ORGANELLE RIBOSOMES AND rRNA IN

OCHROMONAS DANICA

A BIOCHEMICAL AND ULTRASTRUCTURAL STUDY

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ABSTRACT

The effect of 100 μg/ml spectinomycin, a specific inhibitor of translation on organelle ribosomes, on the accumulation of chloroplast rRNA was studied in the Chrysophyte Ochromonas danica. Intact algal rRNAs were isolated and characterized. The molecular weights of the heavy and light cytoplasmic rRNAs are 1.18×10^6 and 0.66×10^6 daltons respectively. The molecular weights of the heavy chloroplast and mitochondrial rRNAs are both 0.94×10^6 daltons, while the light chloroplast and mitochondrial rRNAs have values of 0.50×10^6 and 0.55×10^6 daltons respectively. The thermolabile heavy chloroplast rRNA is probably nicked in three places. Cells exposed to 24 hr light and spectinomycin grow at nearly normal rates, but contain 30 - 40% less chloroplast rRNA than do controls. Electron microscopy showed that spectinomycin disrupts the organization of chloroplast membranes and inhibits the light-induced synthesis of chloroplast ribosomes by 50%. These results strongly suggest that at least some chloroplast ribosomal proteins are synthesized in the chloroplast of Ochromonas.

RESUME DE THESE

L'effet de la spectinomycin (100 µg/ml), un inhibiteur spécifique de la synthèse protéique par les ribosomes d'organelles, sur l'accumulation de l'ARN chloroplastique furent étudiés chez le Chrysophyte, Ochromonas danica. Les ARN ribosomiques intacts de l'algue furent isolés et caracterisés. Les poids moléculaires des ARN ribosomiques lourds et légers du cytoplasme sont de 1.18×10^6 et 0.66×10^6 respectivement. Les poids moléculaires des ARN ribosomiques lourds du chloroplaste et des mitochondries sont tous les deux de 0.94×10^6, tandis que les ARN ribosomiques légers du chloroplaste et des mitochondries ont des valeurs de 0.50×10^6 et 0.55×10^6 daltons respectivement. L'ARN ribosomique lourd du chloroplaste est thermosensible et probablement entaillé à trois sites. Les cellules qui sont exposées à la lumière et à la spectinomycin pendant 24 hr, croissent à des taux presque normaux, main contiennent de 30 -40% moins d'ARN ribosomique du chloroplaste que les témoins. La microscopie électronique a démontrée que la spectinomycin dérange l'organisation des membranes chloroplastiques et inhibe de 50% la synthèse stimulée par la lumière des ribosomes chloroplastique. Ces résultats suggérent fortement qu'au moins certaines protéines ribosomiques du chloroplaste sont formées au chloroplaste même.

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EXPLANATORY NOTE

In the text which follows, the names of a variety of different antibiotics appear frequently. To aid the reader, I have listed the antibiotics according to their mode of action.

a. Inhibitors of protein synthesis on bacterial-type ribosomes:

carbomycin

chloramphenicol

cleocin

erythromycin

lincomycin

neamine

spectinomycin

streptomycin

virginiamycin

b. Inhibitor of protein synthesis on eukaryotic ribosomes:

cycloheximide

c. Analogue of aminoacyl-tRNA causing premature release of peptides from bacterial-type and eukaryotic ribosomes:

puromycin

d. Inhibitor of the replication and transcription of circular DNA molecules:

ethidium bromide

e. Inhibitors of bacterial DNA-dependent RNA polymerase:

rifampicin

rifampin

f. Inhibitor of nucleoplasmic DNA-dependent RNA polymerase in eukaryotic cells:

α-amanitin

g. Inhibitor of oxidative phosphorylation:

oligomycin

In cases where individual ribosomal proteins are identified, the nomenclature used is that of Wittmann et al. (1971).

KEY TO ABBREVIATIONS

Å	angstrom
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
Ci	curie
CM	carboxymethyl
cpm	counts per minute
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetate
EM	electron microscopy
F	sex factor
ft-c	foot-candle
GTP	guanosine triphosphate
hr	hour
K_m	Michaelis constant
lac	lactose
leu	leucine
mA	milliampere
MAK	methylated albumin-kieselguhr
min	minute
mRNA	messenger ribonucleic acid

O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S	Svedberg unit
SDS	sodium dodecyl sulfate
thy	thymine
threo	threonine
tRNA	transfer ribonucleic acid

INTRODUCTION

I. Literature Review

A. Organelle Biogenesis

1. Organelle autonomy

a. Biochemical studies

1. Organelles contain DNA and other components required for autonomy.

Studies conducted during the 1960's established that chloroplasts and mitochondria contain DNA and also the components required for the replication and expression of their genomes. These organelles are, therefore, potentially autonomous with respect to the host cell (Gibor and Granick, 1964; Kirk and Tilney-Bassett, 1967; Whitfield and Spencer, 1968; Smillie and Scott, 1969; Ashwell and Work, 1970; Rabinowitz and Swift, 1970; Tewari, 1971; Sager, 1972). The evidence supporting this conclusion is based on a variety of studies involving cytological and biochemical techniques.

In 1962, Ris and Plaut described DNA-containing areas in the chloroplast of Chlamydomonas moewusii cells, fixed by a procedure originally developed for the preservation of bacterial nucleoplasm (Ryter et al., 1958). Other investigators (e.g. Chiba, 1951) had previously shown the presence of DNA in chloroplasts, but their results were inconclusive since appropriate controls, such as DNase digestion, were not carried out. Ris and Plaut used light and electron microscopy combined with cytochemistry to demonstrate that the fine fibrils approximately 25 Å

in diameter which occupied areas in the chloroplast stainable with acridine orange and the Feulgen reaction were removable by DNase, but not RNase, treatment. The authors therefore concluded that the fine fibrils observed in the chloroplast were DNA molecules. Ris and Plaut also speculated on the possible genetic function of chloroplast DNA and the evolutionary relationship between chloroplasts and blue-green algae.

The presence of DNA fibrils was subsequently reported in chloroplasts of several other algal species. For example, Puiseux-Dao et al. (1967) demonstrated DNase-sensitive fibrils in the chloroplasts of Acetabularia mediterranea. In an ultrastructural study of the brown alga, Egregia menzeisii, Bisalputra and Bisalputra (1967) identified an electron-translucent area at each tip of longitudinally-sectioned chloroplasts. Each area contained fibrils approximately 25 Å thick which were absent following DNase treatment. A similar organization of electron-translucent regions containing fine fibrils was described in the chloroplast of the golden-brown alga, Ochromonas danica (Slankis and Gibbs, 1968).

In higher plants, DNA-containing areas often appear scattered throughout the chloroplast matrix. Gunning (1965) studied the ultrastructure of the chloroplast in oat seedlings (Avena sativa). He observed several areas in each chloroplast section which contained fibrils 25-30 Å thick. Although sections were not incubated with DNase in this study, it is most likely that the fibrillar networks described by Gunning are composed of DNA since they resemble the filamentous nucleoids of bacteria. Kislev et al. (1965) conducted a comprehensive investigation of chloroplast nucleic

acids in Swiss chard (Beta vulgaris). Using light microscope cytochemistry and autoradiography, these authors showed that the chlorqplast component which was Feulgen-positive and which incorporated (^{3}H)thymidine was also removable by DNase treatment. By electron microscopy, they also identified fibrils of chloroplast DNA by their morphology and sensitivity to DNase. This method was also used to demonstrate DNA fibrils in the chloroplasts of Tradescantia albiflora (Sprey, 1966) and spinach (Spinacia oleracea) (Yokomura, 1967a). Odintsova et al. (1970) described three to seven randomly distributed fibril-containing areas per chloroplast section in pea (Pisum sativum). The fibrils were also extracted from isolated chloroplasts lysed by osmotic shock and spread on grids by the Kleinschmidt (1968) technique. When the preparation was exposed to DNase, the fibrils were reduced to a few fragments.

Plastids, other than chloroplasts, also possess DNA. Electron-translucent areas containing filamentous structures have been observed in the chloroamyloplasts from Pellonia daveauana, proteinoplasts from Helleborus corsicus, and amyloplasts from potato tubers (Solanum tuberosum) (Salema and Badenhuizen, 1969). The filaments probably represent DNA molecules, since EM autoradiography showed that each of the three types of plastid incorporated (^{3}H)thymidine. Chromoplasts from Narcissus pseudonarcissus also contain electron-translucent regions occupied by 25-30 Å thick filaments (Kowallik and Herrmann, 1972). In this study, enzyme digestion failed to remove the filaments from fixed material

perhaps for technical reasons, but the identification of these structures as DNA is most likely correct since strands of DNA from chromoplasts were also isolated biochemically (Herrmann, 1972). In an ultrastructural study of Chilomonas paramecium, a colourless cryptomonad flagellate, Sepsenwol (1973) demonstrated that the leucoplast of this organism also contains DNA-like fibrils. The similarity between these fibrils and those in bacterial nucleoids in form and response to fixatives strongly suggested that the leucoplast fibrils represent DNA molecules.

Studies of both algae and higher plants indicate that chloroplast DNA serves a genetic function. In an experiment similar to one designed by Meselson and Stahl (1958), Chiang and Sueoka (1967) demonstrated that in Chlamydomonas reinhardi chloroplast DNA is replicated semiconservatively and at a different time than nuclear DNA. Synchronous cells were labelled during one division cycle with ^{15}N and then transferred to medium containing ^{14}N. Analysis of the buoyant density pattern of DNA extracted from samples taken at different times during the subsequent division cycle showed that the ^{15}N-labelled band containing chloroplast DNA was replaced by a hybrid ^{15}N-^{14}N component early in the light period. A shift in the buoyant density of the nuclear DNA band, however, did not occur until near the end of the dark period. These observations are consistent with the results of the direct assay of total cell DNA, which showed a biphasic increase during the cell cycle of Chlamydomonas. Synthesis of DNA has also been shown in chloroplasts isolated from

Euglena gracilis (Scott et al., 1968), spinach (Spencer and Whitfield, 1967), and tobacco (Tewari and Wildman, 1967). In each case, (^{3}H)thymidine or (^{3}H)adenine was incorporated into an acid-insoluble product which coincided in CsCl gradients with chloroplast DNA synthesized in vivo. Incorporation of the radioactive precursor by the isolated chloroplasts required the presence of the other three nucleotides as well, and was inhibited by DNase and moderate concentrations of actinomycin D. The product of DNA synthesis by isolated tobacco chloroplasts was further characterized by analysis of radioactive hydrolysates and molecular hybridization studies. The results showed that the composition and base sequence of the in vitro product are similar to that of chloroplast DNA, but not nuclear DNA. In summary, these studies suggest that chloroplast DNA is replicated semiconservatively within the organelle to ensure the transmission of genes required for the biogenesis of the daughter chloroplasts.

The existence of ribosomes in chloroplasts was first demonstrated in spinach by Lyttleton in 1962. Chloroplast preparations shown to be free from contaminants by phase microscopy were osmotically shocked to release particles, which, by their spectral properties, sensitivity to ion concentration, and sedimentation behaviour in the analytical ultracentrifuge resembled ribosomes. By comparing the sedimentation profiles of chloroplast and whole cell preparations, Lyttleton was able to identify the chloroplast ribosomes, which sedimented more slowly than the cytoplasmic ribosomes. In a similar study, Brawerman (1963) showed

that ribosome-like particles are also present in algal chloroplasts. When analyzed by ultracentrifugation, the particles from crude Euglena chloroplasts exhibited a sedimentation constant of 43S, which was lower than that of the microsomal particles, and dissociated into two smaller structures when the concentration of magnesium was reduced. The nucleotide composition of RNA extracted from the chloroplast particles differed from that of the microsomal fraction, but was similar to that of RNA extracted from whole chloroplasts purified by flotation. Thus, Brawerman concluded that the particles isolated from Euglena chloroplasts are ribsomes. The low value for the sedimentation coefficient obtained by Brawerman, however, suggests that the 43S component was the large ribosomal subunit, rather than the monomeric chloroplast ribosome.

The sedimentation characteristics of chloroplast ribosomes were subsequently described in several other species of higher plants and algae. Clark et al. (1964) examined ribosomes extracted from Chinese cabbage (Brassica pekinensis) by analytical and sucrose gradient ultracentrifugation. These authors demonstrated the presence of two classes of particles in leaf extracts, with values of 68S and 83S. By appropriate controls, they also verified that the 68S and 83S species were localized in the chloroplast and cytoplasm, respectively, and that the smaller particles were not artifacts resulting from degradation of the 83S component. Two classes of ribosomes, with sedimentation coefficients of approximately 70S and 80S, were also reported in extracts of bean leaves (Phaseolus vulgaris) (Boardman, 1966) and pea

seedlings (Svetailo et al., 1967). Particles were extracted from purified chloroplast fractions disrupted by osmotic shock and analyzed by ultracentrifugation. In agreement with previous observations, the 70S and 80S components were each dissociable into two smaller units and were exclusively located in the chloroplast and cytoplasm, respectively. First attempts to study the chloroplast and cytoplasmic ribosomes of Chlamydomonas indicated the presence of 70S and 80S ribosomal species, but further analysis of the particles was hampered by their instability in the extracting medium (Sager and Hamilton, 1967). Later, Hoober and Blobel (1969) showed that ribosomes from Chlamydomonas are stable in the presence of 20-25 mM magnesium and characterized both chloroplast and cytoplasmic ribosomes with respect to sedimentation, subunits, RNA components and ribosomal proteins.

Evidence for the presence of ribosomes in chloroplasts is also based on light and electron microscopy. Particles resembling ribosomes were detected in proplastids and chloroplasts of maize (Zea mays) by Jacobson et al. (1963). Using light microscope and autoradiography, they showed that material which stained with basophilic dye and contained (^{3}H)cytidine was removable with RNase. By electron microscopy, they found that most, if not all, of the RNase-sensitive material in plastids was in the form of 170 Å particles. By their size and RNA content, these particles were identified as ribosomes. Using the technique of negative staining, Bruskov and Odintsova (1968) studied

ribosomes from pea seedlings and bean leaves. The authors found a significant difference in the size of the purified cytoplasmic and chloroplast ribosomes, which measured approximately 260 Å and 200 Å respectively. The results of these studies involving sedimentation analysis and various visual methods thus illustrate that chloroplasts contain ribosomes which are distinct from those located in the cytoplasm.

Both indirect and direct evidence shows that chloroplasts contain rRNA, tRNA and mRNA. The studies described above, which demonstrate the existence of chloroplast ribosomes, also imply the existence of chloroplast rRNA. Furthermore, Brawerman and Eisenstadt (1964a) analyzed high molecular weight rRNA phenol-extracted from either disrupted cells or chloroplast fractions of Euglena. When examined in sucrose density gradients, the chloroplast rRNA separated into two components (slightly degraded) with sedimentation coefficients of 19S and 14S. Oshio and Hase (1968) characterized chloroplast RNA isolated from non-aqueous extracts of lyophyllized cells of Chlorella protothecoides. They found that the chloroplast rRNA was composed of two components which behaved similarly to E. coli rRNA on MAK columns and sucrose gradients. Chloroplast rRNA from Chlamydomonas was likewise resolved into two components which exhibited a similar sedimentation pattern to E. coli rRNA on sucrose gradients (Hoober and Blobel, 1969). Chloroplast rRNA from higher plants has also been characterized. For example, Hadziyev et al. (1968) compared wheat (Triticum vulgare) leaf and

chloroplast RNA with respect to base composition and elution from MAK columns. Intact wheat chloroplasts, which were isolated in Honda medium (and thus probably free from bacteria) and purified in a discontinuous sucrose gradient, contained an RNA which differed from the corresponding leaf RNA with respect to both elution properties and base composition. The bulk of the RNA in these preparations, by its behaviour on MAK columns, was ribosomal in nature.

Evidence for the existence of mRNA in chloroplasts is based partly on observations of chloroplast polysomes. For example, Gunning (1965) described polysome-like structures in thin sections of oat plastids. Polysomes were also obtained from Chinese cabbage (Clark et al., 1964). Whether or not some of the polysomes were derived from the chloroplast, however, was not determined in this study, since only whole leaf extracts were analyzed. Brawerman and Eisenstadt (1964a) provided more direct evidence for the existence of mRNA in chloroplasts by showing that RNA extracted from isolated Euglena chloroplasts stimulates the amino acid incorporating activity of E. coli ribosomes in vitro. Chloroplasts obtained by differential centrifugation were lysed to yield particulate and soluble RNA fractions. Both fractions exhibited significant messenger activity, which was associated predominantly with a 12S and, to a lesser extent, with a 22S RNA. In contrast to that of chloroplast preparations, most of the putative cytoplasmic mRNA consisted of the 22S component. These results clearly indicate the presence of a chloroplast RNA with messenger-like properties. Chen and Wildman (1967)

later showed that polysomes from isolated washed tobacco chloroplasts incorporate amino acids both before and after liberation from the chloroplasts, and with higher specific activity than monosomes. Furthermore, either increasing the time of incubation or exposure to RNase resulted in conversion of the polysomes to monosomes, indicating that the aggregates were not formed by the non-specific association of ribosomes.

The presence of tRNA in chloroplasts has been demonstrated biochemically. Sissakian et al. (1965) obtained a fraction with the properties of tRNA from pea by extracting osmotically-shocked chloroplasts with phenol and dissolving the ethanol-precipitated RNA in water containing 10% NaCl to precipitate rRNA. The remaining soluble RNA was then purified, freed from attached amino acids, and tested for amino acid acceptor activity by an in vitro assay. The results indicated that radioactive amino acids were transferred to the soluble RNA fraction. Oshio and Hase (1968), in their study of nucleic acids in Chlorella, detected a slowly sedimenting component by sucrose gradient centrifugation of chloroplast RNA preparations. The sedimentation characteristics of this fraction suggested its identity with 4S tRNA.

Evidence suggests that some, if not all, of the RNA present in chloroplasts is synthesized in the organelle. The ability of chloroplasts to synthesize RNA was demonstrated by Schweiger et al. (1967) in enucleated cells of Acetabularia. These investigators used cultures

monitored for sterility by microscopy, so that the incorporation of radioactive precursors into RNA by the enucleated plants was probably not due to contaminating microörganisms. The products labelled *in vivo* were analyzed by sucrose gradient centrifugation, which indicated that the anucleate fragments synthesized rRNA and tRNA. These results were also confirmed by similar experiments with chloroplasts isolated from *Acetabularia* (Berger, 1967). Again, only sterile preparations were used and the products synthesized by the isolated chloroplasts migrated on sucrose gradients to positions coincident with rRNA and tRNA. Isolated chloroplasts from *Euglena* were also shown to be capable of RNA synthesis (Shah and Lyman, 1966). The purity of the chloroplast preparation was checked by microscopy and RNA synthesis was promoted by incubating the chloroplasts in medium containing the required factors. The products of the *in vitro* system were not characterized by sedimentation, but incorporation of radioactive RNA precursors into acid-insoluble material was sensitive to actinomycin D, DNase and RNase. This suggests that the algal chloroplasts were carrying out DNA-dependent RNA synthesis. Kirk (1964) obtained similar results with chloroplasts isolated from broad bean (*Vicia faba*).

Other methods used to demonstrate the ability of chloroplasts to synthesize RNA include EM autoradiography. Gibbs (1968) studied the distribution of RNase-removable grains over various subcellular compartments of *Ochromonas* after cells were allowed to incorporate (^{3}H)uridine. After a 30 min labelling period, approximately two-thirds

of the cytoplasmic grains were located over the chloroplast and mitochondria, whereas after a 2 hr labelling period, the proportion of cytoplasmic grains associated with non-organellar regions increased to one-half of the total grain count. This suggested that there is a lag in the appearance of RNA in the cytoplasm, but that in chloroplasts, as well as in mitochondria, RNA is synthesized in situ. Since the chloroplast nucleoid in Ochromonas is well-defined in appearance and location, Gibbs (1967) was also able to show that chloroplast RNA is synthesized at the site of chloroplast DNA; after short-term labelling, the grains were clustered over the chloroplast nucleoid, whereas, after a longer period of incorporation, grains were more randomly distributed over the chloroplast. Molecular hybridization studies provide more evidence for transcription of chloroplast RNA from chloroplast DNA. Scott and Smillie (1967), for example, showed that ^{32}P-labelled RNA isolated from Euglena chloroplasts hybridized to approximately 1% of the total chloroplast DNA sequences. More recently Stutz and Vandrey (1971) found that the amount of Euglena chloroplast DNA containing rRNA cistrons is closer to 6%, when nucleic acids are extracted by a modified procedure which preserves a heavy chloroplast DNA component. In a study of tobacco, Tewari and Wildman (1968) showed that RNA extracted from 70S ribosomes hybridized with 0.45-0.65% of the chloroplast DNA. They also found that, although no significant binding occurred between chloroplast DNA and RNA extracted from 80S ribosomes, chloroplast rRNA hybridized to about 0.1% of the nuclear DNA. Tewari and Wildman interpreted this finding as evidence for the existence of chloroplast rRNA cistrons in

the nucleus. However, the cross-hybridization between chloroplast rRNA and nuclear DNA most likely reflects the conservation of similar, but non-identical, sequences in chloroplast and cytoplasmic rRNAs (Matsuda and Siegel, 1967; Whitfield, 1973).

The ability of chloroplasts to synthesize proteins is indicated by the incorporation of amino acids into protein by isolated chloroplasts. In the initial experiments, however, most of the amino acid incorporating activity attributed to chloroplasts was probably due to contaminating bacteria. This is indicated, for example, in a report by App and Jagendorf (1963), which showed that amino acid incorporation by isolated spinach chloroplasts was insensitive to RNase and the addition of co-factors such as ATP. Presumably these molecules do not penetrate bacterial cells. Gnanam et al. (1969) showed that the use of 'Honda' medium, which contains Dextran and Ficoll, allows the isolation of intact chloroplasts relatively free from bacteria. Therefore, this method was employed for isolation of chloroplasts from spinach (Spencer, 1965), tomato (Lycopersicum esculentum) (Hall and Cocking, 1966) and wheat (Bamji and Jagendorf, 1966). The characteristics of the amino acid incorporating activity of these systems confirmed the conclusion that chloroplasts are capable of synthesizing proteins. Furthermore, ribosomes extracted from isolated tobacco chloroplasts were also shown to be active in incorporating amino acids into protein (Boardman et al., 1966; Chen and Wildman, 1967).

The independence of chloroplasts would be severely restricted

if the organelle were dependent on the host cell for its supply of amino acids required for protein synthesis. The ability of chloroplasts to synthesize amino acids *in situ*, however, has been demonstrated in *Acetabularia* (Shephard and Levin, 1972). Chloroplasts were isolated in a medium containing Ficoll and purified by passage through a membrane filter. The chloroplast suspension was exposed to ^{14}C-labelled bicarbonate for 6-8 hr, and then the acid-insoluble material was hydrolyzed and finally analyzed by thin-layer chromatography. The results indicated that $^{14}CO_2$ was incorporated into most, if not all, the amino acids comprising the chloroplast protein. Bacterial contamination could not account for this observation since plate counts indicated the presence of very few microörganisms. Furthermore, the relative amounts of different amino acids incorporated *in vitro* resembled the pattern of *in vivo* incorporation, and the amount of radioactivity recovered in protein was 200-fold greater when chloroplasts were illuminated. The possibility that the incorporation of $^{14}CO_2$ into amino acids might result from the addition of carboxyl groups to partially completed carbon skeletons was also ruled out by demonstrating that the amount of label removed by α-decarboxylation was not significantly different from the predicted value for uniformly labelled amino acids. Thus, *Acetabularia* chloroplasts apparently possess the more than fifty enzymes involved in amino acid biosynthesis.

Chloroplasts therefore display considerable autonomy and several attempts have been made to culture these organelles outside the cell. Ridley and Leech (1970), for example, maintained chloroplasts

isolated from immature leaves of Vicia faba for up to 6 days in an artificial medium containing Ficoll and bovine serum albumin. Chloroplast division was monitored by phase microscopy and 'dumb-bell' chloroplasts were observed by electron microscopy. A similar medium containing Ficoll and Dextran was used by Kameya and Takahashi (1971) to culture chloroplasts isolated from young leaves of Nicotiana tabacum. Phase and fluorescence microscopy indicated that the number of tobacco chloroplasts increased over a period of 3 days, although the average size of the organelles decreased. Giles and Sarafis (1971) maintained chloroplasts prepared from the marine alga Caulerpa sedoides and the freshwater alga Nitella gracilis for up to 27 days in hens' eggs, treated with kinetin. The structural integrity of the chloroplasts after 15 days was confirmed by various light microscope methods, as well as electron microscopy. The presence of butterfly-shaped chloroplasts and the correlation between the changes in the proportion of 'butterflies' and increase in size of the plastid population with time was interpreted as evidence for chloroplast division. Although none of these studies demonstrated an increase in the mass of individual chloroplasts, they do indicate that, given a favourable environment, chloroplasts can survive and divide in vitro.

Like chloroplasts, mitochondria also contain DNA, RNA and ribosomes, and thus are at least semi-autonomous organelles (Ashwell and Work, 1970; Rabinowitz and Swift, 1970; Sager, 1972). The evidence establishing the independent nature of mitochondria is similar

to that discussed above concerning the biochemical autonomy of chloroplasts.

In 1963 (a, b), Nass and Nass demonstrated the presence of fibrils 15-30 Å thick in electron-translucent areas of chick embryo mitochondria. The fibrils responded to different fixation and staining procedures in a fashion similar to that of the fine fibers in the nucleoplasm of E. coli. By analogy, this suggested that the intramitochondrial fibrils were composed of DNA, a conclusion supported by the observations that DNase, but not RNase or pepsin, removed the filamentous structures. The presence of DNA-like fibrils in plant mitochondria was shown by thin section electron microscopy in a survey of 18 species of algae and higher plants (Yokomura, 1967b). Mitochondria from dark-grown pea seedlings were also shown to contain DNA-like fibrils occupying electron-translucent regions. When the fibrils were extracted from isolated pea mitochondria and exposed to DNase, they were digested into small fragments (Mikulska et al., 1970). Evidence from studies by Reich and Luck (1966) with Neurospora indicate that mitochondrial DNA serves a genetic function. The results of their density-labelling experiments suggested that mitochondrial DNA is replicated by a semi-conservative mechanism. In another set of experiments, involving crosses between selected strains of Neurospora, these investigators showed that the mitochondrial cytochrome pattern was inherited together with mitochondrial DNA (Reich and Luck, 1966). These results therefore indicated that mitochondrial DNA is a genetic determinant of organelle

phenotype.

Mitochondrial ribosomes were first demonstrated by EM cytochemistry in a variety of adult and embryonic animal tissues (André and Marinozzi, 1965). Lead-stained particles 120-150 Å in diameter were distinguished from larger 'dense granules' and identified as ribosomes by their sensitivity to RNase. Similarly, ribosome-like particles, 150 Å in diameter, were also observed in mitochondria of Swiss chard, and were shown to contain RNase-sensitive material (Kislev et al., 1965). Vignais et al. (1969) characterized mitochondrial ribosomes from yeast (Candida utilis) by sucrose gradient centrifugation. A mitochondrial fraction was obtained which was free from cytoplasmic contamination when checked by electron microscopy. Numerous ribosomes approximately 180 Å in diameter were observed, often in clusters, attached to the inner mitochondrial membrane. The sedimentation profile of ribosomes extracted from the isolated mitochondria showed two main peaks at 52-54S and 77-80S. The larger particles were identified as the mitochondrial monosome on the basis of an experiment in which isolated mitochondria were incubated in the presence of (^{14}C)leucine and bacterial contamination was minimized. Most of the incorporated radioactivity was associated with the 77-80S component. Also, in view of this demonstration of in vitro protein synthesis, the clusters of ribosomes seen in thin sections of the isolated yeast mitochondria most likely represent polysomes. The data of Vignais et al. therefore also provide evidence for the existence of mitochondrial mRNA.

The presence of a complete complement of tRNAs in mitochondria was demonstrated in Neurospora crassa by Barnett and Brown (1967). Transfer RNA was phenol-extracted from cytoplasmic and mitochondrial fractions prepared from disrupted hyphae and eluted from DEAE-cellulose columns. Mitochondrial tRNA exhibited acceptor activity for all eighteen amino acids assayed. Bacterial or cytoplasmic contamination could not account for the observed activity, since only sterile solutions were employed for cell fractionation and no significant amount of cytoplasmic material was detected in thin sections of the mitochondrial pellet. Furthermore, isolated mitochondria were treated with venom phosphodiesterase prior to tRNA extraction to remove any residual cytoplasmic tRNA. Thus, one interpretation of these data is that mitochondria contain a separate set of tRNA molecules.

As discussed above in the case of chloroplasts, attempts to demonstrate that mitochondria synthesize proteins were also hampered by difficulties in preventing bacterial contamination. Kroon et al. (1967), however, succeeded in obtaining preparations of mitochondria from rat liver which were devoid of microörganisms as tested by surface viable counts. This method involved differential fractionation of the experimental material using equipment and solutions sterilized by autoclaving or Millipore filtration. When incubated in the appropriate reaction mixture, the isolated mitochondria incorporated radioactive leucine and also radioactive ATP into acid-insoluble material, thus demonstrating that mitochondria possess all the factors required for the synthesis of RNA and protein. Kroon et al. also reported that both

ATP and leucine incorporation by the sterile mitochondria were resistant to RNase. This finding seems to be inconsistent with the fact that no bacteria were detected in the mitochondrial preparations, since insensitivity to RNase is often interpreted as an indication of bacterial contamination. Perhaps the conditions employed were not optimal for RNase activity or the enzyme did not penetrate the isolated organelles. The notion that mitochondria do carry out RNA and protein synthesis was subsequently confirmed by other investigators, including Vignais et al. (1969), whose experiments are described above.

ii. Some organelle components are distinct from cytoplasmic counterparts

Many components of both chloroplasts and mitochondria are distinct from their counterparts in the surrounding host cell. For example, nuclear and organelle DNAs can be distinguished by whether or not 5-methylcytosine is present. Nuclear DNA from Euglena contains about 2.3 mole % 5-methylcytosine (Brawerman and Eisenstadt, 1964b; Ray and Hanawalt, 1964), while nuclear DNA from HeLa cells and Xenopus laevis consists of 0.7-1.7 mole % of the modified nucleoside (Dawid, 1974). Chloroplast DNA from Euglena and mitochondrial DNA from the animal cells, however, do not contain detectable amounts of 5-methylcytosine. In these studies, the base composition of each nucleic acid preparation was determined directly by paper or thin-layer chromatography of the enzyme-digested DNA. The base composition of organelle DNA has also been analyzed by buoyant density centrifugation. The buoyant density of

chloroplast DNA was a controversial issue during the latter 1960's, but now appears to be generally resolved (Kirk, 1971). Although only a limited number of higher plants and algae have been analyzed, the evidence indicates that chloroplast DNA has an average buoyant density in CsCl of about 1.697 g/cm^3, and, relative to nuclear DNA, is more constant in base composition in different species. The buoyant density of mitochondrial DNA is distinct from that of both chloroplast and nuclear DNA, with a value of approximately 1.706 g/cm^3 in higher plants and 1.713 g/cm^3 in green algae (in Sager, 1972). Another criterion by which nuclear and organelle DNAs can be distinguished is renaturation behaviour. Tewari and Wildman (1966) showed that, whereas chloroplast DNA from tobacco renatures rapidly during slow cooling, nuclear DNA fails to renature completely. This indicates the presence of a higher number of reiterated sequences in chloroplast DNA compared to that in nuclear DNA.

Several enzymes associated with chloroplasts and mitochondria are different from their analogs located in the nucleus or cytoplasm. Polya and Jagendorf (1971) found that chloroplast RNA polymerase from wheat, although similar to nuclear RNA polymerase with respect to cation dependence, thermal and pH optima, differs from the nuclear enzyme in its template specificity. These investigators suggested that the catalytic cores of both enzymes may be identical, but dissimilar subunits modify their respective activities. Smith and Bogorad (1974) characterized RNA polymerases from Zea mays by gel electrophoresis and

found that one of the two polypeptide subunits of the chloroplast enzyme could be resolved from the corresponding subunit of the nuclear enzyme. Küntzel and Schäfer (1971) isolated mitochondrial RNA polymerase from Neurospora and showed it consists of only one polypeptide chain with a molecular weight of 64,000 daltons. They further demonstrated that the mitochondrial enzyme is sensitive to rifampicin, resistant to α-amanitin and discriminates between native and denatured mitochondrial DNA. Mitochondrial RNA polymerase from Neurospora thus differs from nuclear RNA polymerases in its size, monomeric construction and sensitivity to certain antibiotics, as well as its specificity for a mitochondrial DNA template.

Specific tRNAs and acylating enzymes are associated with both chloroplasts and mitochondria. Barnett and Brown (1967) demonstrated that, in Neurospora, one species of aspartic acid tRNA is located exclusively in the mitochondria. They also showed that only one of the two aspartyl-tRNA synthetases present in whole cells reacts with the mitochondrial tRNA species. These data suggest that mitochondria contain unique tRNA synthetases. A study of light- and dark-grown Euglena provides direct evidence for the presence of specific tRNAs and synthetases in chloroplasts. Reger et al. (1970) separated constitutive and light-inducible isoleucyl-tRNA synthetases from whole green cells by reversed-phase chromatography. Only one peak of synthetase activity, identical with the constitutive component, was obtained from dark-grown cells and from W_3BUL, an ultraviolet-bleached mutant lacking chloroplast DNA.

The second component, on the other hand, was present in isolated chloroplasts. Likewise, two different isoleucyl-tRNAs were also found in green cells, only one of which was present in isolated chloroplasts. The isoleucyl-tRNA associated with chloroplasts was acylated only by the chloroplast enzyme, which was identical to the inducible component present in the whole green cells. Multiple phenylalanyl-tRNAs and their synthetases were also found in Euglena with a specific phenylanyl-tRNA acylating enzyme located exclusively in the chloroplasts. In a study comparing light-grown wild-type and streptomycin-bleached cells of Euglena, Kislev et al. (1972) showed that mitochondria also contain a specific isoleucyl-tRNA distinguishable from chloroplast and cytoplasmic species by benzoylated DEAE-cellulose chromatography. Their results failed to demonstrate the presence of a mitochondrial-specific isoleucyl-tRNA synthetase, but do not exclude the possibility that specific mitochondrial aminoacyl-tRNA synthetases do exist in this alga.

Specific tRNAs and synthetases have also been demonstrated in chloroplasts of higher plants. In a study where bacterial and cytoplasmic contamination of preparations was carefully controlled, Burkard et al. (1970) showed that the valyl-tRNA synthetases from chloroplasts and cytoplasm of bean (Phaseolus vulgaris) exhibit different properties when conditions are altered by, for example, the addition of magnesium. Bean chloroplasts also contain three leucyl-tRNAs and one valyl-tRNA species which are charged only by the corresponding chloroplast enzymes. Specific leucyl-tRNAs and phenylalanyl-tRNAs have also been demonstrated in chloroplasts of tobacco

(Nicotiana tabacum) (Guderian et al., 1972) and barley (Hordeum vulgare) (Hiatt and Snyder, 1973), respectively, which are acylated exclusively by chloroplast-specific synthetases. Distinct species of leucyl-tRNA were also found in mitochondria purified from tobacco extracts, but mitochondrial synthetases did not appear to be specific, since they acylated both mitochondrial and cytoplasmic leucyl-tRNAs. This result, however, does not rule out the possibility of unique mitochondrial synthetases.

The presence of a specific methylating activity associated with both chloroplasts and mitochondria of bean (Phaseolus vulgaris) was shown recently by Dubois et al. (1974). Pure yeast aspartyl-tRNA was employed as a substrate for in vitro methylation by enzyme preparations from the isolated organelles and cytoplasm. The regions of the molecule methylated by the three different fractions were determined by nucleotide sequence analysis involving enzymatic digestion followed by electrophoresis or DEAE-cellulose chromatography. The results indicated that one adenylic residue was methylated by enzymes from all three subcellular compartments, but that another adenylic residue was methylated only by the chloroplast and mitochondrial enzymes. These organelles, therefore, contain a methylase which is absent from bean cytoplasm.

Different forms of triose phosphate isomerase have been described in chloroplasts and cytoplasm from pea. Anderson (1971) resolved two isomerases from pea chloroplast and cytoplasmic extracts by isoelectric focussing, and showed that the isoenzymes differ

significantly in their pH optima, Michaelis and inhibitor constants. The existence of such differences between organelle and cytoplasmic components and also those discussed above reflects the independent nature of chloroplasts and mitochondria.

b. Genetic studies

i. Variegated plants

One line of evidence for the genetic autonomy of plastids is derived from studies carried out in the early 1900's on the phenomenon of variegation in higher plants. The major results of investigations, such as those of Rhoades with iojap corn and Renner with Oenothera are reviewed by Sager (1972) in the context of current knowledge regarding the role of plastid DNA in plant genetics. For example, Renner analyzed the results of reciprocal crosses between O. hookeri and O. lamarkiana, which produced green, yellow and variegated progeny, and concluded that plastid phenotype is determined by the interaction of distinct plastid and nuclear genotypes. Neither Renner, nor his contemporaries, however, established the site of the plastid genetic determinant as being in the chloroplasts, themselves. Nonetheless, the extensive body of literature generated by these early studies provides a wealth of descriptive data implicating discrete cytoplasmic genes involved in chloroplast biogenesis.

ii. Division of chloroplasts

Another line of evidence establishing the genetic continuity of organelles is based on the observation of chloroplasts dividing in

vivo. In the late 1800's, the cytologists Schmitz, Meyer and Schimper described chloroplast division in algae and higher plants (in Diers, 1970). More recently, Green (1964) recorded the division of chloroplasts in Nitella by time-lapse cinematography. It is now generally accepted that all plastids are derived from pre-existing plastids.

2. Evolutionary origin of organelles

a. The endosymbiont hypothesis

Aside from the presence of a defined nucleus, eukaryotic cells differ from prokaryotic cells by containing one or more membrane-bound organelles, such as chloroplasts and mitochondria. The acquisition of organelles by eukaryote ancestors during the latter Proterozoic era probably contributed to their rapid development which culminated in the last 600 million years of metazoan evolution (Siever, 1975). One explanation of the evolutionary origin of these organelles is provided by the endosymbiont hypothesis. First formulated by Altmann in 1890 for mitochondria and Mereschkowsky in 1905 for chloroplasts (in Uzzel and Spolsky, 1974), this hypothesis proposes that organelles are modern descendants of prokaryotic cells which were incorporated and maintained by a protoeukaryotic organism. The manner in which the endosymbiotic event might have occurred can be reconstructed from data concerning the earth's history. After condensing from the primitive solar nebula, the earth had a reducing atmosphere and therefore the first forms of life were probably anaerobic heterotrophs

(Siever, 1975). Stromatolite fossil formations indicate that oxygen-producing blue-green algae were present 2.5 billion years ago (in Raven, 1970). The subsequent accumulation of atmospheric oxygen during the Proterozoic era would have allowed the appearance of primitive aerobes. It is possible that an anaerobic protoeukaryotic cell eventually evolved which lacked a rigid cell wall and was able to phagocytize. The eukaryotic ancestor ingested bacteria-like organisms but, perhaps in some cases, the prokaryote was not digested and survived in the host cytoplasm. A symbiotic association could have developed whereby the respiring or photosynthesizing prokaryote provided its anaerobic host with an efficient energy source in exchange for a constant food supply. Of little benefit in an intracellular environment, the symbiont cell wall could have been lost. Since it would ensure the coördination of endosymbiont and host cell functions, control of the prokaryote's development and replication by nuclear genes may have been advantageous. Translocation of the symbiont's genes may have been facilitated by an episome-like mechanism and would result in a reduction in the size of the prokaryote genome. Thus, the endosymbiont could have become so integrated into the host cell biochemistry that it finally assumed the status of an organelle. This sequence of events may have occurred, not just once, but on several separate occasions, as proposed by Raven (1970) regarding the evolution of chloroplasts and mitochondria.

The major weaknesses of the endosymbiont hypothesis are that it

fails to explain the evolution of the nucleus itself, and does not account for the inefficient energy metabolism of the putative protoeukaryote. An alternative explanation for the origin of organelles is that they arose by the elaboration of the plasma membrane with which DNA became associated. The most salient feature of this hypothesis is that eukaryotic cells evolved directly from prokaryotes. Several versions of this 'partition' hypothesis have recently appeared in the literature (Raff and Mahler, 1972; Uzzel and Spolsky, 1974; Bogorad, 1975; Cavelier-Smith, 1975). All suffer, however, from a serious inadequacy; namely, the failure to explain the existence of at least two distinct transcription and translation systems in the eukaryotic cell.

The evidence concerning the hypothetical endosymbiotic origin of organelles has been reviewed by Sagan (1967), Goodenough and Levine (1970a), Taylor (1970), Margulis (1971), Flavell (1972), Lee (1972), and others. Evidence consistent with this hypothesis includes that discussed above indicating the biochemical and genetic autonomy of chloroplasts and mitochondria. Evidence which favours the endosymbiont hypothesis includes examples of modern endosymbiosis and the biochemical homology between contemporary prokaryotes and organelles.

i. Contemporary endosymbiotic relationships

The number and diversity of contemporary endosymbiotic associations suggests that such relationships are easily established.

Eukaryotic algae have formed symbiotic associations with members of more than one hundred and fifty genera of invertebrates, representing eight phyla (in Raven, 1970). Prokaryotic blue-green algae, too, have entered symbiotic relationships with diverse organisms, such as amoebae, flagellated protozoa, green algae, diatoms and fungi (in Raven, 1970). The blue-green algal endosymbiont, or cyanelle, of the cryptomonad Cyanophora paradoxa, has apparently undergone some modification. Electron microscopy shows that the cell wall of the cyanelle is entirely absent and the organism is limited by a single membrane only (Hall and Claus, 1963). Thus, in the context of the endosymbiont hypothesis, the cyanelles of C. paradoxa may represent an intermediate stage in chloroplast evolution.

Endosymbiosis also occurs between nitrogen-fixing bacteria and leguminous plants. This association has been established in vitro, first between Rhizobium and cultured soybean root cells (Holsten et al., 1971), and then with Rhizobium and pea leaf protoplasts (Davey and Cocking, 1972).

The incorporation of organelles into alien host cells has been reported in vitro and also in nature. For example, chloroplasts from the alga Codium fragile are sequestered intracellularly in the hepatopancreas of the marine sacoglossan opisthobranch Elysia viridis (Trench et al., 1973). The precise mechanism by which the algal chloroplasts enter the animal cells is unknown, but neither the ultrastructure nor the photosynthetic capacity of the organelles is

significantly damaged. In the laboratory, both the intraspecific (Potrykus, 1973) and interspecific (Bonnett and Eriksson, 1974) transplantation of chloroplasts into higher plant protoplasts was recently achieved. Also Nass (1969) showed that chloroplasts isolated from spinach and African violets (Saintpaulias) and mitochondria from chicken liver were rapidly taken up by cultured L cells. Although these organelles did not divide, their integrity was maintained even after 5 days residence in L cell cytoplasm.

These examples of natural or artificial endosymbiosis involving contemporary bacteria, blue-green algae, chloroplasts and mitochondria illustrate the flexibility of this biological arrangement. With respect to the endosymbiont hypothesis, these examples of modern endosymbiosis also indicate that such phenomena could also account for the evolution of organelles.

ii. Similarities between prokaryotes and organelles

Current evidence indicates that the biochemistry of organelles is similar to that of free-living prokaryotes. For example, organelle DNA, like bacterial DNA, is not associated with histones, and closely resembles the bacterial nucleoid when viewed in thin section by electron microscopy. In their ultrastructural study of Chlamydomonas, Ris and Plaut (1962) remarked on the similarity of the DNA-containing regions of the chloroplast and the nucleoplasm of blue-green algae. Another property common to both prokaryote and organelle DNA is circularity. Covalently-closed circular DNA molecules have been isolated from both

mitochondria (Borst, 1970) and plastids (e.g. Manning et al., 1971, 1972).

Organelle and bacterial ribosomes are similar in many respects, including their biophysical properties. Analytical centrifugation analysis indicates that the sedimentation coefficient of bacterial ribosomes is approximately 70S, whereas the value for ribosomes from the cytoplasm of eurkaryotic organisms is close to 80S (Taylor and Storck, 1964). Noll and his collaborators showed, by velocity sedimentation in sucrose gradients using E. coli ribosomes as markers, that chloroplast ribosomes from bean and mitochondrial ribosomes from Neurospora belong to the 70S class (Noll, 1970). Measurement of mitochondrial and chloroplast ribosomes in electron micrographs of Ochromonas, also shows that organelle ribosomes are smaller than their cytoplasmic counterparts (Gibbs, 1968). Although chloroplast ribosomes from all species of algae and higher plants analyzed so far are a constant 68S-70S in size, mitochondrial ribosomes from various sources exhibit considerable size differences (Sager, 1972). Mitochondrial ribosomes from eukaryotic microörganisms (Stewart, 1973) and higher plants (Leaver and Harmey, 1973) are larger than those from E. coli, whereas animal mitochondrial ribosomes are relatively small, 55-60S, and thus are often called 'miniribosomes' (Borst and Grivell, 1971). Despite the variations in size, mitochondrial ribosomes resemble those from chloroplasts and bacteria in their requirement for relatively high concentrations of magnesium to remain intact. Like E. coli 70S

ribosomes (Spirin and Gavrilova, 1969), organelle ribosomes dissociate into their subunits at higher magnesium concentrations than do most eukaryotic 80S ribosomes (Boardman et al., 1966; Küntzel, 1969a).

The RNA components from both organelle and bacterial ribosomes have been studied extensively and possess many common features. The two high molecular weight RNAs from chloroplast ribosomes are similar in size to 23S and 16S bacterial rRNAs, and are therefore smaller than the corresponding RNAs of eukaryotic ribosomes, which are 25-28S and 18S (Whitfield, 1973). Mitochondrial ribosomes from yeast also contain 23S and 16S type high molecular weight RNAs; the rRNAs of animal miniribosomes, however, are somewhat smaller, 16-19S and 12-13S (Stewart, 1973). Like bacterial ribosomes, chloroplast (Payne and Dyer, 1971) and mitochondrial ribosomes (Leaver and Harmey, 1973) also contain 5S rRNA. The absence of another low molecular weight ribosomal component, 5.8S rRNA, which is found in 80S ribosomes, further distinguishes bacterial and organelle ribosomes from the eukaryotic type (Payne and Dyer, 1972; Leaver and Harmey, 1973).

Rijven and Zwar (1973) compared the methylation patterns of chloroplast and cytoplasmic high molecular weight rRNAs from (^{3}H)methyl-methionine-labelled fenugreek (Trigonella foenumgraecum) and found a significant difference. Taking advantage of the fact that methylation of the ribose moeity confers stability on the adjacent phosphodiester bond, they determined the ratio of ribose methylation to base methylation

by measuring the relative amounts of mono- and dinucleotides recovered after alkaline hydrolysis of the RNA preparations. The results indicated that 70% of the methyl label in rRNA from cytoplasmic ribosomes was in ribose, whereas the value for rRNA from isolated chloroplasts was only 23%. Citing the data of Fellner (1969), the authors point out that the low level of ribose methylation in chloroplast rRNA is approximately the same as in E. coli rRNA. It is unlikely that contamination of the isolated chloroplasts with bacteria could account for this similarity in methylation pattern, since the plant material was grown and handled under sterile conditions.

Several studies indicate a close relationship between the base sequences of rRNA from chloroplasts and those from blue-green algae. Pigott and Carr (1972) compared the ability of rRNA from seven species of blue-green algae, as well as seven species of bacteria, to hybridize with chloroplast DNA from Euglena. In contrast to cytoplasmic rRNA from Euglena, nearly all the prokaryotic rRNAs bound significant amounts of chloroplast DNA, and rRNA from one species of blue-green algae exhibited 47% sequence homology with Euglena chloroplast rRNA. The binding of heterologous rRNAs to Euglena chloroplast DNA was specific since chloroplast rRNA blocked the binding sites of the other rRNAs in competitive hybridization experiments. Thus, considerable genuine sequence homology appears to exist between the rRNAs from chloroplasts and blue-green algae. This conclusion received further support recently in two papers describing the results of 'fingerprinting' rRNAs

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from chloroplasts and prokaryotes. The technique of fingerprint analysis involves digestion of rRNA molecules into oligomers by T1 ribonuclease followed by two-dimensional electrophoresis. Zablen et al. (1975) examined the 'fingerprint' of 16S rRNA from Euglena chloroplasts and found six out of thirty-one oligomers common to rRNA from Anacystis nidulans, a blue-green alga. Furthermore, from a sequence conservation map of E. coli 16S rRNA showing the frequency of E. coli rRNA oligomers present in the 16S rRNAs from twenty-six other organisms, it can be seen that most of the sequences common to both chloroplast and E. coli rRNAs are conserved in the other prokaryotic rRNAs as well. Three-fourths of the pentamers obtained from Euglena chloroplasts rRNA are also present in chloroplast rRNA from Porphyridium, a red alga, suggesting that the chloroplasts of the two species are closely related. The 'fingerprint' of chloroplast 16S rRNA from Porphyridium was provided by Bonen and Doolittle (1975) who further compared it with the oligomer catalogs known for 16S rRNAs from E. coli, B. subtilis, and A. nidulans. Statistical analysis showed that the number of sequences common to each pairing of chloroplast and prokaryotic rRNAs was significantly greater than that expected for unrelated molecules. On the other hand, relatively few oligomers from either Euglena or Porphyridium chloroplast 16S rRNAs coincided with those from 18S rRNAs of several eukaryotic organisms.

The synthesis of chloroplast rRNA in Chlamydomonas has been shown to be regulated by a mechanism similar to that in bacteria.

Synthesis of RNA in bacteria is normally stringent, since it requires the availability of all amino acids; removal of any one amino acid precipitates an immediate decline in the accumulation of RNA (Edlin and Broda, 1968). Surzycki and Hastings (1968) identified the chloroplast RNA component in profiles of Chlamydomonas RNA fractionated on MAK columns by comparing RNA from wild-type cells with that from isolated chloroplasts and mutant cells lacking normal amounts of chloroplasts ribosomes. By labelling synchronous cultures at various times during the cell cycle, these investigators found that chloroplast RNA is synthesized only during the photosynthetic phase. Since the amino acid pool might be expected to increase under conditions of active photosynthesis, this finding suggests chloroplast RNA synthesis is subject to stringent control. As further evidence for this conclusion, Surzycki and Hastings also point out that the data of Jones et al. (1968) show that chloroplast RNA is not synthesized in arginine-requiring cells of Chlamydomonas during arginine starvation, nor in wild-type cells during gametogenesis, which is triggered by nitrogen starvation. These studies constitute the only information to date concerning the mode of regulation of organelle RNA synthesis. More definitive evidence could be obtained with improved RNA extraction procedures and gel electrophoresis.

The protein component of organelle ribosomes also resembles that of prokaryotic ribosomes more than that of eukaryotic ribosomes. Using two-dimensional gel electrophoresis, Hanson et al. (1974) analyzed

the ribosomal proteins from both the cytoplasmic and chloroplast ribosomes of Chlamydomonas. They found that the total number of different chloroplast ribosomal proteins is considerably less than that comprising eukaryotic ribosomes, and comparable to that present in ribosomes from Neurospora mitochondria and E. coli. Furthermore, the large subunit of Chlamydomonas chloroplast ribosomes contains two acidic proteins which cannot be distinguished electrophoretically from two proteins from the large subunit of E. coli ribosomes. The similarity between chloroplast and prokaryotic ribosomes is limited, however; comparison of ribosomes from pea chloroplasts and Chromatium vinosum indicates that chloroplast ribosomes have a higher protein content than those from photosynthetic bacteria, and that probably all the ribosomal proteins from the two sources are dissimilar (Oparin et al., 1975).

It is likely that the structural homologies between organelle and bacterial ribosomes underlie functional similarities as well. A number of antibiotics, such as chloramphenicol, which inhibit protein synthesis in bacteria, also inhibit protein synthesis in chloroplasts and mitochondria (Eisenstadt and Brawerman, 1964; Lamb et al., 1968). These same antibiotics do not, however, interfere with the synthesis of proteins on eukaryotic 80S ribosomes. The mechanism by which many antibiotics effect their primary action involves their binding to a specific ribosomal protein and subsequent blocking of one of the steps in peptide chain formation. For example, spectinomycin has been shown by reconstitution experiments to bind to protein S5 in the small subunit

of the E. coli ribosome (Bollen et al., 1969), and to interfere with translocation (Pestka, 1971). Radioactive dihydrospectinomycin has also been shown to bind the small subunit of chloroplast ribosomes from Chlamydomonas (Burton, 1972), although the protein responsible has not been directly identified. This indicates the presence of similar sites in bacterial and chloroplast ribosomes which bind spectinomycin. Thus, the similar response of bacterial and organelle ribosomes to certain antibiotics is probably not merely fortuitous, but reflects fundamental homologies in their structure and function.

Direct evidence that the process of protein synthesis is similar in prokaryotes and organelles was provided by Smith and Marcker (1968) who demonstrated that fMet-tRNA, the initiator in bacterial systems, is also present in mitochondria isolated from yeast and rat liver. Burkard et al. (1969) showed that fMet-tRNA is also found in chloroplasts from French bean (Phaseolus vulgaris). In neither case were significant amounts of fMet-tRNA detected in the cytoplasm. Since contaminating bacteria could influence the results of these studies, the sterility of the organelle preparations was monitored by plate counts or electron microscopy. Using an in vitro assay for the synthesis of fMet-puromycin, Sala et al. (1970) showed that ribosomes extracted from Euglena chloroplasts and stripped of initiation factors by high salt treatment, also utilize E. coli initiation factors. With respect to several criteria, such as GTP dependence, the reaction catalyzed by the heterologous system containing chloroplast ribosomes resembled that

catalyzed by the homologous system using E. coli ribosomes. This confirmed that the bacterial initiation factors cooperated with chloroplast ribosomes in genuine peptide bond formation.

Hybrid ribosomes, active in protein synthesis, have been constructed from subunits prepared from organelle and bacterial ribosomes. Lee and Evans (1971) found that when crude chloroplast ribosomes from Euglena were incubated with ^{14}C-labelled E. coli 50S subunits, the radioactivity appearing on sucrose gradients was associated with 70S particles, indicating that E. coli 50S subunits and chloroplast 30S subunits had formed hybrid ribosomes. Under the conditions used in this study, the phenylalanine incorporating activity of the combined Euglena chloroplast 30S and E. coli 50S particles in an in vitro system containing polyuridylic acid exceeded that of intact chloroplast ribosomes. Similar results were obtained in other experiments when the source of chloroplast ribosomal subunits was spinach (Grivell and Walg, 1972). In this case, active hybrid ribosomes were formed from the chloroplast 50S subunit and E. coli 30S subunit. Subunits derived from yeast mitochondrial ribosomes, however, failed to associate with either E. coli or chloroplast ribosomal subunits. These negative results indicate differences exist between mitochondrial and bacterial ribosomes or that technical problems interfere with their hybridization. On the other hand, the observation that chloroplast ribosomal subunits from two different species are interchangeable with those from E. coli indicates homology between at least some of the sites involved in the

association of the large and small subunits and in polypeptide formation.

As discussed above, chloroplasts and mitochondria contain species of tRNA which can be separated from their cytoplasmic counterparts by ion-exchange or reversed-phase chromatography (Barnett and Brown, 1967; Reger et al., 1970). Another property which distinguishes organelle from cytoplasmic tRNAs is the absence of fluorescent bases. Fairfield and Barnett (1971) analyzed the fluorescence emission spectra of phenylalanyl-tRNAs prepared from Euglena, Neurospora, and yeast and showed that, in contrast to the cytoplasmic tRNA species, neither chloroplast nor mitochondrial phenylalanyl-tRNA contains fluorescent bases. The fluorescence emission spectra of the organelle tRNAs does, however, match that of tRNA from E. coli. Thus, the structure of organelle tRNA provides another example of parallels between the biochemistry of organelles and prokaryotes.

B. Sites of Coding and Synthesis of Organelle Proteins

1. Chloroplast

Chloroplast protein is distributed about equally between a water-insoluble phase, which includes membrane polypeptides, and a soluble phase, which contains most chloroplast enzymes. Although the complete biochemical composition of the chloroplast is not known, the number of different proteins composing the organelle can be estimated from available information. Membrane polypeptides from the chloroplast envelope (Joy and Ellis, 1975) and thylakoids (Chua and Bennoun, 1975) have been

characterized by gel electrophoresis and probably total more than fifty. A partial list of enzymes such as those of the Calvin cycle and the photosynthetic electron transfer system indicates these number at least fifty (Smillie and Scott, 1969). An additional fifty enzymes are required for the synthesis of amino acids (Shephard and Levin, 1972). Finally, the protein synthetic machinery of the chloroplast probably contains close to one hundred proteins itself, including at least twenty amino acyl-tRNA synthetases (Reger et al., 1970) and about fifty ribosomal proteins (Hanson et al., 1974). Thus, the chloroplast probably contains a minimum of two hundred to three hundred different proteins.

The number of proteins for which chloroplast DNA can potentially code can be estimated from measurements of its length. Circular chloroplast DNA molecules, 40-45 μm in circumference, have been isolated from both algae (Manning et al., 1971; Manning and Richards, 1972) and higher plants (Manning et al., 1972; Kolodner and Tewari, 1972a; Herrmann et al., 1975). Molecules of this size could code for approximately one hundred different proteins with an average molecular weight of 40,000 daltons (see Appendix). As discussed below, however, very few chloroplast genes have been identified.

a. Sites of synthesis and coding of chloroplast membrane and soluble proteins

Evidence from studies of a variety of algal and higher plant species indicates that genes coding for certain chloroplast membrane components are located in nuclear DNA, whereas others reside in

chloroplast DNA. Nuclear transplantation experiments with two species of Acetabularia showed that the three species-specific peaks in the electrophoretic pattern of a chloroplast membrane fraction from the host species are determined by the donor nucleus (Apel and Schweiger, 1972). The photosystem II chlorophyll-protein complex also appears to be dependent on nuclear genes. Kung et al. (1972) determined the mode of inheritance of this component by analyzing tryptic digests of the protein from hybrids produced by reciprocal crosses between different species of Nicotiana. A Mendelian mode of inheritance generally indicates that the gene coding for a certain protein is transmitted to progeny by both the maternal and paternal parents equally. In non-Mendelian inheritance, a particular gene is transmitted by the maternal parent only. Non-Mendelian inheritance suggests that the gene may reside in chloroplast DNA, since presumably this component is donated exclusively by the egg cell or its equivalent in plant species which reproduce sexually. The mode of inheritance displayed by the photosystem II protein in Nicotiana is Mendelian, however, indicating the participation of nuclear genes. In Chlamydomonas, Chua and Bennoun (1975) demonstrated that a thylakoid polypeptide resolved by SDS-gel electrophoresis is correlated with photosystem II activity. Mutants of Chlamydomonas, which are deficient in this enzyme activity, also lack or contain reduced amounts of this polypeptide. Since the mutants exhibit Mendelian inheritance, the gene coding for the photosystem II polypeptide probably resides in the nucleus.

Evidence suggests that components of the photosystem I complex

are coded by chloroplast DNA. Herrmann and Bauer-Stäb (1969) studied a non-Mendelian mutant of Antirrhinium majus deficient in photosystem I activity. Comparing membrane preparations of chloroplasts isolated from wild-type plants and the mutant albomaculata-1 by gel electrophoresis, these authors found that 2 membrane components are absent in the non-Mendelian mutant. In a detailed study of a similar variegated non-Mendelian mutant of Nicotiana tabacum, Wong-Staal and Wildman (1973) demonstrated that the DNAs purified from chloroplasts isolated from green and white tissue differ slightly in their guanosine-cytosine content. Moreover, electron microscopy of hybrids of DNA from normal and defective chloroplasts revealed the presence of a non-complementary region about 0.15 μm long in some of the heteroduplexes. Thus, the defective condition of the mutant chloroplasts could be due to a deletion of 500-1000 base pairs from chloroplast DNA. The authors suggest that the protein affected by the mutation is associated with photosystem I. Similarly, photosystem I proteins are absent in the electrophoretic profiles of lamellar proteins from two non-Mendelian mutants of Hordeum (Börner et al., 1975). Not all polypeptides comprising the pigment-protein complex may be coded by chloroplast DNA, however, since photosystem I activity is also absent in two nuclear gene mutants of tomato (Börner et al., 1975).

The synthesis of some major membrane polypeptides apparently occurs on cytoplasmic ribosomes. Hoober (1970) studied the effects of chloramphenicol and cycloheximide on the synthesis of chloroplast thylakoid proteins prepared from Chlamydomonas and separated by gel

electrophoresis. The data indicated that over half of the proteins comprising the algal chloroplast membranes are synthesized on the cycloheximide-sensitive ribosomes in the cytoplasm. Two components synthesized in the cytoplasm together represent about 36% of the total protein mass of the chloroplast membranes. Hoober (1972) also found that one of these major membrane components, polypeptide c, is synthesized as a soluble product, since it accumulates in the soluble phase of cells treated with chloramphenicol. Ellis and his associates (Eaglesham and Ellis, 1974) have characterized the membrane polypeptides from pea chloroplasts by SDS-gel electrophoresis. When the distribution of labelled methionine incorporated into membrane protein by isolated chloroplasts was analyzed, they found that only five out of twenty-one polypeptides are apparently synthesized within the organelle. In a more recent study, the synthesis of only two out of twenty-five envelope polypeptides was detected in isolated pea chloroplasts (Joy and Ellis, 1975). Therefore, most thylakoid membrane proteins are probably synthesized in the cytoplasm.

Ultrastructural studies of algae and higher plants show that a membrane-organizing factor is synthesized on chloroplast ribosomes. Inhibition of protein synthesis on 70S ribosomes by chloramphenicol produces a syndrome of similar chloroplast abnormalities in Chlamydomonas (Goodenough, 1971), Ochromonas (Smith-Johannsen and Gibbs, 1972), Euglena (Bishop et al., 1973), Phaseolus (Margulies, 1966), and Pisum (Srivastava et al., 1971); characteristic membrane organization is

disrupted and thylakoids are often arranged in abnormally large stacks. Thus, one or more proteins involved in the control of thylakoid fusion is probably synthesized by chloroplast ribosomes in these organisms.

Analysis of the cytochromes present in chloroplast ribosome-deficient mutants indicates that the synthesis of these membrane-bound proteins occurs in the chloroplast (Levine and Armstrong, 1972). When cultured under mixotrophic conditions, the Chlamydomonas mutant, ac-20, contains few chloroplast ribosomes and is deficient in cytochromes 553 and 563. Moreover, a partial recovery in the number of chloroplast ribosomes following transfer of the mutant cells to phototrophic conditions is accompanied by a 20-30% increase in cytochrome content. The results of several studies using chloramphenicol to disrupt protein synthesis on 70S ribosomes suggest that cytochromes are also synthesized in the chloroplast of Euglena (Smillie et al., 1967), Chlamydomonas (Armstrong et al., 1971) and bean (Gregory and Bradbeer, 1973). Furthermore, these proteins may be coded by chloroplast DNA, since rifampicin, a specific inhibitor of chloroplast DNA-dependent RNA polymerase (Surzycki, 1969), blocks normal increases of cytochromes 553 and 563 in synchronous cultures of Chlamydomonas (Armstrong et al., 1971).

Ferredoxin, another membrane-bound protein, is apparently synthesized on cytoplasmic ribosomes and coded by a nuclear gene. The amount of this iron-sulfur protein is not reduced in Chlamydomonas (Armstrong et al., 1971) or in bean (Haslett et al., 1973) treated with chloramphenicol. Synthesis of ferredoxin in Chlamydomonas is, however,

sensitive to cycloheximide (Armstrong et al., 1971). Interspecific crosses of Nicotiana were used to demonstrate that the structural gene for ferredoxin is located in nuclear DNA (Kwanyuen and Wildman, 1975). The hybrid produced by crossing N. glutinosa ♀ and N. glauca ♂ contained two isozymes of ferredoxin which were resolved by electrofocussing. Amino acid analysis indicated that, whereas ferredoxin from N. glauca contains methionine, ferredoxin from N. glutinosa does not. Since the methionine content of ferredoxin from the hybrid was half that of the N. glauca protein, information for one of the two forms of ferredoxin in the hybrid was transmitted via nuclear genes in the pollen from N. glauca.

One of the Calvin cycle enzymes, ribulosediphosphate (RuDP) carboxylase, alone constitutes 25-50% of the soluble phase of the chloroplast. The enzyme is composed of two types of subunits, one considerably larger than the other (in Givan and Criddle, 1972). A number of early studies suggested that the development of RuDP carboxylase activity is dependent on chloroplast ribosomes (Smillie et al., 1967; Surzycki et al., 1970; Ireland and Bradbeer, 1971), but other studies showed that cytoplasmic ribosomes are also involved (Armstrong et al., 1971). Subsequent investigations have resolved these apparently conflicting results and allow several conclusions regarding the biogenesis of this abundant chloroplast protein.

In vitro amino acid incorporation studies indicate that the large subunit of RuDP carboxylase is synthesized within the chloroplast,

whereas the small subunit is synthesized on cytoplasmic ribosomes. Ellis and his associates (Blair and Ellis, 1973; Siddell and Ellis, 1975) demonstrated that isolated intact pea chloroplasts, using light or ATP as the sole energy source, incorporate labelled amino acids into a polypeptide whose tryptic peptide 'map' corresponds to that of the large subunit of RuDP carboxylase. Other experiments conducted in Ellis' laboratory showed that the large enzyme subunit is also synthesized by E. coli ribosomes programmed with RNA from spinach chloroplasts (Hartley et al., 1975). The large subunit of RuDP carboxylase was identified as a product of the heterologous translation system by comparing the chymotryptic digests of polypeptides labelled in isolated chloroplasts with the large enzyme subunit labelled in vivo. The fidelity of translation by the E. coli ribosomes was confirmed in two ways. First, in the presence of MS2 viral RNA, the bacterial ribosomes synthesized a polypeptide with the same electrophoretic behaviour as MS2 coat protein. Also, the electrophoretic patterns of discrete products translated from spinach chloroplast mRNA in the E. coli extract and in isolated spinach chloroplasts were similar. The small subunit of RuDP carboxylase has been synthesized in an in vitro system using cytoplasmic polysomes from bean (Gray and Kekwick, 1973). The purity of the cytoplasmic polysome preparation was checked by ultracentrifugation with marker ribosomes from rat liver and E. coli, and by electrophoresis of extracted RNAs. No significant contamination by chloroplast ribosomes was detected. Thirty percent of the protein

synthesized *in vitro* was the small RuDP carboxylase subunit as identified by immunoprecipitation. The antibody reaction was probably specific since, in control experiments, anti-γ-globulin antibody precipitated only 1% of the products of *in vitro* protein synthesis. The investigators did not test the products of their *in vitro* system with antisera to RuDP carboxylase large subunit. The results of these studies by Ellis' group and Gray and Kekwick permit the conclusion that the large and small subunits of RuDP carboxylase are synthesized on chloroplast and cytoplasmic ribosomes, respectively.

Evidence from studies using interspecific hybrids of *Nicotiana* shows that both chloroplast and nuclear genes code for RuDP carboxylase. Analysis of peptides derived from the large subunit of the enzyme from plants produced by interspecific crosses indicated that the gene controlling the expression of the large enzyme subunit is transmitted only by the maternal parent (Chan and Wildman, 1972; Sakano *et al.*, 1974). The K_m values of RuDP carboxylase activity also exhibit maternal inheritance (Singh and Wildman, 1973). This mode of inheritance indicates that the gene governing the expression of the large subunit of RuDP carboxylase is located in the chloroplast. The gene coding for the small enzyme subunit, on the other hand, is transmitted by both parents in *Nicotiana*. Kawashima and Wildman (1972) analyzed the tryptic peptides of the smaller component of RuDP carboxylase from hybrids produced by reciprocal crosses of *Nicotiana* and concluded that this enzyme subunit is inherited in a Mendelian manner and thus coded by a nuclear gene. These

studies therefore indicate that in higher plants the biogenesis of RuDP carboxylase requires the coöperation of two spatially separated transcription/translation systems. A comparable analysis of the sites of coding and synthesis of this enzyme in algae is not yet available.

Like the small subunit of RuDP carboxylase, a number of chloroplast enzymes appear to be coded by nuclear DNA and synthesized in the cytoplasm. Except for RuDP carboxylase, the synthesis of Calvin cycle enzymes is not inhibited by lincomycin in pea (Ellis and Hartley, 1971) or by chloramphenicol in bean (Ireland and Bradbeer, 1971). Surzycki (1969) concluded that DNA polymerase is coded by nuclear DNA since rifampicin, which causes loss of chloroplast ribosomes in Chlamydomonas, does not reduce the amount of total DNA per cell. DNA polymerase also appears to be synthesized on cytoplasmic ribosomes in tobacco, since cycloheximide, but not chloramphenicol, inhibits chloroplast DNA synthesis (Drilica and Knight, 1971). Ellis and Hartley (1971) found that lincomycin does not prevent the development of RNA polymerase activity in pea indicating that this enzyme also is synthesized on cytoplasmic ribosomes. The results of studies using chloramphenicol or streptomycin and cycloheximide to distinguish between proteins originating in the chloroplast and cytoplasm, respectively, showed that in Euglena chloroplast amino acyl-tRNA synthetases are synthesized on cytoplasmic ribosomes (Parthier, 1973; Hecker et al., 1974). Furthermore, low levels of phenylalanyl- and valyl-tRNA synthetases were detected in

the ultraviolet-bleached Euglena mutant, W_3BUL, which lacks chloroplast DNA, indicating that the genes for these enzymes are most likely located in nuclear DNA (Hecker et al., 1974). At least some of the enzymes involved in pigment synthesis are also products of extra-chloroplast transcription and translation. Rifampicin does not prevent the accumulation of chlorophyll (Armstrong et al., 1971) or carotenoids (Sirevåg and Levine, 1973) during synchronous growth of Chlamydomonas suggesting that their synthesis does not require enzymes coded by chloroplast DNA. That nuclear genes do code for enzymes in the chlorophyll biosynthetic pathway was indicated in a study of two Chlamydomonas mutants, each exhibiting Mendelian inheritance of a different defect in chlorophyll synthesis (Wang et al., 1974). Finally, evidence that at least some of the enzymes required for pigment synthesis are not only coded by nuclear genes, but also are also synthesized on cytoplasmic ribosomes, is provided by the observation that cycloheximide blocks the synthesis of chlorophyll and carotenoids in synchronized cells of Chlamydomonas (Armstrong et al., 1971; Sirevåg and Levine, 1973).

In summary, although a few chloroplast components, such as membrane polypeptides, cytochromes 553 and 563, and the large subunit of RuDP carboxylase, are coded and synthesized in the chloroplast, many other proteins composing the organelle are products of nuclear genes and cytoplasmic protein synthesis. These proteins, including membrane polypeptides, ferredoxin, Calvin cycle enzymes, DNA and RNA polymerases, amino acyl-tRNA synthetases and enzymes involved in pigment synthesis,

must therefore be somehow imported into the chloroplast.

b. Sites of coding and synthesis of chloroplast ribosomal proteins.

1. Site of coding of chloroplast ribosomal proteins

Most of the evidence regarding whether chloroplast ribosomal genes are coded by plastid or nuclear DNA is based on studies of mutants of the green alga, Chlamydomonas reinhardi, which are resistant to or dependent on various antibiotics. In bacteria, resistance to certain antibiotics has been shown to be due to a change in the amino acid sequence of a ribosomal protein such that it no longer binds the drug in question. For example, in E. coli, resistance to spectinomycin is conferred on the ribosome by single amino acid replacements in S5, a protein in the small ribosomal unit (Funatsu et al., 1971). Since ribosomes from the organelles of eukaryotic organisms resemble bacterial ribosomes in many respects including their sensitivity to inhibitors, such as spectinomycin (as discussed above), it is likely that antibiotic-resistance or -dependence in Chlamydomonas may be caused by alterations in the ribosomal proteins of the chloroplast. The pattern of inheritance in these Chlamydomonas mutants allows one to determine the location of genes affecting their sensitivity to antibiotics; Mendelian inheritance indicates that nuclear genes are involved, and non-Mendelian (alternate terms are cytoplasmic, maternal, and uniparental) inheritance implicates the participation of chloroplast genes (Sager and Ramanis, 1971). Thus, mutants which are resistant to

inhibitors of bacterial protein synthesis and which exhibit non-Mendelian inheritance provide evidence for the coding of chloroplast ribosomal proteins by chloroplast DNA. Since Chlamydomonas contains mitochondria, as well as a chloroplast, the resistance to or dependence on antibiotics by non-Mendelian mutants may also be related to mitochondrial ribosomal proteins and/or mitochondrial DNA. Furthermore, not all antibiotic-resistant or -dependent mutants can be assumed to contain altered ribosomes. For example, some mutants may be capable of de-toxifying the drug as in some strains of bacteria (Beneviste and Davies, 1973). Also, the cell or chloroplast (mitochondrial) membrane may be altered so that it is less permeable to the antibiotic, or accessory factors required for organelle protein synthesis may be missing or non-functional. Even in cases where antibiotic-resistance or -dependence is traced to a ribosomal protein, the mutant gene product may actually be an enzyme which modifies the structural protein. Bearing these various possible levels of antibiotic-resistance and -dependence in mind, I will discuss the evidence regarding the location of genes coding for chloroplast ribosomal proteins.

Evidence for coding of chloroplast ribosomal proteins by chloroplast DNA in Chlamydomonas

As discussed above, the existence of a class of non-Mendelian mutants of Chlamydomonas which display resistance to or dependence on antibiotics suggests that chloroplast DNA may code for chloroplast

ribosomal proteins. According to the classification system proposed by Surzycki and Gillham (1971), Chlamydomonas mutants exhibiting altered sensitivity to antibiotics fall into three distinct classes, depending on whether the chloroplast, mitochondria, or both organelles are involved in phenotype expression. Eleven of the thirteen antibiotic-resistant mutants analyzed by these authors were of the type in which both the chloroplast and mitochondria are affected. Six of these eleven mutants exhibited non-Mendelian inheritance, indicating that organelle DNA was responsible for the phenotype. To determine whether chloroplast or mitochondrial DNA contained the mutation, Surzycki and Gillham treated the antibiotic-resistant or -dependent strain of cells with rifampicin in the presence of the appropriate antibiotic. Rifampicin causes the loss of chloroplast ribosomes, but does not alter mitochondrial ultrastructure, in Chlamydomonas (Goodenough, 1971). Rifampin, a related substance, inhibits the synthesis of RNA in chloroplasts isolated from Chlamydomonas, but does not prevent the heterotrophic growth of cells (Surzycki, 1969). Thus, rifampicin appears to selectively inhibit chloroplast DNA-dependent RNA polymerase, but not the mitochondrial enzyme. Surzycki and Gillham reasoned that if antibiotic-resistance or -dependence in a non-Mendelian mutant was determined by a chloroplast gene, then the mutant cells should lose their resistance or dependence after exposure to rifampicin. They found four mutants (sr-2-60, ery-2y, kan-1, sd-3-18) which became sensitive to antibiotic in the presence of rifampicin, indicating the

the location of the gene conferring antibiotic-resistance and -dependence on the cells in chloroplast DNA. Although this evidence does establish the existence of chloroplast genes involved in resistance to and dependence on antibiotics, it does not indicate whether or not the product of the mutant chloroplast gene is a chloroplast ribosomal protein (a question which was not considered by the authors). If the structural gene for a ribosomal protein is mutated such that the gene product no longer binds a particular antibiotic, then blocking the transcription of this gene would not render the ribosome sensitive to the same antibiotic. Two other non-Mendelian mutants studied by Surzycki and Gillham, spr-1-27 (+) and spr-1-27 (-), did not become sensitive in the presence of rifampicin, suggesting that antibiotic-resistance in these strains might be effected at the ribosome level. Although this observation still does not allow a conclusion regarding which organelle DNA is involved, a subsequent study of spr-1-27 cells (Boynton et al., 1973) showed that the mutation affected the chloroplast only. Thus, the behaviour of the spr-1-27 Chlamydomonas mutant suggests that chloroplast DNA codes for a chloroplast ribosomal protein. The use of rifampicin (Surzycki and Gillham, 1971) as a means of distinguishing between mitochondrial and chloroplast genes, however, can be criticized since the effects of this inhibitor may be related to dilution of chloroplast ribosomes. Furthermore, the light-sensitivity of rifampicin necessitates the use of heterotrophic growth conditions which probably influence the expression of mutant phenotype.

Examples of non-Mendelian mutants of Chlamydomonas with altered sensitivity to antibiotics have been described by other investigators. Behn and Arnold (1972) concluded from the genetic behaviour of neamine-sensitive revertants that the non-Mendelian mutation to neamine-dependence (nd) is localized in the chloroplast. Using a different approach, Lee and Jones (1973) exposed synchronized cultures of Chlamydomonas to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), at various times during the cell cycle. A potent mutagen, MNNG acts by inducing base changes in replicating DNA (Güerola et al., 1971). When mutagenesis coincided with the time of nuclear DNA replication, a 15-30-fold increase in the number of Mendelian streptomycin-resistant mutants (sr-1), but no increase in the number of non-Mendelian streptomycin-resistant mutants (sr-2) was observed. When mutagenesis occurred at the time of chloroplast DNA replication, a 1.5-1.6-fold increase in the number of the non-Mendelian sr-2 mutants was obtained; a 1.5-1.6-fold increase in the number of Mendelian sr-1 mutants was also observed, but was ascribed to a small degree of asynchrony in nuclear DNA synthesis. Thus, the data of Lee and Jones (1973) establish a correlation between a mutation in chloroplast DNA and resistance to streptomycin, and provide suggestive evidence that a chloroplast gene codes for a chloroplast ribosomal protein (which confers sensitivity to streptomycin on wild-type cells).

Some non-Mendelian mutants of Chlamydomonas have been described in which antibiotic-resistance is associated with altered chloroplast

ribosomes. Gillham et al. (1970) analyzed ribosomes from several uniparental mutants by sedimentation velocity in sucrose gradients and found that the 70S ribosomes are replaced by 66S particles in two streptomycin-resistant strains (spr-2-60, sr-2-281) and in two strains resistant to spectinomycin (spr-1-27, nr-2-1 spr-1-1). Furthermore, both streptomycin-resistance and altered ribosomal phenotype segregated together in reciprocal crosses of mutant and wild-type cells. The authors showed that the 66S particles are altered 70S ribosomes by extracting both 23S and 16S rRNAs from the former (electrophoretic profiles of the RNA were not presented in the paper, however). They also showed that the 66S particles originate from the chloroplast by constructing the double mutant ac-20 sr-6-60, which grows in the presence of streptomycin and contains a greatly reduced amount of altered ribosomes. A uniparental mutant dependent on streptomycin (sd-3-18) was also included in this study. Although the sedimentation behaviour of sd-3-18 70S ribosomes is similar to the wild-type, antibiotic-dependence was found to be associated with a reduction in the number of monosomes and accumulation of 54S ribosomal subunits. The 70S ribosomes described in this study can be assumed to be derived from the chloroplast, although mitochondria also contain ribosomes of similar size. Mitochondrial ribosomes comprise only a small fraction of the total ribosome population in Chlamydomonas (Goodenough and Levine, 1970b).

In some non-Mendelian mutants, investigators have demonstrated that antibiotic-resistance is caused by the failure of chloroplast

ribosomes to bind the antibiotic. Burton (1972) assayed the capacity of cytoplasmic and chloroplast ribosomes from wild-type cells to bind (^{3}H)dihydrospectinomycin *in vitro*, and found the antibiotic to interact specifically with the small subunit of chloroplast ribosomes. Chloroplast ribosomes isolated from the uniparental spectinomycin-resistant mutant, *sp2-73*, however, failed to bind the radioactive antibiotic. Therefore, by analogy with *E. coli* in which resistance to spectinomycin is determined by a single protein in the small ribosomal subunit (Funatsu *et al.*, 1971), *sp2-73* may contain a mutation in the chloroplast DNA coding for a protein in the small subunit of chloroplast ribosomes.[1] Burton also assayed the affinity for spectinomycin of chloroplast ribosomes from a Mendelian mutant (*spa-I*) resistant to spectinomycin and found a binding activity equivalent to that of chloroplast ribosomes from the wild-type sensitive strain. Thus, antibiotic-resistance in the Mendelian mutant *spa-I* does not occur at the ribosome level and must be attributed to an alteration in membrane permeability or some other cellular function.

An *in vitro* assay for antibiotic-binding activity was also employed by Boschetti and Bogdanov (1973a) to determine the site of streptomycin-resistance in non-Mendelian (sr_{35}) and Mendelian (sr_3) mutant strains of *Chlamydomonas*. In this study, the 30S subunits of

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[1] The strain *sp2-73* is considered identical to the *spr-1-27* mutant described above in the study of Surzycki and Gillham (1971), and both strains represent subclones of *spr-1-27* originally derived from wild-type cells by exposure to MNNG (Boynton *et al.*, 1973).

chloroplast ribosomes from both mutant stocks failed to bind (^{3}H)dihydrospectinomycin, although the small subunits of ribosomes from a sensitive strain (ss) exhibited considerable binding activity. When intact monosomes were tested, however, the 70S ribosomes from the Mendelian mutant sr_3 bound half as much antibiotic as did ribosomes from the control, whereas those from the non-Mendelian mutant sr_{35} did not bind a significant amount of antibiotic. This finding may help to explain the partial sensitivity of sr_3 cells to streptomycin *in vivo* reported by Boschetti and Bogdanov (1973b). When intact cells were exposed to streptomycin, the protein synthetic activity of chloroplast ribosomes in the Mendelian mutant sr_3 was 40-fold less than that observed in the non-Mendelian mutant sr_{35} as determined by measuring RuDP carboxylase activity. The investigators also found differences in some biophysical properties of the 70S ribosomes from the two mutants. Ultracentrifugation analysis indicated that the tendency of ribosomal subunits to form 70S monosomes and the monosomes to form 100s dimers was greater in the non-Mendelian mutant sr_{35} than in the Mendelian mutant sr_3, and greater in both mutants than the sensitive strain ss. In both resistant and sensitive cells grown in the presence of streptomycin, the proportions of monosomes and dimers in the ribosome population was higher. The magnitude of the relative increase in the incidence of particle association varied, however, such that this 'sticking effect' was minimal in the non-Mendelian strain sr_{35}, moderate in the Mendelian mutant sr_3 and maximal in the

sensitive strain ss. A similar biophysical behaviour has been described for the response of sensitive E. coli ribosomes to streptomycin, as well as a loss of the sticking effect accompanying resistance of ribosomes to the antibiotic (Herzog, 1964). Since streptomycin-resistance in E. coli is determined by a single amino acid substitution in the ribosomal protein S12 of the 30S subunit (Funatsu and Wittmann, 1972), a protein in the small ribosomal subunit of chloroplast ribosomes from each of the two streptomycin-resistant strains may also be the locus responsible for both antibiotic resistance and the 'stickiness' of the 70S ribosomes. Thus, in the case of the uniparental mutant sr_{35}, the mutant ribosomal protein may be coded by chloroplast DNA, whereas in the Mendelian mutant sr_3, a nuclear gene may code for another chloroplast ribosomal protein affecting the binding of streptomycin. Although a particular antibiotic probably binds to only one ribosomal protein, other proteins in the ribosome structure may influence the affinity of the binding site (Cannon and Cundliffe, 1973).

The characteristics of the streptomycin-resistant and sensitive strains were further examined by Boschetti et al. (1974) who tested the effects of streptomycin on growth of the mutants under hetero-, mixo-, and phototrophic conditions. Under heterotrophic conditions and on solid media, strain sr_{35} displays a resistant phenotype (green), but strain sr_3 forms only yellow colonies as do sensitive cells. In the light sr_3 cells are capable of greening. Measurement of RuDP carboxylase

activity in light-grown $\underline{sr}_3$ and $\underline{sr}_{35}$ cells cultured in the presence and absence of streptomycin indicated that protein synthesis in the Mendelian mutant, but not in the uniparental mutant, is inhibited at least 50%. Since the $\underline{sr}_3$ cells appear green, however, they are apparently able to synthesize enough enzymes for chlorophyll formation. Boschetti and Walz (1973) postulated that the differential response of $\underline{sr}_3$ cells to dark and light growth conditions might be caused by a mutation affecting mitochondrial ribosomes. This is an unlikely possibility because the antibiotic-resistance of the $\underline{sr}_3$ mutant has been shown to be correlated with altered biophysical properties of its 70S ribosomes, and mitochondrial ribosomes comprise too small a fraction of the 70S ribosome class to account for this observation. The possibility exists, however, that, although the 30S ribosomal subunit from $\underline{sr}_3$ cells does not bind streptomycin (Boschetti and Bogdanov, 1973a), association of the 30S with the 50S ribosomal subunit alters the conformation of a postulated streptomycin-protein such that intact 70S monosomes do bind sufficient amounts of streptomycin to inhibit chloroplast protein synthesis in dark-grown cells. Moreover, the apparent reduction in the sensitivity of chloroplast ribosomes to streptomycin in $\underline{sr}_3$ cells exposed to light may be explained if the intra-organellar concentration of the streptomycin is reduced by a corresponding change in membrane permeability. Such a change could occur in the plasma membrane or in the chloroplast envelope, which is known to alter in composition in response to illumination (Cobb and

Wellburn, 1974). Since the complete genotype of sr_3 cells is not known, the possibility that this strain also carries another Mendelian mutation closely linked to sr_3 coding for a protein involved in membrane permeability cannot be excluded.

Sager and her colleagues have demonstrated that resistance in a uniparental carbomycin-resistant mutant (Schlanger et al., 1972) and also in four other uniparental antibiotic-resistant strains (Schlanger and Sager, 1974) of Chlamydomonas occurs at the ribosome level. By means of a poly(U)-directed amino acid incorporating assay system in which the activity of the chloroplast ribosomes is favoured by the presence of 25 mM magnesium and 3.3 mM spermidine, these investigators assessed the protein synthesizing capacity of chloroplast ribosomes from streptomycin-, neamine-, spectinomycin-, cleocin- and carbomycin-resistant strains. In all cases, the ribosomes from the antibiotic-resistant mutants incorporate an equivalent amount of phenylalanine in the presence and absence of the corresponding antibiotic, whereas the activity of the ribosomes from wild-type cells is considerably diminished by the antibiotic. By testing the sensitivity of various combinations of subunits from sensitive and resistant ribosomes, Schlanger and Sager (1974) were able to assign the site of resistance to streptomycin, neamine and spectinomycin to the 30S ribosomal subunit, and the site of cleocin- and carbomycin-resistance to the 50S ribosomal subunit. These results implicate the involvement of altered chloroplast ribosomal proteins in the antibiotic-resistance of these strains by analogy with some

resistant forms of bacteria (Otaka et al., 1970). Since each of the mutants exhibits non-Mendelian inheritance, the data also provide indirect evidence for the coding of some chloroplast ribosomal proteins by chloroplast genes. On the other hand, the possibility that an alteration in chloroplast rRNA is responsible for the drug-resistance in one or more of these mutants is not excluded in this study. In a subsequent paper (Ohta et al., 1975), however, Sager's group reports that, in at least the streptomycin-resistant strain, the altered component of the 30S ribosomal subunit is a protein. The presence of an abnormal ribosomal protein was indicated in a double label experiment in which the 30S ribosomal proteins from the wild-type and a streptomycin-resistant strain were co-chromatographed in 6M urea on a CM-cellulose column. The altered chromatographic behaviour of the protein could be caused by an amino acid substitution, but the data does not rule out the possibility of post-translational modification such as methylation.

Likewise, secondary modification may be involved in the alteration of a protein of the large subunit of chloroplast ribosomes from a non-Mendelian erythromycin-resistant mutant (ery-U1a) (Mets and Bogorad, 1972). Detected by two-dimensional gel electrophoresis, the mutant protein appears to form anomolous aggregates, the precise nature of which is unclear. This was the first report of an altered chloroplast ribosomal protein controlled by a uniparentally-inherited gene.

The possibility that at least one protein is common to both

chloroplast and mitochondrial ribosomes and is coded by an extra-nuclear gene in Chlamydomonas is supported by the results of Boynton et al. (1973). These investigators found that, although the uniparental spectinomycin-resistant mutant spr-1-27 displays a resistant phenotype when grown heterotrophically in the presence of 90 µg/ml spectinomycin, its growth is completely inhibited by 60 µg/ml spectinomycin under phototrophic conditions. Thus, only when the medium is supplemented with acetate are the mutant cells able to escape spectinomycin inhibition. Even when cultured mixotrophically in the presence of spectinomycin, the spr-1-27 mutant grows only to heterotrophic cell densities. Electron microscopy of mutant cells grown mixotrophically for five generations in 90 µg/ml spectinomycin showed that chloroplast membrane organization is disrupted, but that mitochondrial ultrastructure is normal. This observation suggests that in spr-1-27 cells, chloroplast ribosomes, but not mitochondrial ribosomes, are sensitive to spectinomycin. Burton (1972) has shown, however, that chloroplast ribosomes isolated from sp2-73 (another subclone of the same spr-1-27-3 mutant from which spr-1-27 was derived) do not bind as much (^{3}H)dihydrospectinomycin as do chloroplast ribosomes from wild-type cells. This observation was also confirmed by Boynton et al. (1973), who demonstrated that the radioactive antibiotic is bound by chloroplast ribosomes from wild-type cells, but not at all by chloroplast ribosomes from either sp2-73 or spr-1-27 cells. Furthermore, when the ribosomal proteins from the small subunit of the chloroplast ribosomes from spr-1-27 and wild-type cells are compared by

one-dimensional gel electrophoresis, one band present in the profile of wild-type proteins is missing in the profile of spr-1-27 proteins. Boynton et al. account for the paradox between the in vivo and in vitro results by postulating that the product of the spr-1-27 gene is a protein common to both chloroplast and mitochondrial ribosomes, but which fails to bind spectinomycin only when incorporated into mitochondrial ribosomes. They suggest that the spr-1-27 protein is only loosely bound to the chloroplast ribosomes and is therefore lost during isolation of 70S ribosomes. An alternative interpretation of the data of Boynton et al. is that the spr-1-27 gene product retains its spectinomycin-binding property, but its conformation is altered such that it cannot be accommodated in the mitochondrial ribosomes, but it is still loosely bound by chloroplast ribosomes, rendering them sensitive to antibiotic in vivo. Finally, since this mutation is uniparentally-inherited and similar mutations to antibiotic-resistance are believed to be localized in the chloroplast, Boynton et al. also hypothesized that spr-1-27 cells contain a mutation in a chloroplast gene specifying a ribosomal protein in chloroplast ribosomes. The data also suggest that since the same mutation may affect mitochondria, the ribosomal protein coded by chloroplast DNA is also a component of mitochondrial ribosomes. The possibility that chloroplast and mitochondrial ribosomes have one or more proteins in common seems remote, however; the putative polypeptide or mRNA would have to diffuse across at least four membranes separating

the respective domaines of chloroplast and mitochondrial ribosomes. Biochemical analysis of ribosomes from purified chloroplasts and mitochondria is required to prove whether the two organelles share ribosomal proteins, but evidence indicates that all proteins from chloroplast ribosomes are distinct from those of cytoplasmic ribosomes (Hanson et al., 1974). Perhaps the spr-1-27 strain described by Boynton et al. is actually a double mutant in which a mutation in mitochondrial DNA leads to spectinomycin-resistance of mitochondrial ribosomes independently of the mutation in chloroplast DNA. Such a mutation would also exhibit a non-Mendelian pattern of inheritance.

Evidence for coding of chloroplast ribosomal proteins by nuclear DNA in Chlamydomonas

Although the results of studies by both Boynton's and Sager's groups on mutants of Chlamydomonas suggest that chloroplast genes code for a number of different ribosomal proteins in the organelle, the most conclusive evidence concerning the coding site of a chloroplast ribosomal protein indicates at least one nuclear gene is involved. Bogorad and his colleagues have characterized several Mendelian (ery-M) and non-Mendelian (ery-U) erythromycin-resistant mutants in Chlamydomonas. In 1971, Mets and Bogorad showed that (^{14}C)erythromycin binds specifically to the large subunit of chloroplast ribosomes from wild-type cells, but fails to bind to chloroplast ribosomes from mutant cells. When the ribosomal proteins from the purified large subunits of chloroplast

ribosomes isolated from wild-type and ery-M2d cells were analyzed by one-dimensional urea-gel electrophoresis, one protein present in the large ribosomal subunit from the ery-M2d strain migrated more slowly than the corresponding ribosomal protein from wild-type cells (Mets and Bogorad, 1972). The authors acknowledge the possibility that the substantial change in electrophoretic mobility of this protein could arise from a post-translational event such as methylation, but favour the alternative idea that ery-M2D is a structural gene in which mutation has altered the relative proportions of acidic and basic amino acids in the polypeptide product. Evidence which further supports the notion that the nucleus contains at least one structural gene for a chloroplast ribosomal protein was recently reported (Davidson et al., 1974). A comprehensive genetic and biochemical analysis of four Mendelian mutants resistant to erythromycin (ery-M1a, ery-M1b, ery-M1c, ery-M1d) indicates the existence of altered forms of chloroplast ribosomal protein LC6. The fact that recombinants are not recovered from crosses between mutants argues that the four ery-M1 mutants are allelic, and thus that the different forms of LC6 result from corresponding mutations in a single nuclear gene. It is indeed difficult to envisage four mutations altering a modifying enzyme such that it produces different changes in the same ribosomal protein.

Other evidence indicating that some chloroplast ribosomal proteins may be coded by nuclear genes has been mentioned above, for example, the data of Lee and Jones (1973). These investigators

obtained many Mendelian streptomycin-resistant mutants (sr-1) by exposing cells to MNNG. Whether resistance was due to a change in the primary structure of a chloroplast ribosomal protein, however, was not determined in this study. Boschetti and Bogdanov (1973a) showed that resistance of the sr_3 mutant to streptomycin does occur at the ribosome level, since the isolated chloroplast ribosomes exhibited altered biophysical properties, including reduced binding of streptomycin. This does not necessarily prove, however, that the nuclear determinant of the chloroplast ribosome is a structural gene for a ribosomal protein.

Studies of Chlamydomonas mutants, other than antibiotic-resistant strains, also suggest that nuclear genes are involved in the synthesis of chloroplast ribosomes. For example, Goodenough and Levine (1970b) described the Mendelian ac-20 mutant which contains only 5-10% the normal amount of chloroplast ribosomes when grown mixotrophically. These authors later showed, however, that in ac-20, the block in chloroplast ribosome formation involves impairment of chloroplast rRNA synthesis (Goodenough and Levine, 1971). On the other hand, four other Mendelian mutants have been characterized in which the synthesis of only the small subunit is affected (Boynton et al., 1970; Harris et al., 1974). The mutant cells accumulate 54S particles which contain 23S RNA and are therefore identified as large ribosomal subunits. In the case of the cr-1 mutant, it is possible that a nuclear-coded protein required for proper assembly of the small ribosomal subunit is altered, although the data do not exclude other interpretations.

Evidence regarding the location of genes coding for chloroplast ribosomal proteins in organisms other than Chlamydomonas

Aside from studies of Chlamydomonas, the evidence concerning the location of genes coding for chloroplast ribosomal proteins is based on only two other organisms, a green alga, Acetabularia, and a higher plant, Nicotiana. In both cases, species-specific differences in chloroplast ribosomal proteins are detectable by one-dimensional gel electrophoresis. Although individual proteins from purified large subunits of ribosomes prepared from isolated chloroplasts (Kloppstech and Schweiger, 1973a) are not completely resolved, the protein patterns displayed by each of three species of Acetabularia can be distinguished by the presence or absence of characteristic peaks (Kloppstech and Schweiger, 1973b). The results of nuclear transplantation experiments indicate that, since the species-specific pattern of chloroplast ribosomal proteins is determined by the donor nucleus rather than the recipient cytoplasm, at least those proteins associated with the species-specific peaks seen in the electrophoretic profiles, are probably coded by the nuclear genome.

In Nicotiana, two species-specific peaks in the electrophoretic profiles of proteins from the large subunits of chloroplast ribosomes from isolated chloroplasts can be distinguished, one (a) associated with preparations from N. glauca and the other (b) with N. tabacum (Bourque and Wildman, 1973). Analysis of the chloroplast ribosomal proteins from the F_1 interspecific hybrids resulting from reciprocal crosses of

the two species shows that both hybrids contained both protein(s) (a) and protein(s) (b). Because of the rigorous standards of purity of the ribosomal subunits established in this study, the possibility that proteins (a) and (b) represent contamination by cytoplasmic ribosomes is remote. Therefore, in Nicotiana, some proteins of the chloroplast ribosomes are apparently coded by nuclear genes.

Evidence is presented in a recent paper which suggests that not all genes coding for the chloroplast ribosomal proteins are located in the nucleus of Nicotiana cells. Maliga et al. (1975) describe experiments with a mutant cell line SR1 of N. tabacum displaying non-Mendelian inheritance of streptomycin-resistance. Electron microscopy showed that, in sensitive strains exposed to streptomycin, few chloroplast membranes are present and there is an increase in the electron opacity of mitochondria. In resistant cells, however, streptomycin neither prevents the formation of chloroplast thylakoids and grana, nor alters mitochondrial ultrastructure. To study the level of resistance to antibiotic, the authors measured the uptake of streptomycin from the culture medium by sensitive and resistant cells. The results of both the chemical determination and bioassay of streptomycin indicate that the concentration of the antibiotic is the same in both resistant and sensitive material. These data therefore rule out the possibilities that resistance of the mutant cells is due to a reduction in membrane permeability to streptomycin or to inactivation of the drug, and suggest, by analogy with some streptomycin-resistant strains of

bacteria (Ozaki et al., 1969), that resistance in N. tabacum SR1 may be caused by a protein in the large ribosomal subunit. Since the ultrastructure of both chloroplasts and mitochondria is apparently normal in the presence of streptomycin, ribosomes from both organelles may contain the same altered protein. Also, whether the non-Mendelian mutation affecting the protein is located in chloroplast or mitochondrial DNA is uncertain. On the other hand, the electron micrographs which provide the evidence that mitochondria are affected by the mutation are not presented by Maliga et al., and their experiments do not rule out the possibility that the SR1 mutant cells do contain streptomycin-sensitive mitochondria but survive because chloroplast ribosomes are resistant to the drug. If this were the case, the SR1 cells would result from a mutation in chloroplast DNA coding for a chloroplast ribosomal protein.

Summary of evidence regarding the site of coding of chloroplast ribsomal proteins

In summary, the evidence indicates that, in Chlamydomonas, the genes coding for chloroplast ribosomal proteins are localized in at least two distinct genomes. The Mendelian mutants ery-M1a, ery-M1b, ery-M1c, ery-M1d (Davidson et al., 1974), and ery-M2d (Mets and Bogorad, 1972) provide the most definitive evidence to date that at least some determinants of chloroplast ribosomal proteins are actually nuclear structural genes. Amino acid sequence analysis of these altered chloroplast ribosomal proteins is required as unequivocal proof. The

results of studies of many uniparental mutants for antibiotic resistance, although suggestive, are less rigorous in the characterization of the altered chloroplast ribosomal proteins, as well as in the identification of the subcellular site of the extra-nuclear genes. Two proteins of the small ribosomal subunit conferring streptomycin-resistance and spectinomycin-sensitivity on chloroplast ribosomes in sm2-r (Ohta et al., 1975) and spr-1-27 cells (Boynton et al., 1973), respectively, however, represent promising candidates for chloroplast gene products. A problem inherent in all these studies of Chlamydomonas mutants is whether the uniparentally-inherited genes are located in chloroplast or mitochondrial DNA.

As in Chlamydomonas, chloroplast ribosomal proteins in Nicotiana may also be coded by genes from two separate genomes (Bourque and Wildman, 1973; Maliga et al., 1975), but the data is only suggestive. The only other organism in which the problem of the location of genes coding for chloroplast ribosomal proteins has been explored is Acetabularia, in which the involvement of nuclear genes is implicated by one report (Kloppstech and Schweiger, 1973b).

ii. Site of synthesis of chloroplast ribosomal proteins

Nearly all the studies of the site of synthesis of chloroplast ribosomal proteins involve the use of antibiotics, such as chloramphenicol, which specifically inhibit the synthesis of proteins on chloroplast (Ellis, 1970) and mitochondrial (Clark-Walker and Linnane, 1967) ribosomes

The results of inhibitor studies must be interpreted with caution for several reasons. Depending upon its concentration, an inhibitor may have non-specific effects or may be toxic to cells. Also, inhibition may be incomplete, or may fail to prevent the accumulation of a particular class of proteins. Despite these difficulties, however, a number of inhibitor studies have yielded meaningful information regarding the site of synthesis of chloroplast ribosomal proteins.

Evidence for synthesis of chloroplast ribosomal proteins in the cytoplasm

In Chlamydomonas, most chloroplast ribosomal proteins appear to be synthesized in the cytoplasm. Ultrastructural studies show that the ability of wild-type cells to form chloroplast ribosomes is not affected by growth in 100 µg/ml chloramphenicol for three generations, whereas both photosynthesis and the organization of chloroplast membranes is disrupted in the presence of the inhibitor (Goodenough, 1971). A similar lack of effect of chloramphenicol on chloroplast ribosome synthesis is observed in the recovery of ac-20 cells following transfer to minimal medium (Goodenough and Levine, 1971). These cells apparently suffer from a lesion in chloroplast rRNA synthesis when grown in light and in medium containing acetate, but begin to accumulate a 4-fold increase in the number of chloroplast ribosomes when cultured in light and medium lacking acetate. Chloramphenicol at 100 µg/ml did not significantly lower the number of chloroplast ribosomes synthesized by the recovering ac-20 cells. Also, the concentration of ribosomes in the chloroplast of

cells harvested after approximately ten generations of growth in medium containing 25 µg/ml spectinomycin is similar to that in control cells (Goodenough, 1971).

Honeycutt and Margulies (1973) tested the effect of 100 µg/ml chloramphenicol on the in vivo incorporation of (^{3}H)arginine into the structural proteins of cytoplasmic and chloroplast ribosomes of an arginine-requiring strain of Chlamydomonas. After a 1 hr labelling period, the cells were harvested and the distribution of radioactivity associated with chloroplast and cytoplasmic ribosomes was examined on sucrose gradients. Although the amount of 67S ribosomes was reduced in the chloramphenicol-treated cells, the level of radioactivity was also decreased, so that the specific radioactivity of chloroplast ribosomes from treated and control cells was the same. The 'loss' of 67S ribosomes in the chloramphenicol-treated cells was not due to inhibition of the synthesis of chloroplast ribosomal protein, but to a tendency for the 67S ribosomes to sediment with the membrane fraction during isolation. In pulse-chase experiments, cells were first treated with chloramphenicol for 1 hr and then allowed to incorporate (^{3}H)arginine for 2 min. Unlabelled arginine was immediately added and the cells were incubated for another 18 min. Sucrose gradient analysis of crude ribosomes prepared from cells at the end of the 2 min pulse showed that the radioactivity sedimented exclusively with the 83S ribosomes; at the end of the chase period, the radioactivity associated with the 87S ribosomes was reduced by an amount comparable to that associated with the 67S

ribosomes. This 'flow' of label was not prevented by a concentration of chloramphenicol sufficient to inhibit chloroplast protein synthesis as indicated by the lack of increase in radioactivity of RuDP carboxylase. These results suggest that the structural proteins of chloroplast ribosomes are synthesized on cytoplasmic ribosomes and that inhibition of chloroplast protein synthesis does not prevent the rapid transport of chloroplast ribosomal proteins to their destination.

Additional evidence concerning the site of synthesis of chloroplast ribosomal proteins in Chlamydomonas is based on the streptomycin-resistant mutant sr_3 described by Boschetti et al. (1974). When grown heterotrophically in the presence of streptomycin, strain sr_3 forms only yellow colonies, and chloroplast protein synthesis is drastically reduced as indicated by an assay for RuDP carboxylase activity. The relative amounts of chloroplast and cytoplasmic ribosomes contained in the mutant cells, however, as shown by electrophoresis of total RNA, is similar to that in wild-type cells grown in the absence of streptomycin. Therefore, since the chloroplast ribosomes of the mutant do not synthesize proteins under heterotrophic conditions, the proteins comprising the chloroplast ribosomes are most likely synthesized in the cytoplasm.

Collectively, these data do not exclude the possibility that some proteins of chloroplast ribosomes in Chlamydomonas are synthesized within the chloroplast. Reconstitution experiments have shown that some structural proteins are not required for the assembly of bacterial ribosomes

(Nomura et al., 1969). Thus, if the synthesis of chloroplast ribosomes can proceed in the absence of the chloroplast ribosomal proteins which are apparently coded by chloroplast DNA (e.g. Ohta et al., 1975), the observed lack of effect of chloramphenicol and spectinomycin on the synthesis of chloroplast ribosomes is explained. On the other hand, mRNAs transcribed from chloroplast genes specifying chloroplast ribosomal proteins may diffuse out of the organelles to be translated on cytoplasmic ribosomes. This possibility, however, seems unlikely. Another interpretation of the results of these inhibitor studies suggests that synthesis of chloroplast ribosomal proteins does occur in the chloroplast of Chlamydomonas, but is not detected in the experiments of Goodenough (1971) due to the presence of a large pool of these proteins in this organism. This argument is weakened by the lack of any dilution effect in the presence of spectinomycin even after ten generations of exposure of the cells to the drug. Furthermore, the labelling experiments of Honeycutt and Margulies (1973) clearly demonstrate the incorporation of radioactivity into the structural protein of chloroplast ribosomes after only 6 min.

Evidence based on an inhibitor study of another alga, Acetabularia, also supports the conclusion that chloroplast ribosomal proteins are synthesized in the cytoplasm. The incorporation of both (^{3}H)uridine and ^{14}C-labelled amino acids into the large 44S subunit of chloroplast ribosomes from Acetabularia is increased by about 50% in the presence of 10 µg/ml chloramphenicol (Kloppstech and Schweiger, 1974). In contrast,

0.5 µg/ml cycloheximide reduces the incorporation of (^{3}H)uridine and ^{14}C-labelled amino acids into the 44S particles by almost 80%. Kloppstech and Schweiger explain the stimulatory effect of chloramphenicol on amino acid incorporation into the large ribosomal subunit by a general increase in protein synthesizing activity in the cytoplasm as a consequence of the inhibition of chloroplast protein synthesis. They also hypothesize that the stimulation of uridine incorporation in turn results from the increased availability of completed ribosomal proteins synthesized on cytoplasmic ribosomes.

Using isolated spinach chloroplasts, Spencer et al. (1971) also obtained evidence for the synthesis of chloroplast ribosomal proteins in the cytoplasm. Preparations of chloroplasts from very young leaves were labelled for 30 min with radioactive amino acids and then treated with puromycin to release nascent proteins. Although 22% of the total radioactivity incorporated was found to be associated with the ribosomal fraction, no label was detected in any of the ribosomal proteins analyzed in urea-polyacrylamide gels. These results suggest that spinach chloroplasts do not synthesize chloroplast ribosomal proteins, but could also be explained by failure to detect low amounts of label incorporated into the structural proteins of the chloroplast ribosomes.

Evidence for synthesis of chloroplast ribosomal proteins in the chloroplast

Evidence based on inhibitor studies with six organisms, three algae and three higher plants, suggests that chloroplast ribosomal

proteins are synthesized on chloroplast ribosomes. In radish seedlings (Raphanus sativus), the accumulation of chloroplast rRNA is almost completely blocked during 7 hr exposure to 100 µg/ml chloramphenicol (Ingle, 1968). Similarly, sucrose gradient centrifugation and polyacrylamide gel electrophoresis show that the amounts of both chloroplast ribosomes and chloroplast rRNA are reduced by 2 µg/ml lincomycin in pea apices (Ellis and Hartley, 1971), by both 25µg/ml lincomycin and 25 µg/ml spectinomycin in Chlorella (Galling et al., 1973) and by virginiamycin (50 µg/ml virginiamycin M and 50 µg/ml virginiamycin S) in Euglena (Van Pel and Cocito, 1973). In contrast, the amount of cytoplasmic ribosomes in these organisms is not affected by these inhibitors.

The data of Detchon and Possingham (1975) suggest that chloroplast ribosomal proteins in spinach are synthesized in the chloroplast. These investigators used a dual-labelling method to study the effect of chloramphenicol and lincomycin on light-stimulated chloroplast rRNA synthesis in spinach leaf discs. Nucleic acids were extracted from a mixture of (^{14}C)uracil-labelled leaf discs cultured in continuous darkness and (^{3}H)uracil-labelled leaf discs incubated first in darkness and then transferred to a light regime. In this way, the RNA synthesized after illumination of the leaf discs is detected on polyacrylamide gels by an increase in the $^{3}H/^{14}C$ ratio in the region of the high molecular weight chloroplast rRNA species. The $^{3}H/^{14}C$ ratio distribution profile of nucleic acids extracted from leaf discs exposed

to either 100 µg/ml lincomycin or 10 µg/ml chloramphenicol during illumination did not contain any peaks in the region of chloroplast rRNA. Since these experiments involved long-term (7 days) exposure of the leaf discs to the antibiotics and non-specific effects of inhibition might have occurred, these results must be interpreted with caution. An observation which favours the validity of the data, however, is that chloroplast rRNA seems to be the only species whose synthesis is affected by either chloramphenicol or lincomycin.

The light-induced development of amino acid-incorporating activity of chloroplasts in pea (Ellis and Hartley, 1971) and Euglena (Reger et al., 1972) is reduced by inhibitors of chloroplast protein synthesis. Chloroplasts isolated from pea apices treated with 2 µg/ml lincomycin for 2 days and from Euglena exposed to 750 µg/ml chloramphenicol for 2 days were assayed for their capacity to incorporate (^{14}C)leucine into protein in vitro. The rate of protein synthesis in the chloroplasts isolated from the drug-treated material was only 5-25% that of chloroplasts from the controls, suggesting that proteins comprising the protein synthetic machinery of the chloroplast are synthesized within the organelle.

Finally, the results of an ultrastructural study of the effects of 300 µg/ml chloramphenicol on Ochromonas indicate that the light-induced increase of chloroplast ribosomes, and thus the synthesis of at least some chloroplast ribosomal proteins, is dependent on chloroplast ribosomes in this organism as well (Smith-Johannsen and Gibbs, 1972).

Summary of evidence regarding the site of synthesis of chloroplast ribosomal proteins

The literature pertaining to the synthesis of chloroplast ribosomal proteins can be summarized as follows. There is evidence for the synthesis of chloroplast ribosomal proteins in the chloroplast of some species, but in the cytoplasm of others. In Chlamydomonas, several lines of evidence show that most chloroplast ribosomal proteins are probably synthesized in the cytoplasm. This conclusion is consistent with the results of others (Davidson et al., 1974), which indicate the existence of structural genes for some chloroplast ribosomal proteins in the nucleus of Chlamydomonas. These studies of Chlamydomonas do not exclude the possibility, however, that a few chloroplast ribosomal proteins are synthesized in the chloroplast. If some chloroplast ribosomal proteins are coded by chloroplast DNA (Boynton et al., 1973; Ohta et al., 1975) as recent evidence suggests, it is more likely that these proteins would be synthesized in situ than on cytoplasmic ribosomes. In Acetabularia, as in Chlamydomonas, most chloroplast ribosomal proteins are probably synthesized in the cytoplasm.

In all other organisms so far studied, evidence suggests that at least some chloroplast ribosomal proteins are probably synthesized in the chloroplast. In the case of spinach, the data are conflicting. Since the results of Spencer et al. (1971) are negative and thus inconclusive, the remaining evidence favours the conclusion that spinach chloroplast ribosomal proteins are synthesized in the chloroplast. The

experiments which support this conclusion for spinach as well as other organisms, demonstrate that low concentrations of chloramphenicol, lincomycin, spectinomycin or virginiamycin inhibit the synthesis of chloroplast ribosomes as measured by sucrose gradient centrifugation, gel electrophoresis of rRNA or electron microscopy. The data from such studies permit more than one interpretation, however. The possible existence of large pools of ribosomal proteins, for example, has been discussed above with reference to Chlamydomonas. Alternatively, these antibiotics may inhibit only the synthesis of chloroplast RNA polymerase. This possibility has been eliminated in the case of the effect of lincomycin on pea apices (Ellis and Hartley, 1971). On the other hand, the synthesis of chloroplast ribosomal proteins on cytoplasmic ribosomes may be simply regulated by a factor dependent on chloroplast ribosomes, as suggested by Galling et al. (1973). For example, if a small number of chloroplast ribosomal proteins or regulatory proteins which are synthesized in the chloroplast are required for the continued synthesis of chloroplast ribosomal proteins in the cytoplasm, then curtailment of protein synthesis on chloroplast ribosomes might result in a secondary inhibition of the cytoplasmic synthesis of chloroplast ribosomal proteins. If this interpretation is accepted, however, then one must assume that in Chlamydomonas and Acetabularia the synthesis of chloroplast ribosomal proteins on cytoplasmic ribosomes is not subject to a similar control mechanism. The most tenable hypothesis which explains the effect of

various antibiotics on radish seedlings (Ingle, 1968), pea apices (Ellis and Hartley, 1971), spinach leaf discs (Detchon and Possingham, 1975), Ochromonas (Smith-Johannsen and Gibbs, 1972), Euglena (Reger et al., 1972; Van Pel and Cocito, 1973) and Chlorella (Galling et al., 1973) is that chloroplast ribosomal proteins are synthesized on the antibiotic-sensitive chloroplast ribosomes in these organisms.

Conclusions regarding the biogenesis of chloroplast ribosomal proteins

In evaluating the evidence summarized in Table I concerning the sites of coding and synthesis of chloroplast ribosomal proteins, it is clear that apparent discrepancies exist between the results of studies based on the same organism, as well as between those based on different organisms. Some of this disparity may arise from different experimental approaches, as in the case of the studies on spinach. Conflicting conclusions regarding the site of coding or synthesis of chloroplast ribosomal proteins in different organisms, however, may reflect genuine species differences. Since organelle evolution proceeds independently within any one species, the location of genes coding for organelle proteins need not be identical in all organisms. Perhaps one of the most significant implications of this research is that, like the polypeptide subunits of RuDP carboxylase, the proteins of chloroplast ribosomes are probably products of at least two distinct transcription (and also translation) systems. The genetic analysis of antibiotic-resistant mutants of Chlamydomonas indicates that some chloroplast ribosomal proteins are coded by nuclear genes and others by chloroplast genes. It remains to be determined whether the distribution of genes

Table I. Evidence for the Sites of Coding and Synthesis Chloroplast Ribosomal Proteins

Organism	Site Coding	Site Synthesis	Experimental Approach
Acetabularia	N		Nuclear transplantation/density gradients/PAGE
		R	In vivo labelling/chloramphenicol, cycloheximide
Chlamydomonas	N		Mutants/density gradients/PAGE/EM/genetic analysis
	c		Mutants/density gradients/PAGE/EM/genetic analysis
	N, c		Antibiotic-resistant mutants/growth conditions/rifampicin
		R	Wild-type and spa-2 cells/chloramphenicol, spectinomycin/EM
		R	ac-20 mutant/chloramphenicol/EM
	c		Mutants/neamine/genetic analysis
	c		(^{3}H)dihydrospectinomycin/in vitro binding assay/density gradients
	N, c		Erythromycin-resistant mutants/density gradients/1-D and 2-D PAGE
	c		Carbomycin-resistant mutants/in vitro protein synthesis
	c		Spectinomycin-resistant mutant/growth conditions/EM/PAGE
	N, c		Streptomycin-resistant mutants/antibiotic-binding assay/density gr
		R	In vivo labelling/density gradients
	N, c		Synchronized cultures/mutagenesis/streptomycin-resistance/genetic
	N, c	R	Streptomycin-resistant mutants/growth conditions/PAGE/in vivo prot
	N		Erythromycin-resistant mutants/genetic analysis/1-D and 2-D PAGE
	N		Ribosome-deficient mutants/genetic analysis/density gradients/EM
	c		Antibiotic-resistant mutants/in vitro protein synthesis/subunit ex
	c		Streptomycin-resistant mutant/double label/CM-cellulose column chr

ıble I. Evidence for the Sites of Coding and Synthesis of Chloroplast Ribosomal Proteins

Experimental Approach	Authors
ransplantation/density gradients/PAGE	Kloppstech and Schweiger, 1973b
ıbelling/chloramphenicol, cycloheximide	Kloppstech and Schweiger, 1974
ensity gradients/PAGE/EM/genetic analysis	Boynton et al., 1970
ensity gradients/PAGE/EM/genetic analysis	Gillham et al., 1970
c-resistant mutants/growth conditions/rifampicin	Surzycki and Gillham, 1970
and spa-2 cells/chloramphenicol, spectinomycin/EM	Goodenough, 1971
ant/chloramphenicol/EM	Goodenough and Levine, 1971
eamine/genetic analysis	Behn and Arnold, 1972
rospectinomycin/in vitro binding assay/density gradients	Burton, 1972
cin-resistant mutants/density gradients/1-D and 2-D PAGE	Mets and Bogorad, 1972
n-resistant mutants/in vitro protein synthesis	Schlanger et al., 1972
ycin-resistant mutant/growth conditions/EM/PAGE	Boynton et al., 1973
cin-resistant mutants/antibiotic-binding assay/density gradients	Boschetti and Bogdanov, 1973a, b
abelling/density gradients	Honeycutt and Margulies, 1973
zed cultures/mutagenesis/streptomycin-resistance/genetic analysis	Lee and Jones, 1973
cin-resistant mutants/growth conditions/PAGE/in vivo protein synthesis	Boschetti et al., 1974
cin-resistant mutants/genetic analysis/1-D and 2-D PAGE	Davidson et al., 1974
deficient mutants/genetic analysis/density gradients/EM	Harris et al., 1974
c-resistant mutants/in vitro protein synthesis/subunit exchange	Schlanger and Sager, 1974
cin-resistant mutant/double label/CM-cellulose column chromatography	Ohta et al., 1975

Table I - continued

Organism	Site Coding	Site Synthesis	Experimental Approach
Chlorella		r	RNA PAGE/chloramphenicol, spectinomycin
Euglena		r	Isolated plastids/in vitro protein synthesis/chloramphenicol pre
		r	Density gradients/viriniamycin
Ochromonas		r	EM/chloramphenicol
Nicotiana	N		Interspecific hybrids/density gradients/PAGE
	c		Streptomycin-resistant mutant/tissue culture/genetic analysis/up
Pisum		r	Lincomycin/isolated plastids/amino acid, uridine incorporation/d gradients/PAGE
Raphanus		r	Chloramphenicol/in vivo uridine incorporation/RNA PAGE
Spinachia		R	Isolated plastids/amino acid incorporation/density gradients/PAG
		r	Chloramphenicol, lincomycin/leaf discs/RNA PAGE

Symbols used: N = nuclear DNA
c = chloroplast DNA
R = cytoplasmic ribosomes
r = chloroplast ribosomes

Experimental Approach	Authors
chloramphenicol, spectinomycin	Galling *et al.*, 1973
plastids/*in vitro* protein synthesis/chloramphenicol pre-treatment	Reger *et al.*, 1972
radients/viriniamycin	Van Pel and Cocito, 1973
mphenicol	Smith-Johannsen and Gibbs, 1972
ific hybrids/density gradients/PAGE	Bourque and Wildman, 1973
cin-resistant mutant/tissue culture/genetic analysis/uptake	Maliga *et al.*, 1975
n/isolated plastids/amino acid, uridine incorporation/density /PAGE	Ellis and Hartley, 1971
enicol/*in vivo* uridine incorporation/RNA PAGE	Ingle, 1968
plastids/amino acid incorporation/density gradients/PAGE	Spencer *et al.*, 1971
enicol, lincomycin/leaf discs/RNA PAGE	Detchon and Possingham, 1975

determining ribosomal proteins and other organelle components in other organisms is similar to that in Chlamydomonas. The results of investigations of the sites of synthesis of chloroplast ribosomal proteins suggest that the location of genes controlling the expression of these proteins may vary among different species.

2. Mitochondria

As for chloroplast DNA the amount of information contained in the mitochondrial genome can be calculated from its physical dimensions (see Appendix). Whereas mitochondrial DNA molecules from animal species average 5 μm in contour length (in Borst, 1970), mitochondrial DNAs from both algae and higher plants are 4 to 6 times larger. For example, Kolodner and Tewari (1972b) found 30 μm circular DNA molecules in mitochondria from pea leaves and Védel and Quétier (1974) obtained linear molecules up to 28 μm in length from potato tuber mitochondria. The size of mitochondrial DNA from Euglena has recently been the subject of controversy; some investigators obtained heterogeneous linear molecules up to 19 μm in length (Manning et al., 1971), but others reported that this mitochondrial DNA occurs in the form of 1 μm circles (Nass et al., 1974). A study of the kinetic complexity of the mitochondrial DNA from this alga, however, indicates a value of 40×10^6 daltons (Talen et al., 1974), which is consistent with a contour length of about 20 μm. Thus, the molecular size of mitochondrial DNA from Euglena is probably in the same range as that of mitochondrial DNAs from

other eukaryotic microörganisms. Mitochondrial DNAs from Neurospora (Clayton and Brambl, 1972) and yeast (Saccharomyces cerevisiae) (Borst, 1970), for example, have been isolated as circular molecules measuring 20 and 25 μm, respectively. Thus, if each circular molecule represents a complete copy of the mitochondrial genome, then the mitochondrial DNA from lower and higher plants is sufficient to code for 40 different proteins having molecular weights of about 40,000 daltons.

a. Sites of coding and synthesis of mitochondrial membrane and soluble proteins

The earliest studies concerning the coding site of mitochondrial proteins were conducted by Ephrussi and his collaborators on petite yeast mutants, in which mitochondrial DNA is grossly altered (in Sager, 1972). These respiratory-deficient mutants lack cytochromes $\underline{a}+\underline{a}_3$ and $\underline{b}$ which suggests that the genes for these components reside in mitochondrial DNA. On the other hand, cytochrome $\underline{c}$ and most soluble proteins, including the Krebs cycle enzymes, are probably coded by nuclear DNA, since these components persist in petite yeast mutants (in Sager, 1972). Using genetic methods, Sherman et al. (1966) demonstrated that the structural gene for yeast cytochrome $\underline{c}$ is located in the nucleus.

In accordance with the low coding potential of mitochondrial DNA, the proportion of mitochondrial proteins synthesized in the organelle is also small. Experiments involving specific inhibitors of translation show that the contribution of the yeast mitochondrial translation system

to total organelle protein is only 4-13%, depending on the physiological conditions (Kellerman et al., 1971). A comparison of in vivo and in vitro labelling patterns indicates that about 90% of the proteins synthesized within mammalian mitochondria are associated with membranes (Coote and Work, 1971), and that, in Neurospora, the products of protein synthesis on mitochondrial ribosomes are incorporated almost exclusively into the inner mitochondrial membrane (Neupert and Ludwig, 1971). Evidence summarized by Sager (1972) shows that mitochondrial proteins synthesized on cytoplasmic ribosomes include cytochrome c, the Krebs cycle enzymes, membrane polypeptides, and mitochondrial DNA polymerase and RNA polymerase.

Recent evidence indicates that the biogenesis of some mitochondrial proteins is more complicated than suggested by earlier studies with, for example, petite yeast mutants. This is illustrated by cytochrome oxidase which is identical with cytochrome $a+a_3$ and has been studied in detail. Cytochrome oxidase can be specifically isolated from crude yeast mitochondrial extract by immunoprecipitation (Rubin and Tzagoloff, 1973), and, when analyzed by SDS-gel electrophoresis, the enzyme is resolved into 7 subunits (Sebald et al., 1973). The sites of synthesis of these polypeptides has been investigated in yeast by using specific inhibitors of translation. Synthesis of the three largest components of cytochrome oxidase is sensitive to chloramphenicol, but insensitive to cycloheximide, and thus occurs on mitochondrial ribosomes (Rubin and Tzagoloff, 1973). In contrast, synthesis of the four smaller

polypeptides is sensitive to cycloheximide, but insensitive to erythromycin, and therefore proceeds on cytoplasmic ribosomes (Mason and Schatz, 1973). The three large polypeptides synthesized on mitochondrial ribosomes are characterized by a relatively high content of nonpolar amino acids (Sebald et al., 1973) and account for up to 25% of the total protein synthesized in the organelle (Ebner et al., 1973). Although the results of analyses of nuclear cytoplasmic yeast mutants lacking cytochrome oxidase activity (Ebner et al., 1973) do not permit a firm conclusion regarding the sites of structural genes for subunits of cytochrome oxidase, they do indicate the interaction of both organelle and nuclear genomes in the assembly of this mitochondrial enzyme complex.

The biogenesis of ATPase, another membrane-bound enzyme, also requires the dual participation of two distinct transcription/translation systems. When analyzed by gel electrophoresis, the purified ATPase complex from yeast is composed of nine subunit polypeptides, five associated with the catalytic function of the enzyme and four with a membrane-binding factor. To determine whether some of these polypeptides are synthesized in the mitochondrion, Tzagoloff and Meagher (1972) conducted reconstitution experiments with radioactive and non-radioactive fractions of the ATPase complex from yeast. The results indicated that at least some of the polypeptides which are involved in the binding of ATPase to membrane are intrinsic subunits of the enzyme complex and are synthesized on mitochondrial ribosomes. Earlier studies (Tzagoloff, 1969) had shown, since ATPase activity accumulates in the soluble phase

of mitochondria of yeast treated with chloramphenicol and this accumulation is prevented in the presence of cycloheximide, that the fraction of the ATPase complex involved in enzyme function is synthesized on cytoplasmic ribosomes. The location of genes controlling the expression of ATPase activity has been studied in oligomycin-sensitive mutants of Saccharomyces cerevisiae (Shannon et al., 1973). The ATPase complex was isolated from a non-Mendelian and several Mendelian mutants and separated into soluble and membrane protein components. Reconstruction of hybrid enzyme complexes from components purified from mutant and wild-type cells indicated that oligomycin-resistance in both types of mutants is a property of the membrane fraction of the ATPase complex. These results suggest that mitochondrial DNA, as well as nuclear DNA, is a genetic determinant of ATPase.

b. Sites of coding and synthesis of mitochondrial ribosomal proteins

i. Site of coding of mitochondrial ribosomal proteins

The evidence regarding the location of the genes coding for mitochondrial ribosomal proteins is limited and controversial. The presence of nuclear genes containing information for at least two mitochondrial ribosomal proteins has been demonstrated by analysis of interspecific hybrids of Xenopus (Leister and Dawid, 1975). However, out of a total of seven species-specific mitochondrial ribosomal proteins

identified in this study, four exhibited a non-Mendelian pattern of inheritance. Although the data can be interpreted to indicate that the genes for these four proteins are associated with mitochondrial DNA, the authors favour the conclusion that nuclear paternal genes for these components are transcribed in the hybrid, but that only the maternally-determined proteins are bound by the corresponding maternally-inherited mitochondrial rRNA. This is a cogent argument since the transcription of mitochondrial RNA from mitochondrial DNA is well documented (Whitfield, 1973). Furthermore, the specificity of rRNA-protein interactions has also been established by reconstitution experiments, in which light cytoplasmic rRNA from yeast and rat liver failed to substitute for 16S rRNA from E. coli in the assembly of E. coli ribosomal proteins into the bacterial 30S ribosomal subunit (Traub and Nomura, 1968).

There are also several reports of non-Mendelian mutations which affect mitochondrial ribosomes. Altered ribosomes, for example, have been detected in mitochondria of erythromycin-resistant mutants of yeast (Grivell et al., 1971) and Paramecium (Tait, 1972). In poky Neurospora mutants, the amount of the small mitochondrial ribosomal subunit is abnormally low, suggesting an instability exists in a ribosomal protein (Neupert et al., 1971; Rifkin and Luck, 1971). However, in a study where mitochondrial ribosomes from several different antibiotic-resistant yeast mutants were analyzed, no differences between the mitochondrial ribosomal proteins from resistant and sensitive strains were detected

(Grivell et al., 1973). Therefore, this observation suggests that the site affected by these non-Mendelian mutations is mitochondrial rRNA. In some strains of bacteria, induced resistance to erythromycin has been correlated with an altered methylation pattern of 23S rRNA (Lai and Weisblum, 1971).

ii. Site of synthesis of mitochondrial ribosomal proteins

The results of inhibitor studies suggest that mitochondrial ribosomal proteins are synthesized outside mitochondria. Using CM-cellulose column chromatography, Küntzel (1969b) showed that, in Neurospora, the synthesis of proteins from both cytoplasmic and mitochondrial ribosomes is sensitive to cycloheximide, whereas chloramphenicol does not inhibit the synthesis of either class of proteins. Davey et al. (1969) found that mitochondria, isolated from yeast which had been grown for five generations in chloramphenicol or lincomycin to dilute out all proteins synthesized on mitochondrial ribosomes, are still active in amino acid incorporation. In Ochromonas, the number of mitochondrial ribosomes is not reduced in the presence of chloramphenicol (Smith-Johannsen and Gibbs, 1972). The results of these studies therefore indicate that most of the proteins of mitochondrial ribosomes are probably synthesized on cytoplasmic ribosomes. Later, Borath and Küntzel (1972) reported that chloramphenicol stimulates the synthesis of mitochondrial ribosomes, and thus ribosomal proteins, in

Neurospora, suggesting that the cytoplasmic synthesis of mitochondrial ribosomal proteins may be regulated by a repressor synthesized in the organelle.

None of these investigations rule out the possibility that a few proteins of mitochondrial ribosomes are synthesized within the organelle. Inhibitor studies on yeast (Schmitt, 1972) and Tetrahymena (Millis and Suyama, 1972) have shown a partial sensitivity of the synthesis of mitochondrial ribosomes to chloramphenicol. This antibiotic is known to affect respiration non-specifically (Freeman and Haldar, 1968), however, and the L-threo isomer of chloramphenicol was not employed in either study as a control (Ireland and Bradbeer, 1971). Therefore, the inhibition of synthesis of mitochondrial ribosomal proteins by chloramphenicol reported in yeast and Tetrahymena may reflect a general depression of energy-dependent protein synthesis on cytoplasmic ribosomes.

The conclusion most compatible with the genetic and biochemical evidence discussed above is that the synthesis of most mitochondrial ribosomal proteins is directed by nuclear genes on cytoplasmic ribosomes. Michel and Neupert (1973) point out that as many as one hundred proteins may be formed by the nuclear-cytoplasmic system for the mitochondrial transcriptional and translational machinery. This machinery, however, generates only a few polypeptides associated with the mitochondrial inner membrane. Apparently, the extreme hydrophobic nature of these components necessitates their synthesis at the site of their incorporation into the membrane.

II. Purpose of the investigation

This investigation was undertaken to answer the question -- are chloroplast ribosomal proteins synthesized in the chloroplast of Ochromonas? This problem is interesting from the point of view of regulation -- how do the host cell and organelle genetic systems interact to control the synthesis of organelle components, and is also important from an evolutionary standpoint -- how did organelles evolve?

Evidence from our previous ultrastructural study, in which chloramphenicol was used to inhibit protein synthesis on chloroplast ribosomes, suggested that chloroplast ribosomal proteins are synthesized in the chloroplast of Ochromonas (Smith-Johannsen and Gibbs, 1972). The results of biochemical studies with some other algae (e.g. Kloppstech and Schweiger, 1974) are conflicting. To resolve the question of whether these discrepancies arise from genuine differences between species or from differences in experimental approaches, the Ochromonas system was studied using biochemical methods.

A procedure was developed for the isolation of intact rRNA from Ochromonas. The electrophoretic profile of the RNA from this organism is unique and contains five peaks of high molecular weight rRNA. A major portion of this study was devoted to identifying and characterizing these rRNA species.

Spectinomycin, a specific inhibitor of translation on 70S ribosomes (Pestka, 1971), was used to block protein synthesis on chloroplast ribosomes in Ochromonas. This antibiotic probably inhibits the functioning of

mitochondrial ribosomes as well, but unlike chloramphenicol, spectinomycin does not have non-specific effects on mitochondrial respiration. Since newly-transcribed rRNA is complexed with proteins (Hamkalo and Miller, 1973) and is probably unstable if not incorporated into ribonucleoprotein particles, the accumulation of chloroplast rRNA can be assumed to reflect the availability of chloroplast ribosomal proteins. The effect of spectinomycin on the synthesis of chloroplast ribosomal proteins was therefore examined by measuring the amount of chloroplast rRNA in control and drug-treated cells. If the amount of chloroplast rRNA in spectinomycin-treated cells is reduced, then one can conclude that the antibiotic probably inhibits the synthesis of chloroplast ribosomal proteins. Ethidium bromide was employed in some experiments to distinguish chloroplast and mitochondrial rRNAs. The effects of spectinomycin and ethidium bromide on the ultrastructure of Ochromonas were also studied.

MATERIALS AND METHODS

A. Biological Material and Growth Conditions

Stocks of Ochromonas danica Pringsheim were obtained from the Culture Collection of Algae at Indiana University (Culture No. 1298). Cells were grown at 29° C in 250 ml Erlenmeyer flasks containing 150 ml of complete Ochromonas medium (Aaronson and Baker, 1959). Dark-grown cultures were maintained in a light-tight incubator; light-grown and greening cultures were maintained on a low speed rotary shaker in a growth chamber equipped with a bank of fluorescent and incandescent lamps adjusted to give a light intensity of 600 ft-c at the culture surface. Inoculations of dark-grown cultures and other dark operations were carried out using safelights covered with both a blue (Rohm and Haas No. 2424) and a green (Rohm and Haas No. 2092) sheet of plexiglass.

The density of cultures at the beginning of experiments where antibiotics were used was $0.2 - 1.5 \times 10^6$ cells/ml. At this concentration both light and dark-grown cells divide at an exponential rate. In the case of greening experiments, the inhibitors were added to dark-grown cells just prior to illumination. D-threo and L-threo chloramphenicol and spectinomycin were added to cultures in powder form; ethidium bromide was added from freshly prepared stock solutions (0.15 mg/ml in distilled water). Cell counts were made with a Model ZB1 Coulter Counter using a 100 µm aperture.

Escherichia coli strain CR34 F^-, thy^-, $threo^-$, leu^-, lac^- was kindly supplied by Dr. D. Lane, Biochemistry Department, McGill University.

(^{3}H)Uridine labelled RNA from sea urchin embryos (Lytichinus pictus) was kindly provided by Dr. D. Fromson, Biology Department, McGill University.

B. Biochemical Studies

1. Labelling of Ochromonas danica RNA

To label the RNA, 50 ml of a dark or light-grown culture containing approximately 1 x 10^6 cells/ml was incubated in the presence of 20 µCi/ml (^{3}H)5-uridine (27 Ci/mM). After a labelling period of 6 hours in the light the cells were harvested, washed once in distilled water, and RNA extracted as described below. Cultures used for labelling studies were routinely tested for bacterial contamination.

2. Extraction of total RNA from Ochromonas danica

A total of 50 - 200 x 10^6 cells which were in the exponential phase of growth were collected in 40 ml plastic tubes by centrifugation at 9000 rpm for 8 min at 4° C using a Beckman J-21 centrifuge and JA-14 rotor. All subsequent operations were carried out at 4° C. The phenol used for isolation of RNA was redistilled and neutralized by three changes of the extraction buffer (0.05 M Tris-acetate, pH 7.6, 0.1 M NaCl, 0.01 M disodium EDTA) in a separatory funnel. An equal volume of chloroform was added to the buffer-saturated phenol and the resulting solution made 1% isoamylalcohol and 0.1% 8-hydroxyquinoline. Equal volumes of extraction buffer, containing 0.5% Sarkosyl NL 97, and phenol-chloroform (1:1) were thoroughly mixed and immediately added to the pelleted cells. The cells were dispersed in 4 ml of the extraction

buffer-phenol-chloroform mixture by vortexing and the slurry was transferred to a 15 ml screw-topped glass tube. Samples were shaken for 15 min at medium speed on a W-8 Twist Action shaker (New Brunswick Scientific, New Brunswick, N.J.) and centrifuged for 15 min at 4500 rpm in an International Model HN centrifuge. The aqueous phase was re-extracted three or four more times with phenol-chloroform, with shaking and centrifugation periods reduced to 10 min each. The RNA was precipitated by adding 0.1 volume 20% potassium acetate, pH 5.5, and 2.5 volumes absolute ethanol to the final supernatant and allowing the solution to stand for a minimum of 18 hr at -20° C.

Precautions were taken to avoid ribonuclease contamination of RNA preparations. Therefore glassware was acid-cleaned and autoclaved; sterilization of buffers and pipets was not, however, found to be necessary.

3. Extraction of labelled RNA from Escherichia coli

To ensure continuous incorporation of radioactive precursor into RNA, 0.1 ml 100 μCi/ml (^{14}C)uracil (0.55 μCi/μM) was added to a 200 ml culture of E. coli at 10 min intervals during a period of 1.5 hr. The culture (3 x 10^8 cells/ml) was centrifuged and washed with an equal volume of Tris-acetate buffer (0.05 M Tris-acetate, pH 7.6, 0.1 M NaCl, 0.01 M $MgCl_2$). The cells were resuspended in Tris-acetate buffer and lysed by adding 1.4 mg/ml lysozyme and incubating at 37° C for 5 min. An equal volume of Tris-acetate buffer-saturated phenol containing 0.1% 8-hydroxyquinoline was added and the suspension agitated for 10 min

by gentle shaking by hand. The aqueous phase was re-extracted with an equal volume of phenol followed by an equal volume of ether. The RNA was precipitated by adding 0.1 volume 20% potassium acetate, pH 5.5, and 2.5 volumes absolute ethanol to the preparation, and stored at -20° C. The specific activity of this RNA was approximately 7500 cpm/μg, assuming $1.0 O.D._{260}$ = 40 μg RNA (Avadhani and Buetow, 1972).

4. Electrophoresis of RNA

a. Preparation of gels and RNA samples

The method used to prepare agarose-acrylamide gels is based on procedures developed by Loening (1969) and Peacock and Dingman (1968). The composition of the gels used in this study was as follows: 2.4% acrylamide, 0.12% bisacrylamide, 0.5% agarose, 1% NNN'N'tetramethyl-ethylenediamine (TEMED) and 1% ammonium persulfate in electrophoresis buffer (0.036 M Tris, pH 7.8, 0.03 M $Na_2H_2PO_4$, 0.001 M disodium EDTA). Stock solutions of electrophoresis buffer and of 15% acrylamide plus 0.75% bisacrylamide were prepared in advance and maintained for no longer than two weeks. Agarose was refluxed as a 1% solution in distilled water immediately before using. Ten percent solutions of ammonium persulfate and TEMED were also freshly prepared. The gel tubes (10 cm long with an inner diameter of 8 mm) were treated with Siliclad and placed vertically in the casting rack, their lower ends sealed with a sheet of parafilm. To prepare gels, appropriate amounts of stock buffer, acrylamide, TEMED, and distilled water were combined in the above order in a small Erlenmeyer flask. The flask was warmed

in a 54° C water bath and an equal volume of 1% agarose (54° C) was added. Finally, 0.1 volume 10% ammonium persulfate was added to the solution which was then gently swirled and rapidly dispensed into the tubes using a Pasteur pipet to avoid introduction of air bubbles. Gels were allowed to polymerize for 10 min at room temperature followed by 10 min at 4° C. The gels were partially extruded from the tubes so that the curved surface formed at the gel-air interface could be cut off; the opposite end which had been in contact with the parafilm provided a smooth, flat loading surface. A small square of cotton gauze was applied to the bottom of each tube to prevent the gel from slipping out. Gels were pre-run for approximately 40 min at 4° C in electrophoresis buffer at 5 mA/gel.

The RNA preparation was centrifuged 5 min at 4° C at 2900 rpm in an IEC Model CL centrifuge using a 221 rotor and washed once with cold absolute ethanol. The pellet was allowed to dry and was resuspended in 0.2 ml cold electrophoresis buffer containing 20% glycerol. Insoluble polysaccharide (chrysolaminarin) was removed by centrifugation for 5 min at 2900 rpm. An aliquot of the supernatant was analyzed in a Beckman DB-GT spectrophotometer: $O.D._{260}/O.D._{280}$ ratios were routinely found to be 2.00. Approximately 10 to 12 µg RNA in 50 µl of buffer was loaded on each gel.

b. Electrophoresis and analysis of gels

Electrophoresis was carried out for 3 hr at 4° C at 5 mA/gel. Under these conditions bromophenol blue, the tracking dye, was located 5 to 10 mm from the anode end of the gel after 3 hr.

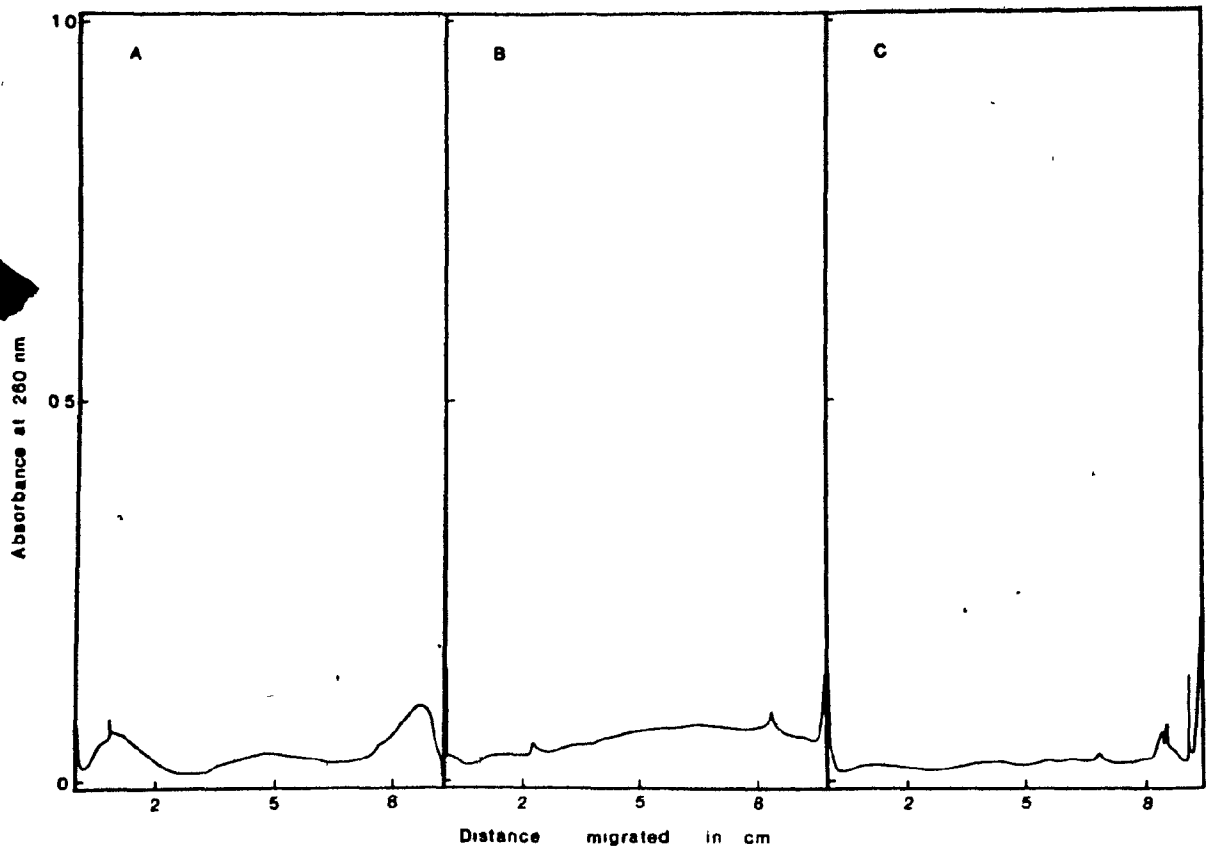
After electrophoresis the gels were removed from the tubes and soaked in distilled water for at least 30 min. They were then scanned at 260 nm in a Gilford Model 240 spectrophotometer equipped with a 2410-S linear transport attachment and 0.2 mm x 2.36 mm aperture plate using a chart speed of 1 in/min.

For determination of radioactivity the gels were fractionated into 1 mm sections using a Gilson gel fractionator. The minced gels were collected in plastic vials and allowed to dry. Protosol (0.3 ml) and 6 ml toluene containing 4 g/l Omnifluor were added to each fraction, the vials were maintained at room temperature overnight, and the radioactivity was measured in an Intertechnique ABAC SL40 liquid scintillation counter.

c. Analysis of polyacrylamide gel electropherograms

Relative proportions of RNA species represented by peaks in the electrophoretic profiles were determined by measuring the areas bounded by the curves. Tracings of three to twelve replicate gels of each sample from one or more separate RNA extractions were Xeroxed and the peaks extrapolated to the base line using a straight edge. Some variability in the background absorbance of gels persisted within and between experiments as shown in Figure 1. Therefore the base line was usually determined by connecting the troughs between the major peaks in the tracing. Individual peaks were then cut out and weighed. Since the heavy cytoplasmic RNA peak often ran off-scale, values for amounts of RNA were normalized with respect to the light cytoplasmic rRNA species.

Figure 1. Variability in background absorbance of agarose-acrylamide gels. Sample buffer (50 µl) was applied to gels and subjected to electrophoresis for 3 hr. A, B and C represent three such gels.


1.0
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A
B
C
Absorbance at 260 nm
2
5
8
2
5
8
2
5
8
Distance migrated in cm

Thus, amounts of organelle rRNA were expressed as a ratio of the organelle RNA peak to the amount of light cytoplasmic rRNA.

d. Statistical analysis

The ratios obtained by analysis of the electrophoretic profiles of RNA samples from control and treated cells were transformed to arcsin values (Sokal and Rohlf, 1969) and the data subjected to statistical analysis. The results from the experiment involving D-threo and L-threo chloramphenicol were subjected to a Student's t-test. The results from the experiment involving ethidium bromide and spectinomycin were subjected to single classification analysis of variance after confirming homogeneity of variance by Bartlett's test (Snedecor, 1956). Dunnett's procedure (Steel and Torrie, 1960) was employed to determine levels of confidence between controls and experimentals.

C. Ultrastructural Studies

1. Electron microscopy

Light-grown and greening cells were treated with 100 µg/ml spectinomycin and/or 1 µg/ml ethidium bromide for 24 hr. Cells were then collected in an IEC clinical centrifuge at 2900 rpm for 3 min and each sample was processed for electron microscopy at room temperature by two different methods. The first method was a standard fixation of 30 min in 2.5% glutaraldehyde in phosphate buffer (0.1 M sodium phosphate, pH 7.2) followed by a rinse in phosphate buffer and 60 min post-fixation in 1% osmium tetroxide in phosphate buffer. The second

method involved suspending cells in a solution containing both glutaraldehyde and osmium tetroxide, a technique Falk (1969) employed to demonstrate 'rough' thylakoids in chloroplasts of Phaseolus vulgaris. Cells were 'co-fixed' for 30 min in freshly mixed 2% glutaraldehyde and 2% osmium tetroxide in 0.05 M phosphate buffer, pH 7.2. After washing in phosphate buffer the cells were post-fixed in 1% osmium tetroxide in phosphate buffer for 60 min.

Samples prepared by both fixations were dehydrated through an ethanol series and embedded in low-viscosity epoxy resin (Spurr, 1969). Sections were cut on a Porter-Blum Mt-2 ultramicrotome, stained for 10 min with lead citrate (Reynolds, 1963), and examined in a Philips EM 200 electron microscope.

Chlorophyll a was extracted in 80% acetone, measured at 663 nm with a Beckman DB-G spectrophotometer and the amount per cell calculated according to the method described by Gibbs (1962).

2. Quantitative analyses

a. Ribosome counts

Chloroplast and mitochondrial ribosomes were counted on electron micrographs of co-fixed cells printed at a total magnification of 73,000 X. A square window representing 1/16 μm^2 in area was placed at random over the organelle and the number of electron dense particles of ribosome size counted. Since membranes usually occupied some fraction of the counting field, the values obtained by this method represent ribosomes/total chloroplast area rather than ribosomes/area chloroplast

matrix. Each value for ribosomes/unit organelle area given in Tables VII and IX is an average of approximately 130 counts from a minimum of 25 chloroplasts or approximately 100 counts from at least 75 mitochondria. The data was subjected to a statistical analysis and levels of confidence were determined by a Student's t-test.

b. Chloroplast volume

The percent volume of the cell occupied by the chloroplast under control and experimental conditions was determined from the fractional area occupied by the chloroplast in approximately 100 sections through as many cells selected at random from each sample. Chloroplast and cell areas were measured with a compensating polar planimeter (Geotec 349-1838) on micrographs printed at a final magnification of 25,900 X.

The percent chloroplast volume was then converted to absolute chloroplast volume by multiplying by the mean cell volume of the population. Cell volumes were measured with a Model ZB1 Coulter Counter, calibrated with paper mulberry pollen. The absolute chloroplast volumes thus obtained were used to compute total ribosomes per chloroplast.

D. Chemicals and Equipment

D-threo chloramphenicol and ethidium bromide were obtained from Sigma Chemical Co., St. Louis, Mo.; L-threo chloramphenicol was purchased from Park, Davis and Co., Detroit, Mich. and spectinomycin from The Upjohn Co., Kalamazoo, Mich..

Radioactive isotopes, Protosol and Omnifluor were secured from New England Nuclear, Boston, Mass.. Sarkosyl NL 97 was a gift from Geigy Limited, Toronto, Ontario. Agarose was obtained from Seakem, Rockland, Maine; agarose obtained from Sigma Chemical Co., St. Louis, Mo. was unsuitable for electrophoresis. Siliclad was bought from Clay Adams, Parsippany, N.J.. Acrylamide, bisacrylamide, ammonium persulfate and TEMED were supplied by Biorad Laboratories, Richmond, Calif., The electrophoresis apparatus including the vertical column, casting rack, and constant-voltage power-pak were provided by the E-C Apparatus Corporation, St. Petersburg, Florida.

Bentonite was obtained from Fisher Scientific Co., Fair Lawn, N.J.. Diethylpyrocarbonate was purchased from Aldrich Chemical Co., Milwaukee, Wis., and sodium thioglycolate from Sigma Chemical Co., St. Louis, Mo..

Glutaraldehyde was obtained from Ladd Research Industries, Burlington, Vermont, and osmium tetroxide was purchased from BDH Chemicals, Toronto, Ontario.

RESULTS

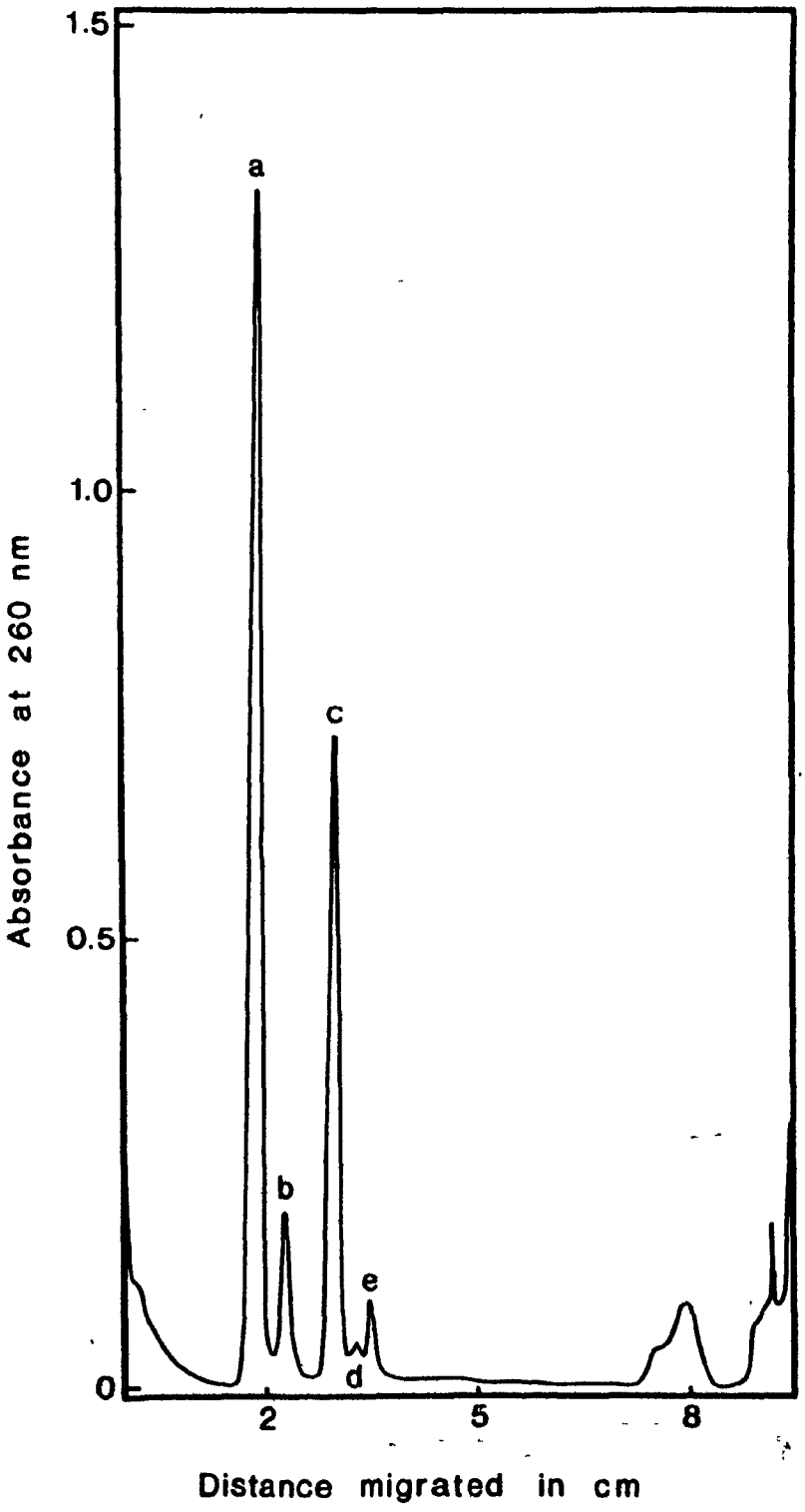
A. Ribosomal RNA in Ochromonas danica

1. Isolation of intact RNA

Cells of Ochromonas were harvested by centrifugation and extracted with an emulsion of Tris-acetate buffer and phenol-chloroform containing 0.5% Sarkosyl. The aqueous phase was re-extracted three times with phenol-chloroform and the RNA precipitated in ethanol overnight. The entire extraction procedure was performed in the cold (0-4° C). The RNA was redissolved in electrophoresis buffer and separated on agarose-acrylamide gels. A representative electrophoretic profile of RNA from light-grown cells appears in Figure 2. Five peaks of rRNA (designated a, b, c, d and e) as well as another peak containing 4S transfer RNA and 5S RNA are resolved. A major portion of this study was devoted to demonstrating the integrity of the high molecular weight rRNA species and their subcellular origin.

The isolation of intact plant rRNAs, particularly the heavy chloroplast rRNA species, is difficult due to their extreme susceptibility to nucleases. The extent of RNA degradation was so great in earlier studies that the absence of heavy chloroplast rRNA was reported in several plant tissues (Spencer and Whitfield, 1966; Woodcock and Bogorad, 1970). More recently the use of inhibitors of ribonuclease activity has allowed the improvement of plant RNA preparations. Results of our initial attempts to isolate rRNA from Ochromonas indicated the potential existence

Figure 2. Representative electrophoretic profile of nucleic acids extracted from light-grown cells of Ochromonas danica at 0-4° C. Samples containing 10-12 µg RNA were applied to 0.5% agarose - 2.4% acrylamide gels and electrophoresis was for 3 hr at 0-4° C. Five peaks of high molecular weight rRNA (designated a, b, c, d, and e) are resolved. The rapidly-migrating 4S and 5S RNAs appear as a single peak on the far right of the electropherogram.


1.5
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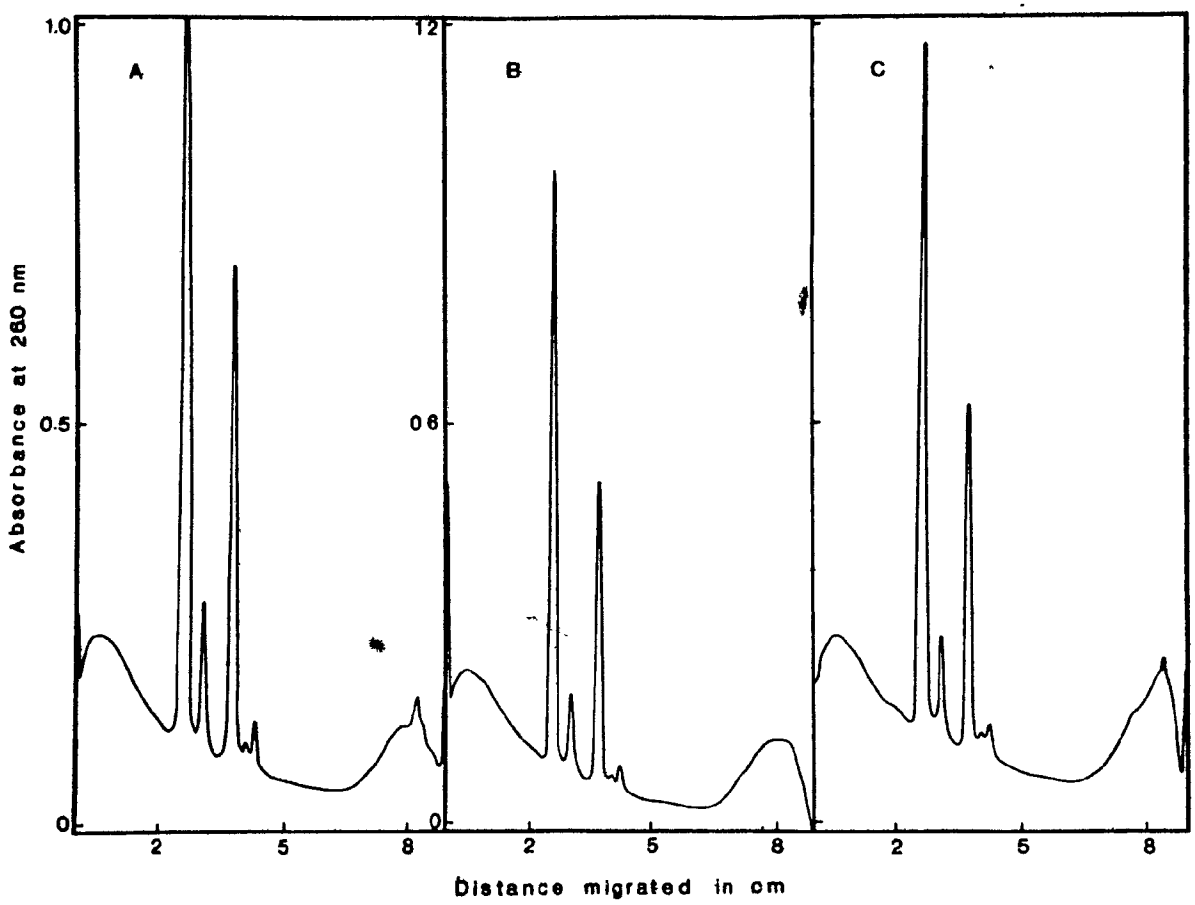
Absorbance at 260 nm
a
b
c
d
e
2
5
8
Distance migrated in cm

of RNA degrading enzymes in this organism. The extraction method eventually developed, however, does not require the inclusion of inhibitors of ribonuclease activity. This was demonstrated by testing the effects of several ribonuclease inhibitors on Ochromonas rRNA. The inhibitors each prevent ribonuclease activity by a different mechanism. Bentonite is a montmorillite clay, $Al_2O_3.4SiO_2.H_2O$, which adsorbs metal ions and basic proteins such as ribonuclease (Singer and Fraenkel-Conrat, 1961). Figure 3A shows that the presence of 50 µg/ml bentonite during extraction does not alter the electrophoretic profile of Ochromonas rRNA. Diethylpyrocarbonate (DEP) inactivates nucleases by its ability to irreversibly denature proteins (Rosén and Fecorcsák, 1966; Solymosy et al., 1968). Since it decomposes rapidly in aqueous solution to ethanol and carbon dioxide, a few drops of DEP were added to saturate the buffer just prior to extraction of RNA. As shown in Figure 3B, however, the electrophoretic pattern of rRNA isolated in the presence of DEP is the same as in Figure 2. Sodium thioglycolate, a reducing agent, inactivates ribonuclease by disrupting disulfide bridges in the native enzyme. Figure 3C shows that, even at 30 mg/ml, sodium thioglycolate does not alter the electrophoretic pattern of Ochromonas rRNA.

The possibility of endogenous ribonuclease activity during the initial stages of extraction was also examined by another method. Cells were first lyophyllized and RNA was isolated from the dry residue. This precaution reduces the susceptibility of RNA to enzymatic attack since

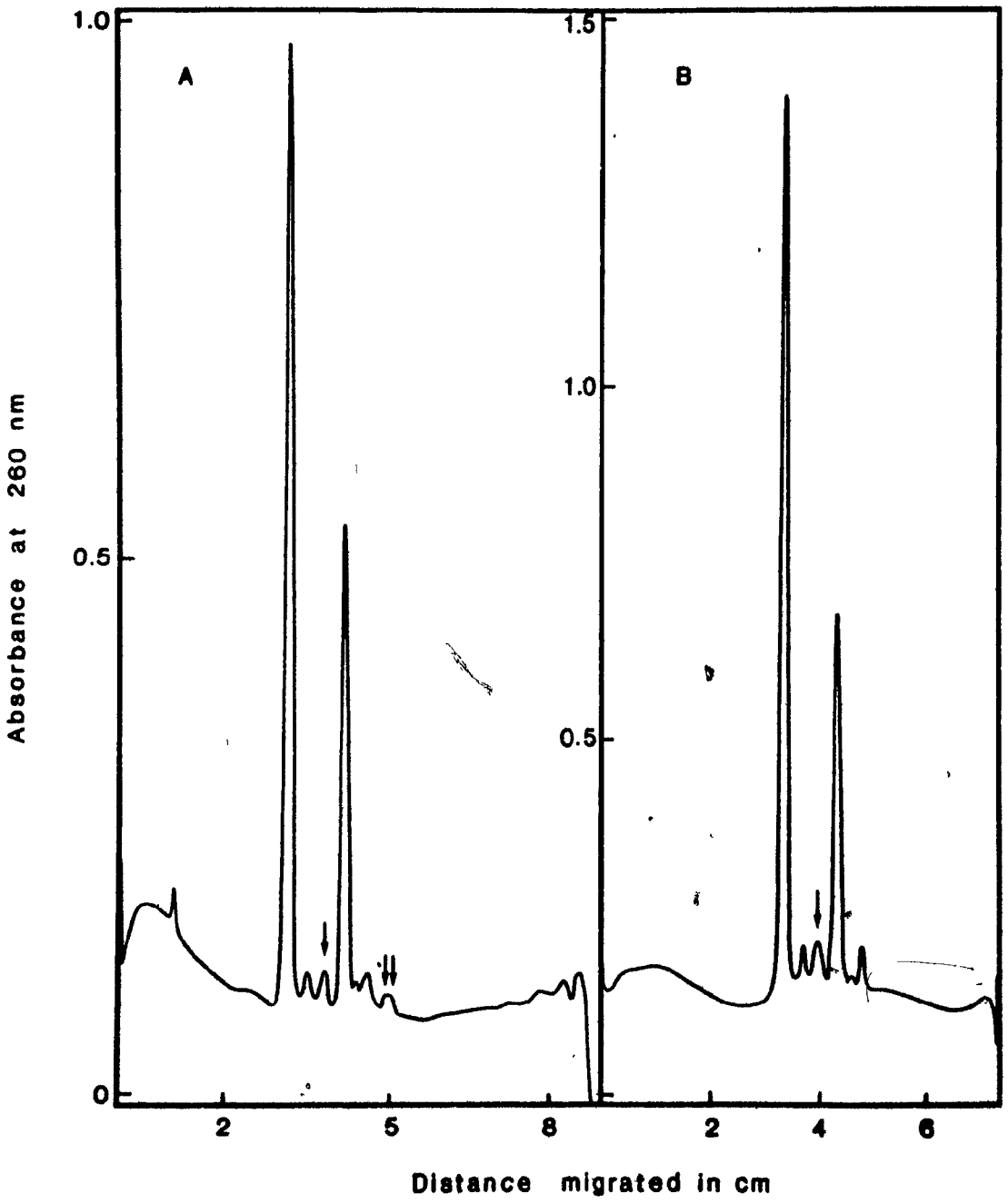
Figure 3. Effects of several RNase inhibitors on Ochromonas rRNA. Isolation of nucleic acids was carried out as described in Materials and Methods except that the inhibitor of RNase activity was included in the extraction buffer: A, 50 µg/ml bentonite; B, DEP; C, 30 mg/ml sodium thioglycolate. Background absorbance in these gels has not been completely removed.


A
B
C
1.0
0.5
0
12
06
0
2
5
8
Absorbance at 280 nm
Distance migrated in cm

the aqueous environment of ribonuclease is rapidly removed. No difference in the electrophoretic profile of Ochromonas RNA extracted from lyophyllized and intact cells is observed (data not shown). Thus variations in the extraction procedure such as addition of ribonuclease inhibitors to the isolation medium and lyophyllization of the cells failed to reduce the number of high molecular weight RNA species in the resulting extract when characterized on gels. These observations suggest that such efforts are not required to obtain undegraded rRNA from Ochromonas under the stated conditions.

Some experiments were therefore conducted to determine which parameters of the extraction method are critical to the isolation of intact rRNA. The maintenance of low temperature (0-4° C) is essential. When RNA is extracted at room temperature (20-22° C) and electrophoresed in the cold, the resulting profile indicates the presence of degraded rRNA: peak b is reduced with the concomitant appearance of a new component migrating between peaks b and c, as well as smaller fragments migrating slightly faster than the RNA species associated with peak e (Figure 4A). A similar pattern results when RNA is extracted at room temperature in the presence of 25 mM magnesium chloride, except that the smaller fragments are not observed (Figure 4B). When RNA extracted in the cold is electrophoresed at room temperature, a characteristically different pattern is observed. Figure 5 shows that no discrete components are present in the region of peak b, while breakdown products heterogeneous in size are indicated by the appearance of broad shoulders

Figure 4. Isolation of Ochromonas rRNA at room temperature in A, extraction buffer containing no magnesium; and B, extraction buffer containing 25 mM magnesium chloride. Arrows indicate components which are not observed in samples extracted in the cold.


1.0
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A
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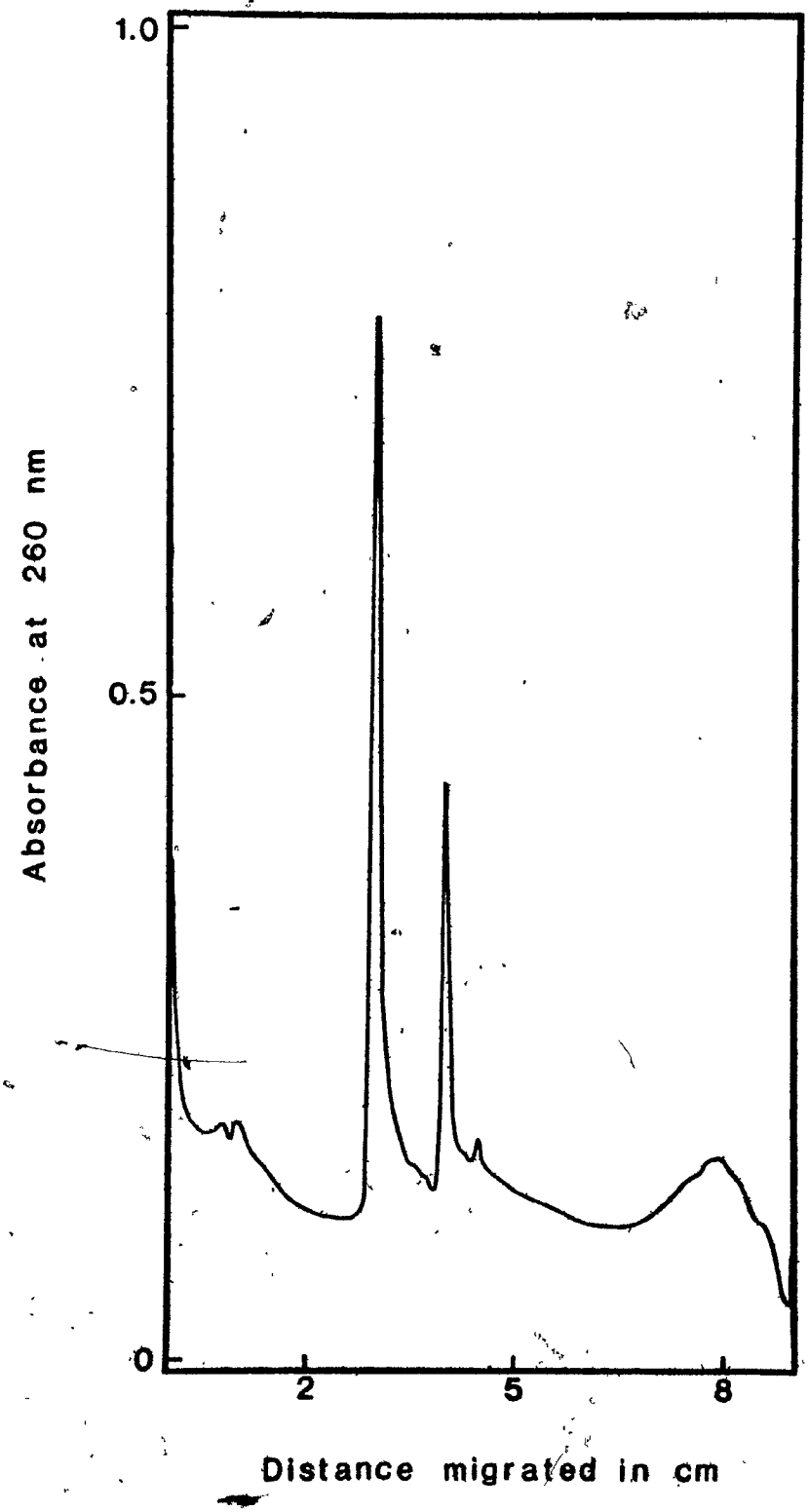
1.0
0.5
B
Absorbance at 260 nm
2
5
8
2
4
6
Distance migrated in cm

Figure 5. Electrophoresis of Ochromonas rRNA at room temperature. Extraction of the RNA was carried out at 0-4° C.


1.0
0.5
0
Absorbance at 260 nm
2
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8
Distance migrated in cm

associated with peaks a and e. The rRNA species represented by peak b is thus less stable than the remaining species since the relative amounts of peaks a, c, e, and probably also d appear unaltered when the rRNA is extracted or electrophoresed at room temperature.

Efficiency of extraction was tested by two methods. (^{3}H)-Uridine labelled RNA from sea urchin embryos (Lytichinus pictus) was added to light and dark-grown cells of Ochromonas and the amount of radioactivity in each sample compared before and after extraction of RNA. The data presented in Table II shows that over 75% of the counts are recovered. That significant amounts of RNA are not lost during the isolation procedure by entrapment in the thick interface usually formed in the first phenol extraction was demonstrated by re-extracting the initial phenol phase and interface with detergent-buffer. When this material was analyzed on gels, very little absorbance was detected in the region of high molecular weight rRNA. Considering that 100% recovery of RNA can never be achieved by sequential phenol extraction (a fraction of the aqueous phase is inevitably lost at each successive extraction), these observations indicate an efficient extraction procedure was used to isolate Ochromonas RNA.

A factor which influences the efficiency of extraction is the order in which the detergent-buffer and phenol-chloroform are added to the cells. In preliminary experiments cells were suspended first in detergent-buffer and then phenol-chloroform was added to the lysate. When the aqueous and organic solutions are added simultaneously, however,

Table II. Efficiency of Extraction of Ochromonas RNA

(^{3}H)Uridine labelled RNA from sea urchin embryos was added to light- and dark-grown cells of Ochromonas and the amount of radioactivity in each sample compared before and after extraction of RNA.

Source of RNA	(^{3}H) cpm in 100λ	
	Cell lysate	Final aqueous phase
Light-grown cells	691	533
Dark-grown cells	799	803

as in all studies reported here, the yield of nucleic acid from comparable numbers of cells is approximately 50% greater. The electrophoretic profile of nucleic acid isolated by the former method is seen in Figure 6. A reproducible peak containing a high molecular weight fraction assumed to be DNA was observed in these preparations; in contrast, this peak is absent from samples extracted by the method chosen for this study (cf. Figure 2).

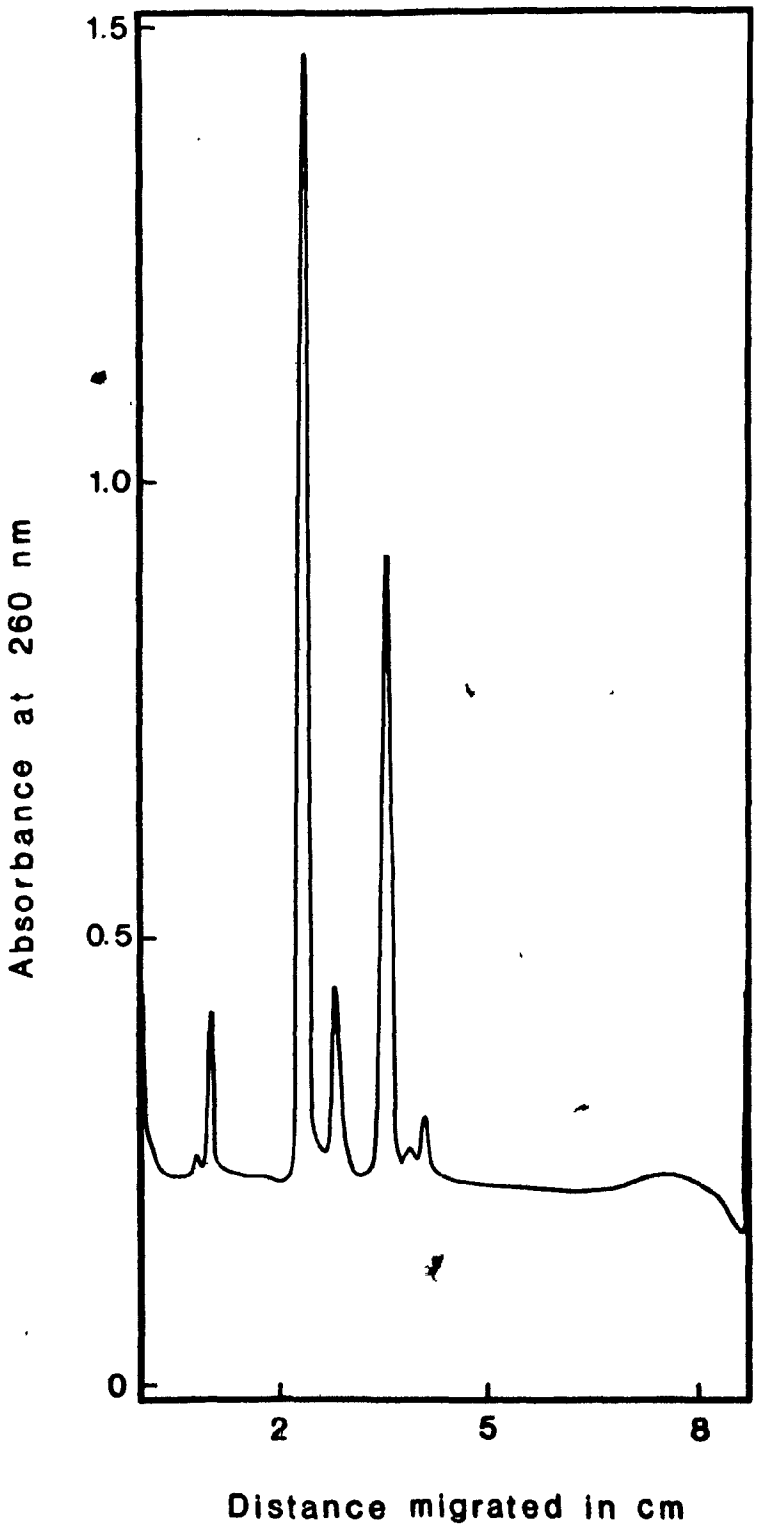
Results of alkaline hydrolysis of the extracted nucleic acids showed that RNA was preferentially extracted by this procedure. A preparation of nucleic acids from Ochromonas was incubated in 0.3 NaOH at 37° C for 21 hr. As can be seen in Figure 7, no high molecular weight material is present in the sample after this treatment. Since DNA, but not RNA, is alkali-resistant, this observation indicates that the nucleic acid extracted from Ochromonas by this method is almost exclusively RNA.

2. Characterization of Ochromonas rRNA

a. Cellular origin of rRNA species

The relative abundance of ribosomes in the chloroplast, mitochondria and cytoplasmic compartments of Ochromonas has been observed by electron microscopy (Smith-Johannsen and Gibbs, 1972). These studies show that cytoplasmic ribosomes predominate in the cell. Therefore peaks a and c were assumed to contain the heavy and light cytoplasmic rRNA respectively, since they comprise the bulk of total Ochromonas RNA. A comparison of RNA from dark and light-grown cells shows clearly that peaks b and e are reduced in the RNA sample from

Figure 6. Effect of the sequential, rather than simultaneous, addition of extractants on the electrophoretic profile of the resulting nucleic acid preparation. Cells were first suspended in detergent-buffer and then phenol-chloroform was added to the lysate.


1.5
1.0
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Absorbance at 260 nm
2
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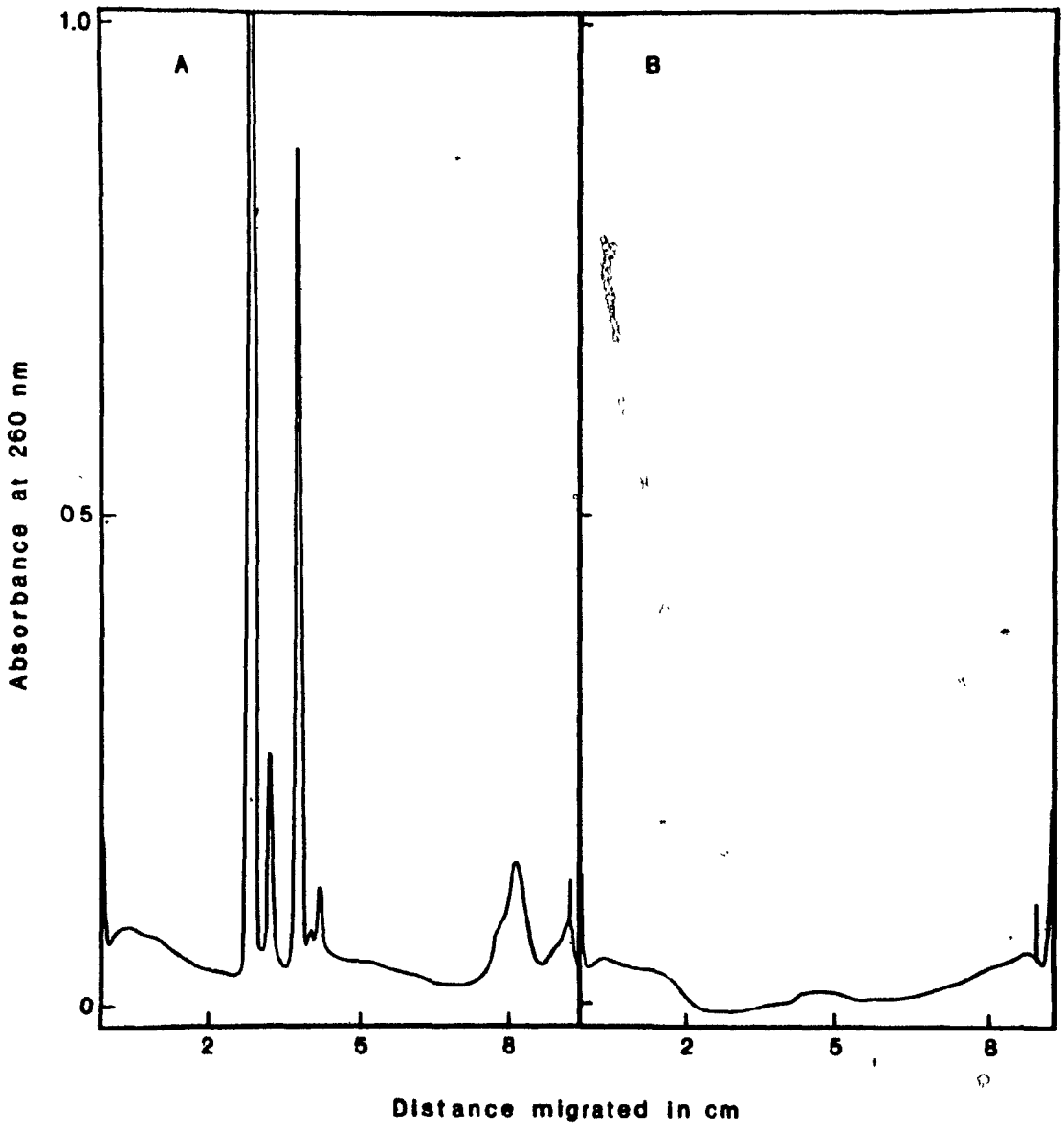
Distance migrated in cm

Figure 7. Alkaline hydrolysis of nucleic acids extracted from light-grown cells of Ochromonas. A, control, kept on ice; B, incubated in 0.3 N NaOH at 37° C for 21 hr.


1.0
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A
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Absorbance at 260 nm
2
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2
5
8
Distance migrated in cm

dark-grown cells (cf. Figure 8A with Figure 2). Cells grown in the dark are characterized by a small proplastid which contains relatively few plastid ribosomes. During greening the number of chloroplast ribosomes per cell increases more than five fold, a much greater change than that occurring in the ribosome number in either mitochondria or the cytoplasm (Smith-Johannsen and Gibbs, 1972). This suggests that peaks b and e represent heavy and light chloroplast rRNA respectively. Mitochondrial rRNA probably accounts for the remaining absorbance peaks occurring in the high molecular weight region of the electrophoretic profile of Ochromonas RNA. To demonstrate the presence of mitochondrial rRNA directly, however, cells were treated with ethidium bromide which is known to interfere with mitochondrial rRNA synthesis (Zylber et al., 1969). RNA extracted from dark and light-grown cells exposed to 1 µg/ml ethidium bromide for 20 hr and 24 hr respectively is analyzed in Figures 8B and 9B. Peaks b and d are markedly reduced in samples from drug-treated cells compared to controls (Figures 8A and 9A). Also, a high proportion of organelle rRNA is ethidium bromide-sensitive in dark-grown cells, which contain almost twice as many mitochondrial as plastid ribosomes.[1] These two lines of evidence strongly suggest that when Ochromonas RNA is subjected to electrophoresis, the heavy and light chloroplast rRNAs migrate as peaks b and e respectively, and the heavy and light mitochondrial rRNAs as peaks b and d respectively. Thus peak

1 Calculated from data in Gibbs, 1968; and Smith-Johannsen and Gibbs, 1972.

Figure 8. Effect of ethidium bromide on rRNA in dark-grown cells of Ochromonas. A, control; B, RNA extracted from cells grown in the presence of 1 µg/ml ethidium bromide for 20 hr.

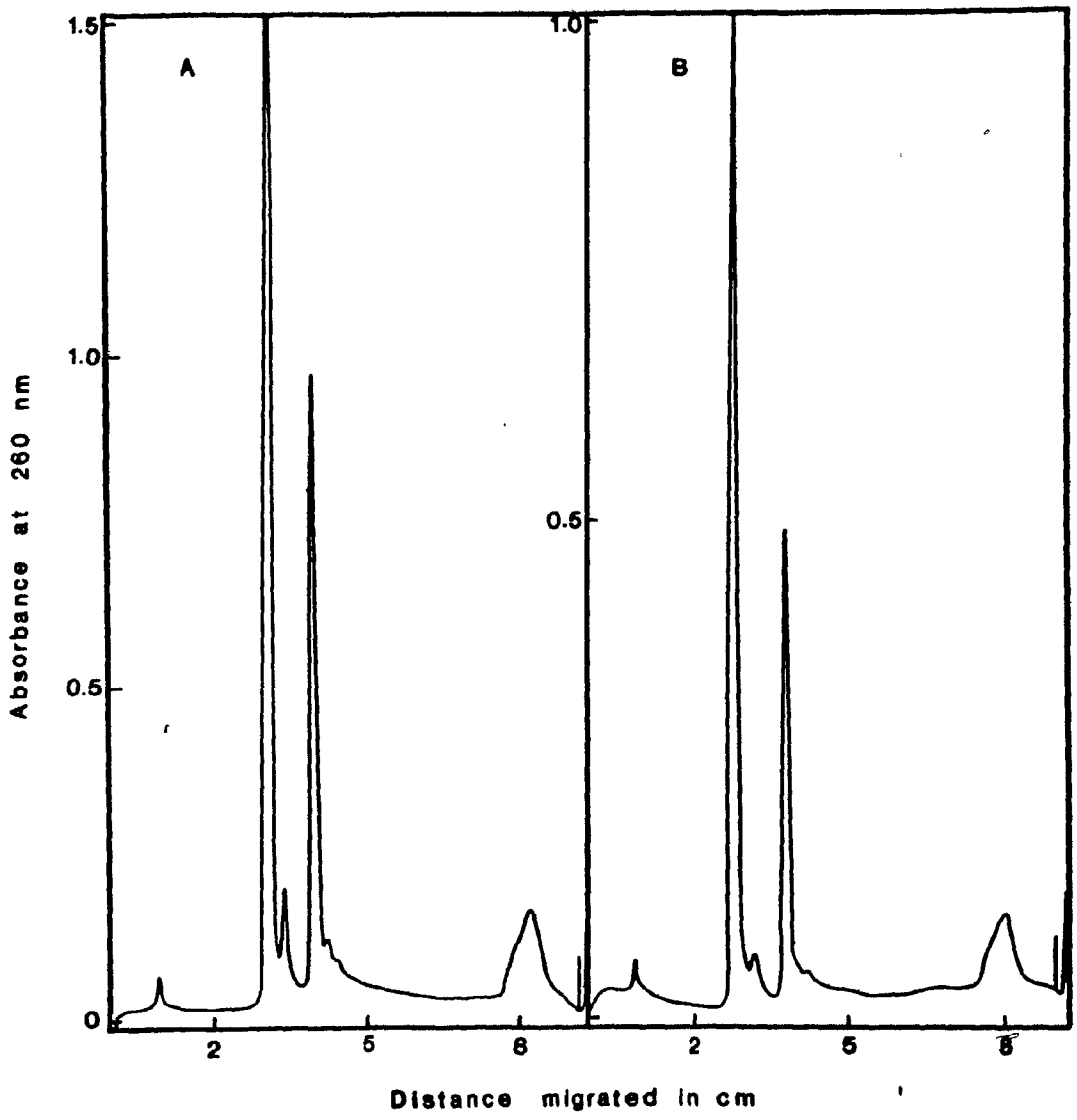
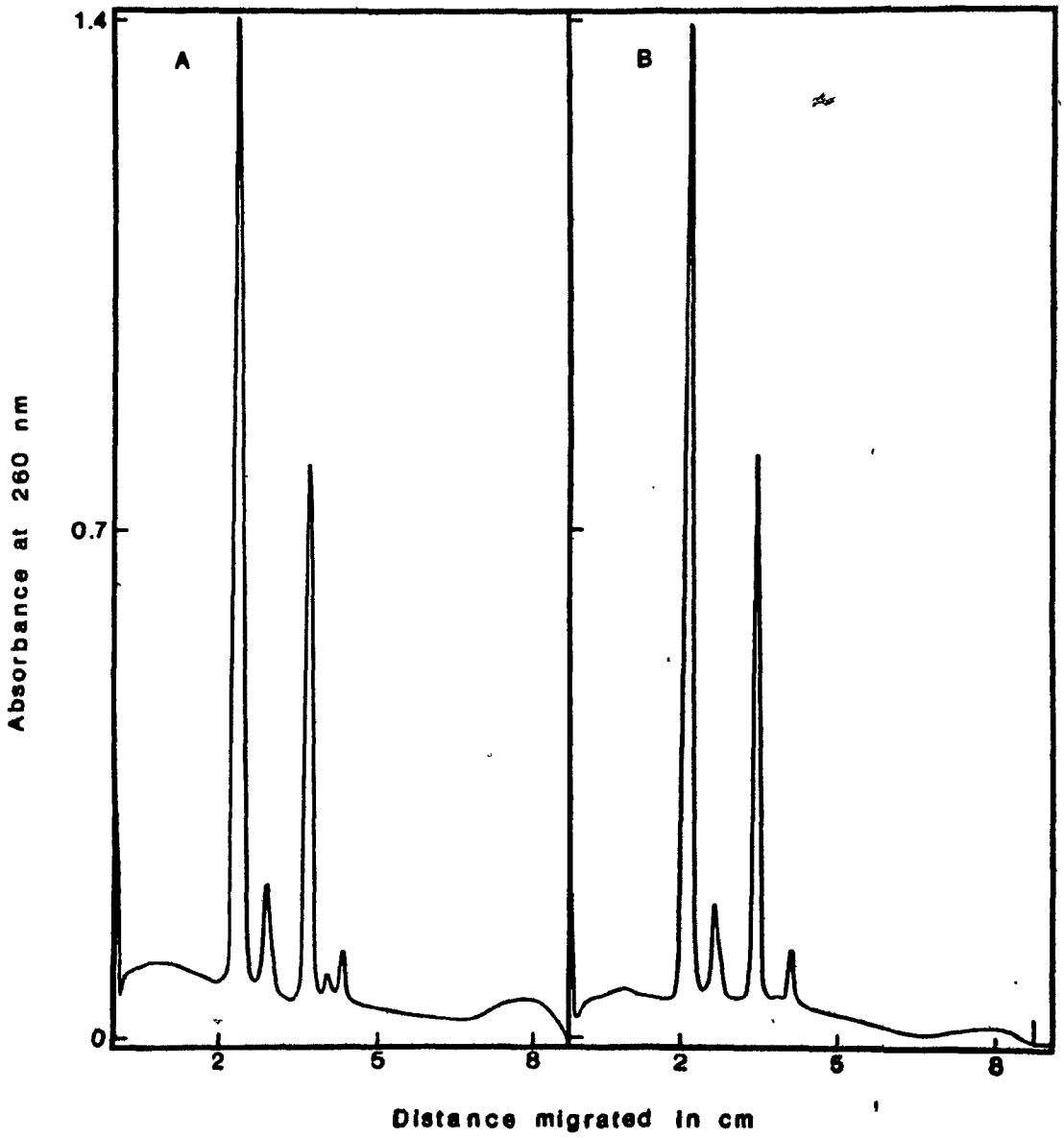

1.5
1.0
0.5
0
A
B
1.0
0.5
Absorbance at 260 nm
2
5
8
2
5
8
Distance migrated in cm

Figure 9. Effect of ethidium bromide on rRNA in light-grown cells of Ochromonas. A, control; B, RNA extracted from cells exposed to 1 µg/ml ethidium bromide for 24 hr.


1.4
0.7
0
A
B
Absorbance at 260 nm
2
5
8
2
5
8
Distance migrated in cm

b is a composite of heavy rRNA from both organelles.

b. Sizes of rRNA species

In order to establish the sizes of Ochromonas rRNA species, (^{3}H)uridine labelled RNA from light-grown cells was co-electrophoresed with (^{14}C)uracil labelled RNA from E. coli. The resulting absorbance and radioactivity distribution profiles are presented in Figure 10. E. coli 23S rRNA migrates faster than the algal heavy cytoplasmic rRNA component and more slowly than the organelle heavy rRNA species. E. coli 16S rRNA appears to be coincident with Ochromonas light mitochondrial rRNA while the light chloroplast rRNA speices migrates slightly faster. For this range of molecular size and the concentration of gel used, the sedimentation constants of RNA components exhibit an inverse linear relationship to their mobilities in gels (Loening and Ingle, 1967). Loening (1968) has also shown empirically that the relationship between the logarithm of molecular weight and electrophoretic mobility is linear. The sizes of Ochromonas rRNA species were therefore determined by plotting either sedimentation constants or logarithms of molecular weight against the distance migrated in the gel by the E. coli rRNA markers. The values for the absolute molecular weights of E. coli 23S and 16S rRNA are 1.07×10^{6} and 0.55×10^{6} daltons respectively (Rodgers, 1974). The results presented in Table III are similar to other values for plant rRNA components reported in the literature.

3. Lability of heavy chloroplast rRNA

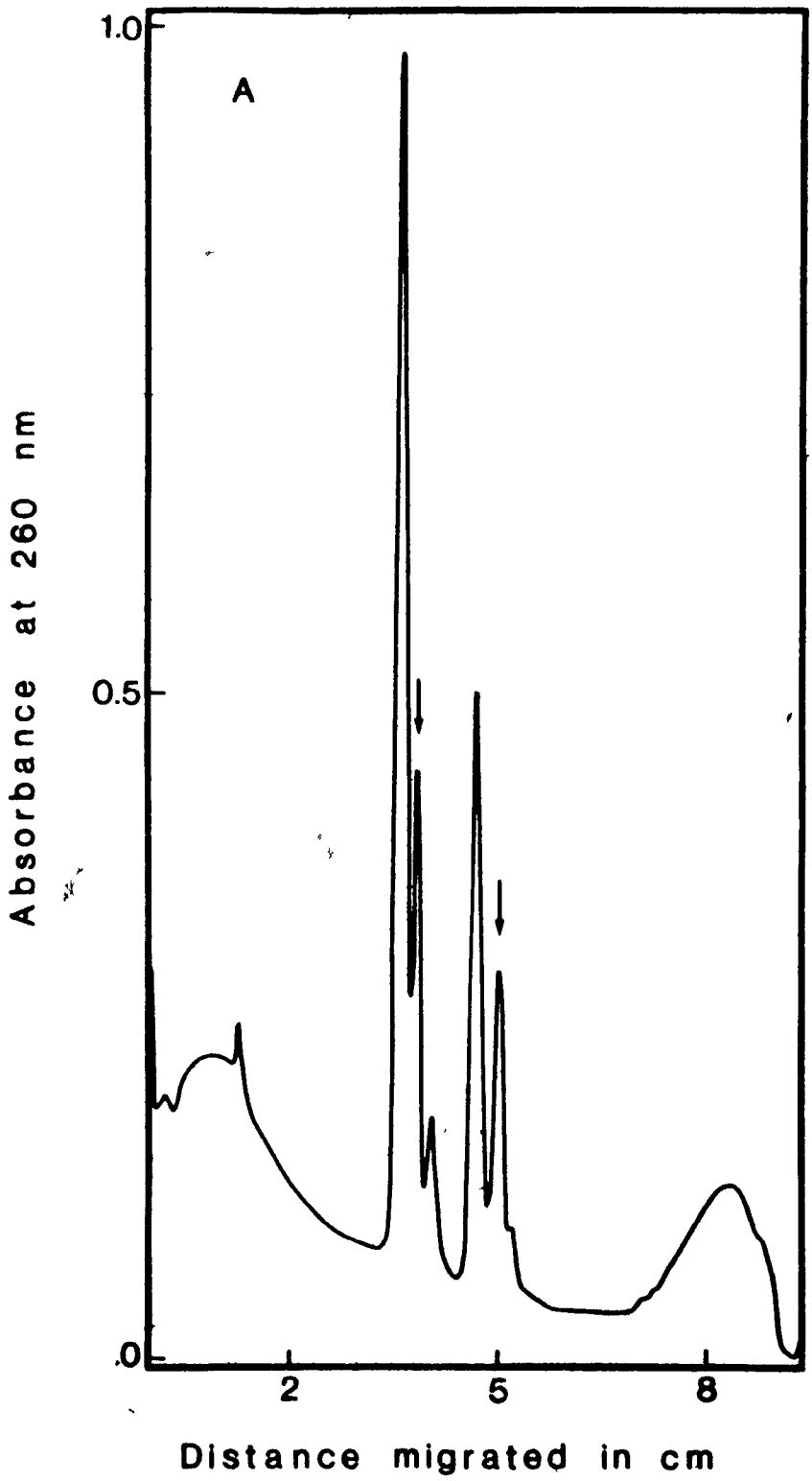
Ochromonas high molecular weight rRNA is resolved into five

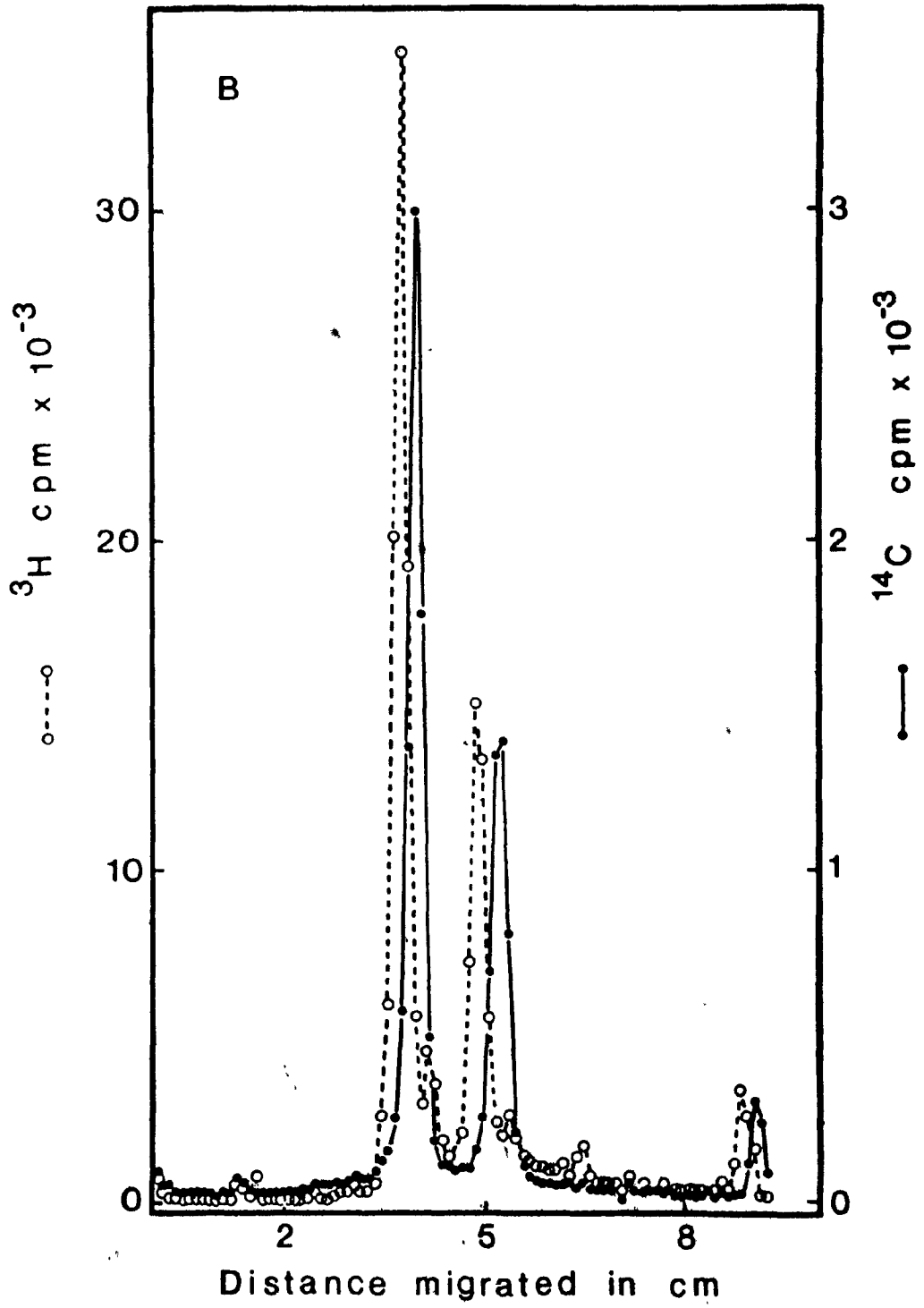
Table III. Sizes of Ochromonas rRNAs

(^{3}H)Uridine labelled RNA from light-grown cells was co-electrophoresed with (^{14}C)uracil labelled RNA from E. coli. The sizes of Ochromonas rRNA were determined by plotting either sedimentation constants or logarithms of molecular weight against the distance migrated in the gel by the E. coli rRNA markers. The values for the absolute molecular weights of heavy and light E. coli rRNAs are 1.07×10^6 and 0.55×10^6 daltons, respectively (Rodgers, 1974).

Species of rRNA		Sedimentation coefficient	Molecular weight (daltons)
Cytoplasmic	heavy	24S	1.18×10^6
Cytoplasmic	light	18S	0.66×10^6
Chloroplast	heavy	22S	0.94×10^6
Chloroplast	light	15S	0.50×10^6
Mitochondrial	heavy	22S	0.94×10^6
Mitochondrial	light	16S	0.55×10^6

Figure 10. Co-electrophoresis of (^{3}H)uridine labelled RNA from light-grown cells of Ochromonas and (^{14}C)uracil labelled RNA from E. coli. A, the ultraviolet absorbance and B, radioactivity distribution profiles of one of two replicate gels are shown. Arrows indicate the absorbance peaks of heavy and light E. coli rRNAs.


1.0
A
0.5
.0
Absorbance at 260 nm
2
5
8
Distance migrated in cm


B
30
20
10
0
3
2
1
0
2
5
8
^{3}H cpm x 10^{-3}
^{14}C cpm x 10^{-3}
Distance migrated in cm

distinct peaks when it is subjected to gel electrophoresis, whereas only four rRNA species have been found in total RNA extracts of other green plant tissues. Since the lability of the heavy chloroplast rRNA species is well documented (Whitfield, 1973), the possibility that the unique electrophoretic profile of Ochromonas RNA may be due to degradation had to be investigated. If one or more of the five rRNA species were the product of incipient breakdown, then subjecting the RNA to stress might augment peak(s) containing the degraded fragment(s). Experiments were conducted to determine the pattern of RNA breakdown induced by heat. The electrophoretic profile of RNA from light-grown cells incubated at 37° C for 10 min and rapidly cooled is shown in Figure 11B. Peak b is reduced compared to the control kept on ice (Figure 11A) and two new peaks representing RNA smaller than 15S are observed. A slight increase in absorbance in the region of mitochondrial 16S rRNA is also detected, whereas no change is apparent in the amount of light chloroplast rRNA species. The magnitude of the decrease in the size of peak b was measured and is comparable to the amount of ultraviolet-absorbing material represented by the two new peaks, suggesting that the latter may be fragments derived from the larger heat-labile RNA species associated with peak b. Incubation of Ochromonas RNA at 54° C (Figure 12A), however, does not result in a further decrease in peak b indicating the presence of a heat-stable 22S component. RNA from dark-grown cells which contain relatively little chloroplast rRNA was also exposed to 37° C for 10 min. In the electrophoretic profile shown in Figure 13B, there is only a slight

loss of absorbance in peak b, compared to the control in Figure 13A. These observations suggest that the heat-labile component in peak b is the heavy chloroplast rRNA molecule and that the heat-resistant rRNA is mitochondrial. Furthermore, the mode of induced breakdown of the chloroplast rRNA argues against the likelihood that one or more of the five species characteristic of Ochromonas rRNA arise by degradation.

Leaver (1973) has shown that the integrity of the heavy chloroplast rRNA molecule in Vicia faba (broad bean) is maintained at elevated temperatures in the presence of adequate concentrations of magnesium. The integrity of Ochromonas rRNA is also preserved by magnesium. Little or no breakdown occurs at 37° C when 10 mM magnesium is added to the RNA sample (Figure 11C). At 54° C, however, 10 mM magnesium is only partially effective (Figure 12B). The RNA molecules migrating slightly ahead of the 22S species probably represent an intermediate stage in the conversion of the heavy chloroplast rRNA into the characteristic low molecular weight products. When the concentration of magnesium is increased to 20 mM, protection of the RNA even at 54° C appears to be restored (Figure 12C). Unfortunately the presence of high levels of magnesium also results in poorer resolution during electrophoresis. The question arose whether the protective effect displayed by magnesium is due to stabilization of RNA during heat stress or to reassociation of the broken molecules when cooled. Similar amounts of Ochromonas RNA were incubated at 37° C for 5 min in the absence of magnesium, and then magnesium was added to 10 or 20 mM. The

Figure 11. Effect of magnesium on the stability of rRNAs. Total RNA was extracted from light-grown cells of Ochromonas and replicate samples were A, kept on ice; B, incubated at 37° C for 10 min; or C, incubated at 37° C for 10 min in the presence of 10 mM magnesium chloride.

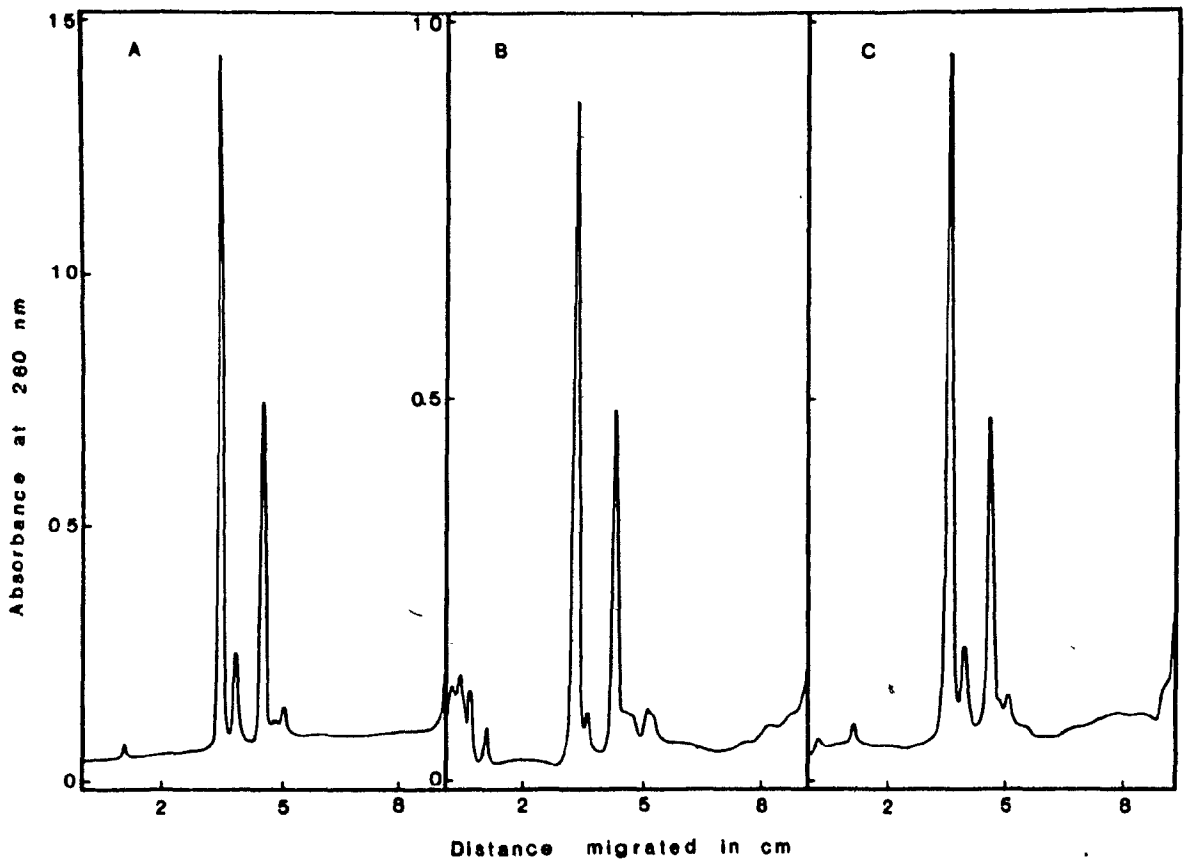

A
B
C
1.5
1.0
0.5
0
1.0
0.5
0
Absorbance at 280 nm
2
5
8
2
5
8
2
5
8
Distance migrated in cm

Figure 12. Effect of magnesium on the stability of rRNAs. Equal amounts of RNA from light-grown cells were heated at 54° C for 10 min A, in the absence of magnesium and B, in the presence of 10 mM magnesium chloride or C, 20 mM magnesium chloride. The absorbance peaks indicated by the arrow in A probably represents an aggregate of rRNA.

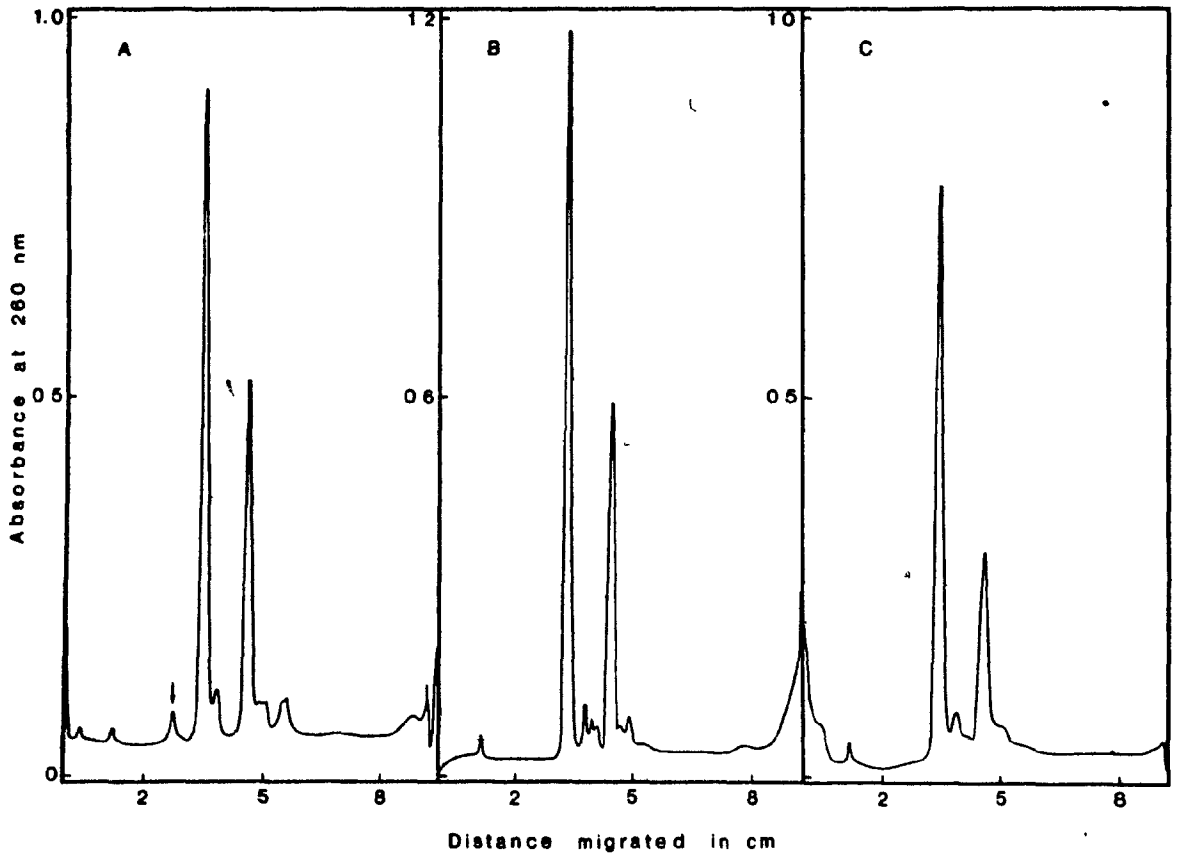
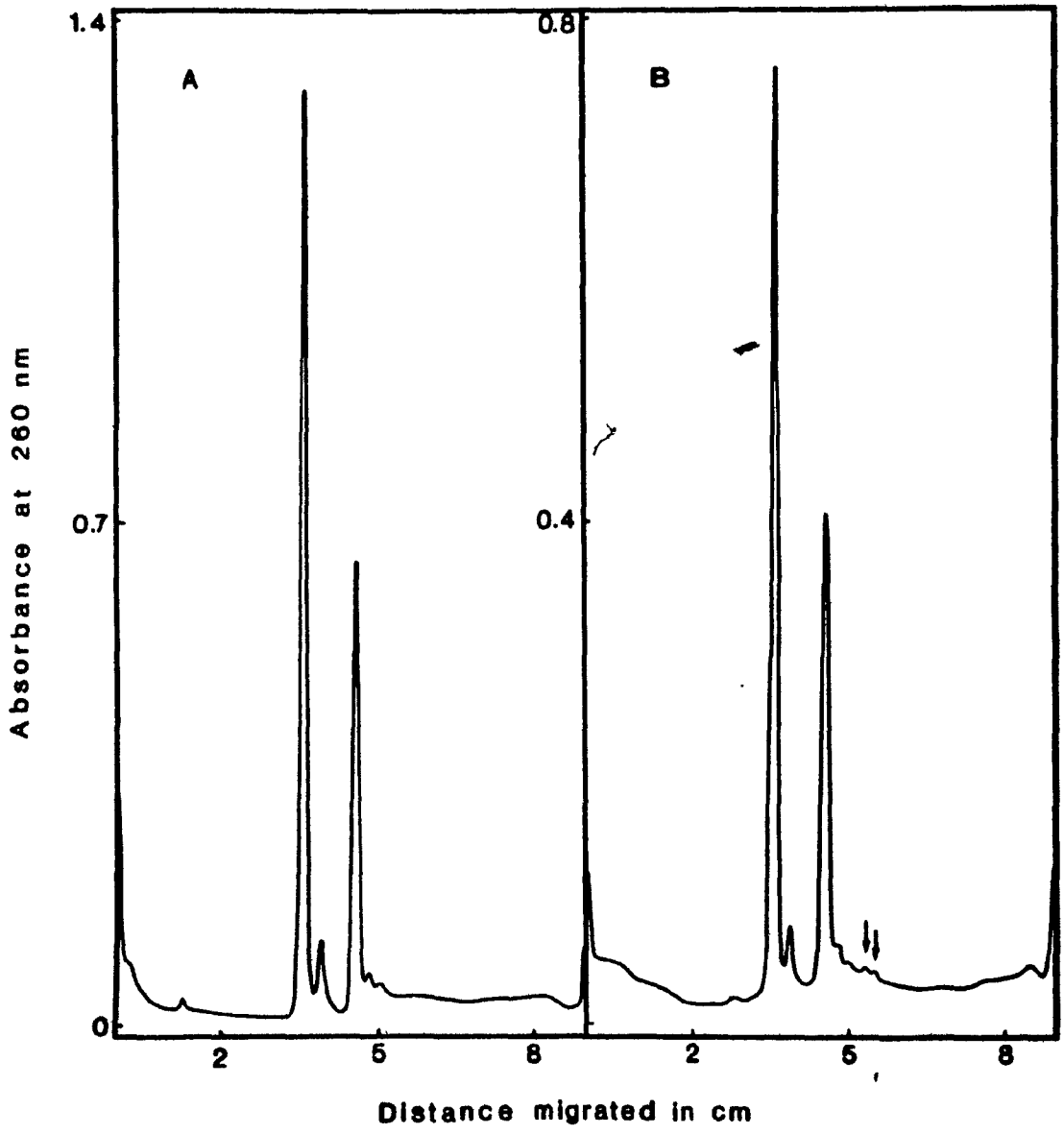

A
B
C
1.0
0.5
0
1.2
0.6
1.0
0.5
Absorbance at 280 nm
2
5
8
Distance migrated in cm

Figure 13. Thermolability of rRNA from dark-grown cells. A, control, kept on ice; B, incubated at 37° C for 10 min. Arrows indicate positions of breakdown products.


1.4
0.7
0
A
B
0.8
0.4
Absorbance at 260 nm
2
5
8
2
5
8
Distance migrated in cm

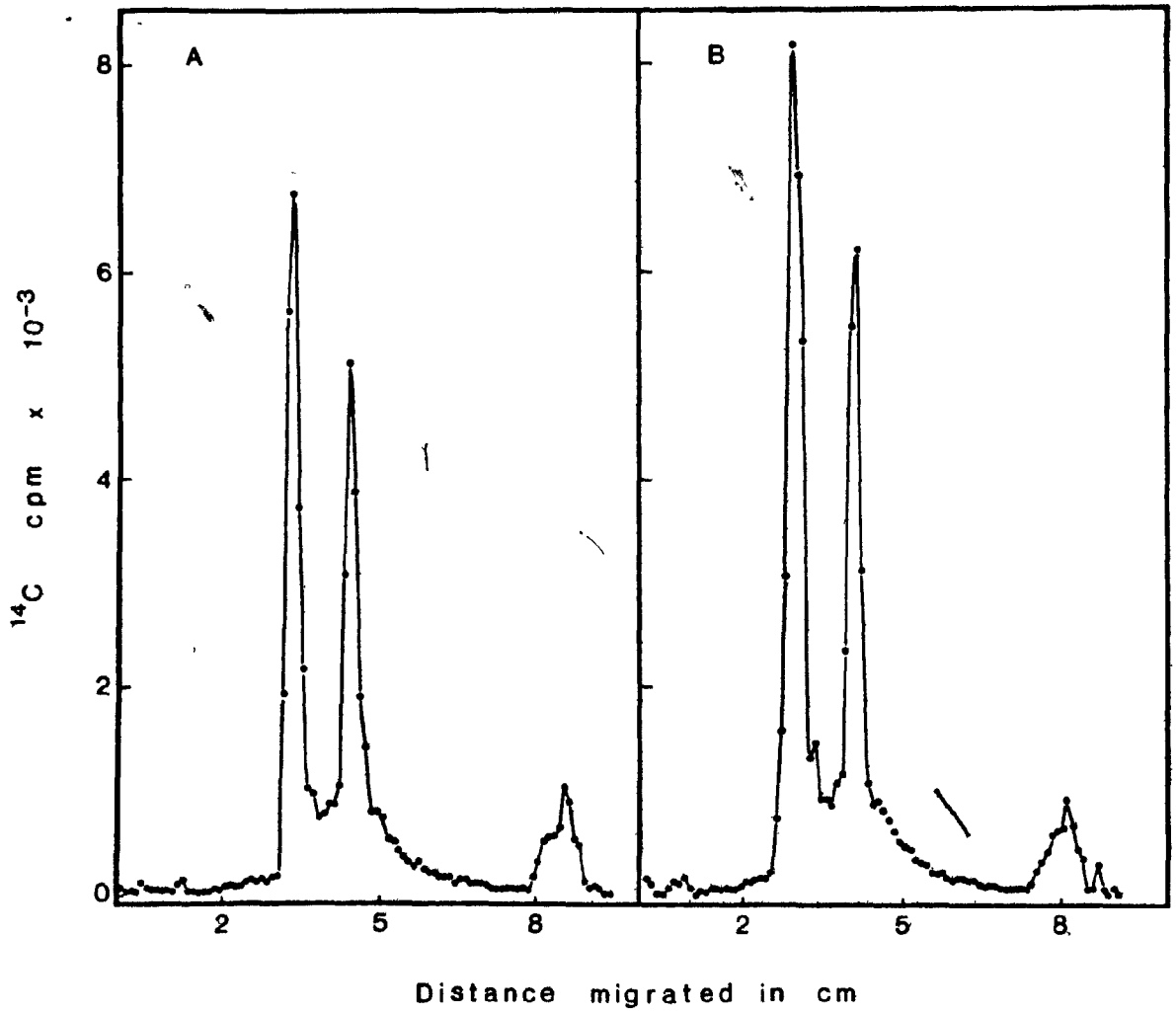
samples were allowed to stand at either room temperature or on ice for 10 min prior to electrophoresis. No protective effect was observed and all samples were equally degraded (data not shown).

The breakdown of the heavy chloroplast rRNA caused by heating may be explained by the presence of one or more non-covalent associations between ribonucleotide chains which are then liberated under conditions interfering with hydrogen bonding. Alternatively, a heat-activated, magnesium-inhibited ribonuclease may persist in these preparations of Ochromonas RNA. To test the possibility of residual ribonuclease activity, (^{14}C)uracil-labelled RNA from E. coli was mixed with unlabelled Ochromonas RNA from light-grown cells at ratios of 1:1, 1:2, and 1:3 and incubated at 37° C for 5 min. The control shown in Figure 14A illustrates the stability of E. coli RNA at 37° C. Addition of an equal amount of Ochromonas RNA to the incubation mixture does not alter the electrophoretic profile of the E. coli RNA (Figure 14B). The samples containing larger amounts of Ochromonas RNA also revealed no evidence of degradation of E. coli RNA. These results suggest that the algal RNA preparations are ribonuclease-free. However, it should be added that RNase may be present, but not active on RNA from E. coli.

4. Incorporation of isotopes

Previous studies on Ochromonas (Gibbs, 1968) and higher plants (Ingle et al., 1970) have demonstrated that light stimulates the synthesis of chloroplast rRNA in greening tissues. The use of radioisotopes provides a sensitive technique for measuring the rates of synthesis of macromolecular components such as nucleic acid. Therefore, preliminary

Figure 14. Stability of E. coli RNA in the presence of RNA from Ochromonas. (^{14}C)Uracil labelled RNA from E. coli was incubated at 37° C for 5 min. A, alone and B, with an equal amount of unlabelled Ochromonas RNA.


A
B
8
6
4
2
0
^{14}C cpm x 10^{-3}
2
5
8
2
5
8
Distance migrated in cm

experiments were performed to determine the appropriate labelling conditions for Ochromonas. It was found that cells incubated in the presence of 20 μCi/ml (^{3}H)uridine incorporate radioactivity at a linear rate for at least 6 hours in the light. The RNA extracted from dark-grown and greening cells labelled for 6 hours with 20 μCi/ml (^{3}H)uridine was analyzed on two separate gels, each of which was also fractionated to permit determination of the distribution of radioactivity. The light chloroplast and mitochondrial rRNAs were not resolved in the radioactivity profiles (data not shown). Furthermore, because of the relatively small amounts of the light chloroplast and mitochondrial rRNAs, these species were not included in specific activity calculations. Therefore, peak b alone was used as a measure of organelle rRNAs. The specific activities of the cytoplasmic and organelle rRNAs from the dark-grown cells were approximately 1250 cpm/μg rRNA and 330 cpm/μg rRNA respectively. In contrast, the specific activity of the organelle rRNAs from the illuminated cells was more than 40-fold higher, while that of the cytoplasmic rRNA species was about 30-fold higher. The amounts of radioactivity associated with the cytoplasmic and organelle rRNAs was roughly proportional to the relative amounts of these rRNA species in both dark-grown and greening cells.

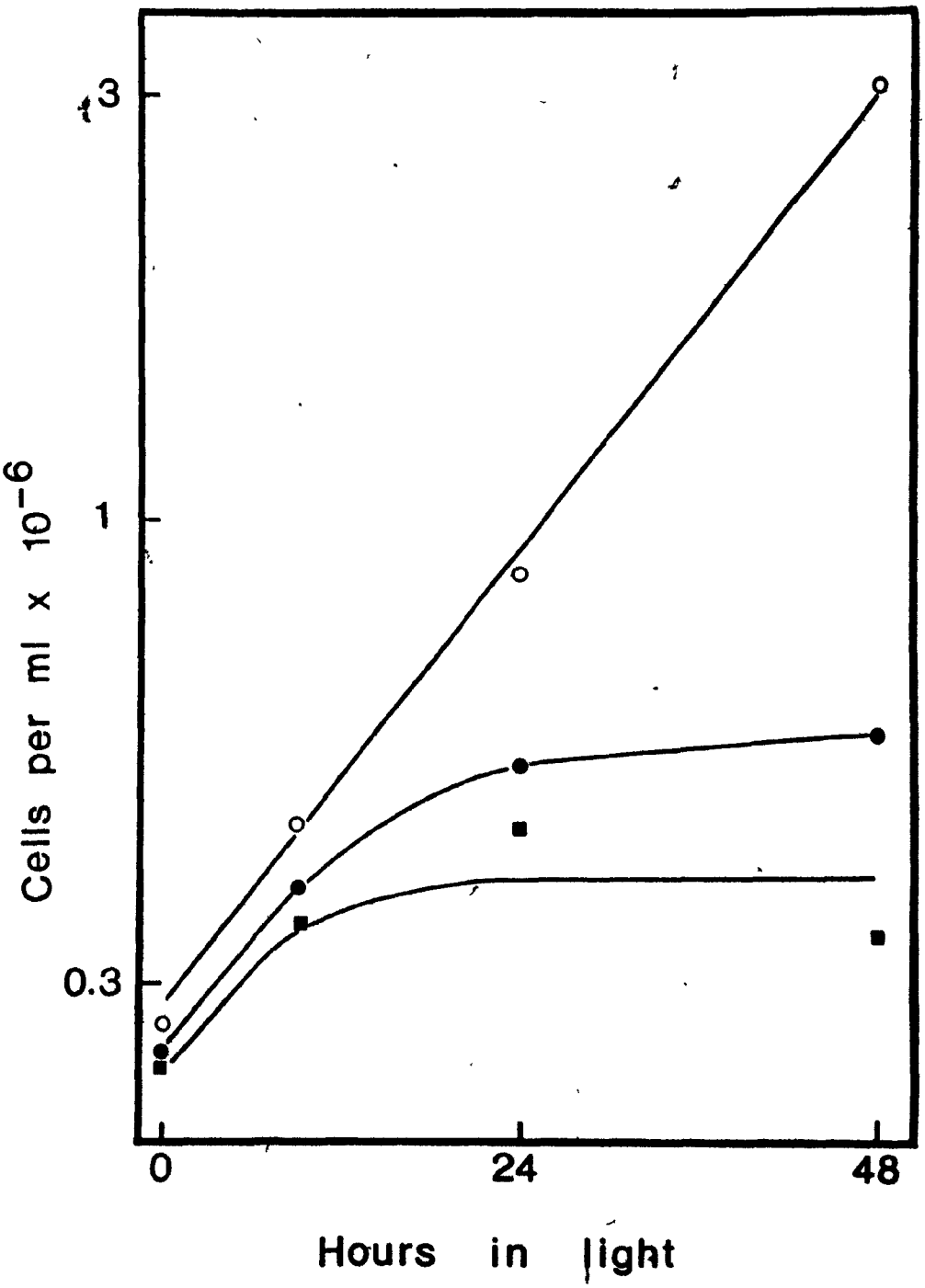
B. Antibiotic Studies

1. Chloramphenicol

In a previous ultrastructural study, I showed that the antibiotic D-threo chloramphenicol inhibits the light-induced synthesis of chloroplast ribosomes in Ochromonas (Smith-Johannsen and Gibbs, 1972). Results based on electron microscopy of fixed specimens may be influenced by artifacts such as the loss of certain cellular components. Therefore, a biochemical analysis of the effects of chloramphenicol on chloroplast rRNA synthesis was made to confirm the ultrastructural observations.

The effect of D-threo chloramphenicol and its isomer, L-threo chloramphenicol, on the growth rate of greening cells is seen in Figure 15. In the presence of 300 µg/ml of either chloramphenicol isomer, cells grow at normal rates during the first 9-12 hr in the light. After a 12 hr exposure to L-threo chloramphenicol, there is a gradual decline in the growth rate until 24 hr when little further cell division takes place. On the other hand, D-threo chloramphenicol causes an abrupt cessation of growth of greening cells after 12 hr. Since inhibition of cell division may in turn affect RNA metabolism, the control and chloramphenicol-treated cells were harvested for RNA extraction after only 12 hr light. Electrophoretic profiles of the RNA from control cells showed that the amount of the light chloroplast rRNA species (peak e) with respect to the light cytoplasmic rRNA species (peak c) is not significantly greater in the 12 hr greening cells than in dark-grown cells. Thus, at this early stage in greening, so little chloroplast rRNA has

Figure 15. Effect of D-*threo* and of L-*threo* chloramphenicol on the division rate of greening cells of *Ochromonas*. Each inhibitor was added at a concentration of 300 µg/ml to dark-grown cells just prior to illumination. Each point is an average of duplicate counts from an individual culture. Control cells, ○ - ○; cells treated with D-*threo* chloramphenicol, ■ - ■; cells treated with L-*threo* chloramphenicol, ● - ●.


3
1
0.3
Cells per ml x 10^{-6}
0
24
48
Hours in light

accumulated that the quantitation of the increase in the amount of these rRNA species in 12 hr greening cells lies within experimental error of measurement.

It was decided to extend the period of illumination and inhibitor treatment, despite the retardation of growth of cells exposed to either chloramphenicol isomer after 12 hr. By 24 hr greening, the ratio of chloroplast rRNA to cytoplasmic rRNA in control cells approaches a maximum. The electrophoretic profiles of RNA extracted from cells exposed to 24 hr light alone, to L-threo chloramphenicol and to D-threo chloramphenicol are shown in Figure 16. Peaks b and e, containing the heavy and light chloroplast rRNA species respectively, are considerably reduced in the electrophoretic profile of RNA from cells treated with D-threo chloramphenicol. In contrast, the relative amounts of the different rRNA species in the extract from L-threo chloramphenicol-treated cells are similar to those in the control. These results are presented quantitatively in Table IV which shows that, in the presence of D-threo chloramphenicol, the decrease in the amount of rRNA in peak e which is comprised exclusively of the light chloroplast rRNA species is about 70%. A reduction in the amount of the light mitochondrial rRNA species (peak d) in the presence of the D-threo isomer was also measured, but this is not statistically significant. Statistical analysis of the amounts of the various rRNA species in the L-threo chloramphenicol-treated cells confirmed the observation that, despite the inhibitory effect of this isomer on cell division after 24 hr, L-threo chloramphenicol does not

Figure 16. Effect of D-*threo* and of L-*threo* chloramphenicol on the accumulation of rRNAs during 24 hr greening in *Ochromonas*. Total RNA was extracted from A, control cells; B, cells exposed to 300 µg/ml L-*threo* chloramphenicol; and C, cells exposed to 300 µg/ml D-*threo* chloramphenicol.

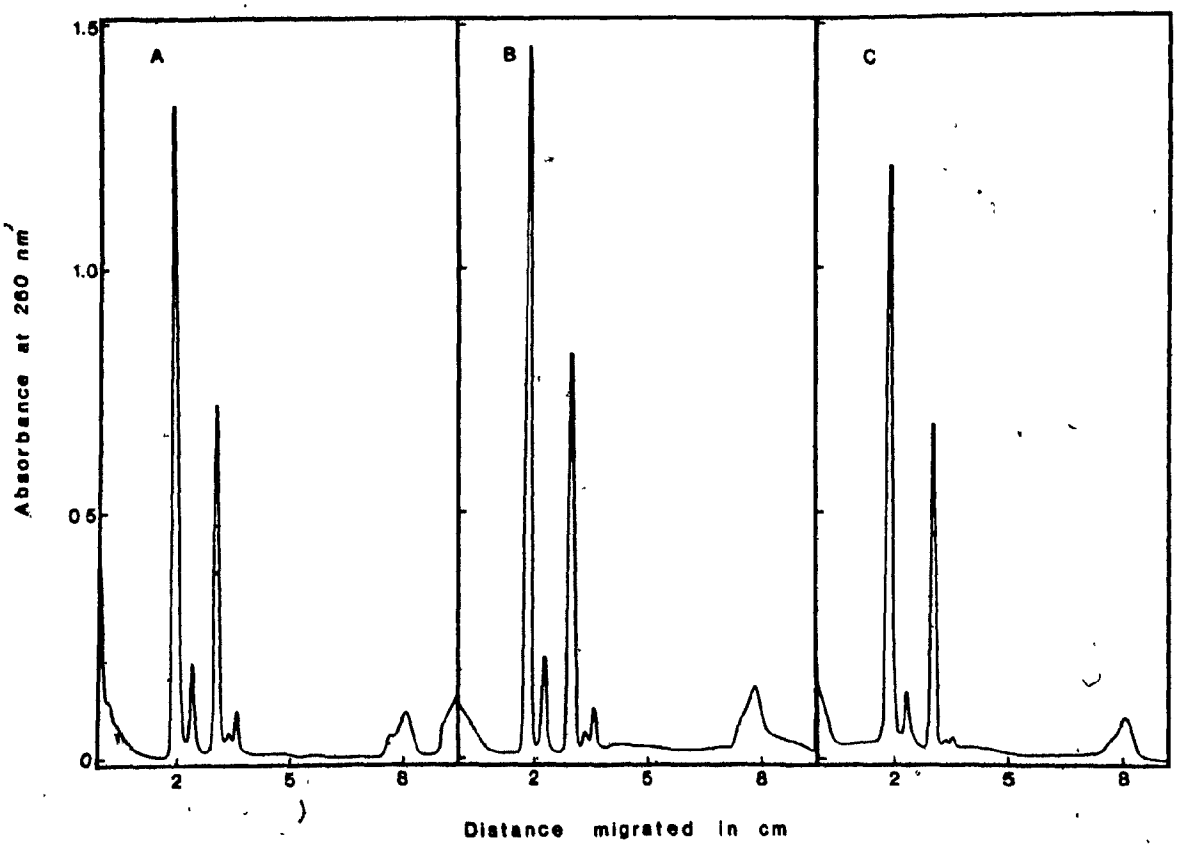

A
B
C
1.5
1.0
0.5
0
Absorbance at 280 nm
2
5
8
Distance migrated in cm

Table IV. Effect of L-*threo* and D-*threo* Chloramphenicol (CAP) on the Accumulation of Organelle rRNAs During Greening.

Ratios of peaks *b*, *d* and *e* respectively to peak *c* were calculated from the values for the area under each peak. These values were determined gravimetrically.

Treatment	Number of RNA extracts	Number of electro-pherograms	Average ratio to peak *c* ± s.e.m.			Difference from Control (%)		
			b	*d*	*e*	*b*	*d*	*e*
24 hr control	4	12	.283±.0001	.048±.0001	.105±.0001	-	-	-
300 µg/ml L-*threo* CAP	1	4	.277±.0001	.051±.0002	.101±.0001	-2.1	+6.2	-3.8
300 µg/ml D-*threo* CAP	1	4	.162±.0008	.029±.0001	.030±.0001	-42.8**	-39.6	-71.4***

** $p > .01$

*** $p > .001$

prevent the light-induced increase in the amount of chloroplast rRNA.

Since the cells treated with D-threo chloramphenicol have ceased to divide before 24 hr,[1] I cannot be certain that the observed reduction in the amount of chloroplast rRNA is due to the direct action of the antibiotic on protein synthesis on chloroplast ribosomes. Therefore, another specific inhibitor of protein synthesis on 70S ribosomes, spectinomycin, which at 100 µg/ml allows continued growth of Ochromonas during at least 24 hr, was used to obtain more conclusive results.

2. Spectinomycin and ethidium bromide

The effects of spectinomycin (100 µg/ml) on greening and light-grown cells of Ochromonas was investigated. In these experiments, ethidium bromide (1 µg/ml) was used in conjunction with spectinomycin in order to distinguish between mitochondrial and chloroplast rRNAs. The effects of ethidium bromide on organelle ultrastructure and chlorophyll synthesis were also examined. Chloroplasts from several species of algae and higher plants contain highly twisted circular DNA (Manning et al.,

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[1] Other observations indicate that although it inhibits growth, D-threo chloramphenicol does not kill the cells. Ochromonas is a flagellated organism, and cells grown in the presence of D-threo chloramphenicol are still motile at 24 hr and even after 7 days when examined by light microscopy. Also, when greening cells, which have been exposed to D-threo chloramphenicol for 24 hr are subcultured in medium which does not contain the drug, the cells resume growth after a lag period. Whether all or only a fraction of the cells regain the capacity to divide is not known, but since cells remaining for the same period of time in the original culture containing chloramphenicol are still motile, it is likely that most of the subcultured cells eventually divide again.

1971; Kolodner and Tewari, 1972a), and ethidium bromide binds preferentially to supercoiled circular DNA (Radloff et al., 1967). In Chlamydomonas, the dye induces the loss of most, but not all, of the chloroplast DNA (Flechtner and Sager, 1973). Therefore, although the results of other experiments included in the present study (see Results Part A) show that in Ochromonas chloroplast rRNA is not affected by ethidium bromide, it was anticipated that ethidium bromide might have other effects on the chloroplast.

a. Cell division

The effects of spectinomycin, ethidium bromide, and spectinomycin plus ethidium bromide on cell division of Ochromonas during greening are shown in Figure 17. The growth rate of cells exposed to spectinomycin is slightly less than the control rate and usually remains constant for 48 hr; however, in some experiments the rate of division of spectinomycin-treated cells declined after 24 hr in the light. Cells treated with ethidium bromide grow normally for almost 24 hr, after which time the rate of cell division decreases. When spectinomycin and ethidium bromide are present in the culture medium simultaneously, cells divide at a rate similar to that of cells treated with spectinomycin alone during the first 24 hr, but then cell division slows down to a rate equivalent to that of the culture containing only ethidium bromide.

The growth curves of light-grown cells exposed to spectinomycin, ethidium bromide and spectinomycin plus ethidium bromide are seen in

Figure 17. Effect of spectinomycin and/or ethidium bromide on the increase in cell number in cultures of greening Ochromonas. Spectinomycin (100 µg/ml) and/or ethidium bromide (1 µg/ml) were added just prior to illumination. Each point is an average of duplicate counts from an individual culture. Control cells, ○ - ○; cells treated with spectinomycin, ■ - ■; cells treated with ethidium bromide, ● - ●; cells treated with both inhibitors simultaneously, ▲ - ▲.

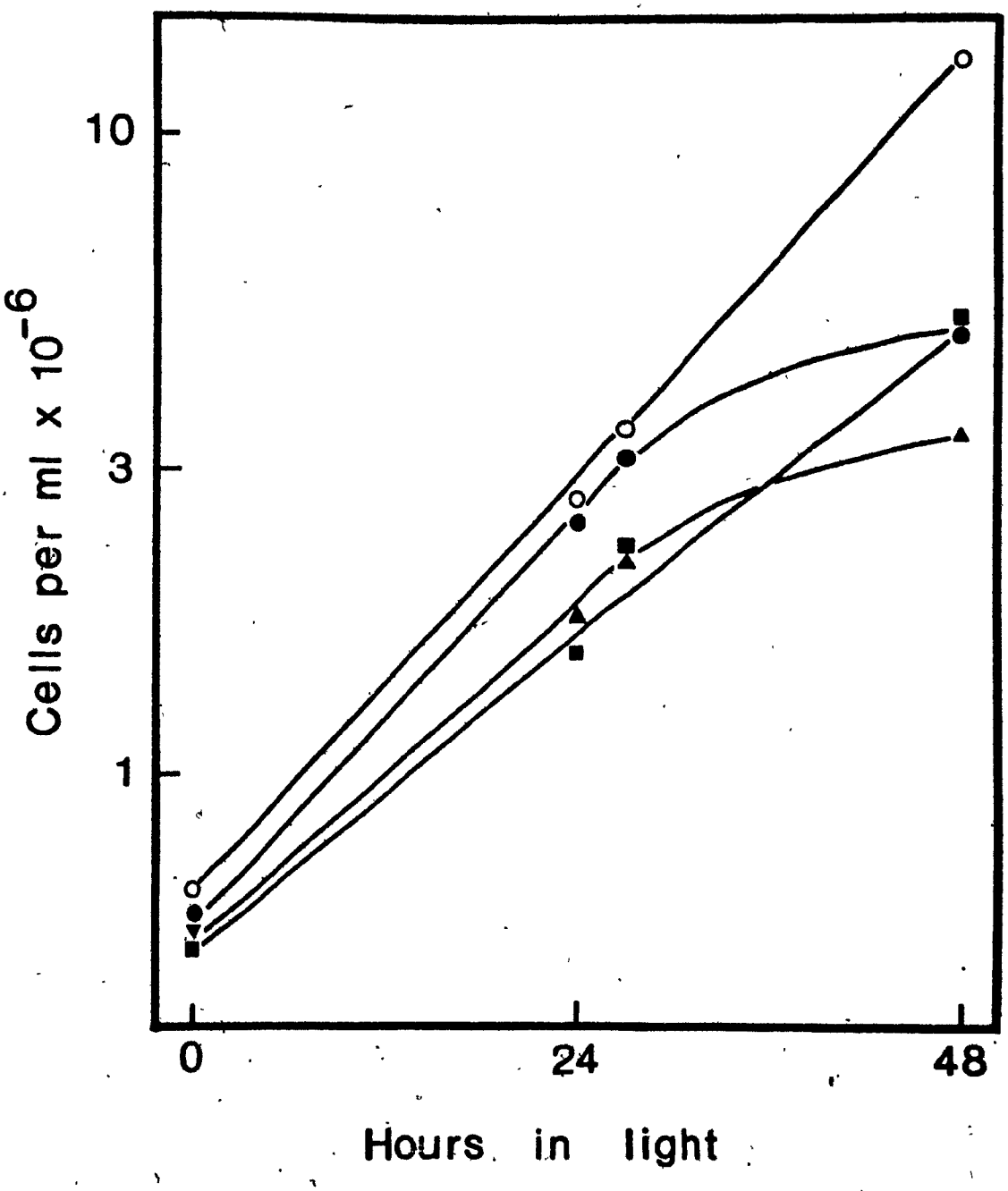

10
3
1
Cells per ml x 10^{-6}
0
24
48
Hours in light

Figure 18. Effect of spectinomycin (100 µg/ml) and/or ethidium bromide (1 µg/ml) on division rate of light-grown cells of Ochromonas. Each point is an average of duplicate counts from a single culture. Control cells, ○ - ○; spectinomycin-treated cells, ■ - ■; ethidium bromide-treated cells, ● - ●; cells exposed to both inhibitors simultaneously, ▲ - ▲.

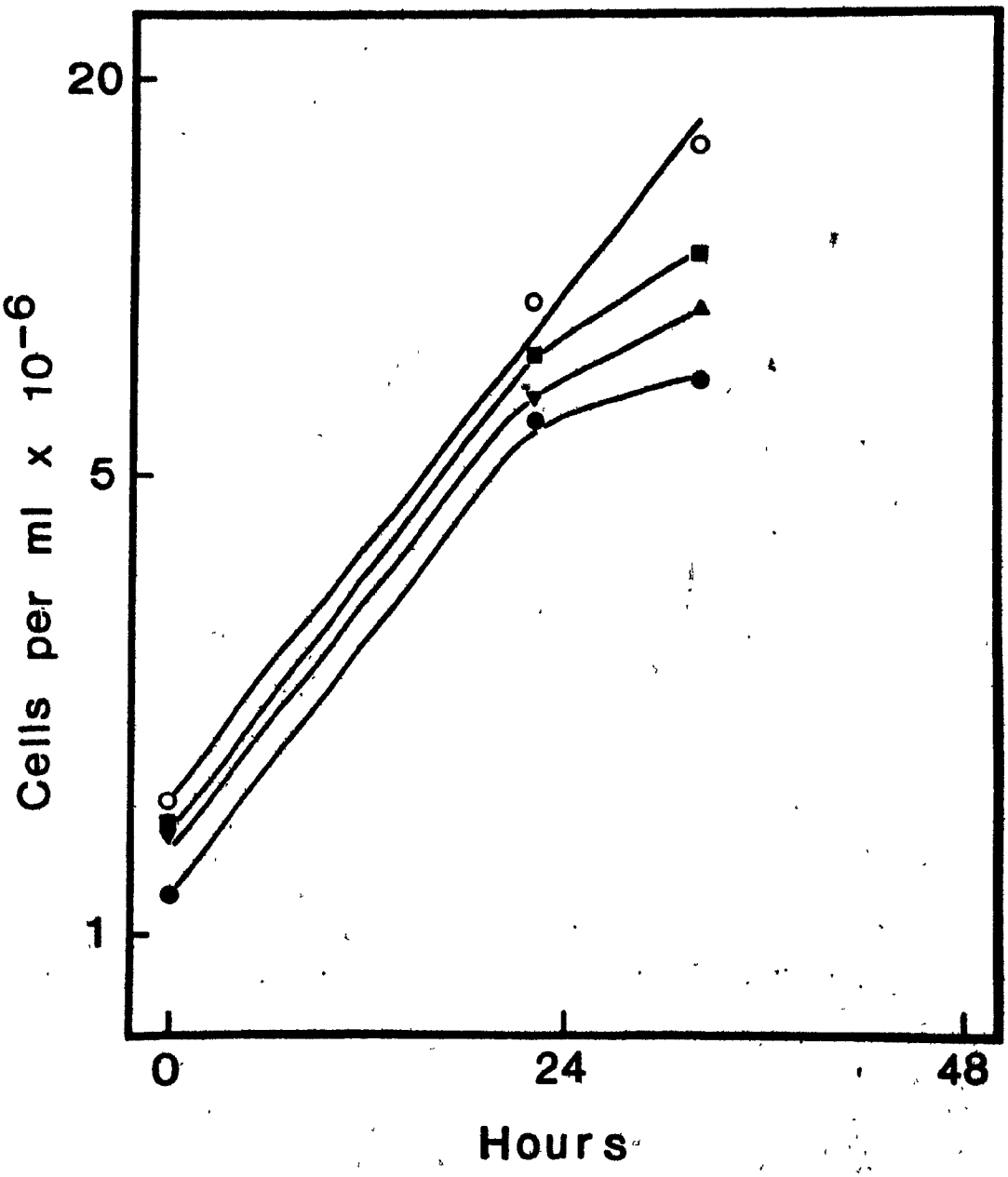

20
5
1
Cells per ml x 10^{-6}
0
24
48
Hours

Figure 18. In contrast with the slightly depressed rate of growth of cells treated with spectinomycin during greening, the rate of cell division in light-grown cultures containing this inhibitor is the same as that of the control culture during the first 24 hr of treatment. After this time, the rate of cell division in light-grown cultures exposed to spectinomycin decreases. In the presence of ethidium bromide, light-grown cells grow at normal rates until 24 hr when the rate of increase in population density begins to decline. Thus, the effect of ethidium bromide on the growth of light-grown cells is similar to that on greening cells. Light-grown cells treated with spectinomycin and ethidium bromide simultaneously grow at rates comparable to that of cells exposed to either inhibitor separately; growth is normal during the first 24 hr and then the rate of cell division declines.

b. Chlorophyll

The effects of spectinomycin and ethidium bromide on the amount of chlorophyll per cell are presented in Table V. In the greening system, cells treated with spectinomycin contain approximately 40% less chlorophyll than controls or cells treated with ethidium bromide. For cells grown in continuous light, the results are quite different. Spectinomycin does not affect the amount of chlorophyll per cell, but there is 20% less chlorophyll in cells grown in the presence of ethidium bromide alone. The chlorophyll content of cultures containing both spectinomycin and ethidium bromide, however, is the same as that of the control.

Table V. Effect of Spectinomycin (Spec) and Ethidium Bromide (EB) on the Amount of Chlorophyll per Cell.

Chlorophyll a was extracted in 80% acetone and measured at 663 nm. The amount of chlorophyll per cell was calculated according to the method described by Gibbs (1962).

Physiological condition	Treatment	Chlorophyll per cell (mg x 10^{-10}) Experiment 1	2*	3**	Average	Difference from control (%)
Greening	24 hr control	4.40	4.5	5.33	4.74	-
	100 µg/ml spec	2.60	2.3	3.04	2.65	-44.1
	1 µg/ml EB	4.61	3.7	5.33	4.55	-4.0
	EB + spec	3.10	2.9	3.08	3.02	-36.3
Continuous light	24 hr control	9.57		9.91	9.74	-
	100 µg/ml spec	8.42		9.66	9.04	-7.2
	1 µg/ml EB	7.35		7.78	7.56	-22.4
	EB + spec	9.57		10.13	9.85	+1.1

* Average of duplicate samples

** Average of triplicate samples

c. Organelle rRNAs

The electrophoretic profiles of RNA extracted from greening cells treated with spectinomycin for the first 24 hr of light and from control greening cells are presented in Figure 19. The amounts of rRNA in peaks b and e are selectively reduced with respect to peaks a and c in the extract from the spectinomycin-treated cells, whereas the amount of rRNA in peak d remains approximately the same. Since peaks b and e contain the heavy and light chloroplast rRNA species respectively, these results indicate that spectinomycin impedes the normal light-induced increase of chloroplast rRNA during greening. Since peak d corresponds to the light mitochondrial rRNA species, these observations also suggest that spectinomycin does not affect the amount of mitochondrial rRNA and that the effect of this inhibitor on the accumulation of the chloroplast rRNA species is specific. In Figure 20 the electrophoretic profiles of RNA from cells treated with ethidium bromide alone and with both ethidium bromide and spectinomycin are compared. Since ethidium bromide treatment eliminates essentially all the mitochondrial rRNA, the effect of spectinomycin on the amount of chloroplast rRNA can be more readily assessed. To quantify the effect of spectinomycin on the accumulation of chloroplast rRNA in greening cells, approximately ten electropherograms of RNA from controls and from cells treated with spectinomycin, ethidium bromide and ethidium bromide plus spectinomycin were quantitatively analyzed. The results are presented in Table VI which shows that in the presence of spectinomycin,

Figure 19. Effect of spectinomycin on the amount of chloroplast rRNA. Total RNA was extracted from A, control greening cells illuminated for 24 hr; and from B, greening cells exposed to 100 µg/ml spectinomycin for 24 hr.

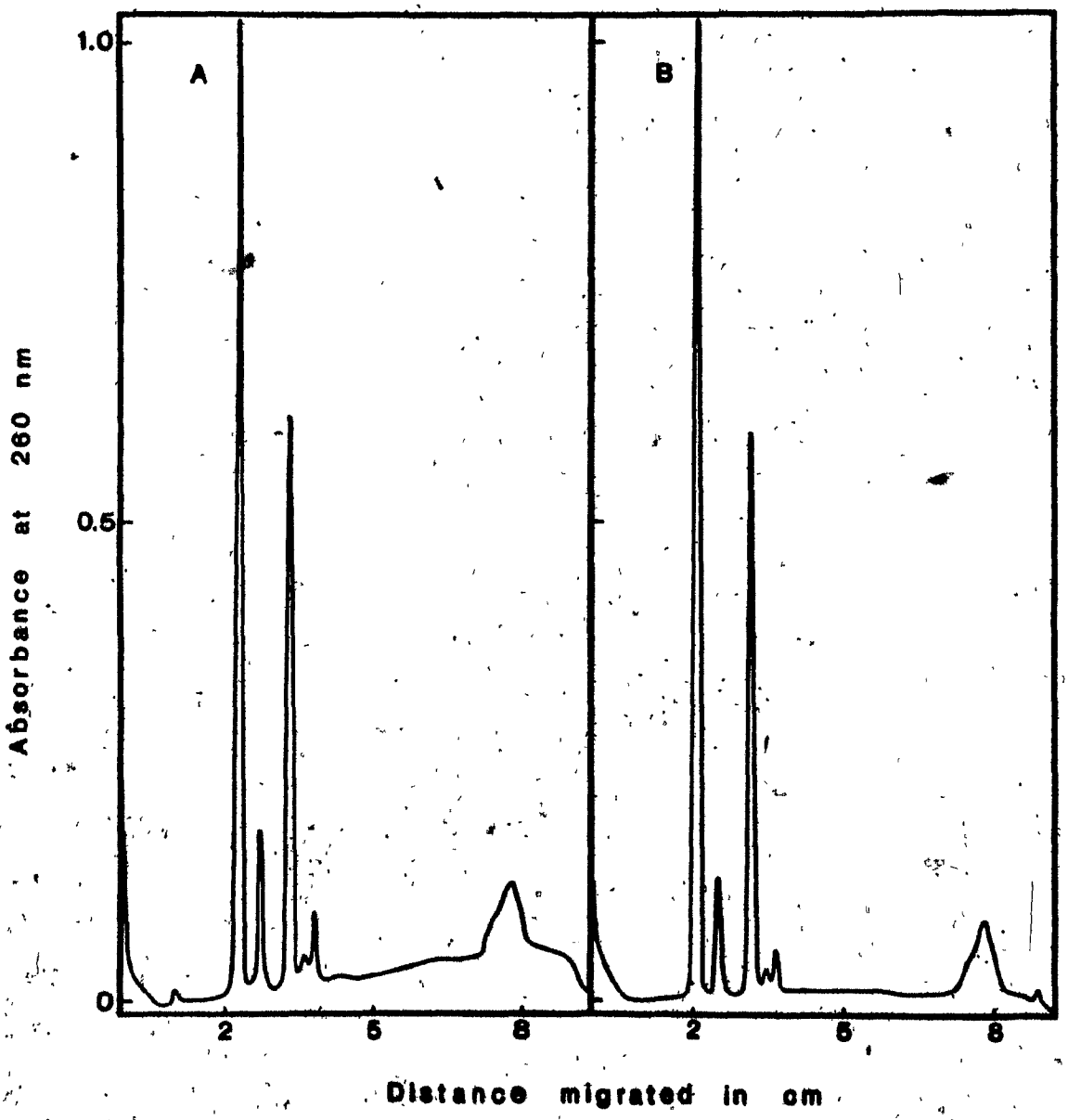

1.0
0.5
0
A
B
Absorbance at 260 nm
2
5
8
2
5
8
Distance migrated in cm

Figure 20. Effect of spectinomycin on the accumulation of chloroplast rRNA under conditions which reduce the amount of mitochondrial rRNA. Total RNA was extracted from A, 24 hr greening cells exposed to 1 µg/ml ethidium bromide for 24 hr; and from B, greening cells treated with both ethidium bromide (1 µg/ml) and spectinomycin (100 µg/ml) for 24 hr.

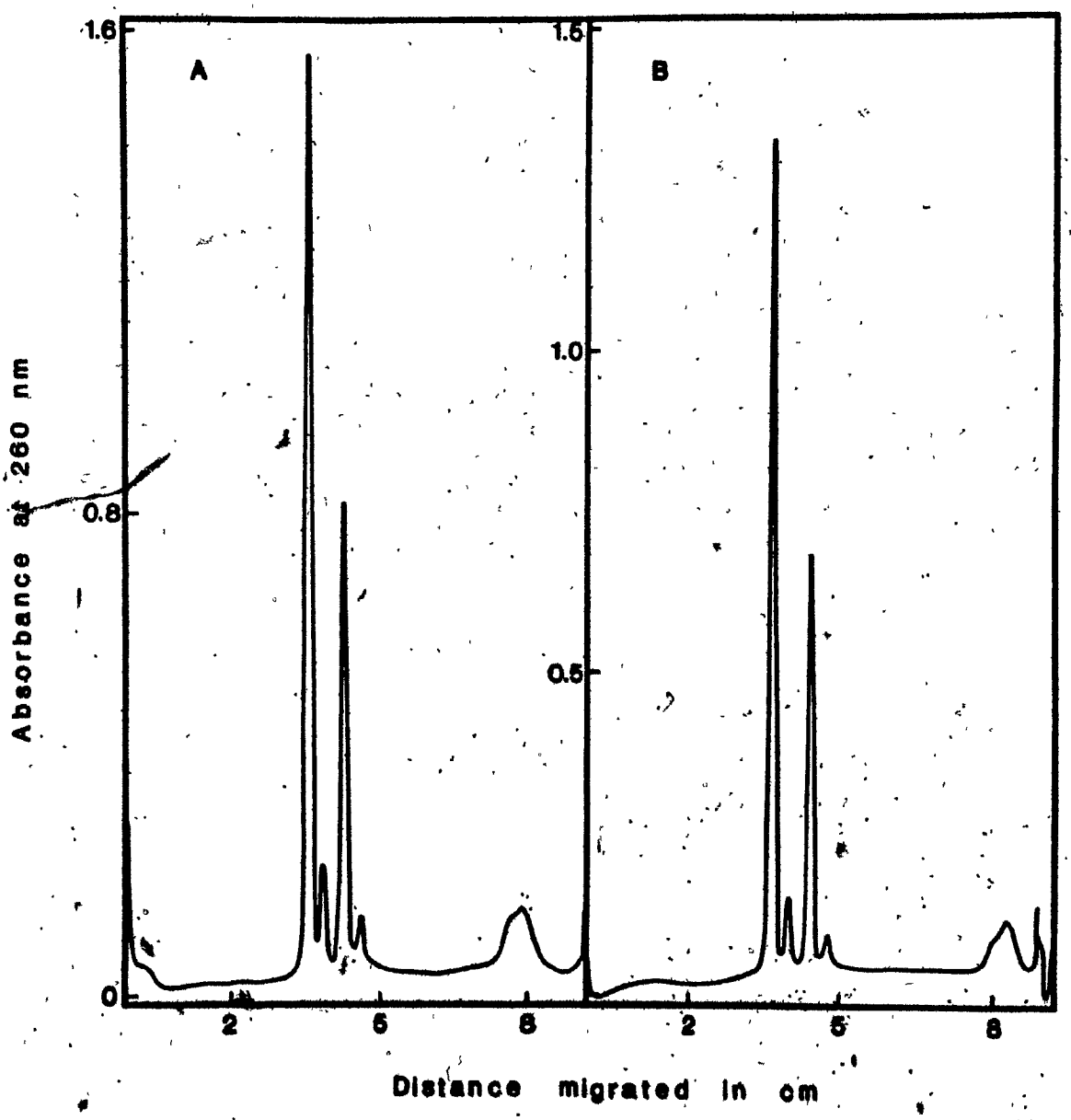

1.6
A
1.5
B
1.0
0.8
0.5
0
Absorbance at 260 nm
2
5
8
2
5
8
Distance migrated in cm

Table VI. Effect of Spectinomycin (Spec) and Ethidium Bromide (EB) on the Accumulation of Organelle rRNAs during Greening.

Ratios of peaks b, d and e respectively to peak c were calculated from values for the area under each peak. These values were determined gravimetrically.

Treatment	Number of RNA extracts	Number of electro-pherograms	Average ratio to peak c ± s.e.m. b	d	e	Difference from control (%) b	d	e
24 hr. control	4	12	.283±.0001	.048±.0001	.105±.0001	-	-	-
100 µg/ml spec	3	10	.227±.0001	.052±.0001	.070±.0001	-19.8***	+8.3	-33.3***
1 µg/ml EB	3	10	.218±.0001	.016±.0001	.105±.0001	-23.0***	-66.7***	±0.0
EB + spec	3	9	.172±.0001	.018±.0001	.078±.0001	-39.2***	-62.5***	-25.7**

** $p > .05$

*** $p > .001$

the amount of chloroplast rRNA is reduced by 30-40%. Statistical analysis of the data indicates that the spectinomycin-induced difference in the amount of chloroplast rRNA in 24 hr greening cells is highly significant.

The electrophoretic profiles of RNA extracted from light-grown cells after 24 hr treatment with spectinomycin, ethidium bromide, and both inhibitors simultaneously are not presented here, but show that the amount of the chloroplast rRNA species is also selectively reduced by spectinomycin in the continuous light system. The magnitude of the reduction in the amount of chloroplast rRNA, however, is less than in the greening system. This is expected since at the beginning of spectinomycin treatment, light-grown cells already possess a full complement of chloroplast rRNA which is reduced only by dilution due to cell division.

Thus, in light-grown cells exposed to spectinomycin, the amount of chloroplast rRNA is slightly reduced, while the rate of cell division and amount of chlorophyll per cell is comparable to that of controls. The effects of spectinomycin on greening cells are more visible: the growth rate of treated cells is slightly reduced, the amount of chlorophyll per cell is decreased by approximately 40% and the accumulation of chloroplast rRNA is selectively reduced by 30-40%.

d. Ultrastructure

The effects of spectinomycin and ethidium bromide on the morphology of both greening and light-grown cells were determined by

electron microscopy. With few exceptions, the ultrastructural alterations induced by these inhibitors in greening cells are also observed in the light-grown cells. Most of the illustrations of these effects are, however, selected from the greening system.

1. Effects of spectinomycin on the chloroplast

Membranes and DNA: Normal chloroplast development has been described in detail previously (Gibbs, 1962; Smith-Johannsen and Gibbs, 1972). During the first 24 hr of greening, three processes occur simultaneously: single thylakoids develop into bands of two and then three appressed thylakoids, the number of three-thylakoid bands per chloroplast increases, and the membrane surface area increases as the entire structure grows in size. After 24 hr illumination, transformation of the small proplastid into a mature chloroplast is nearly complete, although the size of the plastid continues to increase during the subsequent 72 hr. Figure 21 is a longitudinal section of a 24 hr greening cell transecting two lobes of the cell's single chloroplast. A more highly magnified view of one chloroplast lobe in a cell fixed by the standard method is seen in Figure 22. The chloroplast is limited by a double membrane, the chloroplast envelope, and, in addition, is enclosed by a cisternum of the endoplasmic reticulum which is continuous with the nuclear envelope. At this stage most bands consist of three appressed thylakoids. Characteristically, a number of bands form concentric sheets around the periphery of the chloroplast and are designated girdle bands where they loop around the rim of the plate-like chloroplast. The

Figure 21. Longitudinal section of a 24 hr greening cell of Ochromonas danica from an exponentially-growing culture. The cell's single chloroplast is transected through it's two lateral lobes (CL), and at this stage of development is characterized by bands (B) consisting of three appressed thylakoids. This micrograph also illustrates the orientation of the chloroplast with respect to the nucleus (N), as well as several other features typical of Ochromonas, including the prominent nucleolus (Nu), chloroplast ER (CER), chloroplast nucleoid (CN), perinuclear reticulum (PR), Golgi body (G), mitochondria (M), and leucosin vacuole (LV). Standard fixation, 29,600 x. (Neg. 1252-1).

Figure 22. Longitudinal section through a 24 hr greening cell demonstrating the characteristic organization of chloroplast membranes. Some of the bands of thylakoids which traverse the chloroplast loop around the rim of the plate-like lobe to form girdle bands (GB). The chloroplast is enclosed by both a double-membraned envelope (CE) and the chloroplast ER (CER). Nucleus (N), mitochondria (M), osmiophilic granule (O). Standard fixation, 73,010 x. (Neg. 1224-7).

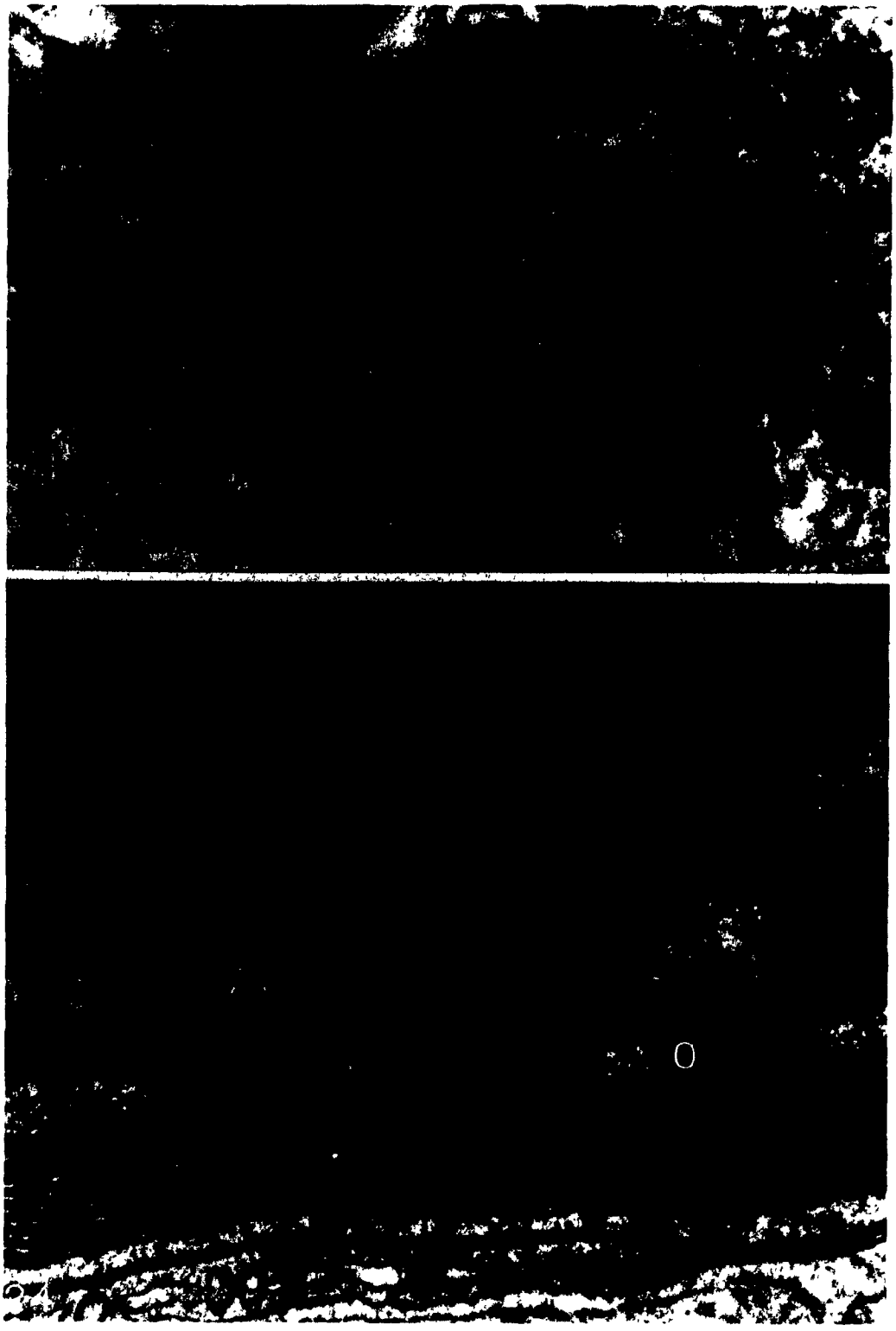
electron-translucent areas lying within the girdle bands at each side of the chloroplast lobe are cross-sections through the ring-shaped nucleoid where the plastid DNA is localized (Gibbs et al., 1974).

An analysis of the amount of thylakoid membrane in random sections of spectinomycin-treated and control greening cells showed that spectinomycin considerably reduces the amount of thylakoid membrane synthesized during the first 24 hr in the light. In contrast, little or no reduction in chloroplast membrane occurs in light-grown cells treated with the antibiotic.

The arrangement of thylakoids in chloroplasts of both greening and light-grown cells treated with spectinomycin is severely perturbed. Whereas bands composed of more than three thylakoids are seldom observed in the chloroplasts of control cells, stacks containing abnormally high numbers of thylakoids are found in the chloroplasts of spectinomycin-treated cells. Most often the abnormal bands contain four to seven thylakoids (Figure 23), but larger aggregations of up to eleven thylakoids are also seen (Figure 24). A survey of greening and light-grown cells treated with spectinomycin showed that only 10% of the abnormally large stacks are composed of multiples of three thylakoids. This suggests that the abnormal bands arise by sequential accumulation of individual thylakoids rather than by association of preformed bands. Compared to the control there is also a higher frequency of single, unfused thylakoids in spectinomycin-treated cells, particularly in the greening system. In sections containing single thylakoids, however, large stacks of membranes

Figure 23. Chloroplast in cell illuminated for 24 hr in the presence of 100 µg/ml spectinomycin showing disruption of chloroplast membrane organization. Girdle bands are absent and abnormal bands composed of five to seven thylakoids are prevalent. Nucleus (N), perinuclear reticulum (PR). Standard fixation, 73,010 x. (Neg. 1227-4).

Figure 24. High magnification of several abnormally large stacks of thylakoids in the chloroplast of a cell exposed to 100 µg/ml spectinomycin during 24 hr greening. Although the number of thylakoids per band is abnormal in spectinomycin-treated cells, both intra- and inter-thylakoid spaces are similar to those in control cells. Perinuclear reticulum (PR), osmiophilic granule (O). Standard fixation, 126,560 x. (Neg. 1230-14).



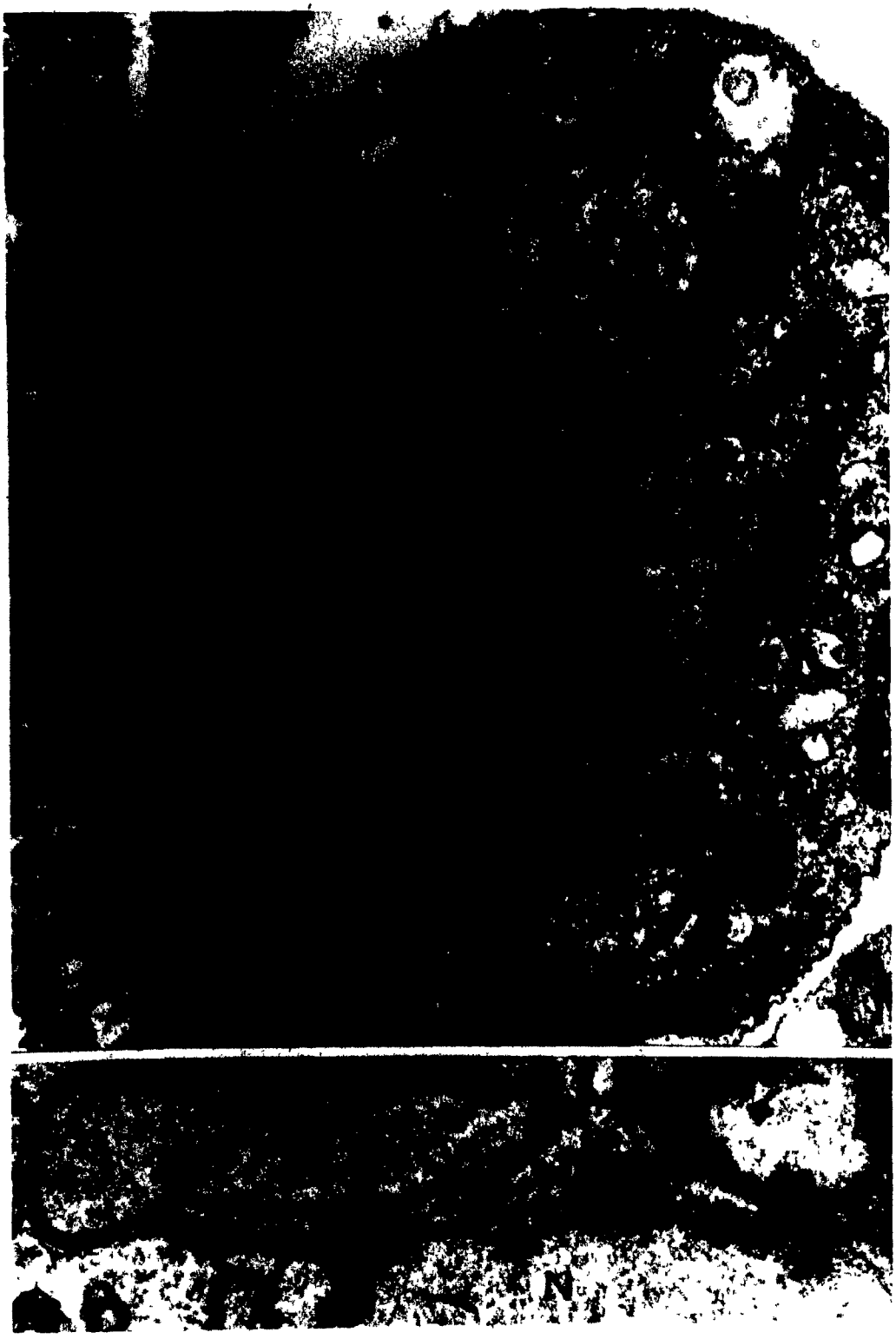
are usually included also (Figure 25). In chloroplasts of light-grown cells exposed to the antibiotic a greater number of three-thylakoid bands persist compared to greening cells, but nearly all sections reveal abnormal stacking as well.

Another dislocation in chloroplast membrane organization induced by spectinomycin is that the bands of thylakoids often terminate abruptly at the rim of the chloroplast instead of looping around the rim to form girdle bands (Figure 23). Consequently, the chloroplast DNA, which normally lies inside the girdle bands, is no longer confined to its characteristic peripheral location and appears dispersed between the truncated thylakoids (Figure 25). In a few sections the electron-translucent chloroplast DNA regions appear scattered throughout the chloroplast of spectinomycin-treated cells even when a girdle band is still present (Figure 28). However, this chloroplast DNA may originate from another region not in the plane of section where the girdle bands are disrupted.

In Ochromonas there is a membrane system closely associated with the chloroplast called the perinuclear reticulum (Smith-Johannsen and Gibbs, 1972). Consisting of one layer of branching tubules interspersed with vesicles, it occupies the narrow space between the nuclear envelope and the chloroplast envelope. In greening cells exposed to spectinomycin for 24 hr the perinuclear reticulum becomes hypertrophied (Figures 25 and 26). In places the perinuclear space is swollen with several layers of the tubules, and occasionally the reticulum is observed

Figure 25. Transverse section of 24 hr greening cell demonstrating other effects of spectinomycin on chloroplast membranes. Although abnormally large stacks of thylakoids are present, many single, unfused thylakoids remain in other regions of the same chloroplast. The absence of girdle bands results in the dispersal of chloroplast DNA, which appears as scattered electron-translucent patches in the chloroplast matrix (black arrows). The perinuclear reticulum normally occupying the channel between the nuclear and chloroplast envelopes, has greatly proliferated, swelling the perinuclear space and, in some places, extending into the space between the chloroplast envelope and the chloroplast ER (white arrow). Standard fixation, 25,900 x. (Neg. 1245-15).

Figure 26. Section through a region of a 24 hr greening cell treated with spectinomycin showing hypertrophy of the perinuclear reticulum in the space between the chloroplast (C) and the nucleus (N). Co-fixation, 43,120 x. (Neg. 1219-3).

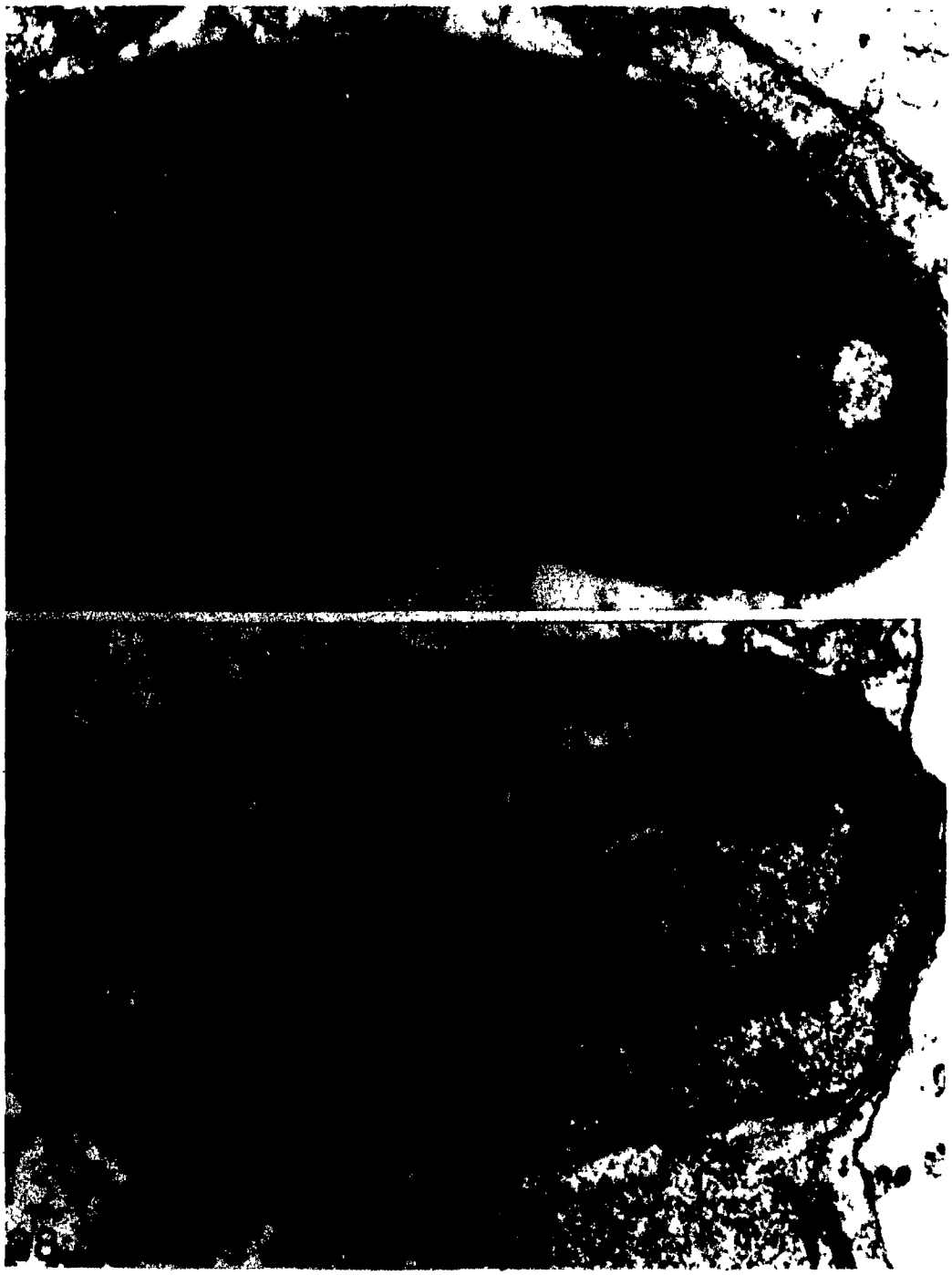

N

to have proliferated beyond the perinuclear region into the space between the chloroplast envelope and the chloroplast ER. Although it is a common feature of greening cells treated with spectinomycin, hypertrophy of the perinuclear reticulum is only rarely observed in light-grown cells exposed to the antibiotic.

Chloroplast ribosomes: After 24 hr greening, the concentration of ribosomes in control chloroplasts approaches its maximum, although the total number of ribosomes continues to increase as the organelle expands in volume. Figure 27 illustrates a section of a chloroplast of a 24 hr greening cell fixed by the simultaneous glutaraldehyde-osmium tetroxide procedure which allows the visualization of chloroplast ribosomes. The standard fixation probably also preserves chloroplast ribosomes but they are obscured by the densely staining matrix. Some matrix material may leach out during co-fixation and increase the contrast of the ribosomes. Figure 28 shows that although ribosomes are present in the chloroplast of a cell exposed to spectinomycin for 24 hr, there are fewer than in the chloroplasts of controls. The total number of chloroplast ribosomes in both greening and light-grown cells treated with spectinomycin for 24 hr was calculated. The results presented in Table VII show that in the presence of the antibiotic the number of chloroplast ribosomes is reduced by approximately 30%. The volume of the chloroplast in cells exposed to spectinomycin is slightly less than in controls but is probably within experimental error. Therefore, assuming chloroplast volume is constant in both experimental and control cells, the reduction in chloroplast

Figure 27. Chloroplast in a control cell illuminated for 24 hr and fixed by a method which renders chloroplast ribosomes visible. The concentration of chloroplast ribosomes reaches a plateau by 24 hr greening, but the absolute number of ribosomes increases as the chloroplast expands in size. Co-fixation, 73,010 x. (Neg. 1241-9).

Figure 28. Chloroplast in a 24 hr greening cell exposed to spectinomycin. Although chloroplast ribosomes are present, they are less concentrated than in control cells (Fig. 27). Co-fixation, 73,010 x. (Neg. 1219-1).



ribosome number observed in the spectinomycin-treated cultures is due exclusively to the lower concentration of ribosomes in the chloroplasts of these cells.

The number of chloroplast ribosomes actually synthesized, however, is determined not only from the number of ribosomes counted in each chloroplast, but also from the rate of dilution of ribosomes by cell division. Since

ribosomes per chloroplast = synthesis / dilution,

and dilution = number of generations X 2,

then chloroplast ribosomes synthesized = (total ribosomes X 2 X generations) - original ribosomes.

In the case of greening cells, the original number of ribosomes is equal to the number of proplastid ribosomes. This was calculated from other data (Smith-Johannsen and Gibbs, 1972) to be 1.62×10^5 ribosomes. The results presented in Table VIII show that, in the presence of spectinomycin, the synthesis of chloroplast ribosomes is inhibited by more than 50% in greening cells and approximately 40% in light-grown cells.

Chloroplast ultrastructure in ethidium bromide-treated cells from both greening and continuous light systems is indistinguishable from that in controls. Neither membrane organization (Figure 29) nor the synthesis of chloroplast ribosomes (Table VIII) is affected by the drug.

ii. Effects of ethidium bromide on mitochondria

Cristae: In Ochromonas mitochondria are abundant and are characterized by numerous tubular cristae. In cells exposed to

Table VII. Effect of Spectinomycin (Spec) and Ethidium (EB) on the Number of Chloroplast Ribosomes

Chloroplast ribosomes were counted on electron micrographs of co-fixed cells pr 73,000 X. A square window 1/16 μm^2 in area was placed at random over the chlor dense particles of ribosome size counted. Chloroplast volume was calculated volume by the mean cell volume. The percent of the cell occupied by the chlor fractional area occupied by this organelle in approximately 100 sections throug from each sample.

Physiological condition	Treatment	Cell volume (μm^3)	Average cell volume (μm^3)	Chloroplast volume (% of cell)	Average chloroplast volume (% of cell)	Aver chlor volu (μm
24 hr greening	Control	343.4		8.2		
	100 µg/ml spec	368.9		7.2		
	1 µg/ml EB	347.0	358.1	8.4	7.88	28
	EB + spec	373.2		7.7		
24 hr light	Control	319.4		11.5		
	100 µg/ml spec	335.8		9.8		
	1 µg/ml EB	302.0	330.0	10.4	10.58	35
	EB + spec	362.6		10.6		

*** $p > .001$

le VII. Effect of Spectinomycin (Spec) and Ethidium Bromide (EB) on the Number of Chloroplast Ribosomes per Cell

e counted on electron micrographs of co-fixed cells printed at a total magnification of ow 1/16 μm^2 in area was placed at random over the chloroplast and the number of electron- me size counted. Chloroplast volume was calculated by multiplying the percent chloroplast olume. The percent of the cell occupied by the chloroplast was determined from the by this organelle in approximately 100 sections through as many cells selected at random

Average cell volume (μm^3)	Chloroplast volume (% of cell)	Average chloroplast volume (% of cell)	Average chloroplast volume (μm^3)	Ribosomes ($\mu m^2/16$) average ± s.e.m.	Total ribosomes per chloroplast	Difference from control %
	8.2			29.74 ± .64	1.76×10^5	-
	7.2			20.96 ± .59	1.24×10^5	-29.5***
358.1	8.4	7.88	28	29.89 ± .64	1.77×10^5	+ 0.5
	7.7			17.58 ± .81	1.04×10^5	-40.9***
	11.5			30.45 ± .57	2.25×10^5	-
	9.8			21.70 ± .59	1.60×10^5	-28.8***
330.0	10.4	10.58	35	30.28 ± .51	2.24×10^5	- 0.4
	10.6			20.76 ± .60	1.53×10^5	-32.0***

Table VIII. Effect of Spectinomycin (Spec) and Ethidium Bromide (EB) on the Synthesis of Chloroplast Ribosomes.

Calculations of the number of chloroplast ribosomes synthesized in control and antibiotic-treated cells are based on values in Table VII and are described in the text.

Physiological condition	Treatment	Total ribosomes per chloroplast ($\times 10^{-5}$)	Generations*	Ribosomes synthesized** ($\times 10^{-5}$)	Difference from control (%)
24 hr greening	Control	1.76	1.96	6.74	-
	100 µg/ml spec	1.24	1.35	3.19	-52.7
	1 µg/ml EB	1.77	2.08	7.20	+6.8
	EB + spec	1.04	1.19	2.31	-65.7
24 hr light	Control	2.25	2.68	9.81	-
	100 µg/ml spec	1.60	2.52	5.81	-40.8
	1 µg/ml EB	2.24	2.47	8.82	-10.1
	EB + spec	1.53	2.50	5.40	-45.0

* Average from 3 experiments

** Number of ribosomes in proplastid assumed to be 0.162×10^5, calculated from data in Smith-Johannsen and Gibbs (1972).

ethidium bromide for 24 hr, the number of mitochondrial cristae is markedly reduced (Figure 29). In some sections the resulting bodies contain only the densely staining inner mitochondrial membrane. Mitochondria from control, ethidium bromide and spectinomycin-treated cells are compared in Figures 30-32. Although chloramphenicol has been shown to cause a gradual loss of cristae in Ochromonas, 24 hr exposure to spectinomycin causes no reduction in the number of mitochondrial cristae (Figure 32).

Mitochondrial ribosomes: Figure 31 also illustrates the dramatic effect of ethidium bromide on the number of mitochondrial ribosomes. As indicated in Table IX, the concentration of ribosomes in mitochondria is reduced over 90% in the presence of ethidium bromide. Mitochondrial volume was not measured in this study, but the average length and diameter of the organelle in ethidium bromide-treated cells is smaller than in controls. Therefore, if the mitochondrial volume is reduced in the presence of ethidium bromide, then the actual degree of reduction in the number of mitochondrial ribosomes by the drug is even greater than shown in Table IX. Spectinomycin, on the other hand, has little or no effect on the number of mitochondrial ribosomes (Figure 32 and Table IX).

iii. Effect of both spectinomycin and ethidium bromide

Figure 33 depicts a cell exposed to spectinomycin and ethidium bromide simultaneously for 24 hr. The chloroplast exhibits all the membrane aberrations characteristic of cells treated with spectinomycin alone, including single, unfused thylakoids and abnormally large stacks of

Figure 29. Longitudinal section through a cell illuminated for 24 hr in the presence of 1 μg/ml ethidium bromide demonstrating the effect of this drug on mitochondrial membranes. The cristae are markedly reduced in number and some mitochondrial profiles (M) are devoid of internal structure. In contrast, the ultrastructure of the chloroplast is unaffected by ethidium bromide. Standard fixation, 56,350 x. (Neg. 1226-4).

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Figures 30-32. Representative mitochondria from control (Fig. 30), ethidium bromide-treated (Fig. 31) and spectinomycin-treated (Fig. 32) cells, illuminated for 24 hr. Mitochondria from cells exposed to spectinomycin during greening (Fig. 32) resemble the mitochondria of control cells (Fig. 30). Both possess tubular cristae, scattered ribosomes and striated cores (SC). Mitochondria from ethidium bromide-treated cells (Fig. 31), however, have very few cristae and almost no ribosomes, although striated cores are observed. Co-fixation, 73,010 x. (Negs. 1241-26, 1226-22, 1233-16).

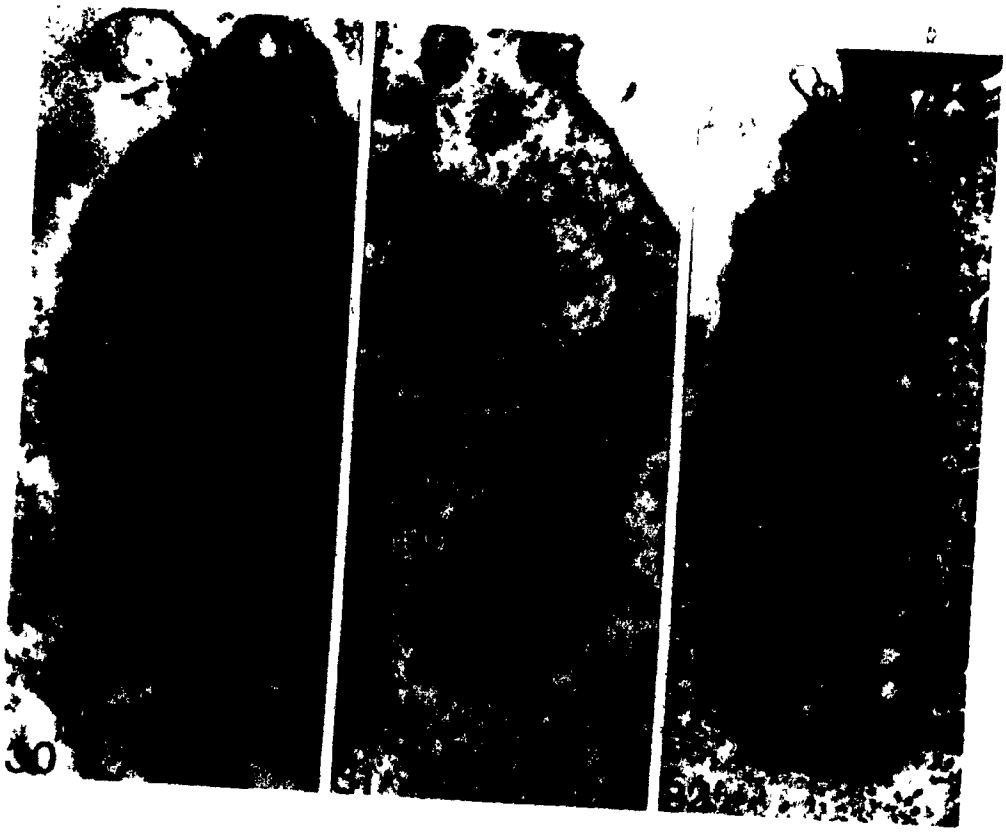


Fig. 33. Longitudinal section through a cell illuminated for 24 hr in the presence of both 100 µg/ml spectinomycin and 1 µg/ml ethidium bromide. The chloroplast exhibits all the membrane aberrations characteristic of cells exposed to spectinomycin alone, including single, unfused thylakoids, abnormally large stacks of thylakoids, absence of girdle bands, and hypertrophy of the perinuclear reticulum. The mitochondria, like those in cells treated with ethidium bromide alone, contain few cristae. Standard fixation, 43,120 x. (Neg 1233-33).

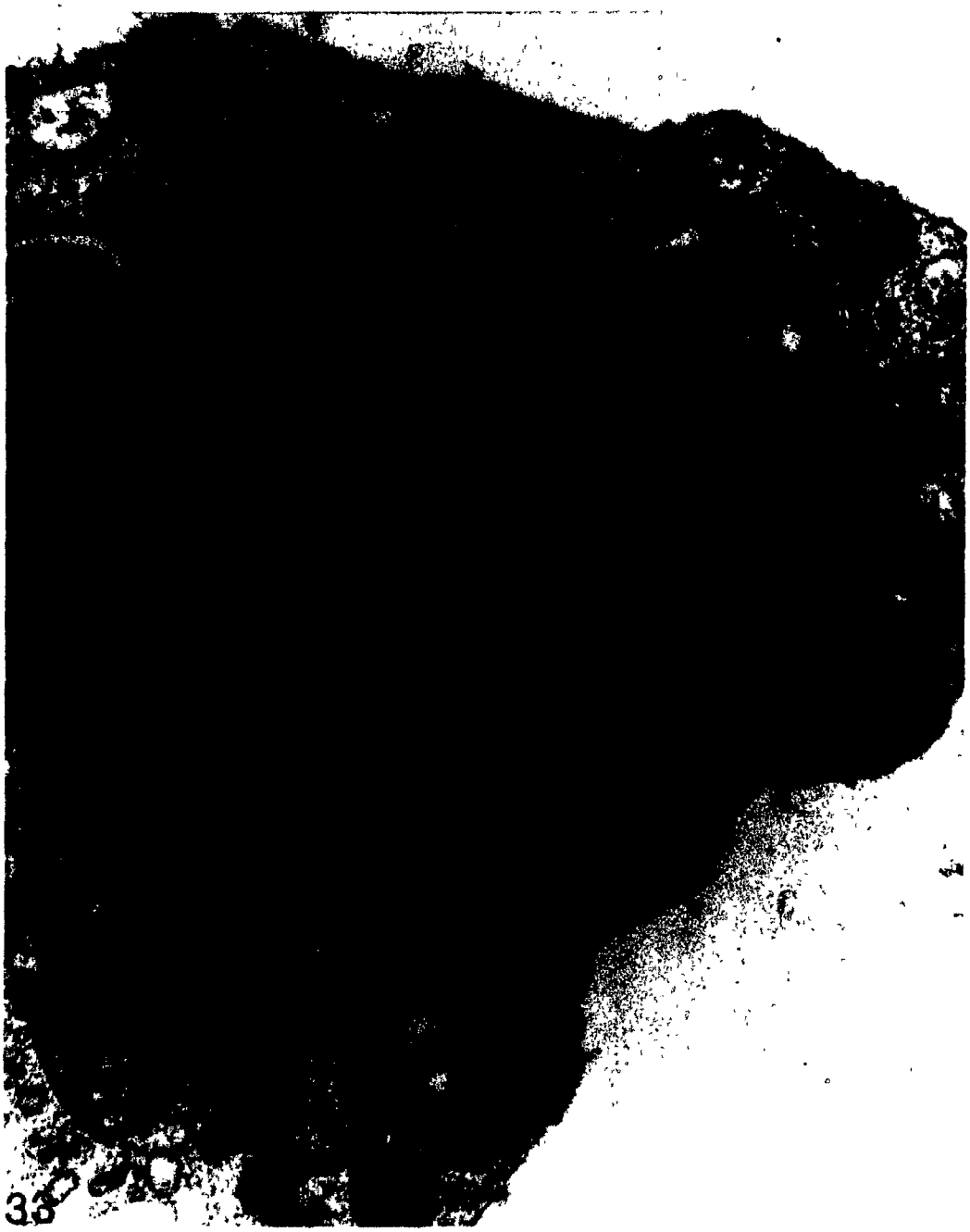

33

Table IX. Effect of Spectinomycin (Spec) and Ethidium Bromide (EB) on the Number of Mitochondrial Ribosomes

Mitochondrial ribosomes were counted on electron micrographs of co-fixed cells printed at a total magnification of 73,000 x. A square window 1/16 μm^2 in area was placed at random over a mitochondrion and the number of electron-dense particles of ribosome size counted. Mitochondrial volume was not determined, but the average length and width of mitochondrial profiles in control and drug-treated cells was measured. The results indicate that the volume occupied by mitochondria in control and spectinomycin-treated cells is similar but that mitochondrial volume may be reduced in cells exposed to ethidium bromide.

Physiological condition	Treatment	Ribosomes (μm^2/16) average ± s.e.m.	Ribosomes Difference from control (%)
24 hr greening	Control	9.87 ± 0.46	-
	100 µg/ml spec	9.67 ± 0.59	- 2.0
	1 µg/ml EB	0.75 ± 0.13	-92.4***
	EB + spec	0.97 ± 0.14	-90.0***
24 hr light	Control	7.84 ± 0.40	-
	100 µg/ml spec	7.60 ± 0.36	- 3.0
	1 µg/ml EB	0.60 ± 0.09	-93.0***
	EB + spec	0.50 ± 0.09	-94.0***

*** $p > .001$

thylakoids, loss of girdle bands with concomitant displacement of plastid DNA, and hypertrophy of the perinuclear reticulum. The mitochondria, like those in cells treated with ethidium bromide alone, are deficient in the number of cristae. The effect of each drug on the number of chloroplast or mitochondrial ribosomes is also unchanged when both drugs are administered at the same time (Tables VII-IX). The number of chloroplast ribosomes in greening cells treated with both antibiotics is approximately 10% lower than in cells treated with spectinomycin alone, but the significance of this difference is doubtful since no additional reduction in chloroplast rRNA is observed under the same conditions (Table VI). Thus, there is no evidence of a synergistic effect when spectinomycin and ethidium bromide are present simultaneously.

DISCUSSION

A. Extraction and Characterization of *Ochromonas* rRNAs

1. Extraction procedure

A major problem encountered in this investigation was the difficulty in obtaining undegraded chloroplast rRNA from *Ochromonas*. The susceptibility of the heavy chloroplast rRNA component to breakdown during extraction has also been noted in a variety of other algae (Woodcock and Bogorad, 1970; Kochert and Sansing, 1971; Howland and Ramus, 1971), a fern (Burns and Ingle, 1970) and higher plants (Spencer and Whitfield, 1966; Ingle *et al.*, 1970; Dyer *et al.*, 1971). The inclusion of inhibitors of RNase in the extraction buffer has been shown to increase the amount of intact chloroplast rRNA recovered from green tissues. For example, sodium thioglycolate was used by Heizman (1970) to isolate total rRNAs from *Euglena* and DEP was required by Cattolico and Jones (1972) to obtain undegraded rRNAs from *Chlamydomonas*. The method developed for the isolation of *Ochromonas* rRNAs does not, however, involve the addition of such RNase inhibitors to the detergent-phenol extraction buffer. Neither sodium thioglycolate, DEP, nor bentonite altered the electrophoretic profile of *Ochromonas* rRNAs. The algal rRNAs were judged to be intact by electrophoresis in EDTA-containing buffer. Structural instabilities in rRNA may not be apparent in preparations analyzed by electrophoresis in the presence of magnesium (Ingle *et al.*, 1970), so that only electrophoresis in an EDTA-containing

buffer can provide information concerning the integrity of rRNA molecules.

The maintenance of cold temperatures (0-5° C) throughout the extraction and fractionation procedures was found to be essential to prevent the degradation of rRNA. The detergent SDS, commonly used in phenol extractions, is insoluble in solutions kept at low temperatures. This may partially explain the breakdown of the heavy chloroplast rRNA extracted at 4° C from cucumber seedlings (Cucumis sativus) by an SDS-phenol procedure (Védel and D'Aoust, 1970). Sarkosyl (sodium dodecyl sarcosinate), which is soluble in aqueous solutions at low temperatures, therefore replaced SDS in the extraction buffer. This anionic detergent was also employed by Woodcock and Bogorad (1970) to isolate rRNAs from Acetabularia, but the chloroplast rRNA in their preparations was still degraded. The results of Woodcock and Bogorad are probably explained by the low pH of their extraction buffer (pH 5.6), which favours the activity of plant RNases (Jervis, 1974). The pH of the Tris-acetate buffer used to extract Ochromonas RNA is 7.2, which is well above the 5-6 pH optima characteristic of plant RNases (Jervis, 1974).

Other properties of the extraction buffer which may influence the structural stability of RNA include ionic strength. When dissolved in buffer of low ionic strength, RNA tends to denature and is more susceptible to nuclease attack (Poulson, 1973). The ionic strength of the extraction buffer was increased by the addition of NaCl, which

also decreases the solubility of phenol in the aqueous phase (Poulson, 1973). Addition of magnesium to the extraction buffer has been shown to decrease the susceptibility of chloroplast rRNA to breakdown (Ingle et al., 1970; Bourque et al., 1973). The use of Sarkosyl in the present study, however, precludes the addition of magnesium to the extraction buffer since the two substances react to form an insoluble precipitate. Fortunately, magnesium is not required to obtain undegraded RNA from Ochromonas. Another precaution taken to reduce the possibility of RNA degradation was to redistill the phenol to remove preservatives and oxidation products and to stabilize it with 8-hydroxyquinoline (Poulson, 1973).

Thus, the combination of the extraction buffer and the cold conditions used in the isolation of rRNA from Ochromonas is sufficient to prevent rRNA breakdown in the absence of magnesium and other inhibitors of RNase. This may be advantageous in view of evidence that some RNase inhibitors interact with RNA itself. For example, bentonite causes the selective loss of chloroplast rRNA during isolation from higher plants (Bourque et al., 1973), and DEP apparently alters the properties of nucleic acid since it abolishes infectivity of TMV RNA (Gulyás and Solymosy, 1970). The use of high concentrations of magnesium has the disadvantage that the resulting RNA preparations are often contaminated with insoluble material (Ingle et al., 1970). Therefore, the procedure developed for the isolation of Ochromonas rRNA may be useful for other studies where a premium is placed on the yield and

purity of the extracted nucleic acids.

2. Characterization of Ochromonas rRNAs

a. Cytoplasmic rRNAs

The sedimentation coefficients of the heavy and light cytoplasmic rRNA molecules from Ochromonas are 24S and 18S respectively, and are thus similar to those of cytoplasmic rRNAs from other algae and higher plants. For example, the heavy and light cytoplasmic rRNAs from French bean leaves and pea root tip have values of 25S and 18S, as determined by comparison with rRNAs from E. coli and Oscillatoria, a blue-green alga (Loening and Ingle, 1967). The cytoplasmic rRNAs in Chlamydomonas are also 25S and 18S (Cattolico and Jones, 1972). The sizes of cytoplasmic rRNAs from Euglena are similar, but the precise values vary: Heizmann (1970) obtained values of 25S and 21S, Rawson et al. (1971) estimated values of 25S and 20S, and Avadhani and Buetow (1972) found values of 24S and 20S. These variations probably result from differences in experimental conditions. Hence, the slightly smaller size of the 24S cytoplasmic rRNA component from Ochromonas compared to the size of most other heavy plant cytoplasmic rRNAs may not be significant. On the other hand, the relatively small size of the heavy cytoplasmic rRNA component in Ochromonas may be indicative of a primitive evolutionary status of this organism, since the heavy rRNA molecule has increased in size during the evolution of eukaryotes (Loening, 1968).

b. Chloroplast rRNAs

The relative amounts of two species of rRNA are greater in light-grown cells than in dark-grown cells. These species were assumed

to be the heavy and light chloroplast rRNAs. Calculation of the proportion of light chloroplast rRNA in dark- and light-grown cells indicates that there is approximately a 2.5-fold increase in the amount of chloroplast rRNA during illumination. Data from this study and others (Gibbs, 1970; Smith-Johannsen, unpublished) suggests that the amount of cytoplasmic rRNA, as well as chloroplast rRNA, increases significantly in response to light. Therefore, the increase of chloroplast rRNA is considerably greater than that indicated by comparing the ratios of chloroplast rRNA to cytoplasmic rRNA in dark- and light-grown cells. The absolute increase in the number of chloroplast ribosomes during 96 hr greening has been calculated to be 27-fold (Smith-Johannsen and Gibbs, 1972). Unfortunately, in the present study the amount of rRNA per cell, and thus the absolute amounts of chloroplast rRNA in dark- and light-grown cells, could not be determined since variable amounts of RNA are lost during the isolation procedure.

Isotope labelling of Ochromonas chloroplast rRNAs with (^{3}H)uridine would allow a more precise measurement of the synthesis of these species than that permitted by the determination of the optical density of accumulated chloroplast rRNAs. Preliminary labelling experiments suggested that the synthesis of organelle rRNA is stimulated 40-fold during 6 hr light. Most of this increase presumably involves the chloroplast rRNA species. However, during the same period, synthesis of cytoplasmic rRNA is stimulated 30-fold. The data agree in

general with a previous autoradiographic study of Ochromonas, in which the amount of (^{3}H)orotic acid incorporated into chloroplast RNA during the initial 30 min of greening was found to be 20 times greater than in dark-grown cells; during the same interval the amount of (^{3}H)orotic acid incorporated into cytoplasmic RNA was about 6 times greater in the greening cells than in the dark-grown cells (Gibbs, 1970).

Although Ochromonas cells readily incorporate (^{3}H)uridine into both organelle and cytoplasmic rRNAs, the isotope labelling method was not adopted for measuring chloroplast rRNA synthesis in the alga. One reason is that a large difference was expected in the light-induced incorporation of (^{3}H)uridine into chloroplast and cytoplasmic rRNA species. The preliminary experiments showed, however, that the profiles of radiolabelled RNA from dark-grown and greening cells closely matched the optical density profiles. Consequently, the proportion of isotope incorporated into the chloroplast rRNA species was relatively small with respect to that incorporated into the cytoplasmic rRNA species. Therefore, the radioisotope approach did not appear to be more sensitive than the optical density method for measuring chloroplast rRNA synthesis. Another consideration, which applies to isotope labelling studies in general, was the difficulty in determining whether an increase in specific activity of rRNA reflects an increased rate of synthesis or an increased permeability of cell membranes to the exogenous radioactive precursor. The possibility that the permeability of the chloroplast in dark-grown Ochromonas is altered during greening is considerable, since

some evidence suggests that certain chloroplast envelope-associated polypeptides in higher plants are released in response to light (Cobb and Wellburn, 1974). Answering the question of whether the precursor pool for chloroplast rRNA changes in cells transferred from dark to light would require determining the kinetics of isotope uptake and incorporation into the chloroplast rRNA of dark-grown and greening cells. This was complicated by the fact that the radiolabelled light chloroplast and light mitochondrial rRNA species were not clearly resolved in the fractionated gels. Therefore, the synthesis of chloroplast rRNA could not be distinguished from that of mitochondrial rRNA (the heavy chloroplast and heavy mitochondrial rRNAs migrate as a single peak). Perhaps, the treatment of cells with ethidium bromide during the labelling period might have minimized this problem. Nevertheless, it was decided to measure the various rRNA species in Ochromonas by their ultraviolet absorbance rather than by radioisotope incorporation.

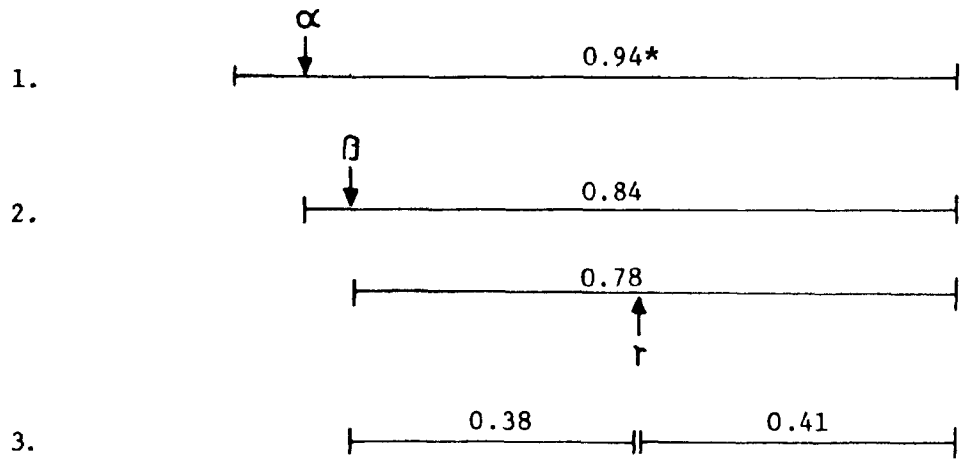
Chloroplast rRNAs from a variety of algae and higher plants are similar in size to the 23S and 16S rRNAs of bacteria and blue-green algae (Whitfield, 1973). The heavy and light chloroplast rRNAs from Ochromonas are 22S and 15S respectively, and are thus relatively small in size. The validity of this conclusion lies in the fact that the chloroplast rRNAs migrated separately and ahead of the E. coli rRNAs which served as internal standards.

The heavy chloroplast rRNA molecule from Ochromonas is similar

to the heavy rRNA component from chloroplasts of other species (e.g. Ingle et al., 1970) in its extreme susceptibility to dissociate under conditions which disrupt hydrogen-bonding. The pattern of breakdown of Ochromonas heavy chloroplast rRNA was initially studied in an effort to demonstrate that one or more of the lower molecular weight rRNA species observed on gels following electrophoresis of total RNA extracts does not represent fragments of degraded chloroplast rRNA. The results confirmed that none of the high molecular weight Ochromonas rRNA species arose from degradation.

The manner in which the heavy chloroplast rRNA breaks down is highly specific and suggests that it may reflect some fundamental structural properties of the molecule. It was therefore of interest to study the breakdown behaviour of Ochromonas chloroplast rRNA more closely, and to compare it with the dissociation pattern of chloroplast rRNA from other species. From an examination of the sizes and stoichiometry of the breakdown products resolved on the gels shown in Figures 11 and 12, it can be deduced that at least three nicks, at α, β and γ, probably exist in Ochromonas heavy chloroplast rRNA, and that cleavage may occur sequentially as illustrated on page 172. Leaver and Ingle (1971) proposed that the heavy chloroplast rRNA components in radish and pea are also nicked at three specific sites, whereas only two nicks occur in the same chloroplast rRNA species in French bean. Grierson (1974) studied the pattern of breakdown of the heavy chloroplast rRNA from mung bean (Phaseolus aureus) and concluded that the observed

SCHEME FOR THE SEQUENTIAL CLEAVAGE OF HEAVY CHLOROPLAST rRNA COMPONENT FROM OCHROMONAS



1. First scission occurs near the end of the intact heavy chloroplast rRNA molecule. The liberated piece representing about 10% of the total rRNA sequences migrates ahead of transfer RNA and is lost from the gel.
2. The α and β loci may be situated at either the same or opposite ends of the molecule. This stage of degradation is seen in Figure 12B.
3. Eventually, all of the chloroplast heavy rRNA component is converted into two low molecular fragments which resist further degradation.

* Numbers denote molecular weight x 10^6 daltons.

degradation products could be produced by dissociation at only two specific sites in the intact molecule.

A similar lability to that of chloroplast rRNA has been noted in the heavy rRNA component from blue-green algae. For example, magnesium is required during extraction to preserve the integrity of the 23S rRNA component from Anacystis nidulans; in the absence of magnesium, a third component appears which migrates slightly ahead of the heavy rRNA on polyacrylamide gels (Szalay et al., 1972; Doolittle, 1973). The degree of instability of the heavy rRNA molecule varies with the species of blue-green alga. For example, under conditions where more than half of the 23S rRNA from Anabaena cylindrica is fragmented, only a small percentage of the heavy rRNA from Tolypothrix distorta is cleaved and the 23S rRNA from Nostoc muscorum is stable (Grierson and Smith, 1973). The heavy rRNA component from another prokaryote, Agrobacterium tumefaciens, provides an example of extreme instability (Grienenberger and Simon, 1975; Schuch and Loening, 1975). Although pulse-chase labelling experiments indicate that the 23S rRNA is transcribed as a continuous chain in this organism, none of the heavy rRNA was present in electrophoretic profiles of nucleic acids extracted at normal salt concentrations and in the absence of magnesium. Evidence suggests that two nicks are first introduced into the Argrobacterium rRNA in vivo at either end of the molecule followed later by a third nick in the middle. Thus, the pattern of nicking of the heavy rRNA component in this prokaryote is very similar to that proposed for the heavy

chloroplast rRNA in Ochromonas.

Labile heavy rRNAs have also been reported in the 80S ribosomes from eukaryotic microörganisms and animals. The heavy cytoplasmic rRNA from Acanthamoeba castellani (Stevens and Pachler, 1972), Euglena (Rawson et al., 1971), and Ilyanassa obsoleta (Koser and Collier, 1971) each dissociates into at least two large fragments under conditions which disrupt hydrogen bonding. The 26S rRNA from insects displays a similar mode of breakdown and has been studied in some detail. Lava-Sanchez and Puppo (1975) examined the sequential breakdown of 26S rRNA from the dipteran Musca carnaria by briefly heating the rRNA preparation at progressively higher temperatures. The resulting series of degradation patterns is compatible with the presence of three nicks in the 26S rRNA molecule.

The variation in breakdown patterns exhibited by heavy chloroplast rRNA from Ochromonas and by heavy rRNAs from other species probably represents species-specific differences in the topography of the ribosomes such that different regions of the rRNA component are susceptible to RNase attack. The notion that scission of the polynucleotide chain occurs at sites which are exposed at the surface of the ribosomes receives support from a study in which E. coli ribosomes were treated with pancreatic RNase (Rodgers, 1974). Gel electrophoresis of the rRNAs from the bacterial ribosomes after mild enzyme treatment indicated that, although the small ribosomal subunit is quite resistant to RNase, endonucleolytic cleavage occurs at a small number of discrete

sites in the rRNA from the large ribosomal subunit. Lava-Sanchez and Puppo (1975) isolated cytoplasmic ribosomes from dipteran larvae pulse-labelled with (^{3}H)uridine. Some of these ribosomes were then incubated with pancreatic RNase. Whereas the (^{3}H)uridine-labelled 26S rRNA extracted from the control ribosomes was intact, the labelled 26S rRNA from the incubated ribosomes contained nicks at the same sites as those produced *in vivo*. This result also supports the idea that the hidden breaks in heavy rRNA occur in regions of the molecule which are exposed or accessible to RNase. Consequently, the breakdown pattern displayed by the heavy rRNA species may provide information concerning the structure of ribosomes and their evolutionary relationships. The similarity between the models proposed for the breakdown of *Ochromonas* heavy chloroplast rRNA and the heavy rRNA species from *Agrobacterium* suggests that the structure of ribosomes from the prokaryote resembles that of chloroplast ribosomes from *Ochromonas*.

The dissociation of the heavy chloroplast rRNA can be prevented in the presence of appropriate amounts of magnesium. This phenomenon was discovered by Ingle and his associates (Ingle *et al.*, 1970; Leaver and Ingle, 1971) and was more recently described by Leaver (1973) for chloroplast rRNA from broad bean. Magnesium also stabilizes the heavy chloroplast rRNA isolated from *Ochromonas*. To explain the effect of magnesium, Leaver (1973) has proposed that the divalent cation mediates the formation of cross-links between

nucleotide residues in neighbouring regions of the rRNA helix. This idea is supported by the observation that calcium, another divalent cation, also stabilizes chloroplast rRNA (Leaver and Ingle, 1971). In this way, 'hidden' breaks or nicks in the polynucleotide chain are masked in the presence of magnesium. In the present study, magnesium was omitted from both the extraction and electrophoresis buffers; presumably, sufficient amounts of intracellular divalent cations remain bound to Ochromonas chloroplast rRNA to preserve its integrity. Atchison et al. (1973) have demonstrated that the thermolability of chloroplast rRNA, isolated from tobacco by a procedure involving the presence of magnesium, is not due to 'hidden' breaks, but rather to 'hidden' nuclease contamination of the RNA preparations. Therefore, it was necessary to determine whether magnesium was acting to inhibit residual RNase present in the nucleic acid extracts of Ochromonas. Ochromonas and E. coli RNAs were mixed at ratios of 1:1, 2:1 and 3:1, incubated at 37° C and subjected to electrophoresis. In contrast to the results for the tobacco rRNA, the algal preparations do not induce breakdown of the E. coli rRNA. This observation suggests that Ochromonas rRNA is free of RNase.

c. Mitochondrial rRNAs

Ochromonas is apparently unique among green plants analyzed to date in that it contains a relatively large amount of mitochondrial rRNA which can be detected in electrophoretic profiles of extracts of total cellular RNA. Identification of the peaks containing mitochondrial rRNA was achieved

by showing that two peaks contained rRNA whose synthesis is inhibited by ethidium bromide. Moreover, the size of the putative light mitochondrial rRNA argues against the possibility that it is merely a degradation product of the labile heavy chloroplast rRNA, since none of the fragments generated by the induced breakdown of the heavy chloroplast rRNA are the same size as the mitochondrial rRNA species. The relative abundance of mitochondrial rRNAs evident in electrophoretic profiles of total Ochromonas rRNA agrees with the relative number of mitochondrial ribosomes present in these cells as calculated from organelle volume measurements (Gibbs, 1968, 1970) and ribosome counts (Smith-Johannsen and Gibbs, 1972). In contrast, Chlamydomonas contains relatively few mitochondrial ribosomes (Goodenough and Levine, 1970b), and thus mitochondrial rRNAs appear to be absent in analyses of total rRNAs from this alga (e.g. Cattolico and Jones, 1972). Loening and Ingle (1967) suggested that some of the minor peaks observed in electropherograms of total RNA from higher plants might represent mitochondrial rRNAs, but these investigators could not exclude the possibility that the minor peaks contained dissociated fragments of chloroplast rRNA.

Therefore, unlike mitochondrial rRNAs from other algae and higher plants, those from Ochromonas could be characterized without prior isolation of mitochondria or mitochondrial ribosomes. This reduces the possibility of rRNA degradation. The sizes of the heavy and light mitochondrial rRNAs from Ochromonas are 22S and 16S, respectively. These values are similar to the 21S and 16S obtained

by Avadhani and Buetow (1972) for mitochondrial rRNAs from Euglena, the only other alga in which these rRNA species have been characterized. Other investigators have reported that Euglena mitochondrial rRNAs are 14S and 11S (Krawiec and Eisenstadt, 1970; Crouse et al., 1974), but it is likely that these smaller molecules are degraded rRNAs. The sizes of mitochondrial rRNAs from Ochromonas and Euglena are therefore in the same range as those reported for these rRNA species in fungi and other eukaryotic microörganisms: 21 - 24S and 14 - 16S for the heavy and light rRNA components, respectively (Stewart, 1973). In contrast, the mitochondrial rRNAs from higher plants have a considerably higher molecular weight and more closely resemble cytoplasmic rRNAs in size. Leaver and Harmey (1973) found that the molecular weights of the rRNAs extracted from purified mitochondria and mitochondrial ribosomes from turnip (Brassica rapa), mung bean, potato, cauliflower (Brassica oleracea var. botrytis) and pea are 1.12 - 1.18 x 10^6 (23.5S)[1] and 0.69 - 0.78 x 10^6 daltons (18S), whereas the molecular weights of the cytoplasmic rRNAs from these dicots are 1.3 x 10^6 (25S) and 0.7 x 10^6 daltons (17.5S). Mitochondrial rRNAs extracted from the purified organelles from etiolated maize shoots have molecular weights of 1.26 x 10^6 (24.5S) and 0.74 x 10^6 daltons (18S), and are thus equal to and larger than the cytoplasmic rRNAs which are 1.26 x 10^6 and 0.70 x 10^6 daltons in this monocotyledonous species (Pring, 1974). The mitochondrial rRNA from Virginia creeper

[1]Sedimentation coefficients in brackets were determined graphically by the candidate.

(Parthenocissus tricuspidata) are exceptionally small compared to those from the other higher plants so far examined, having molecular weights of 0.84×10^6 and 0.42×10^6 daltons, which correspond to sedimentation coefficients of 21S and 13S (Quétier and Védel, 1974).

Mitochondrial rRNAs have some unusual physical properties which influence their electrophoretic mobilities in gels. For example, when subjected to electrophoresis at room temperature (Yu et al., 1972) or in buffer of low ionic strength (Edelman et al., 1971), mitochondrial rRNAs from a variety of fungi migrate more slowly than normally. Evidence suggests that the sensitivity of mitochondrial rRNA to these conditions is related to its conformation, which in turn is partly dependent on its base composition. Base composition analyses indicate that fungal mitochondrial rRNAs contain 13 - 25% less guanosine-cytosine base pairs than do the corresponding cytoplasmic rRNA species (Edelman et al., 1971). Thus, the anomalous behaviour of mitochondrial rRNAs in gels may be explained by their more extended configurations relative to their cytoplasmic counterparts; under mild denaturing conditions, mitochondrial rRNAs may even undergo extensive melting. This possibility is supported by evidence from a study of the circular dichroism of mitochondrial rRNA from the fungus Trichoderma viride (Verma et al., 1971). According to the investigators, the data indicate that at moderate ionic strength, the mitochondrial rRNA has a less ordered structure than the cytoplasmic rRNA, and at low ionic strength, it consists of mostly single-stranded regions.

Thus, determination of the sizes of mitochondrial rRNAs by gel electrophoresis (as well as sedimentation analysis) may be misleading. One way of dealing with the problem posed by the unusual structure of mitochondrial rRNAs is to eliminate the contribution of all conformational effects on migration rate by conducting electrophoresis of rRNAs under denaturing conditions. The molecular weights of maize mitochondrial rRNAs, when electrophoresed with E. coli rRNAs in 8 M urea at 60° C are 1.19×10^6 and 0.67×10^6 daltons (Pring and Thornbury, 1975), which is considerably smaller than the values obtained under non-denaturing conditions (Pring, 1974). The apparent size of mitochondrial rRNAs from Parthenocissus, however, remains constant under a variety of non-denaturing and denaturing conditions (Quétier and Védel, 1974). In the present study, Ochromonas rRNAs were not characterized by electrophoresis in denaturants; however, since low temperatures and moderate ionic strength were maintained during fractionation, the mitochondrial rRNAs would probably tend to assume a relatively compact conformation (Spirin and Gavrilova, 1969). Therefore, the values for the sizes of Ochromonas mitochondrial rRNAs reported here, although tentative, probably represent close approximations.

B. Antibiotic Studies

1. Chloramphenicol

At low concentrations, the D-threo isomer of chloramphenicol inhibits protein synthesis on chloroplast (Eisenstadt and Brawerman, 1964) and mitochondrial ribosomes (Lamb et al., 1968), but not on the

cytoplasmic ribosomes of lower and higher plants. Presumably, the antibiotic blocks translation on the 70S ribosomes of organelles by inhibiting the functional attachment of the aminoacyl-tRNA to the 50S subunit (Pestka, 1971). At higher concentrations, D-threo chloramphenicol may also directly inhibit mitochondrial respiration (Freeman and Haldar, 1968; Firkin and Linnane, 1968). Therefore, L-threo chloramphenicol, which inhibits oxidative phosphorylation (Hanson and Krueger, 1966) but not protein synthesis (Ellis, 1969; Ireland and Bradbeer, 1971), was used in this study to distinguish between the non-specific and specific effects of D-threo chloramphenicol on Ochromonas. The L-threo isomer at 300 µg/ml retards cell division, while the same concentration of D-threo chloramphenicol affects growth of Ochromonas more severely. Since cells treated with D-threo chloramphenicol also suffer a loss in the number of mitochondrial cristae (Smith-Johannsen and Gibbs, 1972), the decline in growth rate in the presence of this antibiotic is probably, at least in part, a secondary effect on mitochondria resulting from the inhibition of the synthesis of cristae proteins. Despite their inability to divide, cells grown in the presence of D-threo chloramphenicol for 24 hr contain normal amounts of mitochondrial rRNAs. The amounts of the chloroplast rRNA species, however, are reduced by at least 70%, in accordance with earlier ultrastructural observations on the effect of D-threo chloramphenicol on the number of chloroplast ribosomes (Smith-Johannsen and Gibbs, 1972). In contrast, cells cultured for 24 hr in medium

containing L-threo chloramphenicol, although also incapable of further division, still possess normal amounts of the chloroplast rRNA species. Thus, the apparent inhibition of chloroplast rRNA synthesis in the cells exposed to D-threo chloramphenicol is most likely a direct consequence of the selective blocking of chloroplast ribosome function. Since D-threo chloramphenicol does cause a marked decrease in the rate of cell division, the possibility that the reduction in the amount of chloroplast rRNAs results instead from the general repression of growth cannot be ruled out.

2. Spectinomycin

a. Chloroplast

Spectinomycin inhibits peptide chain elongation on bacterial ribosomes by interfering with translocation (Pestka, 1971). This aminoglycoside antibiotic has also been shown to be a potent inhibitor of protein synthesis on chloroplast ribosomes from algae (Schlanger and Sager, 1974) and higher plants (Ellis, 1970), while not affecting protein synthesis on cytoplasmic ribosomes.

i. Chloroplast membranes

When protein synthesis on chloroplast ribosomes in Ochromonas is inhibited by spectinomycin, the normal arrangement of thylakoids is altered in a manner similar to that in cells exposed to chloramphenicol (Smith-Johannsen and Gibbs, 1972). Chloroplasts in both light-grown and greening cells treated with spectinomycin contain unfused, single thylakoids as well as abnormally large stacks of thylakoids. Since it

is unlikely that spectinomycin directly interferes with membrane fusion, this suggests that one or more proteins involved in the regulation of thylakoid fusion is synthesized on chloroplast ribosomes in Ochromonas. Other ultrastructural studies show that chloramphenicol also disrupts the organization of thylakoids in Chlamydomonas (Goodenough, 1971), Euglena (Bishop et al., 1973), bean (Margulies, 1966) and pea (Srivastava et al., 1971). Therefore, Ochromonas resembles other algae and higher plants with respect to the site of synthesis of protein(s) involved in the control of chloroplast thylakoid fusion.

The hypertrophy of the perinuclear reticulum in response to spectinomycin was also observed in cells of Ochromonas treated with chloramphenicol (Smith-Johannsen and Gibbs, 1972). The function of the perinuclear reticulum is unknown, but it may play a role similar to that of smooth endoplasmic reticulum which it resembles. In liver cells the smooth endoplasmic reticulum proliferates in response to alcohol and other drugs which are metabolized by enzymes localized in the membrane network (Lieber, 1976). By analogy, the hypertrophy of the perinuclear reticulum in Ochromonas exposed to spectinomycin or chloramphenicol may be related to the induced synthesis of detoxifying enzymes.

ii. Chloroplast ribosomes and rRNAs

Data obtained by both electron microscopy and biochemical analysis indicate that spectinomycin, at a concentration which does not inhibit growth substantially, inhibits the synthesis of chloroplast ribosomes and rRNA in Ochromonas. Although both ribosome counts

(Table VII) and analysis of rRNA electrophoretic profiles (Table VI) show that the magnitude of the reduction of chloroplast ribosomes is about 30% in greening cells, the inhibition of chloroplast ribosome synthesis during greening is actually greater than 50% (Table VIII). In contrast to a previous ultrastructural investigation on the effect of chloramphenicol on Ochromonas in which the growth of the chloroplast itself was markedly inhibited (Smith-Johannsen and Gibbs, 1972), the chloroplast volume was not significantly reduced in the spectinomycin-treated cells described here. Thus, the effect of spectinomycin on the synthesis of chloroplast rRNA and ribosomes cannot be attributed to a feedback inhibition resulting from a more general block in organelle expansion.

A possible explanation for the inhibitory effect of spectinomycin on the accumulation of chloroplast rRNA in Ochromonas could be that the antibiotic blocks the activity or synthesis of chloroplast RNA polymerase. This seems unlikely, however, since Ellis and Hartley (1971) have demonstrated that lincomycin, while preventing an increase in RuDP carboxylase activity in pea, fails to reduce either the amount of chloroplast RNA polymerase synthesized or its activity. This finding suggests that chloroplast RNA polymerase is synthesized on cytoplasmic ribosomes. Assuming the biogenesis of this enzyme in Ochromonas is similar to that in pea, the effect of spectinomycin in the alga cannot be accounted for by a lack of chloroplast RNA polymerase.

The most likely explanation for the reduction of chloroplast rRNA in the presence of spectinomycin is that the antibiotic inhibits the synthesis of ribosomal proteins. Newly-transcribed rRNA is associated with proteins (Hamkalo and Miller, 1973) and, presumably, is rapidly degraded if not incorporated into a ribonucleoprotein complex. Therefore, if chloroplast ribosomal proteins are not available, chloroplast rRNA cannot accumulate. This was demonstrated by Ellis and Hartley (1971) who observed that incorporation of precursors into pea chloroplast rRNAs continued in the presence of lincomycin, while the accumulation of these species was inhibited. The size of the pool of chloroplast ribosomal proteins in Ochromonas is not known, but the data of Honeycutt and Margulies (1973) indicate that in Chlamydomonas the pool is small, since labelled amino acids are incorporated into chloroplast ribosomes after only 6 min. Moreover, in chloroplasts of exponentially-growing algae, the pool of ribosomal proteins would be rapidly exhausted if not continually replenished with newly-synthesized proteins. Therefore, since spectinomycin, which selectively inhibits the synthesis of proteins on chloroplast ribosomes, prevents the light-induced increase of chloroplast rRNA and ribosomes in Ochromonas, I conclude that at least some chloroplast ribosomal proteins in this alga are synthesized in the chloroplast. This supports previous findings based on a study of the effects of chloramphenicol on the ultrastructure of the chloroplast in Ochromonas (Smith-Johannsen and Gibbs, 1972).

Galling et al. (1973) have reported that spectinomycin also causes a reduction of chloroplast ribosomes and rRNAs in Chlorella. These investigators suggest that spectinomycin may inhibit the synthesis of one or a few key chloroplast proteins involved in the regulation of the synthesis of chloroplast ribosomal proteins in the cytoplasm. However, in Chlamydomonas and Acetabularia where evidence indicates that chloroplast ribosomal proteins are synthesized in the cytoplasm (Goodenough, 1971; Kloppstech and Schweiger, 1974), spectinomycin and/or chloramphenicol do not prevent the accumulation of chloroplast ribosomes. Therefore, the data of Galling et al. (1973) could better be interpreted to mean that in Chlorella some chloroplast ribosomal proteins are synthesized in the chloroplast. Similar conclusions can be drawn from studies on other organisms in which inhibitors of translation on chloroplast ribosomes cause a decrease in the amount of chloroplast ribosomes and/or rRNA. Thus, apparently some chloroplast ribosomal proteins are synthesized on chloroplast ribosomes in Euglena (Reger et al., 1972), radish (Ingle, 1968), pea (Ellis and Hartley, 1971) and spinach (Detchon and Possingham, 1975). As stated in the Introduction, the lack of inhibitory effect of chloramphenicol on the synthesis of chloroplast ribosomes and rRNA in Acetabularia (Kloppstech and Schweiger, 1974) and Chlamydomonas (Goodenough, 1971) probably reflects genuine species-specific differences in the biogenesis of this class of proteins.

b. Mitochondria

The only evidence the candidate is aware of that spectinomycin

inhibits protein synthesis on mitochondrial ribosomes is the observation that wild-type cells of Chlamydomonas are killed when cultured heterotrophically in medium containing low concentrations of the antibiotic (Boynton et al., 1973). Moreover, cyanide-sensitive respiration in Chlamydomonas subjected to prolonged exposure to 90 µg/ml spectinomycin is abolished, indicating the absence of mitochondrially-synthesized components.

In contrast to Chlamydomonas, Ochromonas continues to grow at nearly normal rates for up to 48 hr in the presence of 100 µg/ml spectinomycin. Electron microscopy shows that the ultrastructure of the mitochondria in spectinomycin-treated cells (24 hr treatment) appears normal. This is at variance with previous observations of cells treated for 24 hr with chloramphenicol in which the ratio of inner to outer mitochondrial membranes was reduced by over 35%. The concentration of spectinomycin used in the present study is relatively low, however, and the inhibitory effects of the antibiotic may become manifested at a correspondingly slow rate. Thus, it is likely that if cells were examined by electron microscopy after further growth in medium containing spectinomycin, a decrease in the number of mitochondrial cristae would be evident. The difference in the kinetics of the appearance of spectinomycin-induced membrane abnormalities in the chloroplast and mitochondria could be explained by the existence of a relatively larger pool of mitochondrial cristae proteins. This idea is substantiated by the observation that even in

the chloramphenicol-treated cells, the loss of mitochondrial cristae does not occur until after 12 hr of exposure to the antibiotic, whereas chloroplast membranes are immediately affected. Therefore, the absence of an effect of spectinomycin on mitochondrial membrane in Ochromonas does not rule out the likelihood that one or more cristae components are synthesized in the mitochondria of this alga. In fungi, almost all the proteins synthesized on mitochondrial ribosomes are incorporated into the inner membrane of the organelle (Neupert and Ludwig, 1971; Turner, 1973).

In contrast to its inhibitory effect on the accumulation of chloroplast ribosomes, spectinomycin has no apparent effect on the synthesis of mitochondrial ribosomes in Ochromonas. Again, one might argue that mitochondria contain substantial pools of ribosomal proteins. Alternatively, mitochondrial ribosomal proteins in Ochromonas may be synthesized on cytoplasmic ribosomes, as has been demonstrated in fungi (Davey et al., 1969; Küntzel, 1969b; Borath and Küntzel, 1972).

Since 24 hr treatment with spectinomycin fails to alter either mitochondrial ultrastructure or mitochondrial rRNA synthesis in Ochromonas, there is a possibility that the antibiotic does not enter this organelle or does not inhibit translation on mitochondrial ribosomes. The results of a study by Davey et al. (1970) suggest that the sensitivity of mitochondrial ribosomes to aminoglycoside antibiotics varies in different species. Of seven aminoglycosides tested, four failed to inhibit the amino acid incorporating activity of yeast

mitochondrial ribosomes either *in vivo* or *in vitro*. The remaining three antibiotics blocked protein synthesis on both mitochondrial and cytoplasmic ribosomes from yeast, but had no significant effect on amino acid incorporation by mitochondria isolated from rat liver. Although spectinomycin was not included in this study, streptomycin, which is closely related in structure (Beneviste and Davies, 1973), was shown to have little or no effect on protein synthesis on yeast mitochondrial ribosomes. Since *Chlamydomonas*, which does contain spectinomycin-sensitive mitochondrial ribosomes, is more closely related to *Ochromonas* than is yeast it is probable that protein synthesis on mitochondrial ribosomes in *Ochromonas* is also inhibited by this aminoglycoside. Conclusive proof for this assumption requires an *in vitro* assay of the protein synthesizing activity of isolated mitochondrial ribosomes in the presence of spectinomycin.

3. Ethidium bromide

The biological activity of the phenanthridine dye ethidium bromide is correlated with its ability to intercalate between DNA base pairs (Waring, 1968) and, in particular, its selective affinity for closed circular DNA (Bauer and Vinograd, 1968). Using SV40, Bauer and Vinograd (1968) demonstrated that, at low concentrations (less than 5.4 µg/ml), ethidium bromide is preferentially bound by closed, circular viral DNA molecules.

a. Mitochondria

In eukaryotic cells, the target of ethidium bromide action is

the mitochondrion. Mitochondria from nearly all the lower (Borst, 1970) and higher plants (Kolodner and Tewari, 1972b), as well as from animals (Borst, 1970) examined so far, contain covalently-closed circular DNA molecules. Both the replication (Goldring et al., 1970) and transcription (Zylber et al., 1969) of mitochondrial DNA in yeast and mammalian cells respectively, has been shown to be selectively inhibited by the dye. Presumably, binding of ethidium bromide to mitochondrial DNA in vivo blocks the functioning of this component by distorting the tertiary structure of the supercoiled DNA molecules (Smith et al., 1971; Nass, 1972).

Although the growth of Ochromonas cells cultured in medium containing 1 µg/ml ethidium bromide is normal for approximately 24 hr, the morphology of the mitochondria in cells exposed to the dye during this interval is dramatically altered; the number of mitochondrial ribosomes is reduced by more than 90% and the number of cristae is also markedly reduced compared to control cells. The results of quantitative analysis of the electrophoretic profiles of rRNAs from control and ethidium bromide-treated cells are in agreement with the ultrastructural observations and show that the transcription of the mitochondrial rRNA species is severely and exclusively inhibited in the presence of the dye. The possibility that the effect of ethidium bromide on rRNA and ribosome metabolism in Ochromonas when cultured in the light is related in some way to the fluorescence of the phenanthridine dye is ruled out by the fact that the accumulation of mitochondrial rRNAs in dark-grown

cells is equally prevented by ethidium bromide. Also, Pikó and Chase (1973), using electron microscopy and polyacrylamide gel electrophoresis, have demonstrated that 0.1 µg/ml ethidium bromide also induces the selective loss of mitochondrial ribosomes and rRNAs in mammalian embryonic cells.

The abrupt decline in the rate of cell division of ethidium bromide-treated cells after 24 hr is most likely a consequence of a depressed respiration rate due to the loss of the mitochondrial cristae. The effect of ethidium bromide on mitochondrial membrane is probably not direct but is secondary to the decrease in the number of mitochondrial ribosomes on which some cristae proteins are synthesized (e.g. Rubin and Tzagoloff, 1973). A similar loss of mitochondrial cristae was observed when translation on mitochondrial ribosomes was inhibited in Ochromonas by chloramphenicol (Smith-Johannsen and Gibbs, 1972). Low concentrations of ethidium bromide have been shown to mimic the effects of chloramphenicol on mitochondrial membranes in mammalian cells also (King et al., 1972; Pikó and Chase, 1973). In these cells, however, exposure to either drug results in a disorganization of mitochondrial cristae, rather than a reduction in their number. Similarly, 25 µg/ml ethidium bromide induces the dilation of mitochondrial cristae in Acetabularia (Heilporn and Limbosch, 1971), while 1 - 5 µg/ml of the dye causes aberrations in the arrangement of the mitochondrial cristae, as well as a reduction in their number in bleached cells of Euglena (Nass and Ben-Shaul, 1973). The results of a study on Chlamydomonas

indicate that 3 µg/ml ethidium bromide secondarily inhibits mitochondrial protein synthesis in this alga as well (Stegeman and Hoober, 1974).

The effect of ethidium bromide on the synthesis and maintenance of mitochondrial DNA in Ochromonas was not examined in this study. In yeast cells 10 µg/ml ethidium bromide, not only inhibits the replication of mitochondrial DNA, but also induces the degradation of the supercoiled molecules (Goldring et al., 1970). Evidence suggests that ethidium bromide also impairs the synthesis of mitochondrial DNA in Euglena (Nass and Ben-Shaul, 1973), but the effects of the dye in this alga are at least partially reversible. Heilporn and Limbosch (1971) found that, although the amount of mitochondrial DNA is markedly reduced in extracts from organelles isolated from ethidium bromide-treated Acetabularia, the inhibitory effect of the dye is entirely reversible even after 1 month. However, when DNA from total cell extracts was analyzed, no diminution in the amount of mitochondrial DNA was observed. These investigators therefore proposed that ethidium bromide does not degrade pre-existing mitochondrial DNA in Acetabularia, but probably alters the structure of this component such that it is more susceptible to nuclease during extraction. The reversibility of ethidium bromide-induced damage of mitochondrial DNA in Ochromonas could be tested by inoculation of treated cells in media free of the dye. It is important to note that reversibility of the ethidium bromide-induced inhibition does not rule out the possibility that mitochondrial DNA is partially degraded, but that sufficient copies of mitochondrial DNA exist to permit continued

replication of the organelles.

b. Chloroplast

In contrast to its effect on mitochondria, ethidium bromide does not perceptibly alter the ultrastructure of the chloroplast in Ochromonas. Likewise, treated cells contain normal amounts of the chloroplast rRNA species. These observations are therefore in accordance with the lack of effect of ethidium bromide on the ultrastructure of chloroplasts in Euglena (Nass and Ben-Shaul, 1973). Also, although the chloroplasts in Acetabularia become swollen with storage products in the presence of ethidium bromide, their ultrastructure, as well as photosynthetic capacity, is otherwise normal (Heilporn and Limbosch, 1971).

The chlorophyll content of greening cells of Ochromonas is not reduced by ethidium bromide, but the amount of chlorophyll in light-grown cells cultured in the presence of the dye was found to be 20% less than in controls. The differential response of light-grown and greening cells to ethidium bromide may be explained by the fact that the accumulation of chlorophyll in cells grown in continuous light is a function of cell density: chlorophyll per cell increases as cell number increases (Gibbs, 1961). At the time of extraction of chlorophyll, the cultures treated with ethidium bromide happened to contain a slightly lower concentration of cells than the other cultures and thus may not have acquired as much chlorophyll as the more dense cultures. The chlorophyll content of Acetabularia is also unaffected by ethidium bromide (Heilporn and Limbosch,

1971). Nass and Ben-Shaul (1973) reported that in greening cells of Euglena, ethidium bromide depresses chlorophyll formation significantly. The inhibition of chlorophyll synthesis in Euglena occurred only after about 3 days exposure to the dye and can most likely be attributed to a general reduction in cellular metabolism due to the secondary impairment of mitochondrial respiration.

Chloroplasts from Euglena (Manning et al., 1971) and higher plants (Manning et al., 1972) have been shown to contain supercoiled DNA. If one assumes that the closed, circular nature of chloroplast DNA is universal, the apparent insensitivity of the chloroplast in Ochromonas, Acetabularia and Euglena to ethidium bromide seems to be at variance with the mode of action of this dye in eukaryotic cells. The discrepancy between the predicted and observed effects of ethidium bromide on chloroplasts might be explained if chloroplast DNA does not bind the dye. Chloroplast DNA isolated from Antirrhinum, Beta and Spinachia however, has been shown to bind ethidium bromide (Herrmann et al., 1975). Another possible explanation for the lack of effect of ethidium bromide on the chloroplast is that this organelle is impermeable to the dye. This is unlikely since ethidium bromide was shown to inhibit all synthesis of cytoplasmic DNA in Acetabularia under conditions where nuclear DNA synthesis was not affected (Heilporn and Limbosch, 1971). Moreover, Flechtner and Sager (1973) reported that 10 µg/ml ethidium bromide induces the loss of about 85% of the chloroplast DNA in Chlamydomonas. Although the algal cells did not divide during the 12 hr treatment, they

nevertheless were able to resume growth when washed free of the dye, and also regenerated some of their chloroplast DNA. To account for these results, Flechtner and Sager proposed that one or more 'master' copies of chloroplast DNA are sequestered in a region inaccessible to the intercalating dye molecules. Since Acetabularia chloroplasts double in number before their division is arrested directly or indirectly by ethidium bromide (Heilporn and Limbosch, 1971), they may contain at least two 'master' copies of chloroplast DNA. Both greening and light-grown cells of Ochromonas, on the other hand, grow normally for at least two generations in the presence of ethidium bromide, suggesting that, not only the chloroplast, but also the proplastid, contain at least four sequestered copies of plastid DNA. Further studies are required to prove the 'master' copy hypothesis or to explain in some other way the differential response of chloroplast and mitochondrial circular DNAs to ethidium bromide.

C. Future Studies

Perhaps the most important result of this investigation is the evidence that at least some chloroplast ribosomal proteins are synthesized on chloroplast ribosomes in Ochromonas. It would be interesting to know exactly which and how many of the chloroplast ribosomal proteins are synthesized within the organelle. Although initial attempts to isolate ribosomes from Ochromonas were unsuccessful, it may yet be possible. Low temperatures may be as essential to the isolation of ribosomes as they are for the isolation of rRNA from this

alga. Chloroplast ribosomal proteins from control and spectinomycin-treated cells could then be analyzed on two-dimensional polyacrylamide gels.

The effect of spectinomycin (and chloramphenicol) on the perinuclear reticulum of Ochromonas is difficult to interpret since the function of this membrane network is unknown. The hypothesis that the perinuclear reticulum is the site of detoxifying enzymes could be tested by treating cells with radioactive spectinomycin (or chloramphenicol) and analyzing the results by EM autoradiography.

Another important result of the present study is the development of the methodology for the extraction and characterization of intact rRNAs from Ochromonas. The 'nicking' of the heavy chloroplast rRNA component is similar to that in heavy rRNAs from other sources. The time course of this phenomenon in vivo could be examined by isotope labelling studies. The relationship of 'nicking' in vivo and in vitro to chloroplast ribosome structure could be determined by experiments similar to those of Lava-Sanchez and Puppo (1975).

There is some evidence which suggests that rRNA populations in both prokaryotes and eukaryotes are not homogeneous (in Cattolico, 1973). For example, Cattolico (1973) detected a small difference in the oligonucleotide patterns of the 25S rRNA species extracted from Chlamydomonas at two separate stages in the synchronous growth cycle. Ochromonas provides an ideal organism in which to study the question of

rRNA heterogeneity since the alga undergoes a transition when switched from dark to light growth conditions. Moreover, the cytoplasmic, chloroplast and mitochondrial rRNA species can be isolated without agents which may affect their chemical properties, and analyzed independently.

In summary, the results of this investigation have helped to elucidate some aspects of the regulation of organelle biosynthesis.

CONTRIBUTION TO KNOWLEDGE

The candidate considers the following to be contributions to original knowledge in the field of cell and molecular biology:

1. A procedure was developed for the isolation of intact rRNAs from the Chrysophyte, Ochromonas danica. The electrophoretic profile of Ochromonas RNA is unique since it contains, not four, but five peaks of high molecular weight rRNA.

2. Cytoplasmic, chloroplast and mitochondrial rRNA species were identified on the basis of their relative amounts in cells grown in the dark and light, or exposed to ethidium bromide.

3. The sizes of Ochromonas rRNAs were determined by polyacrylamide gel electrophoresis of (^{3}H)RNA from Ochromonas using (^{14}C)rRNAs from E. coli as internal markers. The molecular weights of the heavy and light cytoplasmic rRNAs are 1.18×10^6 and 0.66×10^6 daltons respectively. The molecular weights of the heavy chloroplast and mitochondrial rRNAs are both 0.94×10^6 daltons, while the light chloroplast and mitochondrial rRNAs have values of 0.50×10^6 and 0.55×10^6 daltons respectively.

4. The heavy chloroplast rRNA component from Ochromonas is thermolabile and probably contains three nicks, one at each end of the rRNA molecule and a third nick near the middle.

5. The growth rate of greening cells exposed to either D-threo or L-threo chloramphenicol (300 µg/ml) begins to decline markedly after 12 hr. During 24 hr exposure to 100 µg/ml spectinomycin, greening cells divide at a rate

slightly less than that of controls, whereas spectinomycin treated light-grown cells grow at normal rates. In the presence of 1 μg/ml ethidium bromide, both greening and light-grown cells divide at rates similar to controls for 24 hr.

6. D-threo chloramphenicol (300 μg/ml), but not L-threo chloramphenicol (300 μg/ml), inhibits the 24 hr light-induced increase of chloroplast rRNA in Ochromonas by 70%. Neither chloramphenicol isomer alters the amount of mitochondrial rRNA significantly.

7. Spectinomycin (100 μg/ml) reduces the amount of chloroplast rRNA in 24 hr greening cells by approximately 30% and inhibits the light-induced synthesis of chloroplast ribosomes by 50%. This antibiotic does not affect the amount of mitochondrial rRNA or the concentration of mitochondrial ribosomes in 24 hr greening cells. The effect of 24 hr exposure to 100 μg/ml spectinomycin on the amounts of organelle rRNAs and ribosomes in light-grown cells is similar to that in greening cells, but is less in magnitude.

8. Treatment of greening and light-grown cells with 1 μg/ml ethidium bromide for 24 hr results in the selective loss of mitochondrial rRNA and ribosomes.

9. Greening and light-grown cells exposed to 100 μg/ml spectinomycin for 24 hr contain chloroplasts with disorganized thylakoids. Both single thylakoids and bands consisting of five to eleven thylakoids (instead of the characteristic three thylakoids) are commonly observed. Mitochondrial ultrastructure is normal in spectinomycin-treated cells.

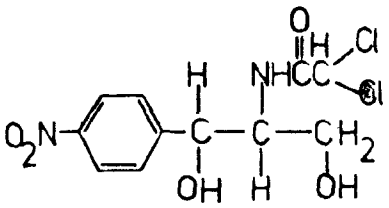
10. Ethidium bromide (1 μg/ml) treatment of both greening and light-grown cells for 24 hr causes a substantial reduction in the number of mitochondrial cristae. The dye has no effect on chloroplast ultrastructure.

11. The effects of D-threo chloramphenicol and spectinomycin on Ochromonas strongly suggest that at least some chloroplast ribosomal proteins are synthesized in the chloroplast of this alga. A protein(s) involved in thylakoid fusion is also probably synthesized on chloroplast ribosomes.

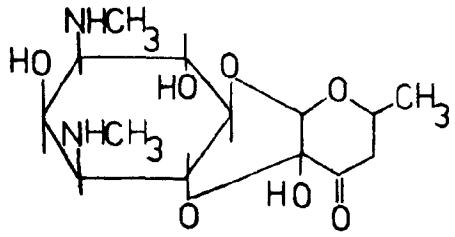
12. The nature of the ethidium bromide induced alterations indicates that Ochromonas mitochondrial rRNAs are transcribed from circular mitochondrial DNA molecules. A protein(s) required for cristae formation is synthesized on mitochondrial ribosomes.

APPENDIX

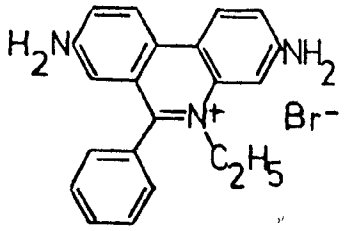
A. Chemical structure of antibiotics used in this study



1. Chloramphenicol



2. Spectinomycin



3. Ethidium bromide

B. Estimation of the coding potential of mitochondrial and chloroplast DNA

Assuming for a nucleotide an average molecular weight = 300 daltons and length along the axis of the DNA helix = 3.4 Å, and for an amino acid an average molecular weight = 125 daltons, one can calculate the approximate number of polypeptides for which organelle DNA can code.

1. Mitochondrial DNA

Animal mitochondria DNA, 5 μm in contour length (Borst, 1970), is the smallest known organelle DNA. The number of nucleotides contained in a strand of DNA 1 μm in length = 2.95×10^3, which = 8.9×10^5 daltons. Although DNA is double-stranded, I assume only one strand is transcribed *in vivo*, so 5 μm of single-stranded DNA = 4.45×10^6 daltons. Mitochondrial DNA contains a cistron each for heavy and light mitochondrial rRNAs and for perhaps twenty tRNAs. Using the following values,

heavy rRNA	= 0.53×10^6 daltons*	tRNA = 10^3 daltons
light rRNA	= 0.30×10^6 daltons*	
	0.83×10^6 daltons	= total rRNA cistrons
20 tRNAs	= 0.50×10^6 daltons	= total tRNA cistrons
	1.33×10^6 daltons	= total RNA cistrons

5 μm single-stranded DNA	= 4.45×10^6 daltons	
and subtracting RNA cistrons	= 1.33×10^6 daltons	
	3.12×10^6 daltons	= remaining sequences.

*Xenopus (Dawid and Chase, 1972)

Therefore, $3.12 \times 10^6/300 = 1.04 \times 10^4$ nucleotides, which = 3.5×10^3 codon triplets = amino acids. Since one amino acid = 125 daltons, 3.5×10^3 amino acids = 436,000 daltons. This represents approximately 11 polypeptides of 40,000 daltons or 22 polypeptides of 20,000 daltons.

Mitochondrial DNA from plants is larger than that from animal cells and can code for a correspondingly greater number of proteins. Euglena mitochondrial DNA is probably about 20 μm in contour length (Talen et al., 1974), which is equivalent to 26 polypeptides of 40,000 daltons, whereas mitochondrial DNA from higher plants is reported to be 30 μm (Kolodner and Tewari, 1972b), which is adequate to code for 42 polypeptides of 40,000 daltons.

In this computation, I have assumed that only one strand of the double DNA helix is transcribed. There is some evidence, however, that at least three tRNA genes are located in the light strand (Aloni and Attardi, 1971). Also, I have used the molecular weight values of the mature mitochondrial rRNA species, although the size of the initial transcription unit(s) is probably larger. With respect to the calculation of the coding capacity of mitochondrial DNA, however, these factors tend to cancel out one another.

2. Chloroplast DNA

Chloroplast DNA from both algae and higher plants averages 40 μm - 44 μm in contour length (Manning and Richards, 1972; Herrmann et al., 1975). In contrast to mitochondrial rRNAs, chloroplast rRNAs

are usually slightly larger and at least two cistrons for each of the two high molecular weight rRNA species are present in chloroplast DNA (Thomas and Tewari, 1974 a+b). These considerations are taken into account in the following calculation of the coding potential of the chloroplast genome:

heavy rRNA = 1.10×10^6 daltons*
light rRNA = 0.56×10^6 daltons*

1.66×10^6 daltons
2 rRNA cistrons = 3.32×10^6 daltons
20 tRNAs = 0.50×10^6 daltons

3.82×10^6 daltons = total RNA cistrons

single-stranded DNA, 40 μm = 3.56×10^7 daltons
total RNA cistrons = 3.82×10^6 daltons

31.78×10^6 daltons = remaining sequences
= 10.60×10^4 nucleotides
= 3.55×10^4 codons = amino acids
= 4.45×10^6 daltons,

which represents a capacity to code for 110 polypeptides of 40,000 daltons. The molecular weight of the large subunit of ribulosediphosphate carboxylase, the most abundant chloroplast protein, is 55,000 daltons (in Givan and Criddle, 1972).

The amount of DNA available for coding for proteins is reduced if one performs this calculation using a value for an early rRNA precursor

*Higher plants (Ingle et al., 1970)

rather than that for mature rRNA forms. Assuming, for example, that the rapidly labelled 2.9×10^6 dalton chloroplast RNA component observed in Phaseolus (Grierson and Loening, 1974) represents a primary polycistronic transcript of rRNA, the coding potential of chloroplast DNA is limited to 100 polypeptides of 40,000 daltons.

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