1979

Studies on Chloroplast Protein Synthesis In Vivo and In Vitro

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STUDIES ON CHLOROPLAST PROTEIN SYNTHESIS IN VIVO AND IN VITRO

A thesis submitted to the Faculty of The Rockefeller University

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

by

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March 8, 1979

The Rockefeller University

New York City

DEDICATION

This thesis is dedicated to Alan and Kate Engelberg without whose friendship this work could never have been written.

ACKNOWLEDGEMENTS

I am very grateful to Nam-Hai Chua for his patience and understanding over the years and for the excellent scientific example he set for me.

I warmly thank Gregory Schmidt, Gianni Piperno, and Paul Lizardi for their assistance and support.

ABSTRACT

Protein synthesis in the Chlamydomonas reinhardtii chloroplast was investigated by in vivo and in vitro approaches. The site of synthesis of chloroplast proteins was examined to determine how many proteins were synthesized inside the chloroplast. Cells were labeled in vivo with [^{35}S]-sulfate in the presence of either chloroplast or cytoplasmic protein synthesis inhibitors, and fractionated into soluble proteins, thylakoid membranes, and chloroplast ribosomal subunits. The use of high specific activity sulfate and improved gel electrophoretic techniques were key features of this study because they permitted minor chloroplast products to be detected for the first time. It was determined that twenty-eight to thirty-one polypeptides are synthesized in the chloroplast. These include the major thylakoid membrane proteins previously reported to be chloroplast products by Chua and Gillham (1977), but also include a number of minor and low-molecular weight thylakoid membrane proteins, three soluble proteins, and at least four chloroplast ribosomal proteins. The synthesis of chloroplast envelope constituents was not examined.

To find out which thylakoid membrane proteins were integral and which peripheral, membranes were extracted with acid, base, urea, and guanidine hydrochloride. It was determined that both integral and peripheral membrane proteins are synthesized in the chloroplast. Integral proteins included constituent polypeptides of chlorophyll-protein complexes and polypeptide D1; the most notable peripheral proteins synthesized in the chloroplast were polypeptides believed to be the α and β coupling factor subunits.

In an effort to gain information about the biosynthetic pathway followed by chloroplast synthesized polypeptides, poly(A)$^-$ mRNA was isolated and translated in the wheat germ cell-free system. One product was identified as the ribulose-1,5-bisphosphate carboxylase large subunit and was synthesized in vitro in a form indistinguishable from the authentic polypeptide. A second product was tentatively identified as the integral membrane protein D1. The in vitro synthesized polypeptide

was slightly different from authentic D1 in a number of ways. The relationship of these differences to a possible precursor *in vivo* is discussed.

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ABBREVIATIONS

ATP	adenosine triphosphate
BPB	bromphenol blue
BSA	bovine serum albumin
CF1	chloroplast coupling factor
CPI-V	chlorophyll-protein complexes I-V
CPK	creatine phosphokinase
DEP	diethyl pyrocarbonate
EDTA	ethylene diamine tetraacetate
GSH	glutathione (reduced)
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high pressure liquid chromatography
NP40	Nonidet P40
PBS	phosphate buffered saline
PC	phosphocreatine
PEP	phosphoenolpyruvate
PMSF	phenylmethionylsulfonyl fluoride
PRS	post-ribosomal supernatant
PVS	polyvinyl sulfate
SDS	sodium dodecyl sulfate
TAP	tris-acetate-phosphate medium
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	N-tris[hydroxylmethyl]methyl-2 aminomethane sulfonic acid
TMF	thylakoid membrane fraction
TPCK	L-(tosylamido 2-phenyl)ethyl chloromethyl ketone
Tris	tris(hydroxy methyl)amino methane
UMP	uridine monophosphate

CHAPTER I

INTRODUCTION

It is well established that chloroplasts contain their own genetic systems and are capable of protein synthesis (Hoober, 1976; Ellis, 1976). It is also known that chloroplasts depend to a very great extent on a contribution from the cytoplasm for their continued functions (Chua and Schmidt, 1979). Exactly how the chloroplast and cytoplasm cooperate and what each gives to the other in this extremely intimate relationship are among the most challenging and important questions in biology today.

The work described here focuses on chloroplast protein synthesis. Over the last few years general characteristics of the chloroplast system have been examined (reviewed by Ellis, 1976). From the outset, the goal of my studies was to investigate the synthesis of specific proteins in the chloroplast to gain a detailed understanding of their biosynthetic pathways. To accomplish this I have first looked at chloroplast protein synthesis *in vivo* using a highly sensitive labeling technique to catalog and partially characterize individual proteins, and then attempted to synthesize these proteins *in vitro* so that their biosynthetic pathways may be dissected.

A Brief Overview of the Chloroplast Protein Synthesizing System

The ability of chloroplasts to synthesize proteins was first suspected from the labeling kinetics of proteins in plant cells (Hoober, 1976). During photosynthesis in the presence of [$^{14}CO_2$], the specific activity of chloroplast proteins increased initially at a more rapid rate than did that of cytoplasmic proteins. These results were extended when isolated chloroplasts were demonstrated to incorporate amino acids into proteins. The most elegant example of these early experiments is that of Blair and Ellis (1973). In this work chloroplasts capable of high CO_2-fixation rates were isolated and shown to synthesize proteins in a light-dependent reaction. This latter characteristic was most significant since it clearly indicated that chloroplasts and not contaminating bacteria were responsible for the observed synthesis and that the isolated chloroplasts were in a biologically functional

condition. Exogenously supplied ribonuclease failed to inhibit protein synthesis insuring not only that the chloroplasts were intact but that only messenger RNA located inside the chloroplasts was being translated. Synthesis was blocked by addition of chloramphenicol or lincomycin but not cycloheximide. The first two substances inhibit protein synthesis only on procaryotic ribosomes while cycloheximide effects cytoplasmic ribosomes.

Chloroplast protein synthesis occurs on 70S ribosomes (Hoober, 1976; Ellis, 1976). This observation is consistent with the antibiotic sensitivity since procaryotic ribosomes are also the smaller 70S type. In Chlamydomonas, about one quarter of the cellular ribosomes are 70S (Hoober and Blobel, 1969; Chua et al, 1973). The 70S population can be dissociated into 50S and 33S subunits by high salt (Chua et al., 1973). Large and small subunit RNAs have sedimentation coefficients of 22.4S and 16.5S, respectively and are present in a 22S:16S ratio of 2.4 when isolated in the presence of high magnesium concentrations (Hoober and Blobel, 1969). Recent results have shown that the chloroplast large subunit also contains 7S, 5S and 3S RNA (Rochaix and Malnoe, 1978). Chloroplast ribosomal RNA genes are localized on chloroplast DNA (Thomas and Tewari, 1979). In maize and Chlamydomonas chloroplasts, each copy of chloroplast DNA contains two 16S and 23S genes in an inverted repeat configuration (Bedbrook et al., 1977; Rochaix and Malnoe, 1978). Isolated subunits from Chlamydomonas chloroplast ribosomes are active in poly(U) directed amino acid incorporation (Chua et al., 1973). This reaction is specifically blocked by chloramphenicol.

A proportion of chloroplast ribosomes are bound to the thylakoid membranes (Chua et al., 1973; Margulies and Michaels, 1974; Alscher et al., 1978). Attachment is observed only when polypeptide chain elongation is inhibited by either rapid cooling of the cultures prior to ribosome isolation or by chloramphenicol treatment (Chua et al., 1973). Addition of chain initiation inhibitors, such as lincomycin or streptogramin, under conditions where ribosomes are cycling between free and membrane-bound pools prevents chloramphenicol from "freezing"

ribosomes to membranes (Margulies and Michaels, 1974; Chua et al., 1976). Although some membrane-bound ribosomes are removed by high KCl concentrations, many can be detached only by a combination of high salt and puromycin (Chua et al., 1973). In membrane preparations isolated from chloramphenicol-treated cells, many ribosomes are in polysomal arrays (Chua et al., 1976). It is, therefore, clear that some thylakoid bound ribosomes are engaged in protein synthesis.

In synchronous cultures of Chlamydomonas, 20 - 30% of the total chloroplast ribosomes are thylakoid membrane-bound in the light, but become detached in the dark (Chua et al., 1976), The light phase coincides with an increase in chlorophyll and membrane synthesis implying that the bound ribosomes are making membrane proteins. Two other experiments are consistent with this hypothesis. When thylakoid membranes bearing attached ribosomes were incubated with protein synthesis factors so that nascent chains might be completed (Margulies et al., 1975; Alscher et al., 1978), radioactive amino acids incorporated in these systems were membrane-bound and, in one case, partially resistant to proteases. The biological significance of these observations is unclear, however, since the synthesis of discrete, identifiable products was not demonstrated.

Other aspects of chloroplast protein synthesis are understood in even less detail. Polypeptide chain initiation is mediated by N-formylmethionyl tRNA (Hoober, 1976; Highfield and Ellis, 1976). Both tRNA and synthetases for amino acid activation are present in chloroplasts. Genes sufficient to code for at least twenty tRNAs have been demonstrated in chloroplast DNA (Haff and Bogorad, 1976), but the uniqueness of the tRNAs or synthetases to the chloroplast has not been established. Although some work indicates that elongation and possibly termination reactions require specific chloroplast factors (Hoober, 1976), precise information will not be forthcoming until an in vitro translation system consisting of chloroplast components is available.

Proteins Synthesized in the Chloroplast: Results from Intact Chloroplast Studies and *In Vivo* Labeling in the Presence of Inhibitors

Two approaches have been used to determine which chloroplast proteins are synthesized inside the organelle. One of these is incubation of isolated chloroplasts with radioactive precursors in the light as mentioned earlier (Ellis, 1976). The other is radioactive labeling *in vivo* in the presence of inhibitors of either cytoplasmic or chloroplast protein synthesis (Chua and Gillham, 1977). Both techniques are dependent on the assumption that chloroplasts will continue to synthesize proteins normally in the absence of contributions from the cytoplasm. This assumption, of course, is only true to a limited degree; the mere existence of enzymes composed of subunits made in both the cytoplasm and the chloroplast implies that synthesis in the chloroplast is coupled to the cytoplasm. Studies with mitochondria, which have a relationship to the cytoplasm analogous to that of the chloroplast, also support this contention (Schatz and Mason, 1974).

The intact chloroplast approach is useful if certain limitations are recognized and stringent controls applied. Protein synthesis in intact chloroplasts proceeds at a rate much lower than that *in vivo* and deteriorates rapidly, possibly because cytoplasmic factors are required for continued incorporation. For this reason, the synthesis of only major products or proteins that turn over rapidly can be detected in this system; polypeptides normally made in small amounts inside the organelle will not be seen. Chloroplasts must be purified free of cytoplasmic contaminants and the observed synthesis must be light-dependent, chloramphenicol sensitive, and ribonuclease resistant. As a final control, it is important that the identification of individual proteins be based on criteria other than comigration on an SDS gel. Under these conditions, all polypeptides synthesized by intact chloroplasts are certain to be true chloroplast products.

Equally strict rules apply when cells are labeled *in vivo*. To qualify as a chloroplast product, it is necessary that a protein be

labeled both under control conditions and in the presence of cytoplasmic synthesis inhibitors, but unlabeled in the presence of chloroplast synthesis inhibitors or both cytoplasmic and chloroplast inhibitors (Chua and Gillham, 1977). It is also important that both preincubation with inhibitors and subsequent labeling be for short periods to avoid problems with secondary inhibitor effects. A cytoplasmic or chloroplast product may be required for the synthesis of a protein in the other compartment; if the pool of this protein is depleted prior to label addition, the synthesis of the second protein will not be observed (Poyton and Kavanagh, 1976; LaPolla and Lambowitz, 1977). Identification of *in vivo* labeled proteins can usually be made simply by comparison to the stained gel profile since the total number of proteins labeled in control samples is conserved. In complex mixtures or instances where polypeptides are not properly assembled, other more definitive means of identification are essential. Since labeling *in vivo* proceeds at least initially at near normal rates, with a high specific activity isotope even minor polypeptides synthesized in the chloroplast can be detected.

Isolated chloroplasts incorporate labeled amino acids into many soluble polypeptides (Ellis, 1977). One product has been identified by tryptic mapping as the large subunit of ribulose bisphosphate carboxylase (Blair and Ellis, 1973). Later studies have confirmed this observation (Bottomley *et al.*, 1974; Morgenthaler and Mendiola-Morgenthaler, 1976). The enzyme is the major soluble protein mass in tobacco chloroplasts (Wildman, 1971). Other soluble proteins synthesized by intact chloroplasts *in vitro* have not been identified; on two-dimensional isoelectric focusing/SDS gels, none of the other faintly labled polypeptides comigrates with known stromal proteins (Ellis, 1977).

Soluble protein synthesis in the chloroplast has also been examined by the selective inhibitor approach. Cashmore (1976) labeled pea seedlings with [^{35}S]-methionine in the presence of either cycloheximide or chloramphenicol. The only soluble protein whose labeling was resistant to chloramphenicol comigrated with carboxylase large subunit on SDS gels, a result consistent with that from intact chloroplasts.

Although their biosynthesis has not been studied directly, other chloroplast localized carbon fixation enzymes are presumably made in the cytoplasm since chloramphenicol does not prevent an increase in their activities during greening (Hoober, 1976).

Subunits of chloroplast coupling factor are synthesized by intact chloroplasts. In spinach chloroplasts polypeptides with mobilities identical to the α, β, and ε subunits are made (Mendiola-Morgenthaler, 1976). Maize chloroplasts also synthesize the α and β subunits (Grebanier et al., 1978). Identification in this case was based on convincing limited-digest maps. The remaining two coupling factor subunits, γ and δ, are made in the cytoplasm (Bouthyette and Jagendorf, 1978). Chloroplast coupling factor is loosely attached to the outer thylakoid surface and is extractable with low ionic strength solutions (Binder et al., 1978). Grebanier observed that newly synthesized α and β subunits were not extracted from thylakoids under conditions known to remove coupling factor (Grebanier et al., 1978). Apparently, isolated maize chloroplasts could synthesize but not properly assemble the subunits in the absence of cytoplasmically made polypeptides or other components.

A recent study has provided additional evidence that α, β, and ε are synthesized in the chloroplast. Bouthyette and Jagendorf (1978) incubated pea seedlings in vivo with [^{3}H]-leucine in the presence of either cycloheximide or chloramphenicol and extracted coupling factor from thylakoid membranes. Only subunits α, β, and ε were labeled in the presence of cycloheximide while δ and γ were labeled in the presence of chloramphenicol. Therefore γ and δ are made in the cytoplasm.

Similar results may have been obtained by Cashmore by in vivo labeling of pea seedlings (Cashmore, 1976). Synthesis of a protein band called PI was totally blocked by chloramphenicol; in the presence of cycloheximide PI was labeled to control levels, but 50% was not membrane bound. If PI was really poorly resolved coupling factor subunits α and β (Gillham et al., 1978), then its failure to attach to the membrane may have been because the cytoplasmically made γ and δ subunits were absent.

Only one other thylakoid membrane protein made by isolated chloroplasts has been identified. Grebanier et al., (1978) reported that a 34,500-dalton polypeptide was made in maize chloroplasts in vitro and had a limited-digest map similar to that of a 32,000-dalton thylakoid membrane protein. Digestion of thylakoid membranes with pronase converted the authentic 32,000-dalton species into a ∿19,000-dalton peptide that was protected from protease presumably by its insertion in the membrane. If thylakoid membranes were isolated from intact chloroplasts which had been incubated with radioactive precursors, and were treated with pronase in vitro, the synthesized 34,500-dalton protein was completely digested. The authors concluded that the high molecular weight form was a precursor that was not processed by intact chloroplasts in vitro.

Pea and spinach intact chloroplasts make a thylakoid membrane polypeptide that may be analogous to the one synthesized in maize chloroplasts. Eaglesham and Ellis (1974) reported that the major membrane associated pea chloroplast product (termed Peak D) had an apparent molecular weight of 32,000 and comigrated with a stained thylakoid band. Polypeptides of similar size were also labeled in spinach chloroplasts (Bottomley et al., 1974; Morgenthaler and Mendiola-Morgenthaler, 1976). None of these in vitro products has been convincingly identified with known thylakoid membrane proteins.

Other proteins that may be synthesized in vitro by intact chloroplasts include cytochrome b559 (Zielinski and Price, 1977) and two or three chloroplast envelope proteins (Joy and Ellis, 1975; Morgenthaler and Mendiola-Morgenthaler, 1976). In each of these cases, identification is based on identical electrophoretic mobilities on SDS gels.

A number of studies have exploited the selective effects of antibiotics to examine the sites of synthesis of chloroplast lamellar proteins (reviewed by Gillham et al., 1978). The clearest of these is that of Chua and Gillham (1977). Cultures of Chlamydomonas were preincubated with either no inhibitor, anisomycin, chloramphenicol, or both inhibitors and labeled for 30 minutes with [^{14}C]-acetate. Aniso-

mycin is a specific cytoplasmic-ribosome protein synthesis inhibitor; Chloramphenicol blocks only 70S ribosomes. Thylakoid membranes were purified and polypeptides displayed on SDS/polyacrylamide gels. Over thirty-three stained bands were resolved. Autoradiographs demonstrated that five stained polypeptides (2, 4.1, 4.2, 5, 6) were labeled in control (no inhibitors) and also anisomycin samples, but were unlabeled when incubated with chloramphenicol or both chloramphenicol and anisomycin. In addition, two diffuse bands (D1 and D2) and two low-molecular weight polypeptides were made in the chloroplasts. In summary, a total of nine thylakoid membrane polypeptides were chloroplast synthesized.

The work of Chua and Gillham was incomplete in two respects. Even though Chlamydomonas readily metabolizes radioactive acetate, the specific activity of the isotope is not high enough to permit detection of minor polypeptides synthesized in the chloroplast. In addition, resolution of low-molecular weight polypeptides was poor on the SDS gels employed in their study.

The functions of polypeptides 2, 4.1, 4.2, 5, and 6 in Chlamydomonas have been established (Table I). Polypeptide 2 is the constituent polypeptide of chlorophyll-protein complex 1 (CPI) and, on the basis of mutant results, functions in PSI reaction center (Chua et al., 1975, Bennoun et al., 1977). The presence of polypeptides 5 and 6 is correlated with PSII reaction center activity (Chua and Bennoun, 1975). In addition, these polypeptides are the sole protein constituents of chlorophyll a containing complexes CPIII and CPIV (Delepelaire and Chua, 1979), a result which is consistent with their functioning in PSII reaction center. Polypeptides 4.1 and 4.2 have recently been shown to comigrate with the α and β subunits of Chlamydomonas coupling factor and to be missing in mutants impaired in photophosphorylation (Bennoun et al., 1978).

Very little information is available on the site of synthesis of chloroplast ribosomal proteins. A number of non-Mendelian mutants have been isolated with chloroplast ribosomes resistant to various antibiotics (Gillham et al., 1978). Since similar mutations affect bacterial ribosomal proteins, it is assumed that the sites of resistance are chloro-

TABLE I

FUNCTIONS OF CHLAMYDOMONAS CHLOROPLAST SYNTHESIZED POLYPEPTIDES

Polypeptide #	Function	Reference
2	CPI†, PSI reaction center	Chua et al., 1975; Bennoun et al., 1977
4.1	α subunit CF1*	Bennoun et al., 1978 Piccioni and Matlin, unpublished data
4.2	β subunit CF1*	Bennoun et al., 1978; Piccioni and Matlin, unpublished data
5	CPIII†, PSII reaction center	Chua and Bennoun, 1975; Delepelaire and Chua, 1979
6	CPIV†, PSII reaction center	Chua and Bennoun, 1975; Delepelaire and Chua 1979
?	ε subunit CF1*	Piccioni and Matlin, unpublished data

*CF1: chloroplast coupling factor

†CPI, CPIII, CPIV: chlorophyll-protein complexes

plast ribosomal proteins rather than ribosomal RNA. It is likely that the resistant proteins are coded by chloroplast genes. Most chloroplast ribosomal proteins, however, are probably cytoplasmically made. In Neurospora, only one mitochondrial ribosomal protein is made in the organelle (Lizardi and Luck, 1972; Lambowitz et al., 1976). Labeling of chloroplast ribosomes with [^{3}H]-arginine in Chlamydomonas whole cells is unaffected by chloramphenicol but inhibited by cycloheximide (Honeycutt and Margulies, 1973). Although this result does not rule out a contribution by the chloroplast in the synthesis of ribosomal proteins, it indicates that the cytoplasmic contribution is more significant. Recently, Freyssinet has found that at least nine Euglena chloroplast ribosomal proteins are made in the chloroplast and twelve are made in the cytoplasm (Freyssinet, 1978). The site of synthesis of eighteen others was not definitively determined.

Proteins Synthesized in the Chloroplast: Synthesis Directed by mRNA In Vitro

1. Chloroplast mRNA

The isolation and characterization of chloroplast messenger RNA is in its early stages. Total mRNA from spinach chloroplasts contains poly(A) tails of less than twenty residues (Wheeler and Hartley, 1975). Specific mRNAs coding for the large subunit of ribulose bisphosphate carboxylase in Euglena, Chlamydomonas and Spirodela, and a 32,000-dalton membrane protein in Spirodela fail to bind to oligo-dT affinity columns and may therefore lack poly(A) or contain very short sequences (Howell et al., 1977; Sagher et al., 1976; Reisfeld et al., 1978). Both mRNAs sediment in the 13S-16S range (Howell et al., 1977; Sagher et al., 1976; Reisfeld et al., 1978). No search has been made for 5' "cap" structures on chloroplast mRNA. Mitochondrial mRNA from HeLa cells, which may resemble chloroplast mRNA to some extent, has recently been shown to lack caps (Grohmann et al., 1978).

2. Proteins synthesized by chloroplast mRNA

Only two proteins that have been synthesized in vitro from chloro-

plast mRNA have been identified. The first of these is carboxylase large subunit. Hartley et al. (1975) isolated mRNA from spinach chloroplasts and translated it in an E. coli system. Although the in vitro product was slightly smaller than the authentic large subunit, their chymotryptic peptides were similar. Howell et al. (1977) also failed to synthesize full-size Chlamydomonas large subunit in the E. coli system with mRNA derived from immunoprecipitated polysomes. Tryptic peptides from immunoprecipitated partial translation products did, however, resemble those of the authentic large subunit digest. Using the eucaryotic wheat germ cell-free system, full-size large subunit was synthesized from Euglena chloroplast poly(A)$^-$ mRNA and identified conclusively by immunoprecipitation and two-dimensional gel electrophoresis. Apparently, the E. coli system is often unable to complete chains directed by chloroplast mRNA. Recently, Howell and Gelvin (1978) translated Chlamydomonas mRNA for the large subunit in the wheat germ cell-free system and obtained a full length product.

The second chloroplast protein to be synthesized in vitro is the 32,000-dalton membrane protein from Spirodela (Edelman and Reisfeld, 1978). The polypeptide synthesized in the wheat germ cell-free system is larger than the authentic protein by daltons. The in vitro product has been identified by two-dimensional gel electrophoresis and limited peptide mapping. Since the larger polypeptide is also seen after pulse-labeling in vivo, it is believed to be a physiological precursor rather than an in vitro artifact. The Spirodela protein may be analogous to the 34,500-dalton polypeptide which is synthesized in isolated maize chloroplasts but not incorporated into the membrane (Grebanier et al., 1978).

Protein Structural Genes in Chloroplast DNA

The structural gene for carboxylase large subunits has been definitively localized in chloroplast DNA by direct molecular methods. Using [^{32}P]-labeled mRNA for Chlamydomonas large subunit, it was demonstrated that the large subunit gene was on a specific EcoRI restriction fragment of chloroplast DNA and that each genome contained one copy (Gelvin

et al., 1977; Howell and Gelvin, 1978). In maize, a BAM-HI restriction fragment of chloroplast DNA coded for the large subunit in a linked transcription-translation system (Coen et al., 1977). Recently, the gene has been localized to a 2500 base pair segment between recognition sites for endonucleases Bgl II and Sma 1 (Link et al., 1978). The large subunit gene is therefore present in only one copy per genome in maize chloroplasts. On a restriction map of the maize chloroplast genome, the large subunit gene is across the circle from one group of ribosomal RNA genes and about a quarter of a circle away from the other ribosomal RNA genes (Link et al., 1978).

Recently, a second gene has been tentatively localized on the maize chloroplast genome (Bedbrook et al., 1978). Labeled RNA isolated from developing plastids hybridized to a BAM fragment of chloroplast DNA distinct from fragments containing ribosomal RNA genes. Translation of this RNA fraction in vitro stimulated the synthesis of a 34,400-dalton polypeptide. Presumably, the mRNA coding for the 34,500-dalton polypeptide hybridizes to the BAM fragment.

No other protein structural genes have been localized on chloroplast DNA. Until it is demonstrated that mRNAs for some chloroplast products are transcribed in the nucleus and shipped to the chloroplast for translation, it is fair to assume that chloroplast DNA contains structural genes coding for all proteins known to be made in the chloroplast. This view is supported by the existence of non-Mendelian mutants showing alterations in chloroplast proteins (Gillham et al., 1978). In Chlamydomonas, a mutant has been characterized which has a variant polypeptide 5 (called 5') slightly larger than the wild-type form (Chua, 1976). The altered polypeptide is apparently incorporated properly into the thylakoid membrane, since the mutant has normal photochemical activities. The mutation is inherited uniparentally and is therefore localized in chloroplast DNA. Hence, it is likely that the structural gene for polypeptide 5 is also on chloroplast DNA. Polypeptide 5 is known to be a chloroplast product (Chua and Gillham, 1977). Other non-Mendelian mutants of Chlamydomonas that are missing chloroplast synthe-

sized polypeptides have been isolated (Bennoun et al., 1977; Bennoun et al., 1978). The interpretation of these mutations is more difficult since altered gene products have not been identified.

Genetic and biochemical evidence also imply that a chloroplast ribosomal protein is coded by chloroplast DNA. A single protein from the large chloroplast ribosomal subunit of an erythromycin-resistant mutant of Chlamydomonas has an altered mobility on two-dimensional gels (Mets and Bogorad, 1972). The mutation is inherited uniparentally. The lesion in chloroplast DNA, therefore, affects either the structural gene for the protein or some secondary modification function. A chloroplastic site of synthesis would support the former alternative.

Scope of These Experiments

In summary, data from a variety of sources indicate that chloroplasts synthesize a number of proteins. Most of these are components of the thylakoid membrane. At least one is soluble and two or three are in the chloroplast envelope. Ribosomal proteins may also be made. Nothing is known about the sequence of events surrounding the biosynthesis and final localization of chloroplast proteins. Membrane-bound ribosomes are present in the chloroplast and may synthesize membrane proteins. Soluble and envelope proteins may be made either on free or on membrane-bound ribosomes. Recent evidence suggests that some chloroplast proteins are made as precursors (e.g., Reisfeld and Edelman, 1978). The nature of these precursors or their function is, however, unknown.

The studies to be described in this thesis are an attempt to extend knowledge about chloroplast protein synthesis in a number of areas. Specifically, these questions will be addressed:

1. How many membrane, soluble, and ribosomal proteins are made in the chloroplast?
2. Which chloroplast synthesized membrane proteins are integral and which peripheral?
3. How many chloroplast products can be synthesized in vitro?
4. Is there evidence that the biosynthetic pathway for membrane and soluble proteins in the chloroplast is different?

Chapter III describes studies directed at the first two questions. Isotopic labeling in the presence of selective protein synthesis inhibitors is used to demonstrate that, in addition to the known major chloroplast products, several minor and low molecular weight membrane proteins, two soluble proteins, and a few chloroplast ribosomal proteins are synthesized in the chloroplast. Thylakoid membranes are extracted to show that chloroplasts synthesize both integral and peripheral membrane proteins.

Chapter IV describes experiments designed to answer the last two questions. Total cell Chlamydomonas mRNA lacking poly(A) is shown to code for at least two known chloroplast products in the wheat germ system. One is identified as the carboxylase large subunit and the other is tentatively identified as the integral membrane protein D1. Differences noted between authentic D1 and the in vitro synthesized protein are discussed in relation to question #4.

The unicellular green alga Chlamydomonas reinhardtii was used throughout these studies. Previous work in our laboratory provided a well-defined foundation for the experiments described here. The ease with which the organism is manipulated and the large amount of genetic information available about chloroplast components were additional advantages.

CHAPTER II

MATERIALS AND METHODS

Growth and Maintenance of Cell Cultures

1. Strains

The wild-type Chlamydomonas reinhardtii strain used in these studies was 137c, mating type +.

2. Media

Cells were grown mixotrophically in the Tris-acetate-phosphate (TAP) medium of Gorman and Levine (1965). TAP medium is a modification of one derived by Sueoka utilizing Hutner's trace elements (Sueoka, 1960; Hutner et al., 1950). The composition of this medium is given in the following:

TAP Medium

Tris base	2.42 g
Glacial acetic acid	1.0 ml
1 M potassium phosphate pH 7.0	1.0 ml
Solution B	50.0 ml
Trace elements solution	1.0 ml
Distilled water to a final volume of 1 liter	

Solution B

NH_4Cl	8.0 g
$CaCl_2 \cdot 2H_2O$	1.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
Distilled water to a final volume of 1 liter	

Trace elements

EDTA, disodium salt	50.0 g
$ZnSO_4 \cdot 7H_2O$	22.0 g
H_3BO_3	11.4 g
$MnCl_3 \cdot 4H_2O$	5.06 g
$FeSO_4 \cdot 7H_2O$	4.99 g
$CoCl_2 \cdot 6H_2O$	1.61 g
$CuSO_4 \cdot 5H_2O$	1.57 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.10 g

Trace elements were prepared by dissolving all components except EDTA in order, one at a time in 550 ml distilled water. The EDTA was dissolved separately by heating in 250 ml water. The first solution was then brought to 70°C and the EDTA solution added. While keeping the complete solution at or above 70°C, the pH was adjusted to 6.5 - 6.8 by adding 20% (w/v) KOH. The final volume was then adjusted to 1 liter and the solution was allowed to stand at room temperature in a cotton-plugged flask. After 4 - 6 weeks a reddish-brown precipitate formed and the solution turned purple. The precipitate was removed by filtration and the solution was stored at 5°C or frozen at -20°C until needed.

For labeling experiments, modified TAP media were utilized. Cells were labeled with [^{14}C]-acetate in minimal (Tris-HCl-phosphate) medium containing 1.5 ml concentrated HCl per liter instead of glacial acetic acid (Surzycki, 1971). For sulfate labeling, cells were transferred to sulfate free-TAP prepared by omitting the trace elements mixture and leaving the magnesium sulfate out of solution B.

3. Stock cultures

Stock cultures were maintained at 15°C under constant illumination on 1.5% agar slants containing TAP medium supplemented with 4 g yeast extract per liter. The cultures were transferred every 3 - 4 months.

4. Working cultures

Plates containing 1.5% agar in TAP medium were inoculated with cells from stock cultures and incubated at 25°C with 3000 - 5000 lux illumination from cool white fluorescent bulbs. Every 2 - 4 weeks cells were transferred to new plates. Liquid 300 ml TAP cultures were routinely inoculated from plates and grown to late log or early stationary phase (2 - 3 days) on a rotary shaker (New Brunswick Scientific). These small "feeder" cultures were used to inoculate larger volumes for experiments.

Isotopic Labeling In Vivo

1. [^{14}C]-acetate labeling

In this and the sulfate labeling procedure to be described, efforts

were made to minimize bacterial contamination during the experiments. All media, glassware, and centrifuge bottles were sterilized and all transfers prior to labeling were performed aseptically.

Log phase cultures (1 x 10^6cells/ml) grown in TAP medium were used. Cells were harvested by low speed centrifugation, washed once with minimal medium and finally resuspended in the same medium to 6 x 10^6 cells/ml. Aliquots (100 ml) were transferred to 500 ml Erlenmeyer flasks and shaken for 15 minutes at 10,000 lux 25°C on a rotary shaker (New Brunswick Scientific) to adapt them to the growth conditions. Inhibitors were added from concentrated stock solutions. Anisomycin (0.125 M in ethanol) was added to a final concentration of 2.5 x 10^{-4} M. Chloramphenicol (100 mg/ml in ethanol) was added to 100 µg/ml. In some experiments lincomycin (100 mg/ml in water) was substituted for chloramphenicol at a final concentration of 300 µg/ml. All flasks received equivalent amounts of ethanol. The final anisomycin and chloramphenicol concentrations were found to be saturating in previous experiments (Chua and Gillham, 1977). The optimal lincomycin concentration was determined by titrating incorporation versus concentration in cell samples already treated with anisomycin (Fig. 1). Flasks were shaken for 5 minutes after inhibitor addition before inclusion of label. Since lincomycin blocks initiation rather than elongation (Pestka, 1974; Chua et al., 1976), preincubation was extended to 10 minutes in experiments in which lincomycin was substituted for chloramphenicol. After preincubation, 1-[^{14}C]-sodium acetate was added to 2 µCi/ml and the samples incubated for an additional 30 minutes. Aliquots (100 µl) were taken to assess incorporation and 50 ml TAP medium added to each flask to dilute the acetate specific activity. Cells were chilled on ice and harvested immediately.

2. Labeling with [^{35}-S]-sulfate

Cells were labeled with [^{35}S]-sulfate in a fashion similar to the [^{14}C]-acetate procedure. After harvesting, cells were washed and resuspended in sulfate-free TAP and incubated for one hour to deplete the intracellular sulfate pools. [^{35}S]-sulfate was added to samples to a final isotope concentration of 0.1 mCi/ml. After 30 minutes labeling,

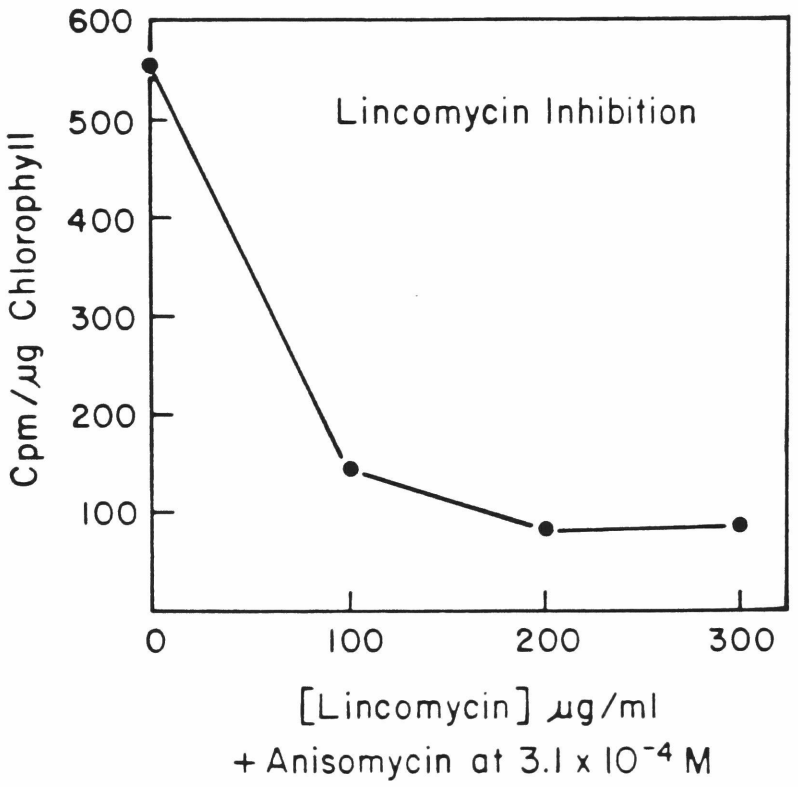


Figure 1. Effect of lincomycin on the incorporation of [^{35}S]-sulfate into thylakoid membrane proteins in the presence of anisomycin.

Chlamydomonas was labeled with 0.01 mCi/ml [^{35}S]-sulfate for 30 minutes in the presence of 3.1×10^{-4} M anisomycin and varying amounts of lincomycin. Lincomycin was added from a stock solution of 100 mg/ml in water. Thylakoid membranes were purified and incorporation determined as described in Materials and Methods.

1 - 2 ml 10% (w/v) $MgSO_4$ was added to limit further isotope uptake.

Cell Fractionation

1. Thylakoid membrane purification

Thylakoid membranes were purified by the flotation procedure of Chua and Bennoun (1975a). TES buffer was substituted for HEPES to permit protein determinations using the Folin reagent (Good and Izawa, 1972). All procedures were performed at 0 - 5°C. Cell pellets from 1 liter of culture was washed once in 25 mM TES-NaOH (pH 7.5)/1 mM $MgCl_2$/0.3 M sucrose and resuspended in 20 ml of the same buffer containing phenylmethionylsulfonyl fluoride (PMSF) (Pringle, 1975). Cells were disrupted by a single passage through a chilled French pressure cell (American Instrument Co.) at 5100 psi. The homogenate was centrifuged at 5000 x g_{max} for 10 minutes. The supernatant, which contained many mitochondria, soluble proteins, and small chloroplast membrane vesicles, was saved for ribosome and soluble protein purification. The pellet, containing mostly thylakoid membranes, was resuspended in 20 ml 5 mM TES-NaOH (pH 7.5)/10 mM EDTA/0.3 M sucrose by homogenization with a motor driven teflon pestle. This step unstacked the thylakoid membranes, releasing trapped starch granules, but did not remove coupling factor or any other major peripheral proteins from the membranes. The suspended membranes were centrifuged at 48,000 x g_{max} for 10 minutes and the supernatant discarded. The pellet was resuspended in 15 ml 5 mM TES-NaOH (pH 7.5)/10 mM EDTA/1.8 M sucrose by homogenization and 5 ml of the suspension overlaid with 2 ml 1.3 M sucrose and 5 ml 0.5 M sucrose in the same buffer. Membranes were floated by centrifugation at 270,000 x g_{max} for 1 hour in an SB283 swinging bucket rotor (International Equipment Co.). The membrane layer at the 1.3 M - 0.5 M interface was collected with a large bore syringe needle, diluted with three volumes 5 mM TES-NaOH (pH 7.5)/10 mM EDTA, and pelleted at 48,000 x g_{max} for 10 minutes. The supernatant was decanted and the membrane pellet was resuspended to 2 - 3 mg chlorophyll/ml in 0.1 M Na carbonate/0.1 M DTT and frozen at -20°C.

2. Isolation of soluble proteins

The 5000 x g_{max} supernatant produced during TMF isolation was centrifuged at 48,000 x g_{max} for 10 minutes to remove small membrane vesicles. Aliquots (1.0 ml) from the resulting supernatant were loaded onto 12.7 ml linear 10 - 30% (w/v) sucrose gradients in 25 mM Tris-HCl (pH 7.5)/0.2 M NaCl/5 mM DTT/1.0 mM EDTA. The gradients were centrifuged for 16 hours at 284,000 x g_{max} at 5°C in an SW 40 rotor (Beckman Instruments). Gradients were fractionated by inserting a stainless steel cannula through the top of the gradient and pumping out the liquid from the bottom while the absorbance was continuously monitored at 280 nm with a UA5 detector (Instrumentation Specialties Co.).

3. Purification of cytoplasmic and chloroplast ribosomal subunits

Ribosomal subunits were purified as described by Chua et al., (1973a). All procedures were at 0 - 5°C. To prepare total ribosomes, cells were washed, resuspended, and broken by passage through a chilled French pressure cell at 5100 psi in 25 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$/25 mM KCl/ 5 mM DTT. The homogenate was centrifuged at 17000 x g_{max} for 10 minutes. The resulting supernatant containing free ribosomes was centrifuged at 48,000 x g_{max} for 10 minutes and the pellet discarded. Aliquots (17 ml) of this supernatant were layered onto a step sucrose cushion consisting of 6 ml 1.95 M sucrose and 3 ml 1.0 M sucrose (same buffer as above) and centrifuged about 18 hours at 161,000 x g_{max} in the 60 Ti rotor (Beckman Instruments). The postribosomal supernatant (PRS) and the sucrose interface containing residual thylakoid membranes were removed. The surface of the ribosome pellet was rinsed with 1 - 2 ml cold distilled water and the pellet frozen at -80°C.

Cytoplasmic (80S) and chloroplast (70S) ribosomes were separated on preparative 12.7 ml linear 10 - 40% (w/v) sucrose gradients in 25 mM Tris-HCl (pH 7.5)/25 mM KCl/5 mM $MgCl_2$/5 mM DTT. Total ribosome pellets were dissolved in a small volume of distilled water and about 10 A260 U were loaded per gradient. Centrifugation was for 15.5 hours at 85,500 x g_{max} (22.5 x 10^3 rpm in the SB283 rotor) at 4°C. Under these conditions

the 70S and 80S ribosomes are well resolved. Gradients were fractionated from the bottom using the cannula technique described earlier. Ribosome peaks were collected and diluted with 10% sucrose in the same buffer. After increasing the Mg^{++} concentration to 25 mM to prevent dissociation into subunits, the ribosomes were pelleted by centrifugation overnight at 143,000 x g_{max}.

Chloroplast ribosomal subunits were purified on high salt gradients. Pellets of 70S ribosomes were dissolved in a small volume of water and a 2X compensating buffer added to make the final concentrations 25 mM Tris-HCl (pH 7.5)/400 mM KCl/25 mM $MgCl_2$/5 mM DTT. These salt concentrations completely dissociate the 70S ribosomes into subunits. An aliquot was loaded onto a 12.7 ml linear 5 - 20% sucrose gradient containing the same high salt buffer. Gradients were centrifuged at 257,000 x g_{max} for 3 hours at 18°C. Subunit pellets were diluted with 25 mM Tris-HCl (pH 7.5)/25 mM $MgCl_2$/5 mM DTT and pelleted by overnight centrifugation as described earlier. Subunit pellets were frozen at -80°C. Cytoplasmic (80S) ribosomal subunits were purified in a similar manner except the KCl concentration in gradient buffers was 500 mM.

Membrane Extraction

Aliquots (0.5 mg chlorophyll) of freshly prepared TMF in 5 mM TES-NaOH (pH 7.5)/10 mM EDTA were placed in 10 ml polycarbonate centrifuge tubes and centrifuged at 48,000 x g_{max} for 10 minutes at 5°C. The clear supernatant was discarded. To the pellets 1.0 ml of either 5 mM TES-NaOH (pH 7.5), 0.5 M acetic acid, 0.025 M NaOH, or 0.1 M NaOH was added and the pellets resuspended by vortexing. The membrane suspensions were incubated on ice and agitated frequently. After 30 minutes the suspensions were centrifuged at 233,000 x g_{max} for 1 hour at 5°C. Supernatants were removed and dialyzed overnight in Spectra/Por #3 (3500 molecular weight cut-off) dialysis bags against 2000 volumes 50 mM TES-NaOH (pH 7.5) at 5°C. After dialysis, supernatants were stored frozen at -20°C until analyzed. The pellets containing membrane residues were resuspended in 0.14 ml 0.2 M sodium carbonate and frozen at -20°C.

2. Extraction with denaturants

Aliquots of fresh thylakoid membranes containing 0.5 mg chlorophyll were prepared as for acid/base extraction. To each membrane pellet 1.0 ml of either 5 mM TES-NaOH (pH 7.5), water, 5 mM TES-NaOH (pH 7.5)/6 M urea, or 5 mM TES-NaOH (pH 7.5)/6 M guanidine hydrochloride was added. To prevent proteolysis artifacts, PMSF (40 mM in ethanol) was added to a final concentration of 1 mM (Pringle, 1975). Membranes were resuspended by vortexing and incubated on ice in the dark for 24 hours with 3 - 4 intermittent mixings. To separate the extracts from the residues, the suspensions were centrifuged for 1 hour at 233,000 x g_{max} at 5°C. Supernatants were withdrawn and dialyzed overnight against 2000 volumes of 5 mM TES-NaOH (pH 7.5) in Spectra/Por #6 (1000 molecular weight cut-off) dialysis bags (Spectrum Medical Industries). Pellets of residual membranes from the TES, water, and urea extractions were frozen at -20°C until further analysis. The membranous residue from the guanidine extraction was resuspended in 1.0 ml 5 mM TES-NaOH (pH 7.5) and immediately centrifuged at 48,000 x g_{max} for 10 minutes at 5°C. The supernatant was discarded and the pellet frozen at -20°C until analysis. This last step was performed to eliminate any residual guanidine which interferes with SDS gel electrophoresis. No protein or chlorophyll was lost in the wash supernatant.

3. Extraction of organic reagents

In some experiments, thylakoid membranes were extracted with chloroform/methanol essentially as described by Chua et al. (1975b). Briefly, membranes (750 µg chlorophyll) in 0.5 ml 0.1 M sodium carbonate/0.1 M DTT were extracted with 10 ml of 2:1 (v/v) chloroform/methanol on ice and the mixture centrifuged at 2000 x g_{max} for 10 minutes. The precipitate was prepared for SDS gel electrophoresis. The extract was dried under a stream of air and pigments extracted from this residue with 30 ml cold diethylether. The material remaining after ether extraction was prepared for electrophoresis. The ether supernatant containing pigments but no protein was discarded.

Digestion of Intact Membranes with Proteases

1. Basic procedure

Freshly isolated thylakoid membranes in 5 mM TES-NaOH (pH 7.5)/10 mM EDTA were centrifuged at 48,000 x g_{max} for 10 minutes and the supernatant discarded. To remove residual EDTA, pellets were resuspended in 25 mM TES-NaOH (pH 7.5) (∿3.0 ml per 1.0 mg chlorophyll) and the suspension recentrifuged. Membranes were resuspended once again in 25 mM TES-NaOH (pH 7.5) to ∿2 mg chlorophyll/ml and distributed into aliquots of 40 µg chlorophyll each (≙ 20 µl). Each aliquot was diluted with the appropriate digestion buffer to 180 µl (0.22 mg chlorophyll/ml) and kept on ice. Proteases were diluted serially from 1 mg/ml stock solutions and 20 µl of the appropriate dilution added to a membrane aliquot. To one aliquot only 20 µl of the appropriate buffer was added (no digestion control). To another aliquot 20 µl of the highest concentration protease dilution 5 µl 10% Nikkol (a non-ionic detergent) were added. The latter was a complete digestion control. The final protease concentrations are given in Figures 20 - 22. All samples were digested for 30 minutes on ice. Appropriate protease inhibitors were immediately added and the samples precipitated with 200 µl 10% TCA on ice for 30 minutes. Precipitates were prepared for electrophoresis as described in Table III.

2. Special procedures for individual proteases

a. Thermolysin

Crystalline thermolysin (10 mg) was dissolved in 10 ml 0.1 M ammonium acetate (pH 8.5)/5 mM $CaCl_2$ (Heinrickson, 1977) and serially diluted in the same solution. Membrane aliquots were diluted to 180 µl with the same buffer. After digestion, 5 µl of 0.2 M EDTA was added to inhibit enzymatic activity (Heinrickson, 1977).

b. Papain

A suspension of mercuripapain (25 mg/ml) was activated by diluting 5 µl to 1 mg/ml in 0.02 M EDTA/0.05 M cysteine (pH 7 - 8) on ice (Arnon, 1970). This solution was serially diluted in additional activating solution. Membrane aliquots were diluted to 180 µl with 25 mM TES-NaOH (pH

7.5). After digestion, papain was inhibited by addition of 5 µl freshly prepared 0.25 M iodoacetamide.

c. Trypsin - TPCK

Crystalline trypsin-TPCK was dissolved in water to 1 mg/ml and frozen in small aliquots at -20°C. Aliquots were never refrozen. The stock trypsin solution and membranes to be digested with trypsin were diluted in 25 mM TES-NaOH (pH 7.5). After incubation, trypsin was blocked by 5 µl of PMSF (40 mM in ethanol).

Isolation and Characterization of "Unbound" Messenger RNA

1. General precautions taken in RNA experiments

To prevent contamination of RNA preparations with exogenous nucleases, several procedures were routinely followed. All glassware and pipettes used in RNA experiments were heat-treated at 170-180°C for at least 3 - 4 hours. Plastic-ware which could not withstand high temperatures were either autoclaved for 20 minutes or soaked in 0.1% diethyl pyrocarbonate (DEP), a potent nuclease inhibitor (Wiener et al., 1972). Solutions were made up in heat-treated glassware and then autoclaved or, when the solution was heat labile, treated with DEP. For the latter procedure, DEP was added to 0.1% (v/v) and the solution stirred in a warm water bath overnight. DEP has a short half-life in aqueous solutions. To prevent contamination from "finger-nucleases", disposable plastic gloves were worn during all RNA experiments.

2. Phenol extraction of whole cells

A log phase culture (6 - 8liters) grown in TAP medium was harvested by low speed centrifugation and the cell pellet washed once in cold 40 mM Tris-HCl (pH 7.5)/0.12 M NaCl. Cells were resuspended at high density in ∿20 ml, 20 mM Tris-HCl (pH 7.5)/0.12 M NaCl/10 mM EDTA. The cell suspension was added slowly to an equal volume of 20 mM Tris-HCl (pH 7.5)/0.12 M NaCl/10 mM EDTA/4% SDS/80 µg polyvinyl sulfate (PVS) which was stirred vigorously at 37°C. Proteinase K was added immediately from a concentrated stock solution to 300 µg/ml and the stirring continued for 5 minutes to lyse the cells and inactivate RNAse. The lysate

volume at this point is defined as the "unit" volume for later reference. The homogenate was added to a teflon bottle containing an equal (unit) volume of 50:48:2 (v/v) phenol (re-distilled)/chloroform/isoamyl alcohol, and the mixture was shaken at room temperature for 15 minutes. To separate phases, the mixture was centrifuged in 150 ml Corex bottles (Corning Glass) at 3800 rpm for 10 minutes at 10°C (PR 6000 centrifuge, International Equipment Co.). The aqueous (upper) phase was transferred with a 25 ml pipette into a second extraction bottle containing 1.2 unit volumes of phenol/chloroform/isoamyl alcohol (same proportions) and mixed briefly. The phenol (lower) layer from the first extraction was re-extracted with 0.5 volume 40 mM Tris-HCl (pH 7.5)/0.12 M NaCl/10 mM EDTA/2% SDS/20 µg PVS by shaking for 5 minutes. After separation of phases as before, the aqueous layer from the two extractions were pooled, shaken with the phenol mixture for 5 minutes, and then centrifuged. If a substantial white interface between the aqueous and organic phases were present after this extraction, the aqueous layer was shaken once more with 1.2 volumes of the phenol mixture for 5 minutes. The NaCl concentration in the final aqueous phase was adjusted to 0.2 M with 4 M NaCl and total nucleic acids precipitated with 2 volumes absolute ethanol at -20°C for at least 12 hours.

3. Lithium chloride precipitation

Total cell nucleic acids, either before or after passage through poly(U) Sepharose, were precipitated with lithium chloride to remove DNA and 4S RNA (Baltimore and Girard, 1966). After ethanol precipitation, nucleic acid pellets were dissolved in water to a concentration of 20 - 100 A260 U/ml adjusted to 1 mM Mg acetate with a 500 mM stock solution. An equal volume of 4 M LiCl was added and the solution refrigerated (0 - 5°C) overnight. The RNA precipitate was recovered by centrifugation at 3600 rpm for 10 minutes at 5°C (PR6000). The pellet was dissolved in water and precipitated overnight after additions of Mg acetate and LiCl as before. The final RNA pellet was dissolved in water, made 0.2 M NaCl, and precipitated with 2 volumes ethanol at -20°C. The ethanol precipitation step was repeated prior to further fractionation or use of the RNA

in translation experiments. In some instances, the DNA-containing supernatant from the first lithium chloride precipitation was precipitated with 2 volumes ethanol after making the solution 0.2 M NaCl.

4. Formamide deionization

Ionic contaminants were removed from formamide by passing 1 liter reagent grade formamide through a column containing 16 g AG 501-X8 mixed bed resin, 26 g AG 50-X8 cation exchange resin, and 6 g Chellex chelator. Deionized formamide was stored at 5°C.

5. Poly(U) Sepharose Chromatography

Nucleic acid preparations were fractionated on poly(U) Sepharose columns to separate poly(A) containing RNAs from those lacking poly(A) (Lindberg and Persson, 1972; Adesnik et al., 1972). Poly(U) Sepharose (1.5 g) was swelled in water for 15 - 20 minutes and packed in a 1 x 10 cm jacketed glass column at a flow rate of about 16 ml/hour. The bed was washed with ∿20 ml 10 mM Tris-HCl (pH 7.4)/5 mM EDTA/ 0.2% SDS/90% (v/v) formamide and equilibrated with 30 - 40 ml 10 mM Tris-HCl (pH 7.4)/ 5 mM EDTA/0.2% SDS/0.4 M NaCl. During equilibration, binding, and elution the column was maintained at 27 - 29°C. The equilibrated bed was 1.0 x 5.5 cm. Elution was monitored with a UA5 detector at 280 nm since formamide has low absorbance at this wavelength. Nucleic acid pellets (200 - 1200 A260 U) either before or after LiCl precipitation were dissolved in 10 mM Tris-HCl (pH 7.4)/5 mM EDTA/0.2% SDS, diluted to <50 A260 U/ml, and made 0.4 M NaCl. The solution was heated to 57 - 60°C for 2 minutes and loaded onto the column at 17 ml/hour. After loading, the column was washed with 10 mM Tris-HCl (pH 7.4)/5 mM EDTA/0.2% SDS/0.4 M NaCl until no 280 nm absorbing material eluted. All absorbing material eluting during either the binding or the washing step was collected and represented the "unbound" nucleic acid fraction used in subsequent experiments. RNA bound to the column was eluted with 10 mM Tris-HCl (pH 7.4)/ 5 mM EDTA/0.2% SDS/90% formamide. In some experiments, bound RNA was eluted with an exponential gradient consisting of 25 ml 10 mM Tris-HCl (pH 7.4)/5 mM EDTA/0.2% SDS and 25 ml 90% formamide in the same buffer.

The gradient was generated in a two-chamber device with a sealed mixing chamber containing a constant 25 ml. Unbound nucleic acids were diluted with an equal volume of water and precipitated with 2 volumes ethanol. The bound RNA solution was diluted to less than 50% formamide, the NaCl concentration made 0.2 M,and RNA precipitated with ethanol.

6. RNA fractionation on SDS/sucrose gradients

Nucleic acid samples were dissolved in 20 mM Tris-HCl (pH 7.5)/0.2% SDS/2 mM EDTA. The solution was heated at 60°C for 2 minutes and 0.5 - 0.75 ml aliquots (6 - 40 A260 U) were loaded on 12.7 ml linear 10 - 30% (w/v) sucrose gradients in 20 mM Tris-HCl (pH 7.5)/0.5% SDS/2 mM EDTA. Gradients were centrifuged at 270,000 x g_{max} for 15 - 18 hours at 18°C in the SB283 swinging bucket rotor. Gradients were fractionated from the bottom as described earlier and monitored at 254 nm. Fractions were ethanol precipitated.

7. RNA fractionation on formamide/sucrose gradients

Nucleic acid samples were dissolved in 20 mM Tris-HCl (pH 7.5)/0.6% SDS/2 mM EDTA/60% formamide. The solution was heated at 60°C for 2 minutes and 1.5 ml aliquots (8 A260 U) loaded on 35.7 ml linear 7 - 23% (w/v) sucr 60% formamide gradients. Gradient solutions were prepared by weighing out eithe 7 or 23 g of solid sucrose, dissolving it in sample buffer (above), and diluting with additional sample buffer to a final volume of 100 ml. Up to four gradients were generated simultaneously by pumping solutions from a two-chamber device through a four-way valve and stainless steel cannulae into the bottom of polyallomer tubes. Centrifugation was for 26 hours at 127,000 x g_{max} and 37°C in the SW27 rotor (Beckman Instruments). Gradients were fractionated from the bottom and monitored continuously at 280 nm as described earlier.

8. RNA gel electrophoresis

RNA was fractionated on an 80% (v/v) formamide gel with a 4 - 10% (w/v) acrylamide gradient. The following solutions were used to prepare the gel:

Stock solutions

20% acrylamide/bis, 80% formamide

144 g acrylamide

16 g bisacrylamide

add formamide and water to a final formamide concentration of 80% (v/v) and a final volume of 800 ml.

Clarify by centrifugation at 3800 rpm for 5 minutes (PR6000).

0.4 M EDTA, pH 7.6

18% (w/v) ammonium persulfate

3.6 M NaCl

20% (w/v) Sarkosyl

formamide (98.5%, commercial, deionized)

Working solutions

1. Heavy (10%) acrylamide/bis, 160 ml final volume

27.2 g sucrose

80.0 ml 20% acrylamide/bis in formamide

0.58 g barbital

0.16 ml EDTA

52.0 ml formamide

9.0 ml water

11-12 µl TEMED

Adjust pH to 8.0 with 2 M NaOH (∿1.6 ml)

2. Light (4.0%) acrylamide/bis, 120 ml final volume

24.0 ml acrylamide/bis in formamide

0.435 g barbital

0.12 ml EDTA

76.5 ml formamide

17.7 ml water

39 µl TEMED

Adjust pH to 8.0 with 2 M NaOH (∿1.2 ml)

3. Slot buffer, 81% formamide, 200 ml final volume

164.5 ml formamide

0.70 g barbital

0.20 ml EDTA

33.8 ml water

Adjust pH to 8.0 with 2 M NaOH (∿1.5 ml)

4. RNA sample buffer, 86% formamide, 50 ml final volume

43.0 ml formamide

0.14 g barbital

0.05 ml EDTA

3.5 g sucrose

0.2 ml Sarkosyl

a few crystals of bromphenol blue

4.7 ml water

Adjust pH to 8.0 with 2 M NaOH (∿0.6 ml)

5. Electrode buffer, 4 liters final volume (20 x)

20 ml NaCl

4 ml EDTA

add water to 4 liters

6. Bottom (apparatus) plug, 200 ml final volume

80 ml 20% acrylamide/bis in formamide

12 ml electrode buffer (#5, above)

0.25 ml TEMED

107.0 ml water

To prepare the gel mold, clean glass plates separated by 3 mm plexiglass spacers were sealed with Scotch tape on the sides and bottom and mounted in a plexiglass trough. A "plug" solution was prepared by adding 0.12 ml additional TEMED to 60 ml of the 10% acrylamide solution. Polymerization of ∿43 ml of this was started by addition of 0.32 ml persulfate and the solution was poured into the sealed glass plates to plug the bottom. After this polymerization, 0.1 ml persulfate was added to 10 ml of the remaining acrylamide to plug the gel sides.

The resolving gradient gel was cast by adding 0.5 ml persulfate to 100 ml 10% acrylamide and 0.80 ml persulfate to 120 ml 4% acrylamide. A two-chamber gradient former was then filled with 101 ml light acrylamide

and 98 ml heavy acrylamide and the acrylamide gradient was generated by pumping the solutions between the plugged plates. After filling the mold, a slot former with 14 teeth 12.5 mm x 2 mm thick was inserted (see appendices). This type of comb was used to prevent samples from leaking between the gel and the glass plates when loaded since formamide/acrylamide adheres poorly to glass. The complete gel was covered with Saran Wrap to prevent drying and polymerized overnight at room temperature. The overall gradient dimensions were about 0.3 cm thick x 32.8 cm wide x 20.0 cm high corresponding to a volume of 199 ml. The bottom plug extended below the gradient for about 1.5 cm.

The slot former was removed on the day of the experiment and the slots filled with the slot buffer. Later the bottom tape was cut-off and the gel placed in a plexiglass apparatus with upper and lower reservoirs connected by an overflow tube so that buffer could be circulated from bottom to top reservoir. The bottom of the gel was sealed in the apparatus with apparatus plug to prevent the gel from slipping from between the glass plates during the run. To do this, the apparatus plug solution was degassed, mixed with 0.6 ml persulfate, and poured into the lower reservoir. The gel apparatus was tipped backward so that the plug solution would rest around the bottom of the gel.

RNA samples (0.35 - 2.05 A260 U) for electrophoresis were ethanol precipitated. On the day of the experiment each precipitate was dissolved in 35 µl RNA sample buffer by warming at 37°C for 10 minutes and refrigerated while the gel was pre-run. The apparatus was filled with electrode buffer. The upper chamber was slowly filled to prevent disturbing the slot buffer until just a little electrode buffer overflowed into the lower chamber. The gel was pre-run with no circulation for 1 hour at constant power, 150 V. RNA samples were heated at 62°C for 3 minutes and loaded with a microliter syringe. Power was again applied to the gel at 30 V for 6 minutes, 45 V for 20 minutes, and then 160 V. Buffer circulation was begun when the power was raised to 160 V. The overall running time was 19 hours.

After the run, the gel was removed from the glass plates under water,

placed in StainsAll for 4 hours, and destained through a series of washes in water, methanol mixture (see below), and ∿25% water + ∿75% methanol mixture. Staining and destaining were done in the dark with slow rotary shaking.

StainsAll mixture

Stock solutions:

0.1% (v/v) StainsAll in crude formamide at pH 7.9

1 M triethanolamine (pH 8.5)

Just before use, mix:

45 ml StainsAll stock

60 ml formamide (pH 9.0)

10 ml triethanolamine stock

100 ml isopropanol

190 ml water

Methanol mixture

41% (v/v) methanol

1% (v/v) glycerol

30 mM triethanolamine (pH 8.4)

9. Poly(A) determination

Poly(A) was measured by [^{3}H]-poly(U) hybridization (Gillespie et al., 1972).

Polynucleotide standards

Poly(A) (heterogeneous length) 100 µg/ml in water

Poly(U) (heterogeneous length) 100 µg/ml in water

[^{3}H]-poly(U)

4.2 Ci/mmol UMP

3.9 µg UMP/ml

2.84 x 10^7 dpm/µg UMP

Incubation buffer

50% formamide

0.45 M NaCl

0.045 M Na citrate

0.01 M Tris-HCl (pH 7.2)

0.02 - 0.04 µg [^{3}H]-poly(U)

50-500 ng mRNA or polynucleotide standard

Digestion buffer

0.01 M Tris-HCl (pH 7.2)

0.01 M $MgCl_2$

0.5 M NaCl

DNAse I (RNAse free), 1 mg/ml in water

RNAse A, 5 mg/ml in water. Heated 100°C for 5 minutes.

All assays were performed in 1500 µl plastic Eppendorf tubes. Incubation buffer (35 µl), [^{3}H]-poly(U), and an RNA sample or standard were added to each tube to a final volume of 50 µl. The samples were incubated for 24 hours at 36°C in a circulating water bath (Haake). After the incubation, samples were cooled to 30°C and 1.0 ml digestion buffer was then added. Some tubes also received 20 µl DNAse and 1-2 µl RNAse solution. All samples were incubated further for 5 hours at 30°C, cooled on ice for 15 minutes, and precipitated with 150 µl 100% TCA/0.2% UMP on ice for 30 minutes. Precipitates were trapped on nitrocelluose membrane filters (25 mm, 0.45 µm) which had been prewashed with TCA/UMP. Sample tubes were rinsed twice with 10% TCA/0.02% UMP (1 - 2 ml) and residual precipitates collected on the same filter. Finally the filters were washed 4 - 5 times with 1 - 5 ml portions of 10% TCA/0.02% UMP, air dried ∿60 minutes, and counted in 5 ml Liquifluor in glass scintillation vials.

Protein Synthesis In Vitro

1. *Escherichia coli* S30 system

a. Extract preparation

E. coli (MRE 600) were grown in a Biogen cultivator to a density of 1 - 2 x 10^8 cells/ml at 37°C. The medium (40 liters) consisted of 454 g bactotryptone, 40 g yeast extract, 40 g glucose, 280 g NaCl, and 50 ml NaOH. Cells were rapidly chilled and harvested in a refrigerated Sharples continuous flow centrifuge. All subsequent procedures were at 0 - 5°C. The cell paste was washed twice in ∿30 ml 50 mM Tris-HCl (pH 7.6)/10 mM

Mg acetate/30 mM NH_4Cl, centrifuged at 12,000 x g for 10 minutes, and frozen in 15 - 18 g aliquots at -80°C. Active extracts could be prepared from cells stored frozen for several months.

To break cells, one aliquot of frozen cells was forced through an Edibo Press, precooled on dry ice for 15 minutes, by >10,000 psi hydraulic pressure. The frozen cell homogenate was thawed on ice in 25 ml 50 mM Tris-HCl (pH 7.6)/10 mM Mg acetate/30 NH_4Cl/1 mM DTT. DNAse I (electrophoretically pure, RNAse free) was added to ∿10 - 20 µg/ml from a stock solution (1 mg/ml in 1 mM $CaCl_2$/1 mM HCl) and the mixture incubated on ice with constant stirring for 30 minutes. The homogenate was centrifuged at 12,000 x g_{max} for 10 minutes to remove cellular debris and centrifuged once again at 30,000 x g_{max} for 30 minutes. The resulting supernatant (S30) was dialyzed against three changes of 2 liters 30 mM Tris-HCl (pH 7.6)/10 mM Mg acetate/30 mM NH_4Cl/0.5 mM DTT for 45 minutes per change to reduce endogenous amino acid concentrations. The dialyzed S30 was frozen in small aliquots at -80°C.

b. Cell-free translation

An energy mix (R_t) and a buffer solution (TM + GSH) were first prepared. R_t contained (final concentrations) reduced glutathione (GSH) 11 mg/ml, phosphoenolpyruvate (tricyclohexylammonium salt) 17 mg/ml, 165 mM Tris-HCl, 48 mM NH_4Cl, 16 mM Mg acetate, 12 mM ATP, 0.8 mM GTP, and 19 amino acids minus methionine (0.4 mM each), and was neutralized with Tris base. TM + GSH contained 7.5 mg/ml GSH, 250 mM Tris-HCl (pH 7.6), 150 mM NH_4Cl, and 50 mM Mg acetate. For incorporation, water, 20 µl R_t, 11 µl TM + GSH, 5 µl 1 M NH_4Cl, and 15 µl S30, were combined in order and preincubated for 20 minutes at 37°C. Reaction tubes were placed on ice and mRNA and 20 µl [^{35}S]-met added so that the final reaction volume was 100 µl. Incubation was then contained at 37°C for 30 minutes. Final concentrations in the reactions were, GSH 3.0 mg/ml, PEP 3.4 mg/ml, Tris 61 mM, NH_4Cl 76 mM, Mg acetate 9 mM, ATP 2 mM, GTP 0.16 mM, amino acids 0.08 mM each, and [^{35}S]-met, 1 mCi/ml (1.2×10^{-3} mM). In most experiments Chlamydomonas reinhardtii unbound mRNA was incubated at 4.1 A260 U/ml. Incorporation was stopped by placing the tubes on ice. A 5 µl ali-

quot was taken for radioactivity determination and the samples were either frozen at -20°C or processed for analysis.

2. The wheat germ system

a. Preparation of a wheat germ cell-free extract (Roman et al., 1976)

All procedures were at 0 - 5°C. A 1.5 x 30 cm Sephadex G25 (medium) column was packed by gravity flow and equilibrated extensively with 1 mM Tris-acetate (pH 7.6)/50 mM KCl/1 mM Mg acetate/4 mM β-mercaptoethanol. Raw wheat germ (1.5 g) was frozen with liquid nitrogen and then ground into a fine powder with a mortar and pestle. During grinding additional liquid nitrogen was added 2 - 3 times. The frozen powder was transferred to a 15 ml Corex centrifuge tube and extracted by extensive vortexing with 10 ml 1 mM Tris-acetate (pH 7.6)/90 mM KCl/3 mM Mg acetate. The resulting suspension was centrifuged at 14,000 x g_{max} for 10 minutes. The supernatant between the large pellet and floating lipid layer was withdrawn. The concentrations of Tris-acetate and Mg acetate in the supernatant were raised to ∿2 mM, respectively, by addition of 200 µl 1 M Tris-acetate (pH 7.6) and 20 µl 0.5 M Mg acetate, and centrifugation was repeated as before. The supernatant was carefully decanted, loaded onto the G25 column, and ∿9 ml of turbid effluent in the void volume collected. The eluate, which contains the wheat-germ cell-free extract, was centrifuged once again, and finally divided into small aliquots and frozen at -80°C. Extract absorbances were typically 48 at 260 nm and 33 at 280 nm. Activities were stable for at least six months at -80°C.

b. Cell-free translation

For cell-free translation in the wheat germ system, an energy mix and a 10X acetate buffer were first prepared. The energy mix contained (final concentrations) 10 mM ATP, 1 mM GTP, 80 mM phosphocreatine (PC), 19 amino acids minus methionine, 1.86 mCi/ml [^{35}S]-met (2.1×10^{-3} mM), and 0.48 mg/ml creatine phosphokinase (CPK). The pH was adjusted to ∿7.5 with KOH prior to dilution to the final volume. CPK was thawed and added to the energy mix just prior to incubation. The standard 10X acetate buffer was composed of 200 mM HEPES-KOH (pH 7.5), 1.1 M K acetate, 16 mM Mg acetate, 10 mM DTT, and 2.5 mM spermine. Concentrated stock

solutions of ATP (0.1 M), GTP (0.01 M), PC (0.8 M) and CPK (8 mg/ml) were stored at -20°C in small aliquots and never re-frozen. No decline in activity was detected after several months storage. Amino acid mixtures (1 mM each) and spermine (neutralized, 31.5 mM) were freeze-thawed many times without deleterious effects. Immediately prior to incubation an aliquot of the wheat germ extract was thawed and combined 4:1 with energy mix. For a 50 µl reaction 0 - 20 µl RNA (RNA + water = 20 µl), 25 µl energy + wheat germ, and 5 µl 10X acetate buffer were mixed and incubated at 26 - 27°C for 60 - 90 minutes. The reaction was terminated by cooling the samples on ice. Aliquots (5 µl) for radioactivity determination were taken and the samples were frozen at -20°C or processed for analysis.

Radioactivity Measurements

1. Disc assay

Incorporation into whole cells, cell fractions, and in vitro synthesized proteins was assayed by a modified paper disc method (Mans and Novelli, 1961). Samples (5 - 100 µl) were pipetted onto 2.3 cm diameter Whatman 3 MM discs suspended on straight pins. The discs were placed in ice cold 10% (w/v) TCA (at least 10 ml per disc) for more than 20 minutes and processed through boiling 5% TCA (10 minutes), two rinses at room temperature of 5% TCA, 50:50 ethanol-ether (10 minutes), and ether (10 minutes). After drying for 5 minutes, discs were counted in glass scintillation vials with 5 ml Liquifluor. A Beckman LS 350 counter with a gain setting of 350 was used to monitor the samples.

2. Aqueous sample determinations

Eluted proteins and column or gradient fractions were counted in plastic minivials with 0.5 ml water and 5 ml of a scintillation cocktail containing 5.5 g/l butyl-PBD and 25% (v/v) Triton X114 in reagent grade xylene. At a gain setting of 265 (Beckman LS 350), the counting efficiency for [^{14}C] was ∿80%.

Protein Gel Electrophoresis Techniques

1. One-dimensional SDS/polyacrylamide gel electrophoresis

a. General procedures

Protein samples were fractionated on one-dimensional SDS/polyacrylamide gels using either the Neville or Laemmli buffer system. The following stock solutions were used to prepare the gels:

Stock solutions

1. 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide
   The solution was decolorized with 1 g activated charcoal per liter and filtered through paper and 1.2 μm Millipore membrane filters.
2. 60% (w/v) acrylamide/0.8% (w/v) bisacrylamide
   The solution was decolorized as above. Since the 60% acrylamide precipitates in the cold, prior to use it was routinely re-dissolved at 37°C.
3. 60% (w/v) sucrose
4. 10% (w/v) ammonium persulfate, freshly prepared every two weeks.
5. Neville system buffers (Neville, 1971)
   a. Upper reservoir buffer (10X)
      Tris 49.6 g
      SDS 10.0 g
      Adjust to pH 8.64 with saturated boric acid and dilute to 1 liter final volume.
   b. Separation and lower reservoir buffer (5X)
      Tris 257.0 g
      Adjust to pH 9.18 with concentrated HCl and dilute to 1 liter final volume.
   c. Stacking gel buffer (2X)
      Tris 13.1 g
      Adjust to pH 6.1 with concentrated sulfuric acid and dilute to 1 liter final volume.

6. Laemmli system buffers (Laemmli, 1970)

   a. Separation gel buffer (8X)

      3 M Tris

      Adjust to pH 8.8 with concentrated HCl.

   b. Stacking gel buffer (4X)

      0.5 M Tris

      Adjust to pH 6.8 with concentrated HCl.

   c. Electrode buffer (5X)

      Tris 302.5 g

      glycine 1440 g

      dissolve and dilute to 10 liters final volume.

For most experiments, protein samples were separated on either a 7.5 - 15% acrylamide gradient or 12 - 18% acrylamide gradient supplemented with 8 M urea. The compositions of these typical gels for both Neville and Laemmli buffers is given in Table II. Gradients were generated from a two-chamber device (Buchler Instruments) suspended approximately 10 cm above glass plates separated by 1 mm thick plexiglass spacers. The plates were sealed with a plug solution consisting of ∿9.0% acrylamide/ ∿0.24% bisacrylamide in water. During polymerization, the top of the resolving gel solution was overlaid with isobutanol. After polymerization, isobutanol was poured off and the surface of the resolving gel was rinsed once with distilled water and once with the stacking gel solution (minus persulfate). The stacking gel was then formed on top of the resolving gel and a plexiglass slot former inserted. The dimensions of glass plates, spacers, and the running apparatus are given in the appendices.

To prepare urea gels, solid urea was dissolved in the acrylamide-buffer-water solutions by stirring at ∿37°C and, after a brief cooling period, the TEMED and persulfate added. The use of crystalline urea and the moderate dissolution temperature limits the generation of isocyanate (O'Farrell, 1975).

Gradient gels without urea were routinely stored for several days

TABLE II

STANDARD GRADIENT GEL COMPOSITIONS

A. 7.5 - 15% Acrylamide Gradient

	Neville Buffers*		Laemmli Buffers*	
	7.5%	15%	7.5%	15%
30% (w/v) Acrylamide/0.8% (w/v) Bis	11.5 ml	22.5 ml	11.5 ml	22.5 ml
Buffer	9.0 ml	9.0 ml	5.6 ml	5.6 ml
60% (w/v) Sucrose	3.8 ml	13.0 ml	3.8 ml	13.0 ml
10% (w/v) SDS	0.45 ml	0.45 ml	-	-
Water	20.0 ml	-	24.0 ml	4.0 ml
TEMED	20.0 µl	20.0 µl	15.0 µl	10.0 µl
10% (w/v) Ammonium Persulfate	0.17 ml	0.17 ml	0.18 ml	0.18 ml
Total Volumes	∿45 ml	∿45 ml	∿45 ml	∿45 ml

B. 12 - 18% Acrylamide Gradient, 8 M Urea

	Neville Buffers*		Laemmli Buffers*	
	12%	18%	12%	18%
60% (w/v) Acrylamide/0.8% (w/v) Bis	9.0 ml	13.5 ml	9.0 ml	13.5 ml
Buffer	9.0 ml	9.0 ml	5.6 ml	5.6 ml
Sucrose (crystals)	-	4.0 g	-	4.0 g
Urea (crystals)	21.6 g	21.6 g	21.6 g	21.6 g
10% (w/v) SDS	0.45 ml	0.45 ml	-	-
Water	10.0 ml	3.0 ml	13.4 ml	6.4 ml
TEMED	15.0 µl	10.0 µl	12.0 µl	9.0 µl
10% (w/v) Ammonium Persulfate	0.1 ml	0.1 ml	60.0 µl	60.0 µl
Total Volumes	∿45 ml	∿45 ml	∿45 ml	∿45 ml

C. Stacking Gel (same for both 12 - 18% and 7.5 - 15% gradients)

	Neville Buffers*	Laemmli Buffers*
	6%	5%
30% (w/v) Acrylamide/0.8% (w/v) Bis	4.0 ml	2.5 ml
Buffer	10.0 ml	3.8 ml
10% (w/v) SDS	0.2 ml	-
Water	5.6 ml	8.5 ml
TEMED	20 µl	20 µl
10% (w/v) Ammonium Persulfate	0.2 ml	0.1 ml
Total Volumes	20 ml	15 ml

*Neville buffers are 5X Tris-HCl (pH 9.2) for the gradient and 2X Tris-sulfate (pH 6.1) for the stacking gel. Laemmli buffers are 8X Tris-HCl (pH 8.8) for the gradient and 4X Tris-HCl (pH 6.8) for the Stacking gel.

Each 1 mm thick slab is made from 38 ml light acrylamide and 35 ml heavy acrylamide. See text and appendix for further details.

before running. The gel top was wrapped in damp paper towels and the entire gel placed in a sealed plastic bag at 0 - 5°C. In general, Neville gels could be stored longer than Laemmli gels. Urea gradient gels were never stored for more than one day and were kept in plastic bags at room temperature since 8 M urea precipitates in the cold.

b. Sample preparation

Samples were prepared for SDS gel electrophoresis by dispersing them in buffer prior to solubilization in SDS. Reducing agents were always included and alkylation was often performed. No difference was detected, however, between samples reduced and alkylated and samples simply reduced. Dispersal and solubilization procedures for various samples are summarized in Table III.

For electrophoresis gels were clamped in an upright plexiglass apparatus (see appendix), the slot-former removed, and the reservoirs filled with buffer. Improved resolution was achieved on Neville gels by making the upper reservoir buffer 1 mM EDTA. Samples were loaded into the slots with a microliter syringe. Electrophoresis was at 10 - 20 mA constant current until the marker dye front eluted from the gel. After electrophoresis, gels were stained for several hours in 0.25% Coomassie brilliant blue R/7% (v/v) acetic acid/50% (v/v) methanol (De St. Groth et al., 1963), and destained through several changes of 7% acetic acid/45% methanol with agitation. Gels were finally equilibrated in destain solution containing ∿5% (v/v) glycerol and vacuum dried overnight on a porous plate covered with Saran Wrap.

2. Two-dimensional isoelectric focusing/SDS polyacrylamide gel electrophoresis

Two-dimensional gels were prepared and electrophoresed essentially as described by O'Farrell (1975). Isoelectric focusing was performed on slabs instead of tubes (Ames, 1976). To prepare a single isoelectric focusing slab, 14.4 g crystalline urea was dissolved in a mixture of 3.99 ml 30% acrylamide stock (28.38% (w/v) acrylamide/1.62 (w/v) bisacrylamide) 6 ml 10% (w/v) Nikkol and 5.9 ml water at ∿37°C. When the urea dissolved, an ampholine mixture consisting of 1.4 ml pH 3.5 - 10, 0.2 ml pH 9 -

TABLE III

SAMPLE PREPARATION FOR SDS GEL ELECTROPHORESIS

Sample Type	Example	Step: 1 Add Buffer	2 Disperse	3 Add	4 Solubilize	5 Alkylate
Membranes	TMF (0.5 mg chlorophyll) TMF extraction residue (0.5 mg chlorophyll)	0.1 M Na carbonate 0.1 M DTT to 0.28 ml	Vortex	0.1 ml 10% SDS 0.1 ml 60% sucrose 0.02 ml 1% BPB	Heat 100°C for 1 min.	-
TCA precipitates	Soluble proteins (∿200 µg) Extracted membrane proteins (≤ 600 µg)	0.1 M Na carbonate 0.1 M DTT 56 µl	Sonicate Vortex	20 µl 10% SDS 20 µl 60% sucrose 4 µl 1% BPB	Heat 37°C for 15 min. Heat 100°C for 2 min.	-
TCA precipitates	TMF (50 µl chlorophyll) Translation samples (50 µl reaction)	0.1 M Tris Base 0.1% BPB 6% sucrose 50 µl	Sonicate Vortex	5 µl 0.1 M DTT 5 µl 20% SDS	Heat 37°C for 15 min. Heat 100°C for 2 min.	5 µl 0.25 M iodoacetamide (fresh) Heat 37°C in dark for 15 - 30 min.

BPB: bromphenol blue

11, 0.1 ml pH 4 - 6, and 0.1 ml pH 5 - 7 was added to the urea/detergent solution. Polymerization was initiated with 0.08 ml 0.14 mg/ml riboflavin/2% (v/v) TEMED (made fresh monthly). The complete solution was poured into glass plates, sealed with standard "plug" solution (∿9.0% acrylamide/∿0.24% bisacrylamide in water) separated by 0.75 mm spaces, a seventeeth slot former was inserted, and polymerization accelerated to completion by standing the entire set-up in front of a fluorescent light bank for two hours.

Samples were prepared while the isoelectric focusing gel was polymerizing. Translation reaction mixes (50 µl) were centrifuged 15 - 30 minutes at 143,000 x g_{max}, 5°C to remove ribosomes and the resulting supernatant treated for 30 minutes on ice with 5 µg pancreatic RNAse A. These steps were performed to prevent streaking from RNA (O'Farrell, 1975) and to reduce the protein load by elimination of ribosomal proteins. The digested translation samples, labeled thylakoid membranes (10 - 20 µg chlorophyll), or other labeled protein samples (10 - 100 µg) were precipitated on ice for 30 - 60 minutes with an equal volume of 10% TCA and centrifuged in a microfuge for 2 minutes. The resulting pellet was washed once with 100 µl cold 1 N HCl to eliminate TCA, and lyophilized briefly to remove excess HCl. To each sample 40 µl of 2% ampholines (pH 3.5 - 10)/2% NP40/10 mM DTT were added and the sample dispersed by sonication. Solid urea (38 mg) was dissolved in the samples for 10 min at 37°C.

After polymerization, the slab gel mold was washed with distilled water to remove crystallized urea and mounted into the vertical gel apparatus (see appendices). The entire sample, prepared as described above, was loaded into a single gel slot. To prevent cross-contamination of samples during isoelectric focusing, only alternate gel slots were used. All sample slots were overlaid with 20 - 50 µl 9 M urea/1% ampholines (0.8% pH 5 - 7/0.2% pH 3.5 - 10). Empty slots were filled with overlay solution. Basic electrode solution (0.2 M NaOH, extensively degassed) was carefully poured into the upper (loading) chamber. The other reservoir was filled with 0.01 M H_3PO_4. Gels were run at 300 V constant voltage for 18 hours and then at 400 V for 1.5 hours.

After completion, isoelectric focusing gels were cut into strips 13 cm long x 1 cm wide, equilibrated with gentle shaking in 40 ml 1X Tris-borate (pH 8.64) (see Neville SDS gel section)/1% (w/v) SDS/1 mM DTT for 5 - 15 minutes, and loaded onto the top of a 1 mm thick Neville SDS polyacrylamide gel prepared with no slots. Strips were sealed on the gels with 0.5% (w/v) agarose in 1X Tris-borate (pH 8.64)/0.1% SDS/ 1 mM EDTA and the gels electrophoresed, stained, destained, and dried as described before. To measure the pH gradient, a strip from an empty isoelectric focusing slot was cut into 1 x 1 cm pieces, incubated at room temperature in 1.0 ml distilled water overnight, and the pH measured with a standard electrode. A typical pH gradient is shown in Figure 2.

3. Detection of radioactive proteins

Dried gels were exposed to Dupont Cronex 2 D/C X-ray film at -20°C. In some instances, sticky gels were lightly dusted with talc to prevent damage to the film emulsion. To amplify low amounts of radioactivity, fluorography was performed exactly as described by Laskey and Mills (1975).

4. Electroelution of proteins from dried gels

Gels containing desired protein bands were fixed in destain solution for 30 - 60 minutes without staining, dried, and autoradiographed. The protein bands were excised using the autoradiograph as a template, and placed in the column of the electroelution apparatus. The column was filled with 1X Tris-borate (pH 8.64, Neville upper reservoir buffer)/ 0.1% SDS/1% (v/v) β-mercaptoethanol/1 mM EDTA and adjusted to ∿1 mM PMSF. The upper column section containing the gel strips was separated from a 3 ml elution chamber by a layer of nylon netting. The bottom of the elution chamber was sealed with dialysis tubing held in place by an O-ring. An exact description of the electroelution apparatus is given in the appendices. The assembled elution column was inserted in a two-reservoir plexiglass support. The upper-reservoir was filled with additional 1X Tris-borate/0.1% SDS (no PMSF) and the bottom reservoir with 1X Neville lower reservoir buffer. Electroelution was carried out at 7 - 8 mA per column for 16 - 18 hours. By this time the eluted protein was

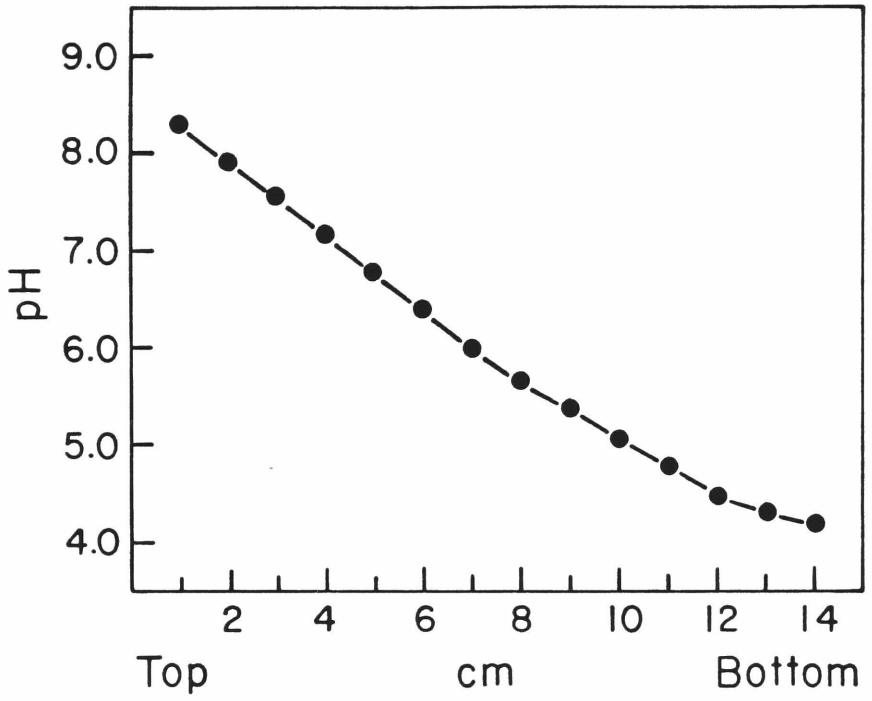


Figure 2. pH gradient of a typical isoelectric focusing gel.

A slab isoelectric focusing gel was prepared and run as described in Materials and Methods. Gel pieces (1cm square) from an unloaded slot were incubated overnight in 1.0 ml distilled water and the pH measured.

concentrated in the elution chamber. The protein solution was removed by sealing the top of the column and removing the dialysis membrane. The eluted protein solution (∿3 ml) was filtered through a Millex 0.22 μm filter to remove bits of acrylamide and stored frozen at -20°C until TCA precipitation.

Immunological Techniques

1. Antibody production and purification

Purified IgG specific for the carboxylase large subunit was a gift of Dr. Nam-Hai Chua. The carboxylase holoenzyme was purified by a published procedure (Iwanij et al., 1974). The holoenzyme was dissociated into subunits in 1% SDS and the large subunit purified by chromatography on Sephadex G100 in SDS. Antibodies were raised in rabbits immunized by either subscapular or intraperitoneal injections, and IgG purified from immune sera by ammonium sulfate precipitation and DEAE-Sephadex chromatography (Harboe and Ingild, 1973).

2. Immunodiffusion on glass slides

Immunodiffusion plates were prepared in 80 mM Tris-acetate (pH 8.6)/ 40 mM Na acetate/1 mM EDTA/1% (w/v) Triton X100/1% (w/v) agarose. The antigen was prepared by precipitating ∿1 mg purified carboxylase large subunit with TCA, dispersing the pellet in PBS/1% (w/v) SDS, and capturing the SDS micelles by making the solution ∿2% (w/v) Triton X100. Plates were incubated 24 - 48 hours in a moist chamber at room temperature and photographed in diffuse light. PBS contains 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 per liter solution (total ionic strength = 0.15 M).

3. Antibody selection by affinity chromatography

a. Large subunit coupling

Purified carboxylase large subunit in water (∿12 mg) was lyophilized and dissolved in 9.5 ml 0.1 M Na carbonate/∿2% (w/v) SDS. CNBr-activated Sepharose 4B (1.0 g) was washed 4 - 5 times with 1 mM HCl (total wash volume >200 ml) on a coarse scintered glass filter (4.0 cm diameter). The Sepharose was sucked dry between each rinse and resuspended gently

with a rubber policeman. The gel was then washed with two 10 ml volumes of 0.1 M Na carbonate, transferred to a 15 ml screw cap centrifuge tube, and centrifuged for 30 seconds in a clinical centrifuge. The packed gel volume was 2.0 - 2.5 ml. After discarding the supernatant, the large subunit solution was added to the packed gel, and the suspension mixed end-over-end for 2 hours at room temperature. The suspension was centrifuged and washed twice with 10 ml 0.1 M Na carbonate. Residual activated groups on the Sepharose were neutralized by incubating with 10 ml 1 M 2-aminoethanol (pH 8.0) for 2 hours. Next, the suspension was washed with 10 ml 0.1 M Na carbonate (pH 8.3)/1% (v/v) Triton X100 to eliminate SDS, centrifuged, and washed three times with cycles of 0.1 M Na borate (pH 8.0) and 0.1 M Na acetate (pH 4) on the scintered glass filter. Finally, the affinity column material was washed with phosphate buffered saline containing 0.02% (w/v) Na azide and stored at 5°C. About 30% of the applied protein was bound to the affinity support.

b. Specific antibody selection

Anti-large subunit IgG (∿420 mg) in 20 mM Tris-HCl (pH 8.6)/0.15 M NaCl/0.04% (w/v) Na azide/Trasylol (1:100 dilution of commercial stock) was diluted with one volume PBS + azide and concentrated on an Amicon PM30 membrane to a final volume of 8 ml. The affinity support was packed into a jacketed 1 cm diameter column at ∿24 ml/hr, 5°C and equilibrated with PBS + azide. The column was washed with 1.0 ml 50 mM Na phosphate (pH 7.3)/3 M Na thiocyanate (recrystallized)* and re-equilibrated with PBS. Half of the IgG solution (4 ml, ∿210 mg) was applied to the column at the same flow rate and 5°C. The unbound material was collected and the column washed with PBS until no 280 nm absorbing material eluted. Specific bound IgG was then released with 50 mM glycine-HCl (pH 2.8). The pH of the eluant (8.7 ml) was immediately adjusted to ∿9 with 1.0 ml 1 M Tris base. After concentration with Aquacide II and dialysis against PBS + azide, the various fractions were examined by immunodiffusion (Fig. 3). Essentially no precipitating antibodies were lost by affinity selec-

* Since the thiocyanate reagent absorbs at 280 nm, it is preferred to use the acidic glycine solution described later for this pre-wash.

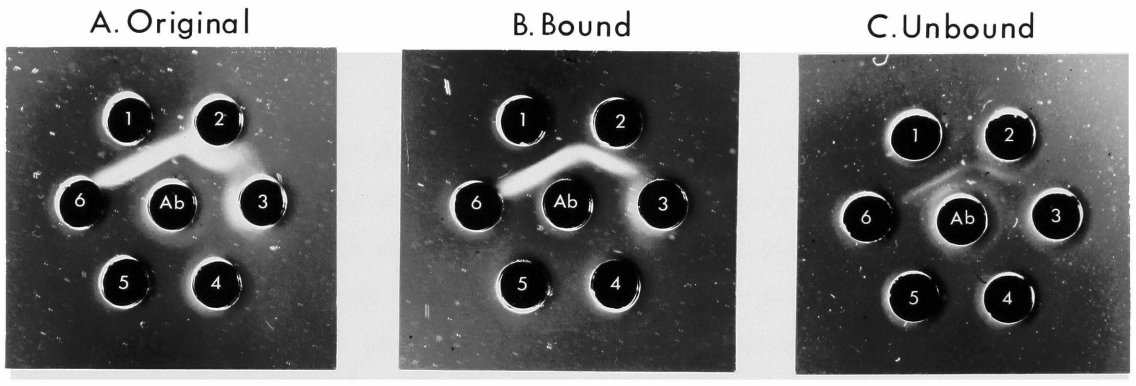


Figure 3. Analysis of immunoselected ribulose bisphosphate carboxylase large subunit antibodies by immunodiffusion.

Antibodies specific for the carboxylase large subunit were selected by chromatography of anti-large subunit IgG on a large subunit affinity column. Volumes of the specific (bound) IgG solution and the non-specific (unbound) IgG solution were normalized relative to the original antibody solution volume and compared by immunodiffusion on agarose plates (see Materials and Methods for composition). Purified carboxylase large subunit was dissolved in PBS with SDS (1.0%) and Triton X-100 (2%) to a final concentration of 1 mg/ml and diluted serially in PBS. Antibody solutions were placed in the central well: A, original solution, 15 µl; B, bound IgG, 9 µl + 6 µl PBS; C, unbound IgG, 12 µl + 3 µl PBS. Outer wells contained the carboxylase large subunit: 1, 10 µg; 2, 2 µg; 3, 0.4 µg; 4, 0.08 µg; 5, 0.016 µg; 6, 0.0032 µg. Plates were photographed wet without staining

tion although only ∿3% of the total IgG was bound (estimated by 1.4 A280 units = 1 mg IgG).

4. Immunoprecipitation with Staphylococcus aureus

Staphylococcus preparation and immunoprecipitation were based on techniques described by Kessler (1975, 1976) and modified by Gregory Schmidt.

A 10% (v/v) suspension of heat-killed, formalin fixed Staphylococcus aureus (Cowans strain) was a generous gift of Gregory Schmidt. An aliquot of Staph was placed in an Eppendorf centrifuge tube and washed three times with NETT buffer (5 mM Tris-HCl (pH 7.5)/0.15 M NaCl/5 mM EDTA/1% (w/v) Triton X100). After each wash, the Staph suspension was centrifuged in a microfuge for 1 - 2 minutes, the supernatant aspirated, and the pellet resuspended by sonication. After washing, the pellet was again made 10% with NETT and frozen at -20°C until immunoprecipitation.

To determine the amount of Staph required to precipitate a given amount of IgG, the washed Staph preparation was titrated with purified IgG and the distribution between bound and unbound monitored by SDS/gel electrophoresis.

A translation sample (100 - 400 µl) from which the ribosomes had been removed by centrifugation was TCA precipitated and resuspended by sonication in 0.17 volumes 0.2 M Tris base/0.02 volumes 0.1 M DTT and solubilized with 0.01 volumes 10% SDS at 100°C for 3 minutes. The term "volume" refers to the original sample size. Finally, 0.8 volumes of 66 mM Tris-HCl (pH 8.6)/0.15 M NaCl/1% Triton X100 and 0.01 volumes Trasylol were added. The final concentrations were 87 mM Tris-HCl (∿pH 8.6)/2 mM DTT/0.1% SDS/0.8% Triton X100/0.12 M NaCl/Trasylol (1:100 dilution of commercial stock).

For immunoprecipitation, excess IgG was added to the sample. The mixture was incubated at 37°C for 1 hour, and placed at 5°C overnight. The next day the sample was added to a pellet of washed 10% Staph sufficient to adsorb all IgG in the mixture (e.g., 0.15 - 0.20 ml 10% Staph per 100 µg IgG) and the suspension incubated at 37°C for 1 hour. The

Staph, with bound immune complexes, was pelleted by centrifugation and the supernatant was saved. The pellet was washed at least three times with ∿1 ml RSB (10 mM Tris-HCl (pH 8.6)/0.1% SDS/0.3 M NaCl) by sonicating in ∿100 µl RSB, diluting the suspension with additional RSB to ∿1 ml, and centrifuging for 1 minute. Immune complexes were released from the final pellet by adding 0.2 - 0.4 volumes 50 mM Tris-HCl (pH 8.6) and 0.025 - 0.05 volumes 0.1 M DTT, sonicating to resuspend, adding 0.25 - 0.5 volumes 5% SDS/24% sucrose/0.1% bromphenol blue, and heating at 100°C for 2 - 3 minutes. The suspension was then centrifuged for 5 minutes and the pellet discarded. The released immunoprecipitate in the supernatant was alkylated with 0.025 - 0.05 volumes of 0.25 M iodoacetamide (freshly prepared) for 15 - 30 minutes at 37°C in the dark. At this point the immunoprecipitate was ready for loading on an SDS gel. The residual supernatant which contained proteins not immunoprecipitated, was prepared for electrophoresis in the same manner as a normal translation sample (see Table III).

Peptide Mapping

1. Precipitation of eluted proteins for peptide mapping

Proteins eluted from SDS gels were precipitated according to Milman et al., (1977). Aliquots (20 - 70 µl, ∿50,000 cpm) were lyophilized on a centrifugal evaporator (Savant Instruments) and dissolved in 150 µl water. Carrier BSA (20 µg = 20 µl) and 60 µl 75% (w/v) TCA were added and samples precipitated for 4 hours at 5°C. Recoveries of counts were 86 - 100%. The highest recoveries were obtained from samples initially having the smallest volumes and precipitated for the longest periods of time. The phenomenon was undoubtedly dependent on the SDS concentration in the sample. Addition of NaCl had no effect on recoveries. The precipitate was pelleted for 30 minutes in a microfuge at 5°C and was washed twice in 200 µl cold 1 N HCl. Residual HCl was removed by lyophilization.

2. One-dimensional limited digestion mapping

This procedure was performed as described by Cleveland et al., (1977).

Protein samples were purified by SDS/gel electrophoresis and electroelution, and concentrated by TCA precipitation. The lyophilized precipitate was dissolved in 0.125 M Tris-HCl (pH 6.8)/10% glycerol/0.5% SDS/0.0001% bromphenol blue and heated for 2 minutes at 100°C. The solution was distributed into 50 µl aliquots (25 - 50,000 cpm each). Serial protease dilutions were added to each aliquot and the samples digested at 37°C for 30 minutes. Aliquots were then supplemented with 6 µl 0.1 M DTT and 6 µl 20% SDS, heated at 100°C for 2 minutes, and immediately electrophoresed on an SDS slab. Peptide bands were visualized by autoradiography.

3. High-pressure liquid chromatography (HPLC) mapping

Precipitated, lyophilized protein samples were performic acid oxidized and trypsin digested. Performic acid reagent was prepared by allowing a mixture of 0.95 ml 99% formic acid and 50 µl 30% hydrogen peroxide to stand at room temperature. Protein precipitates (∿100,000 cpm) were dissolved in 50 µl 99% formic acid, cooled for 30 minutes in a melting ice bath, and oxidized with 100 µl performic acid reagent for 3 hours in the ice bath. The reaction was quenched with 500 µl water and the samples lyophilized. Since the sulfhydryl groups of cysteine were previously alkylated with iodoacetamide, only methionines were oxidized by performic acid. Each sample was redissolved in 50 µl 1% ammonium bicarbonate (pH 8.1) and lyophilized to remove excess performic acid. Finally, samples were dissolved in 50 µl ammonium bicarbonate and digested at room temperature (26 - 28°C) with 2 µl 1 mg/ml trypsin-TPCK for 4.5 hours. An additional 50 µl buffer and 3 µl trypsin solution were added and digestion continued overnight. The digested sample was lyophilized, dissolved in 50 µl water, re-lyophilized, and finally frozen at -20°C.

HPLC peptide mapping was performed exactly as described by Milman et al., (1977) except that in some experiments 0.02 M pyridine acetate was substituted for 0.1 M pyridine acetate and the gradient run from 0.02 - 0.2 M rather than from 0.1 - 1.2 M.

Miscellaneous Methods

1. Protein determination

Protein was estimated by the method of Lowry et al., (1951) using bovine serum albumin as a standard.

2. Chlorophyll determination

Chlorophyll was measured as described by Arnon (1949).

3. The pH of all buffer solutions was adjusted at room temperature.

Sources of Reagents

1. Non-radioactive substances

Acrylamide	Eastman
AG 50-X8 (cation exchange resin)	Bio-Rad
AG 501-X8 (mixed bed resin)	Bio-Rad
Aminex A5	Bio-Rad
Ampholines	LKB
Chellex (chelating resin)	Bio-Rad
CNBr-activated Sepharose 4B	Pharmacia
DEAE-Sephadex	Pharmacia
Deoxyribonuclease I (EC #3.1.4.5)	Worthington
Formamide (reagent grade)	Eastman
Liquifluor	New England Nuclear
N,N'-Methylenebisacrylamide	Eastman
Nikkol	Nikko Chemicals (Japan)
Nonidet P40	Particle Data Labs.
Papain (mercuripapain; EC #3.4.22.2)	Sigma
Phosphoenolpyruvate (tricylohexylammonium)	Boehringer/Mannheim
Poly(A), heterogeneous length	Schwartz/Mann
Poly(U), heterogeneous length	Miles
Poly (U) Sepharose	Pharmacia
Proteinase K	EM Biochemicals
Ribonuclease A (Protease free; EC #3.1.4.22)	Sigma
Sephadex G25	Pharmacia

Sephadex G100	Pharmacia
Spermine	Sigma
StainsAll	Eastman
N,N,N',N'-tetramethylethylenediamine	Sigma
Thermolysin	Calbiochem
Trasylol	FBA Pharmaceuticals
Trypsin-TPCK (EC #3.4.21.4)	Worthington
Wheat germ (raw)	General Mills

2. Isotopes

Acetic acid, sodium salt, [1-^{14}C]-, 40-60 mCi/mmol	New England Nuclear
Polyuridylic acid, sodium salt [uridylate-5,6-^{3}H]-,>500 mCi/mmol UMP	New England Nuclear
Sulfate, sodium salt, [^{35}S], carrier free	New England Nuclear
Methionine, L-[^{35}S]-,400-900 Ci/mmol	Amersham

CHAPTER III

STUDIES ON CHLOROPLAST PROTEIN SYNTHESIS *IN VIVO*

Incorporation of Label into Whole Cells and Cell Fractions

For the work described in this chapter, *Chlamydomonas* was labeled with [^{35}S]-sulfate after a short period of sulfate starvation. Uptake and incorporation of sulfate by cells is active and linear for at least one hour at specific activities varying over at least one order of magnitude (G.M.W. Adams, personal communication). These properties permit cellular proteins to be labeled to very high specific activities in a short time. For example, cells labeled with 0.1 mCi/ml for 30 minutes incorporated ∿15 cpm/cell in a hot TCA insoluble form. Under the same conditions, incorporation into thylakoid membrane proteins was 8.1×10^5 cpm/μg chlorophyll, into soluble proteins ∿3×10^5 cpm/μg protein, and into total ribosomes ∿7×10^6 cpm/A260 U. If the specific activity of [^{35}S]-sulfate in the medium was reduced ten-fold to 0.01 mCi/ml, the incorporation was reduced proportionally to, for instance, 8.4×10^4 cpm/μg chlorophyll for thylakoid membrane proteins.

The effects of the inhibitors anisomycin and chloramphenicol on incorporation of sulfate into cellular proteins are shown in Table IVA. The degree of inhibition observed in similar to that reported earlier (Chua and Gillham, 1977) for [^{14}C]-acetate incorporation using identical inhibitor concentrations and preincubation conditions. Anisomycin, which inhibits elongation on 80S ribosomes (Lizardi and Luck, 1972), blocks all but 16.6% of sulfate incorporation into whole cells. Chloramphenicol, a 70S ribosome inhibitor (Chua *et al.*, 1976), stimulates sulfate incorporation slightly in whole cells and to varying extents in some cell fractions. This effect may be caused by increased intracellular methionine and cysteine specific activities by indirect blockage of sulfolipid synthesis or the synthesis of other sulfur containing molecules.

Synthesis of Soluble Proteins in the Chloroplast

To determine the number and characteristics of soluble proteins synthesized in the chloroplast, cells were labeled with sulfate in the presence of inhibitors and 48,000 x g_{max} supernatants were centrifuged on sucrose

TABLE IV

EFFECTS OF INHIBITORS ON [^{35}S]-SULFATE INCORPORATION

A.	Whole Cells	TMF	48 kg Sup	PRS	Top Fraction	18S Peak	Total Ribosomes
Control	100%	100%	100%	100%	100%	100%	100%
Anisomycin	16.6	50.3	39.1	26	10.6	23.2	20
Chloramphenicol	103	83.5	118	105	94.3	59.4	137
Anisomycin + Chloramphenicol	8.8	19.3	39.2	22	8.9	12.6	16

B.	TMF	Total Ribosomes
Control	100%	100%
Anisomycin	55.1	50.5
Lincomycin	87.2	187
Anisomycin + Lincomycin	10.2	51.9

gradients (Fig. 4) Three peaks were observed. The fastest sedimenting peak was known from earlier work to be a small ribosomal subunit partially unfolded by EDTA in the gradient. Since the subunit probably contained an incomplete set of ribosomal proteins, this fraction was discarded. The second (18S) peak was ribulose-1,5-bisphosphate carboxylase holoenzyme (Iwanij et al., 1974). The broad peak at the top of the gradient contained remaining cellular soluble proteins and proteins released by EDTA from membranes and ribosomes. The gradient fractions from the top of the gradient to the 18S peak were collected and designated as the top fraction. Small thylakoid membrane fragments and remaining ribosomal material sedimented to the bottom of the gradients and were discarded.

From an electrophoretic examination of the 18S peak (Figs. 5 and 6, slots 6-10), it is clear that ribulose bisphosphate carboxylase holoenzyme is the major component in this fraction. As expected, both the large and small subunits are labeled in the control sample. In the presence of anisomycin only the large subunit is labeled whereas with chloramphenicol, only the small subunit is labeled. The doublet migrating slightly slower than the large subunit in the chloramphenicol sample (Fig. 6, slot 9) is believed to be a contaminant from top fraction proteins. Many minor bands are detectable in the control and chloramphenicol autoradiographs; none of these is labeled in the presence of anisomycin. Incorporation of [^{35}S]-sulfate into 18S proteins is completely stopped in the presence of both inhibitors.

While more than one hundred top fraction polypeptides are labeled in the control and chloramphenicol samples, the carboxylase large subunit is the only polypeptide extensively labeled in the presence of anisomycin. Two other slightly labeled bands which migrate just below the carboxylase large subunit are also visible in the anisomycin sample (Fig. 5, slot 3); a third band migrates in a position below carboxylase small subunit. If the gel shown in Figure 6, slot 3 is overexposed, then the bands just below the large subunit are resolved into three polypeptides. On the basis of experiments to be described in a later section, two of these bands have been identified as the peripheral membrane proteins 4.1 and

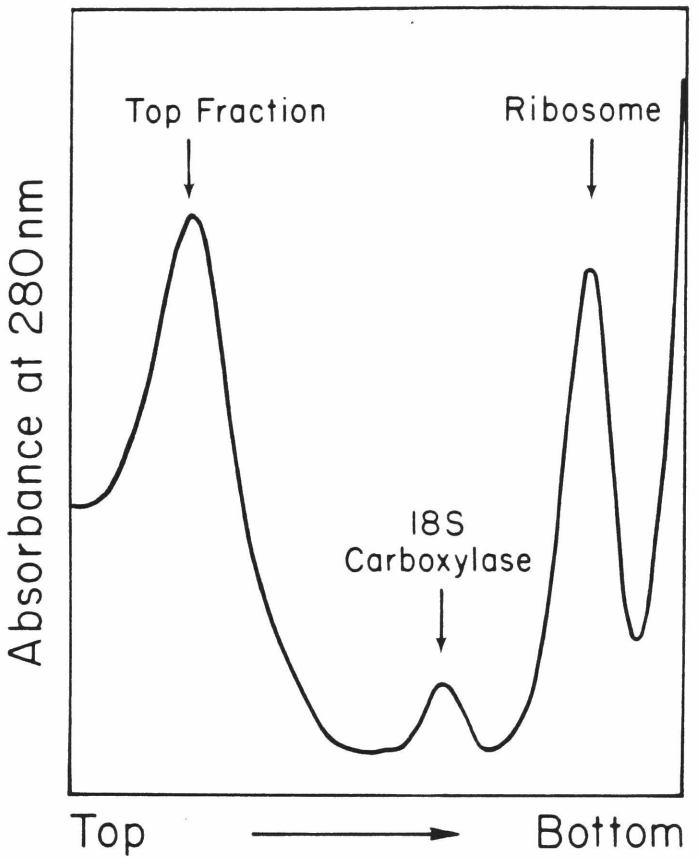


Figure 4. Sucrose gradient fractionation of 48,000 x g_{max} supernatants.

Chlamydomonas reinhardtii was labeled with [^{35}S]-sulfate in the presence of inhibitors and fractionated as described in Materials and Methods. About 1.5 mg protein from a 48,000 x g_{max} supernatant was loaded on a 10 - 30% sucrose gradient in 25 mM Tris-HCl (pH 7.5)/0.2 M NaCl/ 5 mM DTT/1.0 mM EDTA and centrifuged at 284,000 x g_{max} for 16 hours. Three major fractions were detected: a top fraction containing most soluble proteins, an 18S peak containing mostly ribulose-bisphosphate carboxylase holoenzyme, and a small ribosomal subunit derivatized by the EDTA in the buffer. Sedimentation is from left to right. The gradient shown is that of the control sample.

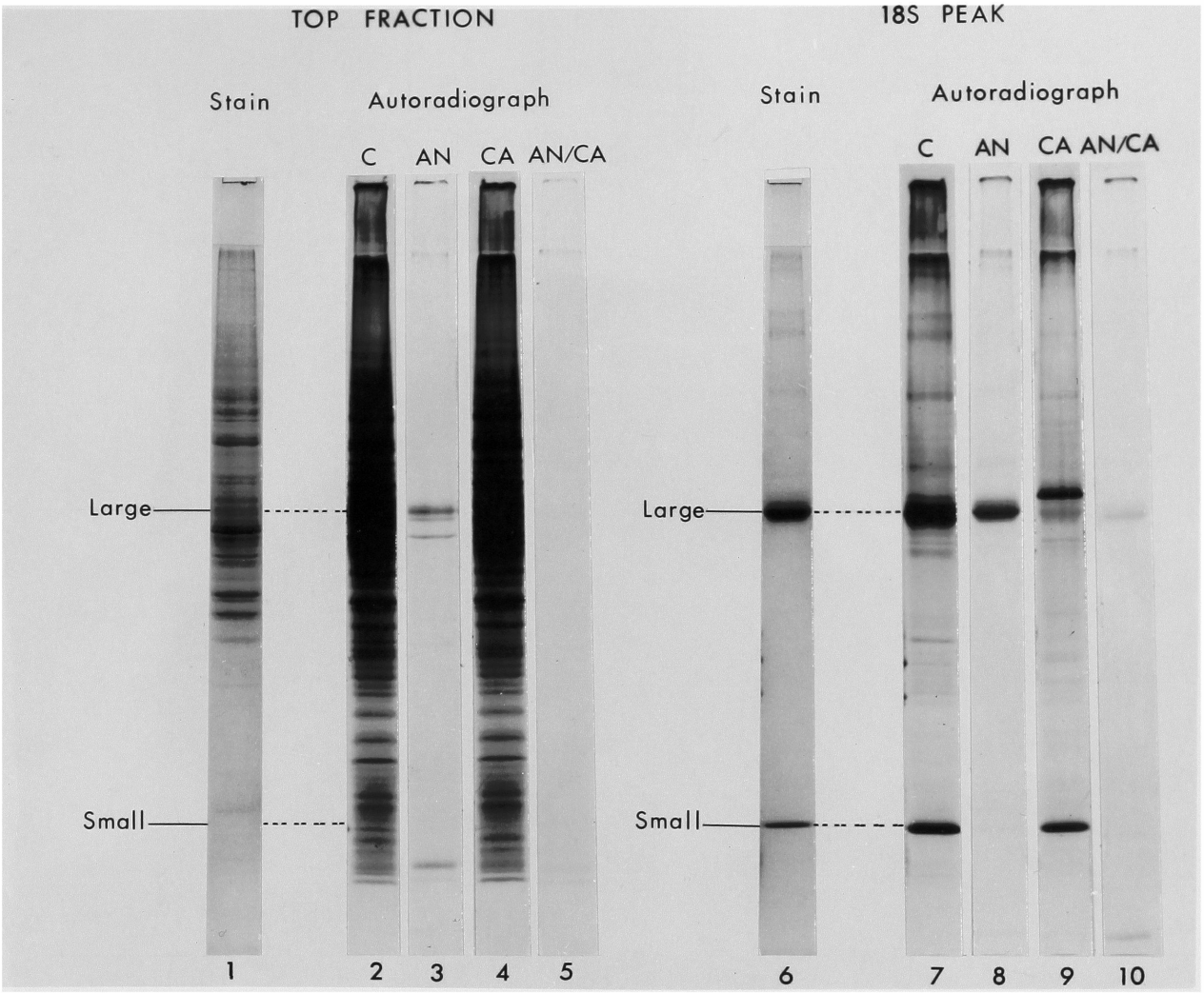


Figure 5. Analysis of soluble protein fractions from sucrose gradients. SDS/polyacrylamide gel electrophoresis: Laemmli 7.5 - 15% gradient.

Labeled protein fractions from the gradients depicted in Figure 4 were precipitated with TCA and prepared for electrophoresis in Na carbonate/SDS/DTT as described in Materials and Methods. Samples were electrophoresed on a Laemmli 7.5 - 15% gradient gel. Top fraction slots contained 60 µg protein each. Slots of 18S peak samples contained 20 µg protein each. Slots 1 and 6 show Coomassie blue stained polypeptides. Slots 2-5 and 7-10 are autoradiographs of [^{35}S]-labeled polypeptides. C, control (no inhibitors); AN, labeled in the presence of anisomycin; CA, labeled in the presence of chloramphenicol; AN/CA, labeled in the presence of both inhibitors.

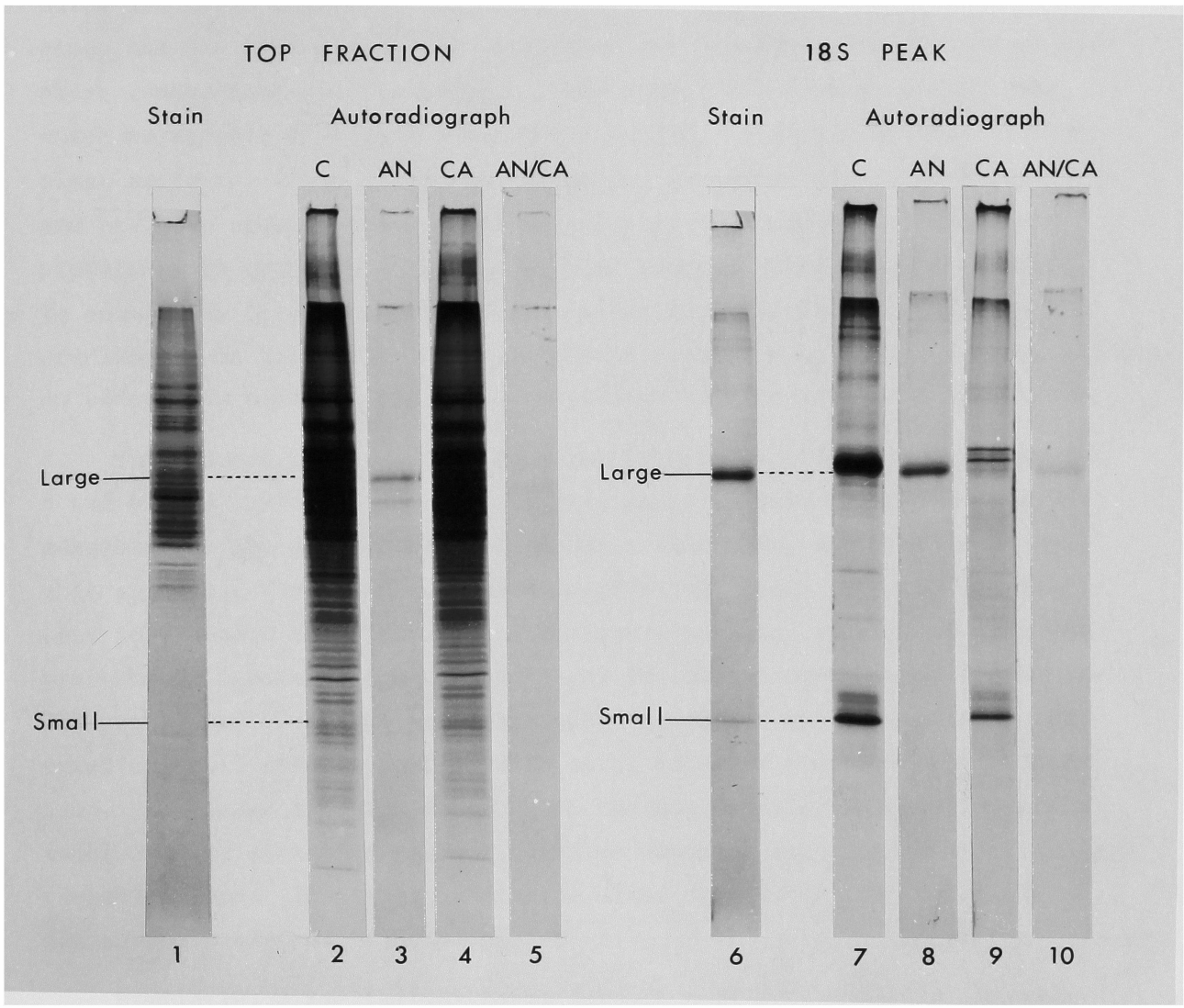


Figure 6. Analysis of soluble protein fractions from sucrose gradients. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

The same samples electrophoresed on the gel shown in Figure 5 were also run on a Laemmli 12 - 18% urea gel. All indications are identical to those used in Figure 5.

4.2. In Figure 5, slot 3, 4.1 and 4.2 comigrate as the band immediately below the large subunit. The third slightly labeled polypeptide in this group has not been previously identified and presumably is another chloroplast synthesized soluble protein. The very low molecular weight band, which is visible in Figure 5 but not Figure 6, is also a soluble chloroplast product. It is detectable in longer exposures of Figure 6 gels and has been seen in independent experiments in which the supernatant proteins were centrifuged over a sucrose cushion rather than a gradient. To summarize, three soluble proteins other than polypeptides 4.1 and 4.2 are labeled in the presence of anisomycin including carboxylase large subunit. As before, no bands in the top fraction are labeled with both inhibitors.

The sucrose gradient fractionation experiment illustrated in Figures 5 and 6 also indicates that carboxylase large subunit is synthesized and assembled in the absence of small subunit synthesis, and that large subunit synthesis continues even when insufficient small subunits are available to assemble the 18S form. The first conclusion is evident from the presence of labeled large subunit alone in the anisomysin sample from the 18S peak. Newly synthesized large subunits either assemble with a preexisting small subunit pool or with small subunits recycled from holoenzyme. The large subunits detected in the top fraction anisomycin sample (slot 3), may either be present as large subunits alone or as a partially assembled form. In either case it is clear that large subunit synthesis and assembly are not tightly coupled.

Similarly, a substantial large subunit pool must exist to assemble with newly made small subunits. In Figures 5 and 6, slot 9, labeled small subunit is present in an 18S form after large subunit synthesis has been blocked with chloramphenicol. Unfortunately, too many polypeptides are present in the chloramphenicol top fraction (slot 4) to allow detection of minute amounts of labeled small subunit or its precursor (Dobberstein et al., 1977). Therefore, it is impossible to determine from these experiments if small subunit synthesis continues when there are no large subunits to assemble with it.

Synthesis of Thylakoid Membrane Proteins in the Chloroplast

1. Site of synthesis of membrane proteins: Experiments on unextracted membranes

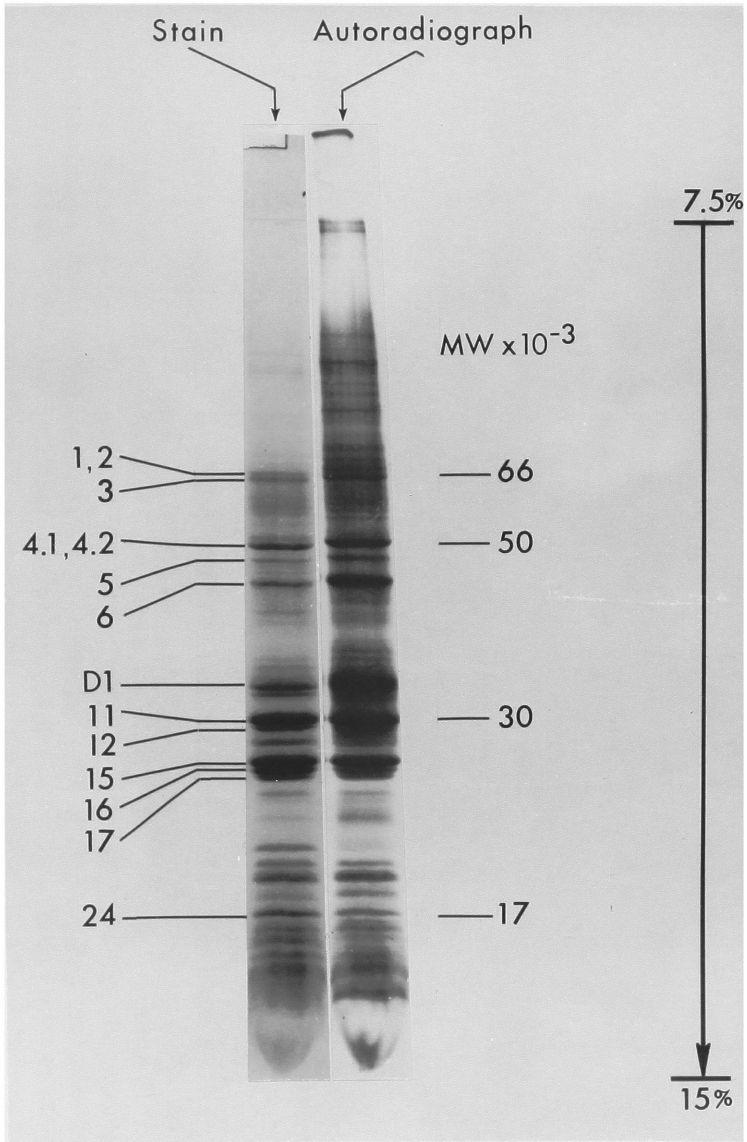
The site of Chlamydomonas thylakoid membrane proteins was examined using high specific activity sulfate labeling and gel electrophoresis. The nomenclature of Chlamydomonas thylakoid membrane polypeptides is shown in Figure 7. The electrophoretic profiles of sulfate labeled thylakoid membranes are illustrated in Figures 8 and 9. From a comparison of slots 1 and 2 in both Figures 8 and 9, it is evident that every band except polypeptide 24 is both stained and labeled. In addition, a number of polypeptides which are not stained are visible in the autoradiograph.

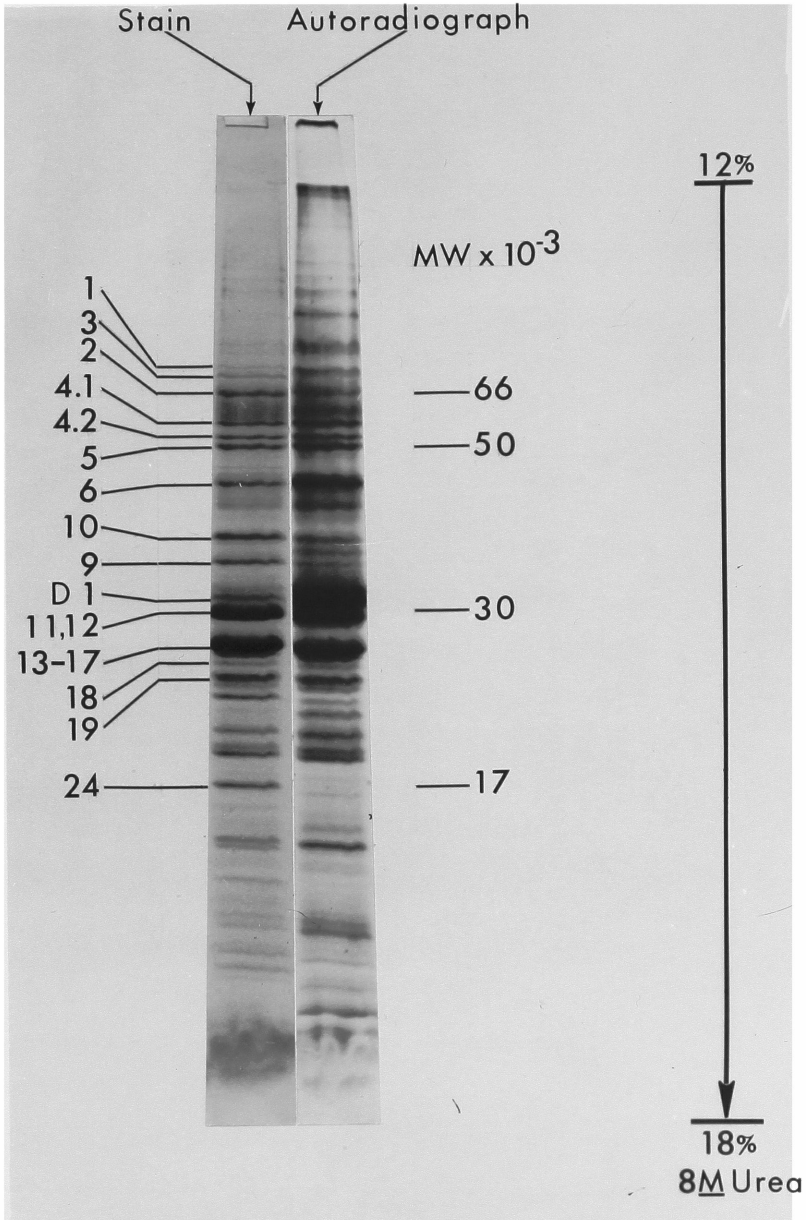
The basic incorporation pattern reported in an earlier study (Chua and Gillham, 1977) is reproduced in this experiment. Polypeptides 2, 4.1, 4.2, 5, 6, D1, and D2 are all labeled in the anisomycin and control samples but not in the presence of chloramphenicol or both inhibitors. Note that 4.1 and 4.2 are resolved only in Figure 9 and that D1 and D2 are resolved only in Figure 8. The small amount of darkening visible in the D1 region of slot 5 is due to incomplete chloramphenicol inhibition, a problem intrinsic to the antibiotic approach, and does not contradict the complementarity of the labeling pattern. Several less prominent polypeptides are also detectable in the anisomycin sample. Some of these are therefore synthesized in the chloroplast since they are not labeled in the presence of chloramphenicol. With others, the site of synthesis is less clear because the gel electrophoretic separation is insufficient to resolve the bands in question. However, all of the faint bands labeled in the presence of anisomycin are probably synthesized in the chloroplast since none is labeled with both anisomycin and chloramphenicol.

At this level of analysis, therefore, it is possible to conclude that polypeptides 2, 4.1, 4.2, 5, 6, D1, and D2 are synthesized on chloroplast ribosomes. Approximately six other polypeptides are labeled faint-

Figure 7. Analysis of thylakoid membranes by SDS/polyacrylamide gel electrophoresis.

Chlamydomonas was labeled with [^{35}S]-sulfate and thylakoid membranes purified as described in Materials and Methods. Membrane aliquots (20 µg chlorophyll) were prepared for electrophoresis (Table III) and loaded on either a 7.5 - 15% acrylamide Laemmli gel or a 12 - 18% acrylamide Laemmli gel supplemented with 8 M urea. Polypeptides were detected both by Coomassie blue staining and by autoradiography. Numbering of polypeptides is according to the nomenclature of Chua and Bennoun (1975a). Molecular weight estimates were made by comparison to protein standards.


Stain
Autoradiograph
7.5%
MW x10^{-3}
1,2
3
4.1,4.2
5
6
D1
11
12
15
16
17
24
66
50
30
17
15%


Stain
Autoradiograph
12%
MW x 10^{-3}
1
3
2
4.1
4.2
5
6
10
9
D 1
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19
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66
50
30
17
18%
8M Urea

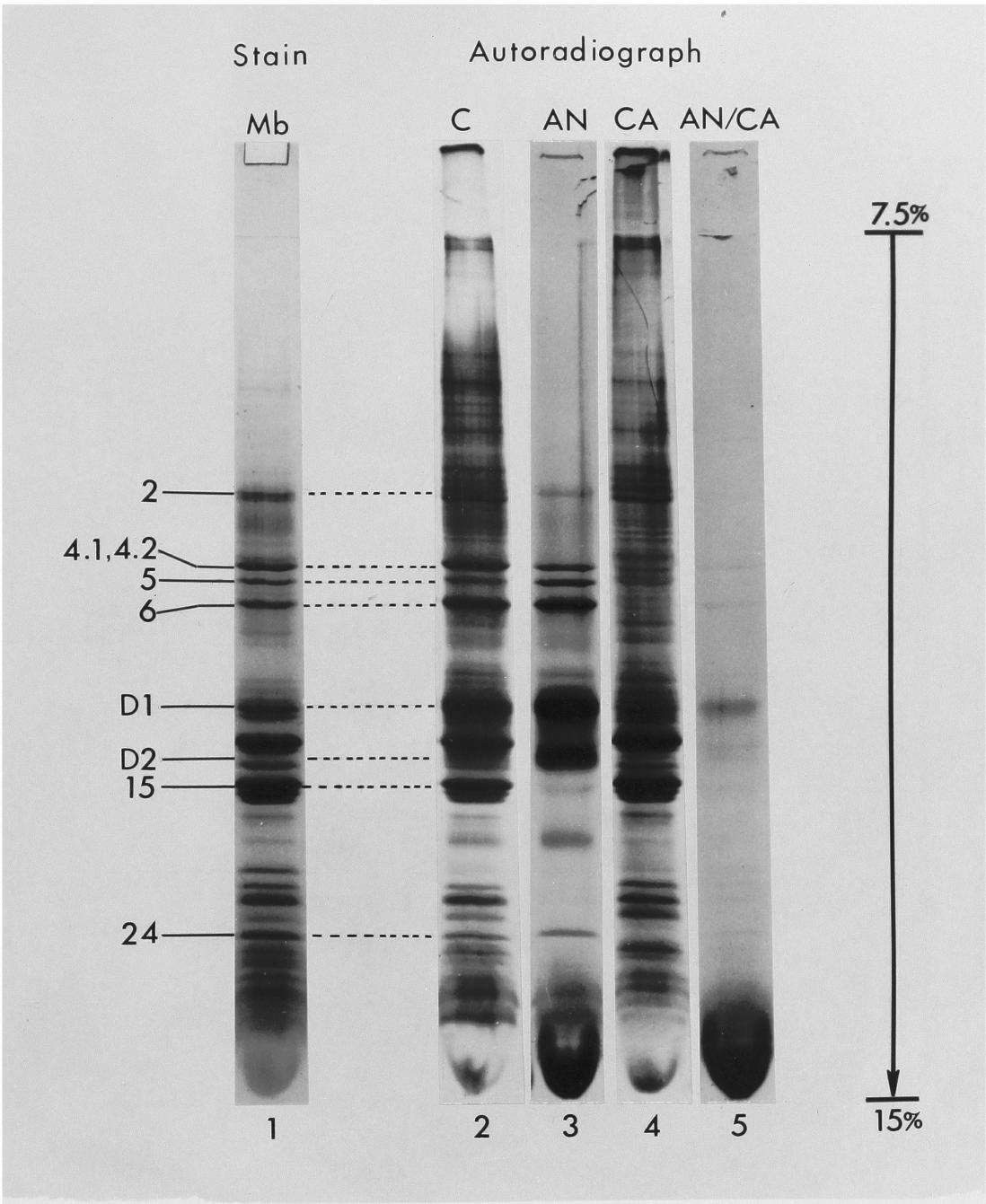


Figure 8. Analysis of thylakoid membranes labeled in the presence of inhibitors. SDS/polyacrylamide gel electrophoresis: Laemmli 7.5 - 15% gradient.

Chlamydomonas was labeled for 30 minutes with 0.1 mCi/ml [^{35}S]-sulfate. Purified thylakoid membrane samples were solubilized in Na carbonate/SDS/DTT (see Materials and Methods) and loaded on a Laemmli 7.5 - 15% gradient gel. The amount loaded in each slot was 20 µg chlorophyll. Polypeptides were detected both by Coomassie blue stain and autoradiography. Slot 1 is the stained pattern; slots 2-5 are the radioactive bands. Major polypeptides are numbered as in Figure 7. C, control; AN, anisomycin; CA, chloramphenicol; AN/CA, both inhibitors.

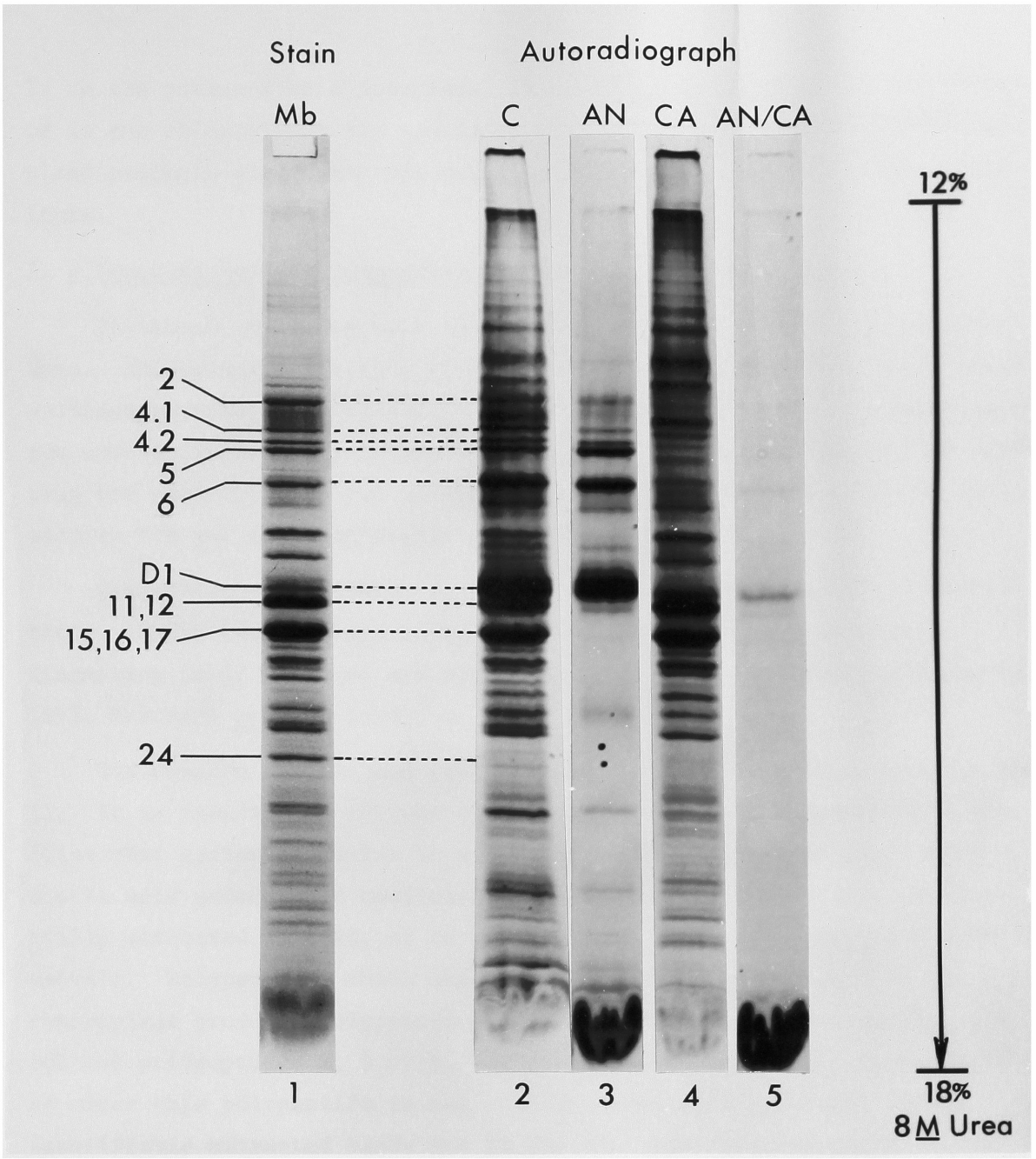


Figure 9. Analysis of thylakoid membranes labeled in the presence of inhibitors. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

The same samples shown in Figure 8 were also run on a Laemmli 12 - 18% urea gel. All designations are identical to those in Figure 8.

ly in the presence of anisomycin. Three of these are clearly synthesized in the chloroplast; the remainder are tentatively designated chloroplast products since they are not labeled in the presence of both inhibitors.

2. Effects of various extraction agents on thylakoid membranes

Thylakoid membranes were extracted with acid and base and denaturants. The primary objective of these extractions was to determine which membrane proteins were integral and which peripheral. The second objective was to simplify the membrane protein electrophoretic profile by splitting the proteins into two groups; this essentially added a second dimension to the gel electrophoretic analysis.

Extraction of thylakoid membranes with acid and base was attempted first. These reagents are believed to remove peripheral proteins by disrupting ionic linkages and unfolding polypeptide chains (Steck and Yu, 1973; Kreibich et al., 1978).

The results of acid and base extractions are shown in Figures 10 and 11. It is immediately obvious from an examination of these stained profiles that sodium hydroxide is a more effective extraction agent than acetic acid under these conditions. While only 4 polypeptides are partially extracted by acid, up to 17 bands are present in the 0.1 N NaOH extract. Polypeptides which are extracted by 0.1 N NaOH include the chloroplast products polypeptides 4.1 and 4.2 (not resolved in Fig. 10) but not polypeptides 2, 5 or 6. It is not known whether D1 is extracted since this polypeptide is not readily detectable by stain. Other identifiable extracted bands are 12 and 24. Low ionic strength buffer removes no detectable protein from the membrane (slots 1 and 2).

In Figures 12 and 13, the results of extraction with water, 6 M urea, and 6 M guanidine are illustrated. Both urea and guanidine remove peripheral proteins by denaturing them, thereby disturbing their normal interaction with the membrane surface (Steck and Yu, 1973). Water was used since low ionic strength is known to extract chloroplast coupling factor from higher plant thylakoids (Strotmann et al., 1973). Water removes a

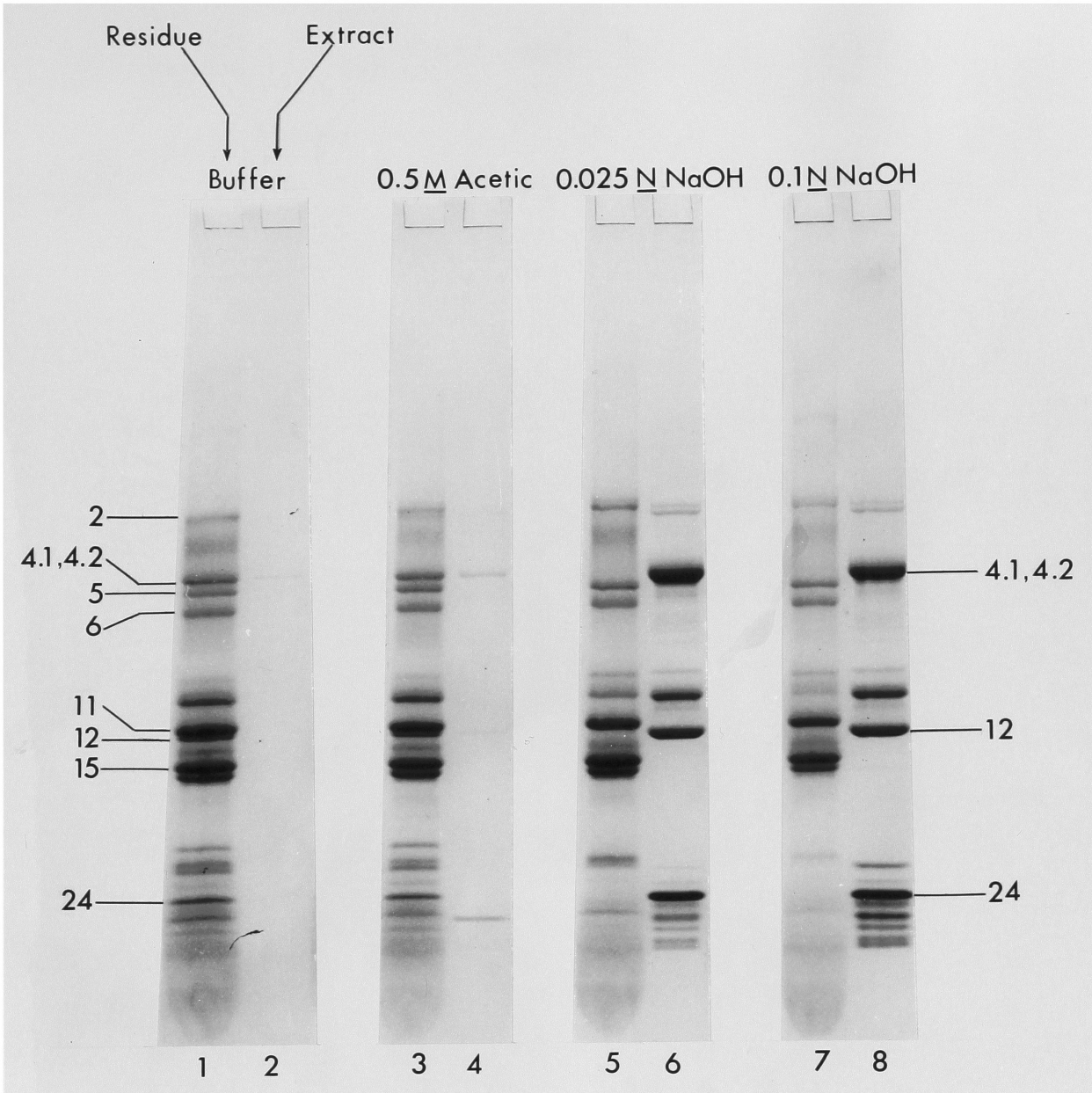


Figure 10. Analysis of fractions derived from acid and base extraction of thylakoid membranes. SDS/polyacrylamide gel electrophoresis: Neville 7.5 - 15% gradient.

Purified thylakoid membranes (0.5 mg chlorophyll per aliquot) were extracted for 30 minutes on ice with either 5 mM TES-NaOH (pH 7.5), 0.5 M acetic acid, 0.025 N NaOH, or 0.1 N NaOH and centrifuged at 233,000 x g_{max} for 1 hour. Residues were solubilized in 500 µl Na carbonate/SDS/DTT as indicated in Materials and Methods assuming that each sample still contained 0.5 mg chlorophyll. Extracts were dialyzed, precipitated with TCA, and finally solubilized in 100 µl Na carbonate/SDS/DTT. Extracts and residues (20 µl per slot) were loaded on a Neville 7.5 - 15% gradient gel. Polypeptides were visualized by Coomassie blue staining. Because of the sample preparation scheme, extracts are concentrated five-fold over residues. Bands are numbered according to the nomenclature of Chua and Bennoun (1975a).

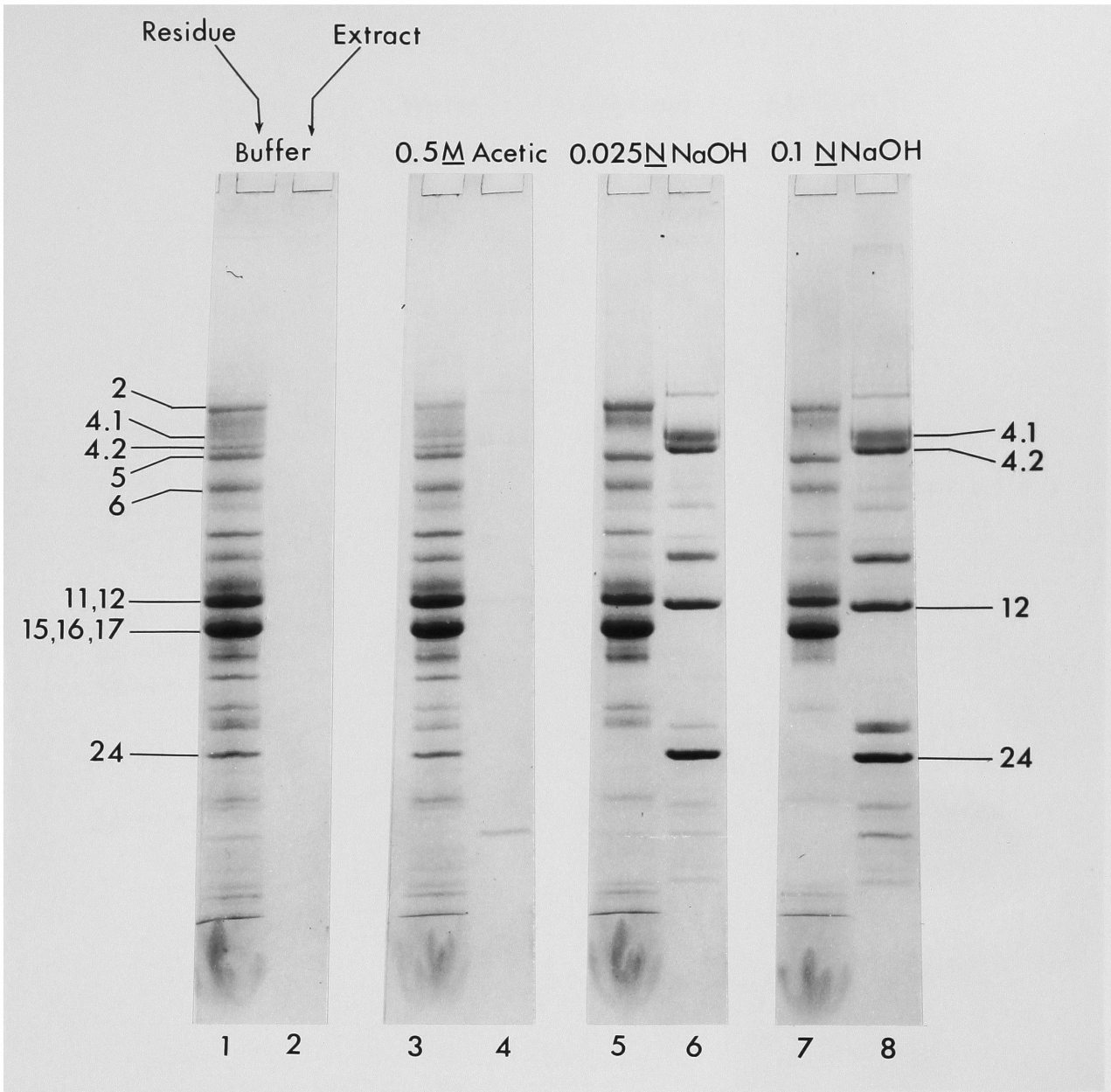


Figure 11. Analysis of fractions derived from acid and base extractions of thylakoid membranes. SDS/polyacrylamide gel electrophoresis: Neville 12 - 18% gradient, 8 M urea.

The same samples electrophoresed on the gel shown in Figure 10 were also run on a Neville 12 - 18% urea gel. Details of sample preparation and analysis may be found in the Figure 10 legend.

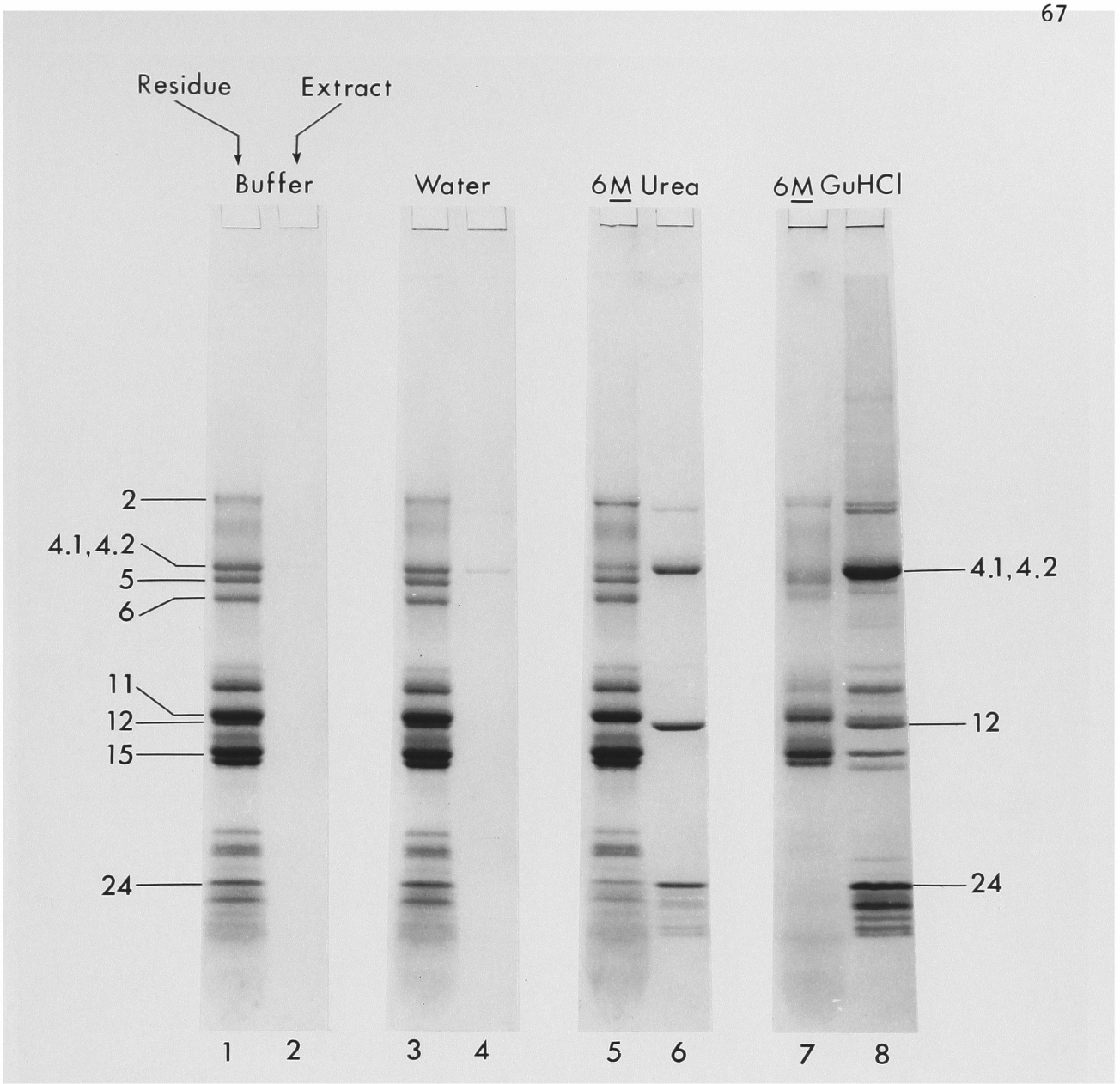


Figure 12. Analysis of fractions derived from water, urea, and guanidine extraction of thylakoid membranes. SDS/polyacrylamide gel electrophoresis: Neville 7.5 - 15% gradient.

Purified thylakoid membranes (0.5 mg chlorophyll per aliquot) were extracted for 24 hours on ice with either 5 mM TES-NaOH (pH 7.5), water, 6 M urea, or 6 M guanidine hydrochloride. The suspensions were centrifuged for 1 hour at 233,000 x g_{max}. Membrane residues (pellets) were solubilized in 500 µl Na carbonate/SDS/DTT assuming that each sample still contained 0.5 mg chlorophyll. Extracts (supernatants) were dialyzed, precipitated with TCA, and solubilized in 100 µl Na carbonate/SDS/DTT. Aliquots of 20 µl were loaded on a Neville 7.5 - 15% gradient gel. Bands were visualized by Coomassie blue staining. Because of the sample preparation scheme, extracts are concentrated five-fold over residues. Polypeptides are numbered according to the nomenclature of Chua and Bennoun (1975a).

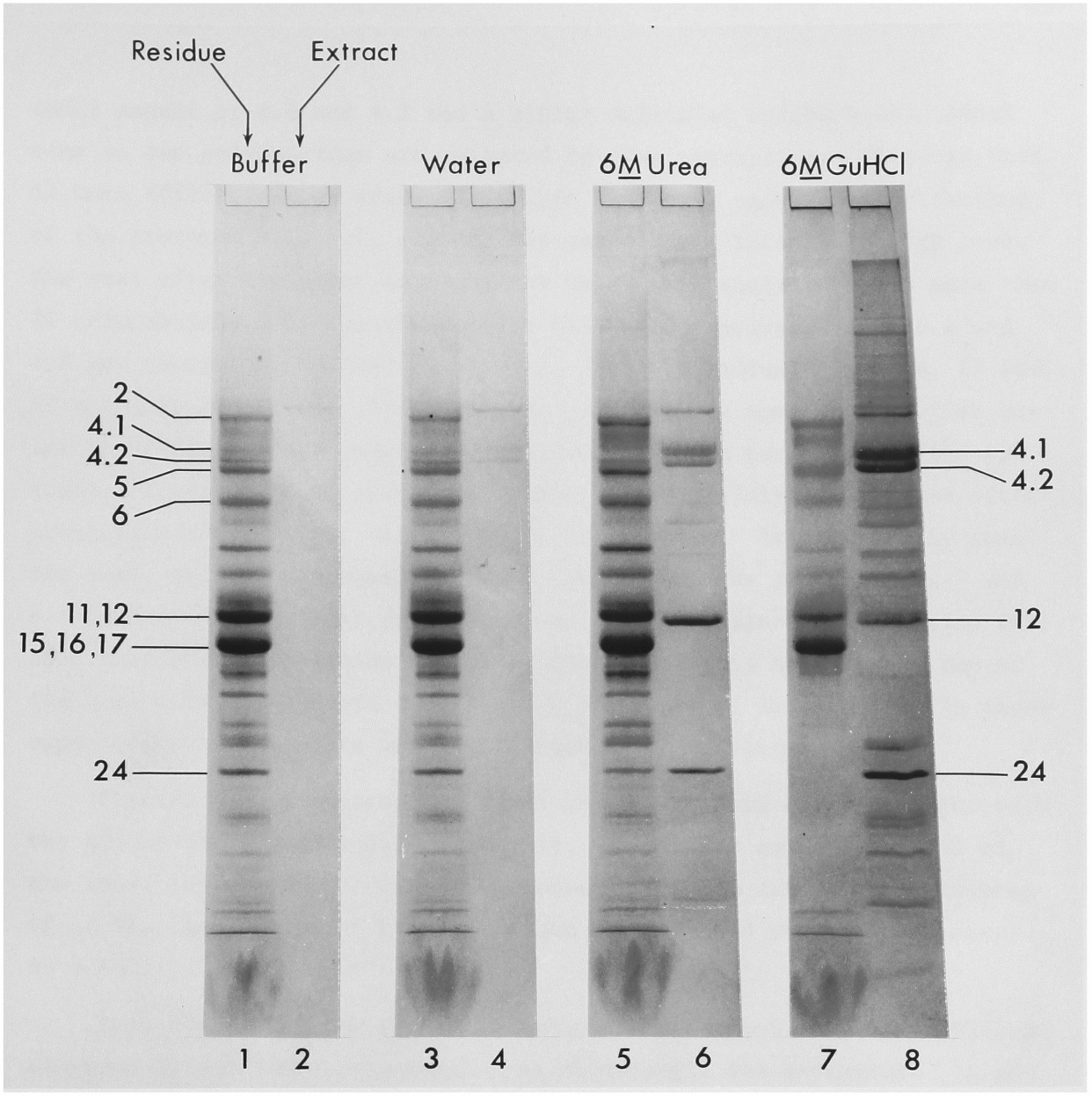


Figure 13. Analysis of fractions derived from water, urea, and guanidine extraction of thylakoid membranes. SDS/polyacrylamide gel electrophoresis: Neville 12 - 18% gradient, 8 M urea.

The same samples shown in Figure 12 were also electrophoresed on a Neville 12 - 18% urea gel. Experimental details may be found in the legend to Figure 12.

small amount of 4.1 and 4.2 and a higher molecular weight band. About nine to ten polypeptides are released by urea extraction. Although urea is more effective than acid, it is less effective than base; extraction of the proteins 4.1, 4.2, and 24, for example, is incomplete with urea. The most effective agent is guanidine which completely extracts more than 20 polypeptides. Of the chloroplast synthesized polypeptides, 4.1 and 4.2 are extracted, but not 2, 5, or 6. As with sodium hydroxide, 12 and 24 are also extracted. Three or four polypeptides that migrate just below 15, 16 and 17 are removed by guanidine but not recovered in the extract. It is possible that these proteins bind avidly to surfaces after extraction and are lost during sample preparation. It is unlikely that the small amount of stained material retained at the top of slots 7 and 8 (Figures 12 and 13) is from these lost proteins since the proteins are also lost after extraction with base and no stain is seen at the top of the base extraction slots (see slots 7 and 8, Figs. 10 and 11). In these experiments buffer again was ineffective as an extraction agent.

Protein determinations performed on the extracts are consistent with the gel electrophoresis data (Table V). Guanidine extracted 20.7% of the total thylakoid proteins while sodium hydroxide extracted an average of 18.9%. Recoveries of protein in the extracts and residues was about 80 - 85%.

Even though a large amount of protein was removed from the thylakoid membrane by guanidine, essentially no chlorophyll was extracted. In addition chlorophyll-protein complexes were intact after guanidine extrations as judged by lithium dodecyl sulfate electrophoresis at $4^{o}C$ (Delepelaire and Chua, 1979). No chlorophyll determinations were performed on extracts from acid and base extraction. Visually, however, it appeared that these agents extracted no chlorophyll.

3. Site of synthesis of membrane proteins: Experiments on guanidine extracted membrane

The site of synthesis question was re-examined using labeled membranes fractionated by guanidine extraction. Extracts are illustrated in Figures 14 and 15; residues are shown

TABLE V

PROTEIN EXTRACTED FROM THYLAKOID MEMBRANES BY VARIOUS AGENTS

A. Acid and Base Extractions (30 minutes)

	% Extracted
Control (5 mM TES-NaOH, pH 7.5)	0.4
0.5 M Acetic Acid	3.7
0.025 M NaOH	17.7
0.1 M NaOH	18.9

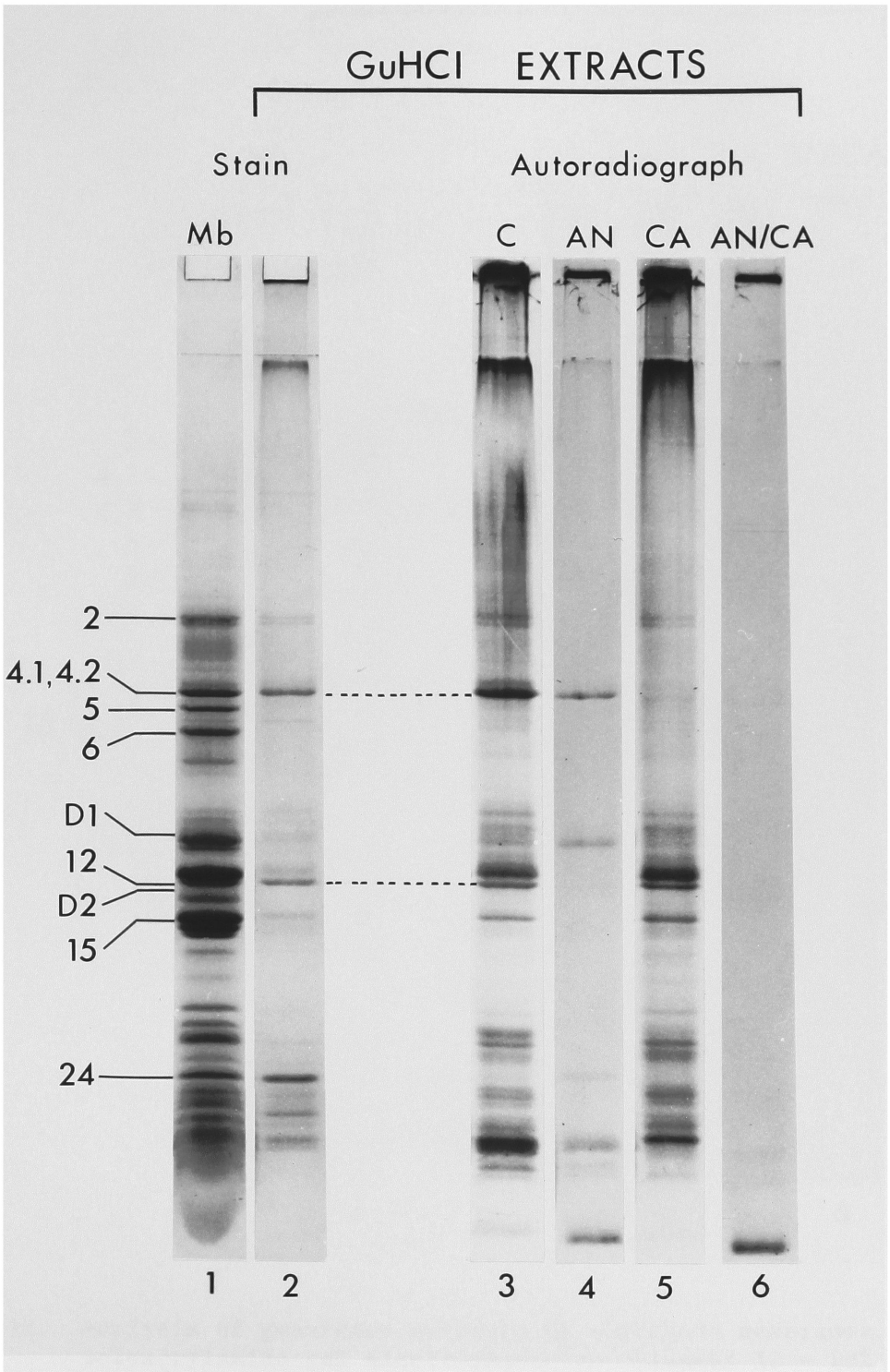
B. Water, Urea, and Guanidine Extraction (24 hours)

	% Extracted
Control (5 mM TES-NaOH, pH 7.5)	0.7
Water	1.2
6 M Urea	9.1
6 M Guanidine-HCl	20.7

Values in A and B are expressed as % of control value (extracted + unextracted).

Figure 14. Analysis of guanidine extracts of thylakoid membranes. SDS/ polyacrylamide gel electrophoresis: Laemmli 7.5 - 15% gradient.

Membranes labeled and purified for the experiment described in Figure 8 were extracted for 24 hours on ice with 6 M guanidine hydrochloride and centrifuged for 1 hour at 233,000 x g_{max}. The extracts (supernatants) were dialyzed, TCA-precipitated, and dissolved in 250 µl Na carbonate/SDS/DTT. Membrane residues (pellets) were dissolved in 500 µl Na carbonate/SDS/DTT assuming a chlorophyll content of 0.5 mg. Samples (20 µl per slot) were electrophoresed on a Laemmli 7.5 - 15% gradient gel and polypeptides detected by Coomassie blue staining and autoradiography. Only the guanidine extracts are shown here; residues are depicted in Figures 16 and 17. Slot 2 shows the polypeptide pattern of the stained extract; slots 3-6 are autoradiographs of samples labeled in no inhibitors (C), anisomycin (AN), chloramphenicol (CA), and both inhibitors (AN/CA). For comparison, the stained polypeptide profile of an unextracted thylakoid membrane preparation is shown in slot 1. Polypeptides are numbered as in Figure 7.


GuHCl EXTRACTS
Stain
Autoradiograph
Mb
C
AN
CA
AN/CA
2
4.1,4.2
5
6
D1
12
D2
15
24
1
2
3
4
5
6

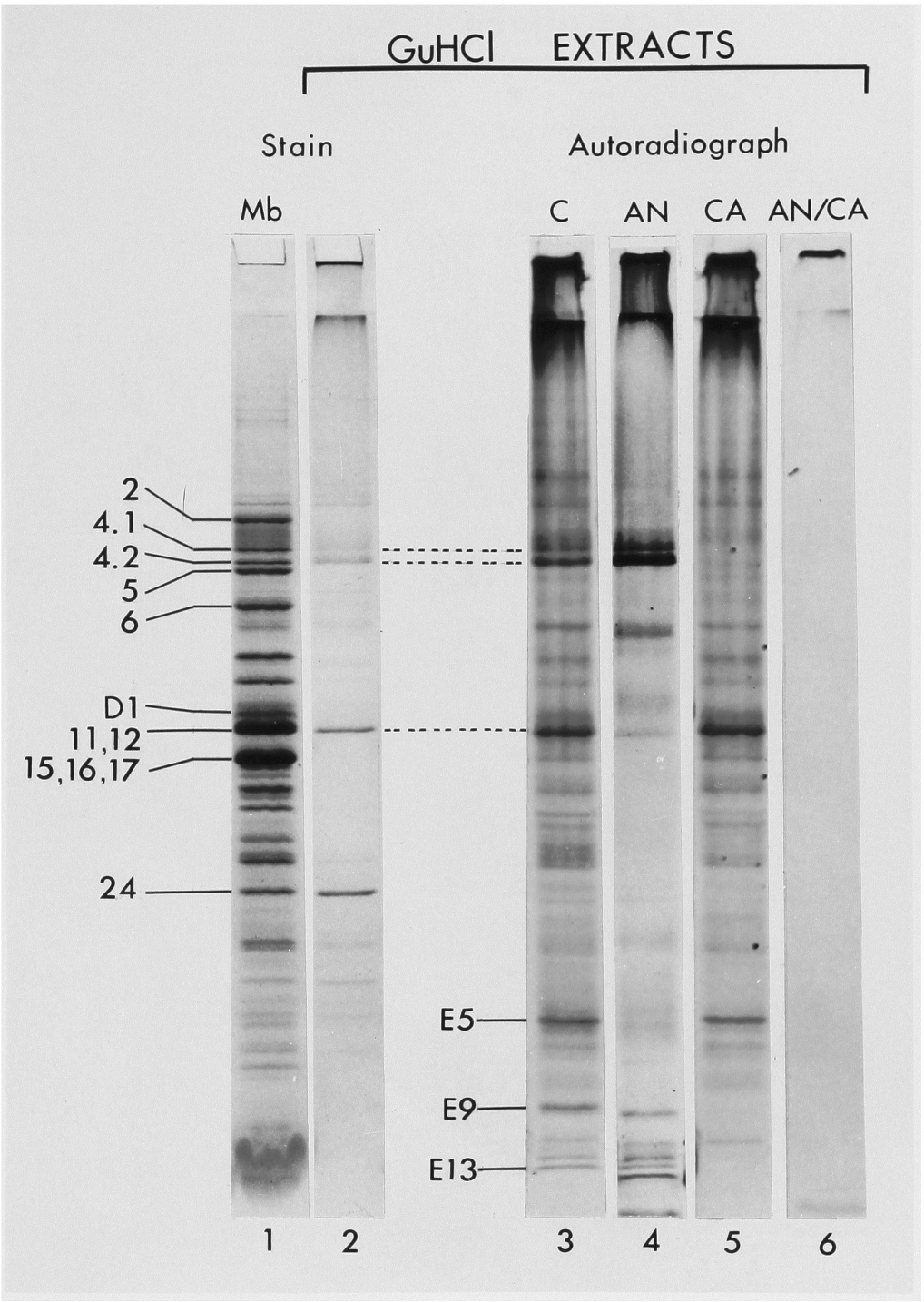


Figure 15. Analysis of guanidine extracts of thylakoid membranes. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

Membrane extracts described in Figure 14 were also run on a Laemmli 12 - 18% urea gel. All designations are the same except for polypeptides numbered with the "E" prefix. "E" refers to minor polypeptides of molecular weight less than 17,000 that are detected in the guanidine extracts. See also Figure 18.

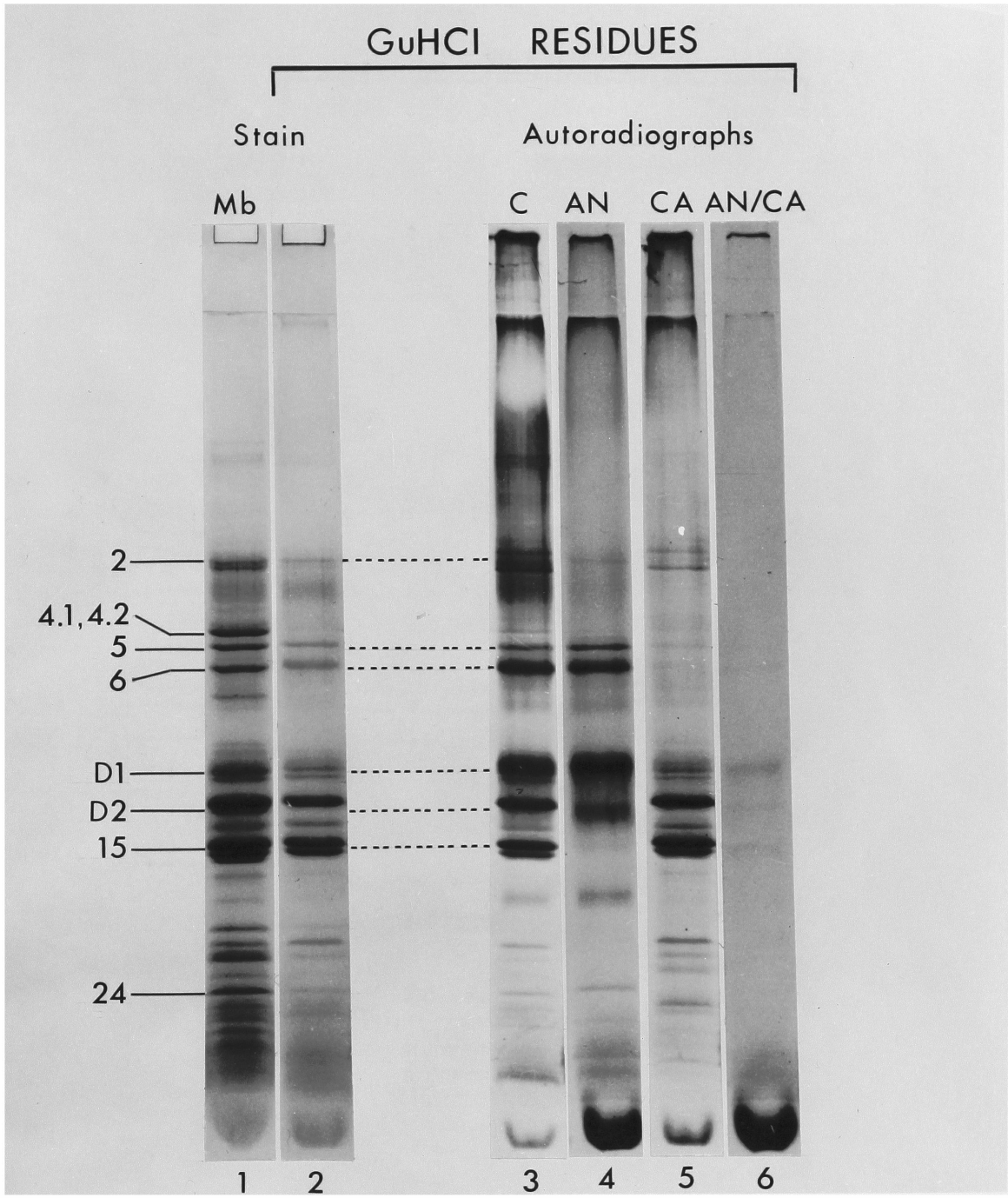


Figure 16. Analysis of guanidine residues of thylakoid membranes. SDS/ polyacrylamide gel electrophoresis: Laemmli 7.5 - 15% gradient.

Membranes were labeled, purified, and extracted with 6 M guanidine hydrochloride as described earlier (see Fig. 14 and Materials and Methods). Samples (20 µl per slot) were electrophoresed on a Laemmli 7.5 - 15% gradient gel. Slot 2 is the stained polypeptide pattern of the membrane residue: slots 3-6 are autoradiographs. For reference, the stained polypeptide profile of an unextracted membrane is shown in slot 1. Band numbering is the same as Figure 7. C, control; AN, anisomycin; CA, chloramphenicol; AN/CA, both inhibitors.

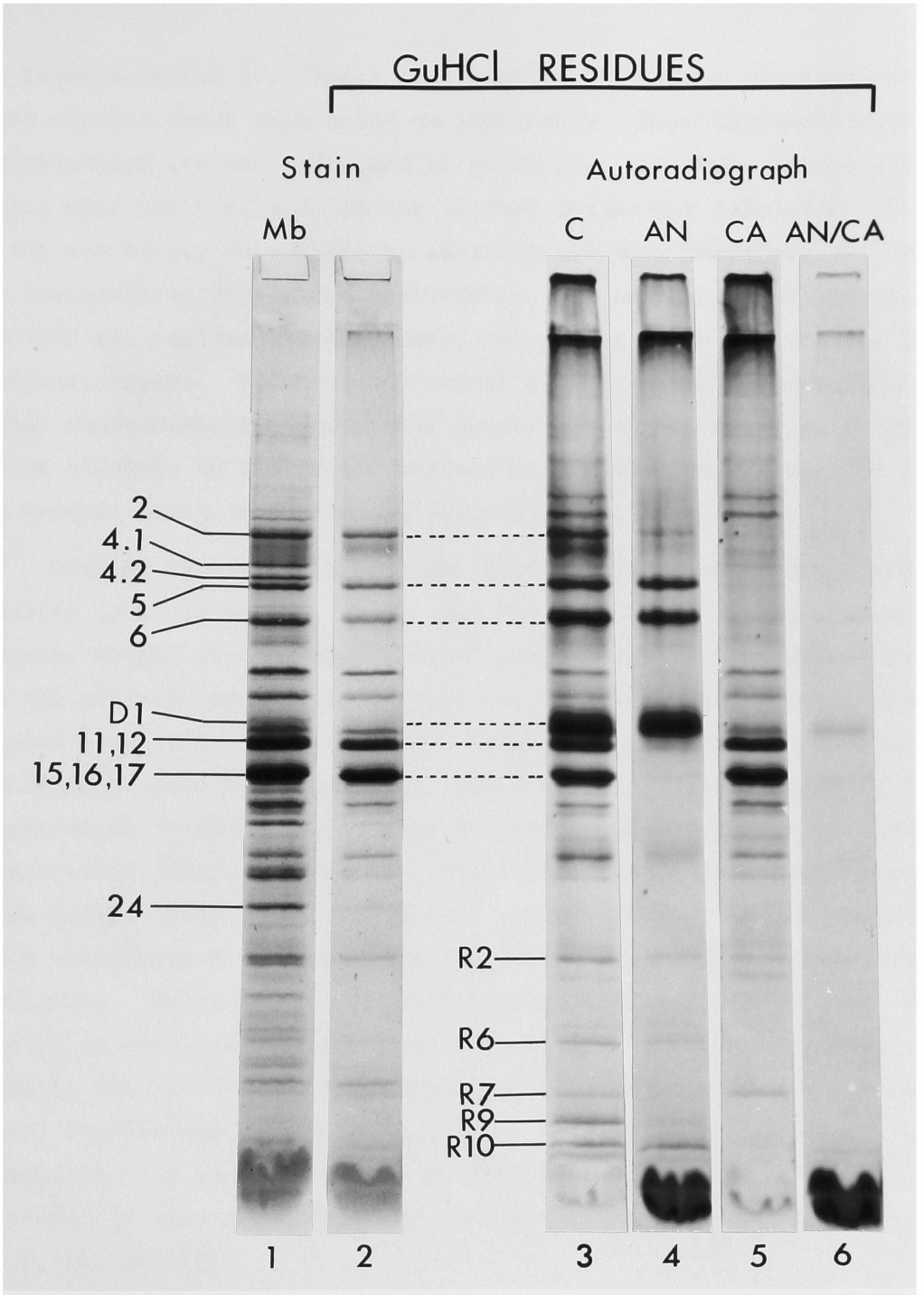


Figure 17. Analysis of guanidine residues of thylakoid membranes. SDS/ polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

Residues shown in Figure 16 were also run on a Laemmli 12 - 18% urea gel. Bands numbered with the prefix "R" are polypeptides of molecular weight less than 17,000 that are detected in guanidine residues. All other designations are identical to Figure 16. See also Figure 19.

in Figures 16 and 17. These data confirm and extend the previous extraction results which were based on stain only. Most thylakoid membrane polypeptides are not extracted by guanidine. Of those extracted, the major ones are 4.1, 4.2, 12 and 24 (the latter not labeled). D1 and D2, which are barely detectable by staining and were therefore not examined in the previous extraction experiments, are not removed by guanidine. As with the earlier labeling data, many minor bands are visible in the autoradiographs. Side-by-side comparison of extracts and residues in other experiments indicates that nearly all of the minor polypeptides occur uniquely in either the extract or residue and are not the result of partial extraction or cross contamination.

Besides the major chloroplast synthesized polypeptides discussed earlier (2, 4.1, 4.2, 5, 6, D1, and D2) an additional four bands of molecular weight greater than that of polypeptide 24 are labeled faintly in the presence of anisomycin. If the polypeptides are designated a, b, c, and d in order of decreasing molecular weight, then a is found just below 6, b above D1, c below D1, and d as a fuzzy band slightly above the position of 24 in Figure 9, slot 3. Polypeptides a and c are guanidine extractable (Fig. 15, slot 4). Their identification as chloroplast products is tentative; even though they are not labeled in the presence of both anisomycin and chloramphenicol, their labeling in chloramphenicol is ambiguous. Polypeptide b is lost upon guanidine extraction but is soluble in chloroform/methanol (data not shown). In Figures 8 and 9, b is clearly not labeled in chloramphenicol or both inhibitors. It is, therefore, synthesized in the chloroplast. Polypeptide d is not extracted by guanidine. It is definitely a chloroplast product since its labeling patterns in chloramphenicol and anisomycin are complementary (see Figs. 8, 9, 16, and 17).

A particularly striking result of guanidine extraction of labeled membranes is the resolution of the low-molecular weight region below polypeptide 24 (est. molecular weight 17,000). Without extraction, approximately twenty labeled bands are resolved, making this region too complex to analyze unambiguously (Fig. 9). In addition, the very-low molecular

weight range is obscured by free chlorophyll and other lipids released from the thylakoids by SDS. Guanidine extracts approximately one-half of the low molecular weight proteins, greatly simplifying the electrophoretic profile. If long exposures of the labeled gels shown in Figures 15 and 17 are examined in detail (Figs. 18 and 19), a total of twenty-four polypeptides are visible in the region below polypeptide 24. Eleven of these are not removed by guanidine (R1-R11); thirteen are found in the guanidine extract (E1-E13). Polypeptides E10 - E13 were formerly hidden by the free chlorophyll zone in the gel. About eight residue proteins (R2, R4-R6, R8-R11) and four to five extract proteins (E9, E10?, E11-E13) are synthesized in the chloroplast since they are labeled in control and anisomycin samples but not in the presence of chloramphenicol or both inhibitors.

To determine if additional very low molecular weight polypeptides were concealed by the pigment zone in the guanidine residues, labeled membranes were extracted with chloroform/methanol and the dried extract washed with ether to remove pigments. This procedure eliminates all pigment without loss of membrane proteins. Both the chloroform/methanol precipitate and ether extracted fraction were examined by gel electrophoresis. No additional very low molecular weight polypeptides were observed (data not shown).

4. Proteolytic digestion of thylakoid membranes labeled in the presence of anisomycin

If intact membrane vesicles are incubated with proteases, only those proteins exposed to the outside are digested. Experiments of this type were performed with thylakoid membranes labeled in the presence of anisomycin to examine the accessibility of chloroplast synthesized polypeptides. Results of papain, trypsin, and thermolysin digestion are shown in Figures 20 - 22.

At low concentrations papain has no effect on chloroplast membrane proteins. Digestion with 5.0 µg/ml papain, however, slightly reduces the amount of polypeptide 2 and appears to shift polypeptide 5 to a faster moving form (Fig. 20, slot 4). With additional papain (slot 5), 2 is re-

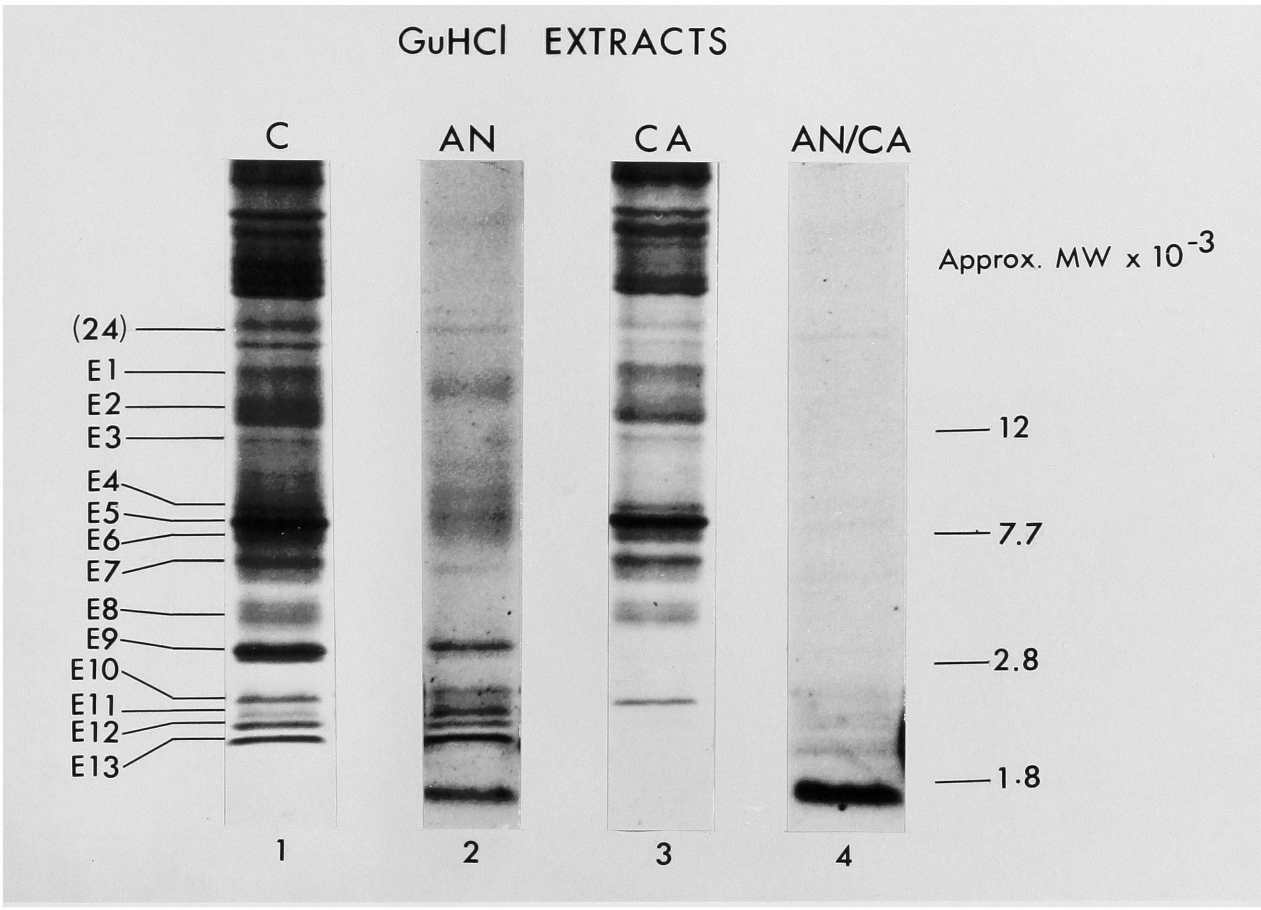


Figure 18. Analysis of guanidine extracts of thylakoid membranes. Low-molecular weight region of Figure 15. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

The gel shown in Figure 15 was exposed for a longer time to enhance detection of faint polypeptides. Slots 1-4 correspond to slots 3-6 in Figure 15. E1-E13 are polypeptides found in guanidine extracts. The position of polypeptide 24 corresponds to an apparent molecular weight of 17,000. Other molecular weight estimates were made by electrophoresis of cytochrome c CNBr fragment and are tentative.

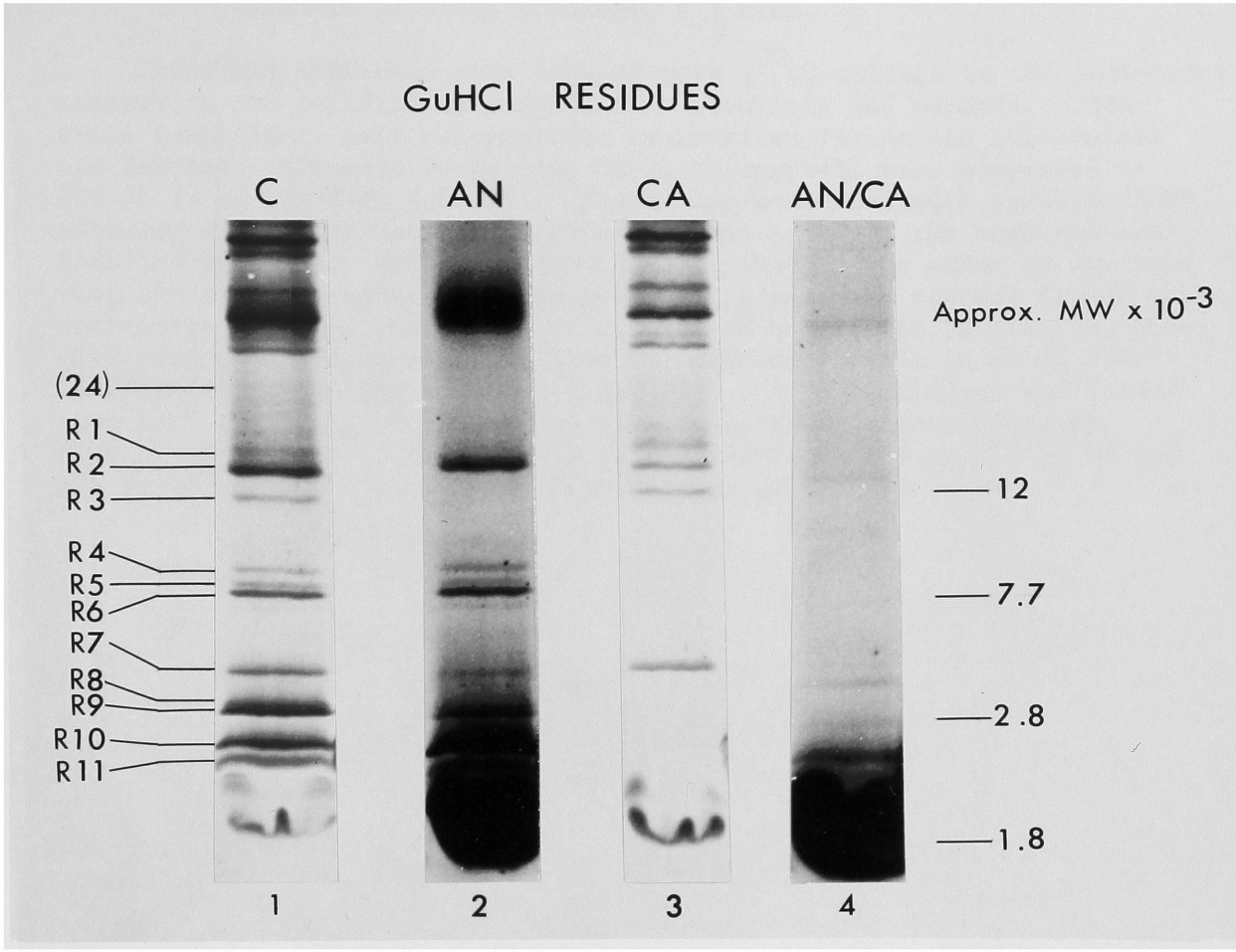


Figure 19. Analysis of guanidine residues of thylakoid membranes. Low-molecular weight region of Figure 17. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

The gel shown in Figure 17 was exposed for a longer time to enhance detection of faint polypeptides. Slots 1-4 correspond to slots 3-6 in Figure 17. R1-R11 are polypeptides found in guanidine residues. The position of polypeptide 24 corresponds to an apparent molecular weight of 17,000. Other molecular weight estimates were made by electrophoresis of cytochrome c CNBr fragments and are tentative.

Figure 20. Papain digestion of thylakoid membranes labeled in the presence of anisomycin. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

Thylakoid membranes were labeled with [^{35}S]-sulfate in the presence of anisomycin and purified as described in Materials and Methods. Under these conditions, only polypeptides synthesized inside the chloroplast are labeled. Aliquots containing 40 µg chlorophyll were suspended in 180 µl 25 mM TES-NaOH (pH 7.5). Papain was activated with cysteine/EDTA, serially diluted in activation solution, and added to the membrane samples (20 µl each). Nikkol (a non-ionic detergent) was added to one tube with the highest protease concentration as a positive control (final concentration ∿0.25%). Membranes were digested on ice for 30 minutes, precipitated with TCA, and solubilized for electrophoresis in 65 µl Tris base/SDS/DTT/iodoacetamide (see Table III). A 25 µl aliquot was loaded into each gel slot. Polypeptides were detected by autoradiography. Slot 1, no papain; 2, 0.05 µg/ml, 3, 0.5 µg/ml; 4, 5.0 µg/ml; 5, 50 µg/ml; 6, 50 µg/ml + Nikkol. See also Figures 20 and 21.

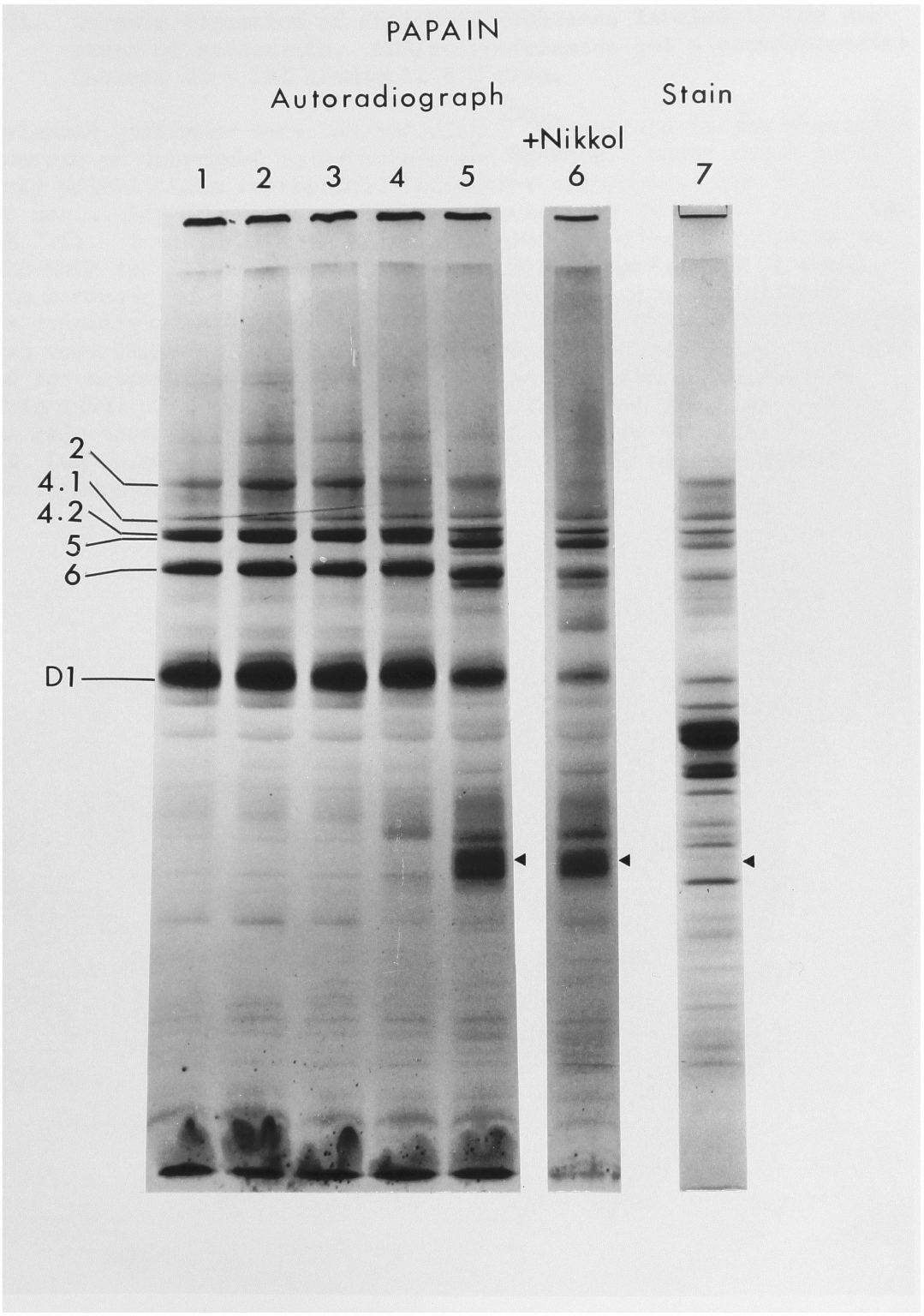

PAPAIN
Autoradiograph
Stain
+Nikkol
1
2
3
4
5
6
7
2
4.1
4.2
5
6
D1

Figure 21. Trypsin digestion of thylakoid membranes labeled in the presence of anisomycin. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

Thylakoid membranes were labeled with [^{35}S]-sulfate in the presence of anisomycin as described in Materials and Methods. Under these conditions only polypeptides synthesized inside the chloroplast are labeled. Aliquots containing 40 µg chlorophyll were suspended in 180 µl 25 mM TES-NaOH (pH 7.5). Trypsin-TPCK (1 mg/ml in water) was diluted serially in 25 mM TES-NaOH (pH 7.5) and added to the membrane samples (20 µl each). Nikkol (a nonionic detergent) was added to one tube with the highest protease concentration as a positive control (final concentration ∿0.25%). Membranes were digested on ice for 30 minutes, TCA precipitated, and solubilized for electrophoresis in 65 µl Tris base/SDS/DTT/iodoacetamide (see Table III). A 25 µl aliquot was loaded into each gel slot. Polypeptides were detected by autoradiography. Slot 1, no trypsin; 2, 0.05 µg/ml; 3, 0.5 µg/ml; 4, 5.0 µg/ml; 5, 50 µg/ml; 6, 50 µg/ml + Nikkol. See also Figures 20 and 22.

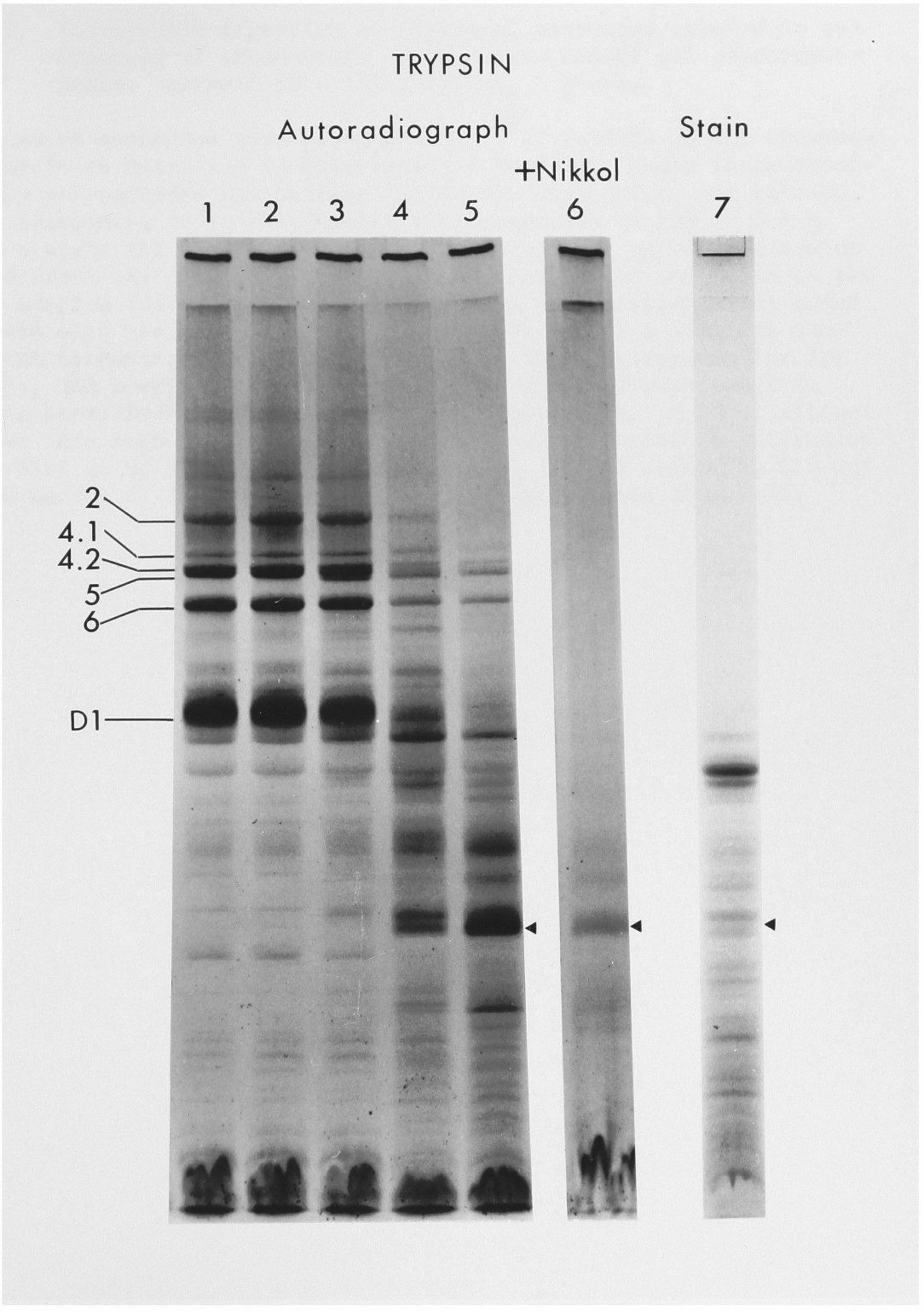
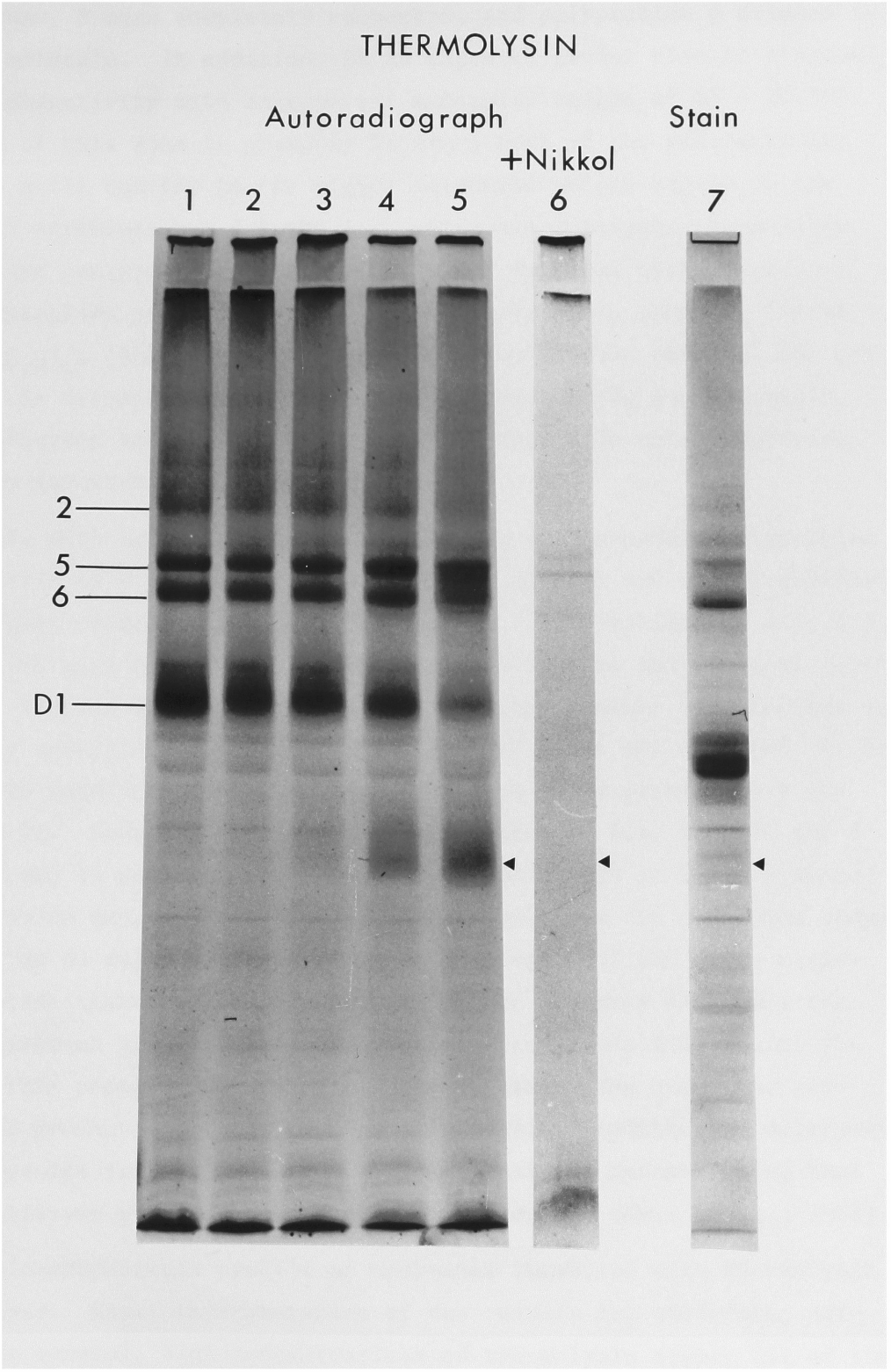

TRYPSIN
Autoradiograph
Stain
+Nikkol
1
2
3
4
5
6
7
2
4.1
4.2
5
6
D1

Figure 22. Thermolysin digestion of thylakoid membranes labeled in the presence of anisomycin. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

Thylakoid membranes were labeled with [^{35}S]-sulfate in the presence of anisomycin as described in Materials and Methods. Under these conditions only polypeptides synthesized inside the chloroplast are labeled. Aliquots containing 40 µg chlorophyll were suspended in 180 µl 0.1 M ammonium acetate (pH 8.5)/5 mM $CaCl_2$. Thermolysin (1 mg/ml in ammonium acetate buffer) was diluted serially in the same buffer and added to the membrane samples (20 µl each). Nikkol (a nonionic detergent) was added to one tube with the highest protease concentration as a positive control (final concentration ∿0.25%). Membranes were digested on ice for 30 minutes, TCA precipitated, and solubilized for electrophoresis in 65 µl Tris base/SDS/DTT/iodoacetamide (see Table III). A 25 µl aliquot was loaded into each gel slot. Polypeptides were detected by autoradiography. Slot 1, no thermolysin; 2, 0.05 µg/ml; 3, 0.5 µg/ml; 4, 5.0 µg/ml; 5, 50 µg/ml; 6, 50 µg/ml + Nikkol. See also Figures 20 and 21.


THERMOLYSIN
Autoradiograph
Stain
+Nikkol
1
2
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4
5
6
7
2
5
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D1

duced further, 5 more completely converted, and polypeptide 6 clipped to a smaller molecule. In addition, D1 is digested giving rise to a broad zone of radioactivity with an apparent molecular weight of 15 - 20,000. The source of this zone is probably D1 since most of the radioactivity in 5 and 6 still remains in the higher molecular weight region of the gel. It is striking that 4.1 and 4.2, which are guanidine extractable and therefore peripherally localized, are not digested with papain. These polypeptides are constituents of the chloroplast coupling factor (Bennoun et al., 1978; Piccioni and Matlin, unpublished results) and are attached to the stromal (external) thylakoid face (Miller and Staehelin, 1976). Detergent addition (slot 6) does not result in total digestion, contrary to expectations.

Results with trypsin are similar to those with papain. Polypeptide 5 is sensitive to 0.5 μg/ml trypsin (Fig. 21, slot 3) and nearly eliminated with higher concentrations (slots 4 and 5). Polypeptides 2, 4.1, 4.2, 6, and D1 are also extensively digested with 5.0 μg/ml and 50 μg/ml (slots 4 and 5). Trypsin also produces smaller peptides similar in molecular weight to the ones generated by papain. Since so many bands are digested, it is difficult to conclude from autoradiographs that these peptides are derived from D1. However, if the observation that 2, 4.1, 4.2, 5, and 6 bind stain and D1 does not is taken into account, then at least a tentative conclusion may be reached. The stained gel from the digestion shown in slot 5 has no major band migrating in the region of the major radioactive peptide (slot 7). Therefore, unless one proposes that only the parts of membrane proteins that are protease accessible bind stain, the source of this peptide must be D1 because peptides from the other proteins would produce a stained band in this region. Addition of detergent (slot 6) results in complete digestion, conclusively demonstrating that the lipid bilayer protected the radioactive fragment (Katz et al., 1977).

The electrophoretic profile of membranes incubated with thermolysin is very fuzzy. Exact interpretation of the results is, therefore, difficult. In general, high concentrations of thermolysin digest all of the major chloroplast synthesized polypeptides (Fig. 22, slot 5). A smeary

polypeptide is presumably produced from D1 and migrates in a position similar to those generated by the other proteases. All proteins are completely digested by thermolysin in the presence of detergent (slot 6).

To summarize, the major chloroplast products, 2, 4.1, 4.2, 5, 6, and D1, are attacked by the three proteases tested. Polypeptide 5 may be the most accessible. In all three cases, D1 is probably reduced to a diffuse peptide migrating with an apparent molecular weight of 15,000 - 20,000. Since D2 was not resolved from D1 in the gels used for this experiment, conclusions about D1 may also apply to D2.

Sites of Synthesis of Chloroplast and Cytoplasmic Ribosomal Proteins

The sites of synthesis of ribosomal proteins were examined by [^{35}S]-sulfate labeling in the presence of inhibitors. In the Chlamydomonas chloroplast, many ribosomes are bound to the thylakoid membranes. Chloramphenicol, an elongation inhibitor, freezes a fraction of chloroplast polysomes to the thylakoid membranes (Chua et al, 1976). To prevent this effect, which would cause selective loss of bound chloroplast ribosomes from the post-mitochondrial fraction, lincomycin was substituted in experiments examining ribosomal protein synthesis. Since linocmycin inhibits chain initiation on 70S ribosomes (Ellis and Hartley, 1971; Pestka, 1974; Chua et al., 1976), in the presence of this drug bound polysomes are expected to fall off the membranes when they complete elongation. The extent of inhibition with lincomycin is similar to that attained with chloramphenicol. In addition, autoradiographs of thylakoid membranes labeled using lincomycin are identical in all respects to those obtained with chloramphenicol (Fig. 23, cf. Fig. 9).

Ribosomal subunits were purified from labeled cell homogenates by the standard published procedure (Chua et al., 1973a). Profiles from typical gradients are shown in Figure 24. Preparative gradients to separate chloroplast and cytoplasmic ribosomes (A) were loaded with only 10 A260 U each instead of 20 as recommended in the procedure to obtain better resolution. In all gradients, ribosome and subunit peaks were collected conservatively to avoid cross contamination. This contention is

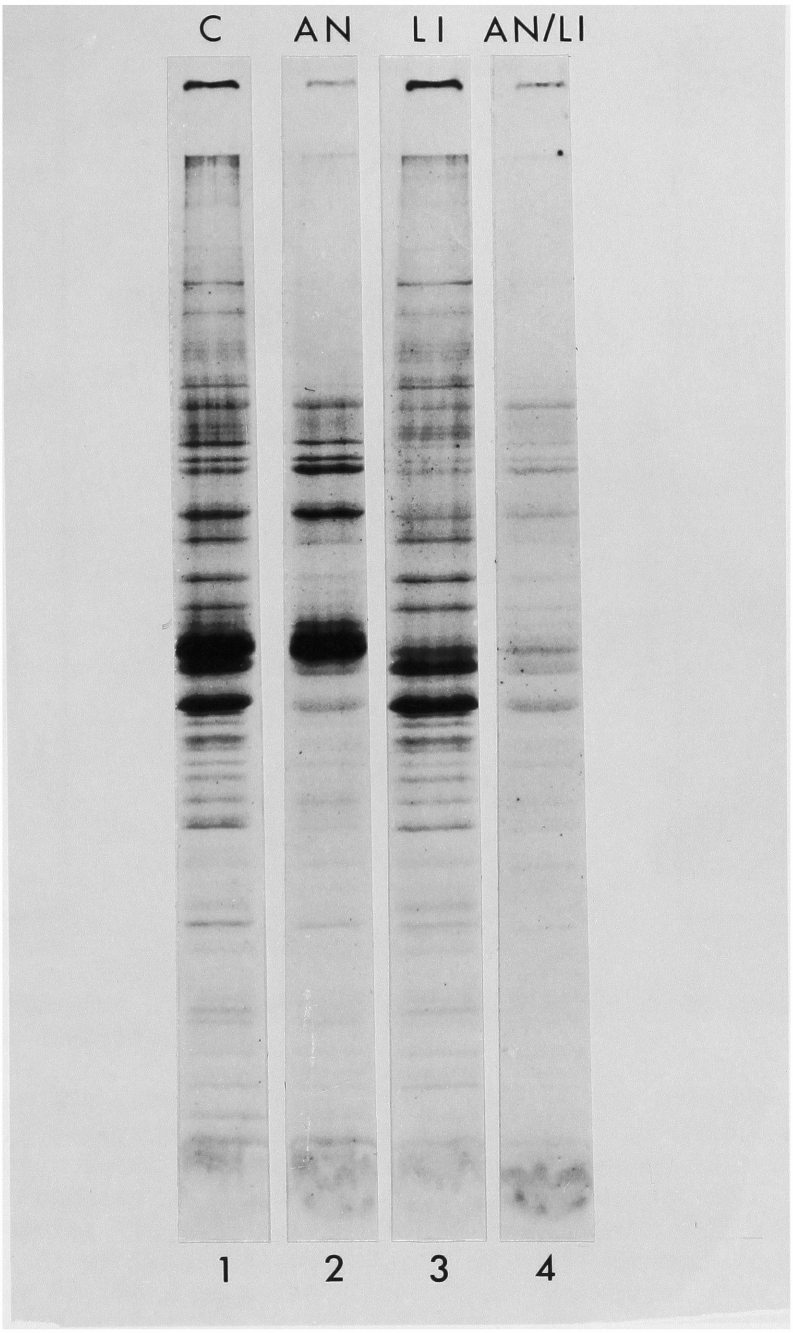


Figure 23. Analysis of thylakoid membranes labeled in the presence of lincomycin and anisomycin. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

Chlamydomonas was labeled with [^{35}S]-sulfate (0.01 mCi/ml) in the presence of inhibitors. In this experiment, lincomycin (300 µg/ml) was substituted for chloramphenicol. Thylakoid membranes were purified and run on a Laemmli 12 - 18% gel (20 µg/slot). Detection is by autoradiography. C, control; AN, anisomycin; LI, lincomycin; AN/LI, both inhibitors.

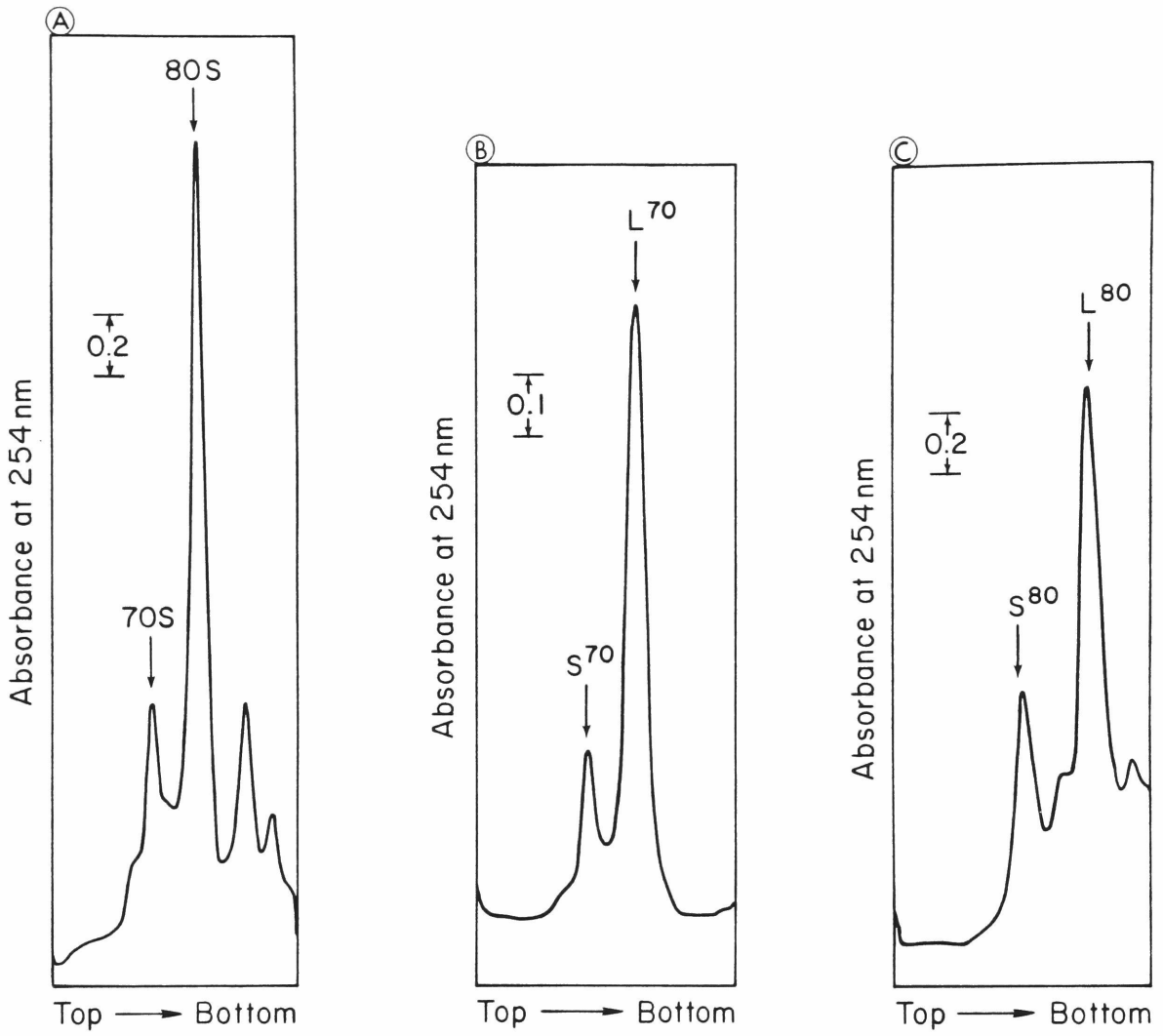


Figure 24. Purification of 70S and 80S ribosomal subunits by sucrose gradient centrifugation.

Chlamydomonas was labeled with [^{35}S]-sulfate in the presence of inhibitors. In this experiment, lincomycin (300 µg/ml) was used instead of chloramphenicol. Total ribosomes were isolated from whole cell supernatants by centrifugation at 161,000 x g_{max}. Aliquots from each inhibitor sample containing ∿10 A260 U were loaded onto linear 10 - 40% sucrose gradients in 25 mM Tris-HCl (pH 7.5)/25 mM KCl/5 mM $MgCl_2$/5 mM DTT and centrifuged at 85,500 x g_{max} for 15.5 hours. Fractions from these gradients containing either 70S or 80S ribosomes were concentrated by centrifugation and separated into subunits on high salt 5 - 20% sucrose gradients (25 mM Tris-HCl, (pH 7.5)/400 or 500 mM KCl/25 mM $MgCl_2$/5 mM $MgCl_2$) at 257,000 x g_{max} for 3 hours. More details are given in Materials and Methods. A, separation of 70S and 80S ribosomes on 10 - 40% gradients; B, purification of large 70S subunits (L 70) and small 70S subunits (S 70) on 400 mM KCl 5 - 20% gradients; C, purification of large 80S subunits (L80) and small 80S subunits (S 80) on 500 mM KCl gradients. The arrows indicate direction of sedimentation.

supported by the gel electrophoretic profiles of the subunits shown in Figures 24 and 25. Each complement of proteins appears, for the most part, to be unique to that particular subunit. Since a few polypeptides from different subunits do co-migrate (e.g., the very high molecular weight species) a small amount of cross contamination cannot be excluded.

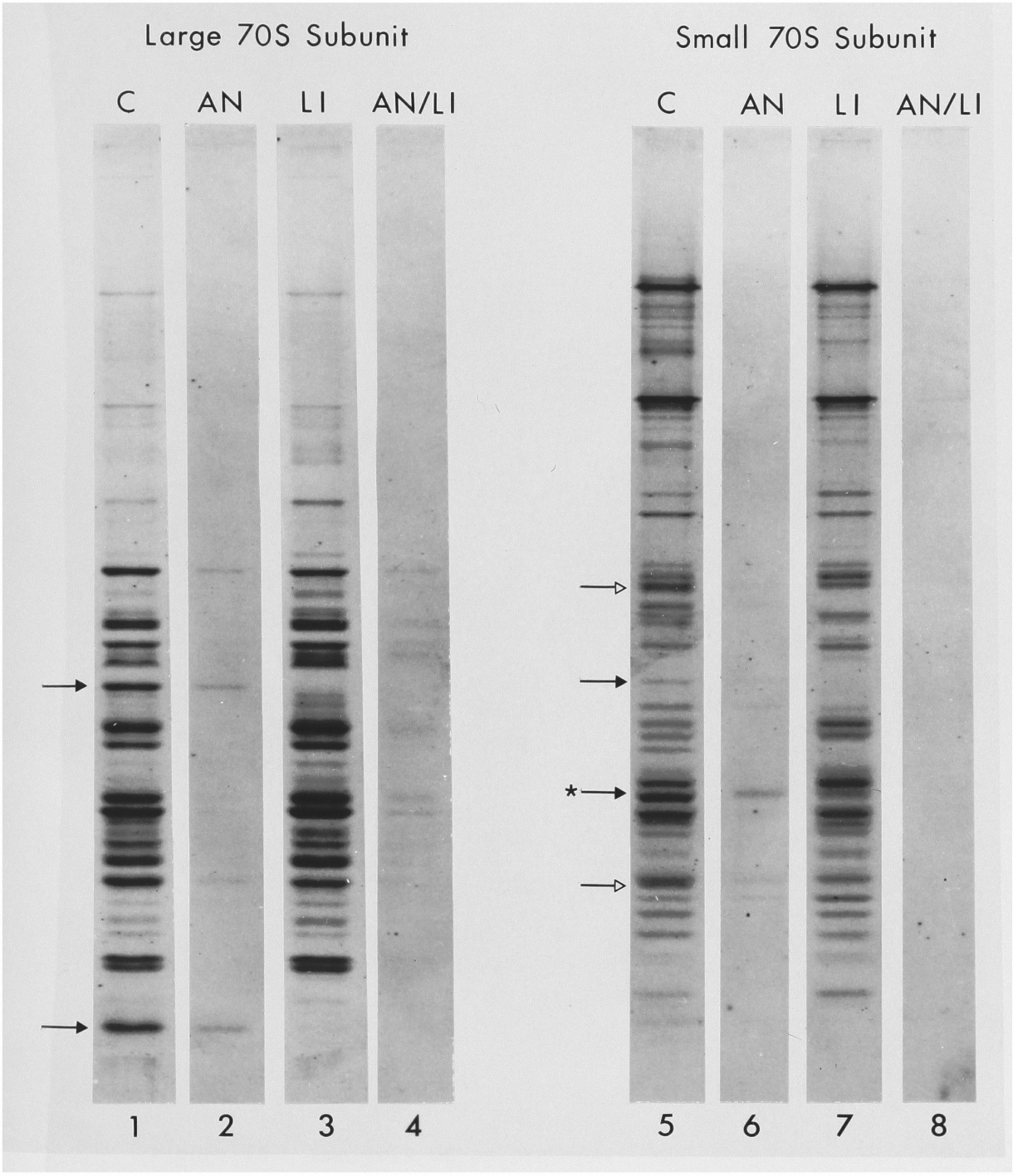
Approximately thirty labeled bands are resolved in the 70S large subunit sample (Fig. 25, slot 1). All but three of these are also labeled in the presence of lincomycin but not extensively in the presence of anisomycin or both inhibitors. These polypeptides are, therefore, cytoplasmically synthesized. Two of the remaining three bands are labeled only in the presence of anisomycin or in the control and hence are chloroplast products (closed arrows). The third polypeptide is not made in the presence of either anisomycin or lincomycin (slots 2 and 3). The appearance of this protein in assembled ribosomes may be coupled to the synthesis of proteins in either the cytoplasm or the chloroplast.

In the 70S small subunit, about thirty-nine labeled bands are resolved (Fig. 25, slot 5). As with the large subunit, most of these polypeptides are cytoplasmically synthesized. One band that is missing in the lincomycin sample (slot 7) is very faintly labeled in the presence of anisomycin (slot 6) and unlabeled with both inhibitors (slot 8). This polypeptide is then a chloroplast product (closed arrow). Another polypeptide made in anisomycin migrates slightly slower than a control band not present in the lincomycin sample (closed arrow with star). Because of this mobility shift, its classification as a chloroplast product is only tentative. Five or six other small subunit polypeptides labeled in the control are not labeled in either lincomycin or anisomysin (e.g., open arrows). These polypeptides are probably subject to the same control mechanisms mentioned earlier.

Figure 26 illustrates analogous polypeptide profiles for the 80S large and small subunits. All bands labeled in control samples were labeled in lincomycin and are therefore cytoplasmic products. Two small subunit proteins are labeled heavily both in control and lincomycin and

Figure 25. Analysis of polypeptides from 70S ribosomal subunits labeled in the presence of inhibitors. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

Ribosomal subunits were labeled with [^{35}S]-sulfate and purified as described earlier (see Fig. 24 and Materials and Methods). Subunit pellets were solubilized in 65 µl Tris base/SDS/DTT/iodoacetamide and electrophoresed on a Laemmli 12 - 18% urea gel (30 µl per slot). Each 70S and 80S subunit preparation labeled in the presence of a particular inhibitor was derived from ∿20 A260 U of total ribosomes. Approximately half of the total preparation was loaded on the subunit gel. Because of the small amount of material, the A260 U concentration of individual subunit preparations was not measured. Bands were detected by autoradiography. Slots 1-4 are the large 70S subunit samples; slots 5-8 are polypeptides of the small 70S subunit. C, control; AN, anisomycin, LI, lincomycin; AN/LI, both anisomycin and lincomycin. Closed arrows point to polypeptides made inside the chloroplast. The one marked with a star (*) is questionable because of mobility differences (see text). Open arrows show some examples of polypeptides unlabeled in the presence of both anisomycin and lincomycin. Polypeptides of 80S ribosomal subunits are shown in Figure 26.


Large 70S Subunit
Small 70S Subunit
C
AN
LI
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LI
AN/LI
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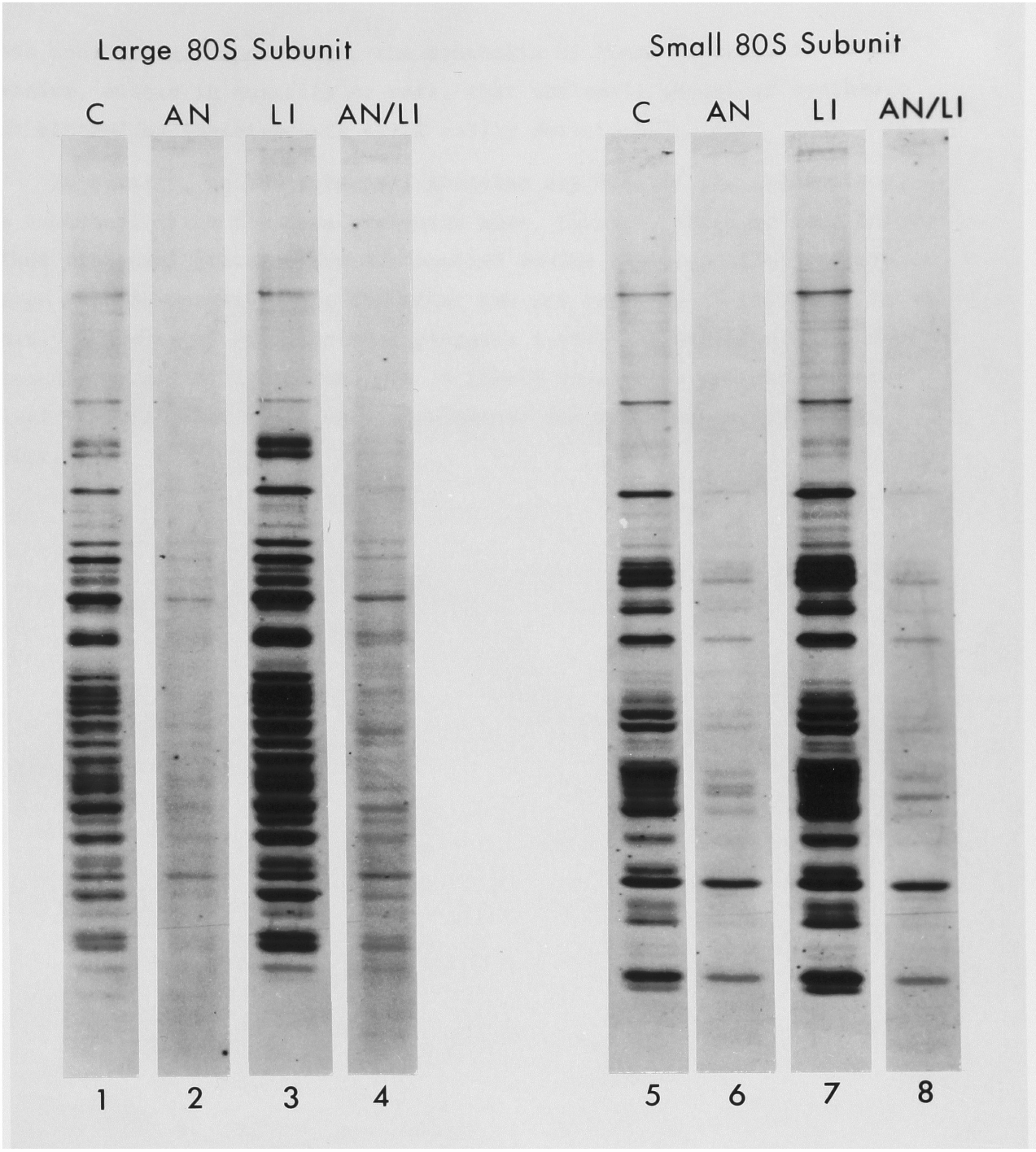


Figure 26. Analysis of polypeptides from 80S ribosomal subunits labeled in the presence of inhibitors. SDS/polyacrylamide gel electrophoresis: Laemmli 12 - 18% gradient, 8 M urea.

Cytoplasmic (80S) ribosomal subunits were labeled with [^{35}S]-sulfate, purified, and electrophoresed as described in the legends to Figures 24 and 25 and Materials and Methods. Slots 1-4 are the large 80S subunit samples; slots 5-8 are polypeptides of the small 80S subunit. C, control; AN, anisomycin; LI, lincomycin; AN/LI, both anisomycin and lincomycin.

both inhibitors. Apparently, the synthesis of these proteins is so extensive, either in quantity or rate, that the small amount of synthesis not blocked by inhibitors is still easily detectable.

In summary, no 80S ribosomal proteins are made in the chloroplast, as expected. From the data presented here, however, three or four chloroplast ribosomal proteins are synthesized inside the organelle; two are large subunit constituents; the other two are proteins of the small subunit. A number of 70S ribosomal proteins (6-7) are not labeled in the presence of either inhibitor. It is likely that these are also chloroplast products, but additional experiments are required to prove this point.

CHAPTER IV

STUDIES ON CHLOROPLAST PROTEIN SYNTHESIS *IN VITRO*

Isolation and Characterization of Messenger RNA

Since intact chloroplasts cannot be isolated from *Chlamydomonas*, messenger RNA specific for polypeptides synthesized in the chloroplast was prepared by fractionating total cellular nucleic acids. Extraction of whole cells with phenol/chloroform/isoamyl alcohol in the presence of RNAse inhibitors was routinely performed on large log phase cultures. Yields of RNA + DNA averaged 190 A260 U per liter of culture. To remove DNA and tRNA from the preparations, single-stranded RNA was specifically precipitated with 2 *M* LiCl. At least 65% of the total A 260 U were recovered by precipitation. Depletion of 4S RNA from the nucleic acid fraction is evident in the gradient profiles shown in Figure 27. After precipitation (B), the large peak running near the top of the gradient is greatly diminished. DNA is pelleted on these gradients and does not show up on the absorbance profile in A.

Either before or after lithium chloride precipitation, poly(A) containing mRNA was removed from the nucleic acid preparation by affinity chromatography on poly(U) Sepharose. Binding was carried out in 0.4 *M* NaCl. At this salt concentration, poly A-U hybrid formation is optimal so that mRNA molecules containing short poly(A) segments were encouraged to bind to the column (Sheldon *et al.*, 1972; P. Lizardi, personal communication). Typically, 1 - 2% of the nucleic acids loaded on the affinity column bound. The unbound fraction, which contains poly(A)$^-$ mRNA, ribosomal RNA, and in some instances DNA, was used in all subsequent experiments.

Nucleic acid preparations were often fractionated on sucrose gradients. Figure 27 illustrates an SDS/sucrose gradient that was used to assess the quality of RNA preparations, monitor LiCl precipitation, and partially purify mRNA from other contaminating RNAs and DNA. The advantage of SDS/sucrose gradients was their simplicity and relatively short running time; their main disadvantage was that they did not prevent RNA aggregation (Reisfeld *et al.*, 1978). Since an aggregated mRNA would ap-

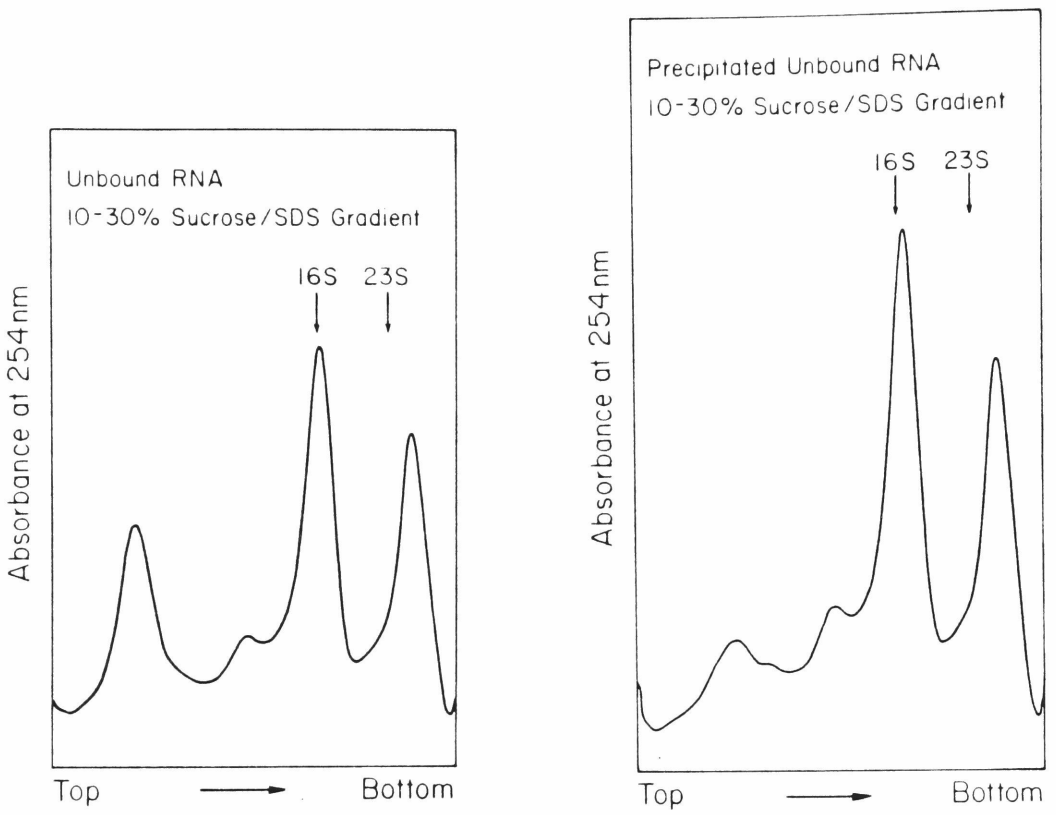


Figure 27. SDS/sucrose gradient fractionation of RNA before and after lithium chloride precipitation.

Chlamydomonas poly(U) Sepharose unbound RNA was precipitated with LiCl as described in Materials and Methods. Samples of the RNA (3.75 A260 U) before and after precipitation were centrifuged on 12.7 ml 10 - 30% sucrose gradients in 20 mM Tris-HCl (pH 7.5)/0.5% SDS/2 mM EDTA at 270,000 x g_{max} 18°C for 15.75 hours. Gradients were fractionated from the bottom and monitored at 254 nm. A, before precipitation; B, after precipitation. Arrows indicate direction of sedimentation.

pear heavier on the gradient than its monomer, these gradients could not be used with confidence to estimate mRNA sizes. Gradients prepared with 60% formamide did not have this disadvantage. A typical profile is shown in Figure 28. Formamide, an RNA denaturant, prevented the formation of aggregates at the 37°C running temperature. Although formamide gradients were very useful for mRNA purification and sizing, their high density lengthened running times.

Gradient profiles of either total nucleic acids (Fig. 27) or poly(U) Sepharose unbound RNA (Fig. 28) always contained two major peaks migrating roughly in the position of 16S and 23S markers. The heavier peak is assumed to be partially degraded 28S (cytoplasmic) ribosomal RNA which is cleaved during isolation (Cattolico and Jones, 1972; Leaver, 1973; Atchison, 1973; Mache, 1978). Its degradation accounts both for its shifted S value and its inverted absorbance ratio relative to the small RNA species. The peak at 16S probably consists of intact or slightly degraded 18S ribosomal RNA together with fragments from the 28S molecule. The degree of degradation was always approximately the same as that illustrated. Although extreme measures were attempted to inhibit the RNAse which was degrading the ribosomal RNA including direct lysis of cells in phenol/chloroform/SDS (Bynum and Ronzig, 1976), intact ribosomal RNA could not be isolated. Published conditions for the isolation of intact Chlamydomonas ribosomal RNA (Cattolico and Jones, 1972) were not suitable for preparing large quantities of translatable mRNA because it was feared that the RNAse inhibitor diethyl pyrocarbonate, would modify mRNA. The intactness of chloroplast ribosomal RNA, which was not resolved on these gradients from cytoplasmic rRNA, was not examined.

RNA fractions from the formamide/sucrose gradient shown in Figure 28 were examined by gradient gel electrophoresis (Fig. 29). Clearly a number of discrete species are present in these fractions and are separated into distinct size classes. The two heavily stained bands (fractions 4 and 5, arrows) correspond to the two ribosomal RNA peaks discussed earlier. Other fractions appear to be free of large quantities of ribosomal RNA but undoubtedly contain many heterogenous degradation

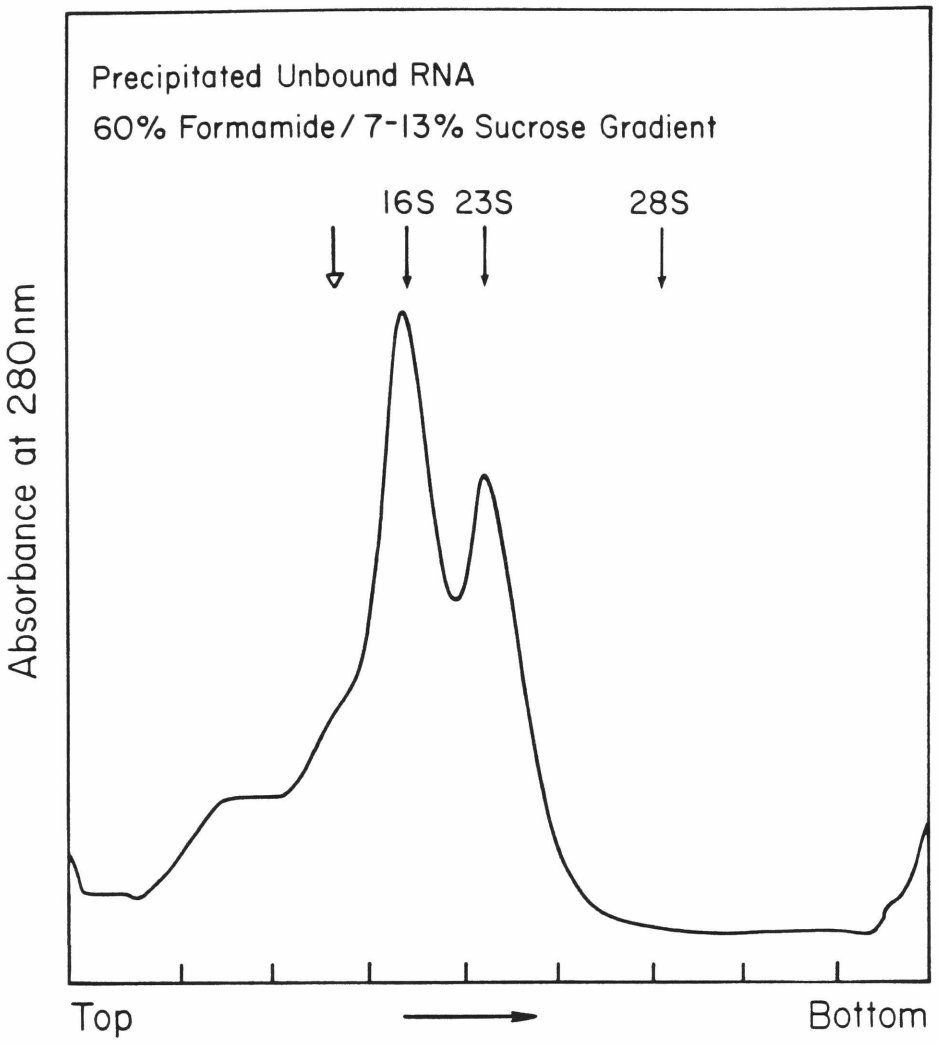


Figure 28. Formamide/sucrose gradient fractionation of poly(U) Sepharose unbound RNA.

Chlamydomonas poly(U) Sepharose unbound RNA was precipitated with LiCl and loaded on 36 ml 60% formamide/7 - 13% sucrose gradients (8 A260 U/gradient). Centrifugation was for 26 hours at 127,000 x g_{max}, 37°C. Gradients were fractionated from the bottom and monitored at 280 nm. E. coli and 3T6 ribosomal RNA standards were run on parallel gradients. The open arrow points to the location of mRNA for the membrane protein D1 (see Fig. 33). The arrows on the abscissa indicates direction of sedimentation.

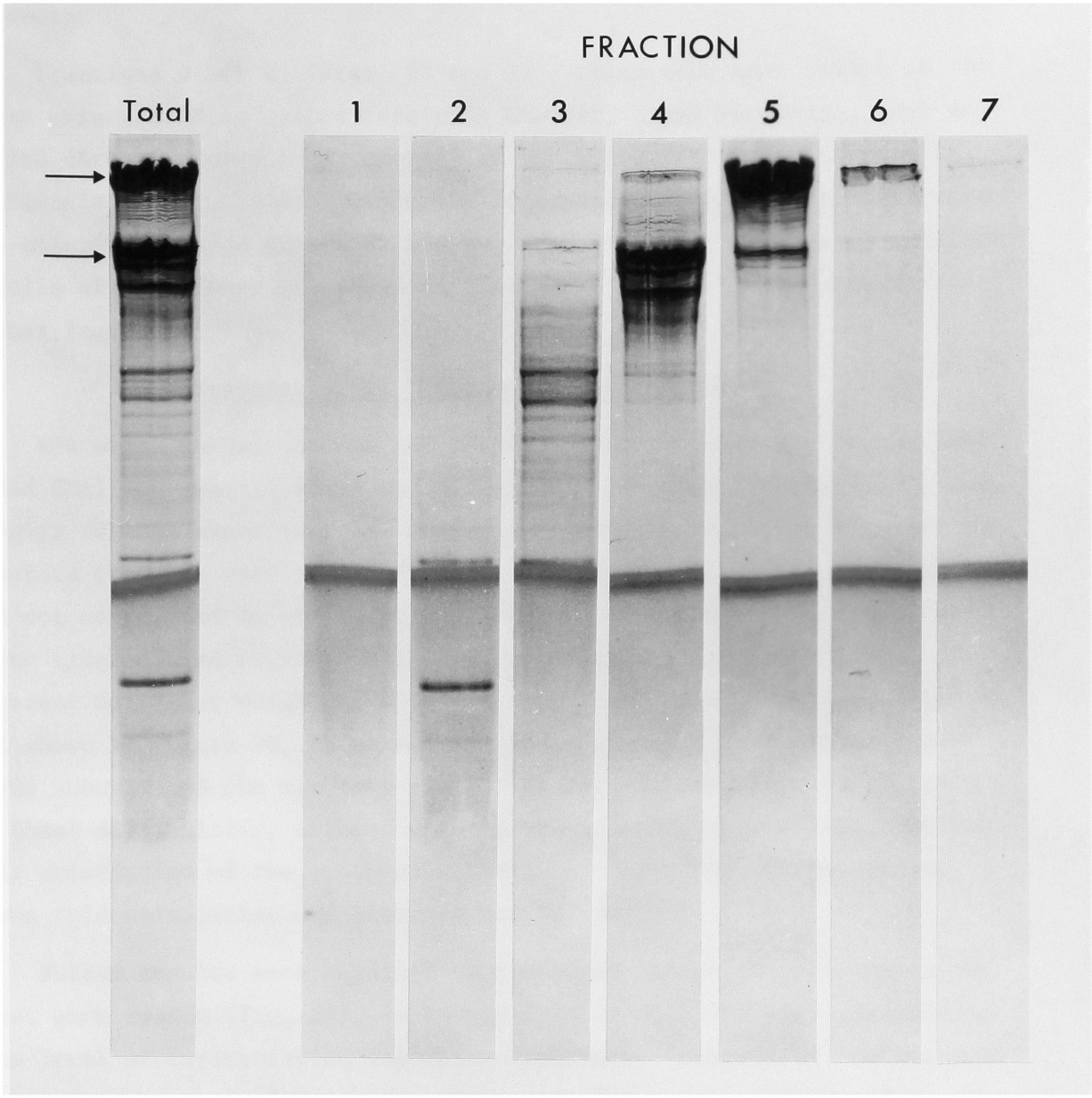


Figure 29. Formamide/acrylamide gradient gel electrophoresis of RNA fractions from formamide/sucrose gradients.

Poly(U) Sepharose unbound RNA was fractionated on 60% formamide/sucrose gradients (see Fig. 28). Equivalent gradient fractions were pooled, ethanol precipitated, and re-dissolved in 0.5 ml water. Aliquots (70 µl) containing a maximum of 1 A260 U were electrophoresed on an 80% formamide/4 - 10% acrylamide gradient gel (see Materials and Methods for details). Total (unfractionated) RNA (2 A260 U) was also run. Bands were visualized with Stainsall. Arrows indicate large and small ribosomal RNA. Fraction numbers correspond to fraction markers in Figure 28 with #1 at top (left) of the gradient.

products.

Fractions 3 and 4, (Figs. 28 and 29), which were most active in the wheat germ cell-free system (see this Chapter, later sections), were analyzed directly for poly(A) content by hybridization with [^{3}H]-poly(U) (Gillespie et al., 1972). Since the proportion of mRNA in each fraction was unknown, a large excess of RNA was used in the hybridization reaction. Results of this assay are shown in Table IV. No poly(A) was detected in either fraction.

Translation of "Unbound" Messenger RNA

RNA which passed through the poly(U) Sepharose affinity column (unbound RNA) was translated in the E. coli S30 in vitro translation system. Results of this experiment are shown in Figure 30. Although a number of discrete products were produced, most were of low molecular weight and did not correspond in mobility to any known chloroplast products. The major labeled band migrated slightly faster than polypeptide 6 with an apparent molecular weight of 50,000. After long exposure of the dried gel shown in Figure 30, faint bands which comigrated with carboxylase large subunit and the membrane protein D1 were detectable. In addition to these difficulties, unbound mRNA in the S30 system gave less than two-fold stimulation of the endogenous level. Under identical conditions, a seven fold stimulation was obtained for MS2 RNA.

Better results were obtained when unbound RNA was translated in the wheat germ system (Fig. 31). A twenty-fold stimulation over the endogenous level of incorporation was often obtained. Analysis of the products show a large number of discrete, high molecular weight polypeptides. The major product comigrated with D1 and another labeled band comigrated with carboxylase large subunit.

The ionic conditions used for translation in the wheat germ system were those optimized for the translation of a number of poly(A)$^+$ mRNAs. Since the message used in these experiments lacked poly(A) and was contaminated with ribosomal RNA, the optimal potassium and magnesium concentrations were re-examined. As the data in Figure 32 indicate, the

TABLE VI

POLY(A) CONTENT OF UNBOUND mRNA

RNA Sample	ng Added	Digestion*	cpm	cpm (-no RNA)	ng Poly(U) / µg RNA	Poly(A) Content (%)
no RNA	0	+	6443.2	-	-	-
no RNA	0	-	187915.4	-	-	-
Poly(U)	500	+	2801.7	-	-	-
Poly(U)	500	-	160955.2	-	-	-
Poly(A)	500	+	122958.4	-	-	-
Poly(A)	500	-	185628.5	-	-	-
mRNA #4	100	+	3089.6	<0	-	0
mRNA #4	50	+	2697.3	<0	-	0
mRNA #3	100	+	2665.2	<0	-	0
mRNA #3	50	+	2978.7	<0	-	0

*(+) = digested with RNAse and DNAse; (-) = not digested

Assay was performed as described in Materials and Methods by the [^{3}H]-poly(U) hybridization technique of Gillespie et al. (1972). Numbers of the RNA preparation refer to formamide gradient fractions (see Figs. 28 and 29).

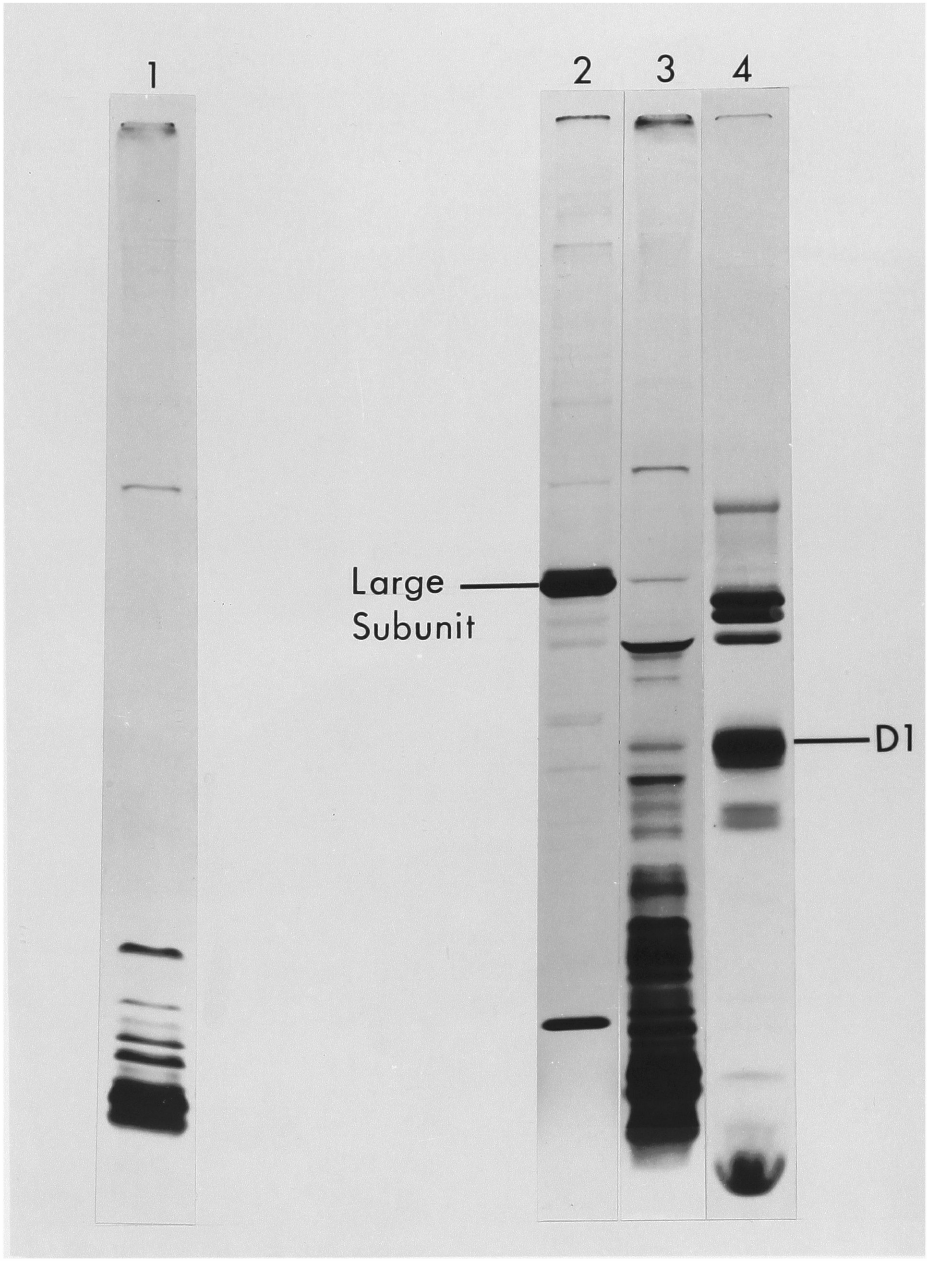


Figure 30. Analysis of products from translation of unbound RNA in the E. coli S30 system. SDS/polyacrylamide gel electrophoresis: Neville 7.5 - 15% gradient.

Chlamydomonas RNA, which failed to bind to poly(U) Sepharose, was precipitated with LiCl and translated in the E. coli S30 system. Products were electrophoresed as described in Materials and Methods. Polypeptides directed by unbound RNA are shown in slot 3; slot 1, no RNA control; slot 2, purified Chlamydomonas carboxylase holoenzyme; slot 4, thylakoid membranes labeled in the presence of anisomycin. Detection is by autoradiography.

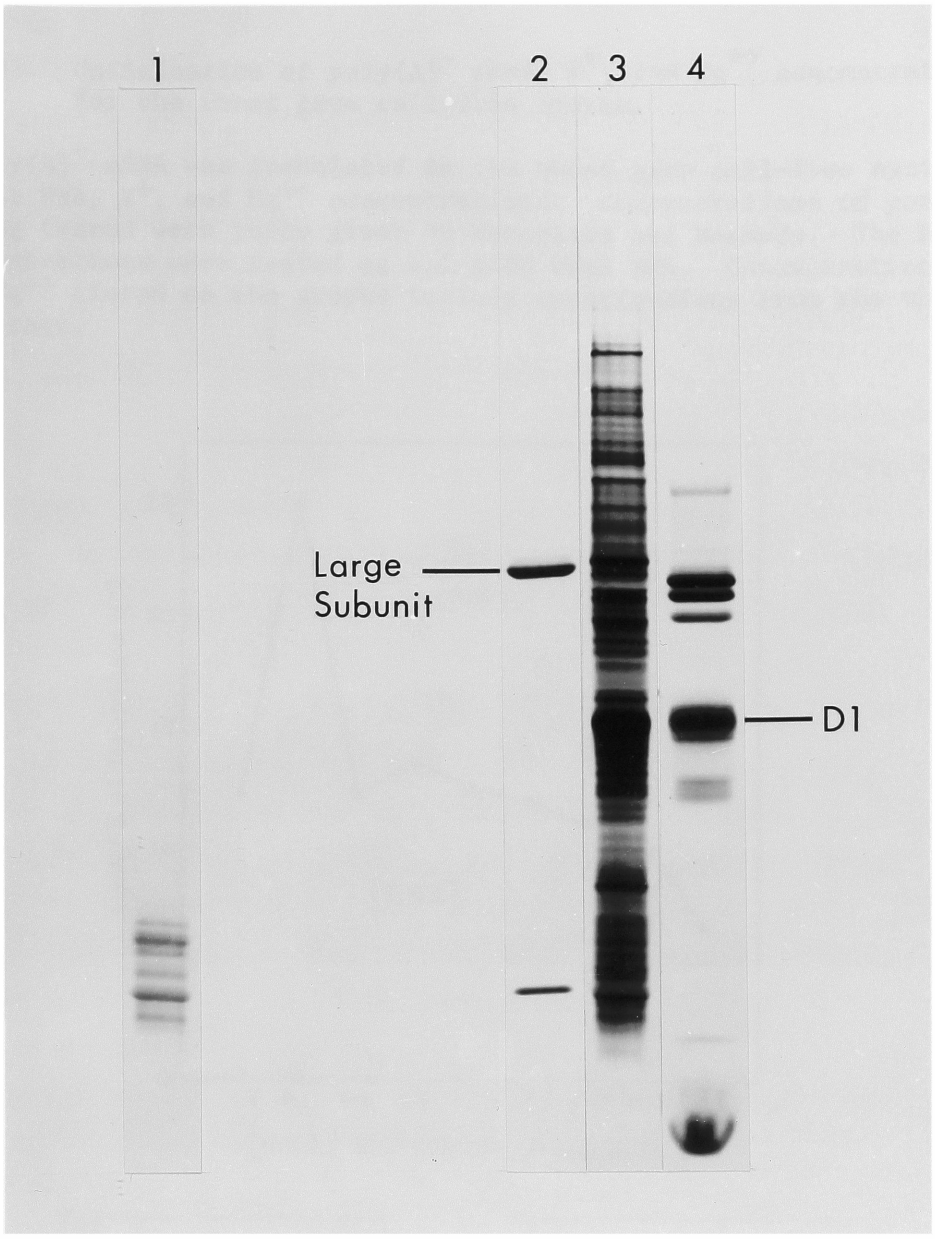
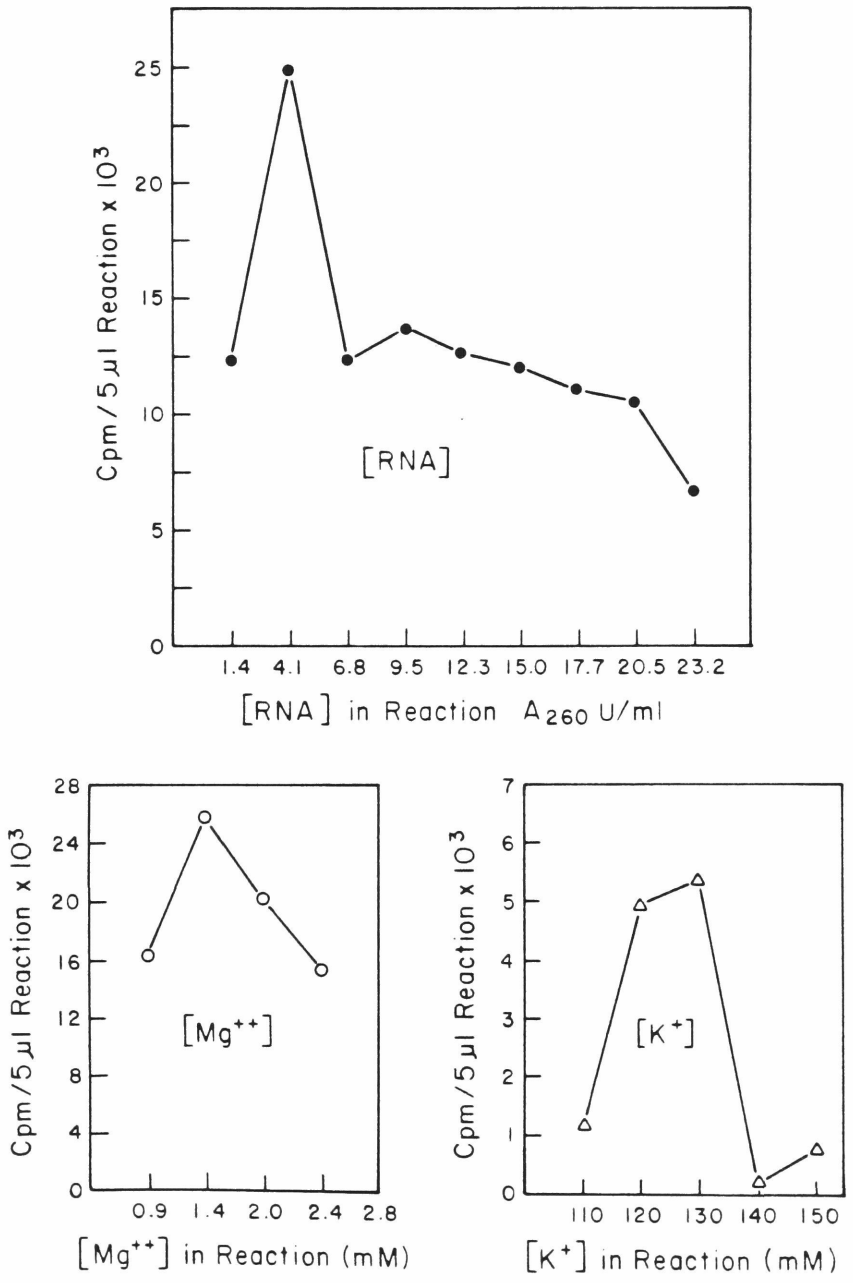


Figure 31. Analysis of products from translation of unbound RNA in the wheat germ system. SDS/polyacrylamide gel electrophoresis: Neville 7.5 - 15% gradient.

Chlamydomonas RNA, which failed to bind to poly(U) Sepharose, was precipitated with LiCl and translated in the wheat germ system. Products were electrophoresed as described in Materials and Methods. Polypeptides directed by unbound mRNA are shown in slot 3; slot 1, no RNA control; slot 2, purified Chlamydomonas holoenzyme; slot 4, thylakoid membranes labeled in the presence of anisomycin. Detection is by autoradiography.

Figure 32. Optimization of poly(A)$^-$ mRNA, K^+, and Mg^{++} concentrations for the wheat germ cell-free system.

Poly(A)$^-$ mRNA was translated in the wheat germ cell-free system at different RNA, K^+, and Mg^{++} concentrations. Concentrations of components not being tested were those given in Materials and Methods. The Mg^{++} and K^+ concentrations were tested at 4.1 A260 U/ml RNA. Concentrations of K^+ and Mg^{++} listed on the graphs include contributions from the wheat germ extract.



previously determined ionic conditions (see Materials and Methods) were also close to optimal for translation of unbound mRNA. While it was possible that radically different concentrations might result in even greater activities, the existing conditions were sufficient to perform the experiments described in the remainder of this chapter. For optimal incorporation, unbound RNA had to be present at a higher concentration than that required for Chlamydomonas polyadenylated RNA. Unbound RNA was most active at 4.1 A260 U/ml while poly(A)$^+$ mRNA activity was greatest at less than 1 A260 U/ml (G. Schmidt, personal communication). At concentrations higher than the optimum, unbound RNA was inhibitory. In contrast, polyadenylated RNA activity shows a linear increase with concentration to a plateau (G. Schmidt, personal communication). Routine incubations were carried out at 25 - 27°C for 90 minutes; close to maximum incorporation was achieved, however, after 60 minutes of incubation.

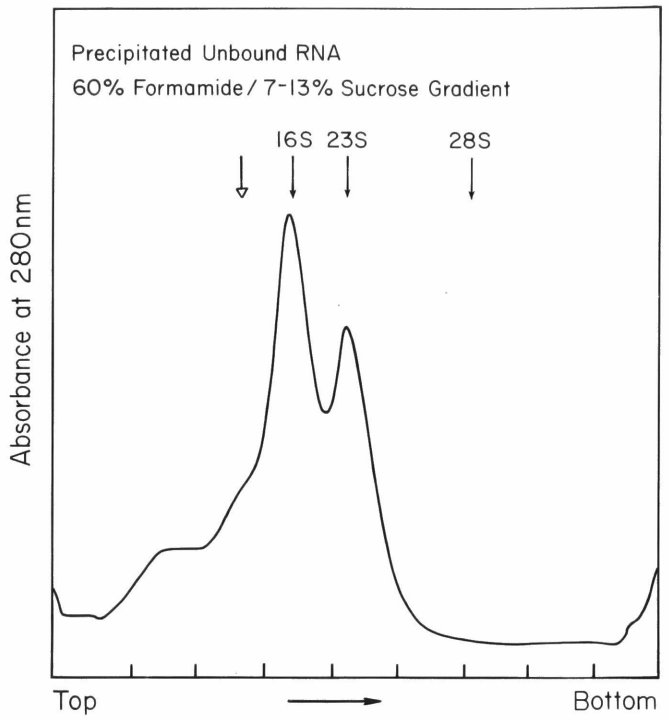
To estimate the size of mRNA coding for specific products, fractions from the formamide/sucrose gradient (Figs. 28 and 29) were translated in the wheat germ system (Fig. 33). Polypeptides of molecular weight less than about 35,000 were translated from mRNA smaller than ∿15S (fraction 3); 16-18S mRNA made products of molecular weight up to about 50,000 (fraction 4) as well as some smaller polypeptides; translation of 23-28S mRNA resulted in polypeptides greater than 50,000 daltons. Essentially all the polypeptides directed by the unfractionated mRNA (total) were also synthesized by gradient fractions. Large mRNAs were apparently translated to completion since very few low molecular weight polypeptides were made in fraction 6 and the bulk of products in fraction 4 were of higher molecular weight. Messenger RNA's for the polypeptides that comigrate with carboxylase large subunit and D1 (arrows) are about 15-16S.

Identification of Products Synthesized in the Wheat Germ System

The in vitro product that comigrates with the carboxylase large subunit on one-dimensional gels was compared with the authentic large subunit by two-dimensional electrophoresis. Authentic large subunit (Fig. 34B) has two to three isoelectric forms (Kung and Rhodes, 1978) and split into two molecular weight species in the SDS gel dimension. The latter

Figure 33. Analysis of products from translation of formamide/sucrose fractions in the wheat germ system. SDS/polyacrylamide gel electrophoresis: Neville 7.5 - 15% gradient.

RNA, which failed to bind to poly(U) Sepharose, was precipitated with LiCl and fractionated on 60% formamide/sucrose gradients (see Fig. 28). Gradient fractions were translated in the wheat germ system and electrophoresed as described in Materials and Methods. The profile of the gradient from which the RNA fractions were derived is shown in the upper panel. "Total" refers to wheat germ translation products synthesized by the unfractionated RNA preparation. Arrows indicate positions of the translation products identified as carboxylase large subunit and the membrane protein D1. Detection is by autoradiography.


Precipitated Unbound RNA
60% Formamide / 7-13% Sucrose Gradient
16S 23S
28S
Absorbance at 280nm
Top
Bottom

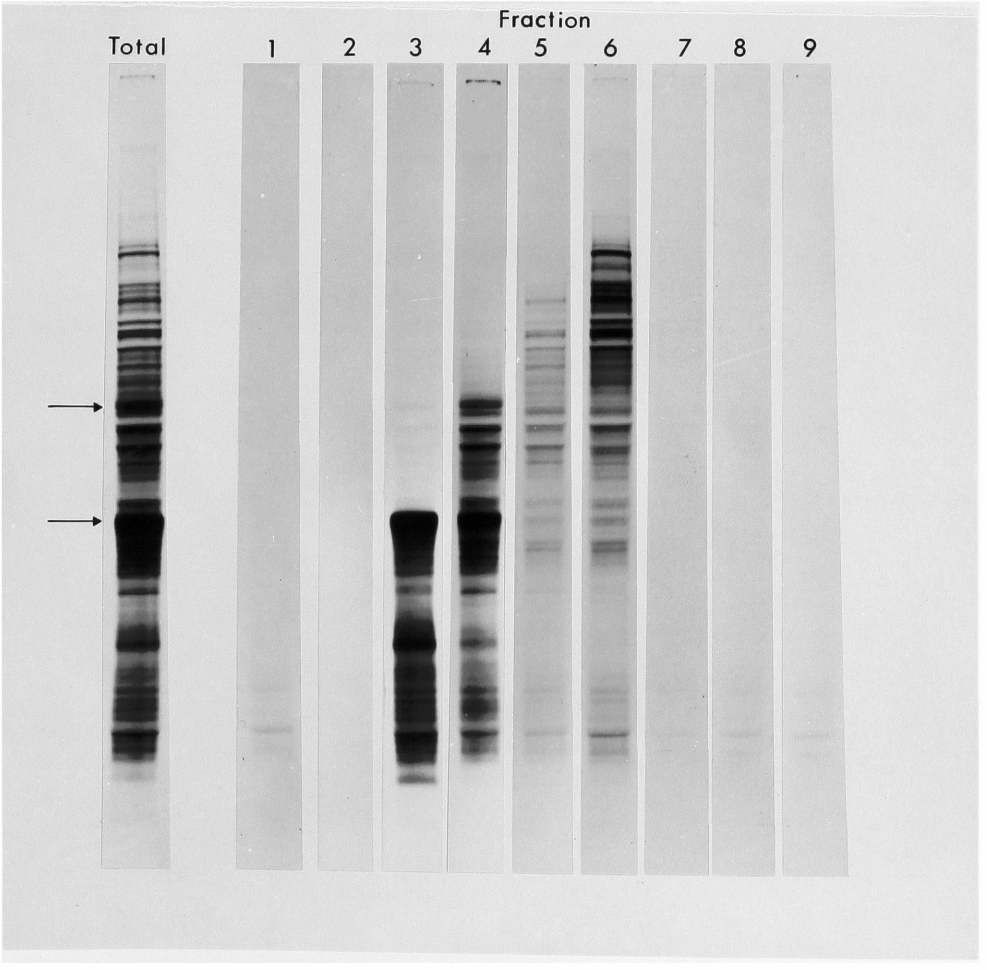
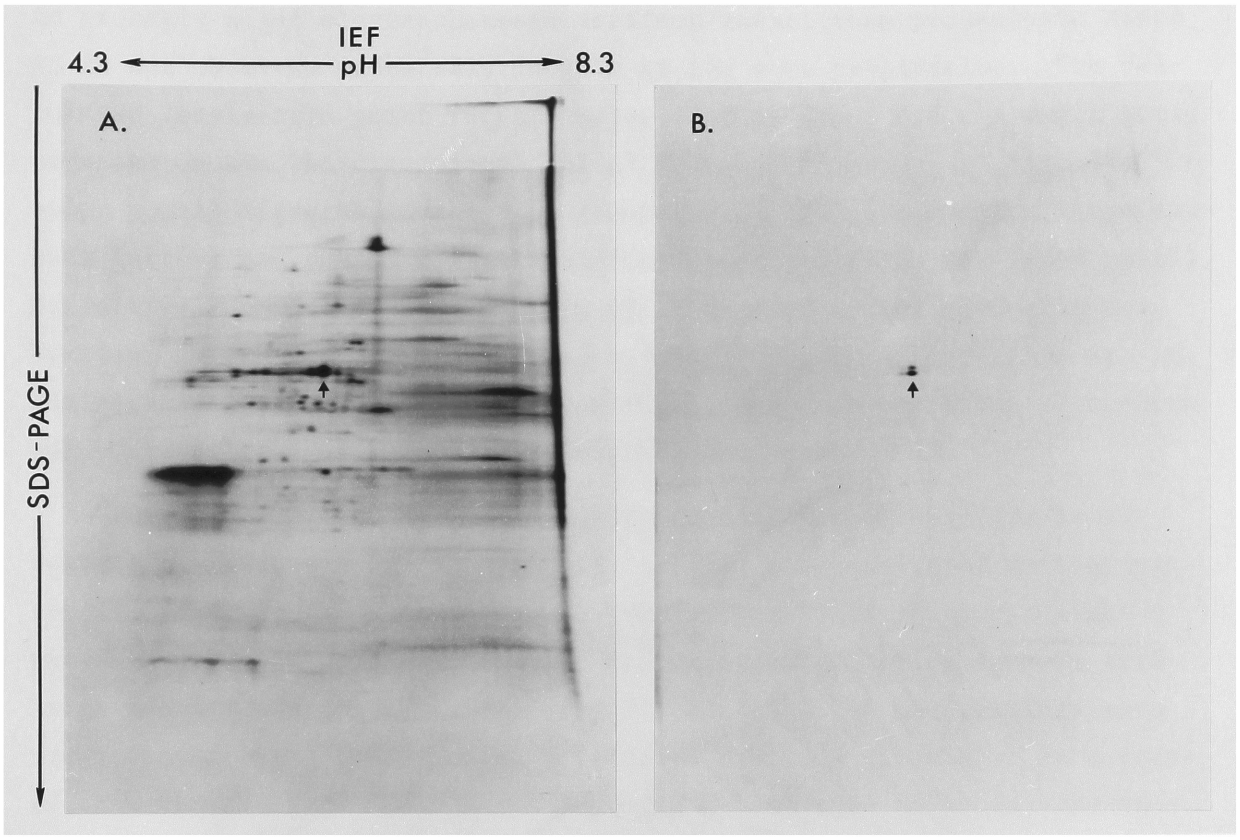

Fraction
Total
1
2
3
4
5
6
7
8
9

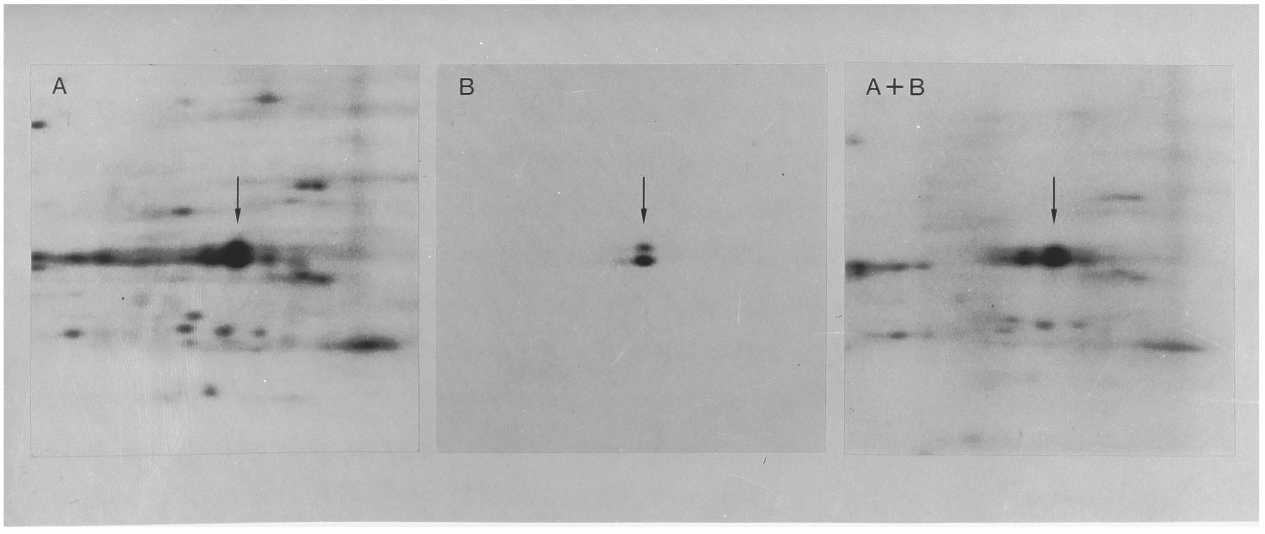
Figure 34. Two-dimensional gel electrophoresis of wheat germ translation products and ribulose 1,5-bisphosphate carboxylase.

Unbound mRNA was translated in the wheat germ system. Translation products (50 µl) and purified carboxylase holoenzyme (200 µl + 50 µl unlabeled carrier proteins) were precipitated and analyzed by two-dimensional isoelectric focusing/SDS gel electrophoresis. In addition, carboxylase (200 µl) and wheat germ translation products (50 µl) were mixed, precipitated, and analyzed similarly. Separation in the first dimension was over a pH range of 4.3 to 8.3. The second dimension was a Neville 7.5 - 15% gradient gel.

The upper panel shows the entire two-dimensional gels. Arrows indicate the position of the carboxylase large subunit. A, wheat germ translation products; B, carboxylase. Detection is by autoradiography. The carboxylase small subunit is too diffusely resolved to appear in this reproduction although it is visible in the original autoradiograph.

The lower panel illustrates a close-up view of the carboxylase large subunit region of gels A and B (above). A + B is the analogous area from the mixing experiment gel.


IEF
4.3
pH
8.3
SDS-PAGE
A.
B.


A
B
A+B

is probably a gel electrophoresis artifact rather than proteolysis since it is not observed repeatedly in gels of the same preparation. The calculated isoelectric point for the major spot is about 6.6. A small group of spots on the two-dimensional gel of translation products (Fig. 34, upper panel) migrates in the same general position as authentic large subunit (arrow) and exhibits identical fine-structure (Fig. 34, lower panel). Definitive identification of the in vitro product as the authentic carboxylase large subunit was obtained by the mixing experiment (A + B). No additional spots are observed demonstrating that the migration of the two polypeptides exactly coincides in both dimensions.

Further evidence that the in vitro product was carboxylase large subunit was provided by immunoprecipitation. Immunoselected antibodies specific for the large subunit were incubated with in vitro translation products directed by unbound mRNA. One major band that comigrated with large subunit was precipitated (Fig. 35, slot 2). No polypeptides were precipitated by an equal amount of control antibody (slot 4). As expected, the residue from the control incubation contained an extra band in the position of the large subunit that was missing in the anti-large subunit residue (slots 3 and 5, arrows). These results demonstrate clearly that the carboxylase large subunit is synthesized by unbound RNA in the wheat germ system.

To identify a second translation product as D1 by two-dimensional electrophoresis, it was first necessary to localize D1 on two-dimensional gels. This was accomplished by analysis of thylakoid membranes labeled in the presence of inhibitors (Fig. 36). Since D1 is the major labeled polypeptide running faster than polypeptides 4.1, 4.2, 5, and 6 (see Fig. 8) in membranes labeled in the presence of anisomycin, its location can be deduced from comparison of the different gels (Fig. 37). D1 always migrates as a diffuse smear, heterogenous in both isoelectric point and molecular weight. This behavior on SDS gels has been noted earlier (Chua and Gillham, 1977). The recovery of D1 on two-dimensional gels was never commensurate with the amount of radioactivity seen in this band on one-dimensional gels. When samples for 2D were solubilized first

Figure 35. Immunoprecipitation of ribulose bisphosphate carboxylase large subunit synthesized *in vitro*.

Unbound mRNA was translated in preparative scale reactions by the wheat germ system. After removal of ribosomes by centrifugation, two aliquots (400 µl each) were TCA precipitated and solubilized in ∿420 µl 87 mM Tris-HCl (pH 8.6)/2 mM DTT/0.1% SDS/0.8% Triton X-100/0.12 *M* NaCl/ Trasylol (see Materials and Methods). Immunoselected antibodies against the large subunit (26 µg) were incubated with one sample. As a control, antibodies against polypeptide 11 (27 µg) were added to the second sample. Immune complexes were adsorbed with 50 µl washed 10% *Staphylococcus aureus* (Cowans) and solubilized in 100 µl Tris/SDS/DTT/iodoacetamide. Residues from the immunoprecipitation mixture were TCA precipitated and solubilized in 420 µl Tris/SDS/DTT/iodoacetamide. For gel electrophoresis, 20 µl of the immunoprecipitate solutions and 15 µl of the residue solutions were loaded on a Neville 7.5 - 15% gel resulting in a greater than four-fold concentration of immunoprecipitates over residues. Slot 2 is the large subunit antibody immunoprecipitate; slot 4 the anti-11 immunoprecipitate; slots 3 and 5 are the respective residues; slot 1 is ribulose bisphosphate carboxylase holoenzyme. Notice the band reduced in slot 3 and present in slot 5 (arrows).

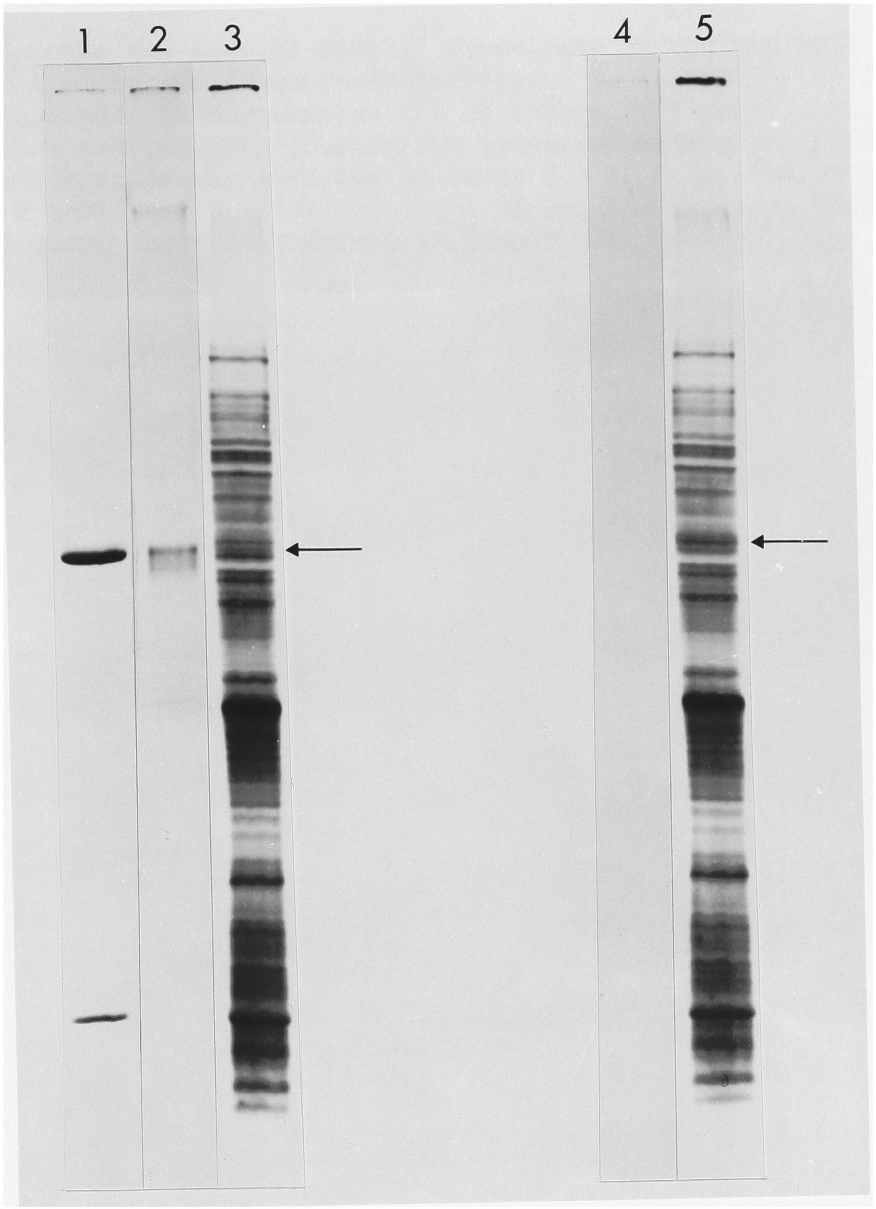
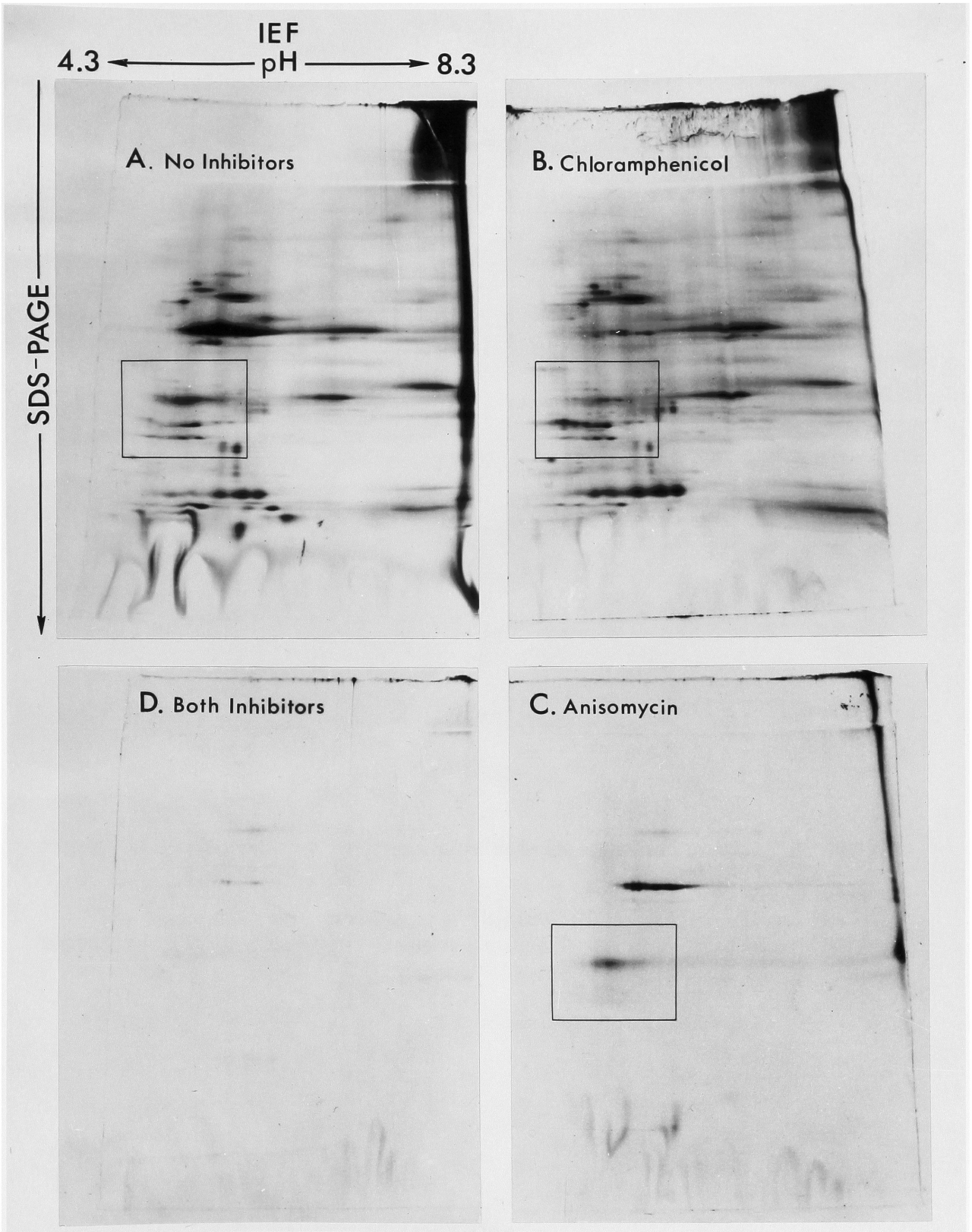

1
2
3
4
5

Figure 36. Two-dimensional gel electrophoresis of labeled thylakoid membranes.

Chlamydomonas was labeled with [^{35}S]-sulfate in the presence of inhibitors and the thylakoid membranes purified. To determine the position of the membrane protein D1, samples (10 µg chlorophyll each) were run on two-dimensional isoelectric focusing/SDS polyacrylamide gels. Separation in the first dimension was over the pH range 4.3 to 8.3. The second dimension was a Neville 7.5 - 15% gel. The D1 region, which is surrounded by a box, is shown in greater detail in Figure 37.


IEF
4.3
pH
8.3
SDS-PAGE
A. No Inhibitors
B. Chloramphenicol
D. Both Inhibitors
C. Anisomycin

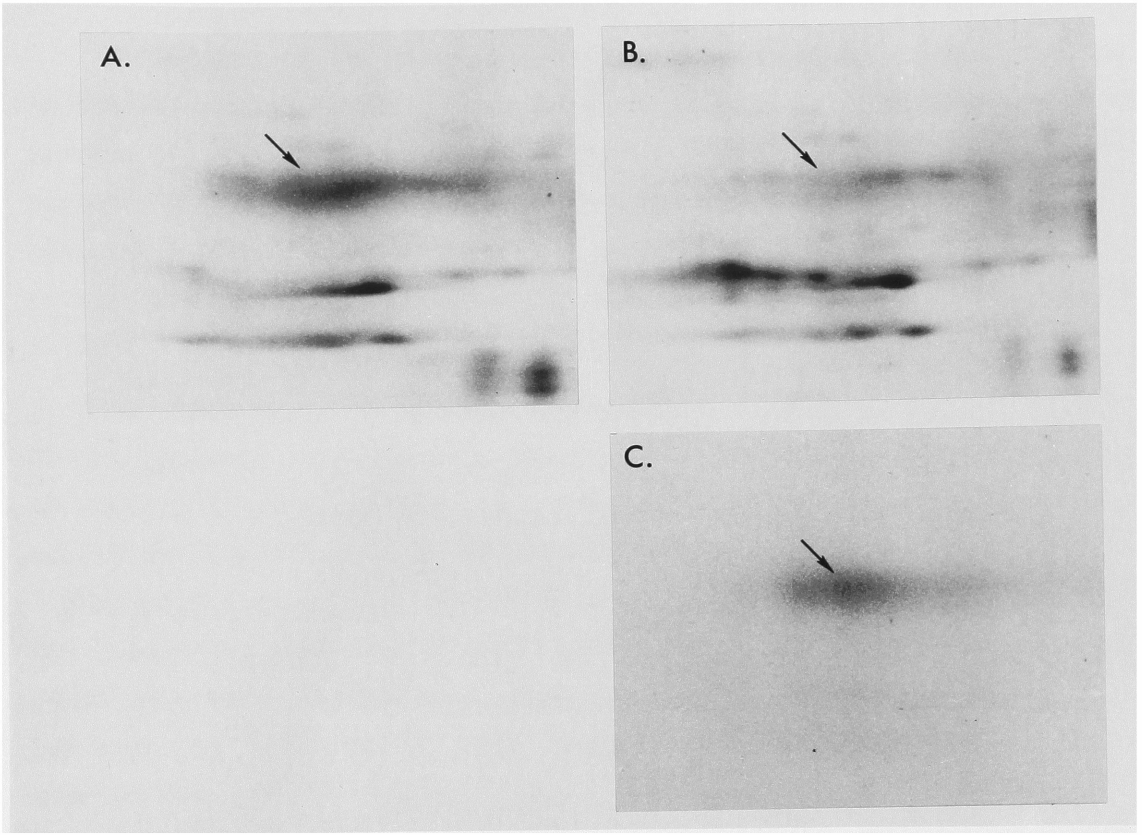


Figure 37. Close-up of D1 region on two-dimensional gels of labeled thylakoid membranes.

See Figure 36 for details. A control (no inhibitors); B, chloramphenicol; C, anisomycin. Arrows point to position of D1.

in SDS then in nonionic detergent and urea instead of just nonionic detergent and urea as illustrated here, the recovery of D1 was somewhat improved but the overall resolution suffered (data not shown).

Comparison of the wheat germ translation products with thylakoid membranes labeled in the presence of anisomycin (Fig. 38), shows that a protein similar to D1 is made in vitro. Presumptive (in vitro) D1 corresponds to authentic D1 both in position and unusual electrophoretic behavior. The calculated isoelectric point ranges, though slightly different, are close. Recovery of presumptive D1 on 2D gels is always excellent.

Peptides produced by limited digestion of presumptive and authentic D1 also provide evidence that the two proteins are related (Fig. 39). At two papain concentrations a series of at least three peptides is generated from both (slots 2 and 3). Authentic D1 digestion products always lag slightly behind those from presumptive D1. This same relationship was seen among protease V8 peptides from presumptive and authentic D1 (data not shown). With more papain, the pattern collapses into one major band and many low molecular weight peptides (slot 4). Close examination of this sample reveals at least three corresponding peptides other than the heavily labeled band. Most peptides from the highest papain concentrations are poorly resolved (slot 5), although some similarities are evident.

Tryptic digests of performic acid oxidized presumptive and authentic D1 were examined by high pressure liquid chromatography (Fig. 40). Few peaks were produced and the yields were low. Major peaks eluted at "breakthrough" positions on the column where new buffers were introduced and were, therefore, not indicative of identity. The maps were, however, unusually similar in their sparsity of peaks. Control protein digests chromatographed under similar conditions gave many peaks as did digests of proteins in published experiments (Capecchi et al., 1977; Milman et al., 1977). Although the HPLC data provide no evidence for the identity of presumptive and authentic D1, they do demonstrate that the two proteins share some characteristics.

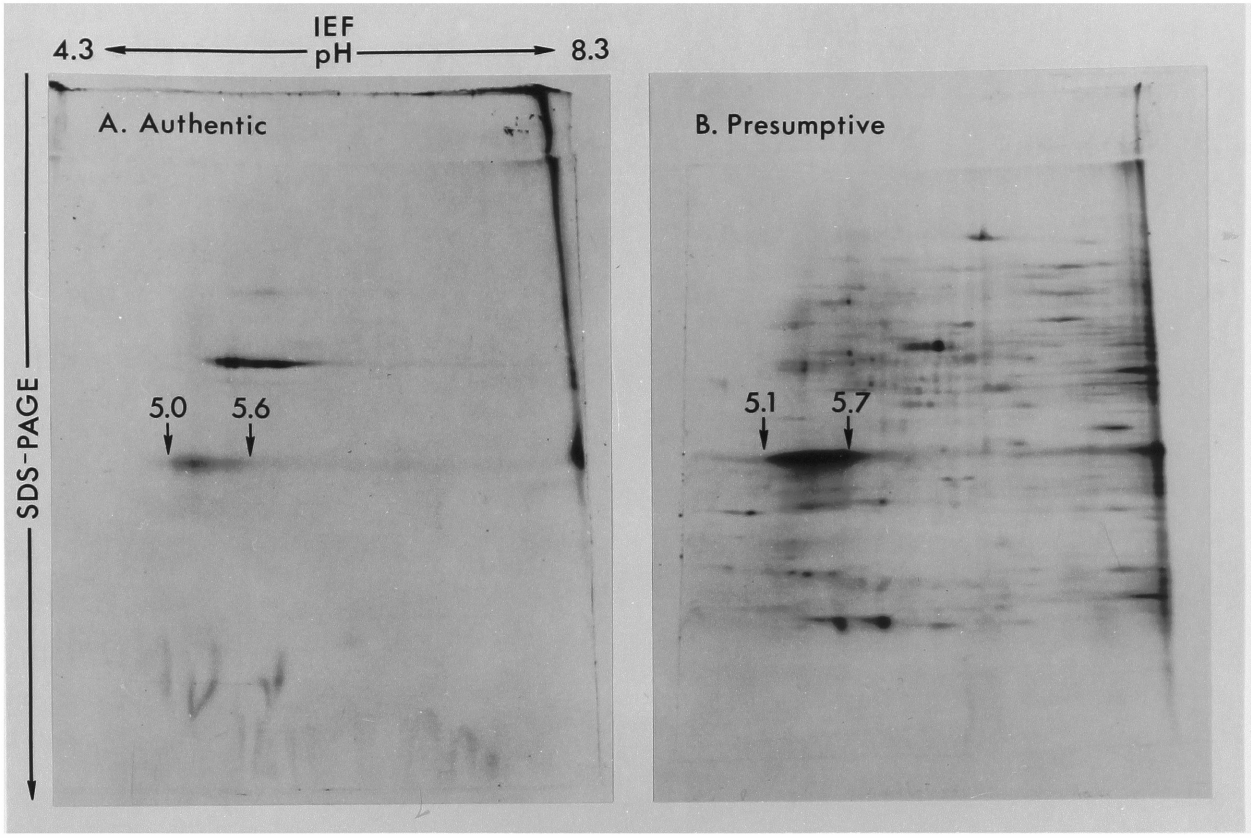


Figure 38. Two-dimensional gel electrophoresis of thylakoid membranes labeled in the presence of anisomycin and wheat germ translation products.

Unbound mRNA was translated in the wheat germ system. Translation products (50 µl) and thylakoid membranes labeled with [^{35}S]-sulfate in the presence of anisomycin (10 µg chlorophyll) were precipitated and analyzed by two-dimensional isoelectric focusing/SDS polyacrylamide gels. Separation in the first dimension was over the pH range 4.3 to 8.3. The second dimension was a Neville 7.5 - 15% gel. A, membranes labeled in the presence of anisomycin; B, wheat germ translation products. Arrows indicate the pH range over which D1 migrates. Notice the slight difference between authentic (in vivo labeled) and presumptive (in vitro synthesized) D1. Isoelectric points were calculated from the gradient shown in Figure 2. See also Figures 36 and 37.

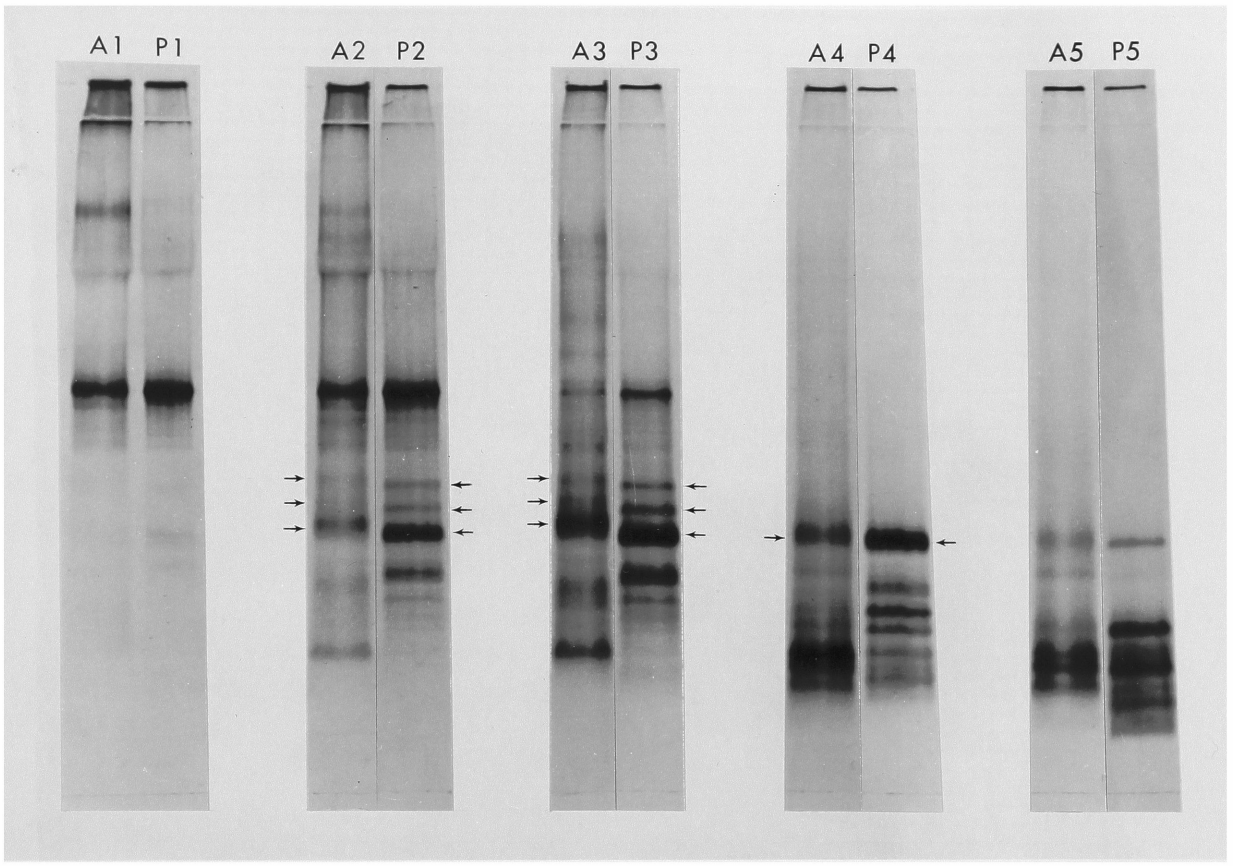


Figure 39. Papain limited digestion of authentic and presumptive D1.

Authentic D1 was purified from [^{35}S]-sulfate labeled membranes by preparative gel electrophoresis and electroelution. Presumptive D1 was synthesized by a large scale wheat germ incorporation using [^{35}S]-methionine and purified on preparative gels. Aliquots (50,000 cpm each) in 50 µl 0.125 M Tris-HCl (pH 6.8)/10% glycerol/0.5% SDS/0.0001% bromphenol blue were digested with serial dilutions of papain for 30 minutes at 37°C. After adding DTT and SDS, samples were electrophoresed on a Neville 12 - 18% gel (no urea). Peptides were detected by autoradiography. A, authentic (in vivo synthesized) D1; P, presumptive (in vitro synthesized) D1. Slot 1, no papain; 2, 0.005 µg; 3, 0.05 µg; 2, 0.5 µg; 3, 5 µg. Arrows point to analogous peptide patterns generated from the authentic and presumptive proteins.

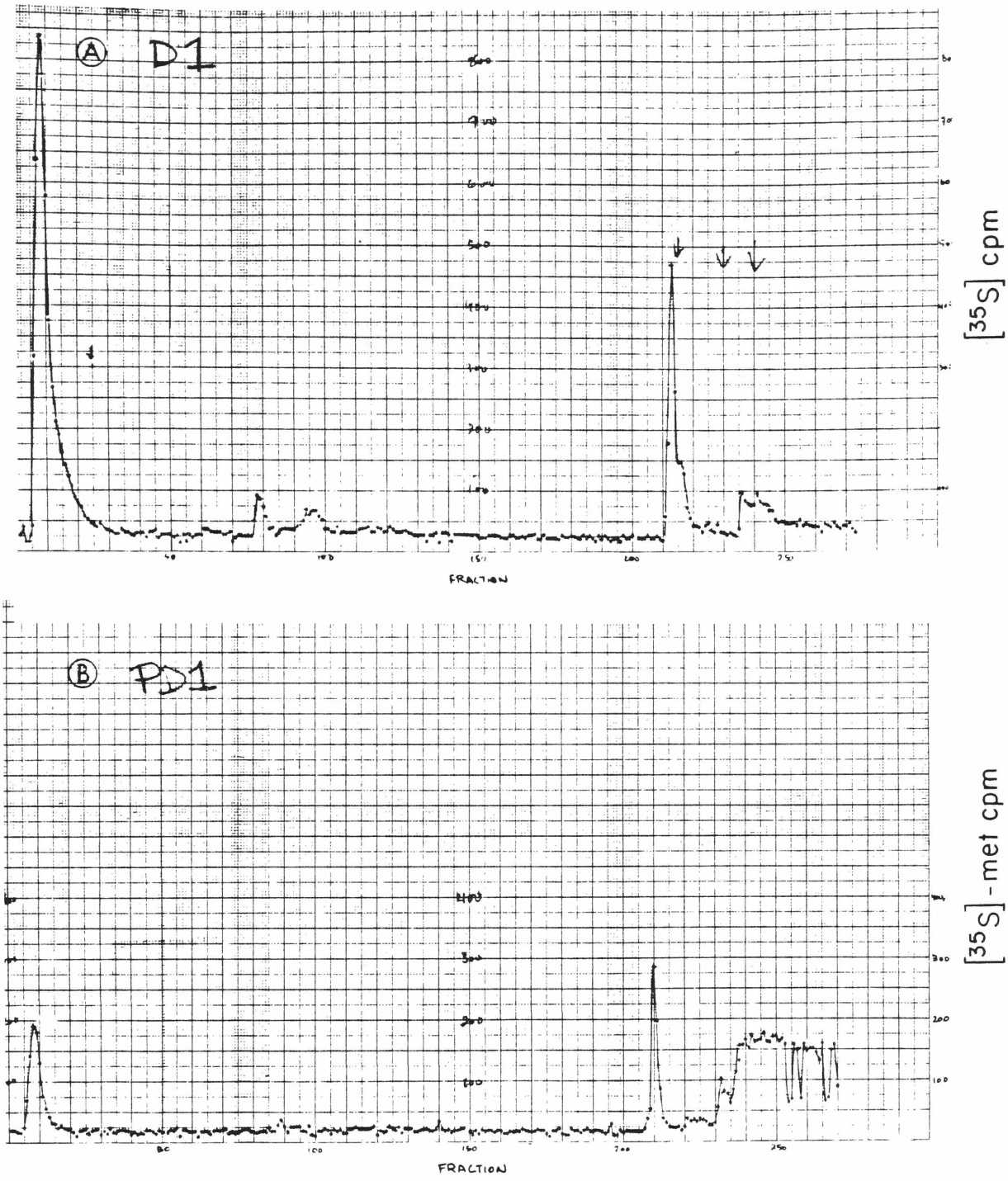


Figure 40. HPLC map of performic acid oxidized tryptic peptides of D1 and presumptive D1.

Polypeptide D1 was labeled *in vivo* with [^{35}S]-sulfate and purified from thylakoid membranes of SDS/polyacrylamide gel electrophoresis and electroelution. Presumptive D1 was synthesized in the wheat germ cell-free system using [^{35}S]-met and purified by SDS/polyacrylamide gel electrophoresis and electroelution. Both proteins were precipitated with TCA, performic acid oxidized, and digested with TPCK-trypsin as described in Materials and Methods. HPLC mapping on Aminex A5 resin was performed as described by Milman *et al* (1977). A, authentic D1 map; B, presumptive D1 map.

CHAPTER V

DISCUSSION

Protein Synthesis In Vivo

1. Sulfate labeling

Sulfate is taken up actively by Chlamydomonas. As mentioned earlier, the rate of transport is linear within one hour. In cells grown on minimal medium (phototrophically), sulfate uptake is dramatically light-dependent (G.M.W. Adams, personal communication). With both light and acetate (mixotrophic growth), the conditions used in these experiments, sulfate uptake does not appear to be strongly influenced by the amount of light and may be stimulated by the presence of acetate (Dunham and Thurston, 1978; unpublished observations). Intracellular sulfate is photosynthetically reduced (Schwenn and Trebst, 1976) and quickly converted to cysteine, methionine, and sulfolipid (Giovanelli, et al., 1978). If Chlamydomonas is labeled for 5 minutes with 0.1 mCi [^{35}S]-sulfate/ml culture and then incubated with a large excess of unlabeled sulfate, proteins and lipids continue to be labeled for several hours (unpublished observations). This result indicates that the ibtracellular sulfate pool cannot be chased.

The sulfate labeling procedure used in these studies has three advantages. (1) Rapid uptake and incorporation insures that cellular proteins will be labeled soon after addition of isotope. This is an advantage when protein synthesis inhibitors are used because it insures that proteins are labeled before their synthesis is curtailed by an internal control or other secondary antibiotic effect. (2) With sulfate it is unlikely that a protein will escape detection if it is synthesized during the labeling period because the high specific activity allows great sensitivity and most proteins are expected to contain cysteine or methionine. (3) A final advantage is that high specific activity sulfate is inexpensively available.

Other radioactive precursors do not have these advantages. In Chlamydomonas, arginine is the only amino acid known to be taken up actively into cells (Kirk and Kirk, 1978). Because radioactive arginine

is not available in high specific activities and is expensive, it is not useful for pulse labeling experiments. Radioactive acetate has been used successfully in a previous study of Chlamydomonas chloroplast protein synthesis (Chua and Gillham, 1977). The specific activity, however, of acetate is much lower than that available for sulfate (e.g., 58 mCi/mmol for [^{14}C]-acetate vs. carrier free [^{35}S]-sulfate).

2. Site of synthesis of chloroplast proteins

Using sulfate labeling in the presence of protein synthesis inhibitors, I have demonstrated that twenty-eight to thirty-one polypeptides are synthesized in the chloroplast. At least twenty-one are thylakoid membrane proteins including twelve of less than ∿17,000-daltons. Three chloroplast products are soluble, and four are chloroplast ribosomal proteins. Two of the latter are found with the large subunit and two with the small.

The number of membrane polypeptides reported here to be made in the chloroplast is more than double that detected by Chua and Gillham (1977). In their investigation of the site of synthesis of major Chlamydomonas thylakoid membrane proteins, they found that seven polypeptides of molecular weight greater than 17,000 (2, 4.1, 4.2, 5, 6, D1, and D2) and two low molecular weight polypeptides (LMW-1 and LMW-2) were synthesized on chloroplast ribosomes. Using a highly sensitive sulfate labeling technique and improved fractionation procedures, I have been able to detect not only the synthesis of the higher molecular weight major polypeptides as described by Chua and Gillham, but also minor polypeptides in the higher molecular weight region and a number of low molecular weight species. The specific activity of whole cell proteins was ∿15 cpm/cell with 0.1 mCi/ml [^{35}S]-sulfate; this is about two orders of magnitude greater than the specific activity achieved by Chua and Gillham with [^{14}C]-acetate.

A second important technical advance was the use of SDS gradient gels supplemented with 8 M urea to resolve membrane polypeptides of less than 17,000-daltons. This procedure permitted me to detect over twenty bands

at least twelve of which were chloroplast products, in the gel region where Chua and Gillham observed the polypeptides designated as LMW-1 and LMW-2. Some of these polypeptides are obscured by the free pigment zone at the bottom of the gel and can only be visualized if the polypeptides are extracted from the membrane with guanidine or the pigment removed with organic solvents.

In addition to the ribulose bisphosphate carboxylase large subunit, two other minor soluble chloroplast products were detected. One is about 50,000-daltons and the other is less than 17,000-daltons. Because the background of polypeptides synthesized in the presence of chloramphenicol is so complex, it is not possible to ascertain whether chloramphenicol alone blocks the synthesis of the two presumptive chloroplast products. Therefore, even though they are not made in the presence of both anisomycin and chloramphenicol, the assignment of their site of synthesis to the chloroplast is tentative.

About thirty large subunit proteins and thirty-nine small subunit proteins were found in chloroplast ribosomes. Both these estimates exceed the numbers reported by Hanson et al. for Chlamydomonas chloroplast ribosomes (1974). After subunit purification, she resolved twenty-six proteins in the large chloroplast subunit and twenty-two proteins in the small subunit by two-dimensional gel electrophoresis. Several explanations of the differences in our results are possible. Since my calculation is based on detection by radioactivity and Hanson's on stain, I am perhaps detecting minor species that were not present in sufficient quantities to see by staining. In my experiments, the number of radioactive bands certainly exceeds the number of stained bands; I have not, however, overloaded the gels to bring out minor stainable species. A second explanation may be that the two-dimensional gels used by Hanson lose some proteins through aggregation. Hanson, in fact, noted selective but not repeatable loss of some proteins due to aggregation. A mitochondrial ribosomal protein in Neurospora also aggregates without SDS (Lambowitz et al., 1976).

A number of cytoplasmic and chloroplastic ribosomal proteins co-

migrate. It is impossible to determine from my experiments if these are cross-contaminants or unique species with similar molecular weights. After excluding a number of irreproducible spots, Hanson also noted some apparent overlap between subunit proteins.

In addition to the four chloroplast synthesized 70S ribosomal proteins, six or seven others were not labeled in the presence of either chloramphenicol or anisomycin. These could either be synthesized in the cytoplasm or the chloroplast. Freyssinet has noted similar labeling characteristics of Euglena chloroplast ribosomal proteins (Freyssinet, 1978). This phenomenon can be explained by relative ribosomal protein pool size differences. Only proteins assembled into complete ribosomes are detectable. If a ribosomal protein which is synthesized in the chloroplast has a large pool, then in the presence of anisomycin the specific activity of the newly made protein will be diluted. If, in addition, a cytoplasmically made ribosomal protein has a very small pool, in the presence of anisomycin its synthesis will be inhibited and ribosome assembly will only proceed until the pool of this protein is exhausted. Since the specific activity of the chloroplast made protein will be low while assembly is still occurring, this protein will not be detected in the ribosomes and its synthesis will appear to be inhibited by both antibiotics. Pool size problems like this can be circumvented by either pre-treating cells with chloramphenicol to enlarge the pool size of cytoplasmically made proteins (Chua and Gillham, 1977), or by chasing labeled proteins into assembled ribosomes after washing away the radioactive precursor and antibiotic (Lizardi and Luck, 1972; Lambowitz et al., 1976).

From my results, it is impossible to determine if either of the chloroplast synthesized large ribosomal subunit proteins is identical to the polypeptide altered in the uniparental mutant of Mets and Bogorad (1972). Hanson et al. (1974) reported that the affected mutant polypeptide has a molecular weight of about 30,000 which is close to the apparent molecular weight of one of the large subunit proteins reported here to be synthesized in the chloroplast (cf. Fig. 25). Direct comparison of

labeled mutant and wild-type chloroplast ribosomal proteins is required to establish that they are, in fact, the same.

The site of synthesis of chloroplast envelope proteins was not examined in this study. It is not possible to isolate chloroplast envelopes from Chlamydomonas because the cell wall and envelope are closely apposed. The envelope is ruptured into small vesicles when the cell wall is broken and these vesicles are lost during an early stage of thylakoid membrane isolation. Chlamydomonas thylakoid membranes purified by flotation (see Materials and Methods) are probably not contaminated with envelopes since no envelope band is obtained when the thylakoids are re-purified on step gradients used to separate higher plant envelopes and thylakoids (N.-H. Chua, personal communication). Higher plant chloroplast envelopes have protein and lipid compositions distinct from those of thylakoid membranes (Poincelot, 1973; Mendiola-Morgenthaler and Morgenthaler, 1974; Joy and Ellis, 1975; Douce et al., 1973). Two to three envelope polypeptides may be made in the chloroplast (Joy and Ellis, 1975; Mendiola-Morgenthaler and Morgenthaler, 1974).

3. Integral and peripheral disposition of chloroplast membrane proteins

Extraction of thylakoid membranes with 6 M guanidine hydrochloride or 0.1 N NaOH releases about 20% of the protein from the membrane but does not extract chlorophyll or disassemble the membrane structure. The major polypeptides extracted are 4.1, 4.2, 12, and 24. At least twenty other minor polypeptides detectable by either radioactivity or stain on overloaded gels are also extracted.

Membrane extraction using these agents is an accepted procedure to determine if membrane proteins are integrally or peripherally disposed (Steck and Yu, 1973; Kreibich et al., 1978). Integral membrane proteins are defined as those that are embedded in the hydrophobic lipid bilayer; peripheral proteins are those attached by ionic or other interactions to protein, carbohydrate, or lipid at the surface of the membrane. Integral proteins may more rigorously be defined as those that contain a hydrophobic domain capable of binding "mild" nonionic detergents

(Helenius and Simons, 1977).

Results from extraction of Chlamydomonas thylakoid membranes are consistent with those from similar experiments with barley (Machold et al., 1977). With barley thylakoid membranes, 6 M guanidine-HCl removed a number of polypeptides including coupling factor subunits, but did not extract chlorophyll protein complexes. Coupling factor is known to be loosely attached to the outer thylakoid surface (Binder et al., 1978; Miller and Staehelin, 1976). Fracture faces of the extracted membranes contained particles, but the particles are arranged in different arrays than those found in unextracted membranes. In Chlamydomonas, polypeptides 4.1 and 4.2, which comigrate with coupling factor α and β subunits of the chloroplast coupling factor (Bennoun et al., 1978) are extracted; chlorophyll-protein complexes I, II, III, IV, and V are, however, still present (Delepelaire and Chua, 1979; unpublished observations).

4. Accessibility of chloroplast synthesized membrane proteins to protease

All major membrane proteins which are synthesized inside the chloroplast are digested to some extent by papain, thermolysin, and trypsin suggesting that all these polypeptides are exposed on the outer (stromal) thylakoid surface. Digestion of 4.1 and 4.2, the Chlamydomonas α and β coupling factor subunits is consistent with the known location of coupling factor (Miller and Staehelin, 1976). The action of the proteases is probably restricted to the outer thylakoid surface since, under experimental conditions similar to those used here immobilized trypsin and soluble trypsin gave identical digestion patterns (N.-H. Chua, personal communication).

Digestion of thylakoid membranes labeled in the presence of anisomycin with three proteases produces a fragment of molecular weight 15,000 - 20,000. The source of this peptide, which is protected by the lipid bilayer, is probably the integral membrane protein D1. A peptide of similar molecular weight, which was generated by pronase digestion of labeled maize thylakoid membranes, was attributed to a 32,000-dalton membrane protein (Grebanier et al., 1978).

Protein Synthesis In Vitro

1. Proteins synthesized in vitro

The results presented here clearly demonstrate that ribulose bisphosphate carboxylase large subunit is synthesized from poly(A)$^-$ mRNA in the wheat germ cell-free system. The polypeptide made in vitro is the same size as authentic Chlamydomonas large subunit and has identical isoelectric heterogeneity. In contrast, recent results have demonstrated that the carboxylase small subunit, which is synthesised on poly(A)$^+$ mRNA in the cytoplasm, is made as a larger precursor (Dobberstein et al., 1977).

A strong relationship has also been demonstrated between the major in vitro product directed by poly(A)$^-$ mRNA and the membrane protein D1. The two proteins show similarities in one-dimensional SDS gel mobility, spot shape and isoelectric point on two-dimensional gels, behavior of tryptic peptides on an HPLC ion exchange column, and papain limited digest maps. In all cases but the tryptic peptide pattern, there are repeatable small differences in the two proteins that prevent the absolute identification of the in vitro product as D1. Presumptive (in vitro) D1 most often comigrates or runs slightly slower than authentic D1 on one-dimensional SDS gels. The diffuse shape of the authentic protein's band, however, makes precise comparison of the two difficult. Attempts to sharpen the band by adding urea to the gel or changing buffer systems have not been successful. In two-dimensions, the isoelectric point of presumptive D1 is shifted about 0.1 pH unit higher than that of authentic D1. The smeary spot structure, however, makes this small difference less than convincing. Peptides generated from authentic D1 always migrate slightly slower than presumptive D1 peptides, even though many features of the overall pattern are similar.

One explanation of these small differences is that the polypeptide synthesized in vitro is a precursor of D1. For instance, if presumptive D1 contained a small, basic peptide extension that was cleaved during assembly of D1 into the membrane, then removal of the extension would

result in a more acidic and perhaps more hydrophobic protein (since a hydrophilic sequence was lost). This would explain the isoelectric point shift and the various mobility differences: as an intact polypeptide, presumptive D1 would migrate slower than the authentic form because of the peptide extension; after limited papain digestion, extremely hydrophobic authentic peptides would migrate (anomolously) behind the more hydrophilic presumptive peptides. Some support for this interpretation of the limited digest map comes from the observation that radically different one-dimensional peptide patterns are produced when two membrane proteins differing only by post-translational proteolytic and glycosidic modifications are compared (Garoff *et al.*, 1978).

The precursor hypothesis is strongly supported by observations in other plants. A 32,000-dalton thylakoid membrane protein from *Spirodela* is made *in vitro* as a 33,500-dalton precursor (Edelman and Reisfeld, 1978). The similarity of this protein and its precursor to D1 and presumptive D1 is striking. Both are acidic proteins and migrate as a smear on two-dimensional isoelectric focusing/SDS polyacrylamide gels. The *in vitro* product from *Spirodela* has an isoelectric point slightly more basic than the authentic protein. Papain limited digest maps of the *Spirodela* proteins are similar although resolution of the peptides is not sufficient to detect the small differences noted between D1 and presumptive D1 patterns. The biological significance of the *Spirodela in vitro* precursor has been verified by detection of the precursor *in vivo*.

The results of Grebanier *et al.* (1978) also support the precursor hypothesis. As mentioned in the Introduction, isolated intact maize chloroplasts synthesize a polypeptide slightly larger than a 32,000-dalton membrane protein. Peptide maps of both the *in vitro* product and the authentic membrane protein are similar. It is believed, therefore, that the polypeptide synthesized by intact chloroplast is a precursor of the 32,000-dalton protein.

2. Chloroplast mRNA

In these studies, my approach has been to identify first chloro-

plast products by *in vivo* labeling and then search for mRNA for these proteins among the total poly(U) Sepharose unbound RNA fraction. This strategy was necessary because intact *Chlamydomonas* chloroplasts cannot be isolated. The rationale for using unbound RNA was based on the observation that large subunit mRNA from *Euglena* did not bind to an oligo-dT column (Sagher *et al.*, 1977) and that most spinach chloroplast mRNAs have very short poly(A) sequences (Wheeler and Hartley, 1975). Localization of *Chlamydomonas* poly(A)$^-$ mRNA in the chloroplast is, of course, an assumption.

Although the mRNAs for large subunit and presumptive D1 appear to lack poly(A), it is possible that mRNA for other chloroplast products may have poly(A) tails long enough to bind to the affinity column. I have not searched carefully among translation products directed by poly(A)$^+$ mRNA for polypeptides known to be synthesized in the *Chlamydomonas* chloroplast. Mitochondria have both poly(A)$^+$ and poly(A)$^-$ mRNA, although no specific polyadenylated message has been demonstrated (Borst and Grivell,1978).

My results indicate that *Chlamydomonas* mRNA is not translated efficiently in the *E. coli* S30 system, but greatly stimulates incorporation in the wheat germ cell-free system. This effect may arise because the *E. coli* system is inhibited by fragments of ribosomal RNA contaminating the poly(A)$^-$ mRNA fraction. Despite early difficulties in obtaining full size products from the *E. coli* (Hartley and Ellis, 1975; Howell *et al.*, 1977), recently published experiments demonstrate that the S30 cell-free system is capable of translating chloroplast mRNA (Reisfeld *et al.*, 1978; Sano *et al.*, 1979). Cell-free systems based on cytoplasmic (80S) ribosomes have, in general, been most successful. For example translation of either *Euglena* chloroplast mRNA in the wheat germ system or a maize chloroplast DNA transcript in the reticulocyte system yielded full-size carboxylase large subunit polypeptides (Sagher *et al.*, 1976; Coen *et al.*, 1977). Recently, Howell has also obtained translation of full size *Chlamydomonas* large subunit in the wheat germ cell-free system (Howell and Gelvin, 1978).

What is clear from all these experiments is that, even though chloroplast ribosomes resemble procaryotic ribosomes in size and antibody specificies, translation of chloroplast mRNA *in vitro* does not proceed more efficiently on procaryotic ribosomes. The reason for this may be that protein synthesis in chloroplasts and perhaps mitochondria is less like that in bacteria than was previously supposed. In fact, genomic organization, tRNA populations, and ribosome structure in organelles may be quite distinct from that in bacteria (Ellis, 1976; Borst and Grivell, 1978). A particularly important recent finding is that the chloroplast 23S rRNA gene contains an intervening sequence (Rochaix and Malnoe, 1978). Previously, intervening sequences had only been found in eucaryotic genes.

Protein Sorting in the Chloroplast

Chloroplasts contain three distinct membrane delimited compartments. These are the space between the envelope double-membrane (intermembrane space), the soluble stroma, and the intrathylakoid space. No connection exists between the intermembrane space and the intrathylakoid space in mature chloroplasts, although the two may be developmentally related (Gunning and Steer, 1975).

Proteins synthesized in the chloroplast have, therefore, several possible final destinations. Some will remain in the soluble stroma. These include carboxylase large subunit and chloroplast ribosomal proteins. Others will become attached, or inserted in the inner membrane of the chloroplast envelope. The bulk of the chloroplast products will become associated with the thylakoid membrane. Proteins synthesized in the chloroplast that finally reach either the intermembrane space or the intrathylakoid space (if such proteins exist) must pass through a membrane. Therefore, from a biosynthetic point of view, the intermembrane and intrathylakoid compartments are equivalent. For the purpose of discussion, it is assumed that no chloroplast product is exported to the cytoplasm.

It is evident, therefore, that some mechanism must exist in the

chloroplast for specifying the final localization of a protein. No information about this mechanism is available. It is tempting, however, to invoke the "signal hypothesis" to explain how some chloroplast products end up in the right place.

The "signal hypothesis" has been developed to explain the transport of secretory proteins through the ER membrane and the insertion of certain integral membrane proteins (Blobel and Dobberstein, 1975; Katz et al., 1977). Briefly, the signal hypothesis states that proteins destined for either the ER cisterna or membrane are synthesized with an N-terminal polpeptide extension (signal). The signal directs the binding of polysomes synthesizing secretory or membrane proteins to the ER and makes possible the co-translational transfer of polypeptides across the membrane. The signal peptide is removed proteolytically during the translocation process. This mechanism has been demonstrated for eucaryotic secretory and membrane proteins and, in an equivalent formulation, for several bacterial proteins (Shields and Blobel, 1977; Smith et al., 1977; Chang et al., 1978; Lin et al., 1978).

Thylakoid membrane bound polysomes are believed to synthesize membrane proteins. In synchronous Chlamydomonas cultures, polysome binding coincides with membrane synthesis (Chua et al., 1973, 1976). Nascent chains released from thylakoid bound polysomes remain membrane associated (Chua et al., 1973; Margulies et al., 1975). In addition, it has been demonstrated that a thylakoid membrane protein is made as a precursor (Grebanier et al., 1978; Edelman and Reisfeld, 1978). Results presented here show that the integral membrane protein D1 may also be synthesized as a precursor in Chlamydomonas chloroplasts. These data appear to be consistent with a signal mechanism by which thylakoid integral and peripheral membrane proteins would be synthesized on thylakoid-bound ribosomes as precursors with signal sequences specifying a thylakoid or intrathylakoid localization.

Synthesis of envelope proteins may not follow a signal mechanism. Envelope-bound polysomes have not been reported. Therefore, envelope proteins may be made on free-polysomes and distributed to their proper

locations post-translationally. A number of precedents for post-translational sorting exist (Chua and Schmidt, 1978; Goldman and Blobel, 1978; Rachubinski et al., 1978).

The discovery, however, that the Spirodela membrane protein precursor is detectable in vivo (Edelman and Reisfeld, 1978) and the observation that a membrane protein precursor is synthesized but not processed in intact maize chloroplasts (Grebanier et al., 1978) appear to be in contradiction with the co-translational processing characteristic of the signal hypothesis. Perhaps processing of the chloroplast precursors has more to do with assembly than simply insertion and, for this reason, cleavage of the signal peptide is separated in time from synthesis. A clear answer to this question must await additional data.

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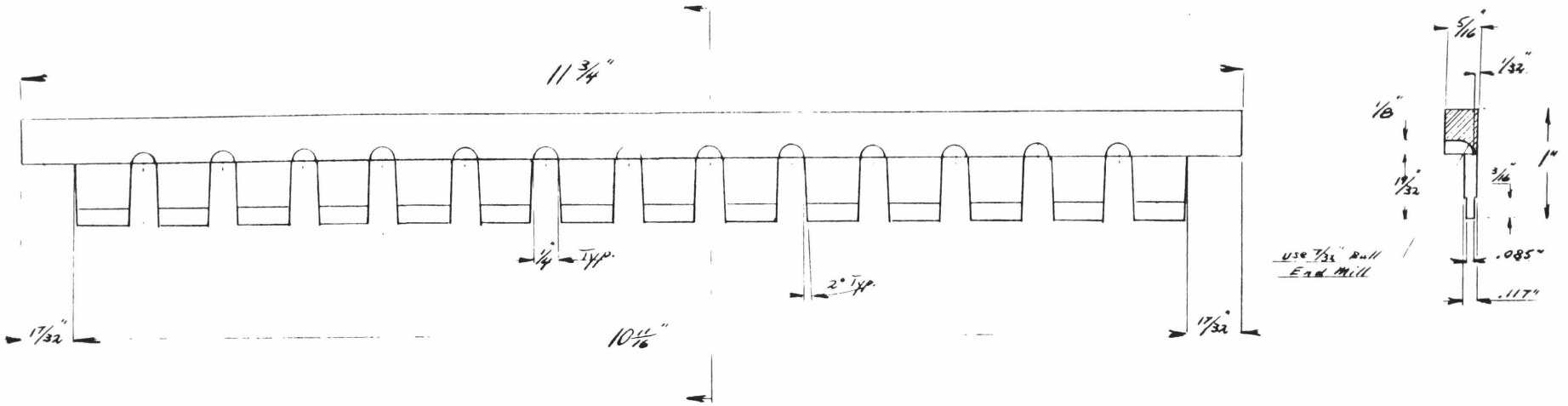
APPENDIX I. SPECIALIZED APPARATUS FOR RNA ELECTROPHORESIS

Slot former for formamide/acrylamide gradient gels



Material: Lucite

14 Teeth Slot Former

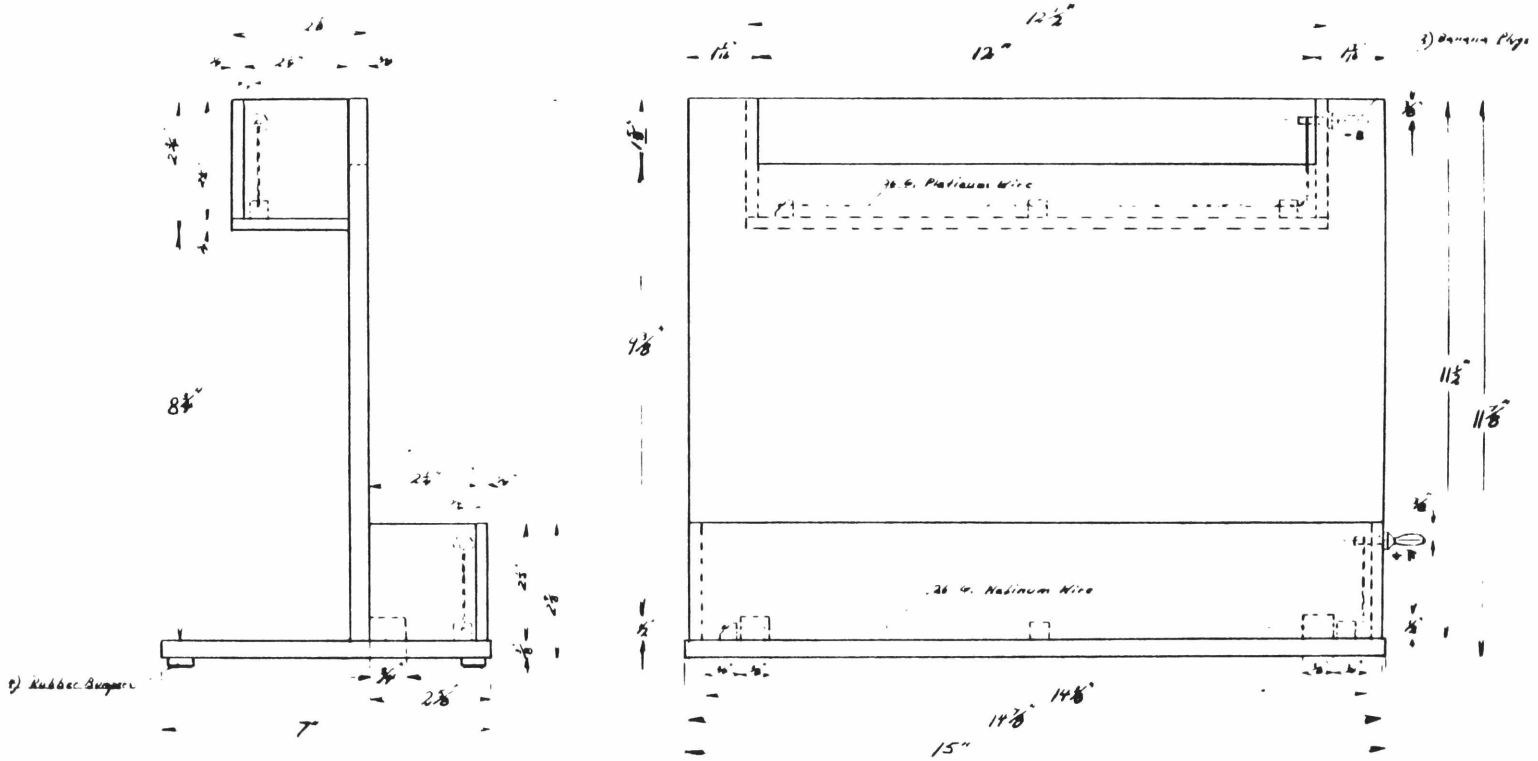
Karl Matlin
R. F. 1/79

APPENDIX II. APPARATUS FOR PROTEIN ELECTROPHORESIS

A. Large gel apparatus

B. Slot former for 1 mm thick large gels

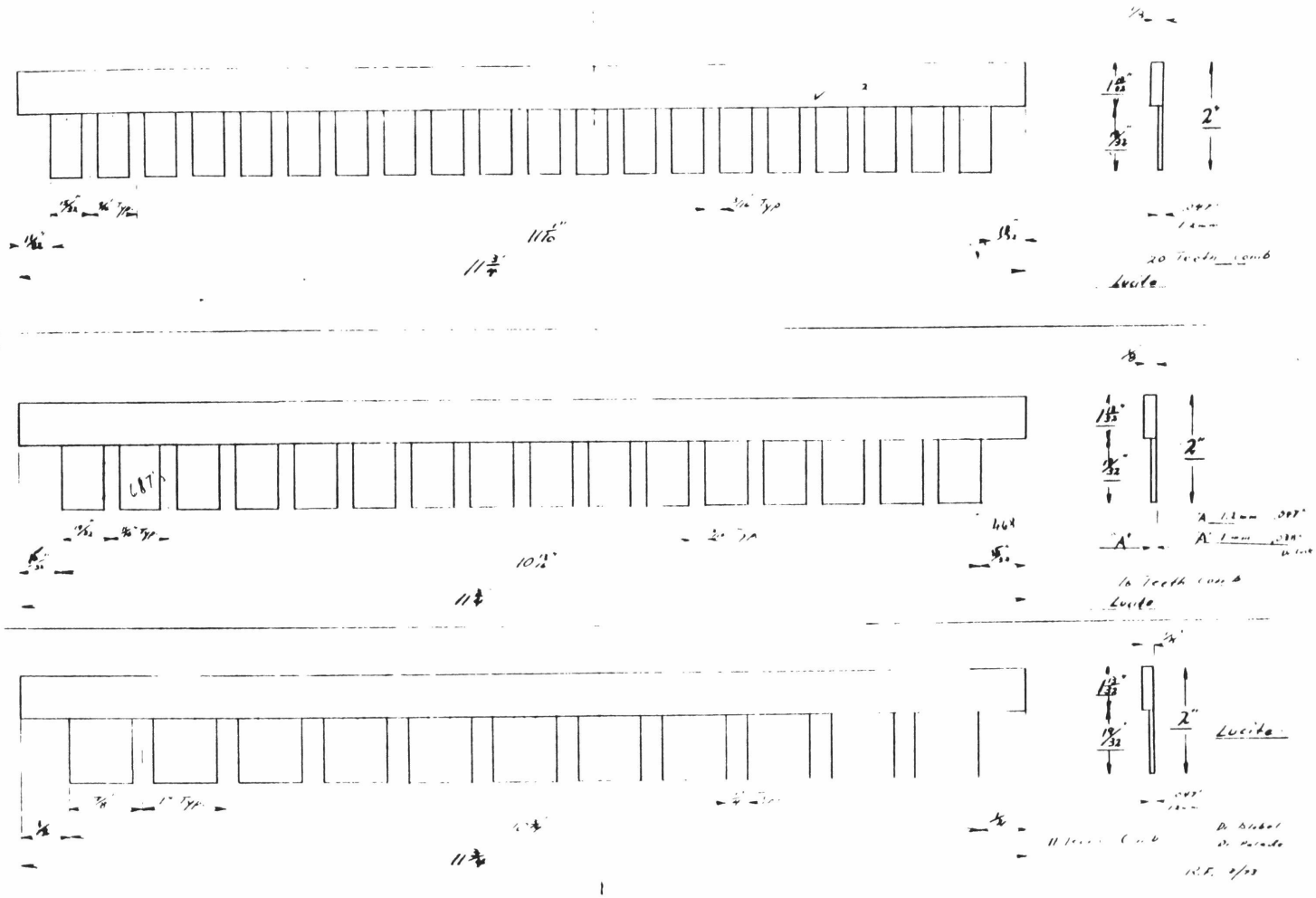
C. Glass plates for large gels

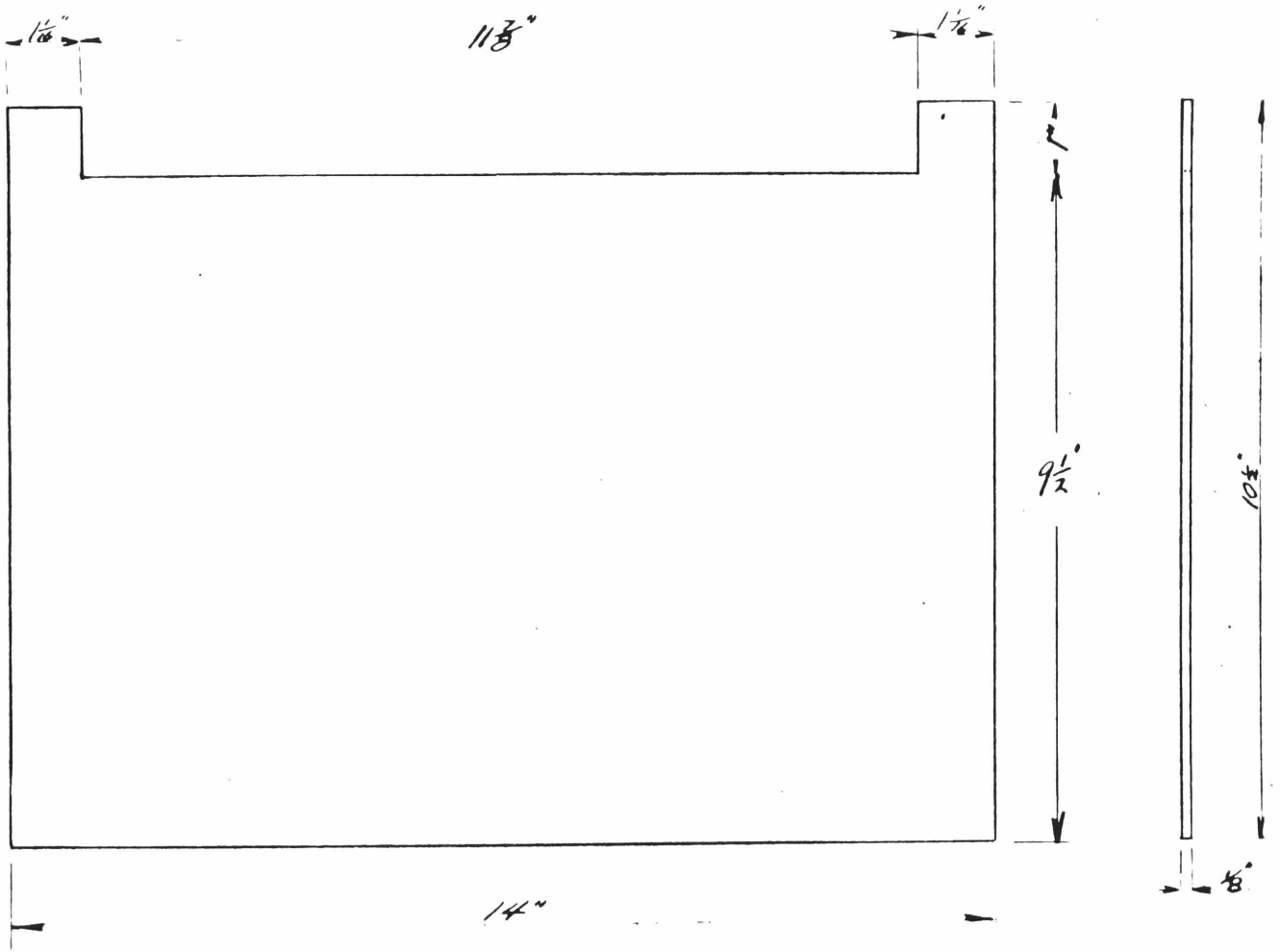


Material: Lucite

Scale: 1/2

R.F. 9/73


20 Teeth comb
Lucite
A
A
Lucite



Note: Make one without

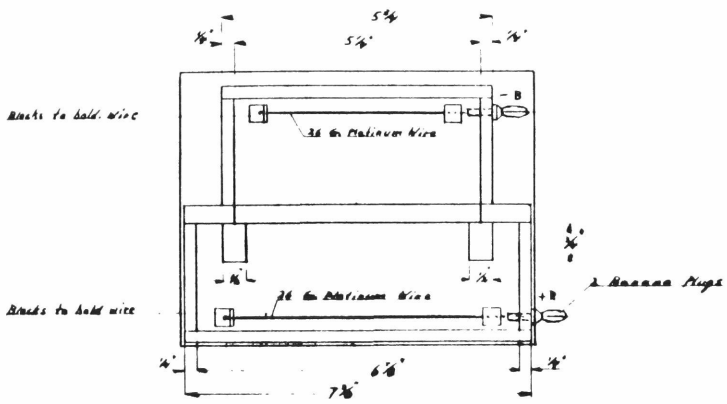
Material: Glass

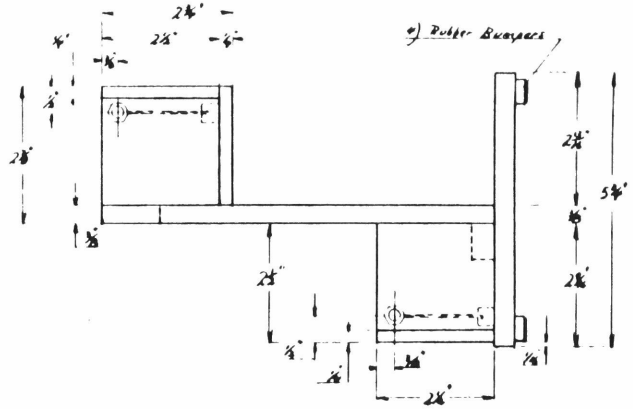
R.F. 8/33

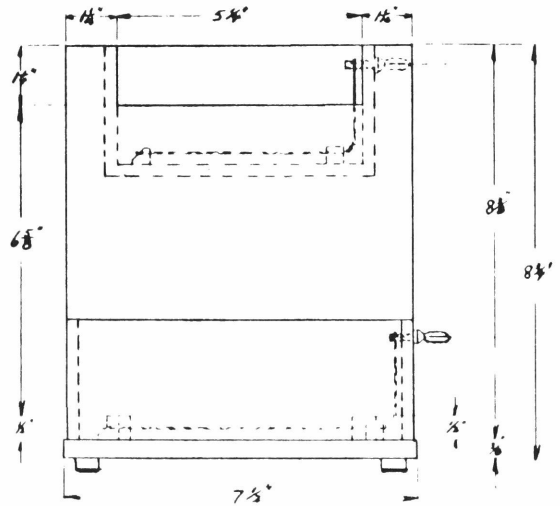
APPENDIX III. APPARATUS FOR SLAB ISOELECTRIC FOCUSING

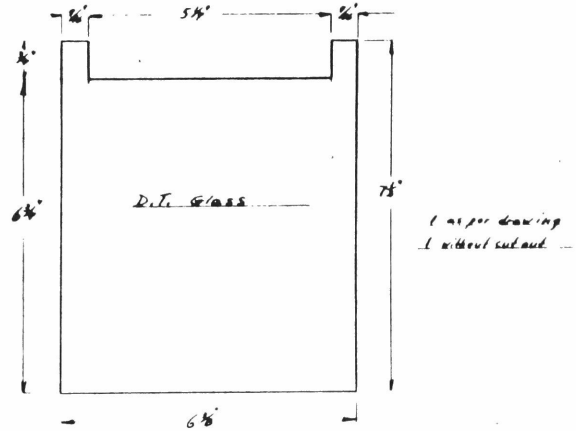
A. Small gel apparatus and glass plates for small gel

B. Slot former for 0.75 mm thick small gels







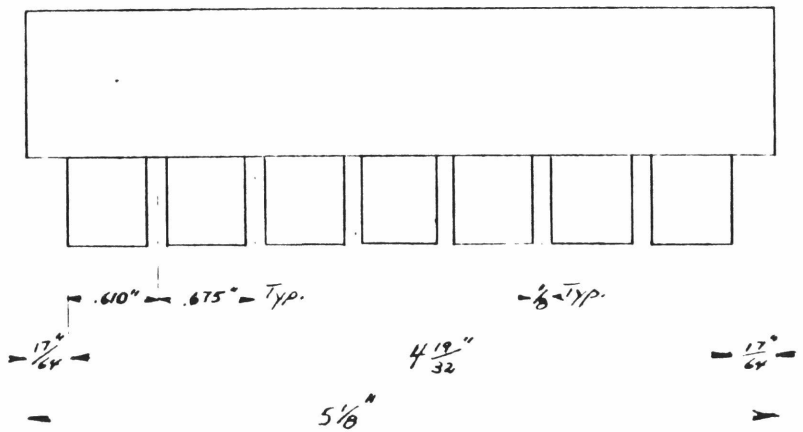


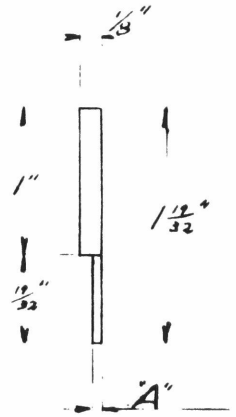
1 as per drawing
1 without cut out

Material: Lucite

Vertical Slab Gel Electrophoresis

R.F. 5/72





Make 1 "A" dim. .089"

" 1 "A" dim. .117"

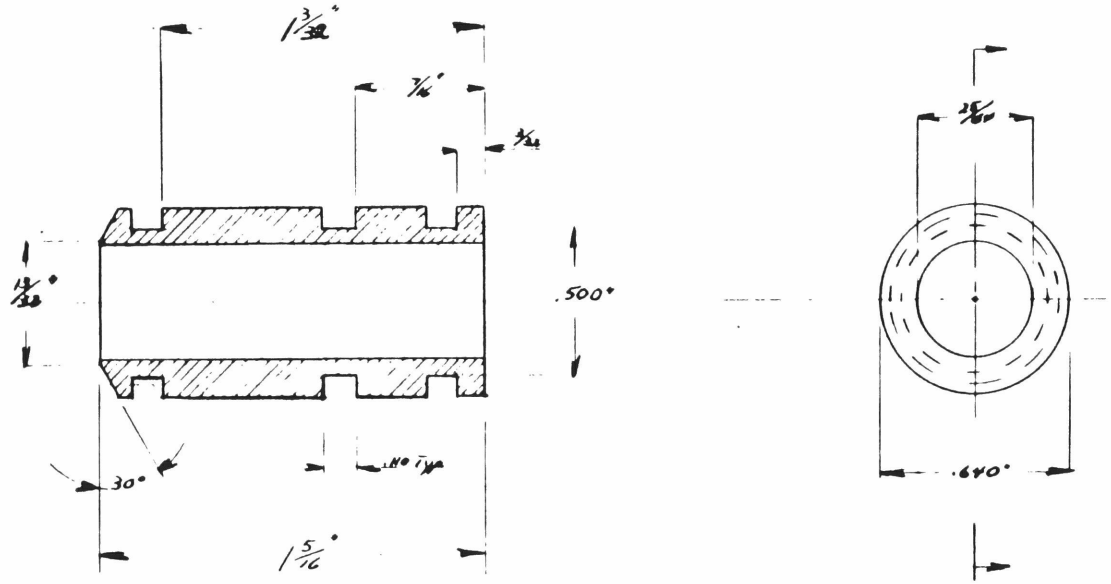
Material: Lucite

APPENDIX IV. APPARATUS FOR ELECTROELUTION

A. Elution chamber

B. Nylon net

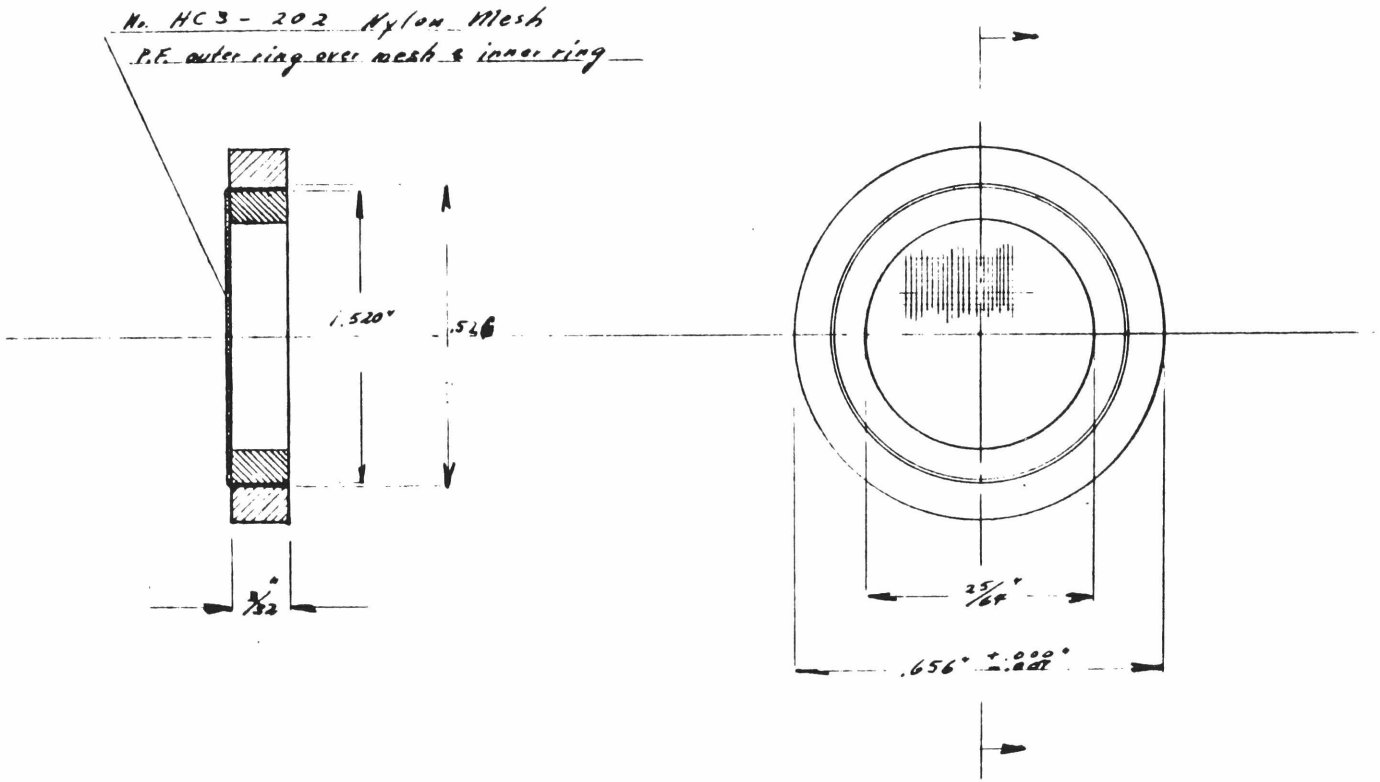
C. Support for elution columns



Material : Teflon
3 No. 2-111 O-Rings

Elution Chamber

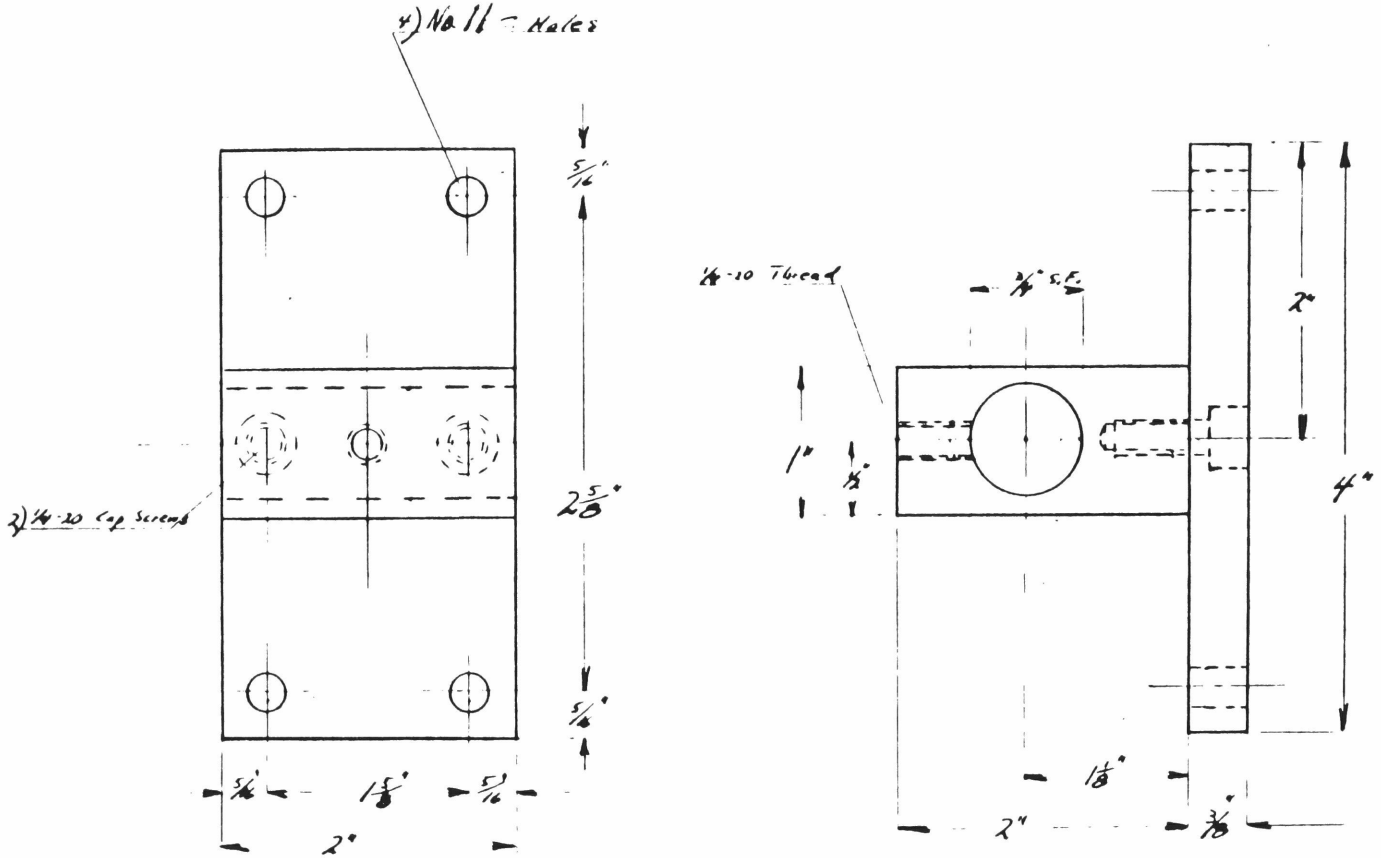
Arde Thierx
Dr. Siekevitz



Material: Kel-F

Anna Thiery

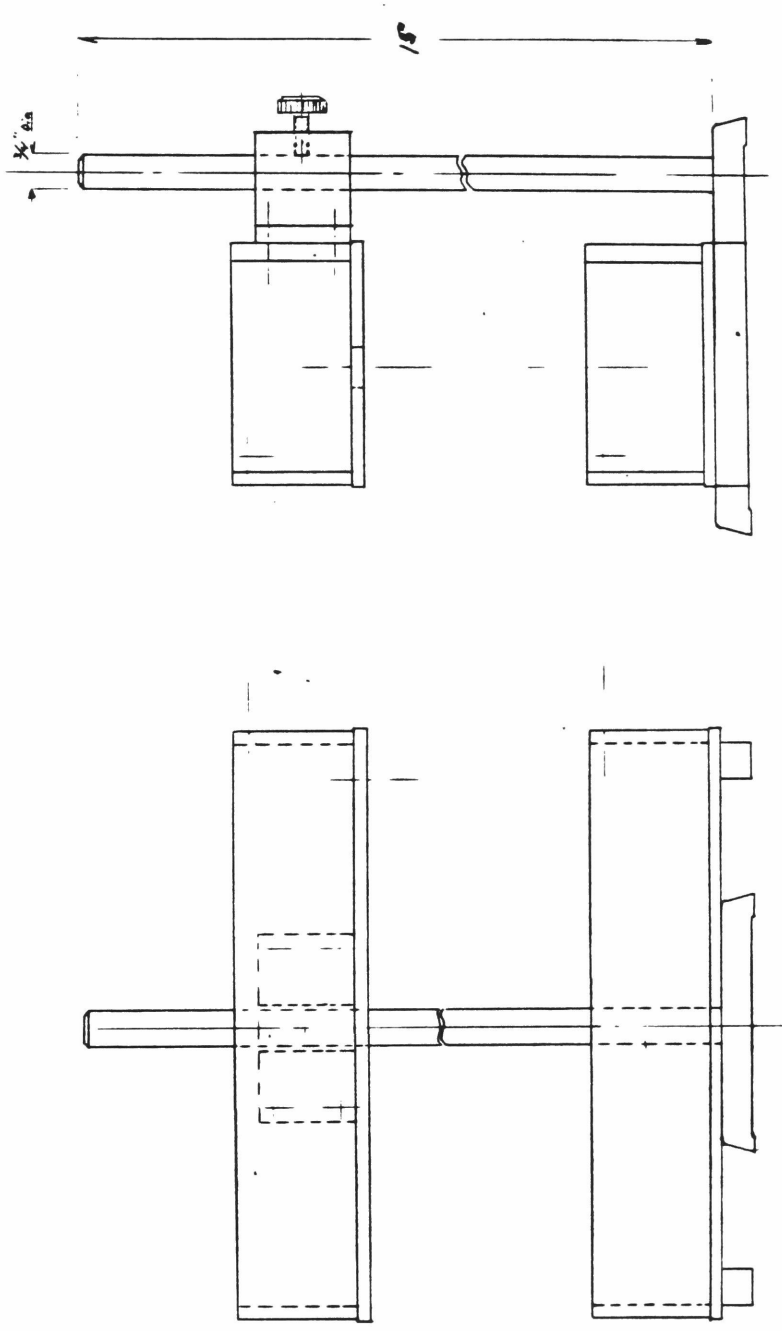
Dr. Sieker

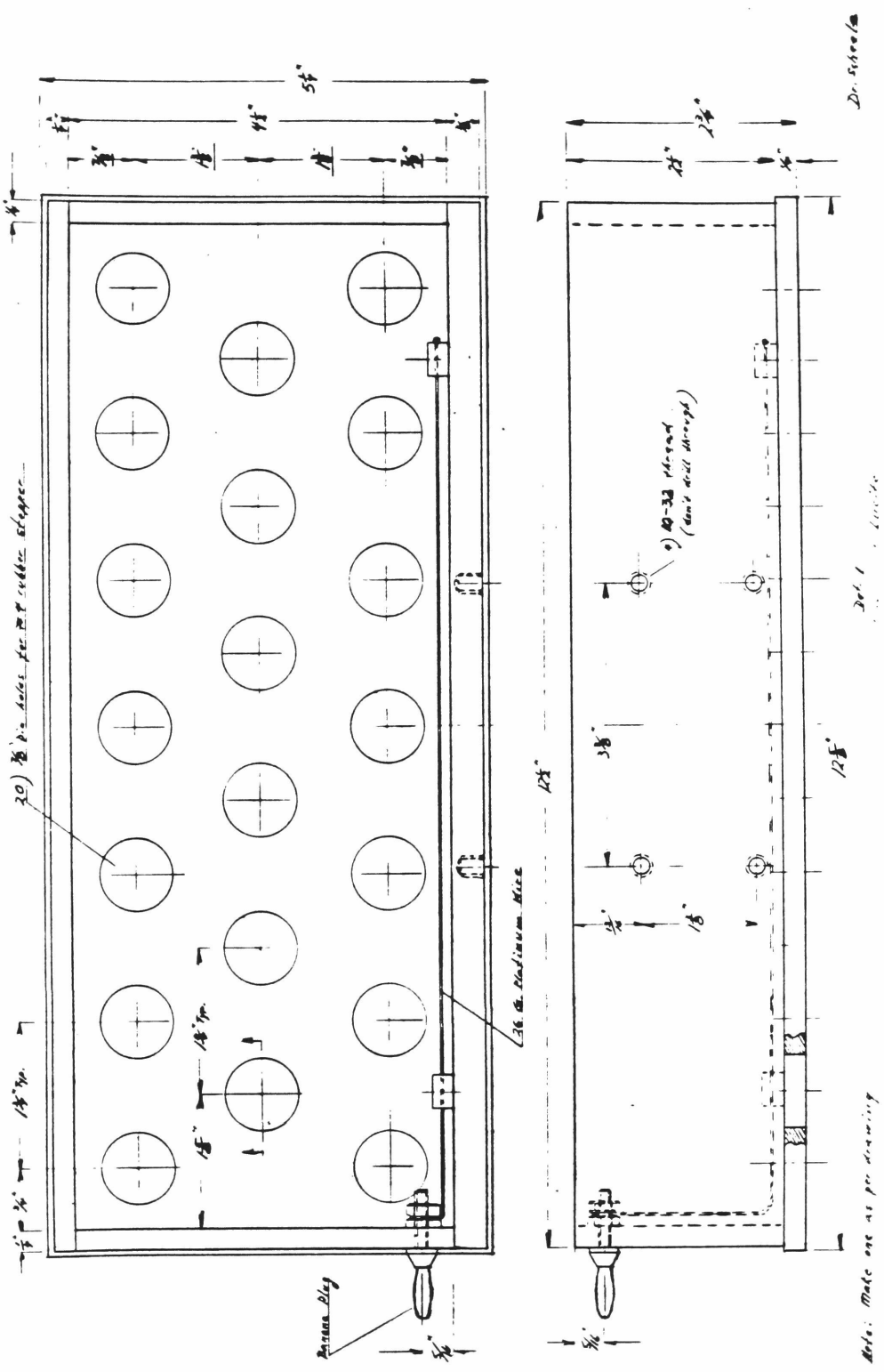


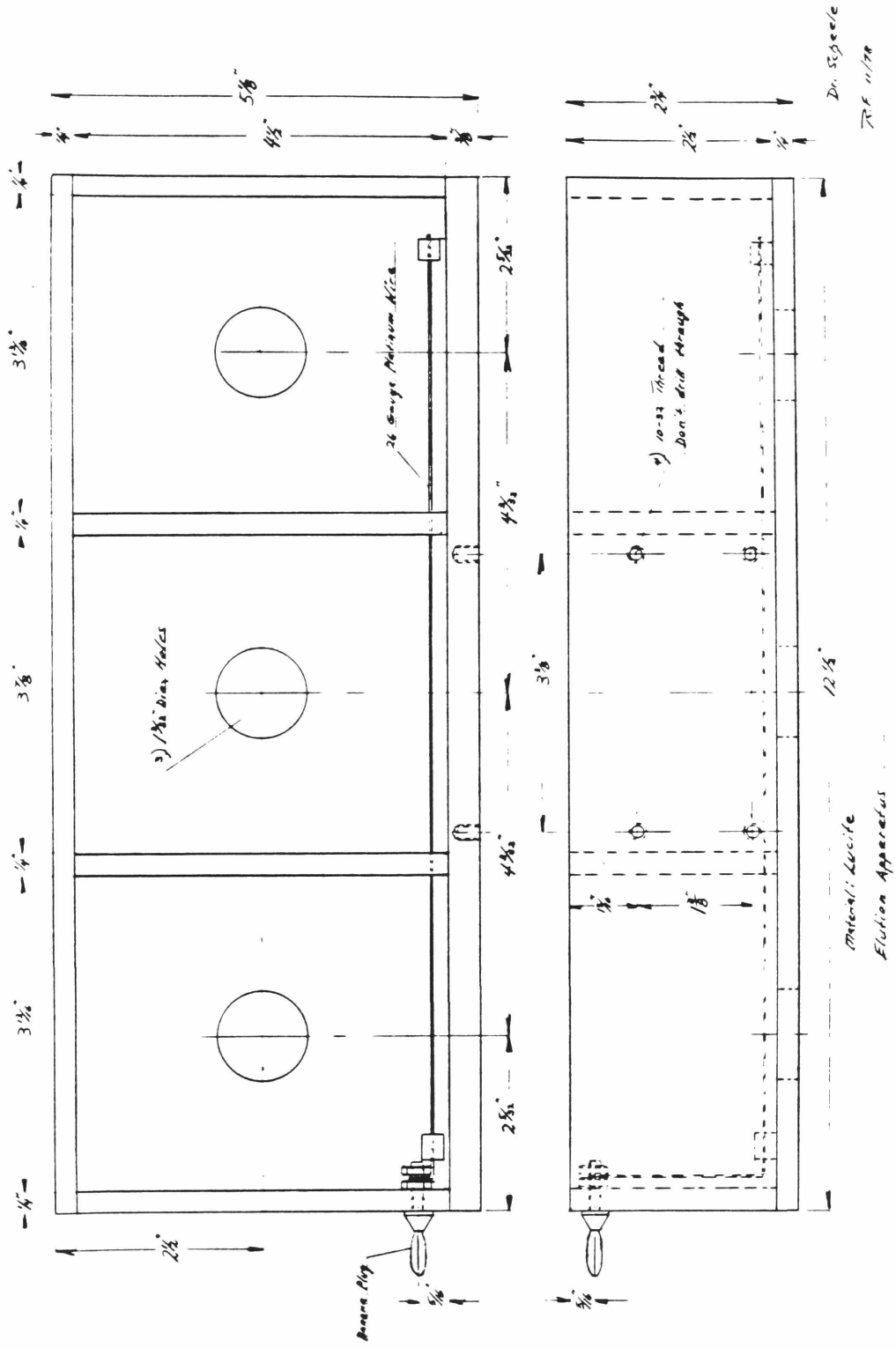
Det

Material - Aluminum

Dr. Schaefer






Dr. Sgreve
Material: Lucite
Elution Apparatus

End