely removes DNA from its characteristic position on the gel and results in low molecular weight fragments of DNA which migrate in the region of soluble RNA.

Purification of DNA

A modification of the procedure described by Marmur (1961) was used. Total nucleic acid was dissolved in 1 x SSC (0.15M sodium chloride, 0.015M tri-sodium citrate) at a concentration of about 2 mg per ml. Ribonuclease was added to a final concentration of 30-50 μg per ml from a stock solution of 500μg per ml in 1 x SSC previously heated to 80°C for 10 minutes and
stored at -20°C. Ribonuclease digestion was for 20-30 minutes at room temperature. The solution was then deproteinised two or three times with phenol mixture. The nucleic acids were then precipitated from the supernatant at 0-4°C for 2-4 hours by the addition of 2 volumes of ethanol. The precipitate, containing DNA and oligoribonucleotides was collected by centrifugation and dissolved in 0.1 x SSC. The salt concentration was then increased by the addition of 0.1 volumes of 3M sodium acetate, pH 7, containing 0.001M ethylene diamine tetraacetate. DNA was selectively precipitated by layering 0.55 volumes of isopropanol above the solution and swirling the tube by hand to slowly mix the contents. The DNA precipitated at the interface between the alcohol and aqueous layers as the solutions mixed and the DNA became associated in a single fibrous mass. This was removed from the solution with forceps, dissolved and reprecipitated with isopropanol as before. The precipitate was drained of alcohol, dissolved in 0.1 x SSC and stored frozen or precipitated with ethanol at -20°C.

**Polyacrylamide gel electrophoresis**

The methods of Loening (1967, 1969) were used with slight modifications. All solutions, except the buffers in the electrophoresis tanks, were milli-pore filtered, particularly when high resolution of ultra-violet (u.v.) scanning was required. Acrylamide and bis acrylamide (obtained from Kodak) were recrystallised from acetone and chloroform respectively before use and stored as a stock solution at 0°C.

- acrylamide 15 g
- bis acrylamide 0.75 g
- water to 100 ml
This solution was used for gels 2.2-5.0 per cent acrylamide. Half the amount of bis acrylamide was used for gels 5-7.5 per cent in acrylamide. Ammonium persulphate solution was made up fresh each time. Old ammonium persulphate crystals were not used successfully.

Three gel buffers were used:

1. **E buffer**
   - 180 mM Tris \(\text{(tris(hydroxymethyl)amino methane, Sigma "Trizma")}\)
   - 150 mM Sodium dihydrogen orthophosphate
   - 5 mM Disodium ethylenediamine tetra-acetate
   - pH 7.6-7.8 at room temperature

2. **Low salt buffer**
   - 150 mM Tris
   - 80 mM Hydrochloric acid
   - 0.1 mM Disodium ethylenediamine tetra-acetate
   - pH 8.0 at room temperature

3. **Mg buffer**
   - as 2. but with the addition of 10 mM magnesium acetate

5 ml of stock acrylamide solution were mixed with the appropriate volume of distilled water and gel buffer and degassed under vacuum until the water started to boil. 25 \(\mu\)l of NNNN tetramethyl ethylene diamine was added, followed by 250 \(\mu\)l of 10 per cent ammonium persulphate. After mixing, the solution was pipetted into the gel tubes which were held vertically in a rack. For concentrated gels the solution was pre-cooled in ice before addition of catalysts in order to prevent polymerisation during pipetting.
Gel concentration (%acrylamide) 2.2 2.4 2.6 5.0 7.5
Volume of buffer (ml) 6.8 6.25 5.8 3.0 2.0
Volume of water (ml) 22.0 19.7 17.8 6.7 2.7

Flat tops to the gels were obtained by carefully layering distilled water over the acrylamide solution immediately after it was pipetted into the gel tubes. After ten minutes the top of the gel showed three layers by visual inspection. These were a layer of water and a layer of polymerising acrylamide separated by a layer of unpolymerised acrylamide. If left longer, the layer of unpolymerised acrylamide set; the polymerisation process is presumably retarded by oxygen diffusing from the water. The slowly polymerising layer could be removed by gently splashing water onto the top of the gel after ten minutes. Perfectly flat gel tops were obtained by this method. Gels were allowed to polymerise for at least two hours before use.

Two sizes of perspex gel tubes were used. Tubes 8 cm long by 0.6 cm internal diameter were fitted with a short plastic cylinder at the bottom which was sealed with a short piece of glass rod while gels were polymerising. Before electrophoresis the glass rod was removed. The plastic washer retained all gels except 2.2 per cent. For these a small piece of filter paper was inserted through the hole in the washer and was used to support the gel.

Tubes 9 cm long by 0.9 cm internal diameter were sealed at the bottom with parafilm or "suba-seal" rubber caps while gels were polymerising. Gels were retained in the tubes during electrophoresis by either a disc of porous polythene inserted into the bottom of the tube, or by a piece of muslin stretched
Figure 7.

Gel electrophoresis tank
over the bottom of the tube and held in place with a rubber band.

The electrophoresis tanks were constructed from perspex, with platinum electrodes mounted separately (Figure 7). Gels were held vertically by rubber grommets in the floor of the upper electrophoresis compartment. The lower and upper compartments of the tank were completely filled with electrophoresis buffer. Gels were run at 50 volts and about 1 milliamp per cm. Sodium dodecyl sulphate was allowed to enter the gel, and catalysts were removed by electrophoresis for about 30 minutes, before RNA was layered on to the gels. Electrophoresis of RNA was for 2-8 hours at room temperature (18-21°C).

The buffers used for electrophoresis were of the same composition as the gel buffers, but diluted five-fold with the addition of 0.2 per cent (buffer 1) or 0.1 per cent (buffers 2 and 3) sodium dodecyl sulphate.

A few experiments were performed with urea gels. These were essentially E buffer gels with 8M urea final concentration in the gels and the electrophoresis buffer.

RNA purified as described was collected by centrifugation, partially dried in vacuo to remove alcohol and dissolved in electrophoresis buffer containing six per cent sucrose ready for layering on gels. The drying step can cause aggregation of rRNA and in place of this, the RNA pellet was drained for two minutes and dissolved in electrophoresis buffer containing 20 per cent sucrose in order to increase the density sufficiently for it to sink to the gel surface. Aggregation of RNA can also occur while it is in solution prior to loading on gels. This is particularly true with buffers containing magnesium, and depends on temperature and RNA concentration. RNA was normally
dissolved at a concentration of about 1 mg per ml for electrophoresis and a sample layered on gels as soon as possible. Excess RNA dissolved in electrophoresis buffer but not loaded on gels was diluted with sodium dodecyl sulphate, sodium acetate solution and reprecipitated with 2 volumes of alcohol at 0-4°C.

Between 10 µl and 100 µl of solution containing 5-200 µg RNA were fractionated per gel. Gels were loaded with the current flowing. After electrophoresis the gels were pushed from the tubes with gentle water pressure from the bottom, placed in distilled water or fresh electrophoresis buffer and washed for 0-4 hours before optical scanning.

Optical scanning

Gels were held in a parallel sided quartz cuvette for U.V. scanning. Transfer of the gels was effected with a pipette of perspex similar to that in which the gels were cast, operated by a 10 ml syringe.

Two instruments for U.V. scanning were used. The Joyce Loebl "Chromoscan" fitted with a mercury vapour lamp, interference filter and liquid filter of para dimethyl amino benzaldehyde (15 mg per 100 ml in methanol) scanned at 265 nm. Four full scale deflection values of 2.0, 1.0, 0.5 and 0.33 optical density were available. The highest of these gave a non-linear response. The recording of the gel scan was three times the actual length of the gel.

The second instrument was the Joyce Loebl "Polyfrac". This had a solid para dimethylaminobenzaldehyde filter, mercury vapour lamp, interference filter and U.V. photocell and also operated at 265 nm. Full scale deflection values of from 0.01 (the highest practicable sensitivity) to 5.0 O.D. were available
using a Servoscribe pen recorder. The response was linear up to at least 3 O.D. By a slight modification of the pen recorder drive, recorded scans of x 2, x 2\(\frac{1}{2}\) and x 4 the length of the gel were available.

U.V. absorbing materials present at the top of the gels after electrophoresis presented little problem. They could be largely avoided by using fresh gels and fresh ammonium persulphate. Before scanning gels with a sensitivity of 0.01 O.D. full-scale deflection it was sometimes necessary to wash the gels in distilled water for one or two hours to remove interfering materials. It was necessary to keep gels absolutely clean for successful high resolution scanning.

**Location and measurement of radioactivity in gels**

After U.V. scanning, gels were placed in aluminium troughs and frozen at the same length as when scanned by placing the troughs horizontally on powdered solid carbon dioxide. Longitudinal expansion of the gel during freezing was prevented by placing stoppers of expanded polystyrene at each end of the gel. Frozen gels were removed from the troughs, frozen to the platform of a Mickle gel slicer and chopped into slices 0.5 mm or 1 mm thick.

If gels were not frozen in an absolutely horizontal position they were compressed slightly at one end and stretched at the other end. This resulted in the U.V. and radioactivity profiles being out of phase (see, for example, Figure 5b). This was avoided by taking care in freezing gels. Occasionally ink marks placed in the gel with a syringe were used to synchronise the absorbance and radioactivity profiles.
Counting radioactivity

Several methods were used for counting $^{32}\text{P}$. The method most frequently used was to place gel slices on alternate frames of 16 mm cine film. The slices were dried and counted by automatically feeding the film past the window of a low background geiger tube shielded in a lead castle. The geiger tube was coupled to a Scaler (Isotope Developments Ltd.) linked with a print-out unit and an automatic sample changing mechanism. Alternatively gel slices were dried on filter paper and counted either in a toluene scintillation mixture (a or b described below) in a Packard liquid scintillation counter, or by sticking the paper to planchettes and counting in a Beckman "Lowbeta" gas flow counter.

For preparative procedures wet slices were counted by Cerenkov counting in a Packard scintillation counter. Slices were placed in polythene tubes inside glass vials, or directly in glass vials with or without additional water. Slices containing $^{3}\text{H}$ and $^{14}\text{C}$ alone, and with $^{32}\text{P}$ were hydrolysed in glass vials with 1 ml 10 per cent piperidine in water containing 1 mM ethylene diamine tetra acetate for four hours at 60°C. The slices were dried at 60°C and swollen by the addition of 0.4 ml water for one hour. 10 ml of scintillation fluid were added and the vials counted in a Packard liquid scintillation counter.

Scintillation mixtures

Non-aqueous

(a) 1,000 ml Toluene

$\frac{4}{g\text{m}}$ 2,5-diphenyloxazole

50 mg 1,4-di-2-(5-phenyloxazolyl)-benzene
(b) 1,000 ml Toluene

5 gm 2-(4‘tert-butyl phenyl)-5(4”-biphenylyl)-1,3,4-oxadiazole

Aqueous

(c) 600 ml Toluene

400 ml Methoxyethanol

5 gm 2,5-diphenyloxazole

0.1 gm 1,4-di-2-(5-phenyloxazolyl)-benzene

(d) 600 ml Toluene

400 ml Methoxyethanol

5 gm 2-(4‘tert-butyl phenyl)-5(4”-biphenylyl)-1,3,4-oxadiazole

32p base compositions

Radioactive RNA components located in gel slices by Cerenkov counting or by counting on cine film was hydrolysed from the slice by incubating it directly in 1-2 ml of:

10% Piperidine

1 mM Disodium ethylene diamine tetra acetate

0.25 mg per ml yeast RNA

for 48 hours at 60°C. The supernatant was removed from the slice and evaporated at 60°C. The mononucleotides were dissolved in a few drops of buffer:

Acetic acid 7.5%

Pyridine 0.75%

pH 3.5

which was then applied as a streak 1 cm long to Whatman 3 mm paper. The paper was moistened with buffer in such a way as to sharpen the bands and placed vertically inside a glass tank filled with white spirit. Each end of the paper dipped into a
Figure 8.

Recovery of rRNA from polyacrylamide gel slices.

RNA labelled with $^3$H uridine was fractionated in 2-4% gels for 3 hours. The RNA peaks were located by u.v. scanning and marked by injecting indiarn ink into the gel. The gel was chopped into 0.5 mm slices and two slices containing 1.3 or 0.7 x 10$^6$ molecular weight rRNA were placed in glass vials containing 1 ml 0.6 M lithium acetate, 0.5% sodium dodecyl sulphate. Elution was at 30°C with shaking. The eluate was removed at regular intervals and replaced with fresh solution. The amount of RNA recovered was determined by aqueous scintillation counting. The amount of RNA left in the slices after 5-5 hours was determined by alkaline hydrolysis of the slices followed by aqueous scintillation counting. The results are expressed as per cent RNA recovered. Each point is the average of two determinations.
trough of buffer connected to a power supply. Electrophoresis was at 60-100 milliamps and 1200-2500 volts for 1½-2 hours depending on the dimensions of the paper.

After electrophoresis the paper was air dried, normally overnight, and the separated nucleotides visualised under a U.V. lamp. The spots were cut out, together with blanks, and were counted in toluene scintillator in a Packard scintillation counter, or stuck to planchettes and counted in a Beckman Lowbeta counter. At least 2,000 counts above background were collected for each sample. The areas between spots were routinely counted as controls.

Base compositions are calculated as the amount of radioactivity present in any single mononucleotide as a percentage of the total radioactivity present as mononucleotides.

**Elution of RNA from gels**

RNA fractions located by ink marks placed in the gel during scanning or by Cerenkov counting after chopping were eluted into various solutions and recovered by centrifugation or by precipitation with alcohol.

The kinetics of elution of the 1.3 and 0.7 x 10⁶ molecular weight rRNAs into 0.6M lithium acetate containing 0.4 per cent sodium dodecyl sulphate are shown in Figure 8. Almost all the RNA can be eluted from the gel slices. In later experiments it was found that it was unnecessary to wash slices with fresh solution several times and that over 90 per cent of the RNA was in solution after simply washing gel slices, with shaking, overnight. The supernatant was removed from the gel slices, millipore filtered and centrifuged at 40,000 rpm in a 10 x 10 ml MSE fixed angle rotor at 16°C overnight. The recovery of
Legend for Figure 9.

RNA fractions separated on a 2·6% gel were recovered by elution with 0·6 M lithium acetate containing 0·5% sodium dodecyl sulphate for six hours at room temperature, followed by centrifugation overnight at 40,000 rpm in an M.S.E. 10 x 10 ml angle rotor at 15°C. The RNA pellets were drained, dissolved in electrophoresis buffer containing sucrose and electrophoresed for three hours in 2·4% gels.

a) 0·56 x 10^6 molecular weight rRNA.
b) 0·9 x 10^6 molecular weight rRNA.
c) 0·7 x 10^6 molecular weight rRNA.
d) 1·3 x 10^6 molecular weight rRNA.
e) 1·1 x 10^6 molecular weight rRNA.
f) marker RNA.
Figure 9.

Re-electrophoresis of RNA fractions recovered from gels. (Legend opposite)
<table>
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<th>Elutant</th>
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<th>0.7</th>
<th>5s × 4s</th>
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<td>89.8 (2)</td>
<td>92.3 (2)</td>
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<td></td>
<td>13.0 (7)</td>
<td>8.4 (7)</td>
<td>6.6 (7)</td>
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<tr>
<td></td>
<td>(5.0)</td>
<td>(1.8)</td>
<td>(1.1)</td>
</tr>
<tr>
<td>3x 0.5 M S.S.C.</td>
<td>81.3 (2)</td>
<td>92.4 (2)</td>
<td>92.9 (2)</td>
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<tr>
<td>0.2 M SDS</td>
<td>13.3 (7)</td>
<td>6.5 (7)</td>
<td>6.0 (7)</td>
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<tr>
<td></td>
<td>(5.4)</td>
<td>(1.1)</td>
<td>(1.1)</td>
</tr>
<tr>
<td>6x 0.5 M S.S.C.</td>
<td>80.7 (2)</td>
<td>91.8 (2)</td>
<td>92.5 (2)</td>
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<tr>
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<td>(1.5)</td>
<td>(1.5)</td>
</tr>
</tbody>
</table>

Table 1. Comparison of the elution of RNA from gel slices using different solutions

(2) RNA eluted after 2 hours
(7) RNA eluted after a further 7 hours

Figures in brackets refer to RNA remaining in the gel slices after 9 hours.

Each vial contained 2 x 0.5 ml gel slices (2.4 ml gel) for each ml of solution. The gel slices contained 32P labelled RNA. Vials were shaken at 30°C. After 2 hours the supernatant was withdrawn and the radioactive RNA eluted was measured by Cerenkov counting. Fresh elution was placed in each vial and the slices washed for a further 7 hours. The amount of RNA eluted after this time was determined together with the amount of RNA remaining in the slices.

The results are expressed as a percentage of the total Cerenkov radiation present at the start of the elution.
radioactive RNA as a pellet varied in several experiments from 92 to 97 per cent. RNAs recovered by this method are intact as judged by their behaviour on electrophoresis (Figure 9). In several experiments between 90 and 97 per cent of the radioactive RNA eluted from gel slices was recovered by centrifugation.

An alternative method used was to elute RNA from gel slices into 3 x SSC containing 0.2 per cent sodium dodecyl sulphate. Table 1 compares the effect of sodium dodecyl sulphate and two salt concentrations on the elution of RNA. Sodium dodecyl sulphate has no effect on elution kinetics and RNA recovered in its absence was not broken down. However it was routinely added as a precaution against ribonuclease. The RNA recovered by this method was millipore filtered and precipitated with two volumes of alcohol overnight at -20°C. In several experiments between 92 and 98 per cent of the eluted radioactivity was precipitated. Re-electrophoresis of the RNA showed it to be intact. With this method acrylamide and sodium citrate are precipitated together with the RNA. These did not interfere with hybridisation or re-electrophoresis of the RNA. However 2 x SSC was routinely used in order to minimise the salt problem.

Alcohol precipitation was used particularly for preparing pre rRNA for hybridisation. It was found that very small amounts of RNA (less than 0.01 μg per ml) can be precipitated from solution. The polyacrylamide that washes out of the gel slices with the RNA may aid in the precipitation. Alcohol precipitation of RNA from lithium acetate makes it difficult to re-dissolve the RNA at high concentrations due to the presence of lithium in the pellet.
Quantitative estimation of RNA components

The total RNA present in an extract was calculated by measuring the absorbance (260-315 nm) in a 1 cm light path of a suitable aliquot dissolved in 1 ml of SSC. One optical density unit was assumed to be equivalent to 40 μg of RNA.

Samples of 10-20 μg of RNA were fractionated on gels retaining the soluble RNA peaks and the peak areas determined by drawing the curves on tracing paper, cutting out the peaks and weighing them. The proportion of a particular RNA component expressed as a percentage of the total RNA was assumed to be directly related to the area of the RNA peak expressed as a percentage of the total peak areas. This method ignores the contribution of polydisperse RNA to the absorbance of total RNA at 260 nm, and does not take account of possible differences in secondary structure of individual RNAs which would affect the absorbance of these molecules at 260 nm.

The amount of radioactivity in a particular component was calculated by adding the counts in each gel slice in the peak and subtracting a baseline value to take into account the background counts and the underlying polydisperse RNA.

Molecular weight determinations

The mobility of an RNA component on polyacrylamide gels is inversely related to the log molecular weight (Loening, 1968). This relationship is in most cases independent of gel concentration. Effects due to base composition can be detected but these are small (Loening, 1969). The relationship does not hold for molecules with very long double stranded regions, i.e. DNA, and possibly TMV RNA (Loening, 1969) and the RNA of the killer particle of Saccharomyces cerevisiae (Berry; Herring, pers.
Figure 10.

The mobility of RNA fractions on polyacrilamide gels as a function of time.

RNA labelled with $^{32}$P for two hours was subjected to electrophoresis for 1-4½ hours in 2-2% gels. The gels were scanned, chopped and counted to locate the pre tRNA and the rRNAs. Mobility of the RNA fractions is plotted as a function of time.

- $\Delta$ $0.7 \times 10^6$ molecular weight rRNA,
- $\blacksquare$ $1.3 \times 10^6$ molecular weight rRNA,
- $\bullet$ $2.9 \times 10^6$ molecular weight pre tRNA.
Figure 11.

Molecular weight determinations of leaf rRNAs.

a) Nucleic acid from 4 day old leaves grown in the light.
b) Leaf nucleic acid plus E.coli nucleic acid.

Electrophoresis was for four hours in 2-6% gels. A molecular weight scale is indicated.
Another exception is mitochondrial RNA of *Xenopus* (Dawid and Chase, 1972) and *Aspergillus* (Edelman, Verma and Littauer, 1970; Verma, Edelman, Hertzberg and Littauer, 1970). The very low content of guanine and cytosine may be responsible for an unusually high degree of unfolding of these molecules. Relative mobility is strongly temperature dependent (Groot, Aij and Borst, 1970) which supports this conclusion.

The relationship does hold providing molecules of similar structure and composition are compared. Unusual molecules such as the mitochondrial rRNAs can be detected by their anomalous behaviour in relation to salt concentration and temperature.

One parameter that may vary is mobility with time. This is important when determining the molecular weight of the pre rRNA by extrapolation. The effective concentration of the top of the gel may be lower than in the middle. There may also be differential heating effects in different parts of the gel, leading to local changes in mobility. Figure 10 shows however that no obvious effect that could interfere with molecular weight estimation by extrapolation can be detected with gels cast as described above. The mobilities of the rRNAs and pre rRNA are linear with respect to time.

The molecular weights of the cytoplasmic rRNAs were calculated as $1.3 \times 10^6$ and $0.7 \times 10^6$ by comparison with *Eschericia coli* rRNAs, molecular weights $1.1$ and $0.56 \times 10^6$. *E. coli* rRNAs were coincident with the chloroplast rRNAs (Figure 11).

**Caesium chloride buoyant density centrifugation**

DNA, purified as described was dissolved in 0.1M tris-HCl buffer, pH 7.4, and mixed with concentrated caesium chloride solution to a final density of 1.6 gm per ml. 8 ml of this
solution containing up to 6 O.D. units of DNA were pipetted into a 30 ml polypropylene centrifuge tube above 8 ml of 1.8 gm per ml caesium chloride solution layered over the top. Densities of solutions were measured with an Abbey refractometer. Tubes were topped up with liquid paraffin and centrifuged in a 8 x 30 ml titanium fixed angle rotor at 26,000 rpm for 2\(\frac{1}{2}\)-3 days at 25°C in an M.S.E. 65 centrifuge. At the end of the run tubes were allowed to stand vertically for about half an hour. A hole was then pierced in the bottom of the tube with a hot needle and 0.3 or 0.5 ml fractions were collected from the bottom of the tube. Fractions were diluted with an equal volume of distilled water and the absorbance at 260 nm measured to locate the DNA.

**DNA-RNA hybridisation**

The method of Gillespie and Spiegelman was used as described by Birnstiel et al. (1968). DNA purified as described and dissolved in 0.1 x SSC or in fractions from caesium chloride gradients was denatured by addition of an equal volume of normal sodium hydroxide solution. After half to one hour at room temperature the solution was adjusted to pH 7.8 by the addition of a tris-HCl-sodium chloride medium in the cold:

- \(N. \text{HCl}\) 100 ml
- \(M. \text{Tris pH 7.8}\) 100 ml
- \(3M \text{NaCl}\) 200 ml

Millipore filters (HAWP, 0.45, 13 mm diameter) cut from large sheets with a punch were washed with distilled water and mounted on the lower half of a Swinney filter holder. This was then screwed into a perspex cylinder of 10 ml capacity which was
held vertically in a rack. The filters were washed with 5-10 ml
2 x SSC and the denatured DNA collected on the filters by
filtration under gravity in the cold. The wet filters were
dried in a vacuum desiccator briefly and then at 80°C in a
vacuum oven for several hours. Filters were normally used
immediately for hybridisation experiments.

RNA fractions eluted from gels and collected by alcohol
precipitation or by centrifugation were dissolved in 6 x SSC.
Occasionally when RNA was eluted into 2 or 3 x SSC, 0.2 per cent
sodium dodecyl sulphate, the salt concentration was increased to
6 x SSC, and the solutions used directly for hybridisation.
Filters were incubated together with blanks in RNA solutions
ranging in volume from 0.3 to 3.0 ml in either Packard vials or
glass screw cap vials 15 mm internal diameter. RNA concentration
and incubation times are given in the legends to the appropriate
figures. The incubation temperature was either 68°C or 70°C.

After incubation filters were removed with forceps and
washed in excess 6 x SSC two or three times, then twice in a
large volume of 2 x SSC for ten minutes followed by a final
incubation in 2 x SSC containing 10-15 µg ribonuclease per ml
for 20-30 minutes. All washes were at room temperature. After
ribonuclease digestion filters were dried in vacuo and counted in
toluene scintillator in a scintillation counter. RNA
concentrations were calculated from the absorbence at 260 nm as
previously described. Activities of RNA were measured by
drying aliquots on millipore filters and counting in toluene
scintillator.

DNA retention on filters was not routinely monitored. In
one experiment, DNA retained on the filters was measured by acid
Hybridisation of $1 \times 10^6$ molecular weight cytoplasmic rRNA to leaf DNA as a function of time.  

$1 \times 10^6$ molecular weight rRNA labelled with $^{32}$P orthophosphate was recovered from gel slices after locating the radioactive peaks by Cerenkov counting. The specific radioactivity of the rRNA was 27,000 Cerenkov cpm. The concentration of the RNA was $1.5 \mu g$ per ml. Incubation was at 65°C in 6 x SSC. Each filter carried $5 \mu g$ DNA. Blank filters had no more than 60 cpm.
Figure 13.

Saturation hybridisation of leaf DNA with 1.3 or 0.7 x 10^6 molecular weight cytoplasmic rRNA in 6 x SSC.

RNA labelled with ^32P orthophosphate was extracted and fractionated in the presence of magnesium in 2-4% gels. The gels were sliced and the cytoplasmic rRNAs located by Cerenkov counting and recovered by elution with 2 x SSC containing 0.2% sodium dodecyl sulphate followed by alcohol precipitation. Hybridisation was for three hours at 68°C in 6 x SSC at the RNA concentrations shown (μg per ml). Each filter carried 10 μg DNA; main band DNA from a cesium chloride gradient was used as a control DNA. The specific radioactivity of the RNA was 30,000 cpm per μg.

- 1.3 x 10^6 molecular weight cytoplasmic rRNA.
- 0.7 x 10^6 molecular weight cytoplasmic rRNA.
- Cytoplasmic rRNAs hybridised to main band DNA.
hydrolysis followed by U.V. absorbance measurements of the supernatant. About 80 per cent of the DNA loaded on the filter was recovered in this way.

Aliquots of DNA solutions used for loading filters were measured by the diphenylamine method (Burton, 1956) by J. Creanor.

The 1.3 x 10^6 molecular weight rRNA when hybridised to total DNA in 6 x SSC at 68°C saturated the DNA after about 1½ hours (Figure 12). Figure 13 shows the hybridisation of both the 1.3 and 0.7 x 10^6 molecular weight rRNAs to total DNA as a function of RNA concentration. It is clear that the 0.7 x 10^6 molecular weight rRNA does not readily saturate the DNA under the same conditions as the 1.3 x 10^6 molecular weight rRNA.

Many experiments suggest that there are equal numbers of genes for 1.3 and 0.7 x 10^6 molecular weight rRNA and therefore one would expect that the two rRNAs would saturate the DNA with a ratio of 1.3:0.7 (1.86:1). This is not the case under these hybridisation conditions. However this is not uncommon. The possible explanations are:

(1) the molecular weight of the 0.7 x 10^6 molecular weight rRNA is grossly underestimated;

(2) the 0.7 x 10^6 molecular weight rRNA is contaminated with another RNA species;

(3) the 0.7 x 10^6 molecular weight rRNA, or part of it, hybridises under these conditions to the 1.3 x 10^6 molecular weight rRNA genes or other genes.

Possibility (1) is probably not the case. Isolation and fractionation of RNA in the presence of magnesium eliminated the possibility that the 0.7 x 10^6 molecular weight chloroplast RNA breakdown product (mentioned later) contaminates the 0.7 x 10^6
Saturation hybridisation of leaf DNA with $1.3 \times 10^6$ and $0.7 \times 10^6$ molecular weight cytoplasmic rRNA in $2 \times$ SSC.

$^{32}$P labelled RNA was extracted and fractionated in the presence of magnesium in $2.4\%$ gels. The cytoplasmic rRNA peaks were located by Cerenkov counting of the sliced gels. The $1.3$ and $0.7 \times 10^6$ molecular weight rRNAs were eluted with $2 \times$ SSC containing $0.2\%$ sodium dodecyl sulphate and precipitated with alcohol. Hybridisation was for three hours at $70^\circ C$ in $2 \times$ SSC at the RNA concentrations shown (µg per ml). Each filter carried $5 \mu g$ DNA. The specific radioactivity of the RNA was $35,000$ cpm per µg.

- $1.3 \times 10^6$ molecular weight cytoplasmic rRNA.
- $0.7 \times 10^6$ molecular weight cytoplasmic rRNA.
molecular weight cytoplasmic rRNA. Under these conditions the same saturation pattern (shown in Figure 13) was obtained. Contamination by the smaller rRNA of the mitochondria cannot be excluded. Possibility (3) seems the most likely. A similar situation has been found in *S. pombe* (Retel and Planta, 1968). This "cross-talk" has consequences for the competitive hybridisation experiments discussed below. It may result from a degree of chemical similarity between the two rRNAs (Van Den Bos and Planta, 1970).

The solution to the problem would be to find hybridisation conditions where the rate of hybridisation of the 0.7 x 10⁶ molecular weight rRNA to genes from which it was not transcribed is very much lower than the rate of hybridisation to its homologous genes. Figure 14 shows that in 2 x SSC at 70°C such a situation is realised. The 0.7 x 10⁶ molecular weight rRNA does not reach a saturation plateau but there is sufficient distinction between the two types of rRNA cystrons to make competitive hybridisation experiments possible.

**Competitive hybridisation**

The two step procedure suggested by Gillespie (1965) was used. This has several advantages. There are no steric effects of competitor RNA other than those due to the formation of specific DNA/RNA duplexes. The method also economises on the use of radioactive RNA. Filters (10 μg total DNA per filter) were incubated in 2 x SSC at 70°C for two hours in solutions of unlabelled competitor RNA at concentrations from 0-5 μg per ml. The 0.7 and 1.3 x 10⁶ molecular weight rRNAs were isolated from magnesium gels. The rRNA from *Schizosaccharomyces pombe* was used as a non-specific competitor.
After incubation the filters were then washed several times in 2 x SSC and dried in a vacuum desiccator. The filters were each marked with pencil and incubated together in a solution of the radioactive test RNA at 70°C in 6 x SSC for two hours. 0.06 per cent phenol was added to inhibit ribonuclease. At the end of the incubation the filters were washed, treated with ribonuclease, dried and counted as described.

Melting of DNA/RNA hybrids

Filters were washed free of toluene scintillator in chloroform at room temperature for a few minutes and air dried. They were then rinsed in 0.1 x SSC at room temperature for 2-3 minutes. Filters were then incubated in glass vials in 1 ml 0.1 x SSC for five minutes at a controlled temperature. They were then removed, placed in a fresh 1 ml of 0.1 x SSC and incubated at a higher temperature for a further five minutes. Temperatures from 30-100°C were used in intervals of 5 or 10°C.

The radioactivity in each vial was determined directly by Cerenkov counting or by reducing the volume of the solution and adding scintillation mixture.

Cell counts

Cell suspensions were counted using a haemocytometer, following maceration of the leaves for 24 hours in 5 per cent chromic acid after Brown and Rickless (1949).
RESULTS
Figure 15.

RNA components of young leaves.

a) Total nucleic acids prepared from primary leaves after soaking dry seeds for six hours in water.
b) Nucleic acids from a) mixed with nucleic acids from leaves of plants grown in the light for four days.

Electrophoresis was for 3 hours in 2.2% gels.
A. THE PROPERTIES OF HIGH MOLECULAR WEIGHT RNAs FROM LEAVES

Results and Discussion

Minor RNA components of young leaves

Figure 15a shows the separation in a 2.2 per cent gel of total nucleic acids extracted from leaves six hours after soaking seeds in water. In addition to the cytoplasmic rRNAs of molecular weight 1.3 and $0.7 \times 10^6$ there are several minor RNA components present. The most prominent of these has a molecular weight of about $1.0 \times 10^6$. Electrophoresis of a sample of the RNA shown in Figure 15a together with RNA from four day old leaves containing chloroplasts shows that the chloroplast rRNAs of molecular weight 1.1 and $0.56 \times 10^6$ and a breakdown product of molecular weight $0.9 \times 10^6$ are distinct from the components in young leaves (Figure 15b). The function of these minor RNAs is not clear. They are not readily detected in RNA extracts from older leaves. This is possibly because they do not accumulate at the same rate as the chloroplast and cytoplasmic rRNAs during leaf development. The $1.0 \times 10^6$ molecular weight RNA is similar to components detected in radish cotyledons (Ingle, 1968a) and barley leaves (Smith, 1970a). The molecular weight estimates are slightly different, but it is striking that in each case the component was detected only in young tissue. In *P. aureus* the $1.0 \times 10^6$ molecular weight RNA detected by optical density has the same mobility on electrophoresis as a rapidly labelled RNA found in both leaves and roots, (Section b). This RNA may represent an intermediate stage in the biosynthesis of the $0.7 \times 10^6$ molecular weight cytoplasmic rRNA. Apart from coincidence during electrophoresis, however, there is no evidence that these molecules are identical.
Comparison of leaf rRNAs labelled for different times.

a) Nucleic acid from leaves of five day old plants labelled for five hours with 120 μCi $^{32}$P per leaf.
b) Nucleic acid from leaves of six day old plants previously labelled for 48 hours with 2 mCi $^{32}$P injected into the sand around the roots.

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$^{32}$P  
Absorbance 265 nm.
It is possible that the $1.16 \times 10^6$ molecular weight RNA described here is of mitochondrial origin. Recent experiments with a range of plant mitochondria have shown that the rRNAs have molecular weights of $1.2$ and $0.7 \times 10^6$ (Harmey and Leaver, 1972).

Synthesis and breakdown of chloroplast rRNA

The synthesis of chloroplast rRNA cannot be detected in young leaves either by optical density or by $^{32}P$ labelling until about two days after germination. Between the third and the fifth day of development radioactivity is incorporated into chloroplast rRNA after prolonged incubation in $^{32}P$ orthophosphate. Figure 16a shows nucleic acids from five day old leaves labelled in vivo for five hours and fractionated in a 2.4 per cent gel. The specific activities of the cytoplasmic rRNAs are approximately the same, which is to be expected if they are synthesised coordinately as part of a larger precursor molecule. The specific activity of the $1.1 \times 10^6$ molecular weight chloroplast rRNA, however, is much greater than that of the $0.56 \times 10^6$ molecular weight chloroplast rRNA. The ratio of radioactivity in the two chloroplast rRNAs is approximately 2:1, the expected value assuming the two rRNAs are synthesised in equal amounts. In contrast the ratio of the chloroplast rRNAs determined by peak area measurements of the optical density scan is about 1.1:1. After 24 hours labelling, however, the radioactivity and the optical density curves become identical (Figure 16b). A similar discrepancy between the optical density scan and the radioactivity measurement has been reported for the chloroplast rRNA of radish cotyledons (Ingle, 1968a). Recent studies have shown that the $1.1 \times 10^6$ molecular weight chloroplast rRNA from
Legend for Figure 17.

Nucleic acids were extracted and purified from leaves of six day old plants in the presence of magnesium and dissolved in electrophoresis buffer containing magnesium. Identical samples were loaded on 2.6% gels. Electrophoresis was for three hours.

(b) E buffer gel.

(a) Mg\(^{++}\)buffer gel.
Figure 17.

The effect of magnesium on the integrity of chloroplast rRNA during electrophoresis.  

(Legend opposite)
a number of plants is unstable and breaks down (Ingle et al., 1970; Leaver and Ingle, 1971). The breakdown can be prevented by extracting and fractionating the RNA in the presence of magnesium ion (Ingle et al., 1970). Figure 17 shows the result of extracting leaf RNA from *P. aureus* in the presence of magnesium ion and fractionating the RNA either in the presence (Figure 17a) or absence (Figure 17b) of magnesium. The differences between the buffer systems used results in altered mobilities of the individual RNA components, but there are also other differences between the two treatments. When magnesium is present during electrophoresis both the chloroplast and cytoplasmic rRNAs are present in a ratio approximating to 2:1 judged by the areas under the optical density curves (Figure 17a). The absence of magnesium during electrophoresis leads to a more complex optical density scan (Figure 17b). The ratio of the 1.3 and 0.7 x 10^6 molecular weight cytoplasmic rRNAs is about 1.8:1 judged by peak area measurements. This is close to the theoretical expectation of 1.86:1. The ratio for the chloroplast rRNAs is about 0.8:1, indicating a reduction in the amount of chloroplast 1.1 x 10^6 molecular weight rRNA. In addition, a number of minor RNA components are also present. These have molecular weights of 0.9, 0.5, 0.4 and 0.2 x 10^6. These results suggest that the chloroplast 1.1 x 10^6 molecular weight rRNA is unstable and gives rise to fragments of lower molecular weight. Magnesium appears to prevent fragmentation of the molecule. However, it is not clear from this experiment whether all the additional RNAs arise by breakdown of the 1.1 x 10^6 molecular weight chloroplast rRNA, or whether some of them are fragments derived from the other rRNAs. The origin of
Legend for Figure 18.

$^{32}$P labelled cytoplasmic and chloroplast rRNA fractions were recovered from Mg$^{2+}$ gels and subjected to electrophoresis in E buffer gels with unlabelled total leaf nucleic acid.

a) $1.1 \times 10^6$ molecular weight rRNA.
b) $0.56 \times 10^6$ molecular weight rRNA.
c) $1.3 \times 10^6$ molecular weight rRNA.
d) $0.7 \times 10^6$ molecular weight rRNA.

$^{32}$P

Absorbance 265 nm.
the fragments was determined directly by the following experiment. RNA from leaves of intact plants previously labelled for 60 hours with $^{32}$P orthophosphate was extracted and fractionated in the presence of magnesium, as in Figure 17a. The individual RNAs were recovered from the gel and co-electrophoresed in the absence of magnesium together with unlabelled leaf RNAs as molecular weight markers. The results are shown in Figure 18. The $1.1 \times 10^6$ molecular weight chloroplast rRNA breaks down on re-electrophoresis in the absence of magnesium to give molecules of molecular weight 0.9, 0.7, 0.5, 0.4 and $0.2 \times 10^6$ (Figure 18a). Only a part of the total population of rRNA molecules breaks down leaving a proportion of apparently intact polynucleotide chains. The $1.1 \times 10^6$ molecular weight chloroplast rRNA is contaminated in this preparation by a small amount of $1.3 \times 10^6$ molecular weight cytoplasmic rRNA. However, the $1.3$ (Figure 18c), the $0.7$ (Figure 18d) and the $0.56$ (Figure 18b) molecular weight rRNAs are all stable in the absence of magnesium. This experiment directly confirms that all the breakdown products arise from the $1.1 \times 10^6$ molecular weight chloroplast rRNA, including the $0.7 \times 10^6$ molecular weight component which was not detected in Figure 17b.

The degree of fragmentation of the larger chloroplast rRNA varies with the age and physiological state of the leaf. Older leaves show the greatest reduction in the amount of this RNA (Figure 19) and leaves grown in the light show more breakdown than leaves grown in the dark. However the breakdown products do not always seem to be preserved in the amounts expected (Figure 19) and it is possible that they are subjected to further hydrolysis. Molecules of the $1.1 \times 10^6$ molecular weight RNA
Figure 19.

Variation in the extent of chloroplast rRNA breakdown with leaf age.

a) Nucleic acid from four day old leaves.
b) Nucleic acid from seven day old leaves.

Electrophoresis was for 2.5 hours in 2.6% gels.
Stability of $1.1 \times 10^6$ molecular weight chloroplast rRNA purified by electrophoresis in the absence of magnesium.

a) $1.1 \times 10^6$ molecular weight chloroplast rRNA eluted from an E buffer gel into lithium acetate and spun down at 40,000 rpm.

b) Marker RNA.

Electrophoresis was in 2.4% gels for 4 hours.

This figure is part of Figure 9 shown previously.
Figure 21.

Model for the breakdown of chloroplast $1.1 \times 10^6$ molecular weight rRNA.

Two labile sites are proposed a) and b). Cleavage at position a) produces fragments of molecular weight $0.2$ and $0.9 \times 10^6$. Cleavage at position b) produces fragments of molecular weight $0.7$ and $0.4 \times 10^6$. Cleavage at both sites produces an additional fragment of molecular weight $0.5 \times 10^6$. 
that appear intact judged by electrophoresis in the absence of magnesium remain intact following isolation and re-electrophoresis without magnesium (Figure 20).

The fragments derived from the larger chloroplast rRNA can be accounted for by assuming that the molecule has two labile sites (Figure 21) and that cleavage of the polynucleotide chain can occur at either one or both of these sites. This model accounts for the production of all the fragments detected in these experiments. The evidence from pulse labelling experiments is that the chloroplast rRNAs are synthesised in a 1:1 molar ratio and that older molecules become susceptible to endonucleolytic attack. It is possible that this hydrolysis occurs during isolation of the RNA. If this were the case it would appear that newly synthesised rRNA is in some way protected from hydrolysis. Nuclease action during extraction seems unlikely however, in view of the fact that the RNA is extracted by directly homogenising the tissue in detergents and nuclease inhibitors. An alternative explanation is that the hydrolysis of chloroplast rRNA occurs in vivo and older chloroplast rRNA is preferentially attacked.

Hidden breaks in cytoplasmic rRNA

In contrast to the $1.1 \times 10^6$ molecular weight chloroplast rRNA, the $1.3$ and $0.7 \times 10^6$ molecular weight cytoplasmic rRNAs are stable during electrophoresis in the absence of magnesium. During experiments involving the manufacture of aggregates of purified rRNAs for use as molecular weight markers it was found that the cytoplasmic rRNAs were capable of dissociating into specific fragments on heating. Figure 22 shows the result of heating purified $1.3$ or $0.7 \times 10^6$ molecular weight cytoplasmic
Figure 22.

RNA fragments produced by heating cytoplasmic rRNAs at 85° in electrophoresis buffer.

a) $1.3 \times 10^6$ molecular weight cytoplasmic rRNA.
b) $0.7 \times 10^6$ molecular weight cytoplasmic rRNA.

rRNAs were eluted from gel slices into $2 \times$ SSC containing 0.2% sodium dodecyl sulphate, precipitated with alcohol, dissolved in electrophoresis buffer containing sucrose, heated to 85°C for 3 minutes, cooled, and 50 μl layered onto 2.4% gels. Electrophoresis was for two hours.
rRNAs at 85°C. In each case small amounts of specific fragments are formed. The degree of fragmentation of the molecule depends to a large extent on the age and physiological state of the tissue from which the RNA was extracted. However, the fragments formed have distinct molecular weights and the 1.3 and 0.7 x 10^6 molecular weight rRNAs form different fragments. The pattern of fragments obtained for each rRNA is similar with RNA prepared from both leaves and roots. In the case of the 1.3 x 10^6 molecular weight RNA the two high molecular weight fragments that are most common have molecular weights of approximately 0.7 and 0.6 x 10^6. A similar situation occurs in peas (Grierson, unpublished results and Higo, Higo and Tanifuji, 1971). Recently the 28s rRNA of silkworm has been shown to dissociate into two fragments of approximately 18s on heating (Ishikawa and Newburgh, 1972). In addition to forming several high molecular weight fragments, the 1.3 x 10^6 molecular weight rRNA produces a molecule of low molecular weight which has a mobility close to that of soluble RNA. This molecule can be distinguished from 5s RNA and transfer RNA in gels 7.5 per cent in acrylamide (Figure 23) and has a molecular weight of about 52,000. This fragment is specific to the 1.3 x 10^6 molecular weight rRNA. Analogous fragments have been found in a variety of higher organisms: In animal cells a low molecular weight RNA is associated with the 28s rRNA (Pene et al., 1967) and a recent survey has shown that a low molecular weight RNA similar to the one reported here is hydrogen bonded to the 1.3 x 10^6 molecular weight rRNA of a variety of higher plants (Payne and Dyer, 1972).

These results show that instability of rRNA is not confined
Legend for Figure 23.

1.3 x 10^6 molecular weight cytoplasmic rRNA was eluted from E buffer gels into 2 x SSC containing 0.2% sodium dodecyl sulphate, precipitated with alcohol, dissolved in electrophoresis buffer containing sucrose and divided into two aliquots. One sample was kept at 0°C (a), and the other was heated at 65°C for three minutes and cooled at 0°C (b). Total nucleic acid carrier was added to each sample. Electrophoresis was for two hours in 7.5% gels.
Production of a specific low molecular weight RNA fragment by heating 1.3 x 10^6 molecular weight cytoplasmic RNA.

Figure 23.

OD 260 nm

(a)

(b)
to the chloroplasts. The differences between chloroplast $1.1 \times 10^6$ molecular weight rRNA and $1.3$ and $0.7 \times 10^6$ molecular weight cytoplasmic rRNA resides in the greater ability of fragments of the latter to remain together during extraction and fractionation. The specific nature of the fragments is presumably a consequence of the arrangement of the rRNA within the ribosome, with certain regions of the molecule being exposed near the ribosome surface and therefore susceptible to attack by ribonuclease. Ribosomal RNA has a high degree of secondary structure (Cox, 1970) and as helical regions of RNA are more resistant to ribonuclease it would seem likely that attack would occur preferentially at single stranded regions. If these labile sites are between double-stranded regions of the RNA, for example in exposed loops of the rRNA at the end of double-stranded helical regions of the molecule, fragmentation of the polynucleotide chain would depend on the dissociation of the hydrogen bonded bases in such helical regions. If this interpretation is correct, the helical regions between fragments of chloroplast $1.1 \times 10^6$ molecular weight rRNA would appear less stable, i.e. shorter than those in cytoplasmic rRNA. The function of magnesium ions in preventing dissociation of the fragments could be to stabilise such helical regions. It has already been pointed out in the case of chloroplast rRNA breakdown that nuclease action during RNA extraction is unlikely. It seems reasonable to conclude that the polynucleotide chains of rRNA may be broken in vivo. The evidence from studies on chloroplast rRNA suggests that old rRNA is more likely to be broken. The physiological significance of this is not clear. It is possible that ribosomes with fragmented rRNA are inactive.
Figure 24.

Detection of pre rRNAs by optical density.

Detached leaves from light grown plants three and a half days old were floated on $^{32}P$ orthophosphate dissolved in distilled water. After two and a half hours total nucleic acids were extracted. DNA was removed by deoxyribonuclease. Electrophoresis was for four hours on a 2.2% gel.

- $^{32}P$
- Absorbance 265 nm, 40 D. full scale.
- Absorbance 265 nm, 0.10 D. full scale.
Legend for Figure 25.

The results of two experiments are shown. Total leaf RNA was used in each case. DNA was removed by prior treatment with deoxyribonuclease.

Experiment 1.

a) Control RNA.

c) RNA dried in vacuo for four minutes.

The high molecular weight region of the gel was scanned with a sensitivity of 0.5 O.D. full scale.

Experiment 2.

b) Control RNA.

d) RNA heated at 60°C for three minutes in electrophoresis buffer.

The high molecular weight region of the gel was scanned with a sensitivity of 0.1 O.D. full scale.
in protein synthesis. Colicin E₃ inhibits protein synthesis in *E. coli* by causing cleavage of the 16s rRNA close to the 3' end (Bowman, Sidikaro and Nomura, 1971). On the other hand, ribosomes with breaks in the rRNA are active in protein synthesis (Jacob, 1961; Furano and Harris, 1971). The proportion of the total rRNA molecules that are fragmented varies considerably. In general the rRNA from older leaves contains more hidden and open breaks than rRNA from young tissues.

**Aggregation of ribosomal RNA**

A number of RNA components heavier than rRNA are detected by optical density scans of RNA preparations fractionated on gels. Among these high molecular weight RNAs are precursors to ERNA which can be identified by pulse labelling (Figure 24 and Section C). Other RNA components migrating in the same area are however not rapidly labelled. The amount of these components varies under different conditions of RNA extraction and purification. Figure 25 shows an extreme case. Nucleic acids were extracted and purified as described and DNA was removed by deoxyribonuclease treatment. The reprecipitated RNA was divided into two parts and one sample was drained for four minutes and then dissolved in electrophoresis buffer containing 15 per cent sucrose. The other RNA sample was drained and dried in vacuo for four minutes and dissolved in the same buffer. The two preparations were immediately fractionated on 2.4 per cent gels. The two samples differ significantly in the region between the origin of the gel and the 1.3 x 10⁶ molecular weight cytoplasmic rRNA. The RNA preparation dried in vacuo shows several minor RNAs in this region (Figure 25c), in contrast to the control preparation which is virtually free
from such high molecular weight RNAs (Figure 25a). Similar RNAs can be produced by heating the RNA solution at 60°C immediately prior to electrophoresis (Figure 25d). The fact that these molecules are produced \textit{in vitro} after RNA extraction strongly suggests that they are artefacts. There are a number of conditions which lead to their formation. These are:

1. hot phenol extraction of RNA at high nucleic acid concentrations;
2. storage of purified RNAs in solution at temperatures of 0-20°C at concentrations of 1 to 2 mg/ml for longer than a few minutes; or heating RNA at concentrations above about 0.5 mg/ml at temperatures above 40°C for a few minutes only;
3. magnesium ions at concentrations of 1 to 10 mM.

From a consideration of the conditions leading to their production, and from molecular weight determinations it seemed likely that these molecules are specific aggregates of rRNA. The identity and composition of some of these aggregates was determined directly by their construction from purified rRNAs \textit{in vitro}. 1.3 and 0.7 x 10^6 molecular weight cytoplasmic rRNAs were recovered from gels and heated in electrophoresis buffer at 65°C either separately or together. The RNA concentration was 0.5 mg/ml. After three minutes the solutions were cooled in ice and unheated total leaf RNA was added and samples of the RNA fractionated on 2.4 per cent gels. The results are shown in Figure 26. When heated together, the 1.3 and 0.7 x 10^6 molecular weight RNAs form aggregates of molecular weight 2.6 and 2.0 x 10^6 (Figure 26a). The 2.6 x 10^6 component is rather broad. This is shown more clearly in Figure 26b where
Legend for Figure 26.

Cytoplasmic rRNAs recovered from gels were heated separately or together in electrophoresis buffer containing sucrose at 65°C for three minutes, cooled to 0°C and total leaf RNA was added. Electrophoresis was in 2.4% gels for four hours.

a) 1.3 and 0.7 x 10^6 molecular weight rRNA heated together at 65°C.

b) 1.3 x 10^6 molecular weight rRNA heated at 65°C.

The high molecular weight region was scanned with a sensitivity of 0.5 O.D. full scale.
Figure 26.

Formation of molecular aggregates from individual rRNAs.

(Legend opposite)
the $1.3 \times 10^6$ molecular weight RNA was heated by itself. There appear to be two components to the peak which has a molecular weight range from $2.5$ to $2.6 \times 10^6$. These results show that the $1.3$ and $0.7 \times 10^6$ molecular weight rRNAs aggregate to form a component which migrates at a position corresponding to a molecular weight of $2.0 \times 10^6$. The $1.3 \times 10^6$ molecular weight RNA forms a broad aggregate on heating. At the temperature used in this experiment ($65^\circ C$) the $1.3 \times 10^6$ molecular weight rRNA loses the RNA of molecular weight $52,000$ shown in Figure 23. The molecular weight of the aggregate ($2.5-2.6 \times 10^6$) is consistent with it being a multiple component of dimers of the $1.3 \times 10^6$ molecular weight RNA which have either retained or lost the $52,000$ molecular weight RNA.

In these experiments the amount of rRNA that forms molecular aggregates is very small and the aggregates are difficult to detect. In addition, the formation of aggregates normally occurs after RNA extraction. This is in marked contrast to the situation in Blastocladiella (Lovett and Leaver, 1969) where aggregation occurs during normal extraction procedures and involves a very high proportion of the total RNA.

Aggregates of known molecular weight can be used as markers during molecular weight determinations of the pre rRNAs (Figure 27b). In general, traces of aggregates of rRNA present in pulse labelled RNA preparations can be distinguished from the precursors to rRNA because the former have a much lower specific activity than the precursors. However, in situations where information about the specific activity of the rRNA precursors is required, involving resolution of these molecules by optical density and radioactivity, any trace of molecular aggregates
Legend for Figure 27.

$^{32}$P labelled leaf RNA was dissolved in electrophoresis buffer at a concentration of approximately 1 mg per ml and divided into two samples. One half was kept at 0°C and the other sample was heated at 50°C for three minutes and then cooled in ice. 25 µl aliquots of each sample were layered onto 2.4% E buffer and low salt gels. Electrophoresis was for 3½ hours.

a) Control sample E buffer.
b) Heated sample E buffer.
c) Control sample low salt buffer.
d) Heated sample low salt buffer.
makes such a measurement impossible. In RNA preparations labelled for a long time so that the majority of radioactivity is in the rRNAs aggregates are significantly labelled. Under these conditions they can be confused with precursors to rRNA. Once they have been formed aggregates are difficult to remove. Those described here are stable to electrophoresis in the presence of EDTA (Figure 27d) although treatments designed to disrupt hydrogen banding such as 8 M urea are successful in removing them. However, such treatments also reveal hidden breaks in the RNA which may make interpretation of the results difficult.
Figure 28.

The accumulation of nucleic acids in primary leaves grown in darkness and light.

The results are expressed as μg nucleic acid per leaf. 1 O.D. unit of nucleic acid was assumed to be equivalent to 40 μg.

- ○ Plants grown in light from day 0.
- □ Plants introduced into the light on day 2.
- △ Plants introduced into the light on day 3.
- ● Plants grown in total darkness.
B. THE ACCUMULATION OF CYTOPLASMIC AND CHLOROPLAST rRNA DURING DEVELOPMENT OF LEAVES IN LIGHT AND DARK

Results

When etiolated leaves are exposed to light they rapidly accumulate nucleic acids (Figure 28), although considerable synthesis of nucleic acid eventually occurs in leaves grown in complete darkness. Gel peak-area measurements show that more than 80 per cent of the leaf nucleic acid is RNA and more than 70 per cent of the RNA is rRNA. In Section A it was established that the chloroplast $1.1 \times 10^6$ molecular weight rRNA is unstable and breaks down, although it is present in equimolar amounts with the $0.56 \times 10^6$ molecular weight rRNA.

Table 2 shows the ratio of the amounts of the larger and smaller rRNAs from chloroplast or cytoplasmic ribosomes in RNA preparations from leaves of different ages. Breakdown of the larger chloroplast rRNA results in a ratio which is much lower than the theoretical value. The loss of the $1.1 \times 10^6$ molecular weight rRNA is greater in RNA preparations from older leaves. In contrast the ratio of the amounts of $1.3:0.7 \times 10^6$ molecular weight rRNAs is close to the theoretical value, irrespective of leaf age. The $0.7 \times 10^6$ molecular weight RNA fragment derived from the $1.1 \times 10^6$ molecular weight chloroplast rRNA is presumably present in such small amounts that it does not influence this ratio.

In order to quantitatively measure the synthesis of cytoplasmic and chloroplast rRNA during leaf development the amount of the $1.3, 0.7$ and $0.56 \times 10^6$ molecular weight rRNAs was determined from gel peak area measurements as described in Materials and Methods. The amount of the $1.1 \times 10^6$ molecular
Table 2. Variation in the ratio of the larger and smaller rRNAs of chloroplast and cytoplasmic ribosomes detected by gel electrophoresis of RNA from leaves of different ages.

RNA was extracted and fractionated in the absence of magnesium. The relative amounts of the ribosomal RNAs were determined by peak-area measurements.
The accumulation of chloroplast and cytoplasmic rRNAs in primary leaves of plants grown in darkness and in the light.

Nucleic acid samples from each treatment were separated on 2.4% gels. The amounts of 1.3, 0.7 and 0.56 x 10^6 molecular weight rRNAs were calculated from gel peak area measurements and related to the total nucleic acid loaded onto each gel. The amounts of the cytoplasmic rRNAs in each sample were added together, and the amount of chloroplast rRNA was calculated by multiplying the amount of 0.56 x 10^6 molecular weight rRNA by three. Results are expressed as µg rRNA per leaf.

- O Cytoplasmic rRNA in the light.
- ● Cytoplasmic rRNA in the dark.
- □ Chloroplast rRNA in the light.
- ■ Chloroplast rRNA in the dark.
Figure 30.

Changes in cell number and fresh weight of primary leaves grown in darkness and in the light.

a) leaf cell number.
b) leaf fresh weight.

- Plants grown in the light.
- Plants introduced into the light on day three.
- Plants introduced into the light on day five.
- Plants grown in the dark.
weight chloroplast rRNA was assumed to be twice that of the 0.56 \times 10^6 molecular weight chloroplast rRNA.

The pattern of accumulation of leaf cytoplasmic and chloroplast rRNA during growth of seedlings in darkness and in light is shown in Figure 29. Both types of rRNA are synthesised during leaf growth in the dark, although most of the rRNA originates from cytoplasmic ribosomes. When seedlings are exposed to light there is a rapid accumulation of cytoplasmic rRNA beginning two days after the start of germination. This is followed by a marked increase in the amount of chloroplast rRNA compared with dark grown leaves. It is clear that light stimulates the accumulation of both classes of rRNA. However the synthesis of cytoplasmic rRNA is promoted more rapidly than chloroplast rRNA.

The increased synthesis of cytoplasmic ribosomes in light grown leaves is correlated with an increased rate of cell division and cell expansion. Figure 30 shows the effect of light on cell division and leaf fresh weight. The leaves are most responsive to light two to three days after germination. Light stimulates the increase in leaf cell number nearly three-fold over the period from day two to day seven compared with the dark control.

In order to assess the importance of light stimulated RNA synthesis in leaf development it is necessary to determine whether it precedes or is a consequence of increased cell division. In some experiments the increased rate of cytoplasmic rRNA accumulation was detected prior to the increase in leaf cell number. In other experiments however cytoplasmic rRNA synthesis and cell division appeared to be stimulated at the same time.
Figure 31.
LIGHT STIMULATION OF $^{32}\text{P}$ UPTAKE AND INCORPORATION INTO rRNA
No periodicity of RNA synthesis or cell division was detected. Labelling experiments with $^{32}$P orthophosphate showed that there was a rapid stimulation of cytoplasmic rRNA labelling by light at a time when no chloroplast rRNA was labelled. Figure 31 shows the results of one such experiment. Dark grown plants were introduced into the light for various times and then returned to darkness and incubated in $^{32}$P orthophosphate for two hours together with dark controls. $^{32}$P uptake by the leaves was determined by washing plants in phosphate buffer for three minutes, grinding the leaves in the medium for RNA extraction and then counting a sample of the supernatant from the centri- fuged leaf homogenate prior to phenol deproteinisation. Nucleic acids were purified and RNA from two leaves was fractionated on gels and the $^{32}$P incorporation into rRNA was determined by counting the radioactivity in each RNA peak. Light stimulated the uptake of $^{32}$P by the leaves and incorporation into rRNA to a similar extent.

The mechanism of cytoplasmic rRNA synthesis and the effect of light

The light stimulated isotope uptake of the leaves makes it difficult to show how quickly light affects RNA synthesis simply by measuring isotope incorporation rates. However a detailed examination of the labelling pattern of RNA in leaves grown in the dark and after a short light treatment reveals considerable differences (Figure 32). The most obvious difference is that there is much more radioactivity in the 1.3 and $0.7 \times 10^6$ molecular weight cytoplasmic rRNAs relative to the pre rRNAs (Section C), in leaves introduced into the light. There are also differences in the relative amounts of the 1.45 and
Legend for Figure 32.

a) Plants grown for 60 hours and labelled for one hour in total darkness by application of $^{32}P$ orthophosphate to the leaves.

b) Leaves of plants grown for 60 hours were introduced into the light for five hours and labelled for the fifth hour with $^3H$ uridine.

c) Plants were grown in the dark for 48 hours, introduced into the light for a further 24 hours and then labelled for 1½ hours with $^3H$ uridine.

Electrophoresis was in 2·4% gels for 4½ hours.
Figure 32.

Effect of light on processing of pre-rRNA in dark grown leaves. (Legend opposite)
<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Dark (1)</th>
<th>Dark (2)</th>
<th>Dark (3)</th>
<th>Light 1 hr</th>
<th>Light 1.6 hr</th>
<th>Light 3.3 hr</th>
<th>Light 6 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.9 x 2.5</td>
<td>1.45, 1.3, 1.0, 0.7</td>
<td>0.56</td>
<td>0.53</td>
<td>0.50</td>
<td>0.45</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Table 3. The effect of light on the processing of pre rRNAs

Leaves of plants grown in darkness or introduced into the light for a short time were incubated with $^{32}$P orthophosphate for one hour. RNA was extracted and DNA removed with deoxribonuclease. Samples were separated on 2% gels and the amount of radioactivity present in pre rRNAs ($2.9 - 2.5 \times 10^6$ molecular weight) and processing products ($1.45, 1.3, 1.0$ and $0.7 \times 10^6$ molecular weight) was measured. The results are expressed as the ratio of pre rRNAs to products after one hour.
1.3 \times 10^6$ molecular weight RNAs. The initial effect of light is to increase the rate of processing of the precursors to cytoplasmic rRNA (Figure 32a, b), although after 24 hours a new pre rRNA species with a much longer half-life can be detected (Figure 32c).

The rapid effect of light on the processing of the pre rRNA was investigated further. The most convenient way to express the relative rates of processing of the pre rRNA is to determine the amount of radioactivity in the precursor region relative to the radioactivity in the $1.45, 1.3, 1.0$ and $0.7 \times 10^6$ molecular weight processing products after a suitable labelling time. This value is the same for plants with a similar rate of synthesis and processing of rRNA and is independent of isotope uptake. Table 3 shows the results of an experiment to determine how rapidly light affected the rate of processing of the pre rRNA.

Plants grown in the dark for 60 hours were introduced into the light for one to four hours. During the last hour of illumination plants were labelled with $^{32}$P orthophosphate in vivo. Leaves of control plants grown in darkness were also labelled for one hour. An increased rate of processing of the pre rRNA was detected in leaves less than two hours after the onset of the light treatment.

Discussion

These results show that in primary leaves of P. aureus, light stimulates the accumulation of cytoplasmic rRNA before the synthesis of chloroplast rRNA is affected. A light stimulated increase in leaf cell number accompanies the accumulation of cytoplasmic rRNA. Phytochrome has been shown to be the
photoreceptor for the initial cell division response in primary leaves of *P. vulgaris* (Dale and Murray, 1968, 1969). Recent evidence suggests that one of the first effects of phytochrome photoactivation may be to increase membrane permeability (Smith, 1970b). The light stimulation of uptake of $^{32}$P orthophosphate by leaves reported in these experiments may be related to this effect.

It is not possible to determine accurately how rapidly light affects the transcription of cytoplasmic rRNA in these experiments. It seems clear that the increased production of cytoplasmic rRNA in the light must involve such an effect on transcription. It is possible that transcription and processing of the pre rRNA are stimulated in a coordinate fashion. However the evidence is that the primary effect of light is to stimulate the processing of the pre rRNA. This can be detected in less than two hours.

These experiments do not show whether the phytochrome itself is involved in the stimulation of RNA synthesis. The rapid response to light in young etiolated tissue suggests that the increased rate of processing of the pre rRNA is not the result of a general stimulation of metabolism by the products of photosynthesis. In support of this conclusion studies with bleached mutants of *Chlorella* suggest that RNA synthesis can be stimulated by light through a mechanism distinct from photosynthesis (Schmidt, 1969). On the other hand, it has recently been shown that cyclic photophosphorylation develops within 15 minutes of illuminating eight day old etiolated leaves of *P. vulgaris* (Ouzle-Karow and Butler, 1972). It is not known how rapidly photophosphorylation develops in young etiolated
leaves of *P. aureus* but it is possible that ATP derived from this process could stimulate processing of pre rRNA.

In several instances involving an alteration in the rate of rRNA synthesis (see Introduction) and in particular in *S. pombe* during a "step-up" incubation (Grierson *et al.*, 1970 and M.-L. Sartirana, pers. comm.) one of the first detectable changes is at the level of processing of the pre rRNA. In addition there is evidence (see Introduction) that newly synthesised rRNA may be destroyed when the rate of rRNA synthesis is low. This suggests that control elements operate at some stage of the post-transcriptional modification of the pre rRNA. Whether control operates at the level of transport of the newly synthesised rRNA to the cytoplasm remains to be seen.
Legend for Figure 33.

Plants were 4-5 days old, grown in the light. 120 µCi $^{32}$P orthophosphate dissolved in 20 µl water was applied to each leaf. Labelling was for 1.5 (a), and 0.5 (b) hours. Electrophoresis was in 2.2% gels for 3.5 hours.

- $^{32}$P radioactivity
- Absorbance at 265 nm.
Figure 33.

Leaf nucleic acids pulse labelled with $^{32}$P orthophosphate. (Legend opposite)
C. THE SYNTHESIS OF RIBOSOMAL RNA

Results

Rapidly labelled RNA components

Figure 33a shows the separation by gel electrophoresis of total nucleic acids isolated from leaves labelled in vivo for one and a half hours with $^{32}$P orthophosphate. The ultraviolet scan shows DNA, not fractionated by molecular weight, at 1.4 cm, the cytoplasmic rRNAs of molecular weight 1.3 and $0.7 \times 10^6$ and chloroplast rRNAs of molecular weight 1.1 and $0.56 \times 10^6$. A minor component of molecular weight $0.9 \times 10^6$ is a breakdown product of the $1.1 \times 10^6$ molecular weight chloroplast rRNA (Section A). There is also a minor component of molecular weight $2.9 \times 10^6$. Rapidly labelled RNA of molecular weight in excess of $7 \times 10^6$ stays near the top of the gel. The DNA and the cytoplasmic rRNAs are labelled. In addition there are peaks of radioactivity corresponding to molecular weights of $2.9, 2.5, 1.45, 1.0$ and $0.45 \times 10^6$ which can be detected above the background of labelled polydisperse RNA. All these RNAs are labelled even after very short radioactive incubations (Figure 33b). All the components on the gel are susceptible to ribonuclease and resistant to deoxyribonuclease, with the exception of the peak at 1.4 cm. Chloroplast rRNA is not labelled under these conditions.

Kinetics of labelling of RNA fractions

The kinetics of labelling of the various RNA components detected in Figure 33 were followed. Plants four and a half days old were labelled for one half to three hours in vivo by direct application of $^{32}$P orthophosphate to the leaves. Purified nucleic acids were fractionated on polyacrylamide gels,
Legend for Figure 34.

Primary leaves of plants grown for five days in the light were labelled with 120 $\mu$Ci $^{32}$P per leaf. Total nucleic acid was extracted from leaves at the times shown and samples fractionated on 2-2% gels for 3-5 hours. The gels were sliced and the radioactivity in each RNA peak was determined.

**Molecular weight of RNA $\times 10^{-6}$**

- $\blacksquare-\blacksquare$ 2.9 - 2.5
- $\square-\square$ 1.45
- $\circ-\circ$ 1.3
- $\bullet-\bullet$ 0.7
- $\blacktriangle-\blacktriangle$ 1.0
- $\triangle-\triangle$ 0.45
- $\blacklozenge$ 1.1
- $\blacklozenge$ 0.56
Figure 34.

The kinetics of labelling of leaf RNA components.

(Legend opposite)
and the radioactivity in each RNA component was determined. The results are shown in Figure 34. It was not possible to completely separate the $2.9 \times 10^6$ and $2.5 \times 10^6$ molecular weight RNA, and the total radioactivity in this region of the gel was determined. After very short labelling times the majority of the radioactivity was in the $2.9$ and $2.5 \times 10^6$ molecular weight RNAs although radioactivity was always detectable in the $1.45$, $1.0$ and $0.48 \times 10^6$ molecular weight components. The amount of radioactivity in the $2.9$ and $2.5 \times 10^6$ molecular weight RNAs did not continue to increase with time but levelled off after about three hours. In contrast the $1.3$ and $0.7 \times 10^6$ molecular weight cytoplasmic rRNAs initially labelled slowly but continued to accumulate label during longer incubations. The labelling rate of the $1.45 \times 10^6$ molecular weight RNA was intermediate between that of the $2.9$ and $2.5 \times 10^6$ molecular weight RNAs and the cytoplasmic rRNAs. The amount of radioactivity present in the $1.0$ and $0.45 \times 10^6$ molecular weight RNAs represented only a small percentage of the total radioactivity in all the preparations examined. Chloroplast rRNA labelled very slowly. Radioactivity was first detected in these RNAs after three hours.

**$^{32}$P base compositions**

The base compositions of the RNA components shown in Figure 33 were determined by elution and hydrolysis of $^{32}$P labelled RNA from the appropriate gel slices followed by electrophoresis and quantitative estimation of the nucleotides. The $^{32}$P base compositions of RNA vary with the length of the incubation in $^{32}$P orthophosphate. Table 4 shows the $^{32}$P base compositions of the $1.3$ and $0.7 \times 10^6$ molecular weight rRNAs labelled for different times. The GMP/AMP ratio determined
<table>
<thead>
<tr>
<th>Labelling time (hours)</th>
<th>1.5</th>
<th>5</th>
<th>60</th>
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</thead>
<tbody>
<tr>
<td>CMP Moles percent.</td>
<td>20.9</td>
<td>21.6</td>
<td>23.0</td>
</tr>
<tr>
<td>AMP Moles percent.</td>
<td>30.1</td>
<td>27.0</td>
<td>24.5</td>
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<tr>
<td>GMP Moles percent.</td>
<td>29.6</td>
<td>31.2</td>
<td>32.4</td>
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<tr>
<td>UMP Moles percent.</td>
<td>19.4</td>
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<tr>
<td>Ratio GMP/AMP</td>
<td>0.98</td>
<td>1.15</td>
<td>1.32</td>
</tr>
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</table>

1.3 x 10^6 molecular weight rRNA

<table>
<thead>
<tr>
<th>Labelling time (hours)</th>
<th>1.5</th>
<th>5</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP Moles percent.</td>
<td>20.1</td>
<td>21.2</td>
<td>20.2</td>
</tr>
<tr>
<td>AMP Moles percent.</td>
<td>27.4</td>
<td>26.4</td>
<td>24.5</td>
</tr>
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<td>GMP Moles percent.</td>
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<tr>
<td>UMP Moles percent.</td>
<td>24.4</td>
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<td>25.1</td>
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<tr>
<td>Ratio GMP/AMP</td>
<td>1.03</td>
<td>1.1</td>
<td>1.23</td>
</tr>
</tbody>
</table>

0.7 x 10^6 molecular weight rRNA

Table 4. ³²P base compositions of rRNA labelled for different lengths of time

CMP = cytidine monophosphate
AMP = adenosine monophosphate
GMP = guanosine monophosphate
UMP = uridine monophosphate
Table 5. $^{32}$P base compositions of pulse labelled RNA fractions

RNA was labelled for 1½ or 3 hours. Two or three peak fractions were analysed for each molecular weight.

*Number of determinations

$\text{CMP} = \text{cytidine monophosphate}$  
$\text{AMP} = \text{adenosine monophosphate}$  
$\text{GMP} = \text{guanosine monophosphate}$  
$\text{UMP} = \text{uridine monophosphate}$
after 60 hours incubation is characteristic of plant rRNAs. The GMP/AMP ratio of the rRNAs is lower during shorter incubations. When a correction is applied for the small fraction of AMP-rich polydisperse RNA contaminating the samples this is insufficient to explain this discrepancy. It presumably results from asymmetric labelling of the triphosphate pools during short pulses. The $^{32}$P base compositions of pulse labelled RNA have been compared with those of rRNA labelled for the same length of time. The results are shown in Table 5.

The 2.9 and $2.5 \times 10^6$ molecular weight RNAs have base compositions similar to the 1.3 and $0.7 \times 10^6$ molecular weight cytoplasmic rRNAs. The $1.45 \times 10^6$ molecular weight RNA has a base composition almost identical to that of the $1.3 \times 10^6$ molecular weight and RNA. The composition of the 1.0 and $0.45 \times 10^6$ molecular weight RNAs is more like that of the polydisperse RNA than that of rRNA. The short half life of these components makes it difficult to obtain samples that are not contaminated with polydisperse RNA of a similar molecular weight. For this reason the figures for the 1.0 and $0.45 \times 10^6$ molecular weight RNAs are probably not accurate. However the indication is that the $0.45 \times 10^6$ molecular weight RNA has a very low GMP/AMP ratio and the $1.0 \times 10^6$ molecular weight RNA has a GMP/AMP ratio intermediate between that of the $0.45 \times 10^6$ molecular weight RNA and rRNA.

The results of the $^{32}$P base composition analysis suggest that at least some of the rapidly labelled RNA components are precursors to cytoplasmic rRNA. The fact that chloroplast rRNA does not become labelled for several hours after cytoplasmic rRNA supports this conclusion (Figure 33). However the base
Table 6. Comparison of $^{32}$P base compositions of chloroplast and cytoplasmic rRNAs

Leaves were labelled *in vivo* for 60 hours with $^{32}$P orthophosphate. Total RNA was extracted and fractionated on gels. Peak fractions from each rRNA were analysed.

<table>
<thead>
<tr>
<th></th>
<th>Moles percent.</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMP</td>
<td>AMP</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>1.3</td>
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<tr>
<td>Cytoplasmic</td>
<td>0.7</td>
<td>20.2</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>0.56</td>
<td>23.1</td>
</tr>
</tbody>
</table>

Molecular weight of rRNA $\times 10^{-6}$

- Cytoplasmic: 1.3
- Chloroplast: 1.1
- Cytoplasmic: 0.7
- Chloroplast: 0.56

**Legend:**
- CMP = cytidine monophosphate
- AMP = adenosine monophosphate
- GMP = guanosine monophosphate
- UMP = uridine monophosphate
compositions of chloroplast rRNA are similar to those of cytoplasmic rRNA when samples from leaves labelled for 60 hours are compared (Table 6). This makes it difficult to distinguish the two types of leaf rRNA by base composition analysis, particularly when the results of the nearest neighbour analysis of pulse labelled RNA appear to depend on the pool sizes within the cell which may vary from one subcellular compartment to another.

Heterogeneity and multiplicity of pre rRNAs

From a consideration of the labelling kinetics and $^{32}$P base compositions it was concluded that the rapidly labelled RNAs migrating in the $2.9-2.5 \times 10^6$ molecular weight region of the gels were precursors to rRNA. The experiments with leaves suggested that there were at least two precursors but there were indications that there might be others. Firstly it was not possible to separate the $2.9$ and $2.5 \times 10^6$ molecular weight pre rRNAs as completely as one might expect if they were two discrete bands in the gel. Secondly the properties of the $0.45 \times 10^6$ molecular weight RNA detected in Figure 33 suggested that it might be an excess piece of RNA cleaved from one end of a pre rRNA. The absence of the $0.45 \times 10^6$ molecular weight RNA in some cases (e.g. see Figure 35) suggested that the parent pre rRNA was also absent. Thirdly the molecular weight estimates of the largest pre rRNA varied with RNA preparations from roots, hypocotyls and leaves of different ages. These differences were very small, about six per cent, which probably approaches the limit of sensitivity of molecular weight estimations by the gel method. For these reasons a comparison of the pre rRNAs from different regions of the plant and from leaves of different ages was carried out by fractionating $^{32}$P and
Legend for Figure 35.

For labelling of the leaves, seedlings were germinated in the dark for 3 days and then illuminated for 24 hours. 120 µCi of $^{32}$P orthophosphate in 10 µl of sterile distilled water was applied directly to each of the primary leaves. After 1·5 hours the leaves were cut off, washed in water and mixed with labelled roots for RNA extraction. For labelling of the roots, seedlings were germinated for 30 hours, when the roots were about 1·5 cm. long. 1 mCi $^3$H uridine in 2 ml steril water was injected into the sand around the roots. After 1·5 hours the seedlings were washed in water, the apical 1 cm of the roots cut off and mixed with labelled leaves and nucleic acids extracted. DNA was removed by deoxyribonuclease treatment. Electrophoresis was in a 9 mm diameter 2.4% gel for 4·5 hours. RNA from 2 leaves and 4·5 roots was loaded onto the gel.

--- Absorbance at 265 nm.

--- $^{32}$P (leaf RNA).

--- $^3$H (root RNA).

The $^3$H counts are corrected for 5% spill-over from $^{32}$P.
Figure 35. Comparison of pulse labelled nucleic acids from leaves and roots.

(Legend opposite)
\( {\text{\( ^3\)H}} \) labelled RNA from different sources in the same gel.

Figure 35 shows \( {\text{\( ^32\)P}} \) labelled RNA from leaves and \( {\text{\( ^3\)H}} \) labelled RNA from roots fractionated together. The radioactivity scan of the leaf RNA shows the components detected previously (Figure 33) including the pre rRNA of molecular weight \( 2.9 \times 10^6 \). A comparison with the \( {\text{\( ^3\)H}} \) labelled root RNA shows that the mobility of the largest root pre rRNA is slightly greater than that of the leaf and has an apparent molecular weight of \( 2.7 \times 10^6 \). In addition the \( 0.45 \times 10^6 \) molecular weight RNA at about 6.5 cm in Figure 35 is detected only in the \( {\text{\( ^32\)P}} \) leaf RNA. The \( 1.45 \) and \( 1.0 \times 10^6 \) molecular weight RNAs are common to both preparations from leaves and roots. Similar results were obtained in a number of separate experiments where the conditions of growth of the plants the labelling of the leaves and roots and the extraction and purification of the RNA were varied. The result shown in Figure 35 was obtained when leaves and roots were mixed prior to RNA extraction and purification and so the difference in mobility of the largest pre rRNA is unlikely to be due to a variation in the conditions of the extraction procedure, e.g. preferential attack of one RNA preparation by ribonuclease or a variation in salt concentration. When leaves were labelled with \( {\text{\( ^3\)H}} \)-uridine and the roots with \( {\text{\( ^32\)P}} \)-orthophosphate the result was the same (Figure 36) eliminating possible differences in labelling between the two isotopes.

The possibility that the different mobilities of the pre rRNAs from leaves and roots results from a conformational difference was examined by coelectrophoresing mixed RNA preparations in buffers of different composition. The results are shown in Figure 36. In buffers containing a low
Legend for Figure 36.

Leaves were labelled with 65 μCi $^3$H uridine per leaf for 1·5 hours. Roots were labelled by placing intact washed seedlings in $^{32}$P orthophosphate (3 mCi per ml) for 1·5 hours. RNA was extracted separately from leaves and roots and the preparations mixed before deoxyribonuclease treatment.

Samples equivalent to two roots plus two leaves were loaded onto 2·4% 0·9 mm diameter gels and electrophoresis was as follows:

a) Low salt buffer 6·5 hours
b) E buffer 4·5 hours
c) Magnesium buffer 6·5 hours.

Other details are as in the legend to Figure 35. Only the precursor region of the gel is shown.

--- $^3$H radioactivity (leaf RNA).
--- $^{32}$P radioactivity (root RNA).
Figure 36.

Comparison of pre tRNAs from leaves and roots under different conditions of electrophoresis.

(Legend opposite)
concentration of sodium the width of the pre rRNA bands is
greater (Figure 36a) than in the normal buffer system (Figure 36b).
This is in sharp contrast to the situation in a buffer containing
magnesium where the pre rRNAs are more distinct and separation of
the $2.9 \times 10^6$ molecular weight pre rRNAs is achieved
(Figure 36c). The ionic environment of the RNA clearly has a
pronounced effect on its mobility. In magnesium one would
expect the molecule to have a high degree of secondary structure
and the peaks are very sharp which is consistent with this view.
The diffuse peaks obtained by electrophoresis in a buffer of low
ionic strength without magnesium are consistent with the view
that under these conditions the RNA molecules unfold and adopt a
number of alternative conformations each with a slightly altered
mobility. The fact that under all conditions of electrophoresis
examined the differences between the $2.9$ and $2.7 \times 10^6$ molecular
weight pre rRNAs from roots and leaves are maintained strongly
suggests that these are indeed two distinct molecular species.

An examination of the pre rRNAs from hypocotyls shows that
three pre rRNAs can be detected (Figure 37). The molecular
weights are $2.9$, $2.7$ and $2.5 \times 10^6$. The root pre rRNAs
coelectrophoresed with the hypocotyl RNA appear very similar to
the $2.7$ and $2.5 \times 10^6$ molecular weight pre rRNAs. The
demonstration that three pre rRNAs could be detected in one RNA
preparation prompted a detailed examination of the situation in
leaves for it had been noted that a pre rRNA with a slightly
lower mobility on gels and with an apparent half life greater
than other leaf pre rRNAs was synthesised in leaves grown in the
light (Figure 32).

The pre rRNAs from young leaves of plants germinated and
Legend for Figure 37.

Labelling of hypocotyls: plants were grown for 2 days in darkness followed by 2 days in continuous light. By this time the hypocotyl hook was unfolded and hypocotyls were about 3 cm long. Segments 2-5 cm long were excised and incubated in $^3$H uridine (1 mCi per ml) for 1-5 hours. Labelling of roots was as described in the legend to Figure 35.

Nucleic acids were extracted from each tissue separately and treated with deoxyribonuclease. RNA from 5 hypocotyl sections and 3 roots was mixed prior to loading onto a 2.4% 0.9 mm diameter gel. Electrophoresis was for 5.5 hours. Only the first 4 cm of gel are shown.

--- $^{32}$P (root RNA).
--- $^3$H (hypocotyl RNA).

The $^3$H counts are corrected for 7% spill-over of $^{32}$P into the $^3$H channel.
Figure 37.

Comparison of pre-RNAs from hypocotyl and roots.

(Legend opposite)
Legend for Figure 38.

Plants were germinated for 60 hours in the dark. One batch was labelled for 1.5 hours with $^{32}$P (----). The remaining plants were illuminated for 24 hours and then labelled for 1.5 hours with $^{3}$H uridine (-----). After nucleic acid extraction and purification the samples were mixed and treated with deoxyribonuclease. Electrophoresis was in a 2.4% gel for 4.5 hours. The gel was sliced and $^{3}$H and $^{32}$P radioactivity measured. About 0% of the $^{32}$P was registered in the $^{3}$H channel. Only the first 3 cm of the gel are shown.
Figure 38.

Comparison of pre rRNAs from green and etiolated leaves. (Legend opposite)
grown in the dark for two and a half days were compared with those from leaves of plants three and a half days old which had previously been introduced into the light for one day. The results are shown in Figure 38. Both RNA preparations appear to contain all three precursors noted previously. In young leaves the $2.7 \times 10^6$ molecular weight pre rRNA is the most highly labelled. In light grown leaves on the other hand the $2.9 \times 10^6$ molecular weight pre rRNA is the most prominent.

**Methylation**

When leaves of light grown plants were labelled with $^3$H-methyl methionine radioactivity was incorporated into the $1.3$ and $0.7 \times 10^6$ molecular weight rRNAs. Studies with other eucaryotes show that the rRNA is methylated with most of the methyl groups on the ribose (see Introduction). The presence of 2-0-methyl ribose yields stable dinucleotides following alkaline hydrolysis because the methyl group prevents formation of the cyclic phosphate intermediate in hydrolysis. Experiments performed with the help of Dr. K. Murray show that similar dinucleotides are present in *P. aureus* cytoplasmic rRNA.

Purified $1.3$ and $0.7 \times 10^6$ molecular weight cytoplasmic rRNAs were subjected to hydrolysis in $0.2N$ potassium hydroxide at $37^\circ C$ overnight. The pH of the digest was adjusted to pH 8.5 with hydrochloric acid and bacterial alkaline phosphatase was added. After one hour at $37^\circ C$ the phosphatase was inactivated at pH 2 with EDTA. The mixture was adjusted to pH 5.5 and treated with $\delta$-labelled $^{32}P$ ATP and polynucleotide kinase. This labelled dinucleotides and trinucleotides. The digest was subjected to electrophoresis at pH 3.5 on AE paper followed by electrophoresis in a second dimension on DEAE paper at pH 2.
Autoradiography detected several radioactive spots in positions characteristic of dinucleotides but the identity and composition of these molecules has not been established by chemical analysis. The results are consistent with the view that $^{3}$H-labelled methyl groups from methionine are incorporated into cytoplasmic rRNA as 2-O-methyl ribose but they do not exclude the possibility that methylation also occurs on the bases.

Radioactivity from $^{3}$H-methyl methionine was incorporated into pre rRNAs and cytoplasmic rRNA although the peaks of radioactivity in the pre rRNAs were never as prominent as they were when $^{3}$H-uridine or $^{32}$P-orthophosphate was used. It is possible that at least one of the pre rRNAs is not methylated to the same extent as rRNA but this is not certain at the present time.

**Competition hybridisation studies**

The relationship between the pre rRNAs and cytoplasmic 1.3 and 0.7 x $10^6$ molecular weight rRNAs was investigated by competition hybridisation experiments. The "two-step" procedure was used under conditions that discriminate between the 0.7 and 1.3 x $10^6$ molecular weight rRNAs from *P. aureus*, as described in Materials and Methods. The pulse labelled RNA used in these experiments was obtained from leaves of seedlings germinated in the dark for two and a half days prior to the addition of $^{32}$P orthophosphate. The labelling with isotope was performed in the light which increases the amount of isotope taken up by the leaf and incorporated into RNA (Section B). The predominant pre rRNA labelled under these conditions has a molecular weight of about 2.7 x $10^6$. The 2.5 x $10^6$ molecular
Legend for Figure 39.

Each filter contained 5 \( \mu \)g of DNA. For the blocking step filters were incubated at 70°C in 2 x SSC for 2 hours at the rRNA concentrations shown (\( \mu \)g per ml). After ribonuclease treatment filters were incubated in \( ^{32} \)P test RNA at 66°C in 6 x SSC for 1·5 hours.

The radioactive RNA was prepared by labelling 20 leaves of 2·5 day old plants with 20 mCi \( ^{32} \)P orthophosphate in vivo for 2 hours in the light. The RNA was extracted, treated with deoxyribonuclease and samples loaded onto 2·0·9 mm diameter 2·4% gels. Electrophoresis was for 5 hours. The gels were sliced, pre rRNAs located by Cerenkov counting and recovered by elution into 2 x SSC, 0·2% sodium dodecyl sulphate followed by alcohol precipitation. The samples were dissolved in 3 ml 6 x SSC. There was insufficient RNA to measure the specific radioactivity. The total Cerenkov radiation from the test solutions was 30,700 cpm for the 2·7 x 10^6 molecular weight pre rRNA and 23,200 cpm for the 2·5 x 10^6 molecular weight pre rRNA.

a) 2·7 x 10^6 molecular weight pre rRNA.
b) 2·5 x 10^6 molecular weight pre rRNA.

- 2:1 mixture S. pombe rRNA.
- 0·7 x 10^6 molecular weight cytoplasmic rRNA.
- 1·3 x 10^6 molecular weight cytoplasmic rRNA.

100% hybridisation = 400 cpm.
Figure 39.
Competitive hybridisation of pre rRNAs with cytoplasmic rRNAs.
Figure 40.

Thermal stability of hybrids between DNA and pre rRNAs.

- $2 \times 10^6$ molecular weight pre rRNA.
- $2 \times 7 \times 10^6$ molecular weight pre rRNA.

The control hybrids from Figure 39 were used. The Tm is 74-75°C (0.1 x SSC).
<table>
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<td>52</td>
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Table 7. Competitive Hybridisation Data: Comparison of the observed results with those expected for polycistronic precursor molecules.
weight pre rRNA is also labelled but the $2.9 \times 10^6$ molecular weight pre rRNA is present only in small amounts (Figure 38).

The results are shown in Figure 39. The $1.3$ and $0.7 \times 10^6$ molecular weight cytoplasmic rRNAs from *P. aureus* each blocked hybrid formation by both pre rRNA fractions tested. The rRNA from *S. pombe* did not compete in the reaction. It is inevitable that the pre rRNA fractions used in these experiments contain those species of polydisperse RNA with a similar molecular weight to the pre rRNAs. Although the conditions of hybridisation of the test RNA favour hybrid formation between nucleic acid molecules present in multiple copies, hybridisation of a significant part of the polydisperse RNA would affect the results shown here. The nature of the hybrids formed was investigated by examining their thermal stability. The melting curves of the hybrids are shown in Figure 40. These show a comparatively sharp transition with a Tm of about $74^\circ\mathrm{C}$. This compares with a Tm of about $78^\circ\mathrm{C}$ for a hybrid between $1.3 \times 10^6$ molecular weight rRNA and ribosomal DNA (Figure 42). The differences in Tm may be due to the different base compositions of the RNAs (Table 5). The results of the competition hybridisation are compared with those expected for polycistronic precursor RNAs of molecular weight $2.7$ and $2.5 \times 10^6$ in Table 7. The experimental results are taken from Figure 39 and the predicted results are those for pre rRNAs of molecular weight $2.7$ and $2.5 \times 10^6$ each assumed to contain one complete sequence of $1.3$ and $0.7 \times 10^6$ molecular weight rRNA and $0.7$ or $0.5 \times 10^6$ molecular weight non-rRNA respectively. The experimental results are in good agreement with the calculated figures.
Hybridisation of cytoplasmic rRNAs to DNA fractions from a caesium chloride gradient.

DNA fractions (0.5 ml) from a preparative gradient were loaded onto filters and the discs cut exactly into 2 halves. Each set of half-discs was incubated in a solution of 1.3 or 0.7 x 10^6 molecular weight ^32P cytoplasmic rRNA (1.5 µg per ml) in 6 x SSC for 2 hours at 68°C.

The specific radioactivity of the RNA was 100,000 Cerenkov cpm per µg.

- - - 1.3 x 10^6 molecular weight rRNA.
- - - - - 0.7 x 10^6 molecular weight rRNA.
- - - - - - Absorbance 260 nm.
Figure 42.

Thermal stability of hybrid between rRNA and ribosomal DNA.

The hybrid between DNA from fraction 13 of Figure 41 and $1 \times 10^6$ molecular weight cytoplasmic rRNA was used.

The Tm is 77-78°C (0.1 x SSC).
Table 8. Hybridisation of various pulse-labelled RNA fractions to ribosomal and main band DNA.

RNA fractions corresponding to the molecular weight shown were eluted into 3x S.S.C. containing 0.2% sodium dodecyl sulphate. The RNA solutions were adjusted to a final volume of 1.5 ml 6x S.S.C. Nitrocellulose filters containing DNA were incubated in the RNA solutions for one hour at 68 C. For ribosomal DNA the heavy fractions (corresponding to the peaks of hybrid formation shown in Figure 41) from three preparative gradients were pooled. Each test filter contained 1/11 of this DNA. For main band DNA the fractions from one preparative gradient were pooled. Each test filter contained 1/11 of this DNA. The filters were treated with ribonuclease, dried and counted in toluene.
RNA-DNA hybridisation studies with the genes for rRNA

The DNA sequences complementary to cytoplasmic rRNA can be separated from the bulk of the DNA by buoyant density centrifugation of *P. aureus* DNA in a gradient of caesium chloride. Hybridisation of purified 1.3 or 0.7 x 10^6 molecular weight cytoplasmic rRNA to various DNA fractions from the gradient shows that the sequences complementary to these rRNAs are denser (approximate density 1.703 g/ml) than main band DNA (approximate density 1.696 g/ml). This result is shown in Figure 41. Both cytoplasmic rRNAs hybridise to DNA sequences of the same buoyant density. The hybrids show a high degree of thermal stability. The Tm of the hybrid formed by the 1.3 x 10^6 molecular weight cytoplasmic rRNA is about 78°C (Figure 42).

The ability of a number of pulse labelled RNA fractions to form hybrids with ribosomal DNA and main band DNA was examined in an attempt to gain further information about the function of these RNAs. The results are shown in Table 8. All the fractions tested hybridised preferentially to ribosomal DNA, including RNA from regions of the gel where no peaks were detected (molecular weight 4.5 and 0.3 x 10^6). The nature of the hybrids formed was examined by determining their thermal stability. Figure 43 shows the melting curves of hybrids with ribosomal DNA and main band DNA compared with the melting curve of the 1.3 x 10^6 molecular weight cytoplasmic rRNA hybridised to ribosomal DNA under the same conditions. The results show that the 2.9-2.7, 1.45, 1.0 and 0.45 x 10^6 molecular weight RNA fractions hybridise more to ribosomal DNA than to main band DNA. The hybrids with main band DNA do not show such a sharp transition. The melting curves of the hybrids with ribosomal DNA are very sharp and resemble those obtained with cytoplasmic rRNA/ribosomal
Legend for Figure 43.

Molecular weight of RNA x $10^{-6}$

a) $2.9 - 2.7$

b) $1.45$

c) $1.0$

d) $0.45$

- ribosomal DNA
- main band DNA
- $1.3 \times 10^6$ molecular weight RNA - ribosomal DNA hybrid (Figure 42) normalised for comparison.

The hybrids from the experiment shown in Table 8 were used.
Figure 43.

Thermal stability of hybrids between rapidly labelled RNA fractions and ribosomal and main bond DNA. (Legend opposite)
DNA hybrids. The curve for the $1.45 \times 10^6$ molecular weight RNA (Figure 43b) indicates a slightly higher Tm than with rRNA. This suggests that this RNA may have a higher content of guanosine and cytosine compared to the $1.3 \times 10^6$ molecular weight rRNA. This conclusion is not supported by the $^{32}$P base composition analysis of these RNAs (Table 5).

When chloroplast and cytoplasmic rRNAs were hybridised to DNA from a preparative cesium chloride gradient the two types of rRNA hybridised to DNA fractions from the same region of the gradients (Figure 44). The peaks of hybrid formation are rather broad suggesting the possibility that the rRNAs are hybridising to DNA with a range of buoyant densities. The melting curves of the hybrids formed between chloroplast and cytoplasmic rRNA and DNA from fractions 9 and 11 in Figure 44 were compared (Figure 45). Cytoplasmic rRNA hybridised to DNA from fraction 9 (Figure 45c) and chloroplast rRNA hybridised to fraction 11 (Figure 45a) gave curves similar to that for $1.3 \times 10^6$ molecular weight cytoplasmic rRNA shown in Figure 42. In each case the Tm is about $75^\circ$C. The curves for chloroplast rRNA hybridised to DNA from fraction 9 (Figure 44b) and cytoplasmic rRNA hybridised to DNA from fraction 11 (Figure 44a) are slightly different. In each case part of the hybrid dissociates at a relatively low temperature and the Tm is lower. This is particularly pronounced in Figure 44d where the Tm is about $68^\circ$C. It seems that chloroplast and cytoplasmic rRNAs do not form identical hybrids with DNA from different positions in the gradient. If it is assumed that the sharpness of the melting curves is an indication of the accuracy of base pairing in the hybrid the results suggest that there may be two types of DNA capable of forming hybrids with rRNA. These can be distinguished
Legend for Figure 44.

DNA fractions (0.3 ml) from a preparative gradient were loaded onto filters and the discs cut exactly into 2 halves. Each set of discs was incubated in either chloroplast or cytoplasmic $^{32}$P RNA. Hybridisation was at 68°C in 6 x SSC for 2 hours.

The RNA concentration was 5 µg per ml of an equimolar solution of chloroplast or cytoplasmic rRNA. The specific radioactivity was 40,000 cpm per µg.

--- Absorbance 260 nm.

--- Cytoplasmic rRNA.

--- Chloroplast rRNA.
Figure 44. Hybridisation of cytoplasmic and chloroplast rRNAs to DNA fractions from a cesium chloride gradient (Legend opposite)
Legend for Figure 45.

The RNA hybrids with DNA fractions 9 and 11 from Figure 44 were used.

a) Chloroplast rRNA hybridised to DNA fraction 11.

b) Chloroplast rRNA hybridised to DNA fraction 9.

c) Cytoplasmic rRNA hybridised to DNA fraction 9.

d) Cytoplasmic rRNA hybridised to DNA fraction 11.
Figure 45.

Thermal stability of hybrids between chloroplast and cytoplasmic rRNAs and ribosomal DNA fractions from caesium chloride gradients. (Legend opposite)
because one type of DNA (fraction 9 in Figure 4) forms better hybrids with chloroplast rRNA than with cytoplasmic rRNA and vice versa.

**Discussion**

Rapidly labelled RNA can be divided into two classes. The first class is heterogeneous in size and the largest molecules have molecular weights in excess of $7 \times 10^6$. The GMP:AMP ratio of this RNA is about 0.6-0.7 (Table 5) and the composition does not vary much with molecular weight. This compares with a GMP:AMP ratio for DNA (computed from the buoyant density in caesium chloride) of 0.56. Presumably at least part of this RNA is mRNA. The second class of RNA molecules appear as distinct peaks superimposed on the background of polydisperse RNA in polyacrylamide gels. Six components are synthesised in leaves; these have molecular weights of 2.9, 2.7, 2.5, 1.45, 1.0 and $0.45 \times 10^6$ (Figures 33 and 38). Roots synthesise all these molecules except the 2.9 and $0.45 \times 10^6$ molecular weight RNAs (Figure 35) and leaves grown in the light synthesise more of the $2.9 \times 10^6$ molecular weight RNA than young leaves grown in the dark.

The kinetic data (Figure 34) suggest that the 2.9, 2.7 and $2.5 \times 10^6$ molecular weight RNAs are macromolecular precursors to rRNA and the hybridisation experiments support this conclusion. The $^{32}P$ base compositions of these fractions show that they are more like rRNA than polydisperse RNA (Table 5). Variation in $^{32}P$ base composition with labelling time (Table 4) is presumably due to differences in the relative specific radioactivities of the nucleoside triphosphate pools during short and long term incubations in $^{32}P$ orthophosphate. Compartmentalisation of the nucleoside triphosphate pools resulting in a slower increase in
specific radioactivity of the ATP pool relative to the pools of GTP, UTP and CTP would lead to the overestimation by the nearest-neighbour analysis of adenylic acid relative to the other nucleotides in samples labelled for a short time. (This assumes a random distribution of nucleotides. If the adenylic acid residues are clustered overestimation would not occur.) Whatever the explanation the observed results raise doubts about the absolute accuracy of the method. Nevertheless it is reasonable to compare \$^{32}\text{P}\$ base compositions of RNA fractions labelled for the same time. When this is done RNA from the 2.9-2.5 x 10\(^6\) molecular weight region is found to be similar but not identical to cytoplasmic rRNA (Table 5). A similar comparison with chloroplast rRNA cannot be made for it is not significantly labelled for several hours (Figure 34). The 2.9 and 2.5 x 10\(^6\) molecular weight fractions from gels are almost certainly contaminated with the 2.7 x 10\(^6\) molecular weight RNA. These fractions have almost identical base compositions (Table 5) suggesting that the 2.9, 2.7 and 2.5 x 10\(^6\) molecular weight RNAs are similar to each other in composition.

The fact that the 2.7 and 2.5 x 10\(^6\) molecular weight RNAs are synthesised in both leaves and roots suggests that they are precursors to cytoplasmic and not chloroplast rRNA. This conclusion is supported by the competitive hybridisation data which shows that both pre rRNAs contain sequences similar to the 1.3 and 0.7 x 10\(^6\) molecular weight cytoplasmic rRNAs. In Table 7 the results are compared with those predicted for each pre rRNA assuming a content of one sequence of the 1.3 and 0.7 x 10\(^6\) molecular weight rRNAs per molecule. The figures are in good agreement and this provides the first evidence that plant pre
rRNAs are polycistronic. The melting curves of the hybrids (figure 40) show a sharp inflection with a Tm of 74-75°C which is close to the Tm of 77-78°C for cytoplasmic rRNA/ribosomal DNA hybrids (Figure 42). This suggests that contaminating poly-disperse RNA does not significantly affect the result. About 28 per cent and 20 per cent respectively of the 2.7 and 2.5 x 10^6 molecular weight pre rRNAs is unlike rRNA. The results in Table 5 suggest that this excess RNA has a lower content of guanylic and cytidylic acid than rRNA. This would explain why the Tm of DNA/pre rRNA hybrids is slightly lower than that for DNA/rRNA hybrids.

The kinetics and base composition of the 2.9 x 10^6 molecular weight RNA suggest that this molecule is also a precursor to rRNA. This view is strengthened by the observation that it hybridises preferentially to ribosomal DNA compared to main band DNA (Table 8, Figure 43). However chloroplast and cytoplasmic rRNA both hybridise to heavy DNA (Figure 44) and so it is possible that the 2.9 x 10^6 molecular weight RNA is a precursor to either chloroplast or cytoplasmic rRNA. The similarity between the 32P base compositions of chloroplast and cytoplasmic rRNA makes it impossible to determine its function from its composition. The only evidence available is of an indirect nature. The 2.9 x 10^6 molecular weight pre rRNA is labelled a long time before chloroplast rRNA (Figure 34) and it is also synthesised in hypocotyls which contain very little chloroplast rRNA (Figure 37). This evidence is consistent with the view that it is a precursor to cytoplasmic rRNA. On the other hand the 2.9 x 10^6 molecular weight pre rRNA is synthesised in the light (Figure 38) and appears to have a much longer
half-life than the other pre rRNAs (Figure 32). The detection of the pre rRNA by optical density in RNA from light grown leaves is consistent with it having a relatively long half-life (Figure 24). On the basis of this evidence it could be argued that the $2.9 \times 10^6$ molecular weight pre rRNA was a precursor to chloroplast rRNA. The long half-life of the precursor would explain why chloroplast rRNA labels only very slowly in leaves, and it would also explain why it is detected in hypocotyls which synthesise very little chloroplast rRNA. Cell fractionation studies combined with a more detailed chemical study of this pre rRNA will be necessary before definite conclusions as to its function can be made.

The fact that the $2.7$ and $2.5 \times 10^6$ molecular weight pre rRNAs contain similar sequences of rRNA is consistent with the view that the larger molecule is a precursor to the smaller. It is difficult to obtain kinetic evidence for this firstly because the pre rRNAs are so similar in molecular weight and secondly because pulse-chase experiments are not really effective in leaves. In *S. pombe* where two large pre rRNAs are synthesised there is evidence from pulse-chase experiments that the larger molecule is converted to the smaller one (Grierson et al., 1970).

The time of labelling of the $1.45 \times 10^6$ molecular weight RNA suggests that it is a processing intermediate of one of the pre rRNAs (Figure 34). This molecule is synthesised in both leaves and roots (Figure 35) and it hybridises preferentially to ribosomal DNA (Figure 43). The $^{32}P$ base composition is almost identical to that of the $1.3 \times 10^6$ molecular weight cytoplasmic rRNA (Table 5) and this suggests that the $1.45 \times 10^6$ molecular
weight RNA is the immediate precursor to the larger cytoplasmic rRNA. Conservative processing of the $2.5 \times 10^6$ molecular weight pre RNA would give two RNA fragments of molecular weight 1.05 and $1.45 \times 10^6$ and the smaller molecule would contain the $0.7 \times 10^6$ molecular weight cytoplasmic rRNA sequence. The $1.0 \times 10^6$ molecular weight RNA detected in both leaves and roots (Figure 35) could be the other processing product. This molecule does hybridise preferentially to ribosomal DNA (Figure 43) but there is no direct evidence that it contains the $0.7 \times 10^6$ molecular weight cytoplasmic rRNA sequence. The kinetic data do not show a typical precursor-product relationship with the $0.7 \times 10^6$ molecular weight rRNA (Figure 34). This could be explained if the $1.0 \times 10^6$ molecular weight RNA had a very short half-life. In carrot (Leaver and Key, 1970) the $0.7 \times 10^6$ molecular weight rRNA is formed very rapidly and transported to the cytoplasm. A RNA component of molecular weight $0.9 \times 10^6$ found in nuclear preparations from pea roots accumulates during inhibition of pre rRNA processing (Rogers, Loening and Fraser, 1970), suggesting that it may be an intermediate in rRNA synthesis.

The $1.0$ and $0.45 \times 10^6$ molecular weight RNAs are distinguished from the breakdown products of the larger chloroplast rRNA firstly by the fact that they become labelled before chloroplast rRNA and secondly because newly labelled chloroplast rRNA does not fragment. The possibility that the $1.0$ and $0.45 \times 10^6$ molecular weight RNAs are mitochondrial rRNAs seems to be ruled out by the report by Harmey and Leaver that mitochondria from a number of higher plants including *P. aureus* have rRNAs of molecular weight $1.2$ and $0.7 \times 10^6$. 

Figure 46.

Suggested stages in processing of precursors to cytoplasmic rRNA.

The RNAs are referred to by their molecular weights in millions.

Solid regions indicate the cytoplasmic rRNA sequences.

The arrangement of the sequences within the 2.7 x 10^6 molecular weight pre rRNA is arbitrary; there are seven other possible arrangements.
Figure 47.

Possible role of $2.9 \times 10^6$ molecular weight pre rRNA in cytoplasmic rRNA synthesis.

Apart from the first processing step the model is identical to that shown in Figure 46.
The evidence that the 2.7, 2.5, 1.45 and 1.0 x 10^6 molecular weight RNAs represent stages in the biosynthesis of cytoplasmic rRNA is quite good. One possible relationship between these molecules is indicated in Figure 46. Further work will be necessary in order to confirm the stages of processing suggested here. The arrangement of the various RNA sequences in the 2.7 x 10^6 molecular weight RNA is arbitrary. There are seven other detailed structures possible.

The scheme shown in Figure 46 does not account for the 2.9 and 0.45 x 10^6 molecular weight RNAs. Hybridisation studies and the 32P base compositions suggest that the larger molecule may also be involved in rRNA synthesis. There is insufficient evidence to decide whether the 2.9 x 10^6 molecular weight pre rRNA is a precursor to cytoplasmic or chloroplast rRNA. From a consideration of the molecular weights it is possible that the 2.9 x 10^6 molecular weight RNA is converted to the 2.5 x 10^6 molecular weight pre rRNA by loss of a piece of RNA of molecular weight 0.45 x 10^6. If this were the case the 0.45 x 10^6 molecular weight RNA would be located at one end of the molecule. The properties of the 0.45 x 10^6 molecular weight RNA detected in leaves are consistent with this proposal. Firstly it hybridises preferentially to ribosomal DNA; secondly it has a low content of guanylic and cytidylic acid and the data in Table 5 suggest that the non-rRNA of the pre rRNAs is low in GC content; thirdly the RNA is rapidly labelled. Figure 47 shows how the 2.9 x 10^6 molecular weight pre rRNA could be involved in cytoplasmic rRNA synthesis. The scheme accounts for the differences between leaves and roots shown in Figure 35. It does predict tissue-specific transcription of the genes for
Models for the transcription of two different precursors to cytoplasmic rRNA.

The arrangement of the cistrons is the same as that shown for the RNA segments in the pre rRNAs in Figures 47 and 46.
cytoplasmic rRNA. This could result in one of two ways, outlined in Figure 48. Firstly two batches of genes for cytoplasmic rRNA could exist (B1 and B2) with one batch giving rise to the $2.9 \times 10^6$ molecular weight pre rRNA and the other to the $2.7 \times 10^6$ molecular weight pre rRNA. Alternatively different transcription products could be produced from identical genes by an alteration in the initiation or termination specificity of RNA polymerase (A). Such a model suggests that the excess RNA transcribed at one end of the pre rRNA is different in different tissues. It is possible that there are also different rRNA sequences but this is not an essential part of the model. A significant feature of this scheme is that it suggests how specific RNA molecules might be produced in relatively large amounts by a specific signal acting at the level of transcription. The concentration of this RNA and the sequence content could control other cellular activities.

The results shown in Figures 44 and 45 show that two types of ribosomal DNA can be distinguished, one forming a better hybrid with chloroplast rRNA and the other forming a better hybrid with cytoplasmic rRNA. The most probable explanation of these results is that chloroplast and cytoplasmic ribosomal DNA both band at positions denser than main band DNA. This has been shown to be the case in a number of plants: (Ingle et al., 1970) where reciprocal DNA/RNA hybridisation experiments show that both types of DNA hybridise to both chloroplast and cytoplasmic rRNA, although the DNAs have different buoyant densities. The apparent similarity between chloroplast and cytoplasmic rRNA judged by hybridisation experiments means that the role of a particular RNA fraction cannot be determined
simply on the basis of its hybridisation behaviour under these conditions. The more stringent conditions used in the competitive hybridisation experiments probably overcame this difficulty.
CONCLUSIONS
The RNA from leaves can be separated into a large number of distinct molecular species. The properties of these molecules are summarised in Table 9. The stable RNAs from chloroplast and cytoplasmic ribosomes constitute the major part of this RNA. The minor components include breakdown products and aggregates of rRNA and macromolecular precursors to rRNA. None of the RNAs has properties expected of a mRNA although several minor components from young leaves have not been characterised. The polydisperse rapidly labelled RNA may include mRNA.

Light stimulates cell division and chloroplast and cytoplasmic rRNA synthesis. The first detectable effect of light is on the processing rate of precursors to cytoplasmic rRNA suggesting that control operates at the post-transcriptional level. This does not rule out the possibility that control also operates at the transcription stage.

Cytoplasmic rRNA is first transcribed as a macromolecular polycistronic precursor which also contains non ribosomal RNA. The available evidence suggests that the excess RNA is discarded during a number of discrete processing stages leading to the production of stable rRNA.

A different precursor is synthesised in leaves grown in the light at the time chloroplast and cytoplasmic rRNA synthesis is increased. It is not possible to conclude which type of rRNA sequences it contains without further study because of the similar properties of chloroplast and cytoplasmic rRNA.
<table>
<thead>
<tr>
<th>Class</th>
<th>Molecular weight of RNA x 10(^{-6})</th>
<th>Function or properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rapidly labelled rRNAs</td>
<td>2.9</td>
<td>Precursor to rRNA, synthesised in leaves grown in the light. It is not clear whether it contains cytoplasmic or chloroplast rRNA sequences.</td>
</tr>
<tr>
<td></td>
<td>Present in small amounts and labelled during short pulses</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>2. Stable rRNAs</td>
<td>1.3, 0.7</td>
<td>Cytoplasmic rRNAs; these constitute 70-99% of leaf rRNA. They contain 'hidden breaks'.</td>
</tr>
<tr>
<td></td>
<td>Present in large amounts</td>
<td>1.1, 0.56</td>
</tr>
<tr>
<td>Class</td>
<td>Molecular weight of RNA x 10^6</td>
<td>Function or properties</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------------------------</td>
<td>------------------------------------------------------------</td>
</tr>
<tr>
<td>3. Aggregates</td>
<td>2.6</td>
<td>Aggregate of two molecules of 1.3 x 10^6 molecular weight cytoplasmic rRNA.</td>
</tr>
<tr>
<td>Artefacts produced by high RNA concentrations, high K⁺ concentrations or high temperatures. Not labelled during short pulses.</td>
<td>2.5</td>
<td>Aggregate of two molecules of 1.3 x 10^6 molecular weight cytoplasmic rRNA each having lost a small piece of RNA by heating - the 52,000 molecular weight fragment.</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>Aggregate of one molecule of 1.3 and one molecule of 0.7 x 10^6 molecular weight cytoplasmic rRNA.</td>
</tr>
<tr>
<td>4. In vivo breakdown products</td>
<td>0.9, 0.7, 0.5, 0.4, 0.2</td>
<td>Derived from chloroplast 1.1 x 10^6 molecular weight rRNA. Held together by Ig.</td>
</tr>
<tr>
<td>Not labelled during short pulses</td>
<td>0.65, 0.58</td>
<td>Functions unknown. Some of these may be mitochondrial rRNA.</td>
</tr>
<tr>
<td>5. Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detected in young leaves by optical density; present in small amounts</td>
<td>1.16, 1.0, 0.8, 0.65, 0.58</td>
<td></td>
</tr>
</tbody>
</table>


- 80 -
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The Synthesis of Ribosomal RNA in Different Organisms: Structure and Evolution of the rRNA Precursor

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The synthesis of ribosomal RNA (rRNA) provides at present the only system in higher organisms in which the activity of a gene and its product can be studied directly. It is the only case in which a gene of known function can be isolated (Birnstiel et al., 1966), the behavior of the corresponding chromosomal region can be studied cytologically (McClintock, 1934), and the direct product of transcription and its maturation and transport into the cytoplasm can be followed in great detail (Perry, 1967; Weinberg, Loening, Willems, and Penman, 1967; Darnell, 1968; Maden, 1970).

We have examined the rRNA precursor and the steps of its processing to rRNA in a range of different species and tissues. We hope that the differences found may give some idea of the function of the excess RNA which is lost during processing, and of the mechanisms controlling the synthesis of rRNA.

Previous work has shown that while the size (Loening, 1968) and to a lesser extent the sequence (Sinclair and Brown, 1969) of rRNA has been strongly conserved through evolution, the size of the precursor has not, and large differences can be found between higher and lower animals and even between closely related species of plants.

The molecular weight of the smaller rRNA component in almost all eucaryotes is identical, about 0.65 to 0.7 \times 10^6 daltons (Peterman and Pavlovic, 1966; McConkey and Hopkins, 1969; Loening, 1968). The larger rRNA in plants and most protozoa has a weight of 1.3 \times 10^6 and in lower animals 1.4 \times 10^6; this component has evolved slightly in size in the higher animals, to 1.5 \times 10^6 in Xenopus and 1.75 \times 10^6 in mammals (Loening, 1969). Thus the combined weights of the rRNA varies from 2.0 \times 10^6 in plants and protozoa to 2.45 \times 10^6 in mammals.

The molecular weight of the rRNA precursor, which contains one each of the rRNA sequences and some non-conserved excess RNA, is 2.3 to 2.9 \times 10^6 in plants, about 2.6 \times 10^6 in Xenopus, and 4.1 to 4.5 \times 10^6 in mammals (Loening, Jones, and Birnstiel, 1969; Weinberg et al., 1967). Birds have a slightly smaller rRNA precursor than mammals (including marsupials) (Perry et al., 1970). The amount of the excess non-ribosomal RNA is thus about 45% of the weight of the precursor in the mammals and birds, and 12 to 30% in the other animals and plants.

These measurements of molecular weight and stages of processing have been obtained by polyacrylamide gel electrophoresis of RNA, and one may question whether this gives valid molecular weight determinations for different types of RNA. We have previously presented evidence that, under suitable conditions, the secondary structure or conformation of the RNA does not lead to errors, and that the estimations are substantially correct (Loening, 1969).

As there are differences in the precursor in different organisms, one can examine the stages of processing and determine which components of the excess RNA have evolved. It is also reasonable to ask whether there are any differences between the tissues of one species which may be correlated with ribosome synthesis in stages of development.

**Processing of the Precursor RNA in Mammals**

The existence of a large amount of non-conserved RNA in the mammalian pre-rRNA has been demonstrated by a variety of different techniques (Weinberg et al., 1967; McConkey and Hopkins, 1969; Jeantur, Amaldi, and Attardi, 1968). The stages of processing and loss of the excess RNA pieces were investigated in detail by gel electrophoresis (Weinberg et al., 1967). Several nucleolar components were identified as follows: 45 S (4.3 \times 10^6) precursor, 41 S (3.3 \times 10^6), and 36 S and 24 S minor components, 32 S (2.2 \times 10^6) precursor to 28 S rRNA (1.7 \times 10^6), 20 S (1.0 \times 10^6) precursor to 18 S rRNA (0.7 \times 10^6). (The sedimentation coefficients are used only as names to identify the molecules; mean molecular weights are given in parentheses, determined by gel electrophoresis.) In a recent paper, Weinberg and Penman (1970) showed that the 41 S component, which accumulates during polio virus infection, contains both 28 S and 18 S rRNA as indicated by the content of 2'-O-methyl ribose. Thus the first
step in processing must be the loss of about $1.0 \times 10^6$ dalton of excess RNA, from 45 S to 41 S (4.3 to $3.3 \times 10^6$), and not the separation of the 18 S from 28 S RNA. The $1 \times 10^6$ excess RNA must therefore be at one end of the molecule. The finding limits the ways in which the minor 36 S and 24 S RNA can arise; it was suggested that these are the result of anomalous scission. The main stages of processing are represented in Diagram 1. The 41 S component is normally split, without loss of excess RNA, into the 32 S and 20 S precursors to rRNA, at A. Splitting at B would give a 36 S and 18 S rRNA, and at C would give 24 S and 28 S rRNA. Thus the 36 S and 24 S components could be the "result of cleavages which are topologically in the correct position but temporally out of order." (Weinberg and Penman, 1970.)

If this interpretation is correct, then it follows that the excess RNA in the 41 S component must be present as one piece in the middle of the molecule with the 18 S and 28 S at the outside, as drawn. It is not known which end of the 45 S precursor is synthesised first, and whether the $1.0 \times 10^6$ daltons of excess RNA which are lost from it are at the 5'- or 3'-end.

Comparison with other mammalian tissues. The nucleolar components found in HeLa cells have been compared with those of rodents. Rat liver nucleolar $^3$H-labeled RNA showed several differences from pulse $^{32}$P-labeled HeLa RNA (Loening, 1970). Figure 1 illustrates a similar difference between RNA of human fibroblasts and mouse myeloma cells in culture.

These results show that (a) the 18 S rRNA and its precursor 20 S (or $1.0 \times 10^6$) in human and rodent are identical; (b) the 28 S rRNA of human is slightly larger by gel electrophoresis; (c) the 32 S RNA has a weight of $2.0 \times 10^6$ in rodents, about $0.2 \times 10^6$ smaller than in man; (d) the rodents have a complex RNA component with a mean mol wt of about $2.7 \times 10^6$; the rat nucleolar RNA (Loening, 1970) showed that this consisted of three components; it compares to the 36 S of HeLa; (e) the 45 S RNA is similar in the two species, possibly the rodent one has a slightly lower mobility indicating a higher molecular weight.

Thus the two mammals differ in the components which are precursors to the 28 S rRNA and not in those to 18 S RNA. It is not possible to fit the apparent stages of processing in the rodents to a simple scheme as in the HeLa cell: the $2.7 \times 10^6$ component is not large enough to be equivalent to the 41 S of HeLa cells, but it could be cleaved to 32 S ($2.0 \times 10^6$) and $0.7 \times 10^6$ of excess RNA. The equivalent of 41 S would then have a weight of $3.7 \times 10^6$.

In all electrophoretic separations of the 45 S precursor, the scanned peak appears broader than expected for a single homogeneous component, suggesting some heterogeneity.

**Diagram 1.** The steps of processing of the rRNA precursor in the HeLa cell. The rRNA regions are indicated in black and the excess RNA in white. "28 S" RNA, which is associated with "28 S" and with "32 S" has been omitted from the diagram. (Adapted from Weinberg and Penman, 1970.)
RIBOSOMAL RNA SYNTHESIS

Figure 1. Comparison of human and mouse pulse-labeled RNA. Human fibroblast cells in monolayer culture were labeled with $^{3}$H-uridine (80 $\mu$Ci/ml) for 2.5 hr. Mouse myeloma cells in suspension culture (strain 6A, kindly given by Prof. G. von Ehrenstein) were labeled with $^{32}$P (about 1 mc/10 ml) for 3.5 hr. RNA was extracted and DNA digested as described previously (Loening et al., 1969). About $\frac{1}{5}$ of the RNA from 1 dish of fibroblast and that from 10 ml of myeloma culture was used for electrophoresis on a 2.0% polyacrylamide gel (10 cm x 9 mm) at 50 v for 4 hr. The gel was scanned at 265 my; the positions of the 28 S and 18 S rRNA peaks are indicated. The gel was frozen and sliced to 0.5 mm slices on a Mickle gel slicer and the two isotopes counted in a scintillator after hydrolysis of the RNA; about 4% of $^{32}$P counts were registered in the $^{3}$H channel and no correction for this has been applied.

PROCESSING IN THE LOWER ANIMALS

The rRNA precursor in Xenopus was found to be 40 S (Gall, 1966; Landesman and Gross, 1969) with a mol. wt of $2.6 \times 10^6$ (Loening et al., 1969; Perry et al., 1970). Thus the amount of excess RNA appears to be only $0.4 \times 10^6$, or about one-fifth of that in mammals. It also appeared slightly heterogeneous, over a range of about $0.1 \times 10^6$. The immediate precursor to the larger rRNA component ($1.5 \times 10^6$) had a weight of $1.6 \times 10^6$; a minor component of about $1.7 \times 10^6$ was probably present. The 20 S precursor ($1.0 \times 10^6$) to the smaller rRNA ($0.7 \times 10^6$) was not seen in scans of total RNA preparations, but probably exists (Rogers, 1968), and has been described for other lower animals such as Drosophila (Edström and Daneholt, 1967).

The precursor RNA in a number of other lower animals has been described, with mol wt up to $2.9 \times 10^6$; the precursor to the larger rRNA was always $0.1 \times 10^6$ larger than the rRNA (Perry et al., 1970).

The simplest scheme of processing suggests that the $2.6 \times 10^6$ precursor is split to 1.6 and $1.0 \times 10^6$ components, which then lose 0.1 and $0.3 \times 10^6$ of excess RNA. The slight heterogeneity in Xenopus, or possible errors of about $0.1 \times 10^6$, and the slightly larger precursors found in some species, suggest that the precursor may first lose a small amount of excess RNA analogous to the loss from 45 S to give 41 S in the HeLa cell.

The rRNA and the precursors appear to be methylated as in mammals (Landesman and Gross, 1969; Perry et al., 1970).

The rRNA of amoeba (Acanthamoeba) differs from that of most other lower animals or Protozoa: both components are about $0.2 \times 10^6$ heavier, and the "28 S" rRNA is very unstable (Loening, 1968). We have found that the precursors, relative to the rRNA, are similar to those in the other lower animals.

THE PRECURSORS IN PLANTS

The rRNA precursor in plants varies in mol wt from about 2.3 to $2.9 \times 10^6$, relative to the total rRNA of $2.0 \times 10^6$ (Rogers, Loening, and Fraser, 1970; Leaver and Key, 1970; Retel and Planta, 1969; Sassella and Loening, in prep.). In most cases the molecule appears heterogeneous, and in some two components can be distinguished. In artichoke (Helianthus) tuber cultures the precursor was homogeneous with a weight of $2.3 \times 10^6$; in pea seedlings at least two components, about 2.4 and $2.3 \times 10^6$, could be distinguished (Rogers, Loening, and Fraser, 1970). In the carrot two distinct components, about 2.8 and $2.2 \times 10^6$, were seen (Leaver and Key, 1970) as also in two species of yeast, $2.6$ and $2.3 \times 10^6$ (Sassella and Loening, in prep.) or 2.9 and $2.4 \times 10^6$ (Retel and Planta, 1970). In bean leaves (Phaseolus aureus) as shown below, the largest precursor has a weight of about $2.9 \times 10^6$ with a shoulder of lower weight. Thus there are large differences in the weights of the precursor in closely related species.

In all plants, as in the lower animals, the immediate precursor to the $1.3 \times 10^6$ daltons rRNA, is $1.4 \times 10^6$. The precursor to the $0.7 \times 10^6$ rRNA was found in nuclear fractions of the artichoke tissue and is similar to that in HeLa cells. All the precursor components accumulated during 2-4 hr in excised pea root tips cultured in 2% sucrose, when processing is inhibited (Rogers et al., 1970).
We have shown that the precursors and rRNA in pea root tips are methylated by labeling with $^3$H-methyl methionine, and Retel and Planta (1970) have found the same for yeast.

**Heterogeneity of the Precursors**

In all the plant species the precursor components appear heterogeneous, even when two components can be detected. The immediate precursors to the rRNA, with mol wt of 1.4–1.45 and 1.0 × 10$^6$, are similar to those in the lower animals. Four situations may be considered which would result in apparently heterogeneous precursor components:

1. The true transcribed length may be the highest molecular weight detected; this may have a short lifetime compared to the products of processing; loss of excess RNA in a single step would yield the smaller component while gradual loss would lead to apparent heterogeneity.

2. If processing started before the synthesis of the molecule was complete, the heterogeneity would represent the unfinished molecules.

3. The transcribed lengths of the multiple copies of the ribosomal genes may not all be identical. There may be (a) variations in the termination or initiation sites, just as there is variability in the lengths of the non-transcribed DNA stretches (Miller and Beatty, 1969) or (b) each length may have multiple initiation or termination sites.

4. There may be some degradation of the molecule during isolation, or differences in conformation but not in molecular weight.

In the results reported previously for yeast, carrot, and pea, the shape of the precursor peak, or ratio of label in the two components, did not change with increasing times of labeling even during times when the amount of label was increasing rapidly (Rogers et al., 1970; Leaver and Key, 1970; Sassella and Loening, in prep.). This would suggest either that the transcribed lengths were different as in (3) above, or that the lifetimes of the precursors were short compared to the times required to increase the specific activities of the nucleotide pools appreciably.

Accordingly, we examined the labeling of the precursor in yeast during very short pulse incubations. Figure 2, a and b, shows the precursor in a preparation of total cell RNA, after labeling for 90 sec and 10 min. It is clear that the large component is labeled first; the trail of radioactivity to lower molecular weights presumably represents the growing chains and some heterogeneous RNA. The smaller (2.3 × 10$^6$) of the two components became labeled after about 2 min; the rRNA (and 1.4 × 10$^6$ precursor) was labeled within 4 or 5 min (not shown). It is probable then that the large precursor yields the smaller one by loss of about 0.3 × 10$^6$ of excess RNA, and that the lifetime of these components is comparable or not much greater than the time required to synthesize the chain. The 2.3 × 10$^6$ daltons component is thus a product of the first precursor (analogous to 41S in HeLa cells). The nature of the heterogeneity is not known, however, and it remains possible that there are small differences between the repeated cistrons.
DETECTION OF ONE OF THE EXCESS RNA PIECES

Figure 3 shows a separation of the RNA of 4 day old mung bean leaves, grown in the light and labeled with $^{32}$P for 1.5 hr. The precursors and rRNA are labeled, but the chloroplast rRNA is not labeled in a leaf of this age: (some chloroplast RNA breakdown products can be seen at 0.9 and sometimes $0.4 \times 10^6$). The labeled components are the rRNA precursor, $2.9 \times 10^6$ with a shoulder on the lighter side, about $2.4 \times 10^6$; the $1.45 \times 10^6$ precursor and $1.3 \times 10^6$ rRNA; the $0.7 \times 10^6$ rRNA is labeled, and its precursor, $1.0 \times 10^6$ is apparent between the chloroplast rRNA of $1.1 \times 10^6$ and its $0.9 \times 10^6$ breakdown product (for a discussion of some chloroplast breakdown products, see Ingle, 1968; the bean leaf has additional ones and details are in preparation). Further, there is a labeled component close to but not identical with the smaller chloroplast degradation products; this has a weight of about $0.48 \times 10^6$. It could therefore be the piece of excess RNA lost in the first step of processing of the $2.9 \times 10^6$ precursor to $2.4 \times 10^6$. Smaller components approximately $0.3$ and $0.15 \times 10^6$, which could be the other excess pieces, have occasionally been observed.

We have attempted to identify the components by hybridization to purified rDNA. Total DNA from the mung bean leaves was centrifuged in CsCl of average density $1.7$ g/ml, in an MSE titanium rotor at $45,000$ g for 60 hr. Ribosomal DNA, detected by hybridization with rRNA, was separated at a density of $1.703$ from the main band at $1.691$. Preparations of rDNA and of the light side of main-band DNA were fixed on Millipore filters (Birnstiel et al., 1968). RNA was eluted from various peak fractions from gels similar to that shown in Fig. 3. The eluted RNA was hybridized to the DNA filters at $65^\circ$C for 1–4 hr in $6 \times$ SSC and the filters washed and digested with RNase as described (Birnstiel et al., 1968). All components hybridized better to the rDNA than to main-band DNA; this was also true of RNA of mol wt greater than $3 \times 10^6$. To test the homology of the hybrids, the filters were heated progressively in $0.1 \times$ SSC, and the radioactivity released plotted against temperature as shown in Fig. 4. The melting of rRNA–rDNA hybrids gave sharp inflections at a $T_m$ of $80^\circ$ (not shown); it is clear that the hybrids of all the precursor components with rDNA show a large proportion of homologous hybridization, compared with those to main-band DNA. Thus the $0.48 \times 10^6$ component behaved similarly to the known precursor RNA components, and may therefore be assumed to be a piece of the excess RNA. Confirmation with a control using heterogeneous RNA is still required.

This result indicates that the $2.9$ precursor loses $0.48 \times 10^6$ daltons and suggests the idea that the first step in processing is the loss of a piece of excess RNA. Evidently the excess piece has a longer lifetime than the corresponding component in animal cells. Similar components have been observed in tobacco leaves (R. S. S. Fraser, pers. commun.). This is the only case in which the excess piece has been found in vivo.

CHANGES IN THE PLANT rRNA PRECURSORS UNDER DIFFERENT GROWTH CONDITIONS

Since large differences between the precursors in closely related plant species have been found and the precursors appear to be heterogeneous, it is reasonable to ask whether any differences under different growth conditions can be found. Similarly, since the components in mouse and man appear to be different, there may also be differences between tissues. No such differences have been found in mammals, but considerable changes are apparent in the plant precursors when rRNA synthesis is stimulated.

In yeast, the $2.6$ and $2.3 \times 10^6$ precursor components occur in approximately equal proportions in cells grown in log phase, as shown in Fig. 2b, indicating that the lifetimes of the two components are comparable. Under these conditions of growth (in EMM 1, and less than $3 \times 10^4$ cells/ml; Mitchison, 1969) the amount of rRNA per cell remains constant until the phosphate content...
of the medium becomes limiting, when the rRNA/cell falls, but cell division continues to about $10^7$ cells/ml. When cells are inoculated from stationary phase into fresh medium, rRNA synthesis is stimulated so that the amount increases up to eightfold during the first 6 to 8 hr. There is no cell division for 6 hr. Pulse incubations from 3 to 15 min show that the $2.6 \times 10^6$ precursor RNA is the major component (Fig. 5); the $2.3 \times 10^6$ precursor evidently has a relatively shorter lifetime under these conditions.

The mung bean leaf provides another example in which rRNA synthesis can be stimulated. Figure 6 shows the amounts of RNA per leaf under different conditions of growth in the light and dark. RNA synthesis increases after 4 to 5 days' growth in the dark; RNA accumulation can be stimulated at least threefold by light given on day 2. This increase is due both to a stimulation of cell division and to an increase in the amount of rRNA per cell. The nucleolar-synthesized cytoplasmic RNA is stimulated first at this age, followed by the
chloroplast rRNA. In older leaves, over 4 days, turnover of cytoplasmic rRNA continues but no radioactivity is incorporated into chloroplast RNA.

Figure 7 shows the heterogeneity of the rRNA precursor under different growth conditions. The gels were scanned at two widely different optical density ranges, so that the heterogeneity of the precursor can be seen and compared with the label.

Three situations are compared: Fig. 7a shows the RNA synthesis in leaves grown in the light and excised from the plant; the 2.9 and 2.45 × 10⁶ daltons precursor component can be seen (similar to those in intact leaves) and the radioactivity follows the optical density. In dark-grown 2 day old plants (Fig. 7b) the 2.9 × 10⁶ precursor is the major component; after 1.5 hr labeling with ³²P the rRNA and 1.45 × 10⁶ dalton RNA is labeled but...
the label on the "18 S" rRNA appears spread on the heavy side (incomplete processing?). When such a plant is illuminated for 4 hr, the amount of the smaller precursor component, the 2.45 × 10^6 daltons RNA, increases (Fig. 7c) so that it becomes the major component and the apparent mean molecular weight of the heterogeneous precursor decreases, as seen in the optical density profile. This effect is transitory; after 4 hr illumination, both 2.9 and 2.45 × 10^6 components are labeled as shown, but the specific activities of the components are not equal. After longer growth in the light, the amount of the 2.45 × 10^6 component is reduced to about the half shown in Fig. 7a. It is also apparent by comparing Fig. 7b and c, that the overall rate of processing of the precursor is increased in the light: there is approximately the same amount of label in the precursors in b and c, but more labeled rRNA has been formed in c. There is no evidence of label on the heavy side of the 0.7 × 10^6 rRNA in the light.

Clearly therefore there are changes in the lifetimes of the precursor components, correlated with a sudden increase in the rate of ribosome synthesis.

**DISCUSSION**

These results suggest that all the eucaryotes synthesize rRNA as a polycistronic molecule containing one each of the rRNA components and some excess non-ribosomal RNA. The amount of excess RNA is very much larger in the warm-blooded animals than in all other eucaryotes. If one assumes that the general structure of the precursor is in all cases similar to that proposed for HeLa (Diagram 1), then it is possible to distinguish the regions that have evolved in size in different species.

Thus all the eucaryotes have in common a ribosomal precursor component of minimum molecular weight sufficient to give the immediate precursors to the two RNA molecules. This minimum is the 41 S (3.2 × 10^6 daltons) RNA in HeLa cells, about 2.6 × 10^6 in Xenopus, and 2.4 × 10^6 in plants. This molecule is cleaved without loss of excess RNA. The smaller of the two components formed is the 1.0 × 10^6 daltons precursor to the 0.7 × 10^6 rRNA; this seems to have been conserved in size in all the eucaryotes as indicated in Diagram 2 (with the exception of a few anomalous species such as Amoeba and Euglena described previously [Loening, 1968]). In all cases the lifetime of this precursor is short and the 0.7 × 10^6 rRNA is rapidly transported to the cytoplasm. The larger component from the cleavage is the precursor to the larger rRNA; it contains 0.1 to 0.15 × 10^6 daltons of excess RNA in plants and cold-blooded animals and about 0.3 to 0.5 × 10^6 daltons in birds and mammals. This component remains for a longer time in the nucleolus (about 5% of the cell cycle).

The molecular weight of the first precursor (45 S in HeLa) is higher than that of the minimum (41 S in HeLa) by an amount that varies in different species. The excess is probably lost in one step in the first stage of processing as indicated by the experiments on the 41 S RNA in HeLa (Weinberg and Penman, 1970) and by the preliminary identification of the cleaved piece of excess RNA in the mung bean. Within the mammals, the size of this excess RNA is probably the same, since the 45 S RNA is similar (but not quite identical) in man and rodents. However, the results shown here for the rodents suggest that there are differences in the processing and that the steps of processing are not readily fitted into a single scheme.

Among the lower animals and in the plants it is clear that the amount of excess RNA over the minimum is less than half that in mammals and can vary even between closely related species (Diagram 2). This would suggest a rapid evolution of those parts of the genes which code for the excess RNA but not of those for the rRNA itself. A simple possibility which would account for this evolution and for the large difference between the lower organisms and the warm-blooded animals, is that the changes are the result of a shift in the initiation or termination sites of transcription and not in the length of the DNA. This suggestion was made previously (Loening et al., 1969; Perry et al., 1970) and would predict that the amount of non-transcribed DNA in the ribosomal cistrons in mammals is less than that which has been found in Xenopus (Birnstiel et al., 1968; Miller and Beatty, 1969). Hybridization of the rRNA and of the precursor to purified rDNA should provide a direct estimate of the amount of non-transcribed DNA.

The reasons for the heterogeneity of the precursor, which is most pronounced in plants, cannot be distinguished at present. However, if the sites of initiation or termination of transcription are easily changed between related species, then it may
be possible that even within one species not all the cistrons are identical.

The differences between closely related species indicates that the excess non-ribosomal RNA in the precursor cannot have a defined function for which a unique size is required, as seems to be the case for the rRNA itself. Thus it is unlikely to be an mRNA coding for the ribosomal proteins (there is not enough in the lower organisms); nor is it likely to be essential for the correct folding of the very similar rRNA components. One could suggest that the unstable excess RNA has some coordinating function in the synthesis of other macromolecules. Thus the rate of ribosome synthesis could control other metabolic processes such as the required synthesis of ribosomal proteins. Since part of the excess RNA can be identified in some tissues, as described here for the bean leaf, it would be interesting to determine both its cellular location after it is cleaved and its lifetime under different physiological conditions.

The relative lifetimes of the plant precursor components appear to vary under different conditions of growth. The experiments with yeast suggest that the lifetime of the smaller \((2.3 \times 10^6)\) component is relatively less under conditions of rapid ribosome synthesis in the "shift-up" incubation. In the bean leaf, where the effect of light is to increase the rate of ribosome synthesis at least threefold (Fig. 6), changes in the relative amounts of the precursors can be detected soon after illumination (Fig. 7). It is probable that the increased rate of ribosome synthesis is associated with a higher rate of transcription, a shorter lifetime or faster processing of the \(2.9 \times 10^6\) daltons precursor and a temporarily increased amount of the \(2.4 \times 10^6\) daltons component. The stimulation of the dark-grown leaf by light is known to be phytochrome mediated; it is probable that this is one of the earliest detectable effects on RNA synthesis.

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REFERENCES

DISCUSSION

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Since this presentation and following discussion indicated that the reasons for "processing" of rRNA precursors are not clear, I would like to point out the analogies between transcription of rRNA genes and of polycistronic operons. In both cases the transcribed RNA molecule is longer than the sum of the gene lengths. The reason for this is that the total length of the RNA molecule (mRNA or rRNA precursor) is determined by the chromosomal location of the transcriptional signals for the initiation (promoter) and termination (terminator), and apparently there is no strong selective pressure to delete all the DNA sequences between the promoter and the first gene, between the genes, and between the last gene and the terminator. In the case of mRNA, the secondary RNA structure and the translational initiation and termination triplets assure synthesis of protein copies from only those RNA sequences that correspond to the specific genes. In the case of rRNA precursors, the analogous recognition sites determine the "processing," i.e., which parts of the RNA are preserved and incorporated into ribosomes, the remainder being degraded. Thus, "processing" of the rRNA precursor with utilization only of its specific parts is analogous to controlled translation only of the specified parts of mono- or polycistronic mRNA.
Distinct Transcription Products of Ribosomal Genes in Two Different Tissues

Most organisms contain multiple copies of the genes which code for ribosomal RNA (rRNA), the number varying from about 160 to 28,000 per nucleus in different eukaryotic species; these genes are clustered in the nucleolus. The repeating unit is a DNA sequence containing the structural genes for the 18S and 28S rRNA together with spacer DNA, only a part of which is transcribed. Ribosomal RNA is transcribed from these genes as a polycistronic precursor molecule (pre-rRNA) which contains the rRNA sequences of the larger and smaller ribosomal subunits together with some additional sequences that are discarded during the maturation to rRNA. The multiple gene copies are identical in the ribosomal regions within the limits detectable by present methods, although there is some evidence that regions of non-transcribed spacer DNA vary in length and may therefore not all be identical. We have suggested that the pre-rRNA may also be heterogeneous in molecular weight.

![Fig. 1 Proposed steps of processing of the pre-rRNA in mung bean leaves. The diagram indicates the molecular weights in millions of RNA components that can be detected by gel electrophoresis; the arrows indicate the proposed relationship between them.](image)

The molecular weight of pre-rRNA seems to be correlated with the evolutionary position of the organism; the amount of excess RNA is very much greater in warm-blooded animals than in any other organism, and most of the difference is thought to be in the first piece of excess RNA to be cleaved during maturation of the pre-rRNA. Among the cold-blooded animals and plants, differences between closely related species may, however, be as large as between distant ones (there is about twice as much excess RNA in the bean precursor as

![Fig. 2 Gel electrophoresis of a mixture of mung bean leaf and root RNA. Seedlings were grown under sterile conditions in sand in glass tubes at 22°C. For labelling of the leaves, seedlings were germinated in the dark for 3 days and then illuminated (with white fluorescent tubes) for 24 h. 120 μCi of 32P-phosphate in 10 μl of sterile distilled water was applied directly to each of the primary leaves of the intact plants (about 3 x 10⁸ cells/leaf). After 1.5 h incubation the leaves were cut off, washed in water and mixed with labelled roots for RNA extraction. For labelling of the roots, seedlings were germinated for 30 h, when the roots were about 1.5 cm long. 1 mCi H-uridine in 2 ml sterile water was injected into the same around the roots. After 1.5 h the seedlings were washed in water, the apical 1 cm of each root cut off and mixed with the leaves. RNA was extracted as described previously. Nine roots and four leaves were ground in detergent and extracted with phenol mixture. The nucleic acids were precipitated and washed with ethanol, and DNA digested with 10 μg/ml of electrophoretically purified DNAase (Sigma) in MES buffer, pH 7.0, containing 2 mM MgCl₂, for 10 min at 0°C. Na acetate 0.15 M and SDS 0.5% were added and the RNA precipitated with ethanol. The precipitate was dissolved in 100 μl of 1 M NaCl, 0.5 mM EDTA and 0.2% SDS) containing 20% sucrose and 30 μl. were layered on a gel. Electrophoresis was in a 9 mm diameter 2.4% polyacrylamide gel 4.5 h at 50 V, 11 mAh. The gel was scanned in a Joyce Loebel Polyfrac ultraviolet scanner, frozen in dry ice and cut into 0.5 mm slices on a Mickle gel slicer. The slices were heated at 60°C for 4 h in 10% piperidine 1 mM EDTA and dried. The slices were swollen and the hydrolysed RNA dissolved in 0.4 ml water for 1 h, and 10 ml of scintillation fluid added (60% toluene, 40% methoxyethanol, 0.5% butyl-PBD). Radioactivity was determined in a Packard Tri-Carb spectrometer, and the 3H counts corrected for 5% spill-over from the 32P. Absorbance at 265 nm; --- --- --- 32P; ---H. Molecular weights are indicated, assuming values for the ribosomal RNA of 1.3 x 10⁶ and 0.7 x 10⁶, which were obtained by comparison with E. coli rRNA of assumed molecular weight 1.1 and 0.56 x 10⁶.

in the pea⁸), suggesting a very rapid evolutionary change within a limiting size. It seemed conceivable that the tissues of one organism may show similar differences; we have now found this for the leaves and roots of the mung bean, Phaseolus aureus.

We have proposed a scheme for the synthesis and processing of the pre-rRNA of the cytoplasmic (not the chloroplast) rRNA of the mung bean (Fig. 1). Evidence for the scheme is based on the molecular weights and rates of labelling of the components determined by gel electrophoresis, 32P base compositions and competitive hybridization experiments. The stages of processing are similar (with very different molecular weights) to those proposed for the HeLa cell by Weinberg and Penman⁹, and probably differ from the scheme proposed for rat tissues by Egawa et al.¹⁰; this difference may be explained by differences between these mammalian species. Mung bean leaves contain an RNA component with a molecular weight of 0.45 x 10⁶ that is rapidly labelled and hybridizes specifically to the isolated ribosomal genes, suggesting that it is the excess RNA first cleaved from the pre-rRNA (Fig. 1). This component was not found in mung bean roots; accordingly, we made a direct comparison of the pre-rRNA in leaves and roots.

Fig. 2 shows a separation by gel electrophoresis of RNA
from leaves labelled with $^{32}$P and RNA from roots labelled with $^3$H. The scan of the optical density shows the two rRNA components with molecular weights of $0.7 \times 10^6$ and $1.3 \times 10^6$ and the chloroplast rRNA with weights of $0.56 \times 10^6$ and $1.1 \times 10^6$. A minor component (molecular weight $1.02 \times 10^6$) in roots and young leaves may be mitochondrial RNA (C. Leaver, personal communication). In the conditions of growth of the seedlings the chloroplast RNA is not labelled. The radioactivity scan of the leaf RNA ($^{32}$P) shows the components previously described with the molecular weights given in Fig. 1. A comparison with the $^3$H RNA of the root shows that: (1) the mobility of the root pre-rRNA is slightly greater than that of the leaf, and has an apparent molecular weight of $2.7 \times 10^6$; (2) the excess piece of RNA, molecular weight $0.45 \times 10^6$, seen in the leaf RNA is missing from the root RNA; (3) the immediate precursors to the mature rRNAs are identical in leaf and root, with molecular weights of $1.45 \times 10^6$ and $1.0 \times 10^6$.

We have obtained similar results with different preparations grown and labelled in various conditions. The following experiments were carried out to check whether the difference between leaf and root could be due to some artefact of labelling, extraction or conformation of the RNA.

(1) As radioactive uridine and phosphate frequently label RNA at different rates, presumably because of differences in the pools in the cell, the comparisons were repeated with the isotopes reversed. Exactly the same result as before was obtained (Fig. 3b).

(2) The method of extraction of RNA from plant tissues has been studied in detail and it is unlikely that any significant degradation occurs. However, many steps, including a deoxyribonuclease digestion which is the only stage at which the RNA is in the absence of detergents, are involved and it is possible that the roots contain some nuclease which survives deproteinization. The separately labelled leaf and root tissues were then mixed before homogenization, so that the whole procedure was carried out on the mixture (Fig. 2). Identical results were obtained with RNA prepared separately from different batches of seedlings. (3) The heavy pre-rRNA molecular weights, $2.9 \times 10^6$ and $2.5 \times 10^6$ in the leaf, seem to be heterogeneous and are not well resolved (Fig. 2), which is a characteristic of this pre-rRNA in the buffer system used. The components are better distinguished by the optical density-profiles on an expanded scale as shown previously, and in buffers containing Mg ion as shown below (Fig. 3c). The apparent heterogeneity is large compared with the difference between root and leaf pre-rRNA, so that the molecular significance of the difference must be examined more closely. Possible errors in the determination of molecular weight by gel electrophoresis can be detected by varying the ionic conditions and the gel concentration. An RNA which unfolds more in low salt concentrations or complexes more in buffers containing Mg$^{2+}$ will vary in mobility relative to a known marker which shows lesser conformational changes. The root and leaf RNA has been analysed by electrophoresis in a buffer containing a very low Na$^+$ concentration and in the same buffer containing Mg$^{2+}$ (Fig. 3). In both cases, the difference between the pre-rRNA of the root and leaf is maintained. In the low ionic strength buffer the apparent heterogeneity is greater than in E buffer, suggesting some random unfolding of the molecules. In the presence of Mg$^{2+}$, the two components of the leaf precursor (molecular weights $2.9 \times 10^6$ and $2.5 \times 10^6$) are better resolved and the peaks are sharper. It seems then that the $2.5 \times 10^6$ component is common to the leaf and root; the major part of the root pre-rRNA has a mobility between the two components of the leaf.

(4) We have also examined the labelled RNA of the hypocotyls from the same bean seedlings, and found that the pre-rRNA is more complex than that of leaf or root, seeming to include the same components as in the leaf, with molecular weights of $2.9 \times 10^6$ as well as others similar in mobility to the root pre-rRNA. We do not know whether all these components occur in a single cell type within the hypocotyl.

These results suggest that there is a true difference of molecular weight between the largest pre-rRNA molecule synthesized in the leaf and that in the root; the molecular weights suggest that the difference is around 600 nucleotides out of a total length of somewhat less than 9,000. We suggest that all of this difference is due to a smaller amount of excess RNA at one end of the pre-rRNA in the root; the evidence for this is that all components other than the pre-rRNA and the $0.45 \times 10^6$ RNA are identical in leaf and root, including the $2.5 \times 10^6$ molecular weight RNA (Fig. 3c).

This is the simplest interpretation of the results, but two other possibilities should be considered: (1) in the root, cleavage of the pre-rRNA starts during or immediately after synthesis, so that the complete transcription product is never
(2) there are other differences in conformation or methylation. Thus a final proof that the primary transcription products in root and leaf differ would require an analysis of the 5' terminus of the pre-rRNA or a detailed finger-print.

The synthesis of different pre-rRNA molecules in roots and leaves could be achieved in two obvious ways: either (i) the reiterated ribosomal genes are not identical and a different group of these genes is active in leaf and root, or (ii) the genes are identical but there are two or more initiation or termination sites for each transcription unit, and these are activated by different control elements in the two tissues.

Many simple suggestions about the significance of the differences can be made. For example, the cleaved excess piece of RNA could control other cellular functions which are different in leaf and root and which are coordinated with ribosome synthesis—the concentration of this RNA would depend on the rate of rRNA synthesis, while its specificity would differ in the two tissues.

During preparation of this manuscript, a report appeared describing heterogeneity of the mammalian pre-rRNA2.

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ABSTRACT OF THESIS

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Title of Thesis: The Synthesis of Ribosomal Ribonucleic Acid in Developing Primary Leaves of Phaseolus aureus

In the Introduction the reasons for studying ribosomal RNA synthesis are outlined and the literature concerning ribosomal RNA metabolism is reviewed.

The results show that the major part of the RNA from young etiolated leaves is cytoplasmic ribosomal RNA. Several unidentified minor RNA components are also present. Chloroplast ribosomal RNA which constitutes about 30 per cent of the total ribosomal RNA in mature leaves is not synthesised until about two days after germination.

Light stimulates cell division and the accumulation of chloroplast and cytoplasmic ribosomal RNA but both types of RNA also accumulate in the dark in the absence of cell division. The first detectable effect of light on RNA synthesis is an increased rate of processing of macromolecular precursors to cytoplasmic ribosomal RNA. This response occurs less than two hours after the onset of light treatment.

The $1.1 \times 10^6$ molecular weight chloroplast ribosomal RNA is unstable. Old as opposed to newly synthesised molecules are hydrolysed in vivo at two specific sites. The resulting fragments dissociate and can be detected separately in polyacrylamide gels. The fragments can be held together by magnesium ions which presumably stabilise base paired regions between the RNA fragments. The $1.3$ and $0.7 \times 10^6$ molecular weight cytoplasmic ribosomal RNAs and the $0.56 \times 10^6$ molecular weight chloroplast ribosomal RNA are all stable during electrophoresis. Hidden breaks can be revealed in the polynucleotide chains of the cytoplasmic ribosomal RNAs by heating the molecules in solution prior to electrophoresis.
Specific molecular aggregates of ribosomal RNA can be formed by heating RNA solutions and under certain conditions of RNA extraction and fractionation.

Rapidly labelled RNA heterogeneous in size has a DNA-like base composition. Six distinct RNA components with molecular weights of 2.9, 2.7, 2.5, 1.45, 1.0 and 0.45 x 10^6 are superimposed on the background of polydisperse RNA separated on acrylamide gels. They are metabolically unstable and the four largest molecules resemble ribosomal RNA in base composition. Labelling kinetics suggest that they are macromolecular precursors to ribosomal RNA. All these molecules hybridise preferentially to DNA fractions containing the genes for cytoplasmic and chloroplast ribosomal RNA which are denser than the bulk of the DNA when banded in caesium chloride.

Young leaves and roots synthesise precursors with molecular weights of 2.7 and 2.5 x 10^6. Competitive hybridisation studies suggest that these RNAs contain sequences of both the 1.3 and 0.7 x 10^6 molecular weight cytoplasmic ribosomal RNAs plus different amounts of non ribosomal RNA which are removed during processing of the precursors to form mature cytoplasmic ribosomal RNAs. RNAs of molecular weight 1.45 and 1.0 x 10^6 may be processing intermediates and the possible stages in precursor processing are discussed.

An additional precursor with a molecular weight of 2.9 x 10^6 is synthesised in leaves grown in the light. It is not clear whether this molecule is a precursor to chloroplast or cytoplasmic ribosomal RNA. It is suggested that the 0.45 x 10^6 molecular weight RNA may be cleaved from one end of this precursor during processing.