THE CORRELATION BETWEEN THE METABOLISM OF RIBOSOMAL RNA AND PROTEIN BIOSYNTHESIS IN HIGHER PLANTS

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ABSTRACT

The main purpose of the investigation described here was to examine the metabolism of chloroplast ribosomal RNA in an attempt to determine the function of the chloroplast ribosomes.

Initial experimentation was devoted to characterizing the chloroplast rRNA. Sucrose density gradient centrifugation, methylated serum albumin - kieselguhr column chromatography, and polyacrylamide gel electrophoresis were the methods employed. The results from polyacrylamide gel electrophoresis indicate that the chloroplast ribosome has one "heavy" (1.1 m) rRNA molecule and one "light" (0.56 m) rRNA molecule, in common with ribosomes from other living organisms. Results differing from this 1:1 ratio are most probably a consequence of the fragmentation of the "heavy" molecule. Possible explanations for the nature of this fragmentation are discussed.

The nature of the plastid rRNA in different tissues of the same plant was then investigated. It was found that the non-green portions of the broad bean plant have proportionately less rRNA and few ribosomes in their plastids than green portions. Their plastids have some internal membranes but no grana. Some ribosomes are present in all the major types of plastids in the normal plant. In comparison, an albino mutant studied had no plastid rRNA or ribosomes, although there was some membrane formation in these plastids. It was thus concluded the cytoplasmic ribosomes are responsible for the synthesis of some plastid proteins, but formation of grana does seem to require the presence of plastid rRNA and ribosomes.

An attempt was made to correlate the development of plastid rRNA with chlorophyll and grana synthesis in etiolated leaves as a response to illumination. The etiolated leaves appear to contain a full complement of cytoplasmic ribosomes. We have shown that the maximum differentiation of the plastid occurs during the period of most rapid synthesis of chloroplast ribosomes. However, we were unable to detect any radioactivity in the separated lamellar proteins made during differentiation of the plastid.

There is a lag in the synthesis of chlorophyll, grana, and chloroplast ribosomes as a response to illumination, indicating that there may be some rate-limiting processes not related to nucleic acid metabolism. Where the individual proteins are made is still not clear.

Abbreviations and Terminology

^{o}A	Angstrom
ATP	adenosine triphosphate
^{o}C	degrees centigrade
cpm	radioactivity counts per minute above background counts
D.C.	direct current
DCMU	3,(3,4-dichlorophenyl)1,1-dimethyl urea
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GTP	guanosine triphosphate
m	million
M	molar
u	micron
mA	milliamperes
MAK	methylated serum albumin coated on kieselguhr
mc	millicuries
mRNA	messenger ribonucleic acid
MW	molecular weight
PPO	2,5,diphenyloxazole
psi	pounds per square inch
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
s	Svedberg unit
TEMED	NNN'N'-tetramethylethylethylene diamine

Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
u.v.	ultraviolet

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INTRODUCTION

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It is now well established that the nucleic acids direct the course of differentiation in living organisms. As outlined in Fig. 1, the differentiation pattern is thought to result from the synthesis of specific enzymes which in turn direct the course of metabolic processes. The synthesis of these proteins from amino acids is brought about by the interaction of the ribosomes, transfer RNA (tRNA), peptide polymerase initiation factors, and amino acyl transferase, the actual sequence of amino acids being determined by messenger RNA (mRNA). The mRNA is polymerized on a DNA template which is the primary carrier of genetic information. Of current interest is the finding that subcellular organelles such as chloroplasts and mitochondria have a protein synthesizing system which differs from that of the rest of the cell, and the question is therefore raised about the degree of genetic autonomy of these organelles. The main purpose of this study has been to compare in higher plants the cytoplasmic and chloroplast RNA's and study their involvement in the processes of plastid differentiation.

Plant RNA - Characterization.

When this study was initiated in October, 1966, little was known of the nature of chloroplast RNA, although there were indications that it differed from that of the rest of the cell. In 1962, Lyttleton reported that spinach chloroplast ribosomes had a

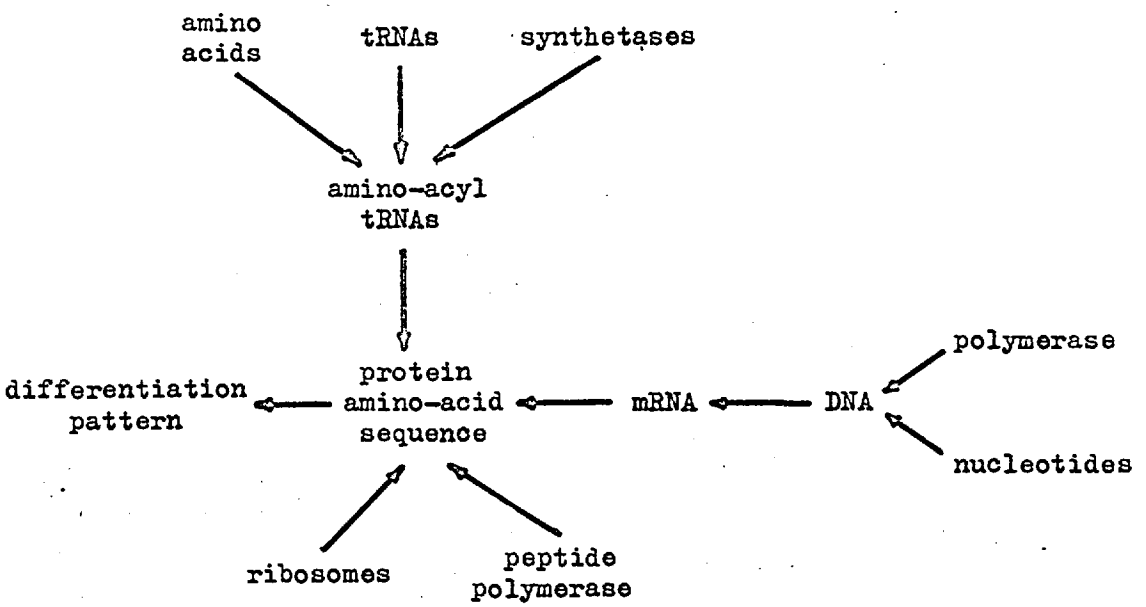


Figure 1. Diagram outlining the mechanism by which the nucleic acids are thought to determine the way in which living organisms differentiate. (as outlined by Drs. T.A. Dyer & C.J. Leaver)

sedimentation coefficient of 70s, whereas that of the cytoplasm was 80s, indicating a larger size in the cytoplasm. An electron microscopic study by Jacobson et al (1963) had also shown that the chloroplast ribosomes were smaller than those of the cytoplasm. Brawerman (1963) described differences in the sedimentation coefficients and base compositions of RNA of Euglena gracilis chloroplast and cytoplasmic ribosomes, but in a similar investigation on higher plants, Odintsova et al (1963) had been unable to detect any differences between the base compositions of these RNA's. However, the results of Dyer and Leech (subsequently published in 1968) showed that there was a difference in the chromatographic properties of higher plant ribosomal RNA's (rRNA) on MAK columns. This study was therefore initiated in an attempt to characterize the plastid RNA further. During the course of this investigation there have been further publications on this topic which have greatly increased our understanding of the subject. (Fig. 2)

One of the first methods used to fractionate the RNA's was sucrose density gradient centrifugation. Most commonly three fractions were obtained, although recent improvements in the technique have increased the resolution of plant leaf high molecular weight rRNA obtainable (Stutz and Noll, 1967; Jacobson and Williams, 1968). The slowest sedimenting fraction is

	ribosomes	ribosome sub-units	RNA
Cytoplasm	80S	60S →	25S + 7S(?) + 5S
		40S →	18S
			tRNA (4S)
			LMW - RNA
Chloroplasts	70S	50S →	23S (18S + 13S) + 5S
		30S →	16S
			tRNA (4S)
			LMW - RNA

Figure 2. Diagram summarizing the sedimentation characteristics of chloroplast and cytoplamsic RNA and ribosomes. (as outlined by Drs. T.A. Dyer & C.J. Leaver)

of low molecular weight, consisting mainly of tRNA with molecular weights of 2.8 to 4.2 x 10^4 and sedimentation coefficients of 4 to 5s. The other two fractions are of high molecular weight rRNA (0.4 to 1.3m M.W.), the one sedimenting more rapidly being referred to as the "heavy" rRNA and the slower as "light" rRNA. In bacterial cells there is one molecule each of a 1.1m and a 0.56m RNA per 70s ribosome, and in animal cells one each of a 1.8m and a 0.7m RNA molecule per 80s ribosome. Since the molecular weight of the larger RNA molecule is approximately twice that of the smaller molecule, the results of the u.v. absorption of the "heavy" fraction is twice that of the "light" in the sucrose denstiy gradients. A similar ratio was found in plants by Click and Hackett (1966) and Click and Tint (1967) in rRNA from etiolated stem apices, the RNA's having sedimentation coefficients corresponding to molecular weights of 1.3m and 0.7m (see Fig.2). However, when the nucleic acids from chloroplast containing tissues have been fractionated, the ratio of "heavy" to "light" RNA was commonly less than 2 to 1. Pollard et al (1966) concluded that there is one RNA molecule of each type present in the cytoplasmic ribosomes, but that there is more of the "light" species in chloroplast ribosomes. Spencer and Whitfield (1966) could not identify a "heavy" RNA at all in RNA isolated from chloroplast ribosomes. They

claimed that the "heavy" ribosomal RNA had not been degraded, since Escherichia coli RNA of comparable size was not degraded when isolated from a mixture of chloroplasts and bacteria. Using convex exponential sucrose gradients, Stutz and Noll (1967) were able to resolve the "heavy" ribosomal RNA's of chloroplast and cytoplasmic ribosomes, but not their "light" components. They derived sedimentation coefficients corresponding to molecular weights of 1.3m and 0.56m for the RNA's of cytoplasmic ribosomes and of 1.1m and 0.56m for that from the chloroplast ribosomes. They also found a relatively higher proportion of "light" chloroplast rRNA than expected.

The technique of fractionating the nucleic acids by weak anion exchange chromatography on methylated serum albumen - kieselguhr (MAK) columns has also been used to study plant nucleic acids. By this method three main fractions are obtained. At low salt concentrations the low molecular weight RNA is eluted. As the salt is increased, the DNA and finally a fraction of high molecular RNA, in which the "heavy" and "light" rRNA are partially resolved, are removed from the column. Wollgiehn et al (1966) and Dyer and Leech (1968) noted differences between the elution profiles of total leaf nucleic acid and chloroplast nucleic acid. As was found with sucrose gradients, the "light" rRNA fractions was proportionately larger in chloroplast RNA preparations

than it was in total leaf RNA. Aoki and Hase (1967) distinguished by MAK column chromatography the appearance of a discrete chloroplast rRNA fraction during the greening of glucose-bleached Chlorella. Four partially resolved high molecular weight rRNA subfractions were obtained by Vedel (1968) in nucleic acids from cotyledons, two of which were of chloroplastic origin.

An explanation for the unexpected behavior of the chloroplast rRNA is suggested by the results of Ingle (1968). Using the high resolution afforded by polyacrylamide gel electrophoresis, he was able to distinguish RNA of 0.7m and 0.4m molecular weight in chloroplast enriched preparations as well as some 1.1m (a "heavy" rRNA) and 0.56m (a "light" rRNA). Ingle suggests that the 0.7m and 0.4m RNA are fragments of the 1.1m RNA and has shown that the ratio of "heavy" to "light" rRNA is 2:1 if a correction for this fragmentation is made. The corresponding cytoplasmic rRNA's were found to have molecular weights of 1.3m and 0.7m. The results to be presented here will show that both fragments of the "heavy" chloroplast rRNA are fractionated with the "light" rRNA in sucrose gradients and on MAK columns, explaining the relatively higher than expected proportion of this fraction that has frequently been found.

Although it has been demonstrated by Brawerman (1963) that in Euglena gracilis chloroplast rRNA contains

higher proortions of adenine and uracil than there is in cytoplasmic rRNA, Odintsova (1963), Spencer and Whitfield (1966), Wollgiehn et al (1966), and Leech and Dyer (in press) found their base compositions to be similar in higher plants. Pollard et al (1966), however, found that the "light" rRNA from lettuce chloroplasts had a different base composition from that of cytoplasmic rRNA, although the differences were not so great as in Euglena. Possibly these discrepancies will only be resolved when both the methods for the preparation of the RNA and determination of base compositions have been improved.

Intact cytoplasmic and chloroplast ribosomes have several distinctive features. In addition to the difference in their sedimentation properties (the chloroplast ribosomes having sedimentation coefficients of 70s and those of the cytoplasm 80s: Lyttleton, 1962; Clark et al, 1964; Sissakian et al, 1965; Boardman et al, 1966; Stutz and Noll, 1967), the proteins of chloroplast and cytoplasmic ribosomes differ from each other in their electrophoretic properties (Lyttleton, 1968; Odintsova, 1969; Hoober and Blobel, 1969). According to Boardman et al (1966), the 70s chloroplast ribosomes require an environment of relatively high magnesium ion concentration (more than 1 mM) in order to maintain their integrity. At lower molarities the ribosomes dissociate into subunits with sedimentation

coefficients of 50 and 30s, which can be re-associated by increasing the magnesium concentration of the medium. To dissociate the cytoplasmic ribosomes to 60 and 40s subunits, it is necessary to remove most of the magnesium, and once they are dissociated they are not reconstituted by increasing the magnesium concentration.

In addition to differences between chloroplast and cytoplasmic rRNA and proteins, Dyer and Leech (1967, 1968) found that the "5s" low molecular weight RNA of chloroplast and cytoplasmic ribosomes could be distinguished from one another in Vicia faba. Chloroplasts were also shown to contain unique soluble RNA's. At least some species of tRNA within chloroplasts are not the same as that in the cytoplasm (Sissakian et al, 1965), and Eisenstadt and Brawerman (1967) have found that, in common with bacterial systems, transcription in Euglena chloroplasts is initiated with N-formyl-methionine.

There have been numerous reports that the DNA of chloroplasts differs from that in the nucleus (cf. Kirk and Tilney-Bassett, 1967). The size of the chloroplast genome is thought to be between 1 and 2 x 10^8 daltons, whereas it is many times this size in the nucleus (Wells and Birnstiel, 1969). The DNA is not associated with histones in the chloroplast, but is in 25 ^{o}A thick fibrils as is found in bacteria (Ris and Plaut, 1962).

Little is known as yet about the mitochondrial nucleic acids of green plants, although the ribosomes

appear to be similar to those in the chloroplasts (Wilson et al, 1968). Studies of these nucleic acids derived from the mitochondria of fungal and animal tissues also suggest that they differ from those of the cytoplasm and have many features in common with the chloroplast nucleic acids (cf. Roodyn and Wilkie, 1968).

Plastid nucleic acids -- Origin.

Since the chloroplasts contain unique nucleic acids, it is necessary when deciding the extent of their genetic autonomy to determine the origin of these nucleic acids. As far as the synthesis of RNA is concerned, isolated chloroplasts have been shown to contain RNA polymerase (Kirk, 1964; Spencer and Whitfield, 1967), and Spencer and Whitfield (1967) have demonstrated their ability to synthesize RNA. However, the RNA made in isolated chloroplasts of higher plants has yet to be fully characterized. The RNA produced *in vitro* appears to be polymorphic in nature (Spencer and Whitfield, 1967; M. Hartley, unpublished results), which is a feature of most rapidly labelled RNA of plant tissues (Ingle et al, 1965). The ability of the isolated chloroplasts to synthesize RNA decreases fairly rapidly with time and has ceased in most preparations after 1 hr. Even in intact tissues little newly synthesized rRNA can be detected after this time, so it is possible that the synthesis of RNA has stopped in isolated chloroplasts before significant quantities of rRNA have

accumulated. Schweiger et al (1967) found that Acetabularia cells continue to make RNA after the removal of the nucleus and that the rRNA synthesized has sedimentation characteristics closely resembling that of Escherichia coli rRNA. It was concluded that nuclear DNA is not necessary for chloroplast rRNA synthesis and that the chloroplast DNA is the template upon which this RNA is manufactured. Berger (1967) also claims to have obtained ribosomal RNA synthesis in isolated Acetabularia chloroplasts. Although these results apparently provide conclusive evidence that the chloroplast rRNA is synthesized within chloroplasts, this cannot be taken as fully proven until these results have been substantiated in a range of plant tissues.

If the chloroplast DNA is the template on which the chloroplast rRNA is made, then this RNA should hybridize with the DNA under suitable conditions. Scott and Smillie (1967) were able to hybridize the total rRNA of light grown Euglena cells with the chloroplast DNA. The total cellular RNA from dark grown cells did not reduce the proportion of this hybridization. From these results it can be inferred that the chloroplast DNA could be the template for chloroplast but not cytoplasmic rRNA synthesis. An understanding of this aspect of RNA metabolism has been complicated by the finding of Tewari and Wildman (1968) that the chloroplast RNA can also be hybridized with nuclear DNA. The degree of hybrid-

ization found implied that there are more cistrons for chloroplast rRNA per cell in the nuclear DNA than there are in the chloroplast DNA. Furthermore, Richards (1967) has shown that Euglena chloroplast and nuclear DNA hybridize with one another. In order to substantiate these findings it will probably be necessary to investigate the purity of the nuclear preparations and to prove that the hybridization is specific.

Plastid RNA's - Function.

It has been suggested that chloroplasts are capable of independent protein synthesis (cf. Kirk and Tilney-Bassett, 1967). Although the incorporation of radioactive amino acids into protein by isolated chloroplasts has been reported extensively, much of the early work is of doubtful significance because of possible contamination of the chloroplast preparations by bacteria. App and Jagendorf (1964) showed that all the synthesis found in an earlier study (App and Jagendorf, 1963) could be accounted for by bacterial contamination. Subsequently, however, Bambi and Jagendorf (1966) demonstrated beyond reasonable doubt the ability of isolated wheat chloroplasts to incorporate amino acids into proteins. A method now commonly used to purify the chloroplast fraction is to treat the incubation mixtures with Triton X-100 detergent before isolating the radioactive product (Spencer, 1965; Parenti and Margulies, 1967). This treatment disperses the chloroplasts but

does not affect bacteria or nuclei present in the preparations.

A characteristic of the *in vitro* protein synthesizing system of isolated chloroplasts is that the rate of amino acid incorporation rapidly declines after about 1 hr (Bambi and Jagendorf, 1966; Spencer, 1965). This is apparently due to a deterioration of the chloroplasts and not to a depletion of a component derived from the nucleus, since Goffeau and Brachet (1965) were able to isolate chloroplasts capable of protein synthesis from *Acetabularia* that had been enucleated 12 days previously. Shephard (1965) in fact showed by autoradiography that enucleation had had little effect on incorporation of ^{14}C-leucine even after 3 weeks. This is consistent with the finding of Haemmerling (1963) that both total and plastid protein increase for at least 2 weeks after enucleation of *Acetabularia*.

The mechanism of plastid protein synthesis appears to be similar to that of other systems. In addition to the ribosomes and amino acids, incorporation is dependent on the presence of an ATP generating system, GTP, potassium, magnesium, and a soluble fraction consisting of protein and tRNA. Spencer (1965) and Eisenstadt and Brawerman (1964) have shown that ATP generated by photosynthesis in intact chloroplasts can be used to energize the synthesis of protein. A difference between the *in vitro* systems isolated from chloro-

plast and cytoplasm is that chloramphenicol inhibits the former but not the latter (Smillie, 1968). Also, Boardman et al (1966) showed that optimal amino acid incorporation is at a magnesium ion concentration of 5 mM in the cytoplasmic system and 15 mM in the chloroplast.

The ability of cell free preparations from the cytoplasm to incorporate amino acids appears to be correlated with their polysome content (Leaver and Key, 1967). Consistent with this, Chen and Wildman (1967) showed that chloroplast polysomes are active in protein synthesis, but they found that the monosomes were also active in vitro without added mRNA. Chinese cabbage polysomes have been characterized by Clark et al (1964) and pinto bean polysomes by Stutz and Noll (1967). Clark (1964) was also able to show that these polysomes were only present in small numbers in plants kept in the dark but appeared rapidly on illumination. This observation correlated well with the findings of Partier (1965) and Wollgiehn and Mothes (1963) that the rate of leaf protein synthesis is greatly enhanced by light.

In an assessment of the role of the chloroplast nucleic acids it is necessary to establish what proteins are made in the isolated chloroplasts. The rates of amino acid incorporation into protein so far achieved with in vitro chloroplast preparations have

been very low, making identification of the products difficult. Spencer (1965) found that radioactive amino acids were incorporated into both stromal and lamellar protein, and Margulies and Parenti (1968) reported that these two protein types were made at approximately the same rate, but that Fraction I protein, a soluble protein, was not made as rapidly as the others.

Since the nucleic acids of chloroplasts and mitochondria have many features in common, a comparison between the proteins synthesized in these organelles is of significance. This is particularly so since mitochondrial protein synthesis has been studied in detail.

Whereas incorporation of labelled amino acids is into both stromal and lamellar protein in chloroplasts, incorporation in isolated mitochondria is almost entirely into a lamellar protein fraction (Kadenbach, 1966). This particular protein fraction, which was first characterized by Criddle et al (1962), was called "structural" since it did not appear to have any enzyme activity and readily formed a complex with itself. It was described as being a water insoluble species of protein with molecular weight betweeen 2 and 3 x 10^4. Criddle et al (1962) suggested that this protein might be the "backbone" of mitochondrial membranes. More recently Haldar et al (1966) and Green et al (1968) were able to fractionate "structural" protein into several components by polyacrylamide gel electrophoresis. A similar protein

has been isolated by Criddle (1966) from chloroplast lamellae, and Schmitz et al (1968) were able to fractionate it into three distinct components by gel electrophoresis.

The mitochondrial "structural" protein was found to be radioactive by Haldar et al (1966) when it was isolated from a mitochondrial preparation that had been incorporating radioactive amino acids into protein. On the other hand, Kadenbach (1966) showed that protein synthesized by cytoplasmic microsomes could be transferred to mitochondria and in a later report (1967) he provided evidence that cytochrome c is one of these mitochondrial proteins made in the cytoplasm. Thus it seems that both mitochondrial and cytoplasmic nucleic acids are involved in mitochondrial protein synthesis.

The question remains whether a similar situation exists in the chloroplasts, and, if so, which proteins are synthesized where. Ohad et al (1967) suggest that the chloroplast ribosomes are concerned primarily with the synthesis of lamellar proteins. In a mutant of Chlamydomonas reinhardii in which the chlorophyll "disappears" and the plastids "de-differentiate" when the cells are placed in the dark, these workers found that the activities of the soluble enzymes were not affected. When returned to the light, the chloroplast structure and photosynthetic capacity of the cells were rapidly restored. It was suggested that the re-assembly

is controlled by lamellar membrane synthesis. However, the results of experiments with Euglena, in which chloramphenicol and cycloheximide were used to selectively inhibit chloroplast and cytoplasmic protein synthesis (Smillie, 1967), indicate that some of the soluble photosynthetic enzymes are made on chloroplast ribosomes, but that the "structural" protein may not be. It should be noted that there is some controversy about the action of these inhibitors (Ellis, 1969). Thus, although there are similarities between the biogenesis of mitochondria and chloroplasts, it is not yet clear where they differ.

The effect of light on etiolated leaves.

The effect of light on etiolated leaves has been widely studied in relation to chloroplast differentiation. It is a suitable system for this purpose since in etiolated leaves the plastids are in a relatively undifferentiated form and they undergo substantial structural changes when the plants are illuminated (Wettstein and Kahn, 1960; Gunning and Jagoe, 1966). The etioplasts are characterized by the presence within them of a prolamellar body which consists of tubular membranes arranged in a crystalline lattice. The prolamellar body disperses almost immediately on illumination, the membranes forming long lamellae in the stroma. This stage is followed by an overlapping of the lamellae, resulting in stacks being formed at regular intervals. Gunning and Jagoe (1966)

have shown that it is not until overlapping begins that there is an increase in membrane content of the plastid. At this stage, also, de novo chlorophyll synthesis commences. It has been suggested that the synthesis of chlorophyll is dependent on the simultaneous production of membranes onto which the chlorophyll is attached (Kirk, 1968; Bergfeld, 1968). Pirson and Boger (1965) have shown, however, that in some algae the development of chlorophyll and membranes is not synchronized, the lamellae requiring more light for their synthesis. As well as membrane reorganization and synthesis, illumination results in an increase in plastid size (Mego and Jagendorf, 1961) and in an increase in the activities of the photosynthetic enzymes. (cf. Kirk and Tilney-Bassett, 1967).

Many kinetic studies have been carried out in order to establish the sequence of events during the differentiation of etioplasts to chloroplasts. In nearly all processes there is a lag period before any changes occur. Although the conversion to chlorophyll of the small amount of protochlorophyll present in etiolated leaves occurs immediately in light, the synthesis of new chlorophyll molecules commences somewhat later (Sisler and Klein, 1963; Gassman and Bogorad, 1967). This lag in de novo synthesis can be abolished by supplying the plants with δ-amino-levulinic acid, which is a precursor of chlorophyll. The development of the

oxygen evolving and carbon dioxide fixing capacities of the tissue also do not commence immediately.

Changes in the nucleic acids on illumination of etiolated plants have been studied extensively, and the results indicate that the nucleic acid metabolism differs considerably from plant to plant. In general, there seems to be an increase in the nucleic acid content of bean and radish in light (Wettstein, 1967; Ingle, 1968), and in maize Mans (1967) found that the newly synthesized RNA had a base composition differing from that of unilluminated plants. As to the nature of the newly synthesized RNA, it appears that both cytoplasmic and chloroplast RNA's are made, but that the chloroplast component accumulates at a greater rate. Bogorad (1967) reported that cytoplasmic ribosome synthesis in maize was only slightly stimulated by light and a similar conclusion was reached by Boardman (1966) in a study on Phaseolus. Boardman (1966) found that there was a 70% increase in chloroplast ribosomes per leaf whereas the cytoplasmic increase was only 30%. These changes were both relatively small compared to the 770% accumulation that occurred in Fraction I protein. Light in itself does not appear to be necessary for chloroplast ribosome synthesis, since ribosome-like particles have been demonstrated to be present in etioplasts by Wettstein (1967) and Bogorad (1967).

It seems likely that the lag in chloroplast

differentiation in etiolated plants when placed in light is the result of an initial rate-limiting process, which might be associated with the nucleic acids. Although Bogorad (1967) reported an increase in chloroplast RNA polymerase activity and in the amounts of all kinds of chloroplast RNA during the lag in maize leaves, Wettstein (1967) found in bean that at first the amount of RNA remained constant, only doubling later. The increase in bean chloroplasts occurred just prior to the synthesis of grana.

In light, after a delay, there is an increase in the polysome content of etiolated leaves together with a rise in the ability of the ribosome population to incorporate amino acids into protein in cell-free systems (Williams and Novelli, 1968). Brown and Gunning (1965) also observed by electron microscopy an increase in the number of ribosomes in polysome configuration on illumination.

The important role of nucleic acid and protein metabolism in chloroplast differentiation is emphasized by inhibitor experiments in which this differentiation in the light is prevented. The inhibitors include chloramphenicol and cycloheximide, which are reputed to inhibit chloroplastic and cytoplasmic protein synthesis, respectively (Smillie et al, 1968), actinomycin D, which stops the synthesis of RNA (Bogorad and Jacobson, 1964), and amino acid and nucleotide analogs

(cf. Kirk and Tilney-Bassett, 1967). Berger and Feierabend (1967) reported that the plastids of chloramphenicol-treated etiolated rye seedlings frequently lack a prolamellar body, thylakoid development is inhibited, and the etioplasts are much smaller than in untreated plants. Synthesis of photosynthetic enzymes such as carboxydismutase and NADP glyceraldehyde-3-phosphate dehydrogenase is also prevented.

A feature of the effect of light on etiolated plants is that only a short pulse of red light is necessary to stimulate the activity of photosynthetic enzymes such as ribulose diphosphate carboxylase, NADP glyceraldehyde-3-phosphate dehydrogenase, and ferredoxin NADP reductase (Graham et al, 1968). The reduction of protochlorophyll to chlorophyll occurs with a short pulse of red light. Increases in fresh weight, protein, and RNA are also initiated. Precursors of chlorophyll accumulate, so that the usual lag in chlorophyll synthesis in white light is eliminated. The effect of the red light can be nullified by subsequent treatment of the plants with far red light, suggesting that these changes are mediated by the phytochrome system (Mego and Jagendorf, 1961; Graham et al, 1968; Filner and Kleim, 1968).

In the study reported here the RNA metabolism during greening of etiolated leaves of higher plants has been further investigated to determine how their metabolism changed and chloroplast ultrastructure

developed. Also, an attempt was made to measure the development of chloroplast lamellar "structural" protein during the same period. By these experiments, it was possible to derive some information as to how the nucleic acids are involved in the initial phase of development during which there is a lag in chlorophyll synthesis and how they change subsequently during the stages of rapid differentiation. "Structural" protein was studied in this context in an attempt to see whether changes in it corresponded to those occurring in the chloroplast nucleic acids. The particular relevance of this is that it has been suggested by Smillie et al (1968), from work with inhibitors on Euglena, that his structural protein is not synthesized on chloroplast ribosomes and therefore the protein and nucleic acid syntheses need not be synchronous. Alternatively, synchrony of syntheses would support the suggestion of Wettstein (1967), based on work on Phaseolus, and Ohad et al (1967), from Chlamydomonas, that the chloroplast ribosomes are involved in the manufacture of this protein.

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The growing of plants in light.

Broad bean. Broad bean seeds (Vicia faba, Carter's variety White Fan, Raynes Park, London S.W.20) were soaked overnight in tap water. The imbibed seeds were then placed in 3 in. of John Innes No.1 soil mixture in wooden seed trays and covered with a thin layer of soil. After being watered, the seed trays were placed in a greenhouse maintained at 20 to 25°C. Light of approximately 600 ft-candles intensity supplementing the daylight was supplied for 16 hr. a day by 80 watt fluorescent tubes. Plants were watered at least twice a week. Unless otherwise stated, plants used for analysis were at least 2 weeks old and never more than 3 weeks old and had developed at least 4 fully expanded leaves.

Pea. Pea seeds (Pisum sativum, Carter's variety Laxton Superb, Raynes Park, London S.W.20) were soaked overnight. They were then planted in "quarter" grade Dupres Vermiculite (Hertford, Herts.) in plastic seed trays. The seeds were covered with a thin layer of Vermiculite, watered, and covered with a plastic sheet. The seeds were grown in a constant temperature cabinet maintained at 25°C, and illuminated by light of 200 ft-candles intensity for 16 hr. per day. After the seeds had germinated, the plastic cover was removed. Growth was for two weeks, the trays being watered well during this time.

Maize. Maize seeds (Zea mays, Carter's variety

Golden Bantam, Raynes Park, London S.W.20) were grown in a way similar to the broad bean. Plants were used for analysis when they had outer leaves approximately 3 in. long.

The growing of plants in the dark and their subsequent illumination. Broad bean and maize seeds were planted in the same manner as for growth in light. Once planted, the seed trays were placed in unilluminated growth cabinets in a constant temperature room set at 25°C. They were watered at least twice a week in total darkness. Broad beans were grown for either 1 or 2 weeks in this manner and the maize for 2 weeks.

Etiolated plants which were to be illuminated were transferred to a constant temperature cabinet at 25°C. and continuously illuminated by fluorescent light of 200 ft-candles intensity. The plants were grown in this cabinet for up to 6 days. For comparison, several plants were maintained in the dark while others were illuminated.

The labelling of plant components with radioactive isotopes.

Nucleic acids. The nucleic acids were labelled by supplying to the plants orotic acid-6-^{14}C monohydrate (specific activity 60.8 mc/mM, from the Radiochemical Center, Amersham), which is a precursor of pyrimidine nucleotides. Aliquots of the orotic acid solution containing 8 μc of radioactivity were dried onto 2 x 15 mm. strips of Millipore filter (AAWP 02500 - 8μ pore

size, Millipore, U.K., Ltd.). A longitudinal slit was made in the stem with a scalpel just below the first leaves. A radioactive strip was inserted into the slit and the slit was sealed by wrapping a 1 cm. wide band of Parafilm around the stem. The Parafilm was then covered with Vaseline.

Incorporation of radioactivity into nucleic acids was studied in etiolated plants and in green plants under dark and light conditions. The radioactive strips were inserted into the etiolated plants by the light of a dark room safe light fitted with a dark green filter (Wratten series, Kodak, Ltd., London). The manipulations were carried out as quickly as possible and the seed trays returned to the dark for a further period of 24 hr. before being illuminated.

Protein. In an attempt to label the "structural" protein of broad bean chloroplasts in green and etiolated plants two different techniques were employed. In the first, either 1 μc valine-^{14}C (U) (specific activity 6.9 mc/mM, Batch 38, the Radiochemical Center, Amersham) or 10 μc L-leucine-4,5-T (specific activity 29.1 c/mM, Batch 15, the Radiochemical Center, Amersham) was dried onto Millipore strips and inserted into the plant stems in the same way as the orotic acid. The plants were then grown for 3 days whilst illuminated continuously by fluorescent light of 200 ft-candles intensity.

In the second method of labelling the plants were grown in the presence of -

grown in the presence of $^{14}CO_2$. Each plant to be used was transferred to a 4 in. pot three days before supplying the radioactivity. To label the plants, the pot was placed under a 10 gal. bell jar on top of a glass plate and all the joints were sealed with Vaseline. The $^{14}CO_2$ was supplied through three glass tubes which passed through a rubber stopper into the top of the jar. A small side arm test tube was joined to the end of each of these tubes. The radioactive carbon, in the form of $Ba^{14}CO_3$ (specific activity 51 mc/mM, Batch 128, the Radiochemical Center, Amersham) had been weighed in equal amounts into each test tube and a serum cap fitted over the mouth. To generate the $^{14}CO_2$, 0.1 ml. of distilled water was injected with a Hamilton syringe (Clark Hamilton Manufacturing AG, Bonaduz GR, Switz.) through the serum cap. This was followed by 0.1 ml. of 50% lactic acid. The liquid was boiled to insure release of all the $^{14}CO_2$.

The radioactivity was supplied at 8 hr. intervals to etiolated plants that had previously received 48 hr. illumination. The total treatment was for 24 hr. under conditions of illumination by fluorescent light of 200 ft-candles intensity. Normal light grown plants were supplied with $^{14}CO_2$ under different conditions. Radioactivity was supplied at the start of the experiment and then 5 and 19 hr. later. For the first 5 hr., the plants were in light, followed by 14 hr. in the dark,

followed by 5 hr. light.

After incorporation of radioactivity, the leaves were removed from the plants and the chloroplasts extracted.

Preparation of tissues from plants.

Roots. Pea and maize root material was prepared by washing the roots free from Vermiculite or soil. The more fibrous portions were discarded and nucleic acids extracted from the remainder. For broad bean, imbibed seeds were germinated for approximately 3 days on 2 layers of Whatman No.1 filter paper in Petri dishes. The young roots were then extracted.

Apical meristems. The white apical meristems were excised under a dissecting microscope after the surrounding green leaves had been removed. They were then stored in ice cold tap water until sufficient numbers had been collected for extraction of nucleic acids.

Leaves. Only the first two fully developed leaves were used. In the study of the effect of light on etiolated plants, progressively fewer leaves were required for extraction as the leaves increased in size and weight. The number of leaves and their weight were recorded.

Isolation of organelles.

Pea and maize chloroplasts. These were extracted as described by Walker (1964). The leaves

(100 g.) were homogenized at 0°C in 250 ml. of semi-frozen extracting medium (0.1 M Na_2HPO_4 - KH_2PO_4 buffer pH 6.8 containing 0.1% NaCl, 0.1% $MgSO_4$, 0.1% sodium ascorbate, and 0.33 M sucrose) by dropping them into the 1 l. chamber of a Waring blender set at half speed. After 1 to 2 sec. they were then homogenized at full speed for 5 sec. The brei was filtered through 2 layers and then 8 layers of absorbent muslin. It was centrifuged in 50 ml. centrifuge tubes in an MSE "High Speed" refrigerated centrifuge at 0°C. When a speed of 6,000 rpm was reached, the centrifuge was rapidly stopped. The supernatant was discarded and the pellet used for RNA extraction.

Broad bean chloroplasts. Road bean chloroplasts were isolated by a method modified from that described by Leech (1964). Fifteen plants that were 12 to 17 days old were destarched by placing them in the dark for 24 to 48 hr. The leaves were chilled and all subsequent procedures carried out at 4°C. Fully expanded leaves were homogenized at full speed for 20 sec. in a 400 ml. chamber of an Omnimixer (Ivan Sorvall, Inc., Norfolk, Conn., U.S.A.) with 120 ml. 0.4 M sucrose in 0.15 M Tris-acetate buffer, pH 7.5. The blade of the homogenizer was replaced with a double- edged razor blade. The brei was filtered through 4 layers of absorbent muslin and the resulting suspension layered onto 8 gradients in 50 ml. clear

plastic centrifuge tubes. The gradients consisted of 12 ml. of 50% sucrose in 0.15 M Tris-acetate buffer, pH 7.5, overlaid with 8 ml. of 46% sucrose in the same buffer. The gradients were then centrifuged in a swing-out rotor in an MSE refrigerated centrifuge at 2,400 to 2,600 rpm (1,000 g) for 20 min. The intact chloroplasts, which accumulated between the 46% and 50% sucrose layers, were carefully removed with a Pasteur pipette. The chloroplast suspension obtained was diluted with an equal volume of the Tris-acetate buffer and the chloroplasts were precipitated by centrifugation at 2,600 rpm (1,000 g) for 10 min. The supernatant was decanted and the resulting pellets stored over ice or frozen until needed for nucleic acid extraction.

Intact chloroplasts could be obtained in this manner in 45 min. The purity of the chloroplast fraction was checked by dark-phase microscopy. At least 50% of the chloroplasts had intact outer membranes and there appeared to be little contamination by other organelles. The yield of ribosomal RNA from these chloroplasts was in the order of 1 mg.

Chloroplast lamellae. Preparation of chloroplast lamellae was by the method of James and Das (1957). Leaves (15 g.) from two-week old broad bean plants were homogenized for 20 sec. at full speed in 120 ml. 0.4 M sucrose in 0.05 M Tris-HCl buffer, pH 7.8, in a 400 ml. chamber Omnimixer. The blade of the homogenizer

was replaced with a double-edged razor blade. The brei was then strained through 4 layers of absorbent muslin and centrifuged in an MSE "Medium" refrigerated centrifuge at 4°C at 1,800 rpm (600 g) for 2 min. The supernatant was decanted and centrifuged at 2,600 rpm (1,000 g) for 10 min. Pellets were resuspended in 32 ml. of the homogenizing medium with a small paint brush. The suspension was layered onto 8 gradients in 50 ml. clear plastic centrifuge tubes. The gradients consisted of 10 ml. of 60% glycerol in 0.05 M Tris-HCl buffer, pH 7.8, overlaid with 10 ml. of 25% glycerol in the same buffer. The gradients were then centrifuged at 2,600 rpm (1,000 g) in the MSE refrigerated centrifuge for 20 min. The chloroplast layer between the 60% and 25% glycerol layers was removed with a Pasteur pipette and diluted with an equal volume of buffer. The chloroplasts were pelleted by centrifugation at 2,600 rpm (1,000 g) for 10 min. The pellet consisted mainly of chloroplasts lacking their outer membranes and stroma. It was stored on ice until needed for chloroplast "structural" protein extraction.

Mitochondria. Mitochondria were isolated from 20 g. of etiolated broad bean leaves. The leaves were homogenized in 120 ml. of 0.4 M sucrose in 0.05 M Tris-HCl buffer, pH 7.8, in the same manner as for the preparation of chloroplast lamellae. The brei was put through muslin, then centrifuged for 2 min. at 600 g. The ...

supernatant was then centrifuged at 1,000 g for 10 min. The supernatant was decanted and centrifuged in an MSE "High Speed 18" centrifuge (8 x 50 ml. rotor) at 20,000 g for 10 min. at 4°C. The "structural" protein was extracted from the resulting mitochondrial pellet.

Extraction of RNA. Unless otherwise stated, the procedure for RNA extraction was the one used by Dyer and Leech (1968). Approximately 5 g. of leaves or 10 g. of roots were homogenized for 30 sec. in an 80 ml. chamber of an Omnimixer at full speed with 10 ml. of a 6% aqueous solution of 4-aminosalicylic acid, Na salt, (British Drug House) and 20 ml. of a redistilled phenol/water mixture (80/20, w/w). The brei was filtered through two layers of nylon muslin. The filtrate was centrifuged in an MSE bench centrifuge at full speed (2,000 g) for 10 min. at 4°C. The upper aqueous layer was removed with a Pasteur pipette, and the nucleic acids in it were precipitated by the addition of ethanol at -20°C and stored at this temperature for between 4 to 24 hr. After this time, the nucleic acids in the suspension were pelleted by centrifugation at 2,000 g for 10 min. at 4°C. The supernatant was discarded and the inner walls of the centrifuge tubes were dried with tissue. The precipitate was resuspended in between 5 to 15 ml. of 3 M sodium acetate, pH 6.0, sufficient to obtain a cloudy suspension of the material. It was stored overnight at 4°C, during which time the low

molecular weight RNA and the DNA were solubilized (Hastings and Kirby, 1966). The high molecular weight rRNA was pelleted by centrifugation at 2,000 g for 10 min. at 4°C. The supernatant was decanted and the tube dried as before. The yield was approximately 5 mg. of rRNA.

In a variation of the above extraction procedure (Loening and Ingle, 1967), 3 g. of tissue were placed in 10 ml. of ice-cold 6% 4-aminosalicylic acid, Na salt, and 1% naphthalene disulphonate in 0.05 M Tris buffer, pH 7.5. Homogenization was for 5 sec. in a 100 ml. chamber of a Virtis "45" (Gardiner, N.Y., U.S.A.) homogenizer. Ten ml. of an ice-cold solution of phenol saturated with the Tris buffer containing 10% cresol and 0.1% 8-hydroxyquinoline was added. The mixture was stirred well in ice. It was centrifuged in a 50 ml. glass centrifuge tube at 1,000 g for 10 min. in an MSE "Medium" refrigerated centrifuge at 4°C. The aqueous layer was then removed with a Pasteur pipette and reextracted with 10 ml. of the phenol mixture. After a second centrifugation, the aqueous layer was removed and 20 ml. of ice-cold absolute ethanol added. Precipitation was carried out at 4°C for at least 1 hr. The nucleic acids were then pelleted by centrifugation at 1,000 g for 10 min. at 4°C. The pellet was washed twice with 10 ml. of ethanol/water (80/20, v/v) at room temperature. It was then

dissolved in 2 ml. of 0.15 M sodium acetate buffer, pH 6.0, containing 0.5% sodium dodecyl sulfate (specially purified, British Drug House). To determine the quantity and purity of the nucleic acid present, the absorbance of the solution between 220 and 320 mμ was determined using an Optika CF4NI recording spectrophotometer. The nucleic acids were then reprecipitated with two volumes of ethanol at 4°C and left overnight at this temperature. After pelleting the nucleic acids by centrifugation, the supernatant was decanted, the walls of the tube dried with tissue, and the material used for polyacrylamide gel electrophoresis.

Nucleic acid fractionation.

Sucrose density centrifugation. The method of sucrose density centrifugation used was that of Tissieres and Hopkins (1961). Gradients were prepared at least 1 hr. prior to centrifugation. Seven solutions containing 20, 17.5, 15, 12.5, 10, 7.5, and 5% sucrose in 0.1 M sodium acetate buffer, pH 6.0, were made. The gradients were prepared in cellulose nitrate tubes (3 in. x 1 in., Beckman, Palo Alto, Calif., U.S.A.). The sucrose solutions were delivered into them through vertically mounted plungerless syringes fitted with 21B hypodermic needles. The needles were bent so that they touched the sides of the tubes. Firstly, 3.4 ml. of the 20% sucrose solution was placed in each syringe, and when nearly drained it was replaced with 3.4 ml. of

the 17.5% sucrose solution. Successively less concentrated solutions were added in sequence, care being taken to insure a continuous flow of liquid during the entire operation.

The rRNA was dissolved in 0.1 M sodium acetate buffer, pH 6.0. The absorbance of an aliquot of the solution was determined at 260 mμ using an Optika CF4NI recording spectrophotometer. Assuming a gram extinction coefficient of 20 for RNA at this wave length, the concentration of the solution was calculated. It was then adjusted so as to contain 1 mg. RNA per ml. of buffer. Then 1 ml. of the nucleic acid solution was layered onto each of the 3 gradients. The gradients were positioned in a prechilled Spinco (Beckman, Palo Alto, Calif., U.S.A.) SW 25.2 rotor. Centrifugation was in a Spinco Model L Ultracentrifuge at 20,5000 rpm (49,000 g) for 16.5 hr. at 0°C.

The centrifuge was allowed to stop without braking. Then the tubes were punctured at the bottom with a No. 1 disposable syringe needle and 10 drop aliquots collected. There were approximately 30 aliquots per gradient. Distilled water (1 ml.) was added to each aliquot and the absorbance was then measured at 260 mμ using an Optika CF4NI spectrophotometer. If the fractions were needed for further analysis, the RNA in them was precipiated with two volumes of ethanol for at least 4 hr. at -20°C.

Methylated serum-albumin - kieselguhr (MAK) chromatography.

Methylated serum - albumin Kieselguhr (MAK) columns were used both to separate the rRNA from the DNA and the low molecular weight RNA and also to analyze the rRNA. The method of chromatography used was that described by Dyer and Leech (1968). To reclaim RNA from the column effluents, fractions located by their absorbance of u.v. light at 254 mμ were collected. Each of these fractions was mixed with an equal volume of absolute ethanol and poured onto a cellulose column. The column had been previously prepared by suspending 5 g. Whatman CF11 powder in 100 ml. ethanol/water (1/1, v/v) and packing the slurry under pressure onto a scintered plate at the bottom of a column 1.7 cm. in diameter. After the nucleic acid solution had drained to the top of the column, 100 ml. of 0.1 M NaCl/ethanol (65/35, v/v) was added. This procedure was required to remove the phosphate buffer used in the elution of the MAK columns, since it precipitates with the nucleic acids at alcohol concentrations greater than 50%. Finally, the adsorbed nucleic acids were removed from the cellulose column with three 10 ml. aliquots of 0.1 M NaCl. RNA was precipitated by adding 60 ml. of ethanol at -20°C. After being left overnight at -20°C, the suspension was centrifuged at 2,000 g for 10 min. at 0°C in an MSE bench centrifuge. The supernatant was decanted and the RNA pellet stored at -20°C until required for electrophoresis.

Agar gel electrophoresis. The method of electrophoresis in 2% agar gels was that of McIndoe and Munro (1967). "Agarose" (1.6 g., Seravac, Maidenhead, Berkshire) was suspended in 15 ml. of 0.02 M Tris-citrate buffer, pH 7.9. The volume was made up to 80 ml. with additional buffer. The "Agarose" was dissolved by heating in an autoclave for 15 min. at 15 psi. The solution was cooled to 40°C before being poured into a 5 in. x 3.5 in. x 0.25 in. Perspex mold. The mold was covered with a Perspex plate excluding all air bubbles. Alternative covers had either 2 or 4 projections of 1/16 in. thick plate which formed sample channels in the gel 0.75 in. from one end. After the gel had set, the cover was removed and 60 µg. of RNA in 60 µl. of buffer was deposited with a Hamilton syringe into each channel. Electrophoresis was carried out in a Shandon (Shandon Scientific Co., Ltd., London) "Universal" electrophoresis apparatus, using 0.2 M Tris-citrate buffer, pH 7.9, in the electrode compartments. Whatman 3MM filter paper, in strips 3.5 in. wide, were used as wicks. A D.C. current of 4 mA/cm^2 (at about 100 volts) was applied across the gel using a Shandon "Vokam" stabilized power supply. After 10 min., the gel was covered with polythene sheeting to prevent condensation of water on top of it. Electrophoresis was carried out for 90 min., after which time the gel was removed from the mold and stained for 1 hr. with 0.01% toluidine blue. Destaining was accomplished

by immersing the gel in several changes of distilled water.

Polyacrylamide gel electrophoresis. A modification of Loening's (1967) method was employed for polyacrylamide gel electrophoresis of the high molecular weight RNA.

Preparation of gels. Solutions were prepared immediately before electrophoresis. The gel buffer contained 180 mM Tris (Analar, British Drug House), 150 mM NaH_2PO_4, and 5 mM EDTA (Na salt). To 12.5 ml. of this buffer, 39.4 ml. distilled water and 10 ml. of an acrylamide solution (15% acrylamide and 0.75% bis-acrylamide in distilled water) were added. The acrylamide had been recrystallized from chloroform and the bis-acrylamide from acetone, as described by Loening (1967).

The solution of buffer and acrylamide was degassed under vacuum and 0.4 ml. of a freshly-prepared 10% ammonium persulphate solution and 0.04 ml. of NNN'N'-tetramethylethylethylene diamine (TEMED) added. (The final concentration of acrylamide in the gel solution was 2.4%.) The solution was swirled gently and placed with a Pasteur pipette into Perspex tubes 13 cm. long and 0.7 cm. in diameter. Dialysis tubing held in place with a rubber ring covered the bottom of each tube. The tubes were filled with gel solution to a mark 10 cm. from the bottom. After removing any trapped air bubbles from the gel solution in the tubes, approximately 0.2 ml.

of distilled water was layered very carefully over each gel with a Pasteur pipette drawn to a fine tip so as not to disturb the gel surface. Once it had been assured that all the tubes were vertical, the gels were left to polymerize. This occurred within ½ hr. Of the 12 gels prepared, 8 were selected for electrophoresis. Gels were rejected if the dialysis tubing had leaked, if the top surface of the gel was not horizontal, if there were any air bubbles or other irregularities in the gel, or if the water layered on top had disturbed the surface during application.

A slight variation of this method was introduced for the electrophoresis of radioactive RNA. The gels were 7 cm. long instead of 10 cm. During preparation of the gels, the bottom end of the tubing was closed with a rubber stopper instead of dialysis tubing. After polymerization, the stopper was replaced with a plastic ring and the gels used for electrophoresis.

Electrophoresis. Electrophoresis was carried out in an Shandon (Shandon Scientific Co., Ltd., London) disc electrophoresis apparatus, modified to accommodate the Perspex tubing which was of larger diameter and longer than that of the original equipment. The tubes were placed vertically between the two electrode chambers and 300 ml. of the reservoir buffer of 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA (Na salt), and 0.2% sodium dodecyl sulphate (specially purified, British Drug House) was

placed in each electrode chamber. The negative electrode of a Shandon "Vokam" stabilized D.C. power supply was attached to the electrode of the upper chamber and the positive to that of the lower chamber. A current of 6 mA/gel was applied. Under these conditions, the voltage was between 60 and 70 volts. The entire system was cooled with a fan. The gels were prerun for ½ hr. before the nucleic acids were applied.

The nucleic acids were dissolved in the reservoir buffer containing 5% sucrose to make the solution more dense than the buffer and thus insure that it formed a discrete band on the top of each gel when applied. If the amount of nucleic acid had not previously been determined, an aliquot was removed for this purpose. The absorbance between 220 and 320 mμ was determined with a Unicam SP800 spectrophotometer. Assuming that 1 mg./ml. of nucleic acids has an absorbance of 20 at 260 mμ, the concentration in the solution was determined. It was adjusted to be between 1 and 4 mg. RNA/ml.

The nucleic acid solution was subsequently layered onto the prerun gels with a Hamilton syringe. Each nucleic acid sample (between 10 and 100 μg. in 10 to 60 μl. of solution) was applied to two separate gels for electrophoresis. To follow the progress of electrophoresis, 20 μl. of 0.01% bromophenol blue in buffered sucrose was applied to one of the gels. The rate of movement of this dye is not reduced by the gel matrix. Current was

passed through the gels for 3 hr. from the time that the last solution was applied.

After electrophoresis, the gels were extruded from the tubing by applying gentle pressure to the top with a rubber teat. To reduce background abosrption in the gels, they were either rinsed overnight with about 25 ml. of 30% ethanol or with the same volume of water for 1 hr. The latter method was employed whenever it was necessary to freeze the gels for determination of radioactivity in the nucleic acids.

Scanning and radioactivity measurements. The nucleic acid bands in the gel were detected by their absorption of light at 260 mμ. The gels were scanned continuously using a Unicam SP500 monochromator and a Hilger-Gilford photometric unit. The gel was placed in a 0.6 cm. wide quartz cuvette which was passed at a uniform rate in front of a vertical beam of light 0.2 mm. wide and 2 mm. long. The absorption was continuously recorded with a pen recorder.

The profile of radioactivity in the gels was determined by cross-sectioning them and detecting the radioactivity in each section. The gels were placed in an aluminum trough 10 c. long and frozen on a bed of crushed solid CO_2. The gels could then be stored at -20°C. Slicing was accomplished on a macrotome based on the design of Dr. U.E. Loening. The gel was secured to the macrotome stage by placing it on a water saturated

piece of filter paper on the stage and surrounding it with crushed solid CO_2. The frozen gel was cross-sectioned into 1 mm. thick slices and the slices were placed in sequence with forceps onto a 1 cm. wide strip of filter paper. The sections were then left to dry on the paper before the strip was cut up into pieces, each of which had an adhering section. The pieces of filter paper were placed in 10 ml. of 0.6% 2,5, diphenyl-oxazole (PPO, Nuclear Enterprises, Edinburgh) in toluene in a scintillation vial. The number of disintegrations occurring during 10 min. was determined for each section in a Nuclear-Chicago model 720 scintillation spectrometer. Background radioactivity was measured in a vial containing filter paper with no attached gel. Efficiency of radioactivity measurements was determined by drying radioactive sucrose of known radioactivity (supplied by Mr. I.F. Bird) onto filter paper and covering the sucrose with a gel slice. After the gel had dried, the strip was placed in scintillation fluid in a vial. The efficiency of measurement under these conditions was found to be 42%.

To assess the relative amounts of each of the nucleic acid fractions in the polyacryamide gels, the peaks on the paper of the optical density absorbance scans of the gels were cut out and weighed. To assess the radioactivity associated with each optical density peak, the disintegrations/min. in each mm. section were

plotted on the same graph as the optical densities, and those sections coinciding with the peaks on the optical density profiles totaled.

Chlorophyll estimations (Arnon, 1949). Approximately 0.5 g. of tissue was homogenized for 30 sec. in 25 ml. of an acetone/water (80/20. v/v) solution in an 80 ml. chamber of an Omnimixer. The extract was clarified by filtering through Whatman No. 1 filter paper. The spectrum of the filtrate in the visible range was determined using a Unicam SP800 spectrophotometer with some of the acetone solution in the reference cell. To calculate the chlorophyll concentration, the following formula was used:

$$c = (20.2 \times OD_{645} + 8.02 \times OD_{663}) \times 0.025$$

where c is mg. chlorophyll in 25 ml. From this the content of chlorophyll per leaf and per g. wet weight of tissue were calculated.

Total RNA content determinations. The RNA content of leaves was determined by the method of Ingle and Hale (1963). Leaves were frozen in liquid nitrogen and stored in solid CO_2 at -20°C. Approximately 1 g. wet weight of leaves was homogenized in 25 ml. of ice-cold deionized water (containing 1 drop ethanol as an anti-foam agent) in a 100 ml. chamber of a Virtis "45" homogenizer at full spped for 30 sec. The brei was filtered through glass wool and 15 ml. of the filtrate was removed and made 0.2 N with respect to perchloric

acid at 0°C. The precipitate was pelleted by centrifugation at 1,000 g for 10 min. at 4°C in an MSE "Medium" refrigerated centrifuge. The pellet was washed with two 10 ml. aliquots of 0.2 N perchloric acid at 0°C. It was then washed at 0°C. in a 0.02 M formic acid solution in methanol. Lipids were removed by washing the pellet twice in 15 ml. ethanol/ether/chloroform (2/2/1, v/v/v) at 37°C for 30 min. on each occasion. The RNA in the pellet was hydrolyzed in 5 ml. of 0.3N KOH for 18 hr. at 37°C. The suspension was subsequently cooled to 0°C and ice-cold perchloric acid added to a final concentration of 0.3 N. The precipitate was pelleted by centrifuging at 1,000 g for 10 min. and reserved for DNA estimations. The ribonucleotide concentration in the supernatant was determined by absorption between 220 and 320 mμ in a Unicam SP800 spectrophotometer, with 0.3 N perchloric acid in the reference cell. It was assumed that a solution of 1 mg./ml. would have an absorption of 25. A portion of the supernatant (10 ml.) was dried down in a scintillation vial and 10 ml. of 0.6% PPO solution in toluene added. The radioactivity in the vials was determined using a Nuclear-Chicago model 720 scintillation counter.

Total DNA content determinations. The perchloric acid precipitate left after hydrolysis of the RNA was washed twice with 10 ml. of 0.3 N perchloric acid at 0°C. It was then suspended in 1 ml. of 5 mM NaOH and an equal

volume of 1 N perchloric acid added. The suspension was heated at 70°C for 15 min. After diluting with an equal volume of 0.5 N perchloric acid, samples were removed for use in the Burton (1956) DNA estimation. Highly polymerized salmon testes DNA (British Drug House) was used as a reference standard. Diphenylamine reagent was added to each standard and sample tube and the tubes incubated for 16 to 20 hr. at 30°C. The absorbance at 600 mμ was measured using a Unicam SP800 spectrophotometer. The amount of DNA in the samples was calculated from a plot of the standard measurements. Radioactivity was determined in the same way as for the RNA.

Fixation and embedding of material for use in electron microscopy. The method used for the fixation and embedding was that suggested by Mr. A.D. Greenwood. The plant tissue was cut into 2 mm. squares with a new razor blade and placed immediately in a fixative consisting of a 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer, ph 7.0. The tissue was infiltrated with fixative by subjecting it to a vacuum four times, quickly returning to atmospheric pressure on each occasion. The tissue was then left in the fixative for 4 hr. at 0°C. Etiolated material was treated in the same way, except in darkness or in dim green light provided by a Safe Light lamp (Kodak, London) fitted with a Wratten "dark green" filter. After fixation, the edges were cut off each square of tissue and it was then cut into 4 pieces.

The fixative was removed with a Pasteur pipette and replaced with a wash solution consisting of 0.1 M sucrose in sodium phosphate buffer, pH 7.0. The tissue was left in this solution for 30 min. at 0°C. This washing procedure was repeated twice. Post-fixation was then carried out by immersing the material for 2 hr. at 0°C in the wash solution containing 1% osmium tetroxide. Afterwards, to dehydrate the material, it was immersed in sequence in 20, 30, 40, and 50% ethanol/water solutions, each for 5 min. at 0°C. It was then immersed in 75% and then 95% ethanol/water for 20 min. each at room temperature, followed by 100% ethanol for 30 min. at room temperature. The tissue was then put in fresh ethanol and left overnight at 0°C. Before embedding the tissue was placed in a further change of ethanol for 30 min. at room temperature. Half the ethanol solution was then replaced with 1,2-epoxypropane and the tissue left in the mixture for 10 min. Half of this solution was replaced with fresh 1,2-epoxypropane and left for an additional 10 min. This step was repeated a third time. Then the material was immersed 3 times in 100% 1,2-epoxypropane for 10 min. on each occasion. Epon resin (Epikote 812, TAAB Laboratories, Reading) (6 parts "B" to 4 parts "A") mixed with 1,2-epoxypropane in the ratio of 1 to 3 was placed with the material in small covered dishes. After 2 hr., the lids were partially opened and the dishes left in this position for 24 hr.

Approximately 12 pieces of tissue were placed in a circle about 6 mm. from the perimeter of 4 cm. diameter plastic cups. Fresh Epon resin was put in the cups to cover the samples. The cups were left for 48 hr. at room temperature, then put into an oven set at 70°C for 48 hr. in order to harden the resin.

The sectioning and staining of the material in 4% lead citrate (according to Reynolds, 1963) and 2% uranyl acetate for 20 min. at 60°C was carried out by Dr. B. Griffiths. Photography was carried out by Mr. A.D. Greenwood and Dr. T.A. Dyer on an MSE "EM6B" electron microscope fitted with a cooled grid stage.

Statistical analysis of electron micrographs. To assess the concentration of ribosomes in both the chloroplast and cytoplasm, electron micrographs of approximately 50,000 magnification were used. The micrographs were covered with a grid dividing the picture into 1 mm. squares. By using random numbers, it was determined what percentage of chloroplast squares contained a ribosome. The same was done for the cytoplasm. To assess the relative volume occupied by both cytoplasm and chloroplast, electron micrographs of approximately 2,000 to 6,000 magnification were used. The micrographs were covered with a grid of 13 wires equally spaced along the length of the picture. The length of wire covering the chloroplasts and cytoplasm was measured and recorded. The ratio of chloroplast length to cyto-

plasm length is equal to the ratio of chloroplast volume to cytoplasm volume (Loud, 1962). Once these two measurements had been made, one could determine the relative contribution of the chloroplast ribosomes to the total ribosome population.

"Structural" protein extraction. The method for the isolation of the "structural" protein from chloroplasts is based on that of Criddle (1966). The chloroplasts purified on glycerol gradients were suspended in 20 ml. water at 0°C. The suspension was slowly added to 200 ml. acetone at -20°C. The acetone solution was kept in ice and stirred continuously during the addition. It was maintained at -20°C for 1 hr. to insure precipitation of all proteins. The suspension was then centrifuged in an MSE "High Speed 18" centrifuge at 10,000 g for 30 min. at 0°C. The pellet was washed once with 50 ml. anhydrous diethyl ether at 0°C. The white powder obtained was stored in a desiccator at 4°C. "Structural" protein was extracted in an identical manner from mitochondria.

When the protein was precipitated with ammonium sulphate, it was dissolved in 100 ml. of a solution of 0.3% sodium dodecyl sulphate and 3% urea in water. Ammonium sulphate was added to a final concentration of 20%. The suspension was left to precipitate 1 hr. at 0°C, after which time it was centrifuged as for the acetone powder. The supernatant and pellet were used

in electrophoresis.

"Structural" protein electrophoresis. Electrophoresis was carried out using 7% polyacrylamide gels, according to the method of Summers et al (1965), and utilizing the Shandon disc electrophoresis apparatus, the Gilford-Unicam SP500 gel scanner, and the gel chopper that were used for the study of the nucleic acids. Cyanogum (British Drug House) (1.75 g.) and 0.75 g. urea were dissolved in 25 ml. of the electrode buffer of 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% sodium dodecyl sulphate (specially purified, British Drug House). TEMED (25 µl.) and 0.2 ml. of a freshly-prepared 5% ammonium persulphate solution were added. The gels were made in the same manner as for the nucleic acid electrophoresis. Gels were either 0.4 x 7 cm. or 0.7 x 10 cm. in size. Samples of the "structural" protein were dissolved to give a concentration of 10% (w/v) in the electrode buffer, which contained 3% urea and 10% sucrose. Between 50 and 100 µl. of this solution was loaded onto each gel. Bromophenol blue was used to indicate the progress of the electrophoresis as in the case of the nucleic acids. Electrophoresis was conducted for 4.5 to 6 hr. at 4 volts per cm. After extrusion of the gels from the tubes with a rubber teat, they were stained overnight in a solution of 1% amido black 10B in 7% acetic acid that had been filtered just before use. Unbound dye was removed by immersing the

gels in several changes of 1% acetic acid until no further dye was removed. The light absorption of protein-bound dye bands was determined at 600 mμ. Gels containing ^{14}C labelled proteins were frozen in solid CO_2 and 1 mm. sections cut. Radioactivity was determined in sections dried onto filter paper and immersed in PPO-toluene scintillation fluid as previously described for the nucleic acids. For the determination of the distribution of tritium labelled protein components in the gels, each gel slice was placed in a scintillation vial with 1 m. of 30% H_2O_2 (Young and Fulhorst, 1965). They were left overnight at 50°C during which time the gel dissolved and the liquid evaporated. Then 10 ml. of the PPO-toluene scintillation fluid was added and the radioactivity determined as for nucleic acids.

Thin-layer chromatography of the amino acids from chloroplast "structural" protein. "Structural" protein (8 mg.) was hydrolyzed by 5 ml. of 6N HCl in a closed vessel in boiling water for 2 hr. It was cooled to 0°C overnight. The vessel was opened and the material dried at 40°C in a rotary evaporator. The residue was then dissolved in 6 ml. water and placed onto an AG 50W-X4 cation exchange resin (hydrogen form, Calbiochem, Los Angeles, Calif., U.S.A.) column 0.7 cm. in diameter and 2 cm.long. The column was washed with 6 ml. of water and the amino acids eluted from the column with 6 ml. of 2N ammonium hydroxide. The eluate was dried using a rotary evaporator

and stored at 0°C. The residue was dissolved in 0.2 ml. water and 50 µl. spotted onto each of two 10 x 20 cm. thin-layer plates coated with a 40 u thick layer of Whatman CF11 thin-layer Chromedia. The chromatographs were developed for 2 hr. in butanol/acetone/ diethylamine/ water (10/10/2/5, v/v/v/v) as described by Arx and Neher (1963). After drying in air, one plate was sprayed with ninhydrin-collidine reagent (0.25% ninhydrin and 0.1% collidine in water-saturated butanol) and the pattern photographed. A sheet of Kodak "Estar" X-ray film was placed in contact with the cellulose layer of the duplicate plate and left for one week in the dark. The autoradiograph was then developed with Kodak 19B developer.

RESULTS

Initial experimentation was devoted to characterizing the chloroplast rRNA. As can be seen in Fig. 3, there are distinct differences in the sedimentation properties of leaf, root, and chloroplast high molecular weight rRNA. Current theory (cf. Loening, 1967) maintains that ribosomes contain one relatively large RNA molecule, one about half the size of the large RNA molecule, and a much smaller RNA having a molecular weight of about 42,000. Since the heavier of the high molecular weight RNA's is approximately double the weight of the lighter one, in absorption profiles of sucrose density gradients on which rRNA has been separated, the ratio areas of the peaks of the "heavy" and "light" components is 2 to 1. The absorbance ratio of these areas in the profiles of the gradient-fractionated chloroplast rRNA differs from this (Fig. 3, 3a). Also, the presence of chloroplast rRNA in the total leaf rRNA modifies the leaf profile so that the ratio is not 2 to 1 in this tissue (Table 1). In the chloroplast fraction, there appear to be two "light" rRNA molecules for every "heavy" molecule. From this it can be concluded that either the chloroplast ribosome is an exception to the general theory of ribosome constitution or the "heavy" molecule is fragmented in some manner. Non-specific breakdown by nucleases would probably result in the dispersion of u.v. absorbing material with an accumulation at the top of the gradient.

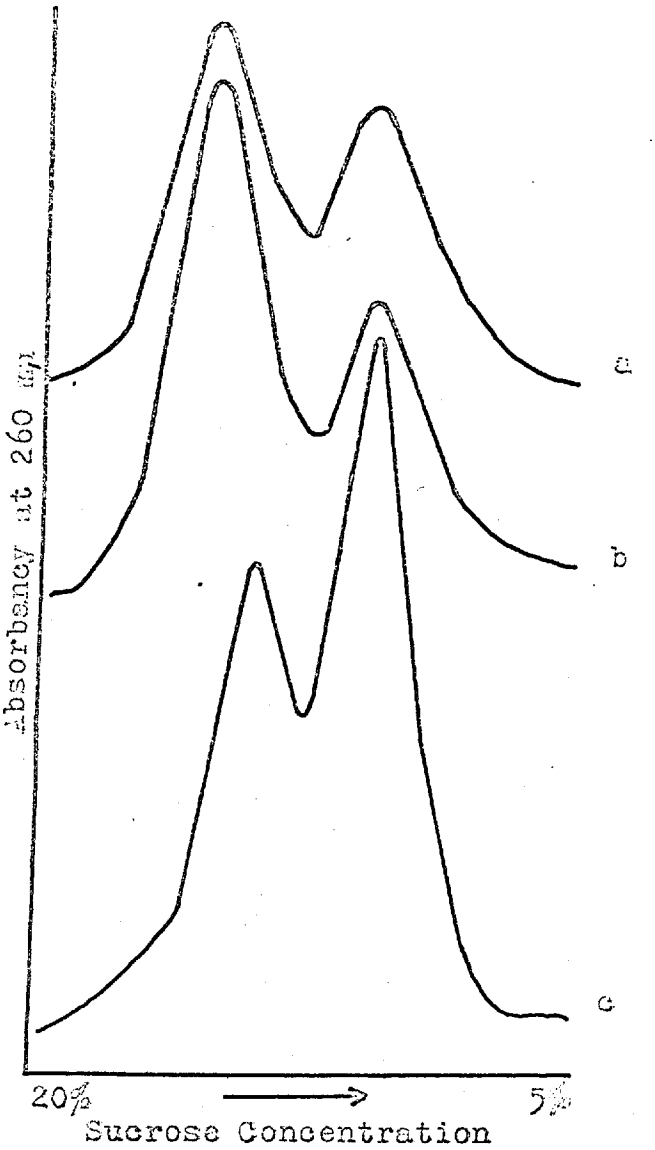


Figure 3. Comparison of abosrbancy profiles of pea rRNA tissues that had been fractionated on sucrose density gradients.

a. Leaf rRNA
b. Root rRNA
c. Chloroplast rRNA

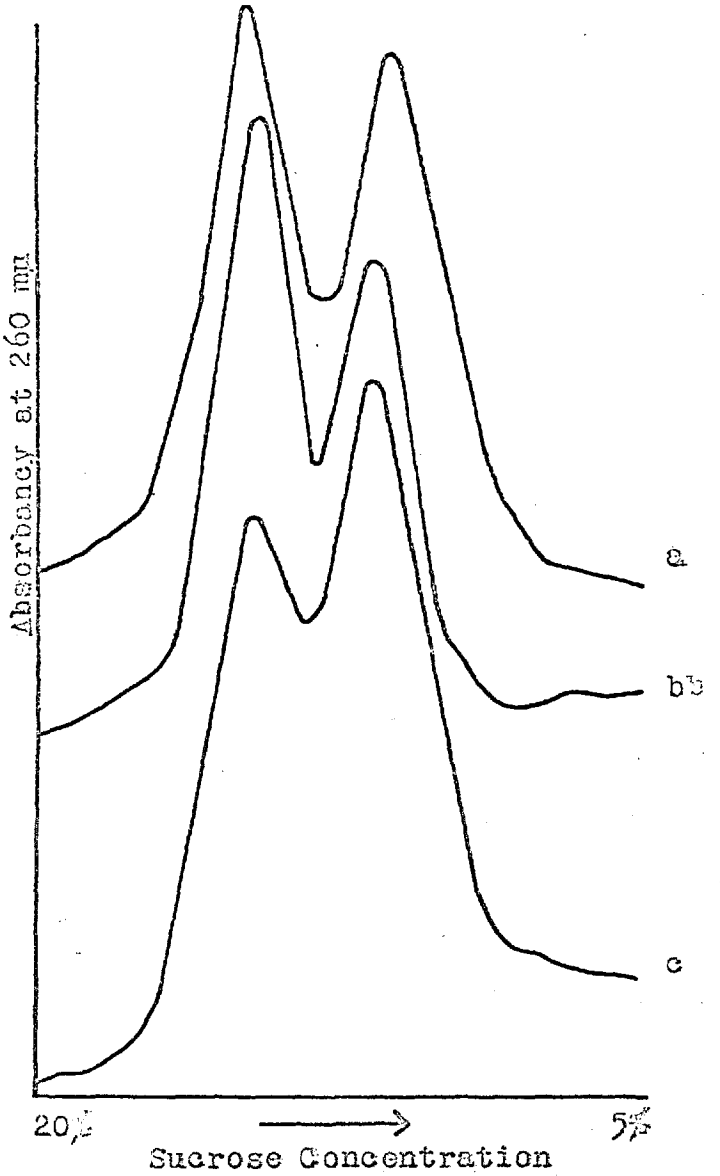


Figure 3a. Comparison of absorbancy profiles of broad bean rRNA tissues that had been fractionated on sucrose density gradients.

a. Leaf rRNA
b. Root rRNA
c. Chloroplast rRNA

Table 1. Ratios of absorbancies at 260 mμ of "heavy" and "light" rRNA fractions separated by sucrose density gradient centrifugation. For comparison with leaf tissue, a value for this ratio was derived by adding the chloroplast and root values.

	Leaf	Root	Chloroplast	Root + Chloroplast
Pea	1.23:1	1.63:1	1:1.35	1.12:1 (2.63:2.35)
Broad bean	1.03:1	2.04:1	1:1.78	1.09:1 (3.04:2.78)

This does not occur. However, a specific cleavage of the heavier chloroplast molecule near its center would produce two fragments of about the same size as the lighter rRNA molecule. A re-run of the "heavy" and "light" fractions from the sucrose density gradients of rRNA from both pea leaf and pea root tisssue (Figs. 4 & 5) show that there is some contamination of each fraction by the other. If the "heavy" chloroplast material is fragmented after isolation, an increase in the "light" fraction contamination of the "heavy" fraction might be found when the nucleic acids of the leaf tissue are fractionated. The results do not show this. The results presented in Fig. 6 are of the elution profiles of "heavy" and "light" rRNA fractions taken from the sucrose gradients and chromatographed on methylated serum albumin-kieselguhr columns. Here also there is no great increase in a "light" component in the "heavy" fraction resulting from fragmentation. It appears, in fact, that the "light" rRNA fraction from the gradients contains more "heavy" rRNA than vice-versa.

The two leaf rRNA fractions separated by sucrose density gradient centrifugation were electrophoresed in "Agarose" gel. These fractions, as well as the corresponding ones from chloroplast rRNA were subjected also to polyacrylamide gel electrophoresis. Similar results by both these methods were obtained. In the

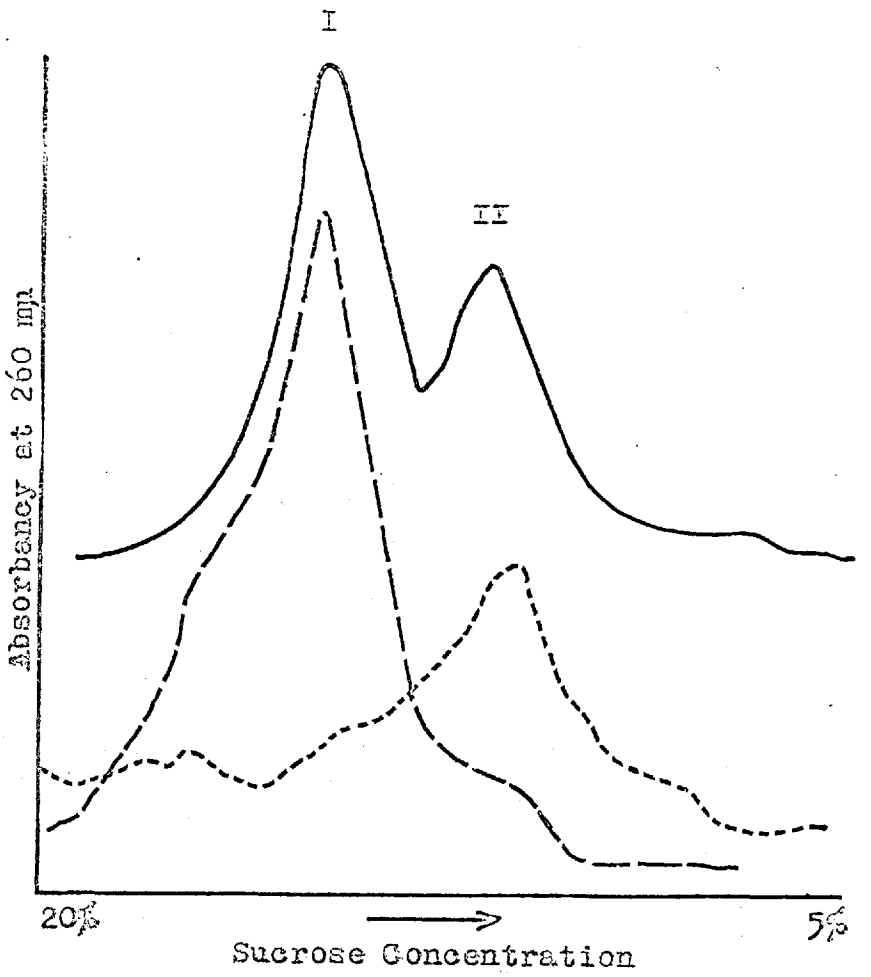


Figure 4. Comparison of absorbancy profiles of pea root rRNA that had been fractionated on sucrose density gradients.
——— Total root rRNA
— — Refractionation of fraction I recovered from a gradient on which the total rRNA had been fractionated
= = = = Refractionation of fraction II

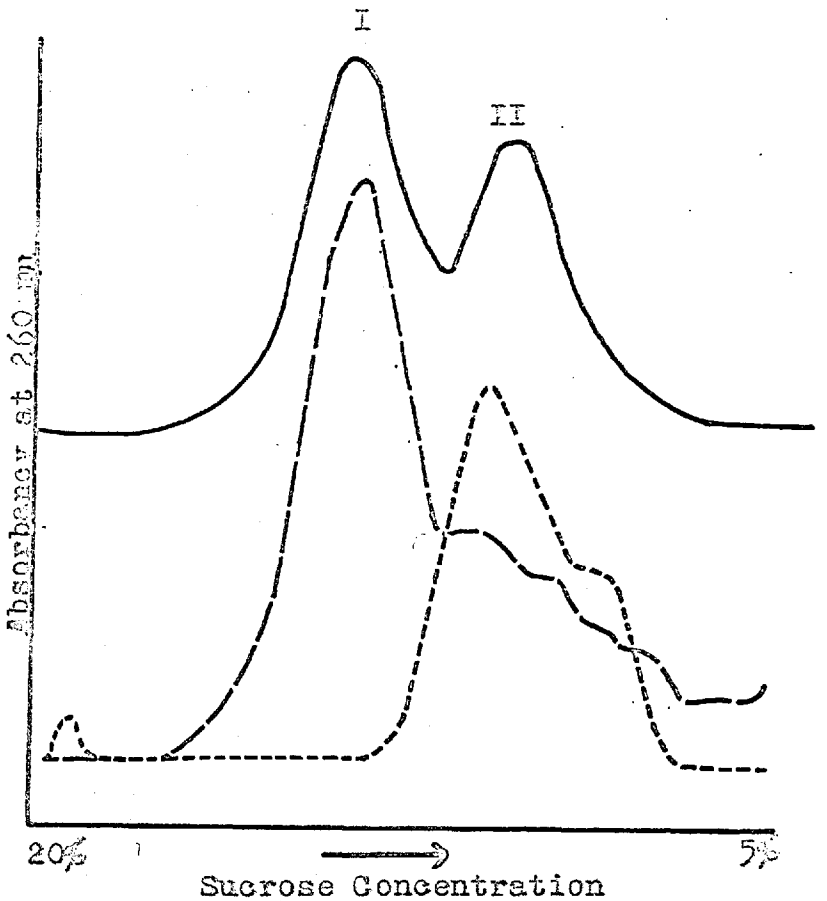


Figure 5. Comparison of absorbancy profiles of pea leaf rRNA that had been fractionated on sucrose density gradients.
——— Total leaf rRNA
— — Refractionation of fraction I recovered from a gradient on which the total rRNA had been fractionated
— — — Refractionation of fraction II

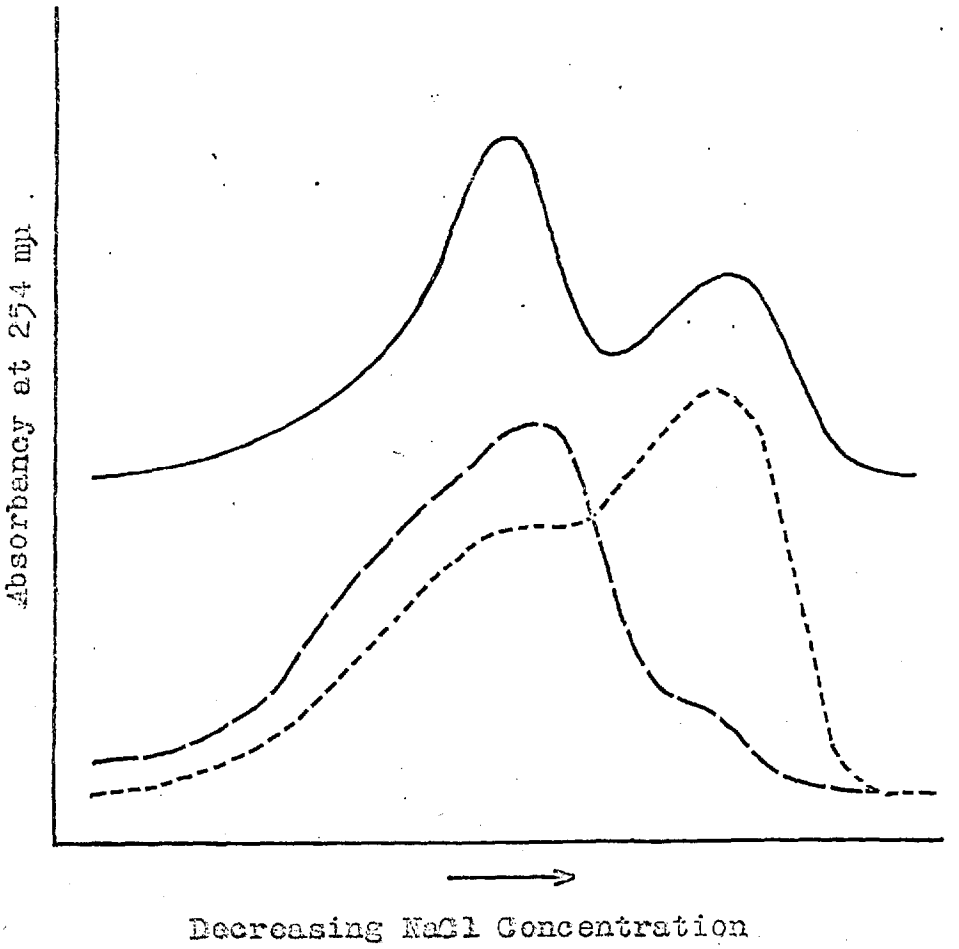


Figure 6. Absorbancy profiles of broad bean leaf rRNA fractionated by chromatography on methylated serum albumin - kieselguhr columns.

——————— Total rRNA

—— —— The "heavy" rRNA fraction derived from total rRNA that had been fractionated on sucrose density gradients

-·—·—·— The "light" rRNA from sucrose density gradients

"Agarose" gels, leaf rRNA is resolved into four components (Fig. 7). The heaviest component, and thus the one with lowest electrophoretic mobility, is present in the "heavy" fraction from the density gradient, and the lighter species were derived from the "light" gradient fraction. The "light" components did not contaminate the "heavy" fraction as much as the "heavy" the "light". In the polyacrylamide gels, an additional component is resolved (Figs. 8 & 9). The large rRNA of relatively low electrophoretic mobility is fractionated into two components which are both present in the "heavy" gradient fraction. Thus, in the high molecular weight leaf rRNA fractionated by sucrose density gradient centrifugation, there are at least two RNA species in the "heavy" gradient peak and three in the "light" gradient peak. The chloroplast fraction high molecular weight rRNA contains a minimum of four components. The presence of a relatively large rRNA molecule results in the "heavy" gradient peak and three smaller types of rRNA in the "light" fraction (Fig. 9). If the "heavy" rRNA from the chloroplast does fragment into two components of about the same size as the "light" rRNA, it would seem that this occurs *in vivo* or during extraction of the RNA. Those molecules remaining intact during extraction appear to be stable during subsequent treatment.

This interpretation is substantiated by analysis



Figure 7. Photograph of an "Agarose" gel on which broad bean leaf rRNA had been fractionated by electrophoresis then stained with toluidine blue.

a. Total rRNA - 60 ug.
b. "Light" rRNA fraction from a sucrose gradient - 30 ug.
c. "Heavy" rRNA fraction from a sucrose gradient - 30 ug.
d. Total rRNA - 30 ug.

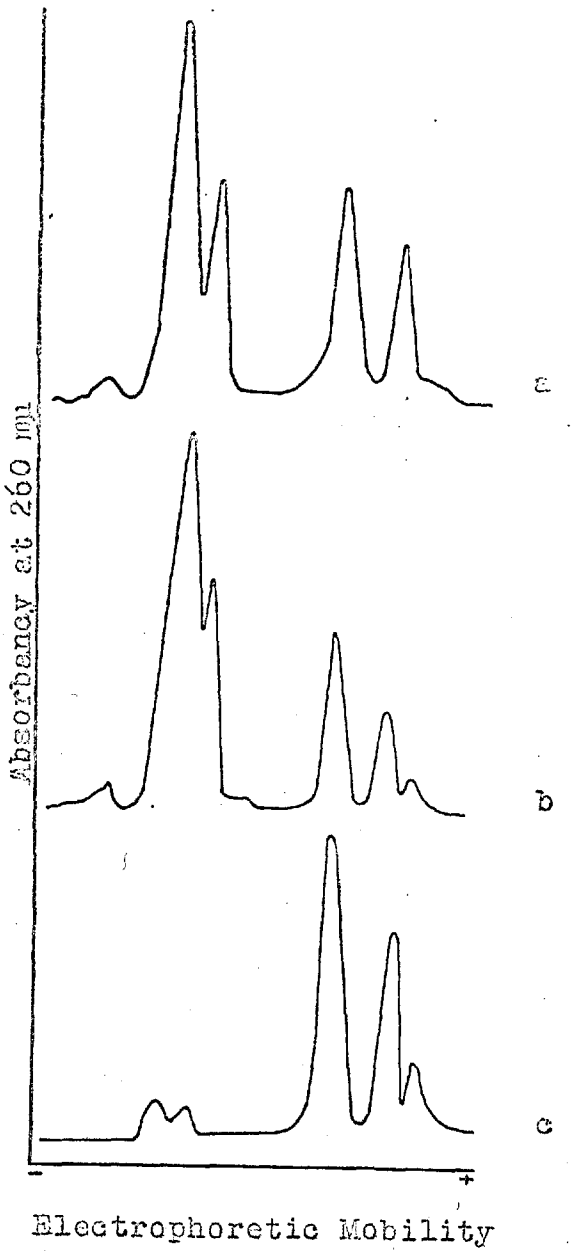


Figure 8. Absorbancy profiles of broad bean leaf rRNA fractionated by polyacrylamide gel electrophoresis.

a. Total rRNA
b. The "heavy" rRNA fraction derived from total rRNA that had been fractionated on sucrose density gradients
c. The "light" rRNA from sucrose density gradients

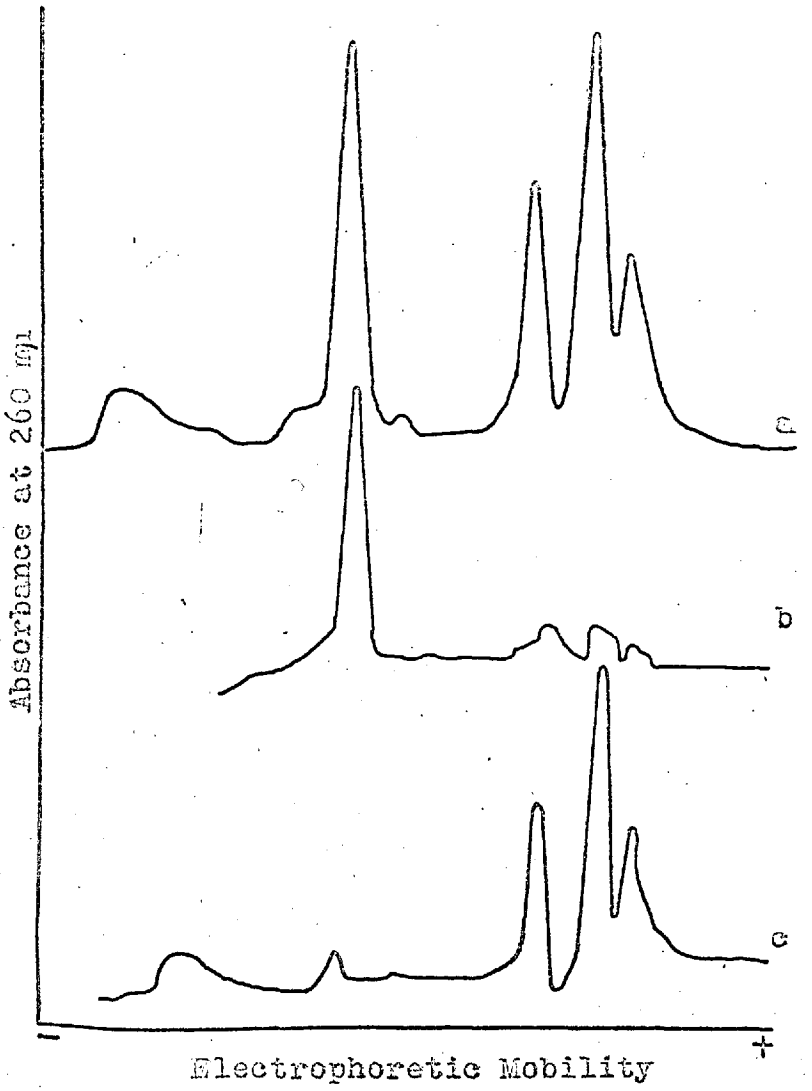


Figure 9. Absorbancy profiles of broad bean leaf chloroplast rRNA fractionated by polyacrylamide gel electrophoresis.

a. Total rRNA
b. The "heavy" rRNA fraction derived from total rRNA that had been fractionated on sucrose density gradients
c. The "light" rRNA from sucrose density gradients

of the results of polyacrylamide gel electrophoresis of chloroplast rRNA. As has been noted previously, three components are resolved from the electrophoresis of the chloroplast "light" rRNA fraction from the sucrose gradients. This is from a highly purified chloroplast fraction (Leech, 1964)) and therefore these species are definitely derived from the chloroplasts. Either the chloroplast has three different varieties of "light" rRNA, or two of the components are formed as a result of the fragmentation of the "heavy" rRNA. Using Escherichia coli rRNA's as markers on the gels, it is possible to determine the molecular weight of various RNA fractions on the gels, according to a relationship between distance travelled and molecular weight (Loening and Ingle, 1967). The molecular weights of the RNA in the "light" gradient fraction were calculated to be 0.4, 0.56, and 0.7 m. If the molecular weights of the largest and smallest of these are totalled, a figure of 1.1 m is obtained which corresponds to the weight of the rRNA derived from the "heavy" gradient fraction of chloroplast rRNA. If one molecule of intact chloroplast "heavy" rRNA splits into one molecule of 0.4 m and one molecule of 0.7 m, the fragments would be present in equimolar amounts. This corresponds with what is found. Also, if the number of moles of 0.4 m or 0.7 m rRNA is added to the number of moles of 1.1 m (or intact) "heavy" rRNA present, the

total is equal to the number of moles of the 0.56 m "light" rRNA present. From this it appears that each chloroplast ribosome has one "heavy" rRNA and one "light" rRNA molecule, in common with ribosomes from other living organisms.

When the conditions for extraction of RNA are altered to contain naphthalene disulphonate, m-cresol, and 8-hydroxyquinoline (Fig. 10), chloroplast-specific rRNA can be maintained largely intact. The "heavy" fraction of leaf rRNA has twice the absorption at 260 mμ of the lighter fraction. When relatively unfragmented rRNA is electrophoresed on polyacrylamide gels (Fig. 11), the component with molecular weight of 0.4 m is no longer present. In Table 2, the relative molar quantities of the RNA components are given. It can be seen that there are approximately equal numbers of "heavy" and "light" chloroplast and cytoplasmic rRNA molecules from both the chloroplast and the cytoplasm. When fragmentation of rRNA molecules from the chloroplast does occur, correction for it must be made when calculating the numbers of chloroplast and cytoplasmic ribosomes or amount of rRNA, and it is only the 1.3 m (cytoplasmic "heavy") and 0.56 m (chloroplast "light") molecules which maintain both integrity and purity.

The absorbancy profiles of the polyacrylamide gel electrophoretograms of leaf, root, and chloroplast are compared in Fig. 12. These results demonstrate in greater

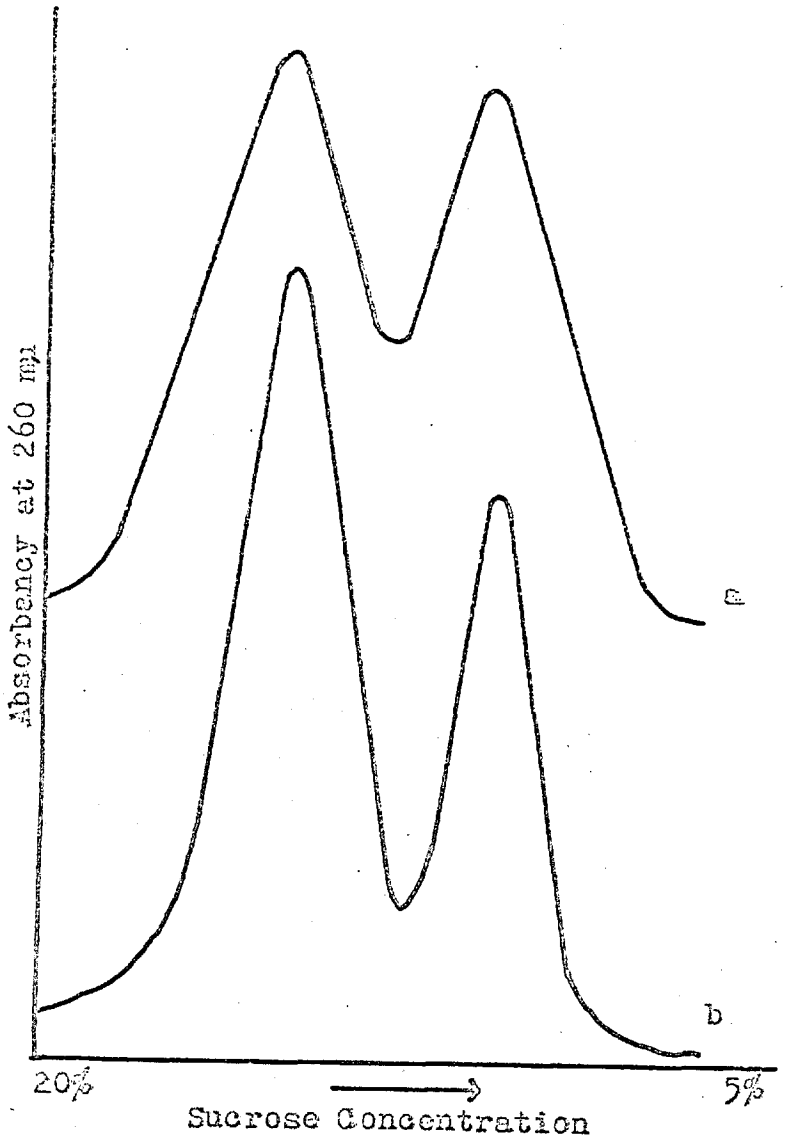


Figure 10. Comparison of the absorbancy profiles of broad bean leaf rRNA that had been extracted by different methods and then fractionated on sucrose density gradients.

a. Extraction method A (see Methods) in which the 1.1 m chloroplast rRNA is largely fragmented
b. Extraction method B in which the 1.1 m chloroplast rRNA largely remained intact

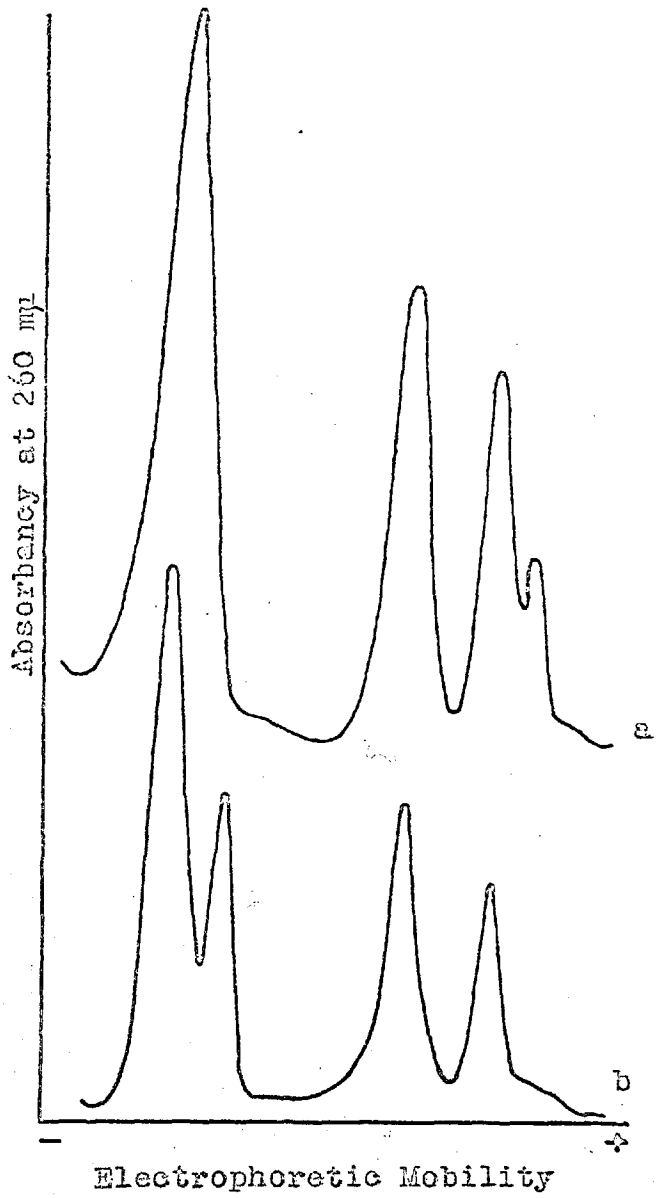


Figure 11. Comparison of the absorbancy profiles of broad bean leaf rRNA that had been extracted by different methods and then fractionated on polyacrylamide gels.

a. Extraction method A (see Methods) in which the 1.1 m chloroplast rRNA is largely fragmented
b. Extraction method B in which the 1.1 m chloroplast rRNA largely remained intact

Table 2. Ratios of "heavy" to "light" rRNA fractions from broad bean leaf determined after their separation by polyacrylamide gel electrophoresis (see Fig. 10). The molar ratio was determined by dividing the respective absorbancy values by their molecular weights.

	Cytoplasmic 1.3 m:0.7 m	Chloroplast 1.1 m:0.56 m
Calculated theoretical absorbancy ratio	1.86:1	1.96:1
Measured absorbancy ratio	1.80:1	1.57:1
Assumed molar ratio	1:1	1:1
Measured molar ratio	1.03:1	1.25:1

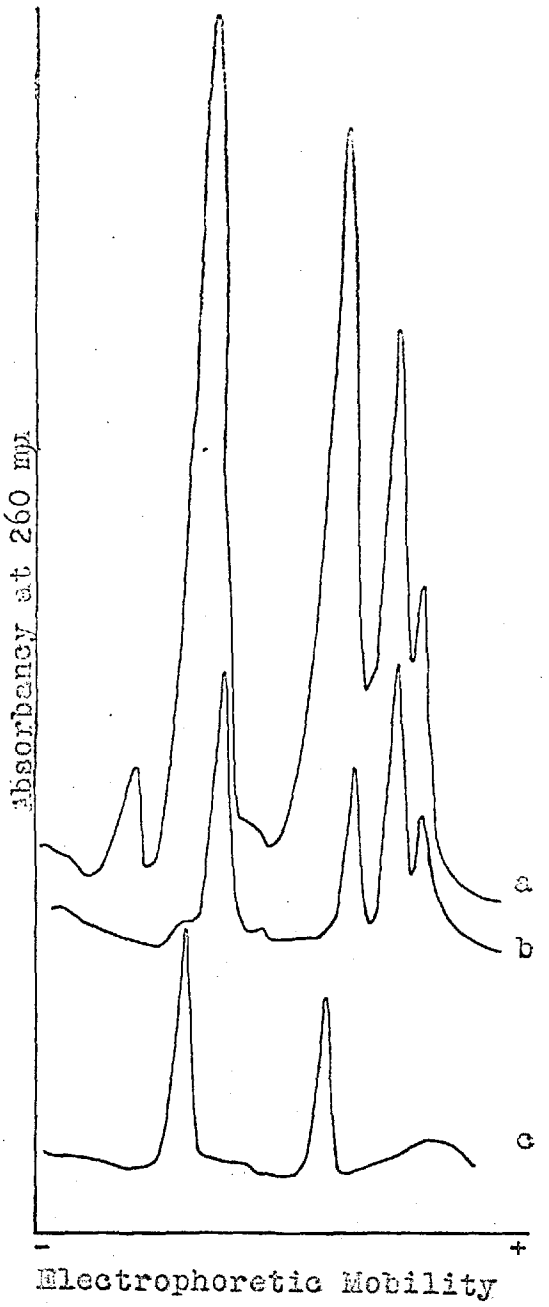


Figure 12. Absorbancy profiles of rRNA that had been extracted from different tissues of broad bean and fractionated by polyacrylamide gel electrophoresis.

a. Leaf rRNA
b. Chloroplast rRNA
c. Root rRNA

detail the differences observed in sedimentation profiles of the nucleic acids of the same tissues. The roots appear to lack the 1.1 m and the 0.56 m chloroplast components, and the ratio of amounts of the cytoplasmic 1.3 m and 0.7 m RNA's is 2 to 1. In the leaf, there are approximately the same molar quantities of chloroplast and cytoplasmic components. This implies that the chloroplast ribosomes constitute about half of the total ribosome population of the green leaf. (Thus, in Table 1, it was possible to add the chloroplast ratio to the root ratio and derive a "heavy" to "light" ratio typical of the green leaf.)

Other tissue besides roots also appear to lack the chloroplast rRNA's (Fig. 13). These include the apical meristems of green plants and the fully-expanded leaves of a spontaneously occurring mutant of the broad bean. The root, meristem, and albino tissue all appeared to contain no chlorophyll (see photograph of mutant in Fig. 14).

It was of interest to know whether these tissues contain any plastid ribosomes. Important differences in the plastid structure and ribosome content between the root, apical meristem, albino leaf, and green leaf of the broad bean are distinguishable by electron microscopy. In the normal green leaf (Fig. 15), the chloroplasts contain well-developed grana and are relatively large (approximately 5 μ in diameter, which is about

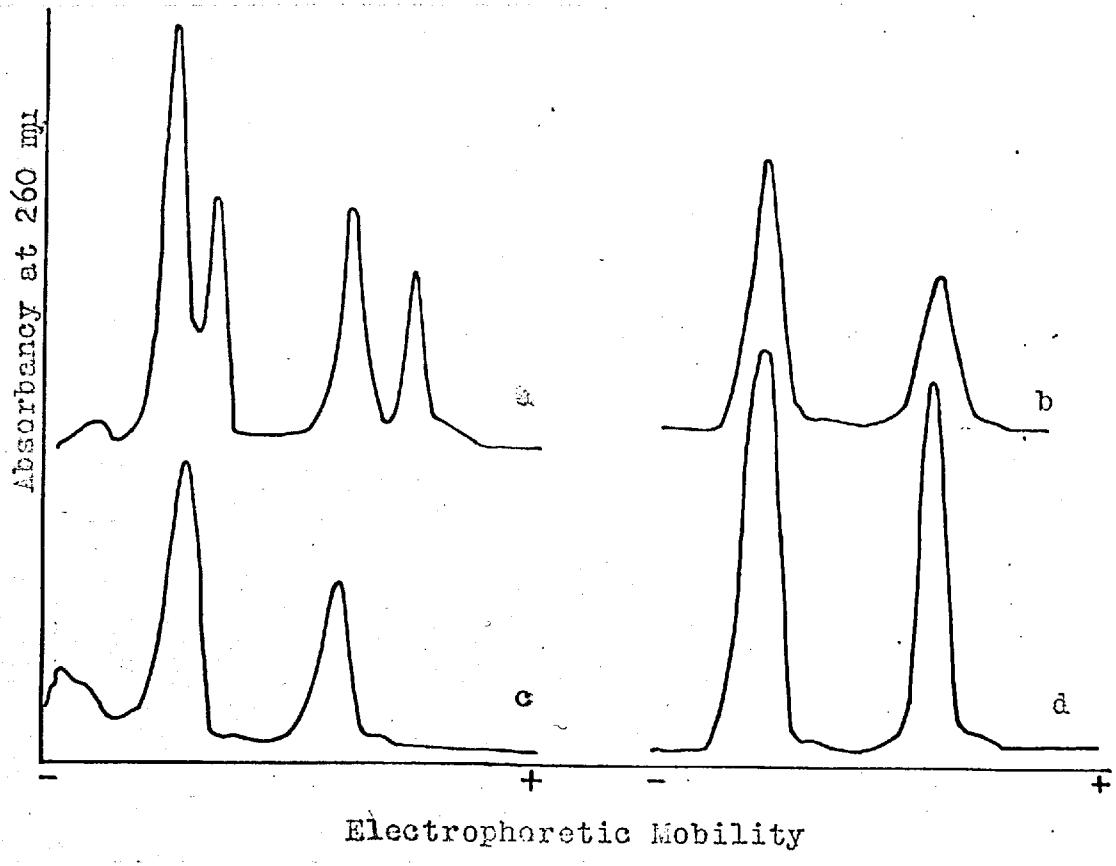


Figure 13. Comparison of the absorbancy profiles of broad bean rRNA extracted from different tissues and fractionated by polyacrylamide gel electrophoresis.

a. Green leaf rRNA
b. Root rRNA
c. Apical meristem rRNA
d. Albino leaf rRNA



Figure 14. Photograph comparing normal broad bean plants 2 weeks old with an albino mutant which occurred spontaneously.

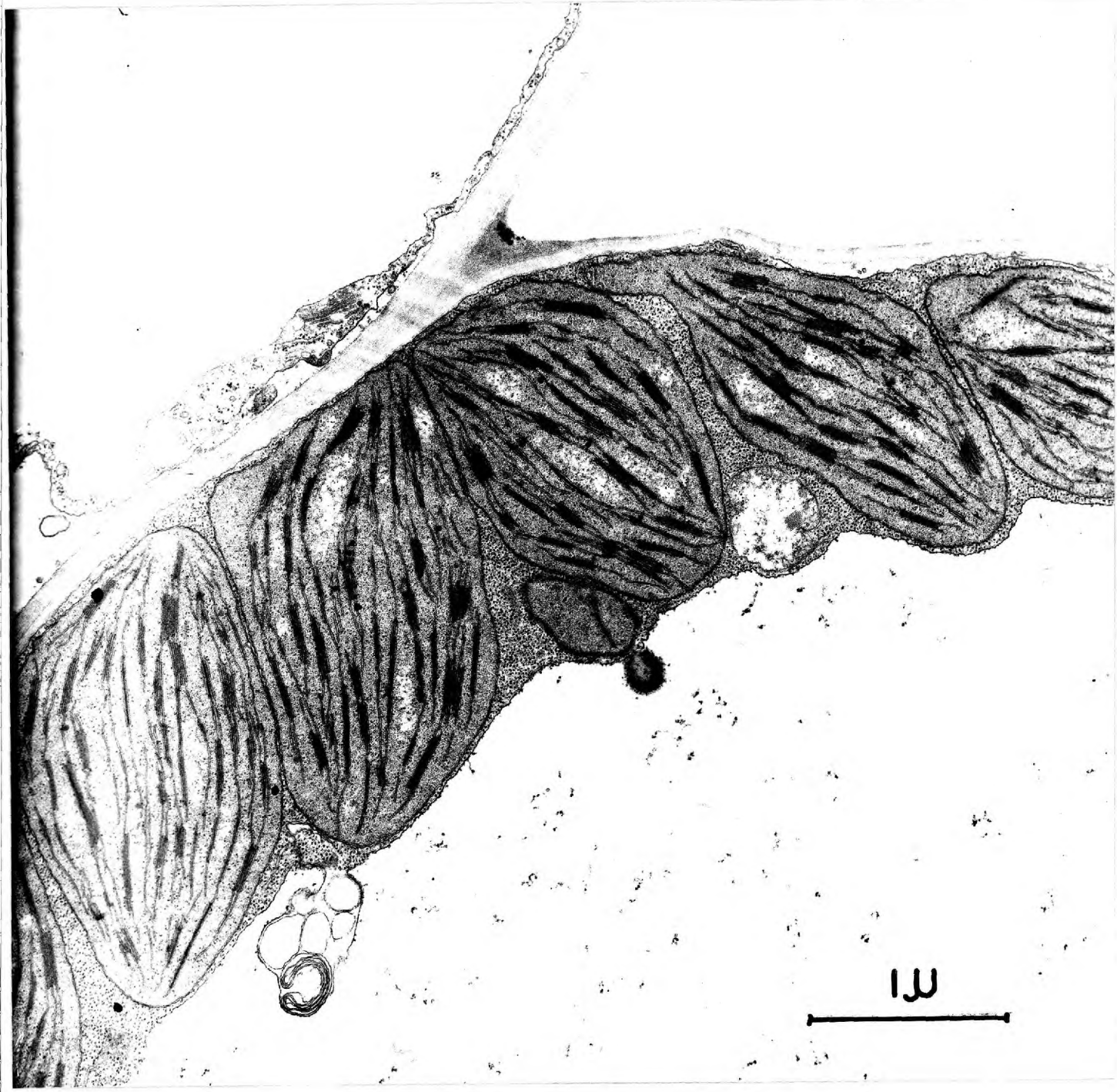


Figure 15. Electron micrograph of a cell from the leaf of a normal green broad bean plant.

5 times that of the mitochondria present). Ribosomes are clearly visible in both the cytoplasm and the chloroplasts. The chloroplast ribosomes represent 50% of the total ribosome population present (Table 3). The root tissue (Fig. 16) has very different plastids. These have very little internal structure and contain a lot of starch. There are ribosomes present in both the plastids and the cytoplasm, but they are in lower concentration in the plastid. Plastid ribosomes are less than 10% of the total ribosomes (Table 3). The cells from the apical meristems of green plants (Fig. 17) contain mostly small (1 - 2 μ diameter) proplastids. The grana have just begun to develop and the ribosome concentration in the proplastids is also lower than that in the cytoplasm. The chloroplast ribosomes constitute less than 10% of all the ribosomes (Table 3). The albino leaf is the only example of tissues investigated where the plastids appear to have no ribosomes (Fig. 18), although ribosomes are readily distinguishable in the cytoplasm of these cells. The plastids are without much internal structure, although they do contain some small vesicles and osmiophilic globules. Thin fibers resembling the DNA in chloroplasts are visible in the stroma.

The results from the polyacrylamide gel electrophoresis of the rRNA correlates well with those obtained from the electron micrographs. Although the concentration

Table 3. Values obtained from electron micrographs for percentages of plastid and cytoplasmic volumes occupied by ribosomes and ratios of plastid to cytoplasmic volumes and ribosome numbers in green and etiolated leaves, roots, and stem apices of broad bean. The relative numbers of ribosomes in the plastid and cytoplasm were determined by multiplying their respective volumes by the percentages of their volumes occupied by ribosomes.

	Percentage of volume occupied by ribosomes		Ratio of plastid to cytoplasmic volume	Ratio of plastid to cytoplasmic ribosome numbers	Percentage of total ribosomes in plastid
	Plastid	Cytoplasm			
Green leaf	32	90	3.09:1	1.09:1	52
Etiolated leaf	30	92	0.661:1	0.22:1	18
Root	24	60	0.108:1	0.04:1	4
Apex of green plants	30	68	0.114:1	0.05:1	5

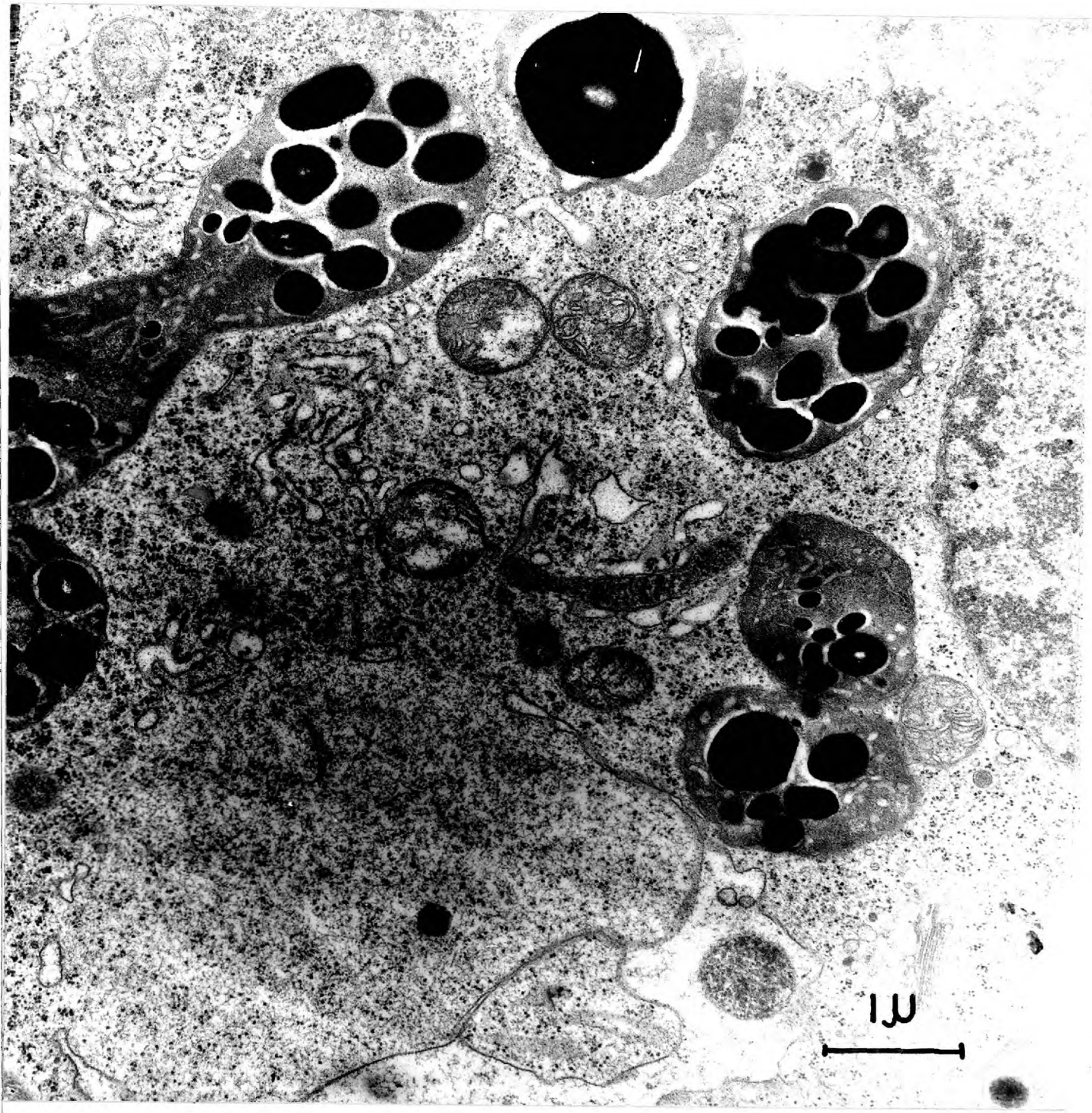


Figure 16. Electron micrograph of a broad bean root cell.

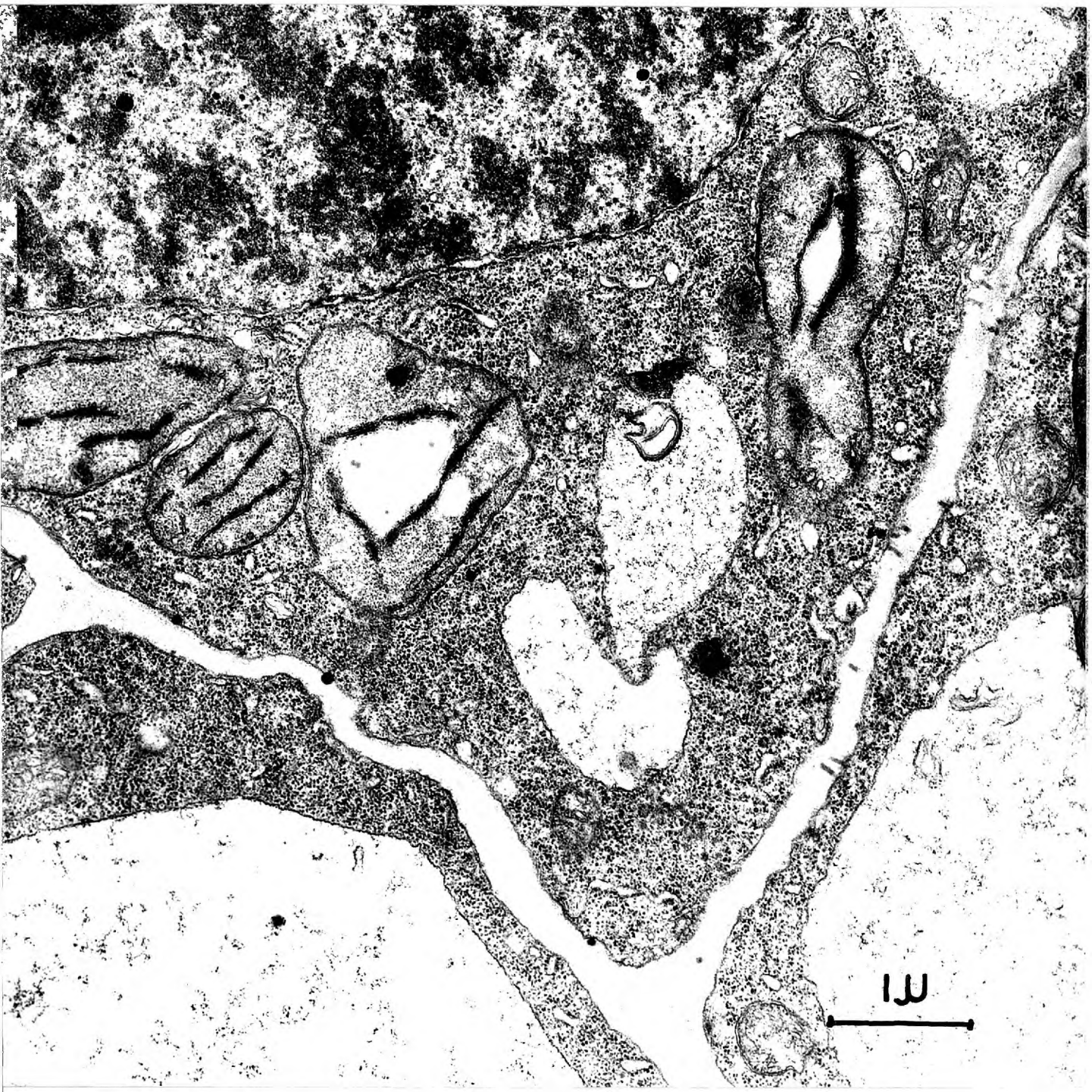


Figure 17. Electron micrograph of cells from the apical meristem of green broad bean plants.

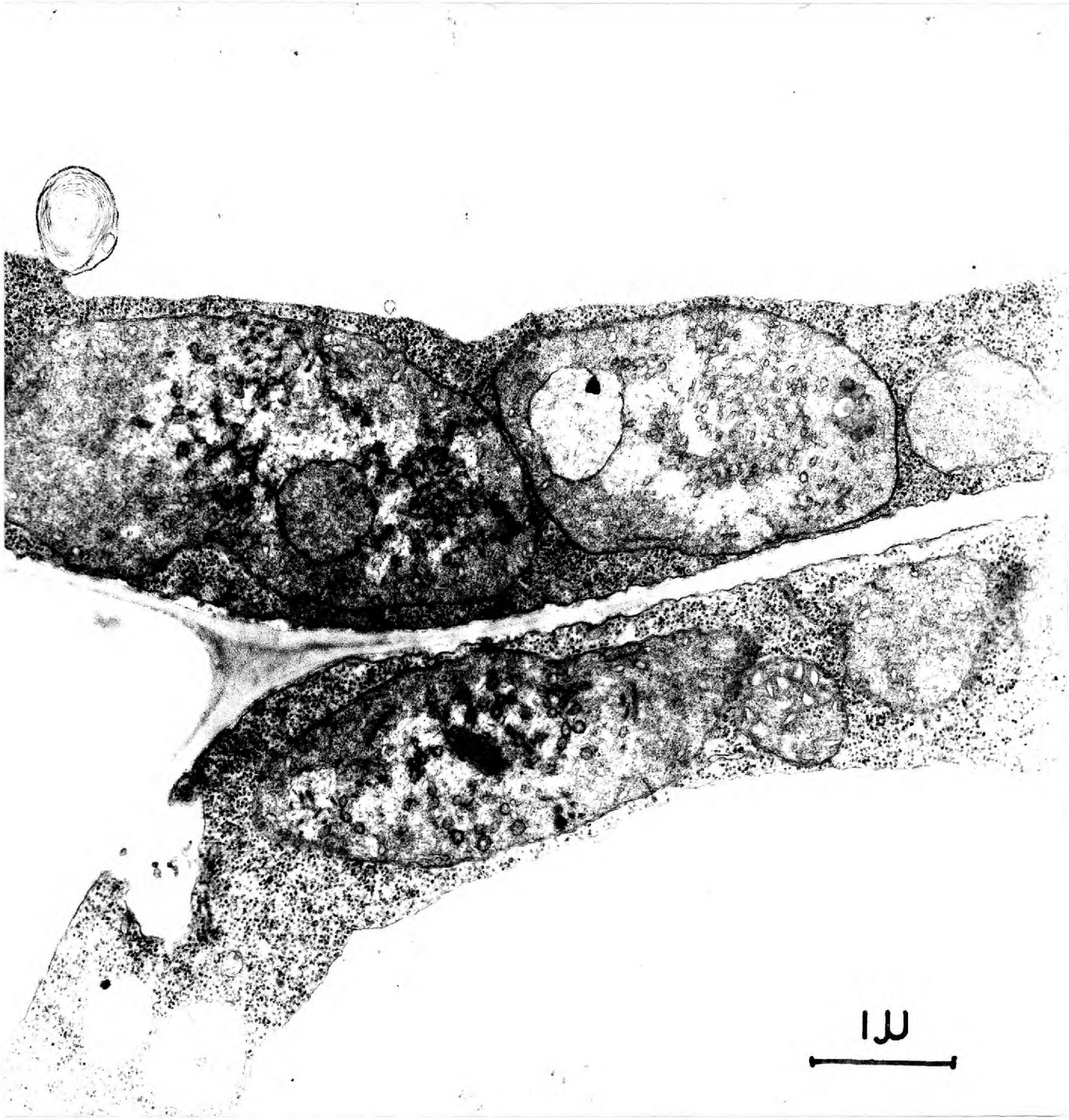


Figure 18. Electron micrograph of cells from the leaf of an albino broad bean plant.

of ribosomes in the mature chloroplast is less than in the cytoplasm surrounding it, the chloroplast takes up more of the cell volume than the cytoplasm. Chloroplast ribosomes constitute one-half of all the ribosomes and chloroplast-specific rRNA is one-half of all the rRNA of the cell (Tables 3 & 4). In the root and the apical meristem, the plastid ribosomes are in a lower concentration than those of the cytoplasm. In addition, these plastids occupy less cell volume than the cytoplasm. The proportion of the total ribosomes in these organelles, and thus the proportion wich their rRNA constitutes, is too low to be distinguishable on the gels. The albino has no chloroplast ribosomes and no chloroplast-specific rRNA, although it does seem to have chloroplast-specific DNA and some plastid structure (Fig. 18).

The sedimentation profile of rRNA from etiolated broad bean leaves is shown in Fig. 19. The absorbancy profile resembles that of root tissue rRNA more than that of green leaf rRNA. The implication is that chloroplast ribosomes are in low numbers in etiolated tissue. Since these leaves develop into green leaves when exposed to light, they provide a useful tissue for the study of the development of the chloroplast ribosomes.

Polyacrylamide gel electrophoresis of the rRNA of etiolated broad beans (Fig. 20, profile A) also shows that there is little chloroplast rRNA in the tissue.

Table 4. Changes in the relative proportions of cytoplasmic and chloroplast rRNA's in etiolated broad bean leaves on illumination. The plants were 2 weeks old when exposed to light. The ratios were determined from the absorbancies at 260 mμ of the 1.3 m and 0.56 m fractions divided by their molecular weights, following separation by polyacrylamide gel electrophoresis.

Hr. illuminated	Molar ratios of cytoplasmic to chloroplast rRNA	Molar percentages Cytoplasm	Chloroplast
0	6.36:1	86.5	13.5
24	7.06:1	87.5	12.5
48	2.69:1	73.0	27.0
72	1.45:1	59.3	40.7
96	1.41:1	58.3	41.5
120	1.61:1	61.5	38.5
144	1.34:1	57.3	42.7
Maintained in dark for 96 hr.	4.38:1	81.5	18.5
Light grown	1.55:1	60.8	39.2

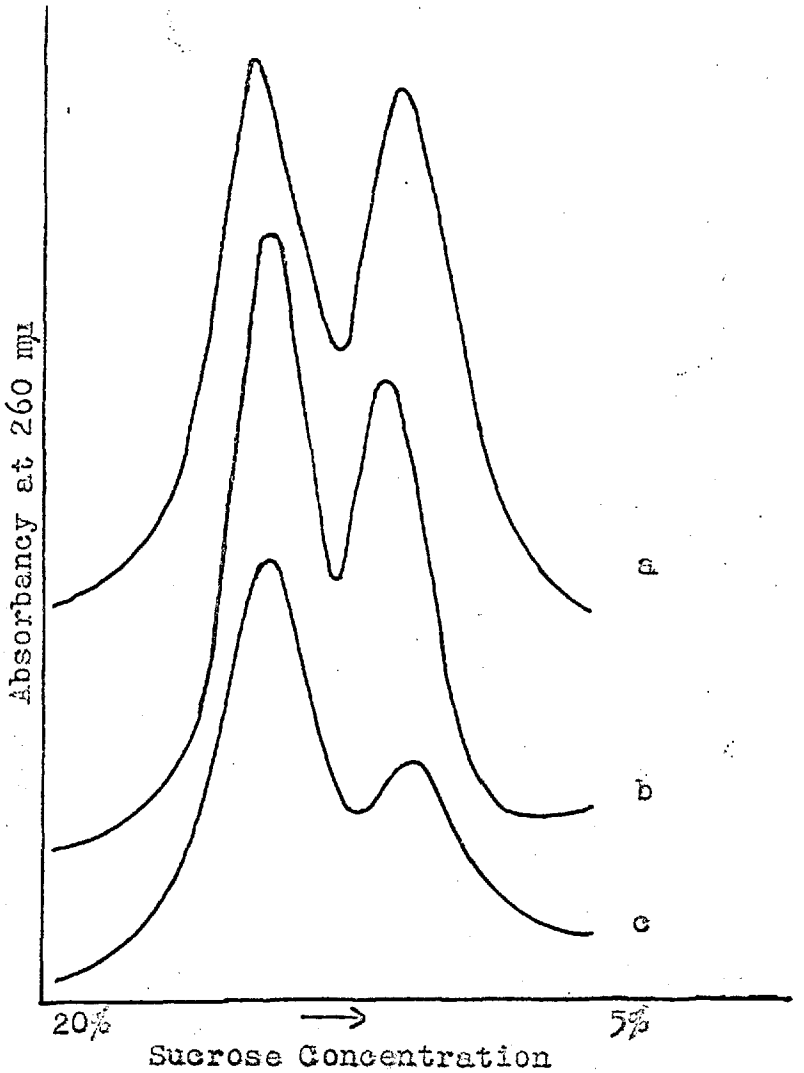


Figure 19. Comparison of the absorbancy profiles of broad bean rRNA extracted from different tissues and fractionated by sucrose density gradient centrifugation.

a. Green leaf rRNA
b. Root rRNA
c. Etiolated leaf rRNA

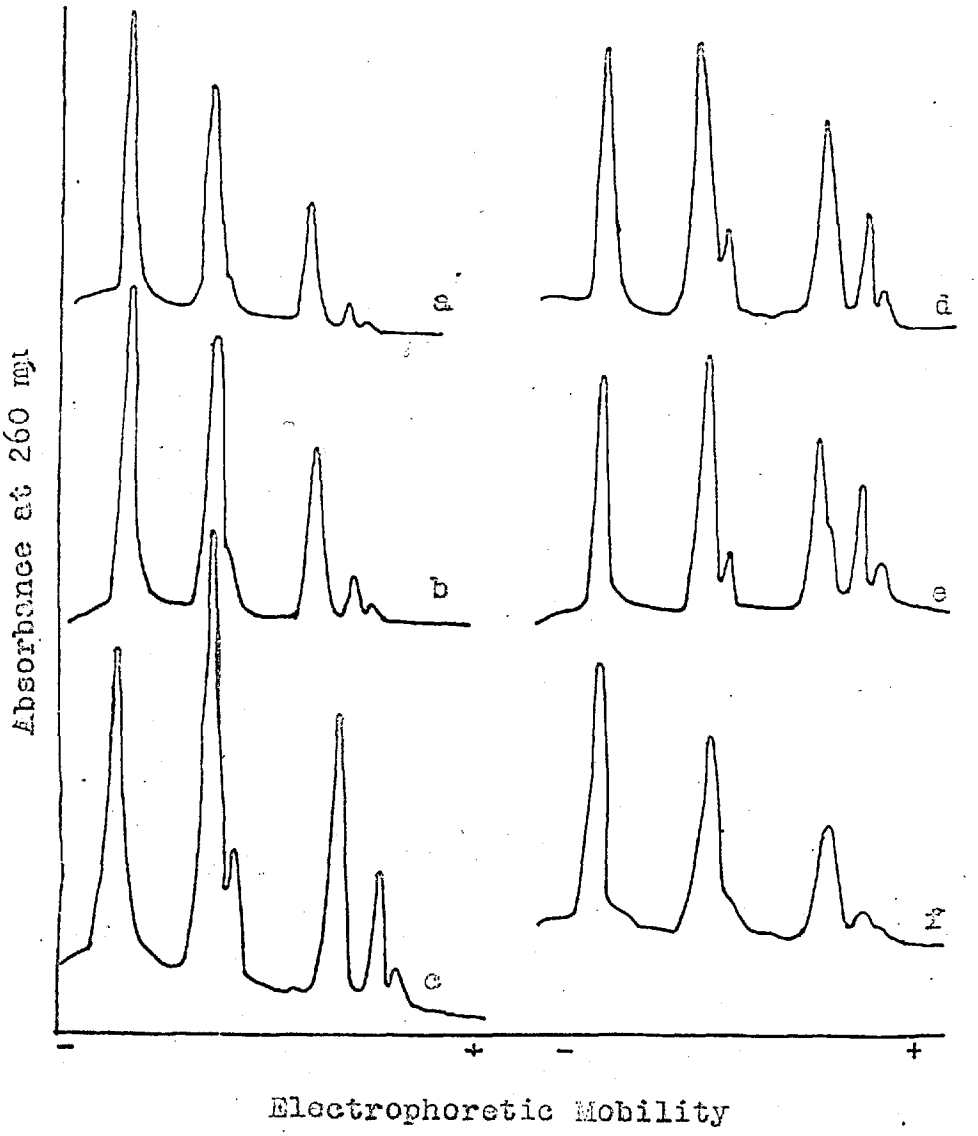


Figure 20. Comparison of the absorbancy profiles of the nucleic acids extracted from etiolated broad bean leaves that had been illuminated for increasing periods of time. The nucleic acids were fractionated by polyacrylamide gel electrophoresis.

a - e. 0, 24, 48, 72, and 96 hr. of illumination, respectively

f. Control - Plants maintained in the dark for an additional 96 hr. while the experimental plants were being illuminated.

An electron microscopic examination of the leaves showed that they contain etioplasts with well-defined prolamellar bodies (Fig. 21). There are ribosomes in the plastids, although they represent approximately only 20% of the total ribosomes of the cell (Table 3). The plastids contain no chlorophyll (Fig. 22).

Many alterations occur when this tissue is placed in the light. It can be seen from Fig. 23 and Table 4 that the chloroplast rRNA components do not increase significantly relative to the cytoplasmic components in the first 24 hr. Between 24 and 72 hr., there is a rapid increase in the proportion of chloroplast rRNA, and by 72 hr. the levels are the same as those of the green leaf. The proportion does not change, even after 144 hr. of illumination. In Fig. 22a, it can be seen that the chlorophyll concentration increases at the greatest rate after 24 and 48 hr. of illumination, and only after 72 hr. does the rate of chlorophyll synthesis begin to level off. Thus there is a lag in both chloroplast rRNA development and chlorophyll synthesis for the first 24 hr. The total RNA levels (Table 5) and the wet weight of the tissue (Table 6) remain constant for 24 hr. Between 24 and 72 hr., the amount of RNA per leaf doubles and the wet weight increases four fold, but the amount of DNA remains constant (Table 7). The increase in total RNA occurs at the same time as the increase in the chloroplast components (Table 8).

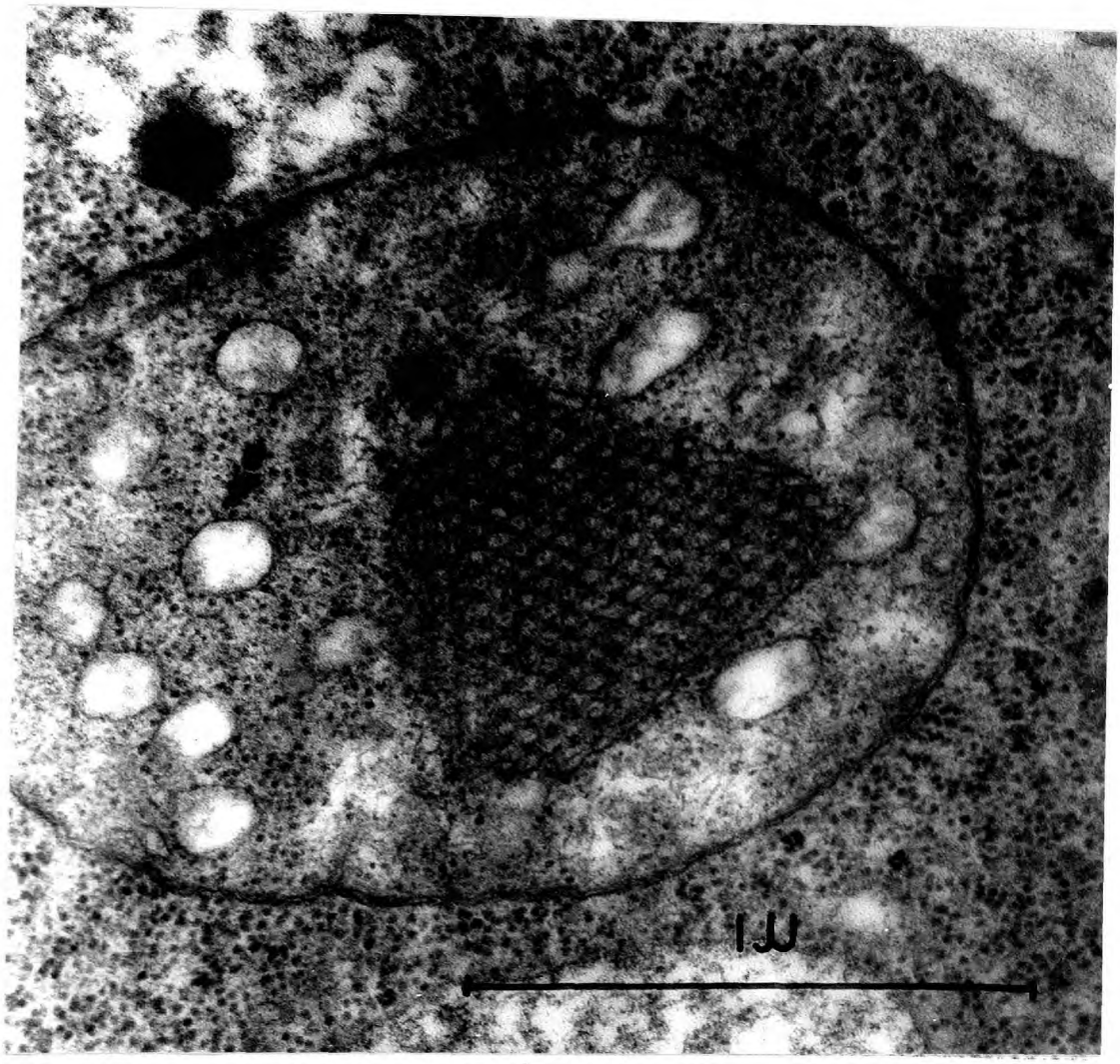


Figure 21. Electron micrograph of a plastid from a leaf cell of an etiolated broad bean plant.

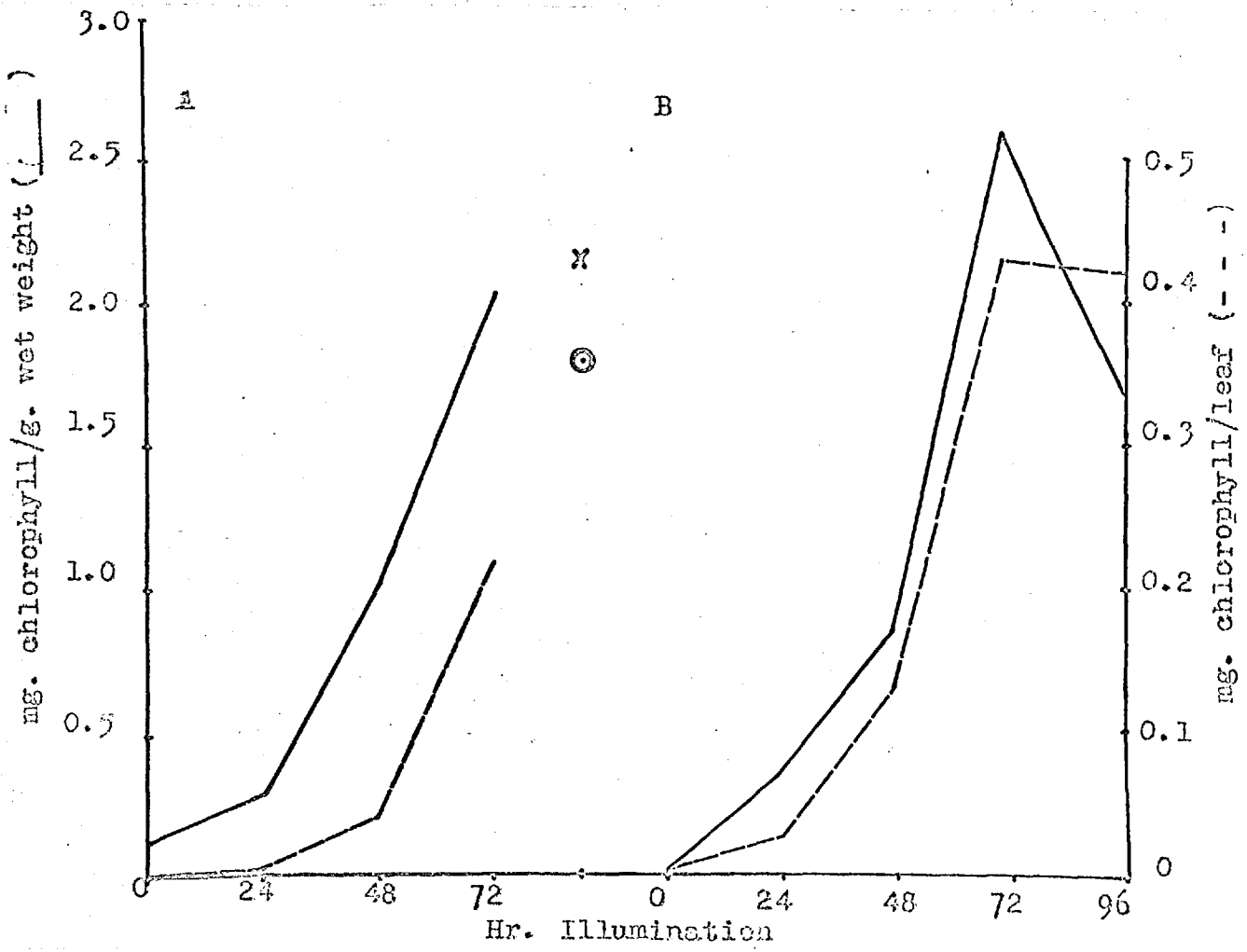


Figure 22. Changes in the chlorophyll content of etiolated broad bean leaves when placed in light.

A. Illumination of 1 - week old plants
B. Illumination of 2 - week old plants

⊙ mg. chlorophyll/g. wet weight, X mg. chlorophyll/leaf in light grown plants

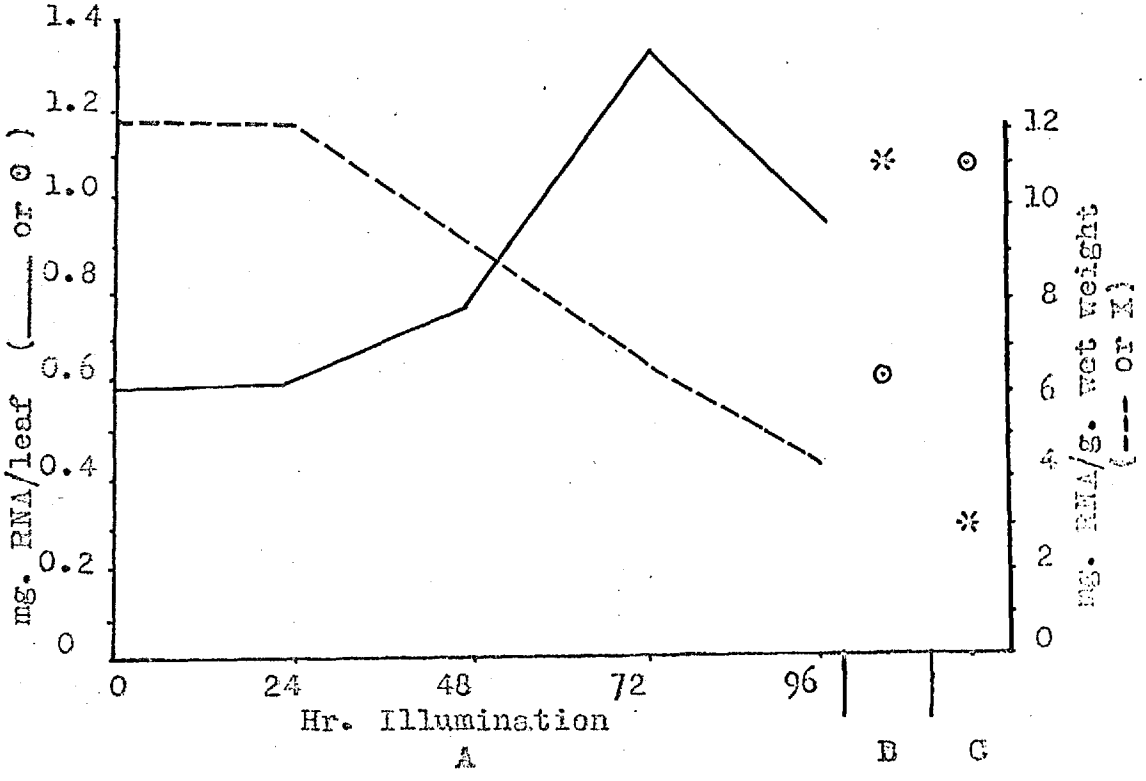


Figure 23. Changes in the RNA content of etiolated broad bean leaves when placed in light.

A. Illumination of etiolated plants
B. Control - Plants maintained in the dark for an additional 96 hr. while the experimental plants were being illuminated
C. Light grown plants

Table 5. Changes in the total amount of RNA in etiolated broad bean leaves on illumination. The radioactivity in the nucleic acids was derived from ^{14}C-orotic acid supplied to the plants.

	RNA		
Hr. illuminated	mg./leaf	mg./g. wet wt.	Specific activity (cpm/µg.)
0	0.60	11.8	24.3
24	0.61	11.8	32.5
48	0.72	9.2	34.8
72	1.35	6.5	25.3
96	0.97	4.5	23.9
Maintained in dark for 96 hr.	0.63	10.9	26.6
Light grown	1.1	3.1	--

Table 6. Changes in wet weight of the first two leaves of etiolated broad bean plants upon illumination.

Hr. illuminated	Leaf weight (mg.)
0	50.8
24	50.9
48	78.3
72	207.4
96	215
Maintained in dark for 96 hr.	57.7
Light grown	355

Table 7. Changes in the total amount of DNA in etiolated broad bean leaves on illumination. The radioactivity in the nucleic acids was derived from ^{14}C-orotic acid supplied to the plants.

	DNA		
Hr. illuminated	mg./leaf	mg./g. wet wt.	Specific activity (cpm/µg.)
0	40.5	0.71	3.32
24	47.5	0.47	6.45
48	45.0	0.44	16.9
72	47.5	0.30	10.5
96	51.7	0.32	4.85
Maintained in dark for 96 hr.	45.0	0.40	10.8
Light grown	57.0	0.41	--

Table 8. Changes in amounts of various rRNA fractions of broad bean leaves on illumination of etiolated plants.

These values were calculated by assessing how much RNA in the total gel volume would give a uniform absorbance of 1 at 260 mμ and then calculating what proportion of this each fraction represents.

	Weight (μg. RNA/fraction/leaf)				
Hr. illuminated	1.3 m	1.1 m	0.7 m	0.56 m	0.4 m
0	280	20	250	40	8
24	290	30	240	40	18
48	270	50	270	100	30
72	450	90	430	300	80
96	300	70	350	190	65
Maintained in dark for 96 hr.	260	20	270	60	13
Percentage increase between 0 and 96 hr.	7%	250%	40%	375%	712%

When the leaf RNA's are labelled with ^{14}C derived from orotic acid, the specific activity of the total RNA increases during 24 hrs. of light treatment (Table 5). By 72 hr., the specific activity has decreased to the same level as it was in the etiolated tissue. This same increase and decrease is found on determination of the specific activity of the individual rRNA's (Fig. 24 and Table 9). The specific activity of the chloroplast components doubles within the first 24 hr. The cytoplasmic components also become more radioactive during the first 48 hr. of illumination, but to a lesser degree than that of the chloroplast. The most radioactive species is chloroplast-specific 1.1 m rRNA.

Ingle (1968) has hypothesized that the newly-synthesized 1.1 m rRNA is more stable than the older 1.1 m rRNA. If this were true it would explain why the 1.1 m chloroplast component has a higher specific activity than the 0.56 m component in these experiments. The older, non-labelled RNA would fragment into the 0.7 and 0.4 m fragments, and thus the 0.7 m peak from the cytoplasm would be contaminated by unlabelled chloroplast rRNA. The cytoplasmic 1.3 m component has a higher specific activity than the 0.7 m component in our system. The decline in specific activity of all components by 96 hr. of continuous illumination might be due to an increase in endogenous synthesis of nucleic acid precursors from the products of photosynthesis.

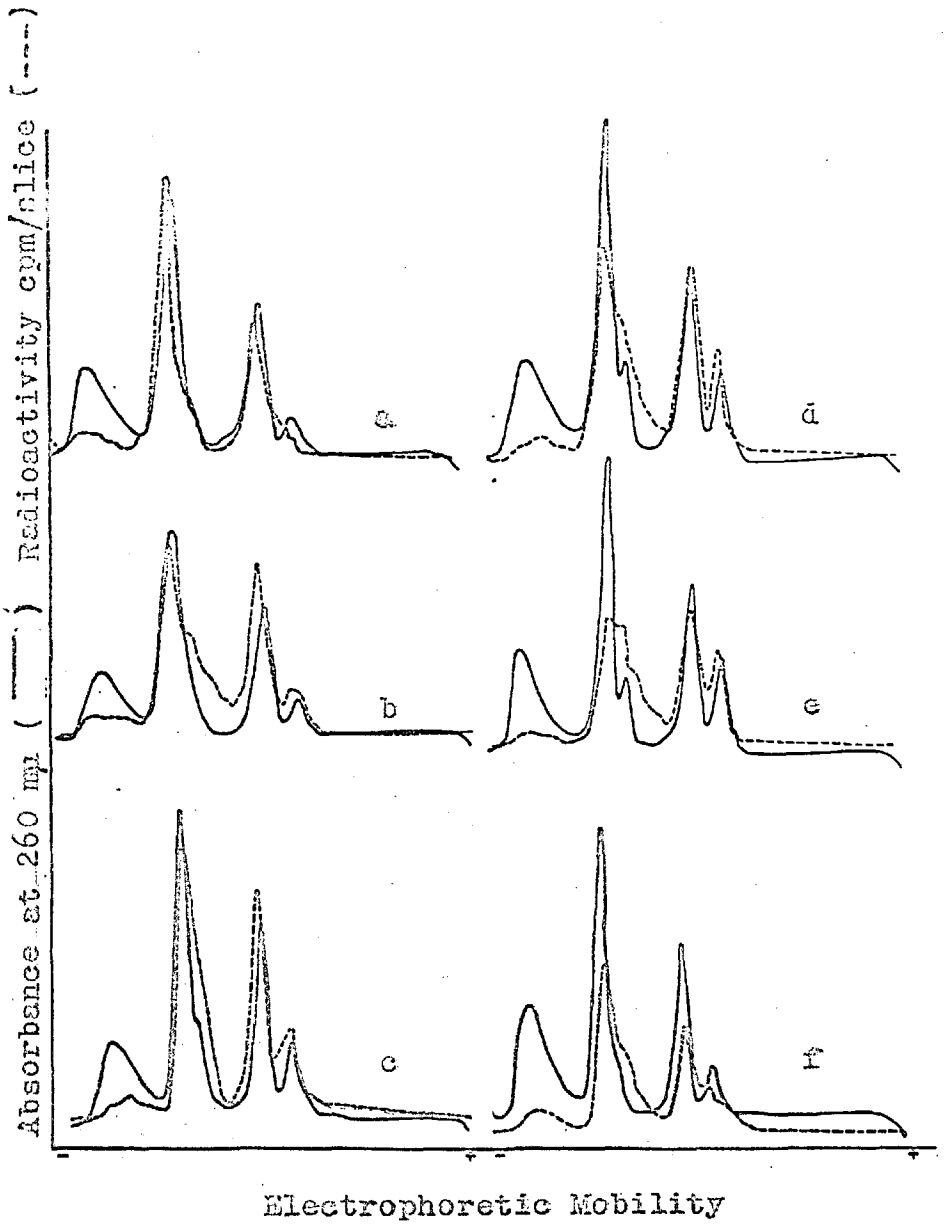


Figure 24. Comparison of the absorbancy and radioactivity profiles of the nucleic acids extracted from etiolated broad bean leaves that had been illuminated for increasing periods of time. The nucleic acids were fractionated by polyacrylamide gel electrophoresis and the radioactivity was supplied to the plants as ^{14}C - orotic acid starting 24 hr. before illumination.

a - e. 0, 24, 48, 72, and 96 hr. of illumination, respectively

f. Control - Plants maintained in the dark for an additional 96 hr. while the experimental plants were being illuminated

Table 9. Changes in specific activity of various broad bean leaf rRNA fractions that had been separated by polyacrylamide gel electrophoresis. Etiolated plants were exposed to light for various periods of time before extraction of the leaf rRNA (see Fig. 24 for calculation of specific activity).

	Specific activity (cpm x 10^7/μmoles RNA)				
Hr. illuminated	1.3 m	1.1 m	0.7 m	0.56 m	0.4 m
0	7.6	7.5	4.4	3.3	--
24	7.3	15.2	5.7	6.1	--
48	9.3	10.6	6.6	5.2	5.1
72	7.0	11.3	5.5	2.9	2.1
96	4.4	13.4	3.8	3.3	1.6
Maintained in dark for 96 hr.	5.2	3.0	2.7	4.5	--

Light-grown plants do not incorporate radioactive nucleic acid precursors into their rRNA when exposed to continuous illumination.

Electron micrographs of etiolated broad bean leaves samples after 0, 24, 48, and 72 hr. of illumination (Figs. 21, 25, 26, & 27) show the gradual transition of the etioplast into the chloroplast. The prolamellar body disperses within 24 hr. Long strings of vesicles radiate into the stroma. By 48 hr., there are numerous grana stacks within the plastids, but they are not all oriented in the same direction as they are in the mature chloroplast. By 72 hr., the grana stacks are orientated and the chloroplasts are morphologically identical to those in light-grown plants (Fig. 15).

In the control material maintained in the dark for an additional 96 hr., there is still no chlorophyll (Fig. 22), but there is additional incorporation of radioactivity into the RNA (Table 4) and a slight increase in the proportion of the total rRNA which the chloroplast rRNA constitutes. (Fig. 23 and Table 4).

Since there is a gradual increase in the chloroplastic rRNA in etiolated plants maintained in the dark, the age of the plants used for experimentation appears to be important in assessing the degree of rRNA development. The rate of changes which occur as a response to light are also dependent upon the age of the etiolated plants. In Fig. 22, it can be seen that when one-week-

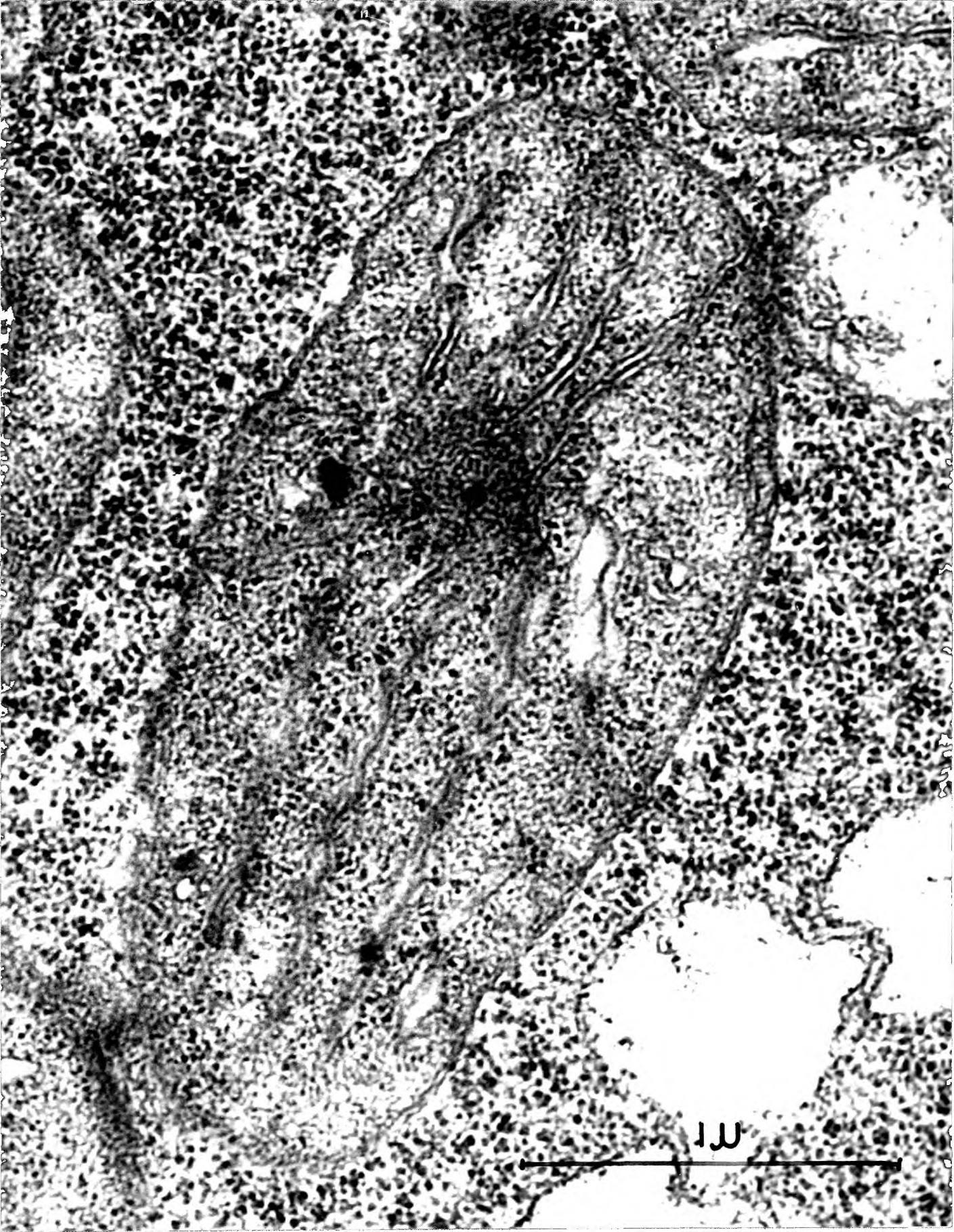

1 µ

Figure 25. Electron micrograph of a plastid from a leaf cell of an etiolated broad bean plant which had been illuminated for 24 hr.

1μ

Figure 26. Electron micrograph of a plastid from a leaf cell of an etiolated broad bean plant which had been illuminated for 48 hr.

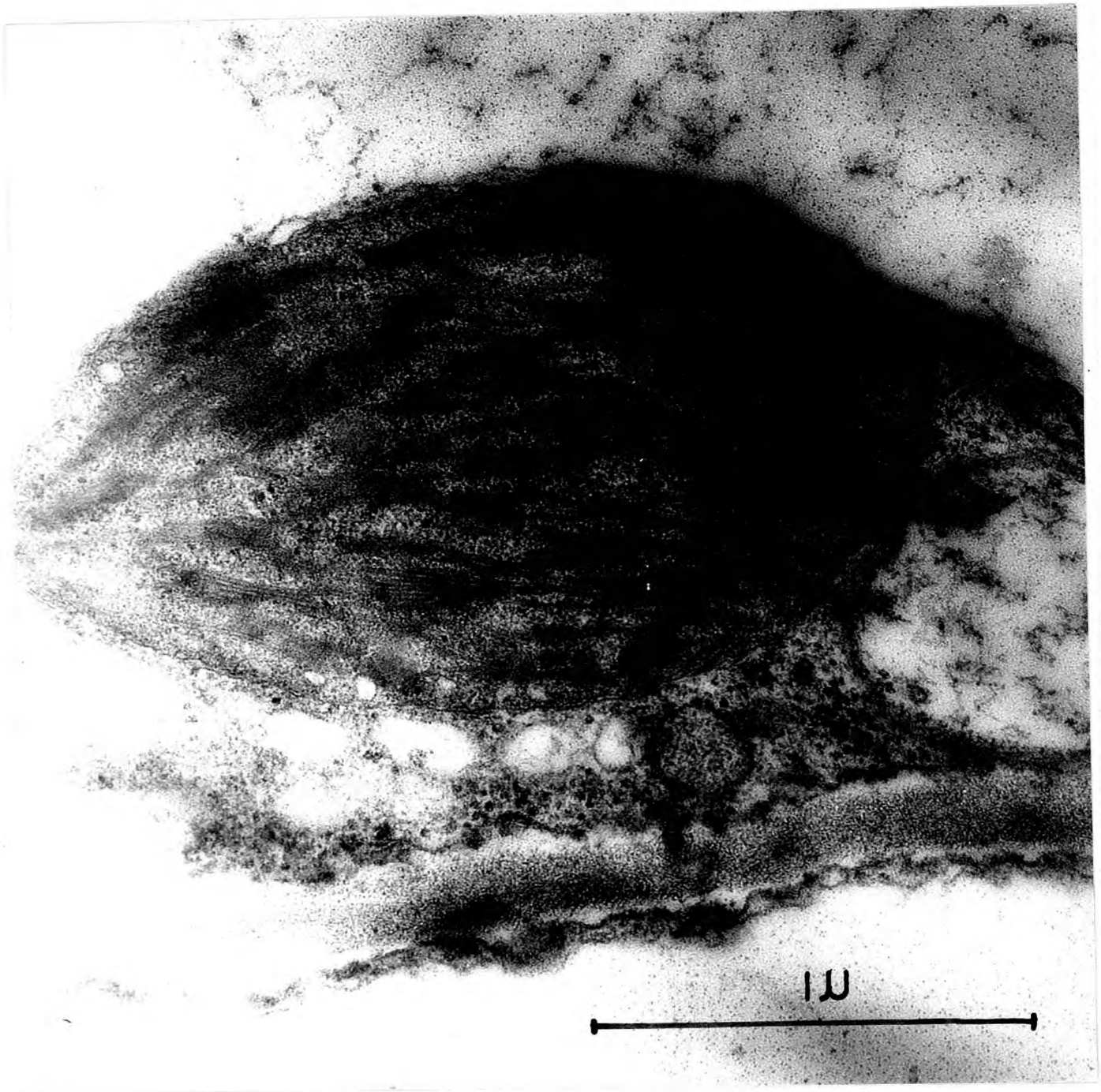


Figure 27. Electron micrograph of a plastid from a leaf cell of an etiolated bread bean plant which had been illuminated for 72 hr.

old etiolated seedlings are exposed to light, the rate of chlorophyll increase is slower than in seedlings grown for two weeks in the dark before illumination. The chloroplast-specific rRNA's also seem to take longer to develop (Fig. 28).

Responses to light also seem to vary between different species of plants. In Zea mays tissue there is no comparable 24 hr. lag in the synthesis of chlorophyll as there is in the broad bean on illumination (Fig. 29). The chloroplast-specific rRNA components are fully developed 48 hr. after placing the plants in light (Fig.29a and Table 10). The etiolated leaves of maize have many more ribosomes in their plastids than comparable broad bean (Jacobson et al, 1963; Wettstein and Kahn, 1961). While chloroplasts of green maize leaves contain many ribosomes, they do not have as well-developed grana stacks as the broad bean.

In summary, it appears that in broad bean leaves grown in darkness for two weeks, the wet weight, chlorophyll, RNA, and chloroplast rRNA levels remain fairly constant for the first 24 hr. of illumination. During this time, there is little new membrane synthesis within the plastids, but dispersion of the prolamellar body does occur. Also, little synthesis of cytoplasmic rRNA seems to occur during this period. Between 24 and 72 hr. after the start of illumination, chloroplast differentiation takes place. Chlorophyll, chloroplastic rRNA and

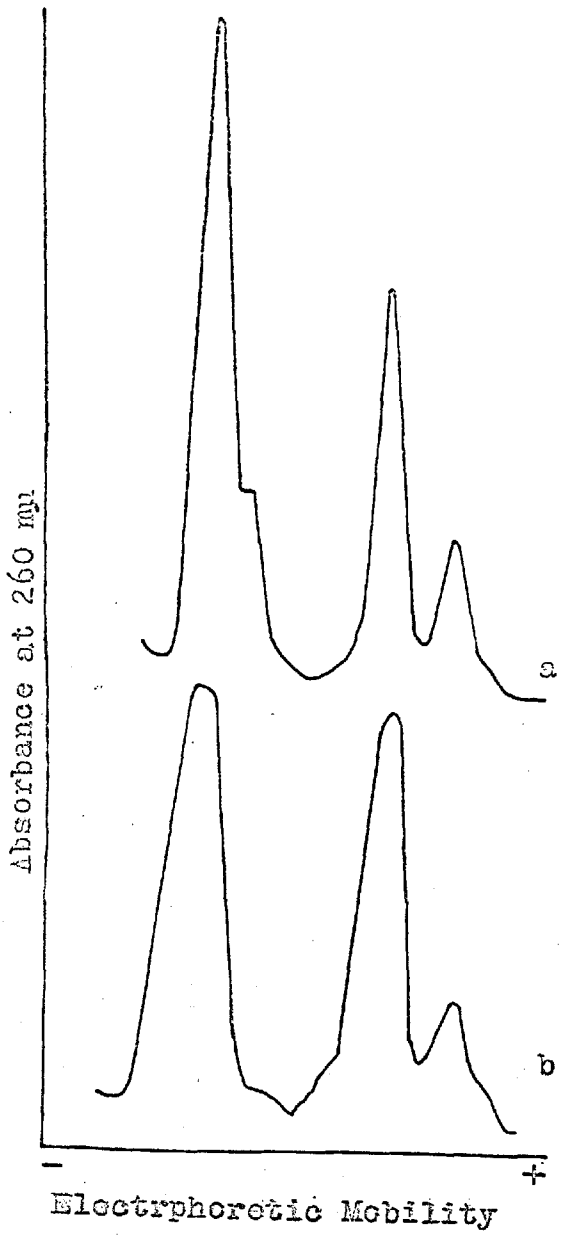


Figure 28. Comparison of absorbancy profiles of broad bean leaf rRNA that had been fractionated by polyacrylamide gel electrophoresis.

a. Leaves from 2 - week old etiolated plants illuminated for 48 hr.
b. Leaves from 1 - week old etiolated plants illuminated for 48 hr.

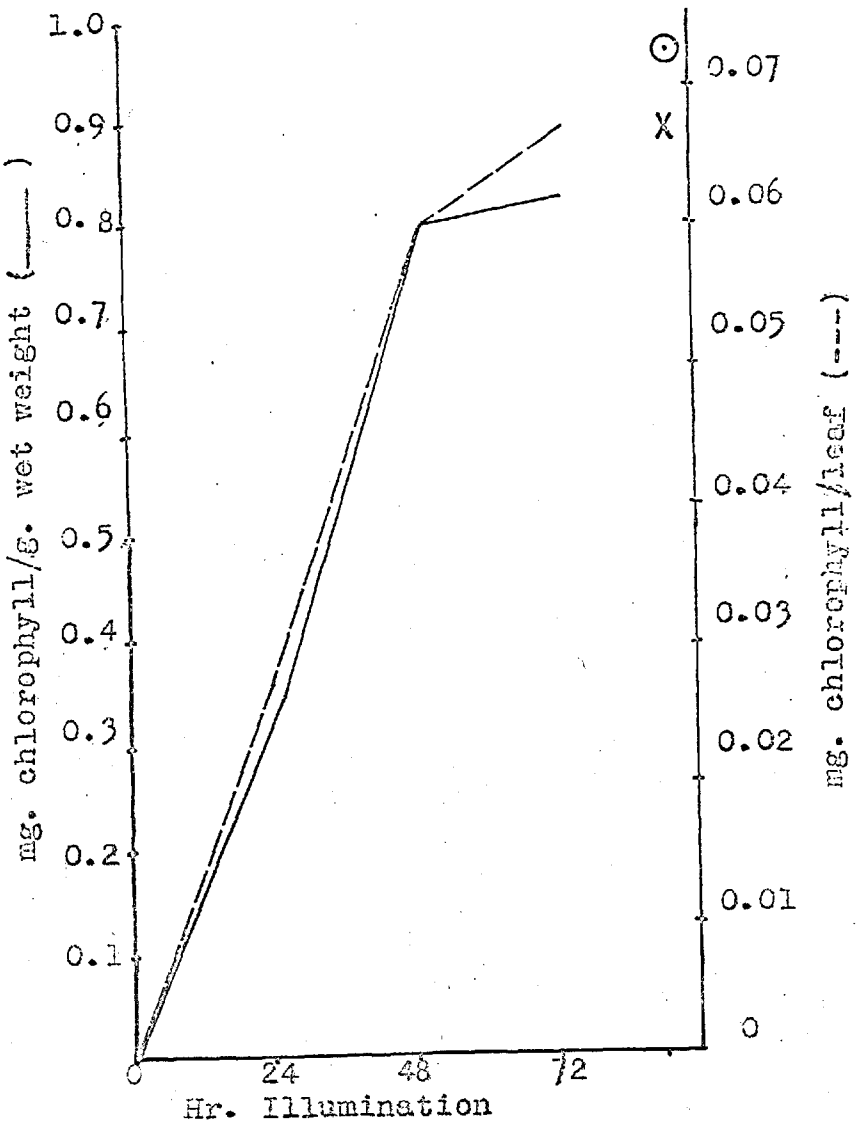


Figure 29. Changes in the chlorophyll content of etiolated maize leaves when placed in light.
⊙ mg. chlorophyll/leaf, X mg. chlorophyll/g. wet weight in light grown leaves

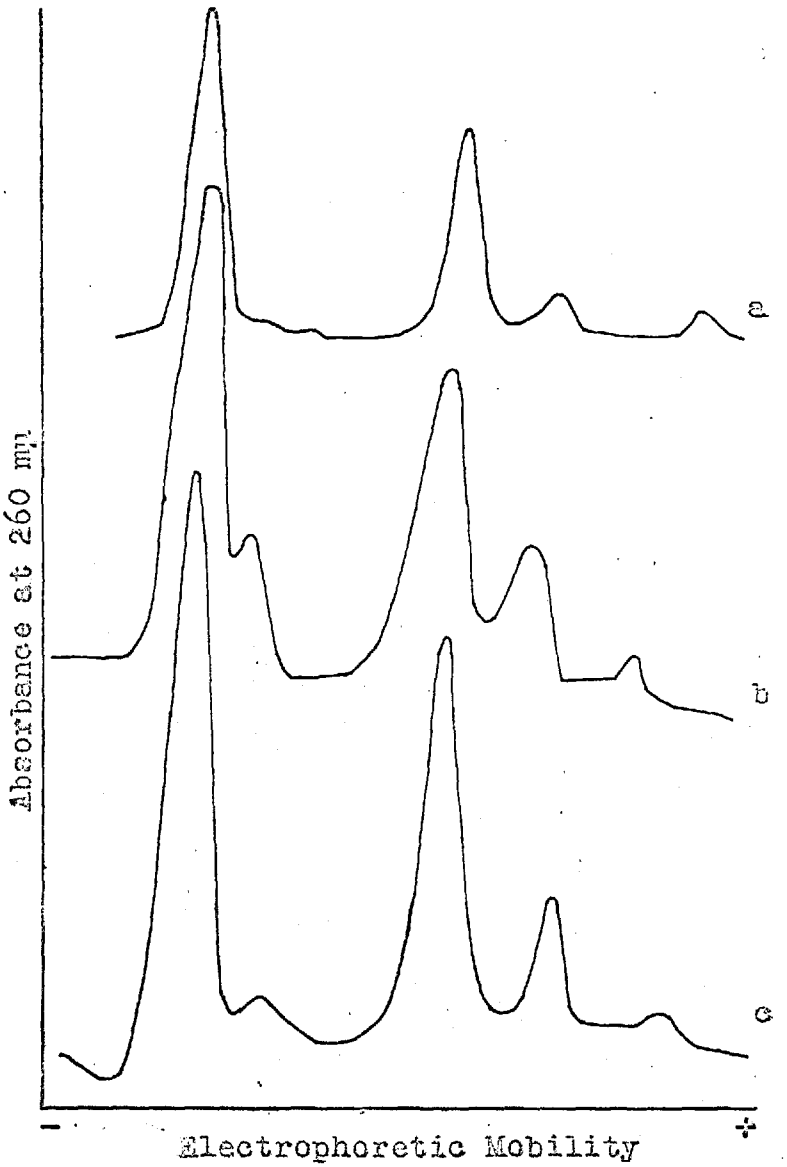


Figure 29a. Comparison of absorbancy profiles of maize leaf rRNA that had been fractionated by polyacrylamide gel electrophoresis.

a. Etiolated plants
b. Etiolated plants that had been illuminated for 48 hr.
c. Light grown plants

Table 10. Changes in the relative proportions of cytoplasmic and chloroplast rRNA's in etiolated maize on illumination. The ratios were determined from the absorbancies at 260 mμ of the 1.3 m and 0.56 m fractions divided by their molecular weights, following separation by polyacrylamide gel electrophoresis.

	Molar ratios of cytoplasmic to chloroplast rRNA	Molar percentages	
		Cytoplasm	Chloroplast
Etiolated maize	2.60:1	72.3	27.7
Light grown maize	1.80:1	64.5	35.5
Etiolated maize illuminated for 48 hr.	1.66:1	62.5	37.5

membranes are rapidly synthesized, but cytoplasmic rRNA is being synthesized at approximately the same rate as it was during the first 24 hr.

During the differentiation of the membranes in the chloroplast, it might be expected that there would be synthesis of the "structural" protein of the chloroplast lamellae. It is only when the membranes differentiate and grana stacks are formed that the chloroplast can participate in photosynthetic processes (Hoober et al, 1969). The development of membrane can be monitored by studying the development of "structural" protein. There are some indications that this protein is made on cytoplasmic ribosomes (Hoober et al, 1969; Smillie et al, 1967). It was of interest to know whether the development of this important protein occurs when there is rapid chloroplast rRNA synthesis.

To characterize the chloroplast "structural" protein, it was electrophoresed on polyacrylamide gels. The absorbancy profile of the electropherogram (Fig. 30) shows that there are several distinct components. To assess which compoents are characteristic of the chloroplasts, various amounts of mitochondrial "structural" protein were added to the gels and electrophoresed with the chloroplast material (Fig. 30). From the results of this experiment, it can be seen that several chloroplast proteins have distinctive electrophoretic mobilities (identified in the figure by asterisks). In his

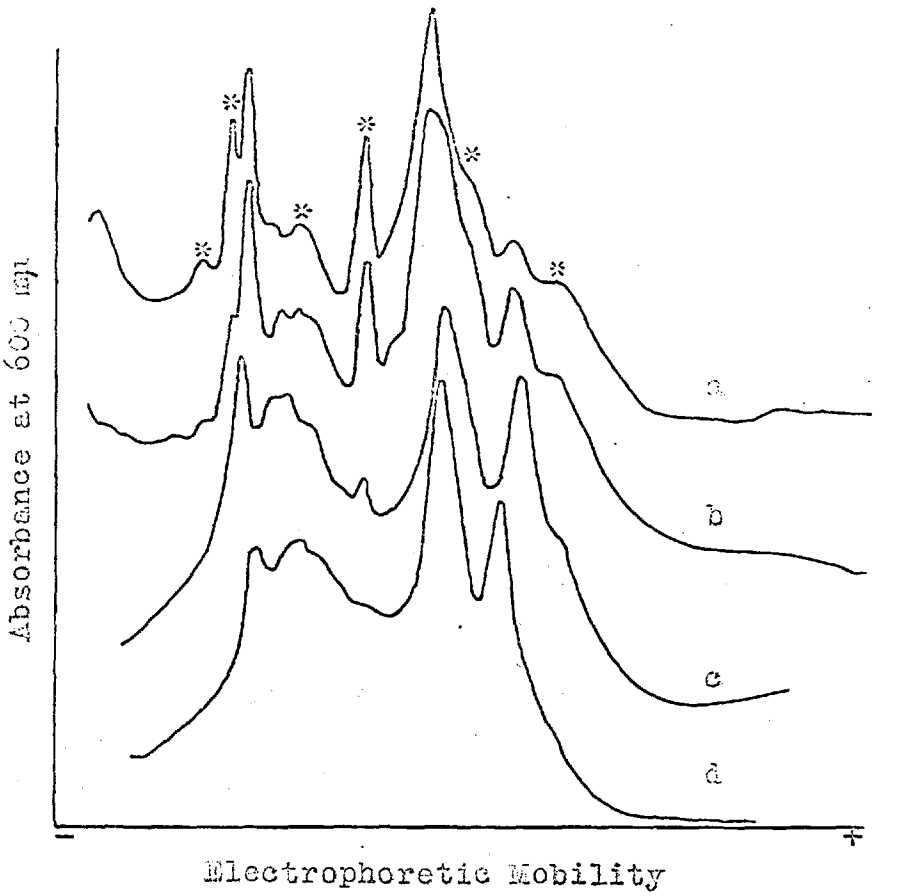


Figure 30. Abosrbancy profiles of broad bean chloroplast and mitochondrial "structural" protein that had been stained with Amido Black following fractionation by polyacrylamide gel electrophoresis.

a. Chloroplast fraction
b. Chloroplast - Mitochondria mixture (4:1, w/w)
c. Chloroplast - Mitochondria mixture (1:4, w/w)
d. Mitochondria fraction

The fractions identified with asterisks appear to be enriched in the chloroplast fraction.

original work on this protein Criddle (1966) subjected the acetone powder derived from the chloroplasts to an ammonium sulphate fractionation. The profiles from gel electrophoresis of the original powder as well as the protein precipitated by ammonium sulphate at 20% of its saturation value and that remaining in the supernatant are presented in Fig. 31. Those components enriched in the precipitate (shown by an asterisk) are "structural" protein, and the other components that are not precipitated are lamellar bound proteins. When the results of Figs. 30 and 31 are compared, it can be seen that there are two characteristic chloroplast "structural" proteins as well as two other components which might also be in this category.

To determine the rate of synthesis of this protein, attempts were made to label it with radioactive precursors. Attempts to label it in vivo with radioactive carbon have been for the most part unsuccessful. In green plants, the most radioactive material obtained contained 1,000 cpm per mg. protein. The maximum amount of protein that can be placed on the gels is 1 mg. due to its extremely low solubility. With approximately 10 components on each gel, there would be an average of 100 cpm per component. Since each protein band in the gel is cut into 5 to 10 gel slices, the maximum number cpm that could be obtained per slice would be between 10 and 20. This is too low to be detected over the

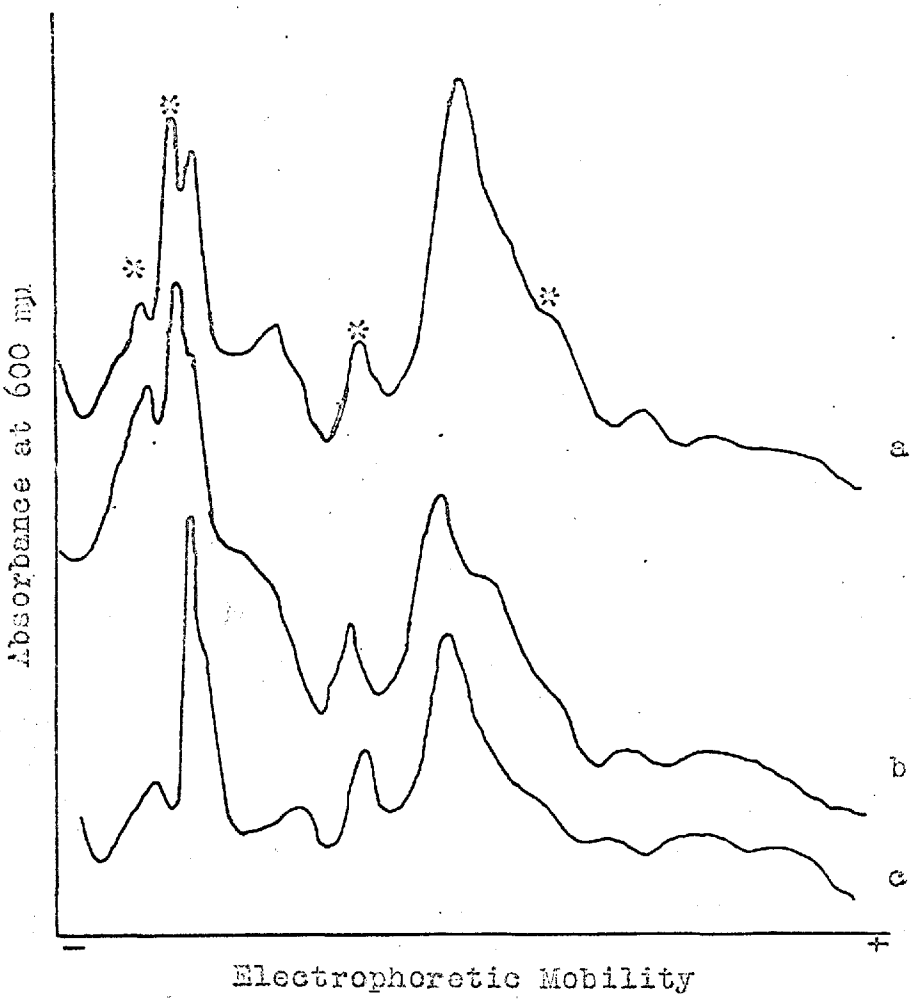


Figure 31.
Absorbancy profiles of broad bean lamellar protein fractionated using ammonium sulphate. The fractions were then separated by polyacrylamide gel electrophoresis.
a. Lamellar protein before fractionation
b. Ammonium sulphate precipitate ("structural" protein)
c. Ammonium sulphate supernatant
Asterisks identify components enriched in the ammonium sulphate precipitate.

background radioactivity. When the most highly labelled green leaf material is hydrolyzed and the constituent amino acids fractionated by thin-layer chromatography, it can be seen that several of the amino acids are indeed radioactive (Fig. 32).

Attempts to obtain more highly labelled chloroplast "structural" protein in green plants by incubating them in the presence of ^{14}C amino acid or $^{14}CO_2$ or ^{3}H amino acid in the dark as well as in light were also unsuccessful. An alternative method was tried in order to label the "structural" protein with radioactivity. Plants that had been etiolated were supplied with $^{14}CO_2$ for the period of 48 to 72 hr. after the start of illumination. Substantial chloroplast differentiation occurs during this period and so it seemed likely that "structural" protein is being manufactured during this time. However, the radioactivity incorporated was also below the limits of detection.

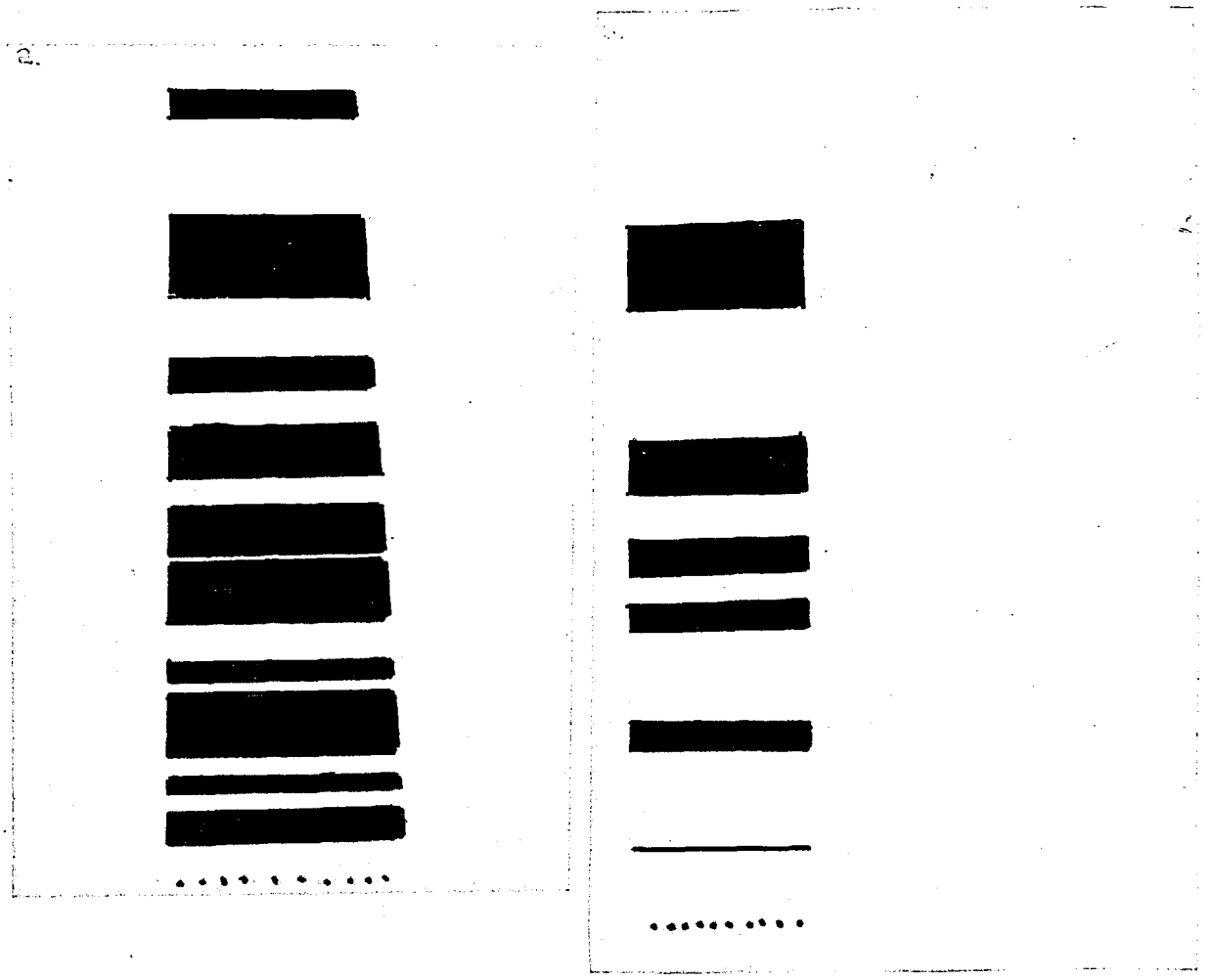


Figure 32. Thin layer chromatographs of the amino acids derived by the hydrolysis of "structural" protein isolated from plants which had been incorporating $^{14}CO_2$.

a. Chromatograph sprayed with ninhydrin reagent
b. Autoradiograph of a chromatograph

DISCUSSION

The main purpose of the investigation described here was to examine the metabolism of chloroplast rRNA in an attempt to determine the function of the chloroplast ribosomes. Initial experimentation was devoted to characterizing the chloroplast rRNA.

The leaf rRNA's were first characterized by means of sucrose density gradient centrifugation. Differences in u.v. absorbancy profiles of the RNA's from different tissues were noted after fractionation in sucrose density gradients (Fig. 3), although chloroplast and cytoplasmic components were not separated from one another. Comparable differences were found by Pollard et al (1966) and by Spencer and Whitfield (1966). The main feature of the results was the unexpectedly high proportion of "light" rRNA from chloroplasts or from tissues containing chloroplasts. Spencer and Whitfield (1966) did not find any "heavy" rRNA in the plastids and thought that degradation was unlikely since Escherichia coli rRNA was not fragmented when mixed with the chloroplast preparation. A similar disproportionately large amount of "light" rRNA was found when the leaf and chloroplast rRNA were fractionated by chromatography on methylated serum albumin- kieselguhr columns (Wollgiehn et al, 1966; Dyer and Leech, 1968).

Only as a consequence of the greatly increased resolving capacity obtainable by polyacrylamide gel electrophoresis has it been possible to characterize

the chloroplst rRNA in detail. The results presented here and those of Loening and Ingle (1967) and Ingle (1968) indicate that the "heavy" (1.1 m) rRNA component is relatively labile. In the broad bean it fragments to produce one 0.7 m and one 0.4 m component which in sucrose density gradients and on MAK columns fractionate with the 0.7 m cytoplasmic and the 0.56 m chloroplast rRNA components, explaining why such a large proportion of the rRNA was found in the "light" fraction.

The nature of the fragmentation of the chloroplast 1.1 m rRNA component is of considerable interest since it may apparently occur even when RNAse activity is rigorously suppressed during isolation of the RNA. The results indicate (Figs. 4 through 9) furthermore that once isolated, little further cleavage of the intact 1.1 m rRNA occurs. The proportion of unfragmented 1.1 m rRNA recovered is very variable. Apparently of considerable importance in the isolation medium is the presence of Mg^{+2} or similar divalent cation although totally unfragmented 1.1 m rRNA may be extracted even in their absence (Fig. 10). It has recently been shown that the 0.7 m and 0.4 m fragments will re-associate in the presence of divalent cations (Dr. J. Ingle and Dr. C.J. Leaver, unpublished results), although if there is much additional degradation, total reconstitution of the 1.1 m rRNA cannot be affected in this way (Dr. C.J. Leaver and M.J. Hartley, unpublished results). These

results indicate a specific association between the 0.4 m and the 0.7 m chloroplast rRNA fragments in the presence of divalent cations.

Two alternative explanations of this fragmentation can be suggested. Either *in vivo* the RNA is composed of two chains that are not covalently linked to one another or that specific degradation of the RNA occurs *in vivo* or during isolation. If the former possibility is the correct one then it should be possible to completely dissociate all the 1.1 m rRNA, a situation which does not usually occur under the conditions so far examined. Also, if the chains are manufactured separately, there will be a 5'-triphosphate at one end of each chain and no phosphate on the 3'-position of the ribose at the other end, since the molecules are formed by polymerization of 5'-nucleotide triphosphates. If the second possibility is the correct one and degradation has occurred as a result of ribonuclease action, then there would be a 5'-triphosphate at one end and a 3'-monophosphate at the other end of one chain and no phosphate on either end of the other chain. Such determinations, although technically feasible, would be quite difficult to perform as judged by the difficulty that was encountered when an attempt was made to label with radioactivity the terminal ribose of the rRNA's in order to determine their chain length (unpublished results). Several factors suggest that the fragmentation

is the result of ribonuclease activity. Latent ribonuclease, the activity of which is apparently inhibited by Mg^{+2}, has been found to be a component of Escherichia coli and several other ribosomes (Petermann, 1964) and specific limited degradation of polynucleotides is known to occur (Gould, 1966). The specific re-association of the 1.1 m rRNA either from two distinct chains or from one that has been disrupted near its center indicates that complementary base sequences might be present at the termini of the fragments, the association of which is stabilized by the divalent cation. A large proportion of rRNA is known to have a double helical configuration and it has been suggested that it may have a series of "hairpin-like" regions in which there is base pairing and that these are linked by lengths of single stranded RNA.



The most likely sites for enzymatic degradation to occur first are where the RNA is single stranded (Gould, 1966; McPhie et al, 1966) either between the haripin loops (A - A) or at their apices (B - B). A break sited in the apical sequences either as a result of enzyme action or because there are two chains could be masked by the base pairing in the helical region which could also make possible specific re-association. A discontinuity between the hairpin-like regions, however, could not be masked and would explain why more extensive degradation results

in fragments which cannot be re-associated.

There have been numerous reports that the "heavy" rRNA from a number of different organisms is either metastable or absent (Petermann, 1964). For example, the fragmentation of the cytoplasmic "heavy" rRNA of Euglena gracilis (Rawson and Stutz, 1968) and of Escherichia coli (Midgley, 1965) have been described. On the basis of the distribution of methylated groups in Escherichia coli rRNA it has been suggested that the "heavy" component is either composed of two sections in which there are considerable base sequence homologies or that it consists of a single chain with duplicated sequences (Fellner and Sanger, 1968). It appears, therefore, that fragmentation of the "heavy" rRNA is a fairly general phenomenon not confined to that from chloroplasts. Although in the broad bean only two components are derived from the 1.1 m rRNA, more extensive fragmentation occurs in maize (Fig. 22) and radish cotyledon as has been found by Ingle(1968). The results presented here confirm the presence within chloroplasts of rRNA which is comparable in size to that of bacteria. Also, in pure chloroplast preparations there is no rRNA similar to that in cytoplasmic ribosomes, which suggests that protein synthesis which occurs in plastids is quite distinct from that taking place in the cytoplasm.

Having established that the rRNA of the chloroplasts differed from that of the cytoplasm, an attempt

was then made to determine if they participated in the synthesis of all or only some of the plastid proteins. In order to do this their metabolism was followed in various tissues and in particular during the greening of etiolated plants when rapid plastid differentiation occurred.

A characteristic of subcellular organization in higher plants is the multiplicity of forms of the plastids found in the cells in various parts of the plant. The plastids appear to be present in all cells in one form or another. Definite developmental lineage is established between proplastids, etioplasts, chloroplasts, and chromoplasts and between proplastids, amyloplasts and chloroplasts and there is no evidence to suggest that all the plastids of a plant are not interrelated.

Despite the omnipresence of plastids in plant cells, the plastid-specific RNA's are not distinguishable in all the tissues of the same plant (Figs. 12 & 13). In the broad bean these RNA's can only be found in leaf or green tissues and are not found in roots or the apical meristem. Ribosomes could, however, be seen in electron micrographs in the plastids from all these tissues. The results obtained by quantitative evaluation of the micrographs suggest that the reason why the chloroplst type rRNA could not be distinguishable in some tissues is that the proportion of the total number of ribosomes which are in the plastids is below the level detectable

by gel electrophoresis of the isolated rRNA. It is therefore emphasised that the gel electropherograms give information only of the relative and not absolute amounts of the various types of nucleic acid.

Although the ribosomes are present in all the major types of plastids in the normal broad bean plant, their presence does not appear to be essential for the synthesis of all the plastid proteins. This conclusion is drawn from an examination of a mutant form which occurred spontaneously among the normal plants (Fig. 14). In the mutant the plastids in the leaves are considerably larger than the proplastids which occur in the apices yet no chloroplast-type rRNA could be detected in gel electropherograms or ribosomes found within the plastids in electron micrographs. From this it would seem that the plastids are similar to mitochondria in that both the ribosomes within these organelles and also those in the cytoplasm are responsible for the synthesis of plastid proteins. If this is the case, it provides a possible explanation for the fact that the great majority of genes characterized which affect plastid processes such as chlorophyll, carotenoid, and starch synthesis have been located in the nucleus (C. Kirk and Tilney-Bassett, 1967). Those results are also in accord with those derived by selective inhibition of chloroplast and cytoplasmic protein syntheses (Smillie et al, 1968). The investigations do not exclude the possibility that

some mRNA synthesized in the nucleus could enter the chloroplast and only there be able to direct protein synthesis. This would attribute some functional significance to the differences between chloroplast and cytoplasmic protein synthesizing systems. While it is clear that chloroplast-type ribosomes are essential for full chloroplast formation, the differentation of the leaves themselves is not affected by their absence, since apart from the lack of chlorophyll the external morphology of this mutant bean was identical to that of normal plants.

The number of broad bean plastid ribosomes present in various tissues appears to be correlated with the size of the plastids (Table 3). During differentiation of chloroplasts either from proplastids or from etioplasts the increase in internal complexity and volume is accompanied by a corresponding increase in the proportion of the total ribosome population which the plastid ribosomes represent. Determinations of the nucleic acid content changes on illumination of etiolated leaves show that the increase in the total RNA is all in the plastid fraction (Tables 5 & 8). From this it may be concluded that etiolated leaves contain their full complement of cytoplasmic ribosomes despite the leaves being greatly reduced in size when compared with those from normal light-grown plants. In maize, in which the leaves are much less reduced in size by etiolation, the decrease in plastid ribosome population is smaller than in the

broad bean. It appears that the effect of light on leaf growth varies considerably among various plants and this may be related to differences in the amount of phytochrome present in each.

Although the increase in plastid-specific nucleic acids is correlated with the differentiation of the photosynthetic mechanism, their development is not dependent upon photosynthesis. This can be concluded from the fact that chloroplast ribosomes are present in appreciable numbers in etiolated leaves and were fully developed in another chlorophyll-deficient mutant of the broad bean (Dyer, unpublished results). This conclusion is also supported by the findings of Schiff et al (1967) who showed that photosynthetic competence in Euglena can be achieved under conditions where carbon fixation is completely inhibited by DCMU during the entire span of development.

The study of the kinetics of the processes culminating in the differentiation of functional chloroplasts from etioplasts indicate that maximum differentiation occurred during the period of most rapid increase in chloroplast ribosomes. While providing further evidence of their role in this process, many details of the differentiation process remain to be elucidated. The lag in the synthesis of chlorophyll and chloroplast ribosomes and in differentiation of structure is of particular interest since it indicates

that there are initially rate-limiting processes not related to nucleic acid metabolism. The particular question posed is of how the light affects differentiation. Little appears to be known as yet as to the mode of action of the phytochrome light receptor molecule. Cumming and Wagner (1968) favor the idea that phytochrome action is based primarily upon the various membranes of cells, including, for example, the plasmalemma and tonoplast, chloroplasts, mitochondria, and the nuclear envelope. They envisage that membrane-bound phytochrome when converted to the active form brings about alterations in membrane permeability, which may then influence processes of translation and enzyme activation and in some cases may eventually lead to gene activation. It seems possible that changes such as these could occur in the etiolated leaves on illumination, since a major constraint of etiolation appears to be on vacuolar and plastid volume increases.

One of the early effects of light on the nucleic acids of etiolated leaves is an appreciable increase in the number of ribosomes in polysome configuration (Williams and Novelli, 1968; Mans, 1967). A detail that has yet to be resolved is whether the mRNA involved in this transition existed before the light treatment or was newly synthesized as a result of it. Other factors which would influence the stimulation of protein synthesis which this increase in polysome level

represents are the supply of energy in the form of ATP and GTP, the presence of amino acids in sufficient quantities, and the presence of a suitable ionic environment. In order to establish whether cytoplasmic protein synthesis is activated before that of the chloroplast it will be necessary to determine whether this increase in polysome levels occurs in the cytoplasm before chloroplast ribosomes are synthesized in quantity.

As was shown in this investigation, there is no increase during the first 24 hr. in total amount of RNA following illumination of etiolated broad bean leaves. However, the rate of incorporation of radioactive ^{14}C-orotic acid into the RNA does increase (Table 5) into what appears to be mainly chloroplast rRNA (Fig. 24 and Table 9). Incorporation into cytoplasmic rRNA is stimulated no more than 3% in response to light (Table 9). confirming that this RNA is little affected by light. Thus there may be a fairly rapid and selective effect of illumination on the synthesis of chloroplast rRNA. Various tissues appear to differ in this respect. Ingle (1968) found a two-fold increase in the amount of cytoplasmic rRNA and 19% stimulation of incorporation of ^{32}P when radish cotyledons were grown for 2 days in light instead of the dary. A possible explanation of this discrepancy is that the radish cotyledons continue to accumulate cytoplasmic rRNA even when maintained in the dark, whereas in the broad bean there is no

further increase during further dark treatment. Therefore the systems are not directly comparable, and the necessity for caution when comparing results from different sources must be emphasized.

With strong evidence that the chloroplast-type ribosomes are not responsible for the synthesis of all chloroplast proteins, a problem to be resolved is which proteins are synthesized where. The approach that has been adopted almost exclusively so far is to measure changes in enzymatic activities on illumination of etiolated cells, distinguishing between chloroplastic and cytoplasmic protein syntheses by means of selective inhibitors. Disadvantages of this approach are that the increases in enzyme activities are not necessarily an indication of protein synthesis and there is reason to doubt the specificity of the inhibitors (Ellis, 1969). These difficulties can be partly overcome by measuring changes in amount of individual proteins and by incorporation of radioactivity into them. In this investigation, the chloroplast lamellar proteins were chosen, since the results of this study as well as that of Wettstein (1967) indicate that the development of lamellae occur concurrently with the formation of the plastid protein synthesizing system. Additional advantages are that this protein can be readily prepared free of contamination, and from the inhibitor studies of Smillie et al (1967) it seems likely that this fraction would con-

tain some protein that is synthesized in the cytoplasm (the structural proteins) and other protein that is made in the chloroplasts (cytochromes). Under conditions favoring either cytoplasmic or chloroplast protein synthesis, one might expect to get selective incorporation of radioactivity into the various proteins depending upon their site of synthesis. The technical difficulty that was not overcome in this investigation is that insufficient radioactivity is incorporated into the proteins both when tritiated and ^{14}C labelled amino acids or $^{14}CO_2$ are used as precursors. This made it impossible to detect radioactivity in the fractionated proteins. A possible reason for the low levels of incorporation of radioactive amino acids in etiolated leaves on illumination is that there is probably a large pool of free amino acids present, as was found to be present by DeBeken-Gronson (1954) in etiolated chicory leaves. When these plants were exposed to light for 2 days, the amount of amino acids was reduced by 90%, presumably by incorporation into protein.

The study of the chloroplast lamellar protein did, however, show that the "structural" protein is a heterogeneous fraction, contrary to what had previously been found by Criddle (1966). At least two components have characteristic electrophoretic mobilities which distinguished them from any in mitochondrial preparations. A similar fractionation of mitochondrial "structural"

protein by Haldar et al (1966) had previously shown that this protein is heterogeneous. Although there is the possibility that the various fractions are the result of either aggregation or fragmentation, the conditions under which the isolation and fractionation were carried out would seem to preclude these possibilities.

In conclusion, this study has provided some information as to the characteristics and function of the plastid nucleic acids. Nevertheless, much remains to be explained, especially with respect to the site of synthesis of the plastid proteins and about the way in which the plastid nucleic acids are synthesized and regulate plastid differentiation.

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